Design and synthesis of metal-based chemotherapeutic agents for targeted DNA interactions or DNA repair pathway modulation

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Chapter 1

Introduction

1.1 Platinum based chemotherapy

One of the first descriptions of cancer was reported in the Hippocratic Corpus (ca. 460–370 BCE) and is attributed to Hippocrates, the father of modern medicine.[1] It describes a case where the inner part of a wild cucumber mixed with honeycomb in water is prescribed to prevent tumor development in the uterus.[2] The field of oncology has been growing ever since and a wide variety of cancers and treatments have been reported. An important class of modern chemotherapy drugs are platinum based therapies. A 2018 case study in a hospital in Australia found that 46% of cancer patients undergoing treatment were prescribed a platinum drug.[3] The cytotoxic properties of cisplatin, the first platinum drug, Figure 1, were reported by Rosenberg *et al.* in 1965[4] and received US Food and Drug Administration (FDA) approval in 1978 for use in testicular and ovarian cancer.[5]

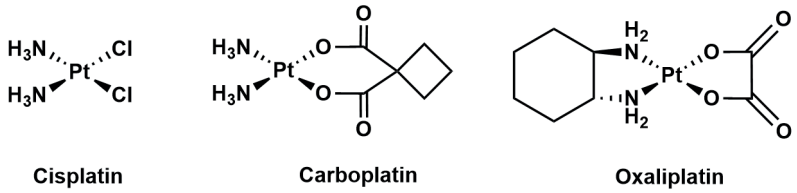

Figure 1. Molecular structure of Cisplatin and its FDA-approved derivatives Carboplatin and Oxaliplatin

The mechanism of cisplatin-induced cytotoxicity is well explored in the literature[6] and excellently reviewed by the Lippard group (figure 2).[7,8] Upon administration, cisplatin remains essentially inert in the bloodstream due to the high chloride concentration that suppresses hydrolysis of the chlorido ligands. Cellular uptake of cisplatin occurs *via* passive diffusion and active uptake by the copper membrane transporter CTR1. Inside the cell, the local lower chloride concentration induces cisplatin hydrolysis and formation of the electrophiles *cis*-$[Pt(NH_3)_2(Cl)(H_2O)]^+$ and *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ which react with a variety of nucleophiles. Although hydrolyzed cisplatin can react with a wide range of biological substrates, it is generally accepted that the main mode of action inducing cytotoxicity is by generating DNA damage *via* crosslinks in DNA. Mono-hydrolyzed cisplatin reacts with the N7 positions on the purine bases of DNA, thus forming coordination bonds between platinum and DNA.[9] The remaining chlorido ligand is subsequently hydrolyzed and substituted by another neighboring purine base, generating primarily 1,2- or 1,3-intrastrand crosslinks and only 1–3% interstrand crosslinks.[10] It is generally accepted that the intrastrand adducts are responsible for the cytotoxicity of cisplatin in cancer cells.[11,12] Despite the widespread success of cisplatin with a 90% cure rate in testicular cancer, it is plagued by side effects commonly associated with chemotherapy such as nephrotoxicity, neurotoxicity, and platinum resistance.[13] The FDA-approved cisplatin analogues, carboplatin and oxaliplatin, were developed to reduce side effects, Figure 1, but their application is also limited by low efficacy on certain forms of tumours and side effects.[14,15]

