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Abstract

RNA interference (RNAi) therapy is an emerging class of biopharmaceutical that has immense potential in cancer medicine. RNAi medicines are based on synthetic oligonucleotides that can suppress a target protein in tumour cells with high specificity. This review explores the attractive prospect of using RNAi as a radiosensitizer by targeting the DNA damage response. There are a multitude of molecular targets involved in the detection and repair of DNA damage that are suitable for this purpose. Recent developments in delivery technologies such nanoparticle carriers and conjugation strategies have allowed RNAi therapeutics to enter clinical trials in the treatment of cancer. With further progress, RNAi targeting of the DNA damage response may hold great promise in guiding radiation oncology into the era of precision medicine.

RNA Interference to Enhance Radiation Therapy: Targeting the DNA Damage Response

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Background

Radiation therapy is a well-established treatment modality for cancer with an important role in the curative and palliative setting. Approximately 50% of all cancer patients will benefit from receiving radiotherapy at some point in their illness (1). In most cancer types the tumour control rate improves with an increasing dose of radiation (2,3). However, radiation causes both early and late reactions in the surrounding normal tissue (4). This leaves a narrow therapeutic window where the challenge is to deliver an adequate radiation dose for tumour control without causing overt toxicity (5).

The introduction of radiosensitizing agents in combination with external beam radiotherapy has greatly improved the efficacy of radiation treatment. The most common agents are cytotoxic chemotherapy agents such as cisplatin and 5-fluorouracil which have been shown to improve tumour control and patient survival outcomes when combined with radiotherapy (6–8). However, due to the non-discriminatory distribution of these highly cytotoxic agents they also sensitise normal tissue to radiation leading to greater toxicity (9).

RNA interference (RNAi) therapies offer an alternative approach to radiosensitization. The mechanism of action of radiation therapy is by causing preferential DNA damage to the tumour by exploiting differences in DNA damage response in cancer and normal cells. By precisely targeting the response to this DNA damage with RNAi, it may be possible to

achieve potent radiosensitization whilst minimising the adverse effects on normal tissues (10). The fundamentals of this technology are developing in the preclinical setting with momentum building for translation to the clinic (11). This review will focus on the use of RNAi to target the DNA damage response for the purpose of radiosensitisation.

Mechanisms of RNA Interference

RNAi with therapeutic oligonucleotides is a hugely promising technology in cancer treatment (12,13). The system uses human genomic data to design and synthesize oligonucleotides that can be introduced into the cell of interest to silence specific genes. There is great potential in targeting and disabling pathways in tumours that are responsible for resistance to radiation, thereby improving the overall efficacy of radiation therapy.

RNAi oligonucleotides are small, non-coding RNA molecules that are usually 20-25 base pairs in length which come in several classes. Antisense oligonucleotides (ASO) are the earliest developed and simplest in structure and are usually single stranded RNA molecules (14). MicroRNA (miRNA) are endogenously occurring RNA, usually with a short hairpin structure, and form the basis for the manufacture of synthetic mimics (15). However, the most widely used form of RNAi are short interfering RNA (siRNA) which are double stranded RNA that can be designed and synthetically produced and are valued for their specificity and efficacy (16).

The mechanism of action of these oligonucleotides relies upon the highly efficient intracellular processing of RNAi present in mammalian cells. The RNAi oligonucleotide is introduced to the cell as a single or double stranded complex (Figure 1). Once in the cytoplasm it is loaded into a set of proteins known as the RNA-inducing silencing complex (RISC) (17). The RISC complex removes the passenger strand of the RNAi if present allowing the remaining single strand to bind to complementary mRNA sequences. Matching mRNA that binds undergoes endonucleolytic cleavage and is degraded, leading to downregulation of the corresponding protein expression.

The main strength of RNAi is that in theory any protein within the cell with a known mRNA sequence becomes a druggable target (18). This also allows for a highly specific knockdown

of the corresponding protein. Moreover, because a single RISC complex will destroy multiple mRNA targets, nanomolar or even picomolar concentrations of siRNA can effectively knockdown proteins for days to weeks (17,19). These features of RNAi can be exploited for radiosensitisation by targeting the complex machinery of the cellular DNA damage response.

Targeting the DNA Damage Response

Clinical radiotherapy uses high-energy ionising radiation, which is delivered with highly conformal techniques to the tumour (4). The biological effects of ionising radiation are caused by damage to DNA which if unable to be repaired leads to the death of the tumour cell by apoptosis or mitotic catastrophe (20). Targeting this mechanism of radiotherapy may be an effective method of enhancing its effect on tumours. Central to this strategy are the inherent features of cancer cells of a greater burden of endogenous DNA damage and abnormalities in the cellular DNA damage response (DDR).

The DDR is a complex, sensitive and interconnected pathway (Figure 2), broadly consisting of sensors, transducer, effector and mediator proteins which undergo numerous post-translational modifications – particularly phosphorylation, ubiquitylation and sumoylation – to trigger a variety of cellular responses (21,22). The DDR has an important role in cancer progression because its dysregulation leads to higher mutation rates, genomic instability, and enhanced intra-tumour heterogeneity (21,22). The differences between DDR pathways in normal cells and cancer cells are an attractive source of targets to be exploited by RNAi therapies. The genomic instability combined with rapid cell turnover leaves cancer cells highly reliant on the DDR (23). Additionally, most cancer cells will have lost one or more DDR pathways leading to a greater dependency on the remaining pathways (24). Hence, the combination of radiation induced DNA damage whilst simultaneously using RNAi to disable the remaining functioning DDR pathways leads to an overwhelmingly and lethal amount of DNA damage to the tumour.

