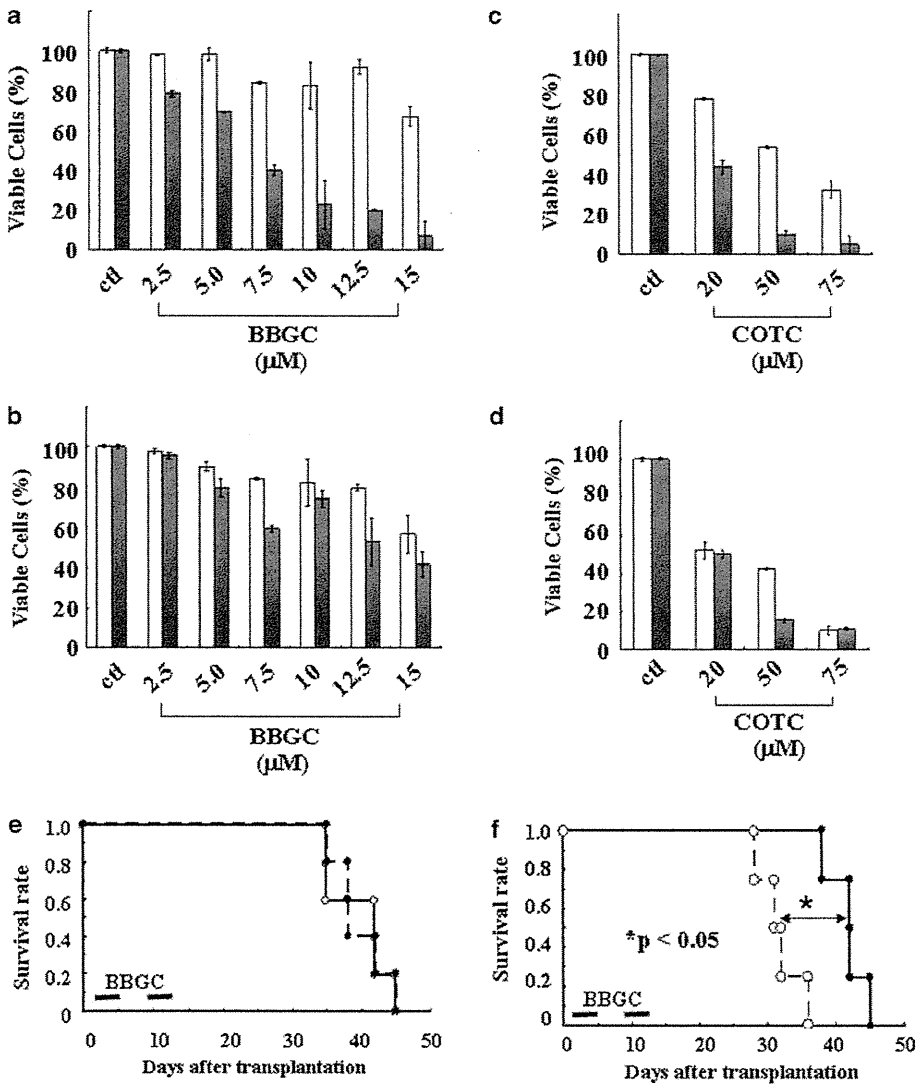

Figure 5 Effect of glyoxalase-I (Glo-I) inhibitors on hypoxia-adapted chronic myeloid leukemia (HA-CML) cells. *In vitro* cytotoxic effects of (**a**) *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) and (**c**) 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone (COTC) on parental K562 (white column) and K562/HA (black column) cells. *In vitro* cytotoxic effects of (**b**) BBGC and (**d**) COTC on parental KCL22 (white column) and KCL22/HA (black column) cells. *In vivo* effects of BBGC on transplanted (**e**) parental K562 and (**f**) K562/HA cells in NOD/SCID/γ_c^{null} (NOG) mice. Vehicle-treated mice, ○; BBGC-treated mice, ●

state.[10,11,29] Hypoxia favors the self-renewal of normal hematopoietic stem cells,[30] and resistance to hypoxia is one of the defining features of leukemic stem cells.[31] Therefore, it may be more important to search for new antileukemic agents that target quiescent CML stem cells residing in the hypoxic BM milieu. To this end, we have established two HA-CML sublines (Figures 2a–c). Previously, investigators have studied the role of hypoxia in leukemia using relatively short-term assays.[30–32] Our results suggest that transient hypoxia may not adequately mimic the physiological environment of CML cells (Figure 3e). Furthermore, in the mouse CML xenograft model, the oxygen concentration of engrafted leukemic cells in the BM was <10 mm Hg ($\sim 1.3\%$ O_2) (Figure 1f). Interestingly, the HA-CML cells exhibited characteristics similar to CML stem cells, including greater numbers of cells in a dormant, side population fraction, higher β-catenin expression, resistance to Abl TKIs and higher transplantation efficiency (Figures 2d, e and 4, Supplementary Figures 8 and 9).

The level of Bcr-Abl phosphorylation was lower in HA-CML cells, which may explain why those cells are less sensitive to Abl TKIs (Figure 2f). Giuntoli *et al.*[32] have also reported that the hypoxic selection of CML cells resulted in decreased cell sensitivity to imatinib and activation of Bcr-Abl-independent survival signaling pathways. Erk, a downstream effector of Bcr-Abl, was also less phosphorylated in HA-CML cells compared with parental cells, whereas the levels of p-Akt and p-Stat5 were similar (Figure 2f). These observations suggest that alternative mechanisms of activation exist for these signaling molecules in HA-CML cells.[33] In addition to

the phosphorylation status of Bcr-Abl, the sensitivity to antileukemic agents depends on the balance of pro- and antiapoptotic molecules.[8] Akt pro-survival effects have been reported to be dependent on the first step in glycolysis.[34] However, Glo-1 induction in HA-CML cells was not directly controlled by Akt because Akt phosphorylation was unchanged in both HA cell lines (Figure 2f). The adaptation to hypoxia may also alter the status of Bcl-2 family members, because the expression levels of several proteins were altered in K562/HA cells. However, there were no changes in KCL22/HA cells. As the parental KCL22 cells are intrinsically resistant to imatinib and exhibit very high Bcl-2 expression, alterations in Bcl-2 family proteins may not be obvious. Further experiments will be required to clarify the hypoxia-induced changes.

We tried to identify a specific target in HA-CML cells that could be inhibited by small molecules with therapeutic potential. The dependence on glycolysis-mediated ATP production for uncontrolled cellular growth under limited O_2 conditions is a hallmark of malignant cells (Figure 3b).[35,36] Reduced ATP production (Figure 3a), as well as increased glucose consumption and lactate production (Supplementary Figure 4) in HA-CML cells, suggested that there was preferential utilization of glycolysis for ATP production in these cells. We focused on the components of glycolysis to identify leukemia cell targets that would circumvent drug resistance acquired through adaptation to hypoxia.[37,38] Glo-I is an enzyme that detoxifies methylglyoxal, a cytotoxic α-oxoaldehyde side product of glycolysis. Accumulation of methylglyoxal damages cells through multi-base DNA deletions and base-pair substitutions. The overexpression of Glo-I induces drug resistance to alkylating agents in leukemia as well as other solid tumors.[26,39] Glo-I activity was elevated in both K562/HA and KCL22/HA cells (Figures 3c and d), possibly because of their increased dependence on glycolysis during adaptation to hypoxia. Both K562/HA and KCL22/HA cells were more sensitive to cell killing by Glo-I inhibitors, indicating that these cells are indeed dependent on Glo-I activity for survival under hypoxic conditions (Figures 5a–d, Supplementary Figures 7b, c, 8 and 9).

All the Glo-I inhibitors examined were effective for killing HA-CML cells *in vitro*. We selected BBGC for *in vivo* analysis because BBGC was the most potent compound *in vitro*, and because BBGC has previously been used against lung cancer *in vivo*.[40] As engrafted K562 cells survive in the hypoxic environment of the BM (Figure 1f), we expected that BBGC would be effective against the parental K562$^{Luc\text{-}EGFP}$ cells, even if Glo-I activity in these cells was low. However, BBGC had no effect on the survival of K562$^{Luc\text{-}EGFP}$-engrafted mice (Figure 5e). This result may be due to the time differences between Glo-I induction by hypoxia and administration of BBGC. BBGC was administered from day 1 to day 11 after transplantation, whereas, in *in vitro* cultures, Glo-I was not induced until 21 days after the initiation of hypoxia (Figure 3e). Intriguingly, BBGC significantly prolonged the survival of K562/HA$^{Luc\text{-}EGFP}$-engrafted mice compared with untreated mice (Figure 5b). Thus, BBGC may be a promising therapeutic agent for use against CML cells with high Glo-I activity, which also more frequently accompanies the quiescent status. As the Abl inhibitor imatinib is currently the drug of choice for CML treatment, we examined the combined effects of BBGC with imatinib *in vitro*. BBGC augmented the effects of imatinib in killing CML cells *in vitro* (Supplementary Figure 10).

In conclusion, the survival of engrafted leukemic cells in the BM under severe hypoxia depends on the induction of Glo-I activity, and adaptation to hypoxia seems to result in the acquisition of Abl TKI resistance in CML cells. Glo-I inhibitors were much more effective against HA-CML cells than parental cells both *in vitro* and *in vivo*. These findings indicate the importance of the hypoxic environment for maintaining quiescent CML cells, and suggest that Glo-I is a novel target for CML treatment.

