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Mechanisms of injury to normal tissue after radiotherapy: a review

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Abstract

Background—The benefits of radiotherapy (RT) for cancer have been well documented for many years. However, even with targeted radiation delivery, many patients treated with radiation develop adverse effects. The purpose of this review was to analyze the current research into the biological basis of RT-induced normal tissue damage.

Methods—The PubMed and EMBASE databases were reviewed for articles on adverse effects of RT on normal tissue published from January 2005 through May 2012. Subsequently, abstracts of these articles were reviewed to identify articles with information relevant to the biological basis of RT-induced DNA damage and DNA repair. In addition, reference lists of the articles identified by the database search were reviewed, and referenced articles that seemed relevant were reviewed with no limitations on publication date.

Results—The database searches yielded 1751 publications. Of these, 1729 were eliminated because they did not address fundamental biology or were duplicates. A total of 22 articles were included. These articles revealed that many adverse effects are driven by chronic oxidative stress that affects the nuclear function of DNA repair mechanisms. Among normal cells undergoing replication, cells in S phase are most radioresistant because of overexpression of DNA repair enzymes, while cells in M phase are especially radiosensitive. Cancer cells exhibit increased radiosensitivity due to a breakdown in cell cycle checkpoints and repair mechanisms, and this increased radiosensitivity leads to accumulation of irreparable DNA lesions and cell death. Irradiated cells have an indirect effect on the cell cycle and survival of co-cultured non-irradiated cells. Method of irradiation and linear energy transfer to cancer cells versus bystander cells is shown to have an effect on cell survival, both cancerous and healthy.

Conclusions—RT-induced increases in reactive oxygen species in irradiated cells may signal healthy cells by increasing metabolic stress and creating DNA lesions. While normal cells express a strong ability to repair RT-induced DNA lesions, the side effects of RT and bystander cell signaling may have a larger impact than previously acknowledged.

1. Introduction

Over 1.6 million new patients in the United States were diagnosed with cancer in 2012, and almost two-thirds of these patients were treated with radiotherapy (RT) [1]. The benefits of RT for cancer have been well documented for many years. These benefits, however, can be

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outweighed by radiation-induced damage to neighboring normal tissues as a result of either direct exposure to radiation or the so-called bystander effect, which refers to biological effects in nonirradiated cells caused by signals from irradiated cells [2,3].

RT is based on the concept that the DNA repair capacity of cells with sublethal damage from RT is generally greater in healthy cells than in cancerous cells. In other words, cancer cells are more susceptible to radiation than are normal cells. The mechanisms underlying RT-induced DNA damage and post-RT DNA repair have been studied in detail; however, there still exist many gaps in knowledge on how these complex systems are entwined.

2. Selection of articles for inclusion in review

The PubMed and EMBASE databases were reviewed for articles on adverse effects of RT on normal tissue published from January 2005 through May 2012. Subsequently, abstracts of these articles were reviewed to identify articles with information relevant to the biological basis of RT-induced DNA damage and DNA repair. In addition, reference lists of the articles identified by the database search were reviewed, and referenced articles that seemed relevant were reviewed with no limitations on publication date. The database searches yielded 1751 publications. Of these, 1729 were eliminated because they did not address fundamental biology or were duplicates. A total of 22 articles were included.

3. Types of radiation-induced DNA damage

To address possible points of intervention to reduce normal tissue toxic effects (NTT), it is essential to first understand the methods by which ionizing radiation damages cells.

Ionizing radiation is naturally encountered as cosmic rays; alpha, beta, and gamma rays; x-rays; and some portions of the ultraviolet spectrum. Visible light is not usually considered ionizing radiation but can cause upwards of 1×10^5 DNA lesions per cell per day [4]. On the basis of the rates at which energy is deposited into cells, the different forms of radiation can be classified as forms of low-linear-energy-transfer (LET) radiation, the most prevalent form used for cancer therapy, or high-LET radiation, which is typically encountered as neutrons, heavy ions, and pions.

Low-LET photon beams (photon radiation) from cesium 137, cobalt 60, iridium 192, and low-LET electron beams from linear accelerators are the most common form of RT. Electron beams produced by linear accelerators are useful for treating superficial lesions because the dose is deposited near the surface. Proton beams have higher LET than photon and electron beams and can be tuned to have low-LET through normal tissues with high LET at the end of the beam. Neutron beams have high LET and are used to treat some inoperable tumors, cancer of the head and neck, and prostate cancer. High-LET RT delivered using carbon ions (also called heavy ion radiation) can be effective against radioresistant cancers but is equally effective against normal tissues, raising risk.

