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Review

Ataxia-Telangiectasia Mutated and the Mre11-Rad50-NBS1 Complex: Promising Targets for Radiosensitization

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Radiotherapy plays a central part in cancer treatment, and use of radiosensitizing agents can greatly enhance this modality. Although studies have shown that several chemotherapeutic agents have the potential to increase the radiosensitivity of tumor cells, investigators have also studied a number of molecularly targeted agents as radiosensitizers in clinical trials based on reasonably promising preclinical data. Recent intense research into the DNA damage-signaling pathway revealed that ataxia-telangiectasia mutated (ATM) and the Mre11-Rad50-NBS1 (MRN) complex play central roles in DNA repair and cell cycle checkpoints and that these molecules are promising targets for radiosensitization. Researchers recently developed three ATM inhibitors (KU-55933, CGK733, and CP466722) and an MRN complex inhibitor (mirin) and showed that they have great potential as radiosensitizers of tumors in preclinical studies. Additionally, we showed that a telomerase-dependent oncolytic adenovirus that we developed (OBP-301 [telomelysin]) produces profound radiosensitizing effects by inhibiting the MRN complex via the adenoviral E1B55kDa protein. A recent Phase I trial in the United States determined that telomelysin was safe and well tolerated in humans, and this agent is about to be tested in combination with radiotherapy in a clinical trial based on intriguing preclinical data demonstrating that telomelysin and ionizing radiation can potentiate each other. In this review, we highlight the great potential of ATM and MRN complex inhibitors, including telomelysin, as radiosensitizing agents.

Key words: ATM (ataxia-telangiectasia mutated), MRN (Mre11-Rad50-NBS1) complex, radiosensitization, adenovirus, E1B55kDa

Radiotherapy is one of the standard treatment options for various malignant cancers and is often combined with surgical resection and/or chemotherapy as a part of multidisciplinary treatment. More than 50% of patients with cancer receive radiotherapy at some point during their treatment process [1]. Like surgical resection, radiotherapy is a local treatment, and it often targets not only primary tumors but also regional lymph nodes. One of the advantages of radiotherapy over surgical resection is that it is less invasive; for that reason, radiotherapy contributes significantly to treatment of cancers in areas of the body in which resection could greatly impair quality of life, such as the esophagus and the head and neck. Although the systemic side effects of radiotherapy are much less severe than those of chemotherapy, radiotherapy sometimes causes severe local adverse effects such as radiodermatitis, because normal tissues adjacent to tumors are usually included in the radiation

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fields. Although both stereotactic and fractionated radiotherapy have contributed to the improvement of irradiation methods in clinical practice, radiotherapy still has plenty of room for improvement [2, 3].

Hypoxia is one of the major limitations of radiotherapy, and researchers have made many attempts to improve it, such as through oxygenation, blood transfusion, and treatment with erythropoietin [4–6]. Although the oxygen level in a tumor is one of the most important factors in its response to radiotherapy, improving the local tumor control and survival rates for radiotherapy using pretreatment oxygenation is controversial. In one study, correction of tumor hypoxia significantly improved the locoregional tumor control and overall survival rates after radiotherapy for head and neck cancer, but was less effective for other types of cancer [7]. Although the rationale for intratumoral oxygenation before radiotherapy appears to be convincing, oxygenation alone does not improve radiotherapy sufficiently.

Many studies have been conducted in an attempt to improve radiotherapy, with much of the work being based on either of 2 hypotheses (Fig. 1). The first is that radiosensitizing agents should increase the cytotoxic effects of radiation on cancer cells by increasing the cells' radiosensitivity. The second is that radioprotective agents should decrease the adverse effects of radiation on normal cells by increasing their radioresistance. In this review, we describe several chemotherapeutic and molecularly targeted agents that have displayed radiosensitizing effects in preclinical and/or clinical studies and then focus on the potential of inhibitors of ataxia-telangiectasia (A-T) mutated (ATM) and the Mre11-Rad50-Nijmegen breakage syndrome (NBS) 1 (MRN) complex as radiosensitizing agents. Furthermore, we highlight the great potential of OBP-301 (telomelysin), a telomerase-dependent oncolytic adenovirus that we developed, as an MRN complex inhibitor.