Materials and Methods

Reagents and cell lines. The Glo-I inhibitors, BBGC, COTC and methylgerfelin, were synthesized and purified as previously described.[26–28] The K562 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The other CML-derived cell lines (KCL22, BV173 and MYL) were kindly provided by Dr Tadashi Nagai (Jichi Medical School, Tochigi, Japan), Dr Oliver G Ottmann (Frankfurt University, Frankfurt, Germany) and Dr Hideo Tanaka (Hiroshima University, Hiroshima, Japan), respectively. The CML cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Vitromex, Vilshofen, Germany) at 37°C in a humidified atmosphere of 20% O_2, 5% CO_2 and 75% N_2. CML cells were subjected to continuous culture in 1.0% O_2 (7.2 mm Hg), 5% CO_2 and 94% N_2, and HA subclones of K562 (K562/HA) and KCL22 (KCL22/HA) were selected and maintained in suspension in low O_2 conditions for more than 6 months. Parental K562 and K562/HA cells were cotransfected with pGL3, a control luciferase (Luc) reporter vector (Promega, Madison, WI, USA) and pCAG.*egfp.neo* using the Nucleofector Kit V, protocol T-03 (Amaxa AG, Cologne, Germany). Stable transfectants (K562$^{Luc\text{-}EGFP}$ and K562/HA$^{Luc\text{-}EGFP}$) were selected by culturing in medium containing G418 (1 mg/ml, Sigma Aldrich, Tokyo, Japan) and isolated by agarose gel cloning assays. Human primary Bcr-Abl$^+$ leukemic cells were obtained from patients with informed consent, according to the Declaration of Helsinki.

***In vivo* engraftment of CML cells and histological analysis.** Animal studies were performed in accordance with the guidelines of the Institutional Review Board for animal studies at Kyoto University. To evaluate the oxygen status of engrafted leukemic cells, 1.0×10^6 cells were injected into sublethally irradiated (2 Gy) male NOG or NOD/SCID mice at 6–8 weeks of age. At 35 days (NOG mice) or 50 days (NOD/SCID mice) post-inoculation, the mice were injected intraperitoneally with pimonidazole hydrochloride (Pimo, Chemicon, Temecula, CA, USA) (60 mg/kg), and 60 min later, the animals were killed and the femur and liver removed. Tissues were subjected to hematoxylin–eosin staining and immunohistochemical analysis using anti-Pimo (Chemicon) and antihuman Ki67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, and examined by microscopy. *In vivo*, leukemia cell proliferation was detected by monitoring luciferase expression using an *in vivo* imaging system (Xenogen, Berkeley, CA, USA), as previously described.[41] For survival analysis, time of death was determined either by spontaneous death, or by date of euthanasia due to pain or suffering, according to established criteria. We also confirmed the oxygen concentration of primary Bcr-Abl$^+$ leukemic cells that were obtained from a Ph$^+$ ALL patient and engrafted in the BM of NOD/SCID mice. Primary Bcr-Abl$^+$ leukemic cells (1×10^6) were transplanted into four NOD/SCID mice, which were killed 50 days after transplantation, and the BM sections were stained as above.

Cell death. Cell viability was measured by incorporation of propidium iodide (PI). Mitochondrial transmembrane potential ($\Delta\psi$m) was determined by staining with 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes, Eugene, OR, USA), as previously described.[9] For analysis of DNA content, cells were fixed with ice-cold 70% ethanol and then incubated with PI as previously described.[42] The percentage of cells that incorporated PI, the $\Delta\psi$m, and the percentage of cells in sub-G_1 of the cell cycle were determined by FACS using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Western blot analysis. Proteins were separated by SDS-PAGE and then electroblotted onto a Hybond-PDVF membrane (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated with 5% (wt/vol) nonfat dry milk in

phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 (Sigma, Saint Louis, MO, USA). Antibodies specific for Akt (#9272, 60 kDa), Erk1/2 (#9102, 42, 44 kDa), phospho (p)-Akt (#9271, 60 kDa), p-Erk1/2 (#9101, 42, 44 kDa) and p-Stat5 (##9351, 90 kDa, Cell Signaling Technologies, Beverly, MA, USA); for Bcl-2 (clone 100, #05–729, 26 kDa) and p-tyrosine (#05-321, Upstate, Lake Placid, NY, USA); as well as for Stat5 (#sc-835, 92 kDa) and c-Abl (#sc-23, 120 kDa, Santa Cruz Biotechnology); Bcl-X_L (#AAM-080, 26 kDa, Stressgen, Victoria, British Columbia, Canada); Glo-I (#H00002739-A01, 28 kDa, Novus Bio, Littleton, CO, USA); β-actin (#A2066, 42 kDa, Sigma); Mcl-1L (38 kDa) and Mcl-1S (30 kDa) (#LS-C43163, Life Span Biosciences, Seattle, WA, USA); and for β-catenin (#610153, 92 kDa, BD Biosciences, San Diego, CA, USA) were used as indicated. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (ECL Advance, Amersham Biosciences).

Measurement of Glo-I activity. Cells were lysed in PBS containing 1 mM phenylmethylsulfonyl fluoride by freezing and thawing, followed by sonication. The lysates were centrifuged at 12 000 $\times$ *g* for 20 min and the supernatant was used as the cytosolic fraction. The Glo-I assay was performed in 0.1 M sodium phosphate (pH 7.0), 7.9 mM methylglyoxal (Sigma), 1 mM GSH and 14.6 mM $MgSO_4$ at 25 °C. An increase in absorbance at 240 nm because of the formation of *S*-D-lactoylglutathione was measured using a temperature-controlled spectrophotometer (Beckman Coulter, Brea, CA, USA, DU640).

ATP assay. ATP levels were measured using an ATP assay kit (TOYO INK, Tokyo, Japan), according to the manufacturer's instructions.

Measurement of glucose consumption and lactate production. Cells were suspended in fresh culture medium. After 6 h, the cells were collected by centrifugation and resuspended in 5 ml of RPMI at a density of 2×10^5cells/ml. Cells were incubated for 24 h, and the culture medium was collected for measurement. Glucose levels were determined using a glucose assay kit (GO, Sigma). Glucose consumption was determined from the difference in glucose concentration compared with the starting medium. Lactate levels were determined using a lactate assay (F-kit L-lactate, JK International, Tokyo, Japan).

Determination of quiescent cells. Numbers of quiescent leukemic cells (in G_0 phase) were determined by double staining with Ki-67 and 7-AAD as previously described.[43] Briefly, 1×10^6 K562 or K562/HA cells were fixed in ice-cold 70% EtOH for at least 12 h, and then resuspended in 100 μl PBS. Cells were stained with 20 μl Ki-67 antibody (BD Biosciences) and incubated for 30 min at RT. Subsequently, 20 μl 7-AAD (BD Biosciences) was added, and the cells were resuspended in 500 μl PBS with 1% FBS and analyzed by FACS. Three independent analyses were performed.

BBGC treatment of CML mice. NOG mice 6–8 weeks of age were sublethally irradiated (2 Gy) and inoculated with 1.0×10^6 K562/HA$^{Luc\text{-}EGFP}$ or K562/HA$^{Luc\text{-}EGFP}$ cells by intravenous tail vein injection. Therapeutic treatments (seven mice per group) were started 1 day (day 1) after transplantation. To prepare the BBGC solution, BBGC dissolved in cremophor EL/DMSO (1 : 1) was diluted to 10 mg/ml using DMSO and PBS. Within each group, half of the mice were administered EL/DMSO only (vehicle controls), and the remainder were administered 10 mg/kg BBGC on days 1 through 4, and days 8 through 11 after transplantation. For survival analysis, the time of death was determined either by spontaneous death or by date of euthanasia due to pain or suffering, according to established criteria.

Statistical analysis. Survival curves were drawn using the Kaplan–Meier method and compared using the log-rank test. *P*-values were derived from two-sided tests and a *P*-value < 0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Future Prospect of RNA Interference for Cancer Therapies

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Abstract: RNA interference (RNAi) is a phenomenon of sequence-specific gene silencing in mammalian cells and its discovery has lead to its wide application as a powerful tool in post-genomic research. Recently, short interfering RNA (siRNA), which induces RNAi, has been experimentally introduced as a cancer therapy and is expected to be developed as a nucleic acid-based medicine.

Selection of appropriate gene targets is an important parameter in the potential success of siRNA cancer therapies. Candidate targets include genes associated with cell proliferation, metastasis, angiogenesis, and drug resistance. Importantly, silencing of such genes must not affect the functions of normal cells. Development of suitable drug delivery systems (DDSs) is also an important issue. Numerous methods to transfect siRNAs into cells have been developed, and the use of non-viral DDSs is preferred because it offers greater safety for clinical application than does the use of viral DDSs. Currently, atelocollagen and cationic liposomes represent the most advantageous non-viral DDSs available.

In this article, we briefly review the mechanism of RNAi and non-viral DDSs. Next we discuss in detail some of the most recent findings concerning the administration of potential nucleic acid-based drugs against polo-like kinase-1 (PLK-1), which regulates the mitotic process in mammalian cells. These promising results demonstrate that PLK-1 is a suitable target for cancer therapy. Finally, several current clinical trials of RNAi therapies against cancers are discussed. Results of current studies and clinical trials demonstrate that manipulation of RNAi mechanism by use of targeted siRNA offers promising strategies for cancer therapies.