Although the DNA damage response relies heavily on post-translational modifications for signal transduction, depletion of a number of key molecules has been shown to effectively

radiosensitise cells both *in vitro and in vivo*. The selection of RNAi targets in the DDR pathway is key to the success of this strategy. Due to the breadth of the DNA damage response, we will focus on components involved in double-strand break repair, because of its major role in ionizing–radiation induced cell death (25). Double-strand breaks (DSB) are the most deleterious of DNA-lesions, and a single break is sufficient to induce cell death. DNA double-strand breaks are repaired via one of two main pathways: Non-Homologous End-Joining (NHEJ) or Homologous Recombination (HR).

Targeting Non-Homologous End-Joining

NHEJ is the major DSB repair pathway that is engaged to repair radiation-induced breaks (26). It can occur at all stages of the cell cycle, and relies on ligation of the broken DNA ends, with or without additional end processing. NHEJ begins with the binding of Ku70/Ku80 heterodimer, flanking each broken end of the genetic insult and forming a platform for the binding of the DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs). Together, Ku70/80 and DNA-PKcs form the DNA-PK holoenzyme, which can self-activate via autophosphorylation, as well as phosphorylate a number of downstream targets involved in signalling and repair of the break (27,28). In mechanisms which will not be discussed in detail here, additional proteins may then be engaged to process the break ends to render them amenable for joining, prior to final ligation of the broken DNA ends by the Ligase IV/XRCC4/XLF complex (26).

The most attractive targets in the NHEJ pathway for radiosensitisation are of the obligate initial and final stages of this process (29). As an important initial step in sensing the DNA break, many studies have investigated the effects of depletion or inactivation of DNA-PKcs. The radiosensitivity conferred by DNA-PKcs depletion or loss stems from its critical role in the NHEJ pathway in recruiting and phosphorylating numerous repair factors and as an important kinase that shares many phosphorylation targets with ATM. Interestingly, the ways in which DNA-PK loss sensitises to radiation may be multifaceted. Depletion of DNA-PKcs by siRNA leads to pronounced radiosensitivity in multiple human cell lines, leading to G2/M arrest and impeded mitotic progression. Interestingly, this effect was more pronounced with use of the DNA-PK inhibitor NU7441, which more severely impeded DDR signalling(30). Delivery of siRNA targeting DNA-PKcs *in vitro* with a commercial liposomal

method has been shown to reduce the capacity for restitution of radiation-induced interphase chromosome breaks and increase the yield of acentric chromosome fragments at post-irradiation mitosis. The result was an increase in radiosensitivity, particularly in p53-mutant lymphoblast-derived cells, which lack functional G1/S checkpoint and exhibited a 2-fold increase in radiosensitivity (31). Similarly, non-replicative adenovirus mediated delivery of short hairpin RNA (shRNA) to target DNA-PKcs in HCT116 colorectal cells *in vitro* demonstrated radiosensitisation. The strategy was initially not successful *in vivo* with a murine xenograft model due to poor transfection, but by introducing a conditionally replicative adenovirus the transfection issues were overcome with potent *in vivo* radiosensitivity demonstrated with DNA-PKcs knockdown (32).

The Ku proteins could also be potential therapeutic RNAi targets as critical mediators of early NHEJ. Currently no small molecule inhibitors have been demonstrated to have inhibitory activity against these proteins. However, multiple studies have demonstrated radiosensitisation upon Ku loss or depletion. Ku70-deficienct embryonic stem cells are sensitised to ionizing radiation, and RNAi-mediated depletion by lentivirus in mammary cells conferred radiosensitivity (33,34). A haemagglutinating virus of Japan envelope vector was used to deliver siRNA targeting Ku80 in A549 and H1299 lung carcinomas and when irradiated demonstrated markedly higher gamma-H2AX foci following DNA damage by IR, indicative of greater DNA damage. Moreover, tumour xenografts using these lines and treated with radiation exhibited greater cytoreduction and slower regrowth compared to radiation alone, illustrating effectiveness of this strategy *in vivo* (35). An alternate strategy to induce radiosensitisation through targeting the NHEJ pathway would be to inactivate the final ligation step of this process which is reliant on the Ligase IV and XRCC4 molecules. Targeting of XRCC4 protein and its co-factor XLF with siRNA in U-2OS osteosarcoma cells led to potent radiosensitisation (36). Similarly, knockout of Ligase IV protein in multiple cultured cell lines or in mice (37,38) causes pronounced radiosensitisation, suggesting its use also as a radiosensitisation target.

Targeting Homologous Recombination

In contrast to NHEJ, Homologous Recombination (HR), is a high-fidelity repair pathway active primarily in the S/G2 phase of the cell cycle, where the sister chromatid can act as a

template for repair. In brief, HR begins with the sensing of the DNA break by the Mre11-Rad50-NBS1 complex. Following the initial sensing of the break, resection occurs by a number of proteins beginning with Mre11, CTIP and including Exo1 and DNA2 to form long 3' single-stranded DNA intermediates which are coated by the Replication Protein A complex (39,40). In subsequent steps, RPA is replaced by Rad51, mediated by BRCA2, BRCA1 and additional proteins to form the synaptic filament. Rad51-coated ssDNA is then able to invade the sister chromatid, which is used as a template for repair (40).