Indirect DNA damage

When the high-energy particles of ionizing radiation penetrate the body and liberate electrons from atoms and molecules, individual chemical bonds are destroyed, which results in highly reactive ions and ion pairs commonly referred to as *reactive oxygen species* (ROS). ROS have been proposed to account for the majority of RT-induced cellular genetic damage [5].

When water, present in high concentrations throughout the entire body, is split by ionizing radiation, free radicals such as the hydroxyl ROS are produced. Hydroxyl radicals are highly

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reactive with DNA, proteins, and lipids. Hydroxyl radicals function in addition reactions across double bonds and are also able to detach hydrogen atoms from the methyl groups of thymine nucleic acids, creating mutations that must be repaired before cell division or transcription to maintain genomic stability [6]. This type of damage is predominantly associated with the linear sequence of nucleotides in DNA (primary structure).

Direct DNA damage

Ionizing radiation is also capable of directly damaging DNA and regulatory proteins. By splitting chemical bonds on the helical backbone, ionizing radiation is capable of creating single-strand breaks (SSBs) and double-strand breaks (DSBs) with any amount of exposure. DSBs are significantly more destabilizing for the genome than SSBs and have a high likelihood of ultimately leading to cell death or leading to poor DNA repair, such as single-strand annealing, which is mutagenic.

Both low-LET and high-LET radiation sources are capable of producing SSBs and DSBs; however, when the high-energy particles of low-LET radiation pass through the cell, fewer ionization events are likely to occur, and most of those events are SSBs. DSBs due to high-LET and low-LET RT are most likely to result from radiation exposure during phases of the cell cycle when chromatin is tightly condensed.

Cellular susceptibility to DNA damage by cell-cycle phase

As radiation exposure increases, the amount of damage increases proportionally, until enough damage is accumulated via direct and indirect means that the repair mechanisms are overcome and the cell becomes senescent, enters apoptosis, or loses the ability to regulate its own growth and becomes tumorigenic.

Proliferating cells have complex, finely regulated systems in place to control each stage of the cell cycle. Cells in the resting phase (G0) must typically receive very specific signals before they begin to prepare for DNA replication, a checkpoint that makes cells in the G0 phase somewhat radioresistant. Cells in the first growth phase (G1), when the cell enlarges, are somewhat radioresistant because of non-condensed DNA and regulated checkpoints. Transition from the G1 phase into the DNA replication phase (S phase) is safeguarded by sequentially activated cyclin-dependent kinases (CDKs) tasked with recruitment and amplification of genes integral for genomic replication during S phase. The G1 to S phase checkpoint prevents cells with damaged DNA from replicating and is called the restriction checkpoint; critical proteins here include P53, P21, and P16, all of which are considered CDK inhibitors [7]. Retinoblastoma (Rb), an important tumor suppressor protein in humans, binds transcription factors required for S-phase genes and prevents translocation of these transcription factors to the nucleus. Phosphorylation of Rb by CDKs releases these transcription factors; however, DNA damage causes accumulation of P53, which in turn activates CDK inhibitors that inhibit phosphorylation of Rb, delaying the transition into S phase until repairs are performed [8].

Cells in S phase are the most radioresistant, because the genes, enzymes, and proteins responsible for accurate genomic replication and repair are overexpressed in S phase compared with the other phases of the cell cycle. Once DNA replication has begun, CDK inhibitors remain prevalent to negatively regulate the progression of cells through the remainder of the cell cycle, and CDKs can stop progression altogether if genomic instability is detected. Mismatch repair (MMR) is also highly active during S phase and is capable of repairing DNA damage due to chemicals and RT as well as natural replication errors [9].

Cells in the second growth phase (G2) retain lingering repair enzymes from S phase and as such are relatively radioresistant. Before mitosis (M phase) begins, the cell must pass

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through the G2 checkpoint, where CDC25 (cell division cycle 25) phosphatase activates M-phase promoting factor (a cyclin-dependent kinase complex) under good genomic conditions in cells without DNA damage. If DNA damage is present, *ATM* (ataxia telangiectasia mutated) kinase phosphorylates CDC25, which in normal cells halts progression to mitosis and can eventually lead to destruction of CDC25 [10]. *ATM* kinase is a critical enzyme for CDK-mediated checkpoints as well as the damage response in cells, interacting with other protein complexes such as P53 and H2AX in response to DSBs [11] (Figure 1).