Keywords: RNAi, siRNA, cancer, PLK-1, DDS, liposome, atelocollagen.

INTRODUCTION

RNA interference (RNAi) is a process of sequence specific post-transcriptional gene silencing induced by double-strand RNA (dsRNA). This phenomenon was discovered in the nematode *Caenorhabditis elegans* (*C. elegans*) by Drs. Fire and Mello [1], and for this condition they were awarded the 2006 Nobel Prize in medicine (http://nobelprize.org/nobel_prizes/medicine/laureates/2006). RNAi has been shown to function in higher organisms including mammals, and methods that exploit RNAi mechanisms have been developing rapidly. RNAi plays important roles in vital responses against the infection of microorganisms as well as post-transcriptional endogenous gene regulation [2, 3]. Aberrant expression of endogenous normal or mutant genes occurs in pathological conditions, resulting in alterations in signal pathways, cellular proliferation, and apoptosis. Post-transcriptional gene regulation by RNAi controls these alterations positively or negatively, and consequently RNAi has now been well-established as a method for experimental analyses of gene function *in vitro* as well as in high-throughput screening [4, 5].

Cytotoxic compounds or antibodies are widely used as strategies for targeting cellular functions or structures of cancerous cells. Recently the application of nucleic acid-based inhibitors such as antisense oligonucleotides (ODNs), RNA aptamars, small interfering RNAs (siRNAs), and locked nucleic acids (LNAs) have been investigated worldwide, and many researchers are searching for ways to use them in therapeutic approaches. To apply the RNAi phenomenon to therapeutics, it is important to select suitable targets for the inhibition of cancer progression and also to develop effective drug delivery systems (DDSs). Polo-like kinase- 1 (PLK-1) belongs to the family of serine/threonine kinases and regulates cell division as several points in the mitotic phase [6]. Because PLK-1 is overexpressed in many types of malignancies and its overexpression is associated with poor prognosis of cancer patients [7, 8], we have developed RNAi therapy against PLK-1 using orthotopic mouse models [9, 10]. In this review, we discuss possible targets as RNAi therapies against cancers and options for non-viral DDSs.

MECHANISMS OF RNAi

Post-transcriptional gene-silencing small RNAs are roughly categorized into two subgroups, siRNAs and microRNAs (miRNAs). siRNAs have been considered to defend the genome in response to the invasive nucleic acids of viruses and transgenes only in lower eukaryotes including *C. elegans* [11, 12] and *Drosophila melanogaster* [13]. However, recent study has demonstrated that mRNA expressions of murine endogenous retrovirus-L and intracisternal A particle, two autonomous long terminal repeat retrotransposons, increased in 2-cell or 8-cell embryo when mouse Dicer siRNAs were microinjected in 1-cell embryo [14]. Moreover, siRNAs were discovered also in mouse oocytes and these siRNAs degraded retrotransposon-derived mRNAs [15]. These findings suggest that siRNAs exist in mammalians and that retrotransposons are suppressed through the

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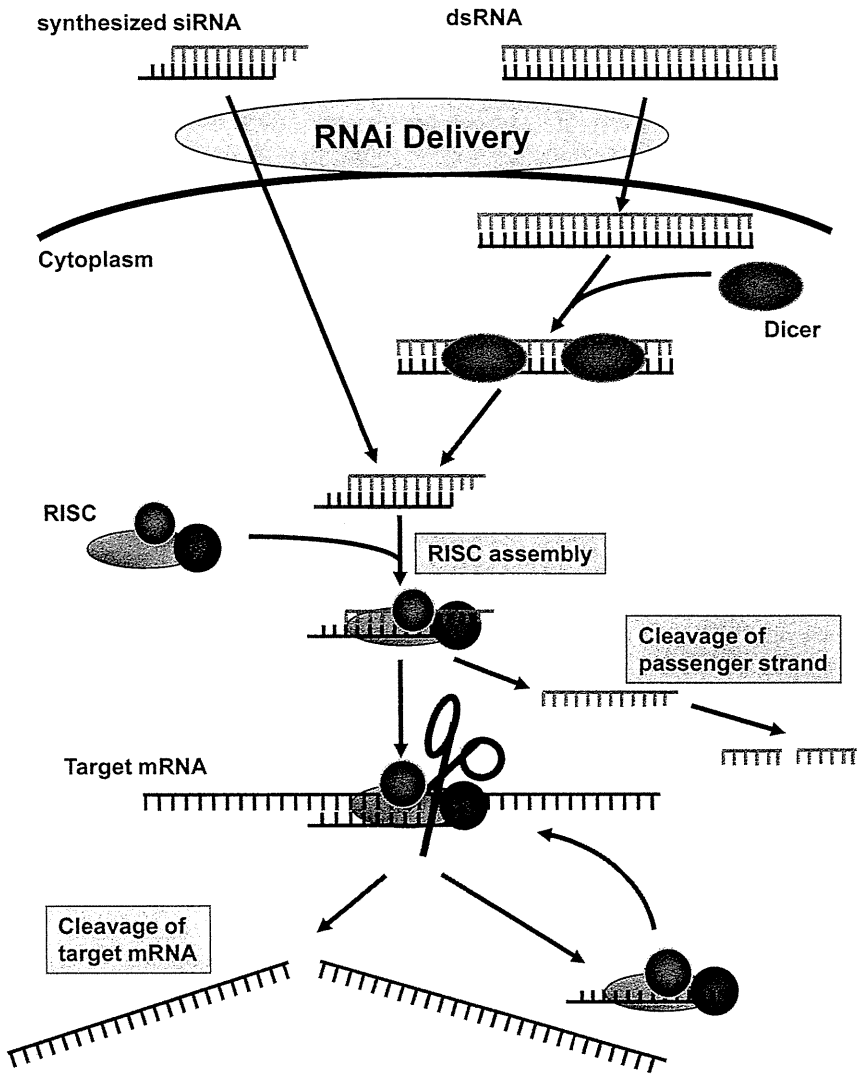

Fig. (1). Mechanisms of RNA interference

After the introduction of dsRNA into a target cell (See Fig. (**2**)), the dsRNA is processed into siRNA length of 21-23 nucletides by Dicer. siRNA then enters an RNA-induced silencing complex (RISC) assembly pathway. The dsRNA unwinds to form two single-strands of RNA. The passenger strand rapidly degrades and the guide strand binds and cleaves the target mRNA, resulting in mRNA degradation.

RNAi pathway in the early stage of embryonic development and mouse oocytes. On the contrary, hundreds of miRNAs, which regulate endogenous gene expression, have been identified in mammalians, but not in lower eukaryotes.

The precise mechanisms of RNAi are discussed in several reviews [5, 16, 17]. RNAi processes can be roughly divided into the initiation phase and the effector phase (Fig. **1**). In the initiation phase, following introduction of dsRNA into a target cell, dsRNA is processed into shorter lengths of 21-23 nucleotides (nts) dsRNAs, termed siRNAs by the ribonuclease activity of a dsDNA-specific RNAse III family ribonuclase Dicer. Dicer consists of the following domains: an N-terminal helicase domain, an RNA-binding Piwi/ Argonaute/Zwille (PAZ) domain, two tandem RNAse III domains, and a dsRNA-binding domain [18, 19]. The number of Dicers present in a cell varies depending on the species of the organism. Mammals and nematodes have only a single Dicer, which acts to produce both siRNAs and miRNAs [20-22], while other organisms have multiple Dicers which perform separate, specialized functions. *Drosophila* has two Dicers: *Drosophila* Dicer-1 is required for generating miRNAs, whereas *Drosophila* Dicer-2 produces siRNAs [17, 23]. dsRNA precursors are sequentially processed by the two RNAse III domains of Dicer, and cleaved into smaller dsRNAs with 3' dinucleotide overhangs [18, 24].

Smaller dsRNAs enter into an RNA-induced silencing complex (RISC) assembly pathway [25]. RISC contains Argonaute (Ago) proteins, a family of proteins characterized by the presence of a PAZ domain and a PIWI domain [26]. The PAZ domain recognizes the 3' terminus of RNA, and the PIWI domain adopts an RNAse H-like structure that can catalyze the cleavage of the guide strand. Most species have multiple Ago proteins, but only Ago2 can cleave its RNA target in humans. The dsRNA is unwound by ATP-dependent RNA helicase activity to form two single-strands of RNA. The strand that directs silencing is called the guide (antisense) strand, and the other is called the passenger

(sense) strand. Ago2 protein selects the guide strand and cleaves its RNA target at the phosphodiester bond positioned between nucleotides 10 and 11 [24, 27]. The resulting products are rapidly degraded because of the unprotected ends, and the passenger strand is also degraded [28, 29]. The targeted RNA dissociates from the siRNA after the cleavage, and the RISC cleaves additional targets, resulting in decrease of expression of the target gene.