As a core component of HR, Rad51 has been the subject of multiple efforts to develop small molecule inhibitors (41–43). Many cancers have been shown to overexpress Rad51, and its overexpression inversely correlates with radiosensitivity (41). A commercial liposomal vector was used to transfect glioma cells with antisense oligonucleotides against RAD51 and was found to significantly enhance the radiation induced cell kill compared to control cells. In a orthotopic murine glioma model, the combination of RAD51 antisense oligonucleotides and radiation led to extended survival times of glioma-bearing mice (44).

Another method under intense investigation over recent years has exploited HR pathway deficiency in cancer cells for therapeutic gain using inhibitors of PARP proteins (45). The PARP proteins are a family of poly-ADP ribosylation factors, which post-translationally modify a number of proteins by PARylation - the synthesis of PAR chains onto substrate proteins (46). When a single-strand DNA break occurs, PARP proteins are one of the earliest responders, binding to DNA and PARylating downstream proteins before auto-PARylation facilitating their release from DNA (47). Single-strand DNA breaks are one of the most common genetic lesions, occurring thousands of times in each cell every day, and PARP proteins are critical factors in their repair (48). However, if single-strand breaks are not appropriately repaired, they can convert to double-strand breaks during S phase. Recently developed small molecule PARP inhibitors allow trapping of PARP at the site of breaks, promoting this occurrence, and resulting in the formation of double-strand breaks. In cancer cells which have inherent deficiencies in HR (such as occurring in BRCA1/2 deficient breast and ovarian cancers), the treatment with PARP inhibitors exploits this deficiency to cause death in the cells, a concept known as synthetic lethality (47,49,50). PARP inhibitors represent one of the rare successful cases of small molecule DNA repair inhibitors with

success in the clinic. However, like with other small molecule inhibitors, development of acquired resistance to PARP inhibitors was ubiquitous, hampering the duration of response, and overall survival benefit in clinic (51–53). However, several combination approaches are being investigated to overcome resistance to PARP inhibitors, including the combination with radiotherapy. PARP inhibitors are being tested in clinical trials for synergism with radiation therapy, not only in HR-deficient cancers, but also for head and neck cancers(54), and glioma(55). Excitingly, this success may open additional avenues for triple-combination therapies, where the tumour could be rendered HR deficient by RNA, and treated with a single or combination therapy of PARP inhibitor and radiation therapy.

Targeting DNA-damage Response Signalling

The HR and NHEJ repair pathways rely on the initial detection of DNA damage. Ataxia-Telangiectasia Mutated (ATM), named after the protein mutated in the genetic disorder Ataxia-Telangiectasia (A-T), is one of the earliest responders to DNA DSBs, and is a kinase belonging to the same family as DNA-PK (56). Patients with A-T and derived cell lines from these patients are exquisitely radiosensitive (57,58). ATM works by co-operating with a trimeric protein complex composed of MRE11-RAD50-NBS1 (59). The importance of these proteins in the DDR is illustrated by their phosphorylation of more than 700 downstream targets in response to DNA damage (60). With its position at the apex of DDR signalling and repair, it represents an ideal target for radiosensitisation.

Efforts to target ATM in the clinic with small molecule inhibitors have been relatively unsuccessful. ATM inhibitors often have off-target effects with other PI3-kinases, are not useful at concentrations required for treatment and have been shown to cause a gastrointestinal syndrome after total body irradiation in preclinical models (61,62). The use of exogenously delivered plasmids encoding siRNA targeting ATM and a related protein ATR was found to be a potent radiosensitizer in DU-145 and PC-3 cells *in vitro*. One study whilst investigating the effects of hyperthermia showed siRNA mediated-downregulation of MRE11 in colon adenocarcinoma cells induces radiosensitisation *in vitro*. Another study targeted another protein of the trimeric complex, NBS1 in H1299 lung cancer cells leading to increase radiosensitivity *in vitro* (63). Furthermore, RNAi targeting of ATM and the Mre11-

Rad50-NBS1 complex has been shown to be effective at sensitising prostate, colon and lung cancer cells to radiation (61,63,64).

Downstream of the ATM and ATR kinases: targeting CHK1 and CHK2

Finally, the checkpoint kinases serve as important downstream transducers of the DDR and act predominantly downstream of the ATR and ATM kinases (60,65). Checkpoint kinases serve to halt the cell cycle in response to DNA damage, until the damage is appropriately addressed (via repair or induction of cell death) (66). Cancer cells often have inherent defects in p53-dependent G1/S DNA damage checkpoint, one of the most commonly mutated tumour suppressor gene in cancers, resulting in an increased dependency on the remaining functional checkpoint machinery. A lentivirus-delivered shRNA targeting Chk1 in human glioblastoma stem-like cells in combination with radiation increased the apoptosis rate, decreased the degree of G2/M arrest and was associated with increased radiosensitivity (67). Targeting of CHK1 by RNAi in particular warrants further investigation due to the failure of inhibitors in the clinic (68).