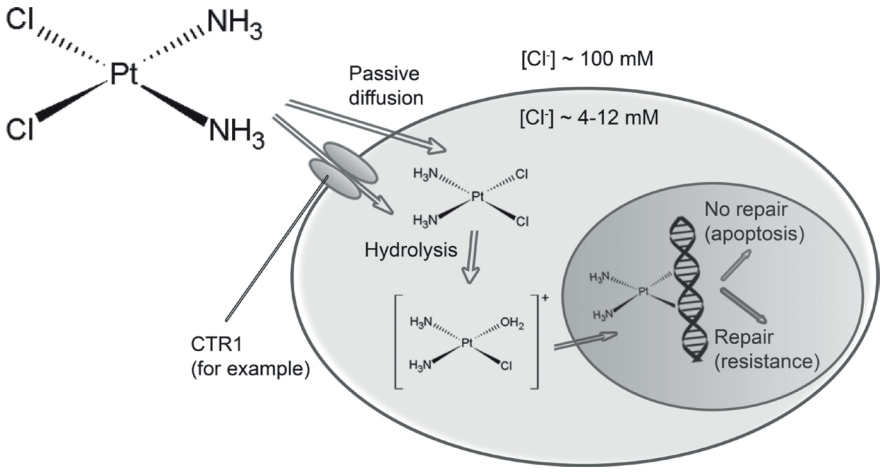

Figure 2. Mechanism of action for cisplatin, where the low intercellular chloride concentration facilitates hydrolysis and activates the platinum compound for DNA binding and the formation of crosslinks. Figure taken from Browning 2017.[6]

1.2 Photoactivated chemotherapy

A promising alternative to platinum-based therapies to increase selectivity is the photo activation of metal-based prodrugs. These therapies use a compound that remains inert in the dark, but upon light activation generates local cytotoxicity. Light-activated prodrugs can be divided in two distinct classes, those for photodynamic therapy (PDT) and those for photoactivated chemotherapy (PACT).

In PDT a compound, called a photosensitizer (PS), generates reactive oxygen species (ROS) upon light activation inducing cytotoxicity in an oxygen-dependent fashion.[16] PDT is further subdivided in type I and II, in type I the activated PS reacts with biological substrates by electron transfer to form radicals that in turn interact with oxygen to produce oxygenated products ($O_2^{\bullet-}$, $HO_2^{\bullet}$ and $HO^{\bullet}$),[17] while in type II the activated PS reacts directly with molecular oxygen by energy transfer generating cytotoxic singlet oxygen ($^1O_2$).[18] Their photophysical mechanisms are shown in Figure 3 and both start with the promotion from the ground state to the singlet Metal-to-Ligand-Charge-Transfer (^{1}MLCT) excited state, followed by intersystem crossing to the ^{3}MLCT. The ^{3}MLCT then reacts with substrates forming ROS, for type I, or via triplet-triplet annihilation with oxygen forming $^1O_2$ for type II. Ruthenium-polypyridyl PDT agent TLD1433, figure 4, was reported by the McFarland group for its near-unity singlet oxygen generation quantum yield and high phototherapeutic index (PI) of 1500 in HL-60 normoxic cells,[19] where PI is the ratio between the dark EC_{50} and the light EC_{50}. The compound is currently in phase II clinical study for non-muscle-invasive bladder cancer treatment.[20] The same group recently published a series of compounds that are based upon the [Ru(dnp)(dppn)(4-pic)]$^{2+}$ scaffold, where dnp = 2,6-di(1,8-naphthyridin-2-yl)pyridine, dppn = benzo[i]dipyrido[3,2-a:2',3'-c]phenazine, and 4-pic = 4-methylpyridine. The series

proved highly potent toward melanoma cells with EC_{50} ranging between 0.29–0.60 μM upon low energy near-infrared irradiation (NIR, 733 nm).[21] Ruthenium-dppn complexes are remarkable singlet-oxygen-generating PS due to the long-lived, ligand-based $^3\pi\pi^*$ excited state that is stabilized by the extended π-system.[22–24] Other compounds react by the type I mechanism. For example, Zhou *et al.* reported cyclometalated palladium compounds that self-assemble into nanorods. These self-assembling sensitizers exhibited excellent photodynamic properties with nanomolar EC_{50} values upon green light activation (515 nm) via a PDT type I mechanism.[25] One limitation of PDT therapy is that it relies on the presence of molecular oxygen in the light-irradiated tumor tissues: in a range of tumors, called hypoxic tumors, the O_2 concentration in the tumor tissue is low, in which case PDT can fail. Hypoxia is a characteristic property of several types of solid tumors, for which other therapies must be developed.[26]

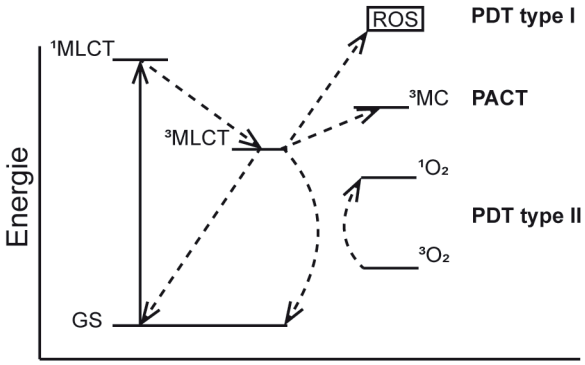

Figure 3. Photophysical mechanisms of photodynamic therapy and photo activated chemotherapy for ruthenium polypyridyl compounds.