Since the discovery of the first miRNA *lin-4* [30, 31], miRNAs, some of which are non-coding RNA species, have been shown to regulate diverse biological processes in eukaryotes. miRNAs are derived from stem-loop-structured primary miRNAs (pri-miRNAs) by the cleavage activity of Drocha, a nuclear-localized member of the RNAse III family, to yield short precursor miRNAs called pre-miRNAs. Pre-miRNAs comprising 60-70 nts exhibit a hairpin structure with a 5'-phosphate and a 3'-2 nts overhang. After translocation from the nucleus to the cytoplasm by Exportin-5, pre-miRNAs are processed by Dicer into miRNAs of 20-24 nts. miRNAs as well as siRNAs enter into RISC assembly pathway. Once associated with Ago, the guide strands of miRNAs bind to the sequence-specific basepairs of target RNAs, resulting in translational repression or mRNA degradation (Fig. **2**) [32]. Unlike siRNAs, the complimentarity of miRNAs are most often imperfect. Bioinformatic studies reveal that a single miRNA might bind to as many as 200 gene targets which have diverse functions [33-35]. In case of the perfect complimentarity, the target mRNA can be cleaved by an Ago protein. When complimentarity is imperfect, the translation is suppressed without RISC-mediated cleavage [36-38]. Early studies indicated that repression of animal miRNAs occurred without corresponding decrease of mRNA [31, 36]. However, recent studies have demonstrated that miRNAs can reduce the mRNA levels in mammalian cells and *C. elegans* [39, 40]. Furthermore, mRNA degradation is controlled by cytoplasmic processing body (P-body) [41-43]. Although the complete components of cytoplasmic P-body are not identified yet, some proteins involving in nonsense-mediated decay, target mRNA, and miRNAs are observed. miRNAs colocalize with their target transcripts in sites of mRNA degradation, resulting in repression of mRNA translation [41, 44, 45].

While synthesized siRNAs that are directly transfected into cells, short-hairpin RNAs (shRNAs) are synthesized in the nucleus, and they are transcribed by either RNA polymerase II or III [2, 46]. shRNAs have a stem-loop structure and are processed through the endogenous RNAi machinery similar to miRNA maturation pathway (Fig. **2**) [32]. shRNAs are usually expressed from plasmids or viral-based expression vectors [47]. Adenoviral vectors are widely used both *in vitro* and *in vivo*, however, the main drawback associated with their use is vector-mediated immunogenicity. Adeno-associated viral (AAV) vectors have low inflammatory potential and they have an advantage in terms of safety for *in vivo* application [48]. Therefore, clinical application of AAV vectors to RNAi is expected. Retroviral vectors can induce long-term gene silencing and have low inflammatory potential. However, they can transduce shRNAs into only

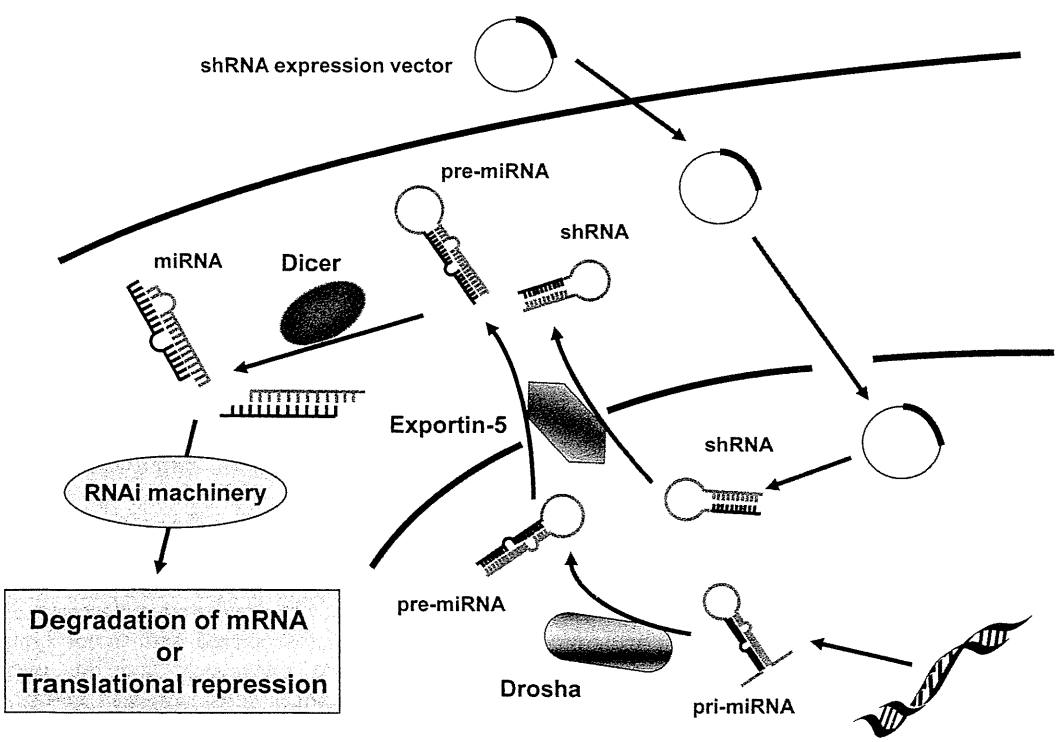

Fig. (2). Processing of miRNAs and shRNAs

pri-miRNAs are cleaved by Drosha and pre-miRNAs are produced. pre-miRNAs are translocated from nucleus to cytopolam by Exprotin-5, and are processed by Dicer into miRNAs. Through the RNAi machinery, miRNAs repress translation or degrade mRNAs (See Fig. (**1**)). After shRNA expression vector is delivered into the cytoplasm, the vector is transported into the nucleus for transcription. Thereafter, primary shRNA is generated and this has a hairpin-like stem-loop structure similar to pri-miRNA. The primary shRNA transcript is translocated into the cytpoplasm by Exportin-5. Then, shRNA is processed by Dicer and enter the endogenous RNAi machinery.

dividing cells. On the contrary, lentiviral vectors can transduce into non-dividing cells and can induce persistent gene-silencing in most tissues. Lentiviral vectors will be important delivery systems for wide range of diseases including hematopoitic disorders. However, these integrating viral vectors have major concerns of insertional mutagenesis [49, 50]. The development of site-specific insertional machinery [51, 52] is expected to prevent the vector-insertional tumorigenesis.

TARGETING CANCER-ASSOCIATED GENES BY RNAi

Because of its high specificity and high efficiency in binding gene products, RNAi has recently been evaluated as a therapeutic strategy for cancer treatment. To develop nuclear medicine against cancers, it is essential that suitable gene targets are selected. Such targets include antiapoptotic proteins, cell cycle regulators, transcription factors, signal transduction proteins, and factors associated with malignant biological behaviors of cancer cells, all of these genes are associated with the poor prognosis of cancer patients.

siRNA's candidate targets include molecules of apoptosis/cell cycle regulation. The antiapoptotic Bcl-2 protein contributes to resistance to apoptosis against external stimuli, including cytotoxic agents. Bcl-2 is upregulated in a variety of cancers and participates in tumorigenesis and progression [53-58]. Overexpression of Bcl-2 in tumor cells correlates with the poor prognosis of the patients [59-61]. Many studies have demonstrated that siRNA treatment against Bcl-2 inhibited the proliferation of tumor cells. Administration of synthetic Bcl-2 siRNA, using a cationic liposome, suppressed tumor progression in a xenograft mouse model [62-64], indicating that Bcl-2 is a suitable target for cancer therapy. Survivin is a member of the inhibitors of apoptosis proteins (IAPs) family and plays a role in chromatin cleavage associated with spindle formation in cell division [65]. Survivin is almost undetectable in adult tissues [66]. However, overexpression of survivin has been shown in cancers [66, 67] and to correlate with poor prognosis of cancer patients [68-73]. Moreover, survivin is associated with resistance to chemotherapy and radiotherapy [74-77]. Therefore, a siRNAs-mediated decrease of the expression of survivin represents a potential target for siRNA anticancer therapy and may improve the clinical outcomes of cancer patients.

Another category of siRNA candidate targets are molecules of signal transduction. Members of the signal transducer and activator of transcription (STAT) family act as key components of cytokine signaling pathways that regulate gene expression [78, 79]. Among seven types of STATs, STAT3 is the most strongly implicated in carcinogenesis. Whereas STAT3 is transiently activated in normal cells, a constitutively active form is detected in a variety of cancers [80] and dysregulates the downstream target genes of cell proliferation and survival [81-83]. Knockdown of STAT3 protein expression inhibited tumor growth and invasion [84-86]. Bcr-Abl fusion protein, which is created by the molecular consequence of the t(9;22) translocation, is a constitutively active tyrosine kinase that causes leukemias [87]. Imatinib mesylate (IM; Gleevec™, Glivec™) was developed as a first generation tyrosine kinase inhibitor (TKI) and its emergence has dramatically changed the outcomes of therapies against Philadelphia (Ph)–positive leukemia, especially chronic myelogenous leukemia (CML) [88-91]. Moreover, the use of several second generation TKIs developed to overcome resistance to IM have yielded excellent outcomes [92-95]. These findings indicate that targeting Bcr-Abl protein is a promising strategy to eliminate Bcr-Abl-positive leukemic cells. The approach to downregulate the expression of Bcr-Abl mRNA by RNAi was investigated *in vitro* [96-98], and further studies are needed for its introduction to the clinical field. β-catenin is a downstream protein of the canonical Wnt signaling pathway that has been shown to play an important role in the process of development, proliferation, and differentiation [99]. In the absence of Wnt, intracellular levels of β-catenin are regulated by multiple proteins, such as glycogen synthase kinase-3, casein kinase 1 and adenomatous polyposis coli, and β-catenin is phosphorylated and is degraded by the ubiquitin-proteasome pathway. In the presence of the Wnt ligand, intracellular β-catenin is stabilized in the cytoplasm and the subsequent intracellular accumulation of β-catenin results in its translocation to the nucleus. In the nucleus, β-catenin binds to the T cell factor transcription factor and target genes such as *c-Myc* and *Cyclin D1*, which are upregulated, resulting in cell proliferation [100]. Recently, this pathway has been focused on as it is involved in cancer development. Overexpression of β-catenin was detected in many types of cancers [101-105]. Treatment of siRNAs against β-catenin successfully suppressed the proliferation of colon cancer cells and myeloma cells by inducing caspase-dependent apoptosis [105, 106]. Thus, β-catenin represents a suitable target for RNAi therapy.