Risks and Rationale of Targeting the DDR with RNAi

RNAi targeting of DDR however is not without risk of toxicity. From clinical experience with small molecule DDR inhibitors we know that this approach can have associated adverse effects including myelosuppression(69), cardiac toxicity (70), gastrointestinal toxicity (61) and neurotoxicity (71) and rarely can cause second malignancies such as acute myeloid leukaemia (72). The RNAi technology has its own inherent toxicities such as off-target effected caused by cross-hybridisation with unintended transcripts that contain partial identity to the RNAi sequence leading to unintentional silencing of other protein(73). RNAi can also stimulate an immune response and in some cases can oversaturate the RISC complex (74,75). Many of these can be minimised with sequence optimisation and oligonucleotide structure modification.

The rationale in targeting the DDR is validated by several small molecule inhibitors which are the subject of pre-clinical and clinical trials (22,76). However, to date most of these drugs have not been successfully adopted in clinical practice. They remain costly to develop,

can have poor bioavailability and can have off-targets *in vivo* with associated toxicity. Hence these targets may be suited to targeting with RNAi, with the added benefit that multiple RNAi targets could be easily combined for therapeutic advantage.

Delivery Technology for RNA Interference

Despite the abundance of targets for radiosensitization the clinical translation of RNAi for therapy poses some significant challenges (Figure 3) (77). RNAi oligonucleotides are not stable in circulation due to the presence of ribonucleases with more than 99% degrading in human blood within minutes of incubation (78). Their size is small enough to be rapidly cleared through the kidneys but large enough to be targeted by the reticuloendothelial system (79). Their polyanionic and hydrophilic properties leads to difficulty in penetrating the phospholipid bilayer making up the cell wall (80). Even once taken into the cell, they are entrapped in endosomes and only a small fraction are released into the cytoplasm to be therapeutically active (81).

However, delivery technology for RNAi has been rapidly developing to meet these challenges with examples including viral vectors, nanoparticles and aptamers. A comprehensive review of delivery technologies for RNA interference is outside the scope of this article, but there are several recent reviews on the topic (82–85). To our knowledge, there are currently no clinical trials investigating the use of RNAi delivery in combination with radiotherapy. However, the following selection of RNAi delivery technologies may act as a gateway to future trials of RNAi in combination with ionising radiation.

One of the earliest delivery methods was using viral constructs that encapsulate a modified genome that carries a therapeutic gene cassette in place of the original viral genome (86). These viral particles are incapable of replicating and instead only function to infect a cell leading to the expression of the therapeutic genetic information. The most common viral vectors are adenoviruses (87) and we have already provided some examples in the previous sections of proteins responsible that have been targeted successfully with this strategy in the preclinical setting (32,63,88). Examples outside of the DDR pathway include using adenovirus-mediated strategies for radiosensitisation targeting matrix metalloproteinase-2

(MMP-2) in glioma (89), anti-apoptosis protein survivin in lung cancer (90) and the Mcl-1 gene in pancreatic carcinomas (91). There are some barriers to the clinical translation of adenovirus vectors including the potential for inflammatory, immunogenic and mutagenic effect, which makes them a safety risk for translation to humans (86–88). Viral vectors also have a high cost of production, which can be a barrier when scaling up manufacturing (92). Nevertheless, oncolytic adenoviruses has been used as non-RNAi gene therapy vector in combination with radiotherapy in a Phase II clinical trial in intermediate risk prostate cancer (93).

Nanoparticle delivery vehicles are another promising method for RNAi delivery (94). Nanoparticles are clusters of atoms of molecules ranging usually from 1 nm to almost 1000 nm in size and can be composed of lipids, polymers, proteins and inorganic compounds. RNAi nanoparticle platforms are designed to encapsulate and protect the oligonucleotide cargo from degradation thereby greatly enhancing the delivery to the tumour. In addition they benefit from the enhanced permeability and retention (EPR effect), a biophysical phenomenon where abnormal vasculature of solid tumours with wide fenestrations allows molecules within the nanoparticle size range to preferentially accumulate within solid tumours and be retained there by high lymphatic pressures (95). The result is a higher concentration of the radiosensitiser within the tumour and lower concentration within the normal tissue (Figure 4). These advantages have led to a number of preclinical studies. Iron oxide nanoparticles coated with polyethylene glycol (PEG) and polyethyleneimine (PEI) were used to delivery siRNA against the DNA base excision repair protein Ape1 in medulloblastoma and ependymoma cells as a radiosensitiser(96). Similarly, a PEG-PEI copolymer was used to deliver siRNA against sCLU in a MCF-7 breast cancer cell line with evidence of radiosensitisation (97).

A number of nanoparticles are approved for clinical use in oncology including liposomal doxorubicin, liposomal irinotecan and albumin-bound paclitaxel (98). Nanoparticles for RNAi delivery are being investigated in clinical trials (Table 1). Lipid-based nanoparticles are the mainstay of RNAi cell transfections for life science research with a number of commercially available formulations (99). Early nanoparticles had difficulty travelling beyond the liver leading to interest in using cationic liposomes to deliver siRNA to sensitize hepatoma cells to