M-phase cells are especially sensitive to RT. Upon entry into M phase, chromosomes condense, and the mitotic spindle begins to pull chromosomes apart. An ionizing event that occurs when DNA is in a condensed state has a higher probably of causing a DSB than does an event that occurs when DNA is not condensed. The only currently understood checkpoint in M-phase cells is based on tension in the spindle apparatus.

Susceptibility of cancer cells versus normal cells to DNA damage

Cancer cells resemble undifferentiated stem cells more closely than they resemble the mature, differentiated cells around them. This is a result of genomic instability and failures in genetic checkpoints that allow cancer cells to escape the normal controls of the cell cycle, leading to an increase in the cells' proliferative capacity. Failed checkpoint controls can allow damaged DNA to be passed on to daughter cells. RT is effective in killing cancer cells because dividing cells (M-phase cells) are especially radiosensitive and cancer cells divide more often.

Additionally, cancer cells generally have reduced capacity to repair genomic lesions because cancer mutations are often related to the DNA repair process, allowing for DNA damage from other stages of the cell cycle to accumulate and be passed on to daughter cells [12,13]. In contrast, healthy cells subjected to low levels of ionizing radiation are generally capable of halting any ongoing replication and repairing the damage over time or, if the damage is irreparable, activating proper apoptosis pathways. Fractionated RT capitalizes on this concept by spreading out treatments over time, allowing normal cells to regenerate before additional doses are given.

4. Cellular response to DNA damage

Most types of human cells handle DNA damage with a standard response, regardless of whether the damage is direct damage in the form of SSBs and DSBs or indirect damage caused by ROS. Cells utilize a sensor-signal-effector mechanism driven by the initial perception of a DNA break or conformational change in the helical structure. The details of the sensor-signal-effector mechanism differ according to the type of DNA lesion being processed, but the overall sensor-signal-effector pattern holds true in each case. The DNA repair pathways discussed in this section are illustrated in Figure 2.

Response to DSBs

When a DSB occurs in a normal human cell, it elicits a reaction from *ATM* kinase (the sensor). *ATM* kinase recruits the autophosphorylated form of *ATM* and DNA-dependent protein kinase [14]. These kinases, in addition to other PI3 family kinases, phosphorylate histone H2AX on serine 139 [15]. The phosphorylated form is known as γ-H2AX and is an early reporter of DSBs. When γ-H2AX is present, signaling a DSB, local DNA structure relaxes and residues become less condensed, allowing space for repair proteins to bind to the DNA and facilitate nonhomologous end joining (NHEJ) or, if the cell is in S phase or G2 phase, homologous recombination (HR). In HR, the strand is first resected from the 5′ end. This produces an overhang that the broken strand is matched to. NHEJ is similar but without

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homologous pairs. The γ-H2AX signal protein can be visualized within seconds of initiation of a DSB and persists until repair is complete—for typical cells, approximately 24 to 48 hours. Other proteins (the effectors) are also present within minutes of detection of a DSB, including P53 binding protein 53BP1, and either guide the cell toward repair or ultimately force apoptosis by stimulating release of mitochondrial cytochrome c and subsequent activation of caspase-3 [16]. DSBs are the most difficult lesion for a cell to accurately repair and can lead to mutations such as deletions and insertions.

Response to SSBs

The cellular response to SSBs follows the same general pattern as the cellular response to DSBs. The main pathways used by human and eukaryotic cells in response to single strand damage are base excision repair (BER), which is favored for minor DNA repair; nucleotide excision repair (NER), which is favored when the helix is disrupted; and MMR. Most minor DNA lesions— including most SSBs, minor oxidative damage, and lesions resulting from light chemical mutagens and alkylation products—are processed with BER. Damaged residues are sensed by a glycosylase (e.g., methylpurine DNA glycosylase or uracil DNA glycosylase). The damaged base is then removed, preserving the DNA backbone and creating an apurinic/apyrimidinic site. Endonucleases then excise a portion of the injured DNA strand, generating a SSB. BER machinery then binds to the damage site and finishes the repair by synthesizing new DNA [17]. If the intermediate enzymes are disrupted and uncoupled from the repair process early, the damage site is treated as a SSB; otherwise, repair progresses to completion. NER is similar to BER for damage such as bulky adducts and pyrimidine dimers. MMR is used primarily to address erroneous incorporation of bases during replication but also plays a role in repairing some forms of DNA damage.