Other candidate siRNA targets are molecules that define the behavior of cancerous cells, such as metastasis or resistance to cytotoxic agents. P-glycoprotein (P-gp) is a product of *multidrug resistance* (*MDR1*) gene, and the overexpression of P-gp induces cross-resistance against structurally unrelated cytotoxic agents [107]. Depletion of P-gp by knockdown of MDR1 mRNA reversed the sensitivity to adriamycin or vincristine in cancer cell lines [108, 109]. In addition, chemosensitivity was increased in cancer cell lines by treatment with siRNAs against other molecules associated with chemoresistance, including multidrug resistance protein 1 (MRP1), ABCG2, and MRP7/ABCC10 [110-113]. The vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) axis plays an important role in angio- and lymphangiogenesis. VEGF-A stimulates anginogenesis in tumor vessels, enhances the permeability of the blood vessels, and promotes the motility of cancer cells, which results in metastases [114-116]. Treatment with VEGF-A siRNAs successfully prevented metastases of cancers [117-119]. VEGF-C is associated with tumor lymphangiogenesis and lymph node metastasis, and the overexpression of VEGF-C/D in cancer cells increased metastases via lymph vessels [120-123]. VEGF-C siRNA inhibited metastasis of breast cancer in a mouse xenograft model [124].

POLO-LIKE KINASE-1

PLKs belong to the family of serine/threonine kinases and are highly conserved among eukaryotes. PLK family has identified PLK-1, PLK-2 (SNK), PLK-3 (FNK), and PLK-4

(SAK) in mammalians so far and PLKs function as regulators of both cell cycle progression and cellular response to DNA damage [6, 125-127]. PLK-1 is the best characterized among the four PLKs identified to date. PLK-1 has an N-terminal serine/threonine protein kinase domain and two polo box domains at the C-terminal region. Polo box domains regulate the kinase activity of PLK-1 [7, 128]. PLK-1 regulates cell division at several points in the mitotic phase: mitotic entry through CDK1 activation, bipolar spindle formation, chromosome alignment, segregation of chromosomes, and cytokinesis [6, 129]. PLK-1 gene expression is regulated during cell cycle progression, with a peak level occurring at M phase [130, 131]. Similar to its gene expression, PLK-1 protein expression and its activity are low in G0, G1, and S phases, and begin to increase in G2 phase with peak in M phase [130-133]. When cells exit from mitosis, PLK-1 is degraded through the ubiquitin-proteasome pathway as a substrate of ubiquitin-ligase, known as the anaphase-promoting complex/cyclosome [134, 135]. PLK-1 is highly expressed in tissues with actively proliferating cells, placenta, spleen, and testis, whereas PLK-1 is scarcely detectable in most other adult tissues [130, 136, 137].

Many reports have described that PLK-1 is overexpressed in cancerous tissues and that PLK-1 expression levels were tightly correlated with histological grades of tumors, clinical stages, and prognosis of the patients. PLK-1 mRNA levels were elevated in non-small cell lung cancer (NSLC) tissues and this transcript levels were correlated with the survivals of cancer patients [138]. Moreover, the immunohistoligical study showed that PLK-1 protein was overexpressed in NSLC tissues in patients at progressed stages of cancer (postsurgical stage ≥II) and in patients with poorly differentiated NSLCs [10]. Patients with urinary bladder cancers expressing high levels of PLK-1 have a poor prognosis compared with patients with its low expression. Moreover, the histologically high-grade, deeply invasive, lymphatic-invasive, and venous-invasive bladder cancers demonstrated significantly higher PLK-1 expression [9]. Elevated expression of PLK-1 is observed in colorectal cancers. Overexpression of PLK-1 correlated positively with clinicopathological features including Dukes stage, tumor stage, and nodal status. Additionally, PLK-1 expression was a prognostic marker in univariate survival analysis [139]. PLK-1 overexpression is detected in other various cancers and, therefore, PLK-1 overexpression is a prognostic biomarker for cancer patients (Table **1**).

Inhibition of PLK-1 activity induces mitotic arrest and tumor cell apoptosis [140-142]. Depletion of PLK-1 mRNA also inhibits the functions of PLK-1 protein in DNA damages and spindle formation and causes the inhibition of the cell proliferation in a time- and a dose-dependent manner. PLK-1 siRNA treatment induces an arrest at the G2/M phase in the cell cycle with the increase of CDC2/Cyclin B1 [9, 10, 117, 143-145]. PLK-1 siRNA-transfected cells had dumbbell-like and misaligned nuclei, indicating that PLK-1 depletion induced abnormalities of cell division during M phase, and these cells were shown to yield to caspase-dependent apoptosis [9, 10, 143]. PLK-1 silencing inhibits the cell proliferation of other various malignancies including prostate cancer [145], esophageal cancer [146], hepatocellular carcinoma [144], breast cancer [147], gastric cancer [148], and leukemia [149, 150]. Moreover, preclinical studies using xenograft mouse models demonstrated that the administration of PLK-1 siRNA suppressed the growth of cancers [10, 144 , 151-154].

Interestingly, depletion of PLK-1 increases the susceptibility of anticancer agents [147, 155]. Polymerized microtubles attach to kinetochores and their dynamics carry out cell division [156]. Paclitaxel targets microtubule dymanics [156, 157]. PLK-1 monitors the tension, and its depletion by siRNA reduced expression of some spindle checkpoint proteins including Mad2, Cenp-E, Hec/Ndc80, and Spc [158]. Consequently, PLK-1 siRNA acts synergistically with paclitaxel to inhibit the proliferation of breast cancer cells [147, 153]. Cyclin-dependent kinase inhibitor $p27^{kip1}$ is an atypical tumor suppressor that regulates G0 to S phase transition, and reduced $p27^{kip1}$ expression is associated with poor prognosis of cancer patients [159, 160]. Herceptin is a humanized monoclonal antibody agent against Her2-positive

Table 1. Cancers whose PLK-1 Overexpression is Correlated with the Prognosis of Patients

Cancer	Authors
Oropharyngeal carcinoma	Knecht, *et al.* [235, 236]
Esophageal carcinoma	Tokumitsu, *et al.* [237], Feng, *et al.* [238]
Non-small cell lung cancer	Wolf, *et al.* [138]
Breast cancer	Wolf, *et al.* [239], Ahar, *et al.*[240], Weichert, *et al.* [241], Loddo, *et al.*[242]
Gastric cancer	Weichert, *et al.* [243], Kanaji, *et al.* [244]
Hepatoblastoma	Yamada, *et al.* [245]
Colorectal cancer	Takahashi, *et al.* [246], Weichert, *et al.* [139], Macmillan, *et al.* [247]
Urinary bladder cancer	Nogawa, *et al.* [151], Yamamoto, *et al.* [248]
Ovarian cancer	Weichert, *et al.* [249]
Melanona	Strebhart, *et al.* [250]
Non-Hodgkin's lymphoma	Mito, *et al.* [251], Liu, *et al.* [252]

cancers. Herceptin-resistant breast cancer cells decreased the p27 ^{kipl} expression, moreover, exogenous addition of p27 ^{kipl} increased the sensitivity of herceptin [161]. Because PLK-1 siRNA treatment induced p27 ^{kipl} expression and seems to facilitate the induction of apoptosis by the cleavage of caspases in HER2-positive breast cancer cells, PLK-1 siRNA acts synergistically with herceptin [147].

Because PLK family cooperatively acts in mitosis, depletion of PLK-1 alters the expressions of other PLKs. PLK-2 and PLK-3 transcripts are increased after the PLK-1 siRNA treatment [10]. Unlike PLK-1, PLK-2 and PLK-3 play inhibitory roles in cell proliferation. PLK-2 is transcriptionally regulated by p53 in response to DNA damage by irradiation and PLK-3 is activated by DNA damage checkpoint [126]. These observations suggest that PLK-1 depletion induces mitotic catastrophe and activation of DNA damage checkpoint and spindle checkpoint, resulting in increase of PLK-2 and PLK-3 transcripts. Although PLK-1 plays an important role in cell division, depletion of PLK-1 does not affect the proliferation of normal cells [10, 140, 162]. This suggests that some other kinases compensate loss of PLK-1 function during mitosis in normal cells [10, 162]. Because depletion of PLK-1 induces mitotic catastrophe and cell death in cancer cells specifically, PLK-1 could be an excellent target for cancer therapy.