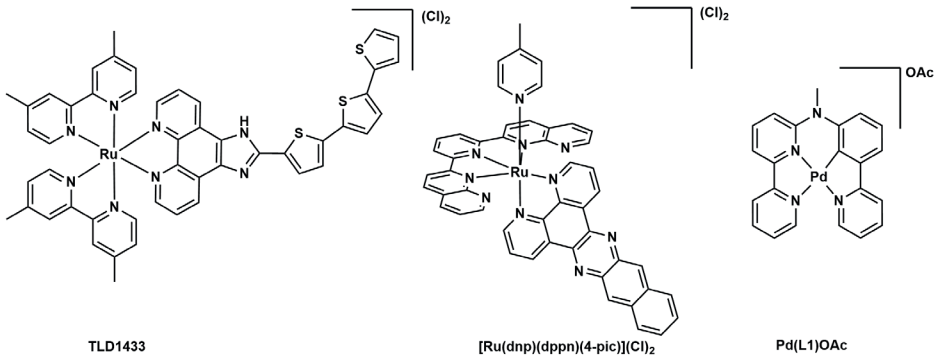

Figure 4. Chemical structure of three photodynamic therapy compounds.

PACT acts via a different mechanism by inducing photocytotoxicity in an oxygen-independent activation mechanism. It has been thoroughly reviewed elsewhere.[27] In this thesis, we will focus on ruthenium polypyridyl compounds which induce biological effects by a ligand photosubstitution reaction. The photophysical mechanism of this reaction starts, similar to PDT, with the photochemical promotion of the ground-state molecule to the ^{1}MLCT state followed by intersystem crossing to the ^{3}MLCT state. From the ^{3}MLCT state the mechanism of PACT deviates from PDT as in PACT compounds a triplet Metal-Centered excited state (^{3}MC) is low enough to be thermally promoted from the ^{3}MLCT state. This characteristic can be explained by an eg molecular orbital that is stabilized by a distorted geometry of the first coordination sphere, which is induced by the polypyridyl ligands.[28] The antibonding character of the e.g. orbital lengthens and weakens one of the coordination bonds in the excited state, thus facilitating photosubstitution. PACT compounds must be inert in the dark (photocaged), while upon light activation a ligand is photosubstituted (uncaged) thus generating an available coordination site on the metal complex, which potentially reacts with biological substrates. The therapeutic activity of a PACT drug can be ascribed solely to the uncaged metal complex, solely by the uncaged ligand, or by a combination of both fragments (figure 5). Cuello-Garibo *et al.* reported a in detailed study the mechanism inducing cytotoxicity for the ruthenium compounds $[Ru(Ph_2Phen)_2(mtmp)](Cl)_2$, where Ph_2Phen = 4,7-diphenyl-1,10-phenanthroline, mtmp = 2-methylthiomethylpyridine, and $[Ru(bpy)_2(dmbpy)](Cl)_2$, where bpy = 2,2'-bipyridine and dmbpy = 6,6'-dimethyl-2,2'-bipyridine. The phototoxicity of the former was solely caused by the uncaged ruthenium center as the uncaged mtmp ligand proved to be non-toxic upon blue light (455 nm) irradiation. Interestingly, the latter compound demonstrated the exact opposite as the uncaged ruthenium center appeared to be non-toxic while cytotoxicity was induced by the uncaged dmbpy ligand with an EC_{50} value of 10.9 µM.[29]