In one mechanism of SSB repair, poly(ADP-ribose) polymerase (PARP) acts as a damage sensor and signaling protein [18]. Upon binding to a SSB, PARP begins synthesis of ADP-ribose polymer chains that signal DNA-repairing machinery to form a repair complex. PARP can induce apoptosis in cells with excessive DNA damage by depleting the cell of NAD+ nucleosidase, which is required for every link in the growing ADP-ribose polymer chain.

PARP can be involved in DSB repair pathways but is not required for HR [19]. PARP is a popular target for inhibition by new radiosensitizing drugs (Figure 3): when a cell's ability to repair SSBs through BER is reduced, DNA damage accumulates, especially in cancer cells, many of which have degraded repair pathways to begin with. Ongoing research at The University of Texas MD Anderson Cancer Center has focused on PARP activation as a major pathway that can be damaged not only by pharmacologic agents but by RT itself.

5. Effects of radiation on bystander cells and tissues

Bystander effects, defined as biological effects in nonirradiated cells as a result of signals from irradiated cells, have been increasingly studied as an important consequence of RT that may lead to NTT [20,21]. Damage in bystander tissues and cells can take years to manifest [22].

Mechanisms of signaling from irradiated to nonirradiated cells ("bystander signaling") are still mostly unknown; however, a number of mechanisms have been proposed as integral to such signaling, including signaling due to small soluble chemical factors, electrical currents, ion concentrations, and even pressure waves [2,3,20,23]. *In vitro* studies looking at cell-cell communication pathways such as gap junctions have shed some light on the mechanisms of bystander signaling [24].

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In one study showing the bystander effect, P53, which accumulates in response to DNA damage [8], was analyzed in cultured fibroblasts after irradiation with high-LET alpha particles [25]. P53 was observed in a higher fraction of bystander cells than cells directly hit by alpha radiation. Gap junction inhibitors reduced this effect. When medium was transferred from irradiated cells to non-irradiated cells, P53 levels remained low in the non-irradiated cells, highlighting the significance of cell-cell interactions through gap junctions as the means of propagating stress responses in cells. However, other studies have found that medium transferred from irradiated cells to non-irradiated cells can have a detrimental effect on cell viability [26]. Current research at MD Anderson is focused on determining whether bystander signaling is mediated by a chemical that can be found in the medium. Our current research has focused on co-culture with inserts, which allows for medium exchange through a semipermeable membrane without cell-cell contact, eliminating the need to transfer the medium. This improved system has shown promise in determining the effects of bystander and stress signaling on apoptosis between isolated groups of cells.

Moving to living systems to look for bystander effects of RT and their contribution to NTT adds additional complexity. One study showed quantifiable DNA damage responses in shielded tissues *in vivo* when mice were administered targeted RT [27]. Shortly after exposure, γ-H2AX staining showed significant increases in DSB foci in tissues that had been shielded from direct exposure above levels of controls. This study is interesting because it showed similar results in two different strains of mice that conventionally react very differently to radiation. Results such as these are in line with results of other studies on the bystander effect *in vivo* [28]. From a clinical standpoint, such results help to explain the wide patient-to-patient variability in NTT by showing that radiation administration can elicit a far-reaching, invisible effect in patients regardless of genetic predisposition [29]. Mechanisms underlying different organs' reacting to the same isolated radiation event are unlikely to be based on cell-cell interactions, which suggests that soluble factors and ROS are more likely than direct contact to have played roles in signaling to other cells in the mice-with-shielded-tissues study described above.

More research is necessary to clarify the implications of RT downstream effects such as ROS in the context of genetic background and increases of DSBs. While most healthy (noncancer) cells tolerate SSBs very well, DSBs in healthy cells have negative implications in terms of genomic stability and cell survival [30]. Thus, better understanding of DSBs in bystander cells and tissues is critical.

6. Impact of LET on RT-induced tissue injury

As radiation interacts with matter, it loses its energy through interactions with atoms in its direct path. In RT, LET is defined as the average amount of energy lost per defined distance in tissue, as in the energy deposited into a handful of cells. LET occurs at different rates in different tissues, and quantification of LET in cellular systems is an important component of determining correct dosage in radiology.

Brahme recently published a thorough review of mechanisms, equations, and models of cell death in relation to LET, radiation type, DSB probability per dose, and more [31]. The models proposed in this study illuminate differences in the impact of radiation type and dose on different cell types under different metabolic conditions. Oxygen is a great radiosensitizer through mechanisms including ROS, so hypoxia, such as commonly exists in tumors, can drastically decrease response to RT, whereas the normoxia prevailing in normal tissues makes them susceptible to NTT.