DEVELOPMENT OF NON-VIRAL DDSs

Although siRNA target molecules are overexpressed in cancer cells, most of them are essential to maintain homeostasis of physiological functions in humans. Therefore, siRNAs must be delivered selectively into cancer cells. Moreover, naked siRNAs are degraded by endogenous nucleases when administered *in vivo*, so that delivery methods that protect siRNAs from such degradation are essential. For these reasons, safer and more effective DDSs must be developed. DDSs are divided into two categories: viral vector based carriers, and non-viral based carriers. Viral

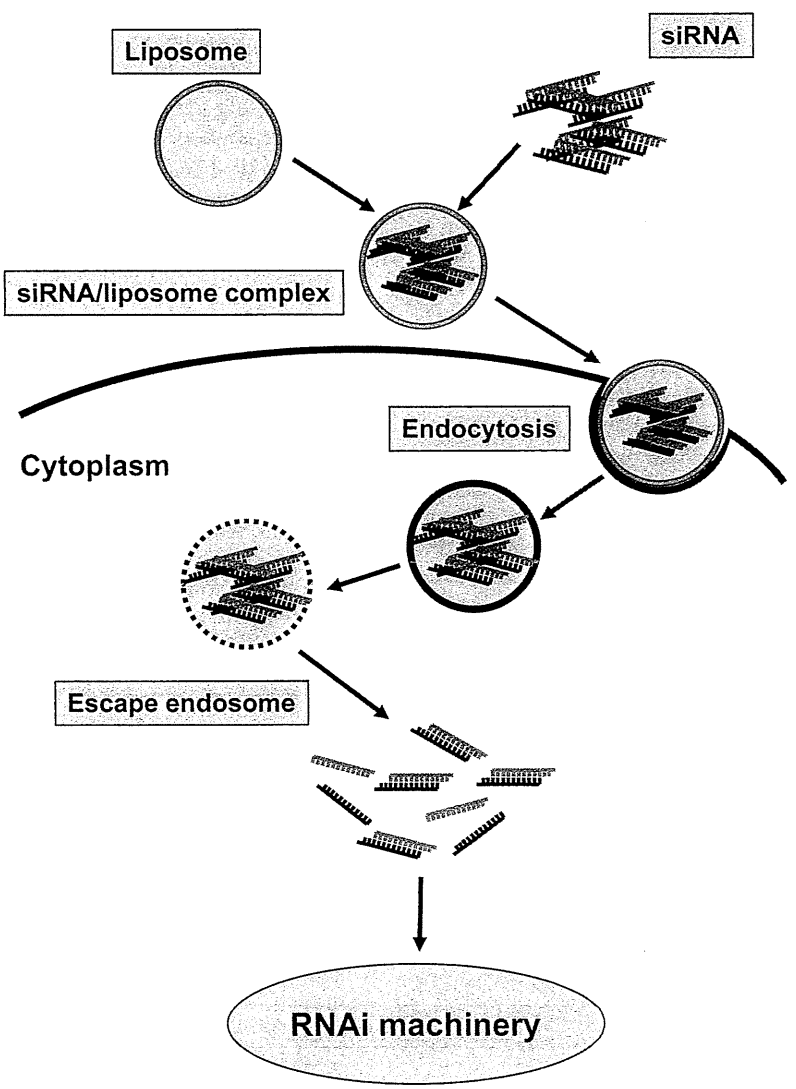

Fig. (3). Delivery of siRNA/liposome complex

Positively charged liposomes facilitate complex formation with negatively charged siRNAs and binding to cell membrane. After binding to the cell membrane, the siRNA/liposome complex enters the cytoplasm of the cell via endocytosis. The complex then escapes the endosome and releases its siRNA to the RNAi machinery (See Fig. **1**).

vectors are highly efficient delivery systems and they are the most powerful tools for transfection so far. However, their clinical application is limited by toxic immune responses and random integration into host cells' DNA followed by insertional mutagenesis [49, 50, 163]. Thus, we will discuss non-viral DDS, especially lipid-based carriers and polymers below.

Cationic liposomes represent one of the most attractive carriers used for the delivery of nucleic acid-based drugs. Positively charged liposomes facilitate complex formation with negatively charged siRNAs and bind to cell membranes. After binding to the cell membrane, a siRNA/liposome complex enters the cytoplasm via endocytosis after which the complex escapes from the endosome and releases its siRNA to the RNAi machinery [164, 165] (Fig. (**3**)). N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), a synthetic cationic lipid, was first used to transfer plasmid DNA into mammalian cells [166]. Since then, numerous cationic liposomes have been generated for DDSs of DNAs and siRNAs. LIC contains the cationic lipid analogue 2-O-(2-DAME)-carbamoyl-1,3-O-dioleoylglycerol and phosphatidylcholine derived from egg. LIC nanoparticles are uniform in size, and the diameter of the siRNA/LIC complex is around 200 nm [167]. siRNA/LIC complexes were shown to inhibit the progression of urinary bladder cancer tumors and lung cancer tumors in xenograft mouse models [9, 64].

To protect the therapeutic nucleic acid in the siRNA/liposome complex from nuclease degradation in the bloodstream and to deliver the complex to the targeted sites, several liposome modifications have been tested. A modified cationic liposome comprising polyethylene glycol (PEG) residues was formulated [63]. Treatment using this pegylated liposome resulted in increased concentrations of siRNA in plasma as well as in the targeted tumors, and consequently the progression of tumors was inhibited and the survival rates of cancer-bearing mice were prolonged.

A stable nucleic-acid-liposome particle (SNALP) has been generated for systemic *in vivo* delivery of siRNAs. SNALPs are composed of a lipid bilayer containing a mixture of cationic and fusogenic lipids that enables cellular uptake and endosomal release of siRNAs. The SNALP surface is coated with a diffusible PEG-lipid conjugate that provides a neutral, hydropholic exterior and stabilizes the particle during formation [168]. Complexes formed with SNALPs and siRNAs against targeted mRNAs show excellent outcomes in several preclinical studies using mice and nonhuman primates [168-170], and it is expected that SNALPs will be suitable for application in cancer therapy.

Active, targeted delivery of siRNAs is necessary to obtain maximum benefits and minimum adverse effects. However, hematopoietic cells are intractable to the delivery methods, and to overcome this difficulty, Antibody (Ab)-combined DDSs have been developed. Nanometer-scale (around 80 nm in diameter) liposomes were formed from neutral phospholipids, and hyauronan was attached to the liposome outer surface and then modified with monoclonal Abs. The lyophilized lipid nanoparticles were rehydrated with water containing protamine-condensed siRNAs. Following intravenous administration of β7-integrin Ab-conjugated nanoparticles containing Cyclin D1 siRNAs, leukocyte proliferation and the expression of T helper cell 1 cytokines were suppressed in mice with colitis [171]. Ab-mediated delivery carriers are formed by generating a fusion protein containing cationic protamine and single-chain Ab fragment, and represent another type of DDS. HIV-1 envelope (gp160) Ab-loaded fusion protein combined with targeted siRNAs inhibited the growth of gp160-expressing B16 tumors [172]. These Ab-combined nanoparticles are expected to be applied to clinical settings.

In contrast to many kinds of cationic liposomes, anionic liposomes bound by Sialyl Lewis X (SLX) have been developed [173]. SLX is a sugar chain expressed on the surface of leukocytes that binds with E-selectin expressed on the surface of endothelial cells. Because SLX-combined liposomes (SLX-Lipo) are negatively charged, as are vascular endothelial cells and cells such as erythrocytes and leukocytes, these liposomes are sparred from the reticuloendothelial system, phagocytosis by macrophages, and nonspecific absorption with endothelial cells. In addition, due to the hydrophlilization of the liposome surface, retention of these liposomes in the bloodstream is prolonged. Consequently, Cy5.5-conjugated SLX-Lipo was shown to accumulate in tumors and at the sites of inflammation such as arthritis [173]. This unique liposome will be useful as a systemic delivery carrier for siRNAs.

Linear or branched cationic polymers can bind and condense large nucleic acids and have been used as transfection agents [174, 175]. Polymer complexes with siRNAs are taken up by cells into the cytoplasm *via* endocytosis and escape from the endosome. siRNAs are released into the cytoplasm by action of the proposed "proton sponge effect" mechanism in which polymers buffer the low endosomal pH through enhanced influx of protons and water, resulting in endosomal rupture [176]. Polyethyleneimine (PEI) is a synthetic polymer used broadly as a delivery carrier for nucleic acids, including DNAs, siRNAs, and oligonucleotides [177-179]. The intraperitoneal treatment of PEI/human epidermal growth factor 2 siRNA complex showed an inhibition of subcutaneous tumors [178]. Arg-Gly-Asp peptide-conjugated PEGylated PEI successfully delivered VEGFR2 siRNA to subcutaneous tumors by intravenous injection [180]. Cyclodextrin polymers have also been used as effective DDSs. The growth of Ewing's sarcoma tumors in a metastatic model was inhibited by the administration of EWS-FL11 siRNA in a cyclodextrin-containing polycation (CDP) assembly [181]. In another study, CDP nanoparticles with transferrin and PEG were loaded with the M2 subunit of ribonucleotide reductase (RRM2) siRNA and administered intravenously to monkeys in escalating doses. The results demonstrated the efficacy of CPDs self-assemble in a non-human primate [182], and in other studies it was shown that this particle is effective in knocking down *RRM2* mRNA in mice [183].