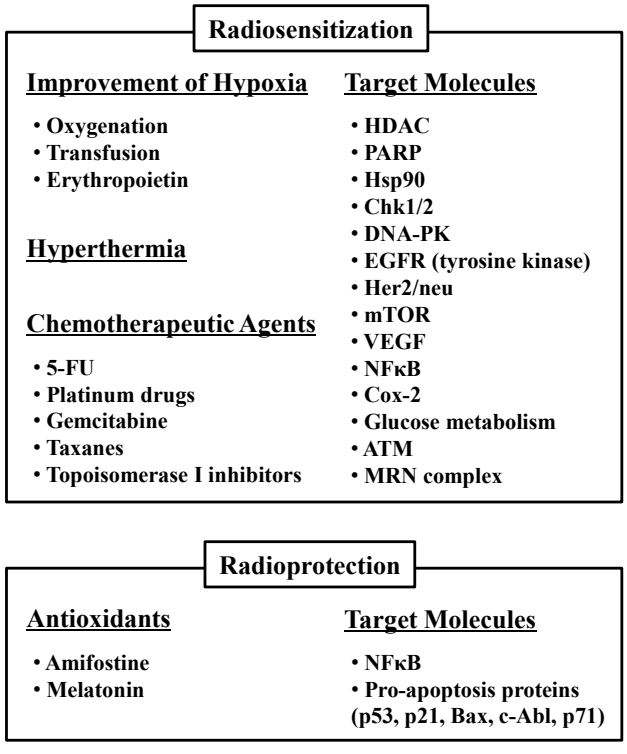

Fig. 1 Approaches to improvement of radiotherapy. Radiosensitizing agents are designed to increase the cytotoxic effects of radiation on cancer cells, and radioprotective agents are designed to decrease the adverse effects of radiation on normal cells. Hsp90, heat shock protein 90; NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2.

DNA Double-Strand Break Response: DNA Repair and Cell Cycle Checkpoints

Following DNA double strand-breaks (DSBs) induced by ionizing radiation, DNA repair and cell cycle checkpoints are the main mechanisms of maintenance of genomic stability [8]. Cells have several checkpoints that function at various phases of the cell cycle. Specifically, the G1/S and intra-S checkpoints prevent inappropriate DNA replication, whereas the G2/M checkpoint prevents cells with DNA damage from entering mitosis. When these checkpoints detect DNA damage at each phase, they induce cell cycle arrest and make time for repair of DNA damage. ATM plays a central role in the DNA damage response pathway by controlling the checkpoints via effector proteins such as Chk1, Chk2, p53 and BRCA1.

Homologous recombination (HR) and nonhomologous end joining (NHEJ) are major DNA DSB repair pathways, and cells use them according to the phase of the cell cycle and condition of the DSB ends [9, 10]. HR provides accurate genetic recombination using a sister chromatid as a template, which is essential for maintenance of genomic stability. Although HR is a desirable method of DNA DSB

repair, it is limited in cells during the S and G2 phases because of the need for a sister chromatid. NHEJ is a simple method of directly connecting the DSB ends. Although NHEJ is not as accurate as HR, it plays an important role in minimizing DNA damage, especially in cells in the G0 and G1 phases, in which HR is not available. Ku70/80, the DNA-dependent protein kinase, catalytic subunit, and DNA ligase IV are major contributors to NHEJ.

DNA repair and cell cycle checkpoints must cooperate closely to repair DNA damage and maintain genomic stability. Defects in this network produce dysfunction in the repair of DNA damage induced by ionizing radiation, which results in enhancement of the cytotoxic activity of radiation. Thus, molecules involved in these mechanisms can be suitable targets for radiosensitization.

Chemotherapeutic Agents as Radiosensitizers

As described above, radiotherapy is often combined with chemotherapy, and several chemotherapeutic agents are known to enhance the radiosensitivity of cancer cells [11, 12]. 5-Fluorouracil (5-FU), one of the most commonly used chemotherapeutic agents, is a member of the thymidylate synthase inhibitor family; these inhibitors produce cytotoxic effects by interfering with DNA synthesis [13]. Researchers have tested the combination of 5-FU and ionizing radiation and shown it to be effective against various types of cancers. This combination is a central component of current chemoradiation regimens [14].

Cisplatin, another commonly used chemotherapeutic agent, causes cytotoxicity by cross-linking DNA and interfering with cell division. Although cisplatin use is often combined with radiotherapy, oxaliplatin, another platinum derivative, has displayed more profound radiosensitizing effects [14, 15].

Gemcitabine, which is a nucleoside analogue that produces cytotoxic activity by blocking DNA replication, is another chemotherapeutic agent that is considered to be a radiosensitizer [16]. In preclinical studies, gemcitabine produced radiosensitization by interfering with Rad51 function and HR repair [17] as well as by redistributing cells into S phase by correlating with Chk1 and Chk2 [18]. Gemcitabine and radiotherapy have been shown to exert synergistic effects against cancers of the lung, pancreas, and head and neck in several clinical trials [19–21].