Ruthenium centers that remain non-toxic upon light activation offer the possibility to photocage a wide range of enzyme inhibitors, which allows for locally inducing a wide range of biological effects that can be much more refined than cell death. This principle was demonstrated by the group of Etchenique, who reported the photocaging of several neurotransmitters where the ruthenium fragments remain non-toxic after photoactivation. In another example, Lameijer *et al.* reported the caging of the nicotinamide phosphoribosyltransferase (NAMPT) inhibitor X=STF31 by $[Ru(tpy)(biq)(X)](Cl)_2$ scaffold, where biq = 2,2'-biquinoline and tpy=2,2':6',2''-terpyridine.[30] STF31 was uncaged upon red light irradiation demonstrating EC_{50} values in the same order of magnitude as for the uncoordinated inhibitor control. Furthermore, they found identical PI values under normoxic (21% O^2) and hypoxic (1% O^2) conditions, underlining the likelihood of an oxygen-independent mechanism associated with PACT. The group of Glazer reported a ruthenium compound where both the uncaged ruthenium center and the uncaged ligand induce biological effect.[31] Upon light activation compound

$[Ru(bpy)_2(L)_2]^{2+}$ photo-released the cytochrome P450 inhibitor L, which led to a 136-fold increase in protein inhibition in comparison to dark conditions. Meanwhile, the uncaged ruthenium center was shown to bind to DNA, thus generating two biological effects following a single light trigger.

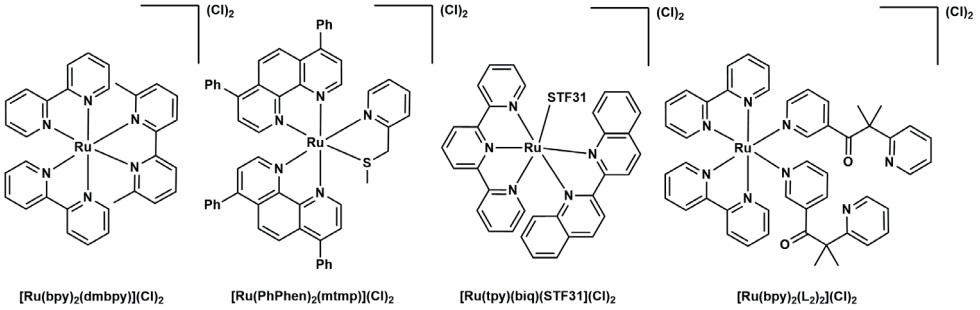

Figure 5. Ruthenium photoactivated chemotherapy agents, where either the ligand, the ruthenium core, or both, induce cytotoxicity upon light activation.

1.3 Small molecule interactions with DNA

DNA as a target for chemotherapy drugs has been a popular theme for the last 80 years and today a myriad of studies exists describing the interactions between small molecules and duplex DNA. The different kind of interactions can be categorized in covalent bonding, intercalation and groove binding, as displayed in Figure 6. As mentioned previously, cisplatin interacts via coordination with DNA. Takahara *et al.* reported a crystal structure of duplex DNA, d(CCTCTG*G*TCTCC)·d(GGAGACCAGAGG), G-G crosslinked by the platinum electrophile.[33] The binding disrupts DNA base stacking significantly and induces a formidable degree of distortion of the phosphate backbone, resulting in a strong bend toward the major groove facilitated by the widening and flattening of the minor groove. Cisplatin is not the only drug to bind to DNA directly. In spite of the widespread use of cisplatin, the first group of covalent DNA binders studied for their anti-cancer properties were actually carbon-based alkylating agents, such as nitrogen mustards or sulfur mustards based upon $RSCH_2CH_2X$ and $RNCH_2CH_2X$ scaffolds, where X is a halide ion. These covalent DNA crosslinkers had a macabre start with their horrid application as nitrogen mustard gas during World War I. Later during World War II an infamous event occurred at Bari, Italy that would start the application of DNA crosslinking agents as chemotherapy drugs. The US merchant ship S.S john Harvey was docked in Bari, loaded with 2000 M47A1 hundred-pound mustard bombs, when it was bombed by a German air raid.[34] The cargo detonated leading to a death toll of up to 2000. During the aftermath medical personnel recorded a remarkable white blood cell count of 100 cells/μL in patients exposed to the hazardous chemical.[35] After declassification in 1946, Goodman *et al.* reported the use of mustard gas and prototype alkylating agent chlormethine, for the treatment of lymphoma.[36] More