Atelocollagen is a natural polymer and its efficacy as an *in vivo* siRNA delivery system has been reported in many studies (Table **2**). Atelocollagen is type I collagen obtained from calf dermis. Amino acid sequences at the N- and C-termini of the collagen molecules are called telopeptide, and they have antigenecity of collagen molecules. As the telopeptide is removed from collagen molecules by pepsin treatment, atelocollagen shows low immunogenicity. There-

fore, atelocollagen has been shown to be a suitable biomaterial with an excellent safety profile and it is used clinically for a wide range of purposes. The molecular weight of atelocollagen is approximately 300,000 and the length is 300 nm. It forms a helix of 3 polypeptide chains [184]. Atelocollagen is positively charged, which enable binding to nucleic acid molecules. Moreover, at low temperature atelocollagen exists in liquid form, which facilitates easy mixing with nucleic acid solutions. The size of the atelocollagen-nucleic acid complex can be varied by altering the ratio of siRNA to atelocollagen. Because atelocollagen naturally forms a fiber-like structure under physiological conditions, particles formed a high concen-tration of atelocollagen persist for an extended period of time at the site of introduction, which is advantageous to achieve a sustained release of the associated nucleic acid (Table **2**). It has been confirmed that atelocollagen is eliminated through a process of degradation and absorption similar to the metabolism of endogenous collagen [184]. Alternatively, particles formed under conditions of low atelocollagen concentrations result in siRNA/atelocollagen complexes approximately 300 nm in size that are suitable for systemic delivery by intravenous administration (Table **2**). As atelocollagen accumulates in the liver, such atelocollagen could be used as a liver-directed DDS [10]. Atelocollagen complexes protect siRNA from degradation by nucleases and are transduced efficiently into cells, resulting in long-term gene silencing. Atelocollagen is an attractive non-viral DDS for siRNAs.

In the development of nucleic acids for use in combination with such delivery carriers, many efforts have been made to identify siRNA modifications to overcome the following problems: (1) nuclease degradation in the bloodstream, (2) rapid uptake by reticuloendotherial systems, and (3) nonspecific activation of the immune system mediated by Toll-like receptors (TLRs) (described below). Modification of the sugar structures found in nucleotides by incorporation of 2'-O-methyl, 2'-O-fluoro, or 2'-O-methoxyetyl has been tested [185-188]. These modifications impart resistance to endonuclease activity and avoid the activation of nonspecific immunity. A reduction in exonuclease susceptibility was demonstrated when the 3'-end phosphodiester group was replaced with phosphorothioate [188]. A locked nucleic acid (LNA) is a bicyclic nucleic acid in which a ribonucleoside is linked between the 2'-oxygen and the 4'-carbon with a methylene unit. This modification was shown to increase serum stability and reduce off-target effects [185, 189].

Conjunction of siRNA with small molecules or peptides is another form of nucleic acid modification. Conjugation of cholesterol to the sense strand of the siRNA duplex increases binding to serum albumin and improves the distribution of

Table 2. Experimental RNAi Therapies Using Atelocollagen

Target genes	Cancers	Models	Administration	References
VEGF	prostate cancer	subcutaneous	local	[253]
FGF-4	testicular cancer	orthotopic	local	[254]
EZH2, p100-α	bone metastatic tumor (prostate cancer)	orthotopic	systemic (i.v.)	[255]
EGFR	head and neck cancer	subcutaneous	local	[256]
HPV18E6E7	cervical cancer	subcutaneous	local	[257]
Midkine	prostate cancer	subcutaneous	local	[258]
GST-pi	prostate cancer (androgen-independent)	subcutaneous	local	[259]
HPV16E6E7	cervical cancer	subcutaneous	local	[260]
PAR-2	pancreatic cancer	subcutaneous	local	[261]
TS	salivary gland cancer	subcutaneous	local	[262]
RPN2*	breast cancer	subcutaneous	local	[233]
PLK-1	liver metastatic tumor (non-small cell lung cancer)	orthotopic	systemic (i.v.)	[10]
syndecan-1 (CD138)	prostate cancer	subcutaneous	local	[263]
β-catenin	multiple myeloma	subcutaneous	local	[105]
miR-16	bone metastatic tumor (prostate cancer)	orthotopic	systemic (i.v.)	[264]
Bcl-$_x$L	prostate cancer	subcutaneous	local, systemic (i.v.)	[234]
VEGF-A	Ewing's sarcoma	subcutaneous	local	[265]
mutant p53 promoter/enhancer	fibrosarcoma (chemical-induced)	orthotopic	local	[266]

VEGF-A; vascular endothelial growth factor-A, FGF-4; fibroblast growth factor-4, EZH2; enhancer of zeste homolog 2, p100-α; phosphoinositide 3'-hydroxykinase p100α subunit, EGFR; epidermal growth factor receptor, HPV; Human Papillomavirus, GST-Pi; glutathione S-transferase-Pi, PAR-2; proteinase-activated receptor-2, TS; thymidylate synthase, RPN2; ribophorin II, PLK-1; polo-like kinase-1, *RPN2 expression induces resistance to docetaxel. Although RPN2 siRNA alone does not induce apoptosis in breast cancer cells, RPN2 siRNA treatment increases the susceptibility of docetaxel on breast cancer cells.

siRNA to the targeted organ including the liver. Cholesterol-conjugated apolipoprotein B siRNAs were used to knockdown apolipoprotein B expression in liver and jejunum of mice, resulting in a decrease in cholesterol levels [190]. In another study, cholesterol-conjugated siRNAs against mRNAs associated with Huntington's disease delayed the abnormal behavioral phenotype observed in a rapid-onset mouse model [191].

BIOLUMINESCENT *IN VIVO* IMAGING ANIMAL MODELS

With an aim to establish RNAi strategies for cancer therapy, researchers have investigated the therapeutic effects in animal models. Previously, novel antineoplastic compounds have been usually screened using mouse models implanted human tumor cells subcutaneously. However, as these subcutaneous xenograft models do not clearly represent the primary sites of cancers or the metastatic sites, these models do not sufficiently represent the essential features of clinical cancers and surgical orthotopic models have been developed [192-194]. Whereas surgical orthotopic cancer models mimic the clinical situations, the assessment of cancer therapy and tumor growth required the sacrifice of the animals at a certain time point after the implantation of cancer cells and the evaluation of tumor load at death. Advances in molecular biology and medical technology have brought us non-invasive *in vivo* imaging of tumor load in small animals [195-197]. Several non-invasive imaging approaches including magnetic resonance imaging, computerized tomography, positron emission tomography, fluorescence imaging (FLI), and bioluminescence imaging (BLI) enable us to evaluate tumor sizes at serial time points from the same individual subject.

The BLI signals are produced by the reactions of luciferases with their substrate luciferin. Luciferases compromise a family of photoproteins isolated from various species such as firefly and sea pansy and they release photons by the reaction of luciferin under the existence of ATP [198]. Cancer cells are transfected with the gene for luciferase (Luc) and the bioluminescence signal from tumor-bearing mice is detected with a sensitive charge-coupled device (CCD) camera. Whereas FLI detects approximately 1,000-10,000 GFP expressing tumor cells inoculated intraperitoneously in mice [199], BLI can detect approximately 400-1,000 Luc-labeled cells inoculated subcutaneously or intraperitoneously and 1,000-10,000 cells inoculated intravenously [197, 200]. BLI is the most sensitive non-invasive approach to date. Moreover, there is much lower level of background noise in BLI compared with that in FLI because autofluorescence of non-labelled cells increases noise in FLI [195]. After luciferin is injected intraperitoneally in intracranial tumor-bearing mice, BLI signals of luciferase-bearing cancer cells increase during the first minutes, reaching a plateau after 10-15 minutes, and remains stable for additional 15-20 minutes [201]. Because intensity of BLI signals is correlated with tumor load, this approach is suitable for real time spatiotemporal analysis of tumor growth that reveals the dynamics of cancers and the therapeutic effects in experimental mouse models [151, 200, 202, 203]. However, BLI has some disadvantages. It is necessary to modify cancer cells transgenically for monitoring BLI as FLI. BLI shows anatomical low-resolution and it is difficult to detect signals released from deeper regions. Therefore, it is not likely to be applied to a clinical setting [195, 196].

PRECLINICAL APPLICATION OF RNAI THERAPY AGAINST PLK-1 IN MURINE ORTHOTOPIC MODELS OF CANCER

Here we introduce two applications of PLK-1 siRNA against urinary bladder cancer and metastatic liver tumors. As described above, PLK-1 is overexpressed in tumors of urinary bladder and in non-small cell lung cancer. PLK-1 expression levels are tightly correlated with histological grades of tumors, clinical stages, and prognosis of the patients. Superficial urinary bladder cancers, which compromise approximately 70% of urinary bladder cancers at initial diagnosis, are usually managed with transuretheral resection, followed by intravesical administration of agents such as Bacillus Calmette-Guerin (BCG), mitomycin C, and adriamycin. Although intravesical administration of BCG is considered to be the most effective strategy for the eradication and prophylaxis of the recurrent superficial cancers, this strategy causes irritation voiding symptoms, and life-threatening BCG sepsis. Therefore, other options of intravesical therapy for bladder cancer treatment are under investigation [9, 204]. Clinical trials of siRNA therapeutics often rely upon localized drug delivery [205], because maintenance of higher siRNAs concentrations is necessary for efficacy against the targeted diseases. Therefore, we investigated the efficacy of intravesical therapy of PLK-1 siRNA against urinary bladder cancers by using an orthotopic mouse model. Bladder cancer-bearing mice were established by the implantation of Luc-labeled UM-UC-3 bladder cancer cells into the murine bladder cavity through the urethra. Four days after the implantation, engraftment in the bladder was evaluated by using the *In vivo* Imaging System (IVIS) of BLI [151]. Mice were treated by intravesical administration, with 100 μl of PLK-1 siRNA/LIC-101 liposome complex (6, 0.6 μM) for 5 days, and progression of urinary bladder cancer was successfully inhibited in a dose-dependent manner [9].