Taxanes such as paclitaxel and docetaxel produce cytotoxic activity by disrupting the function of microtubules that lead to cell division. A remarkable point is that taxanes arrest cells at the G2/M phase, which is the phase at which ionizing radiation is most effective [22]. Not only preclinical studies but also several clinical trials of regimens including taxanes and ionizing radiation used to treat cancers of the head and neck, esophagus, and lung have shown that taxanes are effective radiosensitizers [23–27].

Topoisomerase I inhibitors such as irinotecan, topotecan, and camptothecin interfere with topoisomerases, which are enzymes that are essential for winding and unwinding the DNA double helix during DNA replication and repair. Considering that ionizing radiation targets DNA and causes DNA DSBs, the combination of a topoisomerase I inhibitor and ionizing radiation may produce synergistic effects. Many preclinical studies using cultured cells and animal models have supported the synergy of this combination, although the specific mechanism of the synergistic effects remains unclear [28]. Also, many clinical trials have shown that these combinations are effective against various solid tumors, including head and neck, esophageal, lung, and brain tumors [29–32].

Molecularly Targeted Therapy for Radiosensitization

Although traditional chemotherapeutic agents that target rapidly dividing cells are still central to current cancer therapy, the attention of scientists is moving toward targeted therapy, which is expected to increase the effectiveness of treatment against cancer cells while reducing its harmfulness to normal cells [33]. Several small molecules and monoclonal antibodies that target epidermal growth factor receptor (EGFR), Her2/neu receptor, and vascular endothelial growth factor (VEGF) are currently in clinical use, and investigators have developed various types of molecularly targeted agents and are currently testing them in clinical trials [34, 35]. Some examples of molecularly targeted agents that are undergoing testing in clinical trials and expected to be used as radiosensitizers of tumors are described below.

Histone deacetylases (HDACs) are enzymes that control histone acetylation in coordination with the

opposing actions of histone acetyltransferases and play important roles in the regulation of gene expression. Physicians have long employed HDAC inhibitors such as valproic acid as anticonvulsants and mood-stabilizing drugs in the clinic, and use of these agents recently has generated a great deal of interest in their potential as antitumor drugs [36]. HDAC inhibitors have induced tumor-selective apoptosis and growth arrest in preclinical studies and exhibited effectiveness against tumors alone or in combination with chemotherapy in many clinical trials [37, 38]. To date, two HDAC inhibitors approved by the U.S. Food and Drug Administration—vorinostat and romidepsin—are in clinical use for treatment of T-cell lymphoma. Regarding the potential radiosensitizing effect of HDAC inhibitors, histone hyperacetylation induced by HDAC inhibitors appears to increase the cytotoxic activity of ionizing radiation [39, 40], and several clinical trials are testing these inhibitors in combination with radiotherapy for many types of cancer [41, 42].

Poly (ADP-ribose) polymerase (PARP) enzymes are proteins that play critical roles in DNA repair and replication. PARP1, which is the most abundant PARP and accounts for most PARP activities in cancer cells, binds to both DNA single-strand breaks (SSBs) and DSBs, but its role in SSB repair is better established. Although PARP inhibitors mainly contribute to SSB repair and often do not directly contribute to DSB repair, which is more critical for cell survival, defects in HR brought about by PARP inhibitors appear to increase the cytotoxic activity of ionizing radiation, especially in cells that are defective in DSB repair or NHEJ function [43–46]. Many PARP inhibitors are currently in clinical trials as single agents or in combination with DNA damage-inducing chemotherapeutic agents, and the PARP inhibitor ABK-888 administered in combination with radiotherapy recently entered clinical trials [47].

In addition, inhibitors of heat shock protein 90 or Chk1/2, some of which are currently in clinical trials as monotherapy or in combination with chemotherapeutic agents, have exhibited potential as radiosensitizers in preclinical studies, although combinations of them with radiotherapy have yet to be tested in clinical trials as far as we know [48–50]. Some EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib and VEGF inhibitors such as bevacizumab, which are currently in clinical use for cancer therapy, also have displayed radiosensitizing effects in many preclinical studies and clinical trials [51].