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Medium-LET ions such as proton and neutron beams are most effective for causing DSBs in radioresistant cancer cells while minimizing damage to normal cells. Common low-LET radiation sources (photon and electron beams from cesium, cobalt, iridium, and linear accelerators) transfer too little energy for many deep or radioresistant cancers and require many fractions, while high-LET radiation from carbon ions and other heavy ion radiation can produce a high frequency of DSBs in normal cells, leading to NTT [31]. A proper radiation source should be chosen when available, and dose and fractionation should be determined on the basis of the tumor type, the tumor location, and the LET of the radiation source.

7. Discussion

The cellular response to DNA damage has been studied exhaustively; however, new discoveries are still being made, and connections between cellular signaling pathways involved in the DNA damage response are slowly being elucidated. ROS are a primary method of RT-induced cellular damage, if not the primary method of RT-induced cellular damage [24]. ROS can serve as more than locally destructive agents by inducing damage response mechanisms in, and possibly even directly damaging, tissues far removed from the treatment zone [23]. As most tumors are relatively hypoxic, ROS-induced damage occurs primarily within normal tissues and is thus of great concern clinically.

Mechanisms of DNA damage repair are complex; some repair pathways, such as PARP signaling, have been highlighted in this article. While DNA damage repair pathways have some redundancy, a failure of any repair system increases the chances of NTT and increases metabolic stress. DNA repair mechanisms exhibit feedback mechanisms balanced between damage repair and apoptotic pathways; by reducing the oxidative stress in non-target cells, the likelihood of unintentional cell death and NTT can be reduced.

Important questions remain regarding the long-term consequences of bystander signaling with various forms of RT as few forms of bystander signaling have been characterized. Research focusing on minor differences in repair mechanisms between different types of RT will be an important step to increase understanding of the DNA repair process. NTT begin in the cell; thus, any advances in understanding processes at the cellular level can help spur innovations that improve patient outcomes.

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Glossary of Terms

ATM	Ataxia telangiectasia mutated gene BER: Base excision repair
CDC25	Cell division cycle 25
CDK	Cyclin-dependent kinase
DSB	Double-strand break
HR	Homologous recombination
LET	Linear energy transfer
MMR	Mismatch repair
NER	Nucleotide excision repair

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NHEJ	Non-homologous end joining
NTT	Normal tissue toxic effects
PARP	Poly(ADP-ribose) polymerase
Rb	Retinoblastoma
ROS	Reactive oxygen species
RT	Radiotherapy
SSB	Single-strand break

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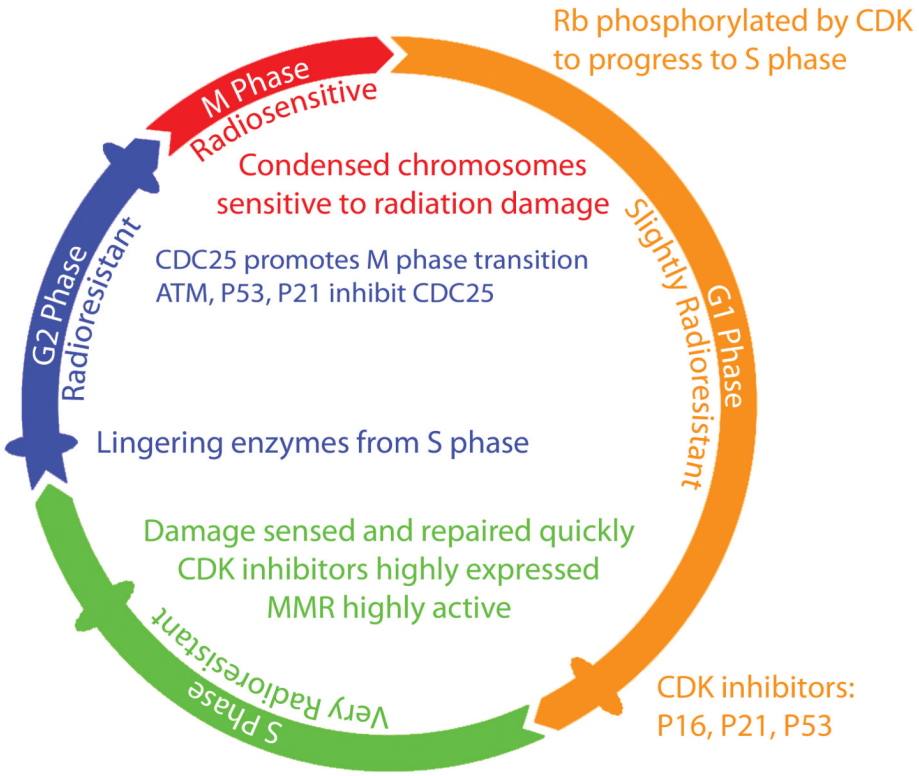

Figure 1.