Liver metastasis is one of the most important prognostic factors in lung cancer patients. However, despite the development of new chemotherapeutic agents, current therapies are not sufficient to inhibit liver metastasis. We investigated the effects of PLK-1 siRNA on the liver metastasis of lung cancers. We first established a mouse model of liver metastasis. Spleens were exposed to allow direct intrasplenic injections of Luc-labeled A549 lung cancer cells. Ten minutes after injections of tumor cells, the spleens were removed. After confirmation by IVIS visualization of Luc-labeled A549 cell engraftment, 200 μl of PLK-1 siRNA/atelocollagen complex (25 μg of siRNA), nonsense siRNA/atelocollagen complex (25 μg of nonsense siRNA), or PBS/atelocollagen complex was administered by intravenous injection for 10 consecutive days following day 1 of transplantation. The final concentration of atelocollagen was 0.05%. On day 35, mice treated with nonsense siRNA/atelocollagen complex or PBS/atelocollagen complex showed extensive metastases in the liver when compared to mice treated with PLK-1 siRNA/atelocollagen complex. More-

over, on day 70 after the inoculation of tumor cells, livers of mice treated with nonsense siRNA/atelocollagen or PBS/ atelocollagen complex had numerous large tumor nodules, whereas the livers of mice treated with PLK-1 siRNA/ atelocollagen complex showed a much lower number of smaller nodules. These findings indicate that PLK-1 siRNA/ atelocollagen complex is an attractive therapeutic tool for further development as a treatment against liver metastasis of lung cancer [10]. Consequently, our preclinical applications suggest that PLK-1 siRNA is a promising tool for cancer therapy.

ADVERSE EFFECTS OF RNAi IN *IN VIVO* APPLICATION

Although RNAi shows excellent specificity in gene-silencing, several adverse effects are brought in *in vivo* application. One probable adverse effect is activation of immune reaction [189, 206]. Mammalian immune cells express family of TLRs, which recognize pathogen-associated molecules including unmethylated CpG DNA and viral dsRNA. Among 13 TLRs, TLR7 and TLR8 recognize ssRNA sequence-dependently and produce IFNs and inflammatory cytokines such as IL-12 and TNF-α through the activation of NF-κB and IFN regulatory factor (IRF) -7 [207]. Furthermore, these TLRs recognize uridine- or guanosine-rich ssRNA [208, 209]. TLR3 recognizes unmethylated CpG DNA but not ssRNA [207]. dsRNA is directly binds to TLR3 [210] and this signaling pathway is activated sequence-independently [207, 211]. The size of ssRNA is also important for the immune reaction. Twelve nt ssRNAs containing the immunostimulatory motif (5'-GUCCUUCA A-3') merely induced IFN-α, however, 16 to 19 nt ssRNA restored IFN production [186]. The administration of siRNAs into mammalian cells activates the immune systems also sequence-independently. siRNAs induce dsRNA-activated protein kinase (PKR) autophosphorylation and PKR produces IFNs through the activation of NK-κB and IRF-3 [212]. One possible strategy to prevent the immune activation is chemical modification of siRNA. The 2' position of nucleotides is within TLR-7-interacting sequences, and 2' O-methyl or 2' fluoro- modification abrogate immune response. Furthermore, the uridine or guanosine modification is most effective [213]. LNA can also reduce the immunostimulatory effects [188]. Although the cationic lipid-based DDSs are efficient for the trasnduciton of siRNAs into cells, the complexes of siRNAs with cationic lipids induce the production of inflammatory cytokines [186, 187]. siRNAs conjugated to cholesterol have no significant activation of immune system and improve the distribution of siRNA to the targeted organ including the liver. Cholesterol-conjugated apolipoprotein B siRNAs induce a decrease of apolipoprotein B expression in liver and jejunum of mice, resulting in a decrease in cholesterol levels without the activation of immune systems [190].

Although RNAi mediated gene silencing is induced by perfect complenetarity in target RNA sequence, partially complementary sequences in unintended RNAs induce gene silencing (off-target effect). This off-target effect is induced by the sequence complementarity in the seed region of siRNAs or shRNAs [214]. Moreover, the 7 nt motif complimentary to 2-8 nt at the 5' end of antisense strands of siRNAs has been shown to be a key determinant in directin off-target effects because an siRNA with 1 nt mutated in th 7 nts region failed to induce off-target effects [215]. Ther are several ways to control the off-target effects. Methods fc *in silico* screening of siRNA constructs are useful fc optimization to prevent the off-target effects and severa groups have been developing algorithm [216-218]. Chemica modification is also useful. The O-methyl modification c the 2'-position of the ribose within the seed region of siRN/ sense strands reduces sense strand-mediated off-targe activity [219]. Asymmetrically designed siRNAs reduce off target effects compared to symmetric siRNAs [220] Asymmetric siRNAs with 15 nt of the sense strand induc sequence-specific gene silencing without silencing of non target genes. The incorporation of the sense strand into RISC can also cause off-target effects [221, 222]. It is speculate that the asymmetric siRNAs enter RISC with high efficienc and persist in Ago 2 protein longer compared to siRNAs Asymmetric siRNAs reduce off-target silencing by reducin incorporation of the sense strand into RISC.

Although shRNAs can induce stable gene silencing, ther is a possibility that long-term silencing by shRNA overexpression causes fatal adverse effects. As shRNA i processed through the miRNA pathway, the miRNA maturation is blocked in response to shRNA concentration The sustained high-level shRNA expression in the liver c mice by AAV vector downregulated liver-derived miRNAs resulting in hepatic injury and death [223]. One explanatio is that saturation of Exportin-5 whose function is nuclea transport inhibited the miRNA maturation pathway Overexpression of Exportin-5 can tolerate higher doses c shRNA without its toxicity [224]. However, another stud shows that the administration of synthesized siRNA induced acute and long-term gene silencing withou interrupting the endogenous miRNA biogenesis [225] Considering these findings, careful modification an formulation of siRNAs could avoid the competition betwee siRNA and miRNA. Usage of inducible siRNA vectors [226 227] or selection of promoter such as tissue-specifi polymerase II promoters [224] would be other options t avoid this adverse effect.

CLINICAL TRIALS OF RNAi TOWARDS CANCEI THERAPIES

The first systemic administration of an siRNA drug i humans was performed in a patient with CML in a experimental setting of a traditional clinical study [228]. A 47-year-old, Ph-positive and IM resistant, female patient with CML received allogeneic bone marrow transplantation However, an extramedullary relapse of pleural effusions an subcutaneous nodes occurred. The patient received furthe intravenous or subcutaneous administration of siRNA against *Bcr-Abl* mRNA. In combination with IM an cytosine-arabinoside, the siRNA was delivered usin liposomes at doses of 10 or 30 μg/kg as a systemic treatmen and at 300 μg as a local treatment. Levels of *Bcr-Abl* mRNA expression were dramatically decreased following the firs administration; however, this effect was not observed afte further more administrations of the siRNA. The siRNA treatment was well tolerated without any adverse effects.

siRNA cancer therapies have been conducted in clinical settings, but few clinical trials for cancer therapy are ongoing (http://clinicaltrials.gov/ct2/home) as many of the current siRNA therapeutics in advanced clinical trials rely on localized drug delivery [205]. Alnylam Pharmaceuticals is developing ALN-VSP01 targeting kinase spindle protein and VEGF, and conducting a Phase I study in patients with advanced tumors with liver involvement. Calando Pharmaceuticals is conducting a Phase I study of CALAA-01 in patients with solid tumors refractory to standard-of-care therapies. CALAA-01 is composed of RRM2 siRNA and CDP nanoparticles called Rondel™, and CALAA-01 has been proven safe and effective in mice and nonhuman primates studies [182, 183]. Clinical studies using LNAs are also ongoing. Santaris Pharma has developed LNA against Bcl-2, SPC2996, for use in an ongoing Phase I/II study in patients with relapsed or refractory chronic lymphocytic leukemia is ongoing. Enzon Pharmaceuticals has developed a LNA against hypoxia-inducible factor-1α and a Phase I/II study in patients with advanced solid tumors or lymphoma is ongoing.

CONCLUSION

Studies to establish the pharmacokinetics and pharmacodynamics of siRNAs on the administration are necessary step in the potential approval of siRNA as a tool for cancer therapy. Moreover, to maximize efficacy and to minimize adverse effects of RNAi, it should be determined whether siRNAs are best administered alone or in combination with chemotherapeutic agents [77, 229], and whether it is better to administer a single specific siRNA or multiple specific siRNAs [144, 230-234]. In conclusion, RNAi therapy represents a powerful strategy against cancers and may offer a novel and attractive therapeutic option. The success of RNAi depends on the suitable selection of target genes and the development of DDSs. We anticipate that the continued development of effective DDSs and the accumulation of evidence further proving the success of siRNA treatment will advance siRNA as a promising strategy for cancer therapy.

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