radiation *in vitro* and *in vivo* (100). However, adaptation of liposomal delivery vehicles with polyethylene glycol (PEG) to reduce liver accumulation and fusogenic elements (101) to enhance cellular uptake have led to platforms like Atu027 developed by Silence Therapeutics, with evidence of efficacy in clinical trials (69,102). Another highly novel, recently developed RNAi nanoparticle delivery platform is a system of bacterially derived minicells each approximately 400 nm in diameter (103). These non-viable particles are produced by de-repressing polar sites of cell division in bacteria and can be loaded with RNAi oligonucleotides (104). They are coated with bispecific antibodies (BsAbs), which allow them to be targeted to a receptor that is overexpressed on the cancer cell (105). There are multiple preclinical studies to deliver siRNA and miRNA to tumour *in vivo* (106–108) and Phase 1 clinical trial in malignant mesothelioma deliver miR-16-based mimic microRNA were effective stabilising tumour growth in the majority of these patients. Even with these advances the challenges of nanoparticle delivery of RNAi beyond the liver(83) have not been overcome completely and clinical toxicity is unique and known to be present for each of the platforms(109) without being fully elucidated in clinical trials. Thus further work remains in developing nanoparticle delivery of RNAi as a monotherapy and in combination with radiotherapy.

Lastly, developments in RNAi conjugates are being explored as a method of delivery (110). Advances in chemical modifications to RNAi oligonucleotides have enhanced their resistance to serum nucleases reducing the need for protective carriers such as nanoparticles (111,112). This allows conjugation with molecules such as antibodies and aptamers that allow active targeting to the intended tissue. Conjugation with monoclonal antibodies have been shown to effective in targeting siRNA to overexpressed tumour surface antigens (113). Aptamers are oligonucleotides designed through a special selection process that function comparably to traditional antibodies, and are especially suited to this purpose (114). Aptamer/siRNA chimeras have the ability to target cellular receptors, promote uptake and deliver the siRNA payload (81). An aptamer-siRNA chimera was used to target the DDR pathway in prostate cancer. This approached used anti-DNA-PKcs siRNA with an aptamer targeting PSMA, a protein which is overexpressed in prostate cancer cells (115). It was shown to effectively deliver the siRNA in murine xenograft model leading to downregulation of DNA-PKcs and resulting in radiosensitisation of the tumour (116).

Conclusion and Future Directions

The field of radiation oncology has experienced a quantum leap in technology with advances in imaging, dosimetric planning and treatment delivery (117). However, progress in the application of cancer biology to clinical radiotherapy has been much slower. Though conventional cytotoxic agents are associated with better outcomes, this comes at the cost of indiscriminate toxicity to normal tissue (118). The recent revolution in targeted constructs and immunotherapies may provide many opportunities for combination with radiation, however these agents are not designed with the purpose of being radiosensitizers (119).

Gene therapy with RNAi holds great promise in combination with radiotherapy. There is a clear abundance of molecular targets in the DNA damage response pathway that can result in dramatic radiosensitisation in tumours. Although challenging to implement, the major attraction of siRNA-based drugs is that any of these genes may be targeted, which may otherwise not be possible with small molecule or protein based drugs. It also opens the exciting possibility of using multiple siRNAs targeting different pathways simultaneously silencing several genes responsible for radioresistance and leading to an even more potent effect.

The challenge with RNA interference remains in the delivery. However, progress is being made with increasingly robust delivery platforms being developed to safely and efficiently deliver the RNAi payload to the cancer cells. This approach would take advantage of decades of radiobiology research on the DNA damage response to be applied in a way that has a direct clinical impact. RNA interference may aid in the development of truly personalised medicine in radiation oncology.

Conflicts of Interest

There are no conflicts of interest to disclose.

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Table 1 Recent clinical trials of RNA interference therapeutics for cancer treatment

Indications	Name	Delivery Route	Target	Delivery System	Development Phase	Clinical Trial Number
Advanced solid tumors	siRNA-EphA2-DOPC	Intravenous (I.V.) injection	EphA2	Lipid-based nanoparticles	Phase I, Recruiting	NCT01591356
Advanced solid tumors	Atu027	I.V. infusion	PKN3	Lipid-based nanoparticles	Phase I, completed	NCT00938574
Pancreatic ductal adenocarcinoma; Pancreatic cancer	siG12D LODER	Intratumoral implantation	KRASG12D	LODER polymer	Phase I, completed	NCT01188785
Primary or secondary liver cancer	TKM-080,301	Hepatic intra-arterial injection	PLK1	Lipid-based nanoparticles	Phase I, completed	NCT01437007
METAVIR F3–4	ND-L02-s0201	I.V. injection	HSP47	Lipid-based nanoparticles	Phase I, completed	NCT02227459
Solid tumors; multiple myeloma; non-Hodgkin's lymphoma	DCR-MYC	I.V. infusion	MYC	Lipid-based nanoparticles	Phase I, terminated	NCT02110563
Cancer; solid tumour	CALAA-01	I.V. injection	RRM2	Cyclodextrin-containing polymer	Phase I, terminated	NCT00689065

Neuroendocrine tumors; adrenocortical carcinoma	TKM 080301	I.V. infusion	PLK1	Lipid-based nanoparticles	Phase I/II, completed	NCT01262235
Solid tumors	ALN-VSP02	I.V. injection	KSP VEGF	Lipid-based nanoparticles	Phase I, completed	NCT01158079