than 70 years later chlormethine and its derivatives, such as Cyclophosphamide and Melfalan, are still used in clinics as chemotherapy agents and are listed on the World Health Organization's List of Essential Medicines.[37] It is generally accepted that the cytotoxicity of alkylating agents is induced by their interactions with DNA. Electron rich nitrogen atoms of DNA are alkylated by nucleophilic substitution reactions, where neighboring group participation enhances the reactivity of mustard compounds. Early studies showed increased antitumor activity for bifunctional agents over mono- or trifunctional agents,[38] resulting in the theory that the cytotoxicity of alkylating agents was induced by crosslinking DNA. For selectivity, chlormethine, showed preference for binding the N-7 position of guanine and forms mostly monoadducts with DNA (90%). Both inter- and intrastrand crosslinks are also formed but to a lesser extent.[39] The cytotoxicity of nitrogen mustards, however, is mainly induced by the formation of interstrand DNA crosslinks. This mechanism is in stark contrast with that of cisplatin, for which cytotoxicity is induced mainly by intrastrand crosslinks.[12] Furthermore, chloromethine demonstrated crosslink selectivity for the 1,3 intrastrand crosslink sequence 5'-**G**NC-3'/3'-CN**G**-5' (N= G or C) over 1,2 intrastrand 5'-**G**C-3'/3'-C**G**-5' crosslinks.[40,41]

The second type of interactions between small molecular drugs and DNA is intercalation. This binding mode is defined by planar aromatic systems inserting between consecutive base pairs in duplex DNA. This supramolecular interaction, stabilized by π-π interactions, leads to unwinding of the DNA helix. Factors that influence intercalation into DNA are size,[42] pH, protonation state,[43] charge,[44] and overall electrostatic interactions.[45] Figure 6 shows the crystal structure of ellipticine intercalated into duplex DNA $d(CGATCG)_2$,[46] where two ellipticine molecules intercalate by stacking between both terminal CpG steps. Furthermore, the intercalation leads to an extension of the DNA length and an unwinding angle of 14° in comparison to standard duplex DNA. Ellipticine itself is a potent chemotherapy agent characterized by its intercalation into DNA and subsequent inhibition of topoisomerase II. Another potent chemotherapy intercalator is doxorubicin. It is also one of the most widely applied chemotherapy drug, as its effectiveness extends to a wide array of malignancies, such as (but not only) breast, esophagus, prostate, uterus, stomach and liver cancers.[47] The two main mechanisms of action of doxorubicin are, (I) free radical formation and oxidative damages,[48] (II) intercalation into DNA and the subsequent hindering of DNA replication and transcription.[49] The latter mechanism results in the formation of the highly toxic DNA double-stranded breaks, resulting in apoptosis when they accumulate.[50] Ethidium bromide is another widely used intercalator and fluorophore, the fluorescence of which is significantly increased upon intercalation into DNA. This compound is therefore mainly used as a molecular probe to visualize DNA in biochemistry techniques such as agarose gel electrophoresis.[51] A metal complex with similar properties is $[Ru(bpy)_2(dppz)]^{2+}$ (bpy =

2,2'-bipyridine, dppz = dipyridophenazine),[52] which is well known for its "light switch" effect. When dissolved in aqueous solutions the complex is not fluorescent in absence of DNA, but it demonstrates remarkable red luminescence upon intercalation of the dppz moiety into DNA.

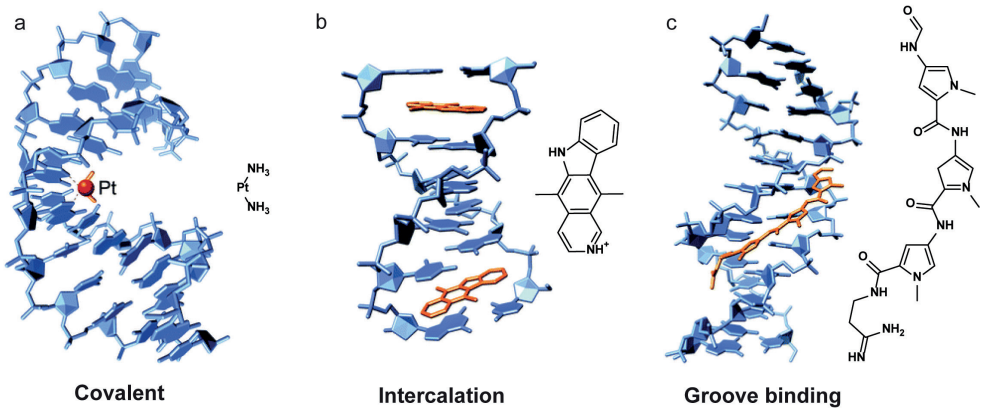

Figure 6. Three categories of small molecule interactions with DNA of the X-ray structure of three classical DNA-binding drugs (orange) in complex with their target DNA (blue). (a) Cross-linker cisplatin. (b) Intercalator ellipticine. (c) Minor groove binder distamycin a. Figure adapted from Boer 2009.[32]