ATM as a Target for Radiosensitization

As described above, molecules involved in DNA repair or cell cycle checkpoints can be targets to enhance tumor radiosensitivity. Interest in molecularly targeted therapy has deepened our understanding of the signaling pathways for DNA repair and cell cycle checkpoints, and ATM has been revealed to play a central in these signaling pathways. Studies originally identified the *ATM* gene in A-T, a disease that causes several severe disabilities, such as cerebellar degeneration, immunodeficiency, hypersensitivity to radiation and genomic instability, and increased incidence of malignancies [52, 53]. All patients with A-T have mutations in the *ATM* gene, and intensive investigation of such patients and A-T cells has contributed to the elucidation of ATM function. The construction of the ATM protein is similar to that of ATM- and RAD3-related (ATR), the DNA-dependent protein kinase, catalytic subunit, and mammalian target of rapamycin (mTOR), and ATM belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family.

Following DNA damage, ATM immediately activates signaling pathways for DNA repair and cell cycle checkpoints. Although recent studies have shown that downstream signaling of ATM is becoming increasingly complicated, p53 and Chk2 are undoubtedly the main targets of ATM and control the G1/S and G2/M checkpoints while interacting with each other. Also, inhibition of these checkpoints allows damaged cells to move to the mitotic phase without undergoing proper DNA repair, leading to mitotic catastrophe, which is currently considered a main cause of cell death induced by radiotherapy [54–56]. Moreover, ATM is known to affect HR repair by directly or indirectly phosphorylating at least 12 targets, such as BRCA1/2 and NBS1, and defects in ATM function lead to dysfunction in HR repair [57, 58]. These findings indicate that targeted ATM inhibition is an attractive approach to enhancing tumor radiosensitivity.

Caffeine and wortmannin, which are nonspecific PI3K inhibitors, have been widely used in studies related to ATM/ATR functions [59, 60]. However,

some of the effects of caffeine and wortmannin in cells, such as apoptosis and checkpoint abrogation, are caused not only by ATM/ATR inhibition but also by other factors in the PI3K family [60, 61]. Recently, researchers developed several more specific ATM and ATM/ATR inhibitors—KU-55933, CGK733, and CP466722—and tested their potential as radiosensitizers in preclinical studies. KU-55933 was found to exhibit a specific inhibitory effect on ATM but not on other PI3K-family proteins, such as PI3K, DNA-PK, ATR, and mTOR, and sensitized cells to ionizing radiation by blocking phosphorylation of γH2AX, NBS1, and Chk1 [62]. CGK733 demonstrated selective inhibition of ATM and ATR, which led to blockage of the checkpoint signaling pathways, and researchers showed that its inhibitory effects were more beneficial than its small interfering RNA-mediated inhibition [63]. CP466722 exhibited inhibition of ATM and its downstream signaling pathways in the same way that KU-55933 did, and investigators emphasized that transient (4 h or less) inhibition of ATM expression was sufficient to increase the radiosensitivity of tumor cells [64]. Small interfering RNAs and antisense DNA for ATM also exhibited potent radiosensitizing effects [65, 66]. Based on this preclinical evidence, ATM inhibitors are expected to be promising candidate radiosensitizers.

The MRN Complex as a Target for Radiosensitization

Although the importance of the ATM signaling pathway in DNA repair and cell cycle checkpoints has been established, the MRN complex has emerged as an essential factor in ATM activation. Mre11 and Rad50 were originally isolated from the yeast *Saccharomyces cerevisiae* in genetic screens in which an Mre11 mutant was defective in meiotic recombination [67] and a Rad50 mutant was sensitive to DNA damage [68]. NBS1 was isolated as a member of the complex that binds with Mre11 and Rad50, and mutations in this gene cause NBS, which is characterized by high cancer incidence, cell-cycle-checkpoint defects, and radiosensitivity [69]. Mutations in the *Mre11* gene have been reported to cause A-T like disorder [70], and deficiency of the *Rad50* gene causes NBS-like disorder [71]. The indispensability of the MRN complex to cells is emphasized by the fact that null mutations of either of these genes cause embryonic lethality in mice [72]. The Mre11 protein is uniformly distributed in the nucleus under undamaged conditions, but it migrates to sites of damage within 30 minutes after DNA DSB induction and forms a complex with Rad50 and NBS1, which is visualized as nuclear foci [73].