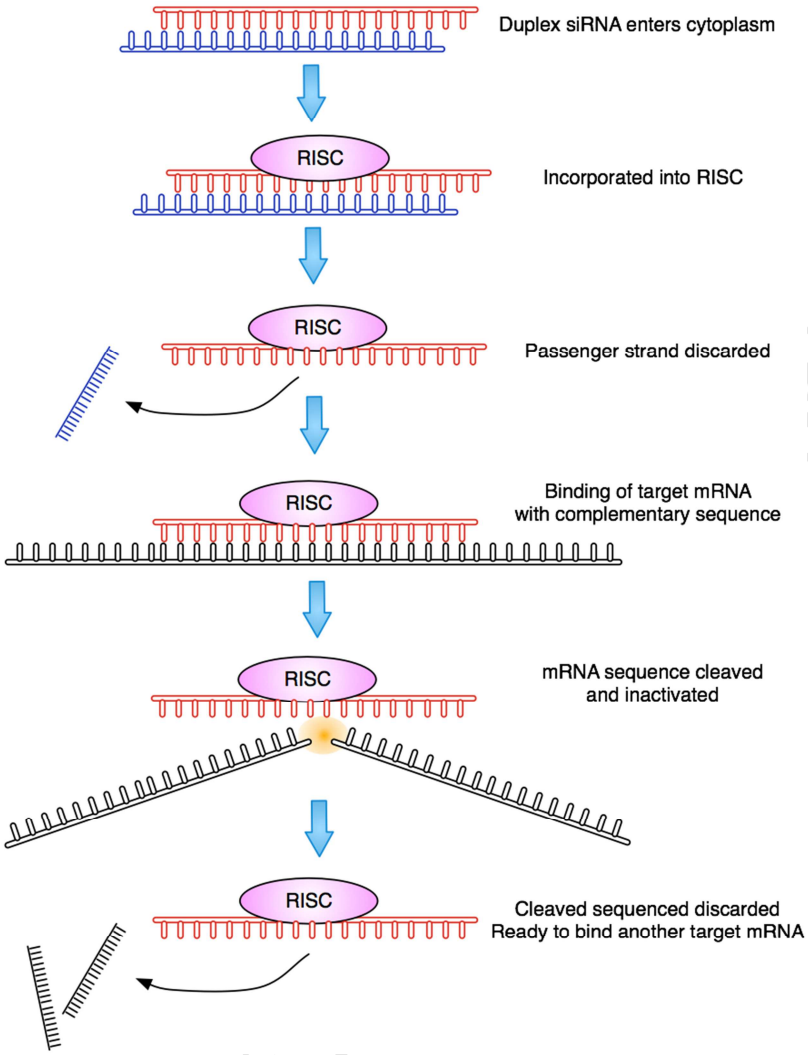


Figure 1 Mechanism of RNA interference from exogenous siRNA

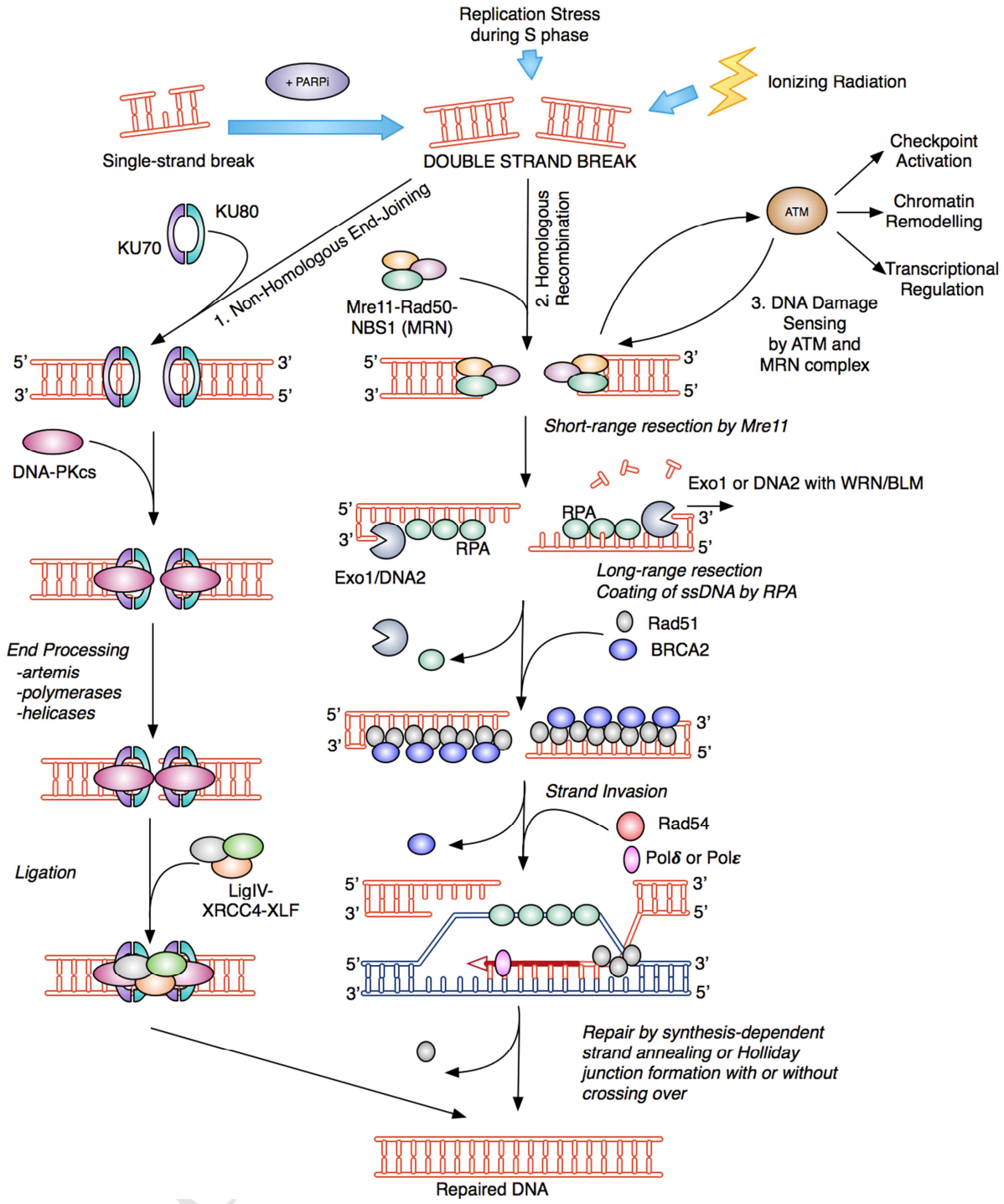
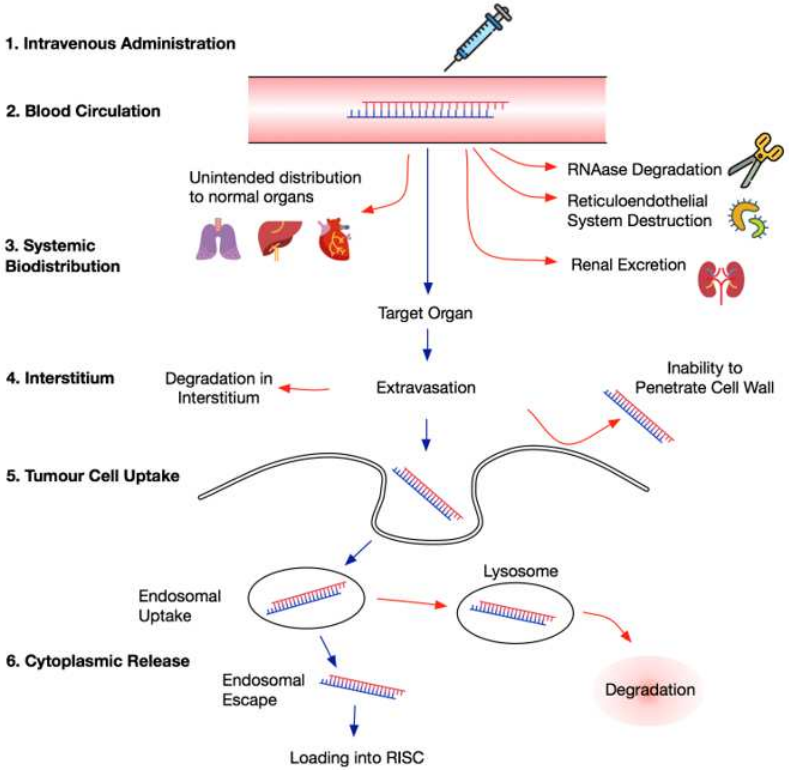


Figure 2 DNA Double-strand break repair Double-strand breaks can be repaired by two major pathways: Non-homologous end-joining or Homologous Recombination. 1. Non-homologous end-joining begins with the binding of the Ku70/80 heterodimer followed by the DNA-PK catalytic subunit. Depending on the nature of the break, it may then be further processed by a combination of polymerases or helicases prior to final ligation of the break ends by the Ligase IV-XRCC4 complex, catalysed by XLF. 2. Homologous Recombination begins with the binding of the Mre11-Rad50-NBS1 complex to the break ends. Mre11

performs short-range resection, followed by long range resection by Exo1 or DNA2/WRN/BLM to form long, 3' ssDNA intermediates. RPA binds the long ssDNA tracts formed by resection and Rad51 is loaded onto the ssDNA, assisted by BRCA2 and associated proteins. The Rad51-bound DNA forms a synaptic filament which is able to perform a homology search, invading the sister chromatid and using it as a template for repair. Final repair products may then arise via formation of several intermediates 3. The ATM-kinase is an important signalling event activated by double-strand breaks and performs multiple regulatory functions including checkpoint activation, chromatin remodelling and transcriptional regulation. Please note that this diagram is a simplified schematic of the DSB repair process, and for a more in-depth review, the reader is referred to several excellent review articles cited in the text.