The stages of the cell cycle differ with respect to radioresistance. CDK inhibitors such as P16, P21, and P53 are common checkpoint proteins expressed in radioresistant phases. In the G1 phase, where the cell is expanding, these CDK inhibitors are expressed to safeguard progression into the DNA synthesis phase (S phase). Once the cell enters S phase, these CDK inhibitors are relatively overexpressed, as is the enzymatic machinery responsible for DNA repair, to make sure that DNA synthesis is accurate and complete. Cells in the G2 phase are considered radioresistant because of lingering enzymes and CDK complexes from S phase. PI3 family kinases activated by *ATM* and others proteins lead to CDK inhibition and cell cycle arrest when DNA damage is detected. During early M phase (prophase), when chromosomes condense, the DNA becomes highly susceptible to radiation damage. In M phase generally, a relative lack of repair mechanisms, no major checkpoints to prevent progression to cell division, and thick, bulky, condensed DNA provide the perfect target for ionizing radiation. This is the basis of fractionated radiation treatments; to catch dividing cells at this phase of the cell cycle.

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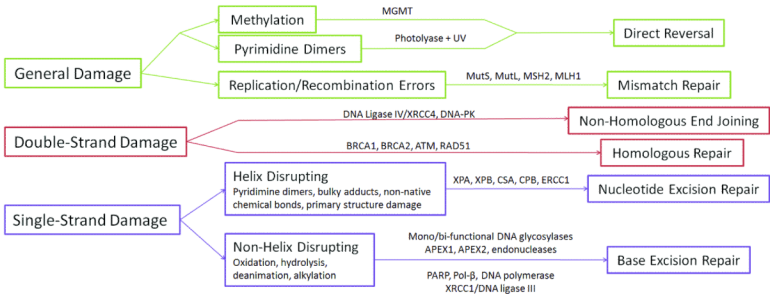

Figure 2.

DNA repair pathways with important proteins and enzymes at each stage. Some types of tumors have characteristic damage to specific repair pathways: breast, ovarian, and pancreatic cancer commonly lack functional proteins for HR, while colorectal cancer lacks functionality of MMR because of mutated hMLH1. Many cancer drugs target specific pathways that are functional in cancers to maximize DNA damage accumulation in response to chemotherapy and RT. Through better understanding of the genetic repair pathways listed (and others not listed), drugs can be better designed to target specific cancer genotypes instead of relying on multiple drugs to interrupt multiple pathways, which greatly increases NTT and side effects. UV, ultraviolet radiation.

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PARP Inhibitor Name	Phase	Information
First Generation		
3-aminobenzamide	Experimental	Early observations on PARP inhibition
Benzimidazole-4-carboxamide	Experimental	Precursor for drugs such as veliparib
Second Generation		
BMN-6T3	Phase I clinical	Solid tumors
MK-4827	Phase Ib clinical	Inhibits PARP1 & PARP2, solid tumors, melanoma, glioblastoma
CEP-9722	Phase II clinical	Non-small cell lung cancer
Veliparib	Phase II clinical	Solid tumors such as ovarian, breast, gastric, pancreatic, metastatic melanoma
Rucaparib	Phase II clinical	Solid tumors such as ovarian and breast
Iniparib	Phase III clinical	Solid tumors such as breast, squamous cell lung cancer
Olaparib	Phase III on hold	Solid tumors, non-Hodgkin's lymphoma, multiple myeloma, ovarian, colorectal

Figure 3.

Drugs targeting specific DNA repair pathways have proven effective as treatments for cancer, as many cancers have broken and/or ineffective alternate repair pathways. We focus here on PARP inhibitors, which have progressed to phase II and III clinical testing. This list of relatively new drugs based on PARP pathways highlights the importance of understanding repair mechanisms of cells. By designing PARP inhibitors specific for certain aspects of a single pathway, NTT are greatly reduced because of redundant pathways present in normal cells that are not present in their cancerous counterparts.

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