Even though DNA can adopt several conformations, the B form is the most frequent and relevant one in biology. This arrangement of the base pairs in the double helix results in the formation of a major and a minor groove, Figure 7a. Both binding sites are molecularly distinct and variations in hydrogen bond acceptor/donor patterns lead to selective interactions between proteins and DNA that are essential for a variety of biological processes.[53] Although it is well established that the exact size of both grooves is sequence-dependent, as seen for A/T and G/C rich minor groove in Figure 7b-c, the general width of the major groove is 11.6 Å with a depth of 8.5 Å, while the width of the minor groove is only 6.0 Å, it has a similar depth of 8.2 Å.[54] Most DNA-binding proteins interact with the major groove, which has a higher number of hydrogen bonding sites, compared to the minor groove, and a larger available space to accommodate proteins.[55] The less spacious minor groove, however, offers enough room to be targeted by small molecules for medicinal purposes like antibiotic or anticancer compounds. In general, minor groove binders have a crescent shape, electron-donating and -accepting groups capable of entering into hydrogen bonding networks, a cationic charge, and selectivity towards A/T regions.[56] These structural and electronic properties combined with strong van der Waals interactions, result in the stabilization of the DNA-ligand complex.[57,58] The molecularly-related compounds, netropsin and distamycin A, have been studied since their isolation from *streptomyces netropsis* in 1951[59] and *Streptomyces distallicus* in 1964,[60] respectively. They are paradigms of minor groove binders, even though their high toxicity obstructed clinical application.[61] Figure 6c shows a crystal structure of

distamycin A in the minor groove of A/T rich DNA dodecamer d(CGCAAATTTGCG). [62] Minor groove binder pentamidine showed more clinical success treating trypanosomal infections in humans and animals.[63] However, systemic administration causes severe side effects, such as nephrotoxicity.[64] Other commonly used minor groove binders are the fluorescent stains DAPI and Hoechst 33258, both are used in fluorescence microscopy and flow cytometry in molecular biology.[65,66]

Interestingly, minor groove binders have also been chemically combined with alkylating agents for combination therapy. Tallimustine, a distamycin and nitrogen mustards derivative, showed both the DNA-alkylating capabilities as well as minor groove A/T selectivity.[67] However, it appeared inactive in phase II clinical trials against lung and colorectal cancer.[68,69]

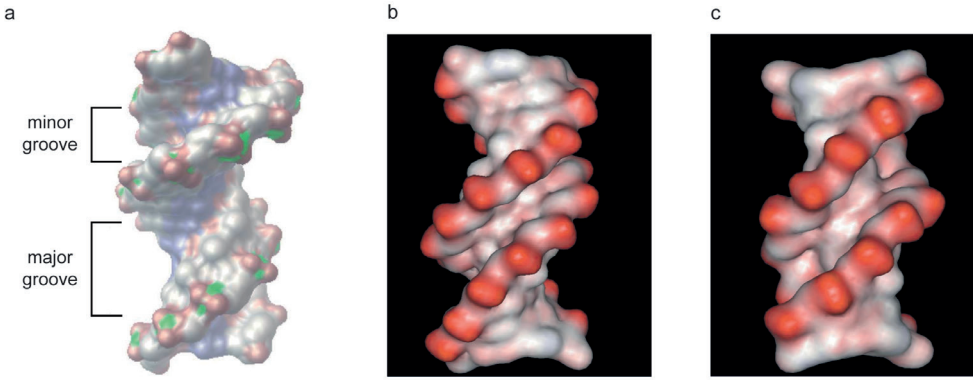

Figure 7. (a) Distinction between minor and major groove of B-DNA. (b) Crystal structure of DNA sequence d(CGCAAATTTGCG) view of the narrow A/T minor groove. (c) Crystal structure of DNA sequence d(C-CAGGCCTGG) view of the wide G/C minor groove. Figure adapted from Hamilton 2012 and Neidle 2001. [53,54]