The MRN complex plays important roles in signal transduction related to DNA repair and cell cycle checkpoints [10]. One of these roles is activation of the ATM/ATR signaling pathway. Dysfunction of the MRN complex results in impairment of the ATM signaling pathway, which leads to hypersensitivity to DNA-damaging agents. The MRN complex has also been reported to contribute to the DNA DSB-repair pathway directly or indirectly via ATM activation [9]. In the HR repair process, the MRN complex serves as a primary damage sensor and is involved in the early steps of HR repair, which include processing of the broken DNA ends: in other words, removal of the 5' strand to uncover the 3' single strand [74]. Whereas Ku70/80 and DNA-PK are well known to be the main components in NHEJ, the importance of the MRN complex to NHEJ has only recently been demonstrated, and whether the MRN complex is correlated with Ku70/80 and DNA-PK in NHEJ remains unclear [10, 75].

As might be expected from the fact that mutations in members of the MRN complex are hypersensitive to DNA DSBs, inhibitors of the MRN complex enhance the cytotoxic activity of ionizing radiation. Although disruptions of the MRN complex by gene therapy have been reported to be effective in combination with radiotherapy, researchers recently isolated a novel small-molecule inhibitor of the MRN complex called mirin from a chemical genetic screen [76, 77]. Mirin inhibited MRN complex-dependent ATM activation and Mre11-associated exonuclease activity, leading to abolishment of the G2/M checkpoint and impairment of HR repair. These results are consistent with the known and anticipated functions of the MRN complex. Considering the importance of the MRN complex in DNA repair and cell cycle checkpoints, MRN complex inhibitors appear to be very promising as radiosensitizers.

The Radiosensitizing Effect of the Adenoviral E1B55kDa Protein

We recently demonstrated that telomelysin sensitizes cancer cells to the cytotoxic activity of ionizing radiation [78]. Telomelysin is a telomerase-dependent oncolytic adenoviral agent whose replication is controlled by the human telomerase reverse transcriptase (hTERT) promoter. Telomelysin can thus induce cell death via oncolysis by replicating only in cancer cells whose hTERT activity is high [79–81]. An American Phase I clinical trial of single-agent telomelysin evaluated the clinical safety and pharmacokinetics of the agent in the human body following its approval by the U.S. Food and Drug Administration in 2006. When injected intratumorally in patients with various solid tumors such as melanoma, sarcoma, lung cancer, breast cancer, and head and neck cancer, telomelysin proved to be effective and well-tolerated without any severe adverse events [82].

The adenoviral E1B55kDa protein has been reported to play an important role in creating the optimal intracellular environment for adenoviral protein synthesis by inhibiting the function of the MRN complex and p53 in cooperation with the adenoviral E4 protein [83]. Inhibition of the MRN complex is also considered to be a self-defense response to concatemer formation of the double-strand DNA genome of adenovirus by the MRN complex [84–86]. We showed that expression of the MRN complex in cancer cells began to decrease about 24 h after telomelysin treatment, when the E1B55kDa protein began to be expressed, which led to inhibition of ATM phosphorylation by ionizing radiation and inhibition of DNA repair. We determined the importance of the presence of E1B55kDa in regard to this inhibitory effect by comparing telomelysin with the E1B-defective oncolytic adenovirus dl1520 (onyx-015), which has been used in many clinical trials [87].

We demonstrated that inhibition of the MRN complex by telomelysin via the E1B55kDa protein produced a profound radiosensitizing effect *in vitro*; interestingly, on the other hand, ionizing radiation increased the cytotoxic activity of telomelysin, presumably by increasing viral uptake into cancer cells, which means that telomelysin and ionizing radiation potentiate each other. Furthermore, combined therapy with telomelysin and ionizing radiation exhibited a strong synergistic antitumor effect in animal studies [78]. A clinical study of the combination of telomelysin and ionizing radiation against cancers of the head and neck and esophagus is currently under consideration in Japan, and additional telomelysin-based treatment is expected to contribute to improvement of the survival rates and quality of life in patients with these cancers. Moreover, this inhibitory effect on the MRN complex via the E1B55kDa protein may apply to not only telomelysin but also all of the other oncolytic adenoviruses that produce this protein, which may provide new clues to clinical applications of oncolytic adenovirotherapy (Fig. 2).

Perspectives on ATM and MRN Complex Inhibitors

Precise cellular responses to DNA DSBs require efficient recognition of the damaged DNA sites and organized activation of the signaling pathways leading to DNA repair and cell cycle checkpoints. Numerous preclinical studies have shown that ATM and the MRN complex play critical roles in this response, which indicates that these molecules are promising targets for radiosensitization. In fact, the ATM and MRN complex inhibitors described above have exhibited profound radiosensitizing effects in preclinical studies. The next step should be to test these inhibitors toward clinical application is to be tested in clinical settings, but to our knowledge, none of them have entered clinical trials.