Barriers	Viral Vectors	Nanoparticles	RNAi Conjugates
RNAase Degradation	Viral envelope encapsulation protects from nucleases	Complexation within nanoparticle protects from nucleases	Chemical modification of oligonucleotide structure for nuclease resistance
Liver Uptake	*Weakness: Causes adenoviral mediated hepatic toxicity*	Polyethylene glycol (PEG), monoclonal antibody decoration	Less for smaller, neutral charge conjugates
Renal Excretion	Large particle size (90-100 nm) reduces excretion	Large particle size (and sometimes charge) reduces excretion	*Weakness: small size leads to efficient removal by glomerular filtration*
Organ/Tumour Targeting	Directable tissue tropism by pseudotyping	Enhanced permeation and retention effect, active targeting (e.g. antibodies)	Conjugation with antibodies, aptamers, peptide ligands
Cell Wall Penetration	Receptor-mediated endocytosis	Decoration with antibodies, aptamers, peptides, ligands etc.	Conjugation with antibodies, aptamers, peptide ligands
Endosomal Escape	Fusion with endosomal membrane	Fusogenic lipids/proteins, "proton sponge" effect	Cell penetrating peptides

Figure 3 Barriers to Systemic siRNA Delivery There are several challenges to the efficient delivery of RNAi therapeutics to tumours following systemic administration of the drug into the circulation (left). RNAi delivery technologies have developed a variety of strategies to address these barriers (right).