So far, our discussion has primarily focused on the interactions of compounds with B-form duplex DNA, however, DNA can adopt multiple structures and interactions of small molecules with other non-canonical DNA foldings have been extensively described for G-quadruplexes,[70–72] 3-way junctions[73,74] and 4-way junctions.[75] Our group reported the synthesis of the square-planar platinum(II) compound $[Pt(H_2bapbpy)]^{2+}$, where H_2bapbpy = 6,6'-bis(2"-aminopyridyl)-2,2'-bipyridine. The compound was found to be highly toxic in MCF7 and MDA-MB231 cell lines and a crystal structure was obtained of this complex interacting with a small palindromic DNA oligomer 5'-d(CGTACG)-3. In this crystal structure, the DNA formed a 4WJ-like motif, with the metal complex binding at its core through an intercalation type mechanism, as seen in Figure 8.[76] The planar structural arrangement of the platinum H_2bapbpy compounds appears to offer a conducive environment for the conventional type of intercalation within B-form duplex DNA. However, it is worth noting that this particular interaction, as well as any potential connections between the compound induced toxicity and DNA 4-way junctions interactions, have not been documented.

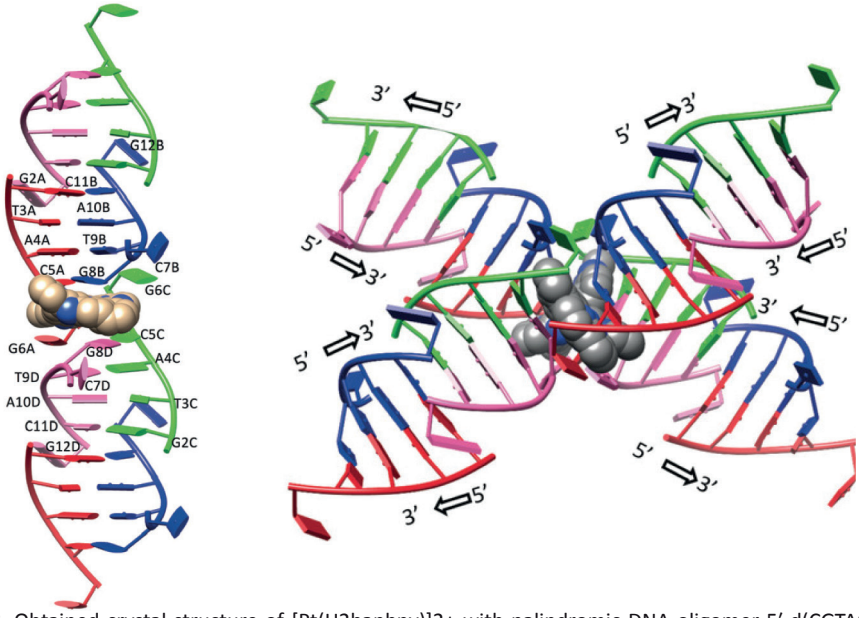

Figure 8. Obtained crystal structure of [Pt(H2bapbpy)]2+ with palindromic DNA oligomer 5′-d(CGTACG)-3 forming a 4-way like junction. Figure adapted from Van Rixel 2019.[76]

1.4 DNA repair

It is estimated that a human cell suffers approximately 70,000 DNA lesions per day.[77] DNA damage can arise from endogenous sources, occurring during normal cellular functions, such as metabolism,[78] which involves processes like hydrolysis, oxidation, alkylation, and DNA base mismatches.[79] Additionally, it can also result from exogenous sources like UV light, ionizing radiation, and the aforementioned small molecules.[80] Apart from single-strand breaks (SSB) and double-strand breaks (DSB), several other types of lesions, such as base damage, sugar damage, and DNA cross-linking, occur regularly.[81] To combat these lesions, human cells have evolved sophisticated DNA repair pathways that ensure genome integrity. These pathways include direct reversal, base excision repair, nucleotide excision repair, mismatch repair, SSB repair, and DSB repair. The DSB repair pathways, Single Strand Annealing, and microhomology repair, are outside the scope of this introduction and are reviewed elsewhere.[82]