One of the factors that could impede the success of ATM and MRN complex inhibitors in clinical trials is tumor selectivity. The expression and functions of ATM and the MRN complex do not appear to differ much in cancer cells and normal cells, which means that unless these inhibitors are delivered to tumors selectively, severe adverse events may occur when they are combined with radiotherapy. Recent developments in the field of drug delivery could have remarkable outcomes when combined with developments in the field of drug discovery. For example, nanomedicine has revolutionized drug delivery, and nanosized carriers such as liposomes, polymers, and micelles increase the stability of therapeutic drugs in the bloodstream [88]. Moreover, these carriers can acquire tumor-targeting potential by being equipped with antibodies or peptides that target biomarkers that are overex-

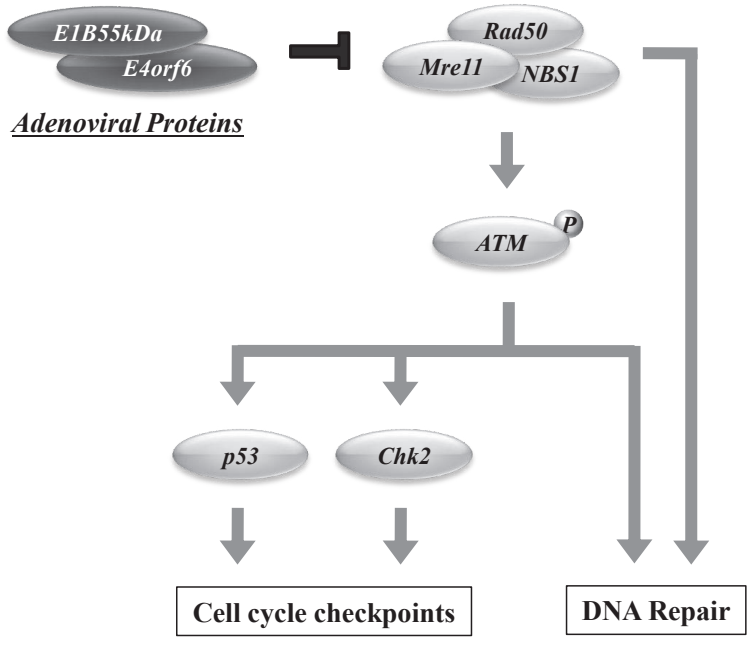

Fig. 2 The molecular mechanism of radiosensitization via the adenoviral E1B55kDa protein. E1B55kDa inhibits the function of the MRN complex in cooperation with the adenoviral E4orf6 protein, which inhibits the ATM signaling pathway and leads to cell-cycle-checkpoint abrogation and DNA-repair dysfunction.

pressed in tumors [89]. This type of improvement in drug delivery may be necessary for the use of ATM or MRN complex inhibitors before they enter clinical trials.

Regarding tumor-targeting potential, telomelysin may be a step ahead of these ATM or MRN complex inhibitors because its effect is strictly limited to cancer cells with high telomerase activity levels. Moreover, Phase I clinical trials in the United States have already determined the safety of monotherapy with telomelysin, and this agent is about to undergo testing in combination with ionizing radiation in a clinical trial in Japan.

However, telomelysin also has some challenging drawbacks that must be overcome in order to increase its attractiveness and its application as a cancer therapeutic agent. One of these issues is that telomelysin currently can only be administered via local injection and not systemically. The majority of intravenously administered adenoviruses become trapped in the liver, and thus they are not present at sufficient levels at the tumor sites [90]. In addition, most people have neutralizing antibodies against adenovirus type 5, which is one of the common cold viruses. Therefore, telomelysin, which consists of this adenovirus, is removed by the immune system immediately after systemic administration. For this reason, application of telomelysin is currently limited to cancers confined within locoregional areas, and improvements in telomelysin that would facilitate its systemic delivery will be needed before the drug can be used in the treatment of distant metastases.

In summary, the field of targeted radiosensitization of tumors is developing rapidly and drawing much attention. ATM and the MRN complex play central roles in the DNA DSB-response pathways, and inhibitors of these molecules are promising candidate radiosensitizing agents. An upcoming clinical trial of telomelysin combined with ionizing radiation will test this agent's function as an MRN complex inhibitor, and the outcome of this trial is expected to open new opportunities for other oncolytic adenoviruses that produce the E1B55kDa protein as promising radiosensitizers.

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