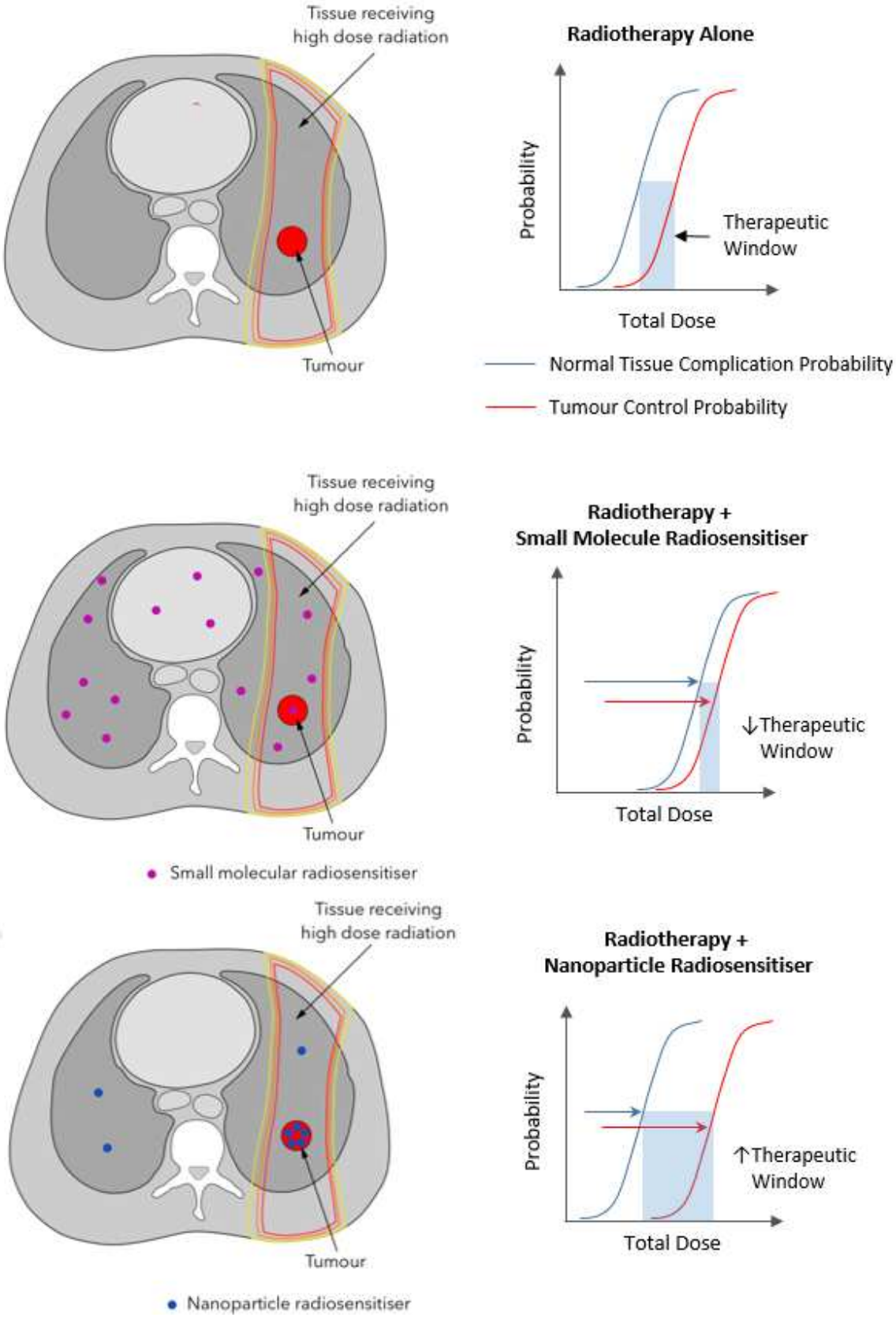


Figure 4 Radiation therapy combined with nanoparticle delivery of RNAi to tumour

Radiotherapy delivered to a lung tumour (left panel) seen as an axial cross section with dosimetry. Comparison between no radiosensitiser, small molecule radiosensitiser and a nanoparticle radiosensitiser. Graphs (right) illustrate concept of therapeutic ratio as a relationship between normal tissue complication probability (NTCP) and tumour control (TC) dose-response curves. Biodistribution of small molecule radiosensitisers to both tumour and normal tissue shift both TCP and NTCP curve not improving therapeutic ratio. Biodistribution of nanoparticle radiosensitiser preferentially to the tumour shifts TCP more than NTCP thereby widening the therapeutic window.

- RNA interference (RNAi) is an emerging therapy that can precisely suppress proteins in a tumour cell
- Proteins involved in the detection and repair of DNA damage can be targeted by RNAi to sensitize cancers to radiotherapy
- Newly developed delivery platforms are surmounting the obstacles to the clinical translation of RNAi