Direct reversal repair (DRR) enables efficient and accurate DNA repair by specifically reversing three covalent DNA adducts. These adducts include UV-induced pyrimidine dimers, which are repaired by photolyases; O6-alkylated G adducts, repaired by alkyltransferases; and N-alkylated N1 A and N3 C adducts, repaired by AlkB family dioxygenases.[83] The repair of O6-alkylated G adducts demonstrates the elegance of this pathways as the alkyl moiety is simply transferred from the damaged DNA to a cysteine group of the alkyltransferase protein in an irreversible reaction, directly reversing the DNA damage without excision.[84]

Base-Excision Repair (BER) repairs DNA lesions of the base caused by reactive oxygen

species (ROS), resulting in DNA base oxidation, alkylation, or deamination.[85] BER can repair one nucleotide (short patch) or longer chains of nucleotides (long patch), as seen in Figure 9. Both pathways start with the recognition of the lesion by DNA glycosylases and the subsequent excision of the damaged base, forming an abasic site. The second step induces a nick at the lesion site by AP endonuclease APE1.[86] The last step of DNA synthesis and ligation differ for both pathways. In the short patch pathway Pol β fills a single nucleotide gap and is ligated by DNA ligase IIIα(LIG3) and cofactor XRCC1, while the first nucleotide in the long patch is also filled by Pol β. Further elongation is carried out by Pol δ and or Pol ε.[87,88] Other proteins involved are proliferating cell nuclear antigen (PCNA) and flap endonuclease-1 (FEN-1) before the long patch repair is finally ligated by DNA ligase 1 (LIG1).[89]

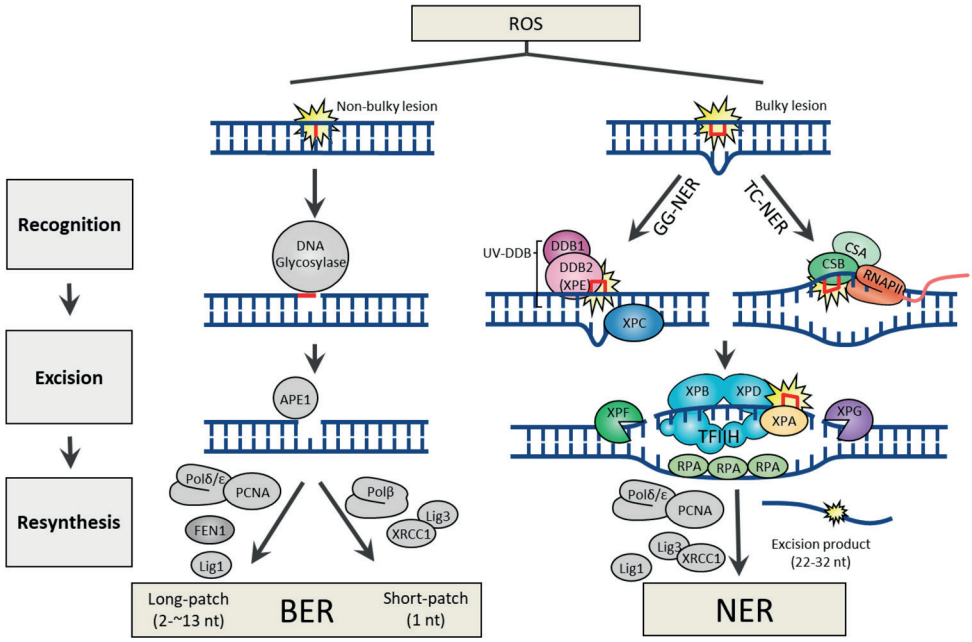

Figure 9. Schematic overview of the BER and NER pathways. Figure taken from Lee 2019.[90]

Nucleotide excision repair (NER) is responsible for repairing helix-distorting DNA damage such as bulky adducts and cross-linking.[91] It consists of two pathways, the global genome repair (GGR)-NER, which searches the entire genome for DNA damage, and the transcription-coupled repair, which repairs transcribed strands of active genes.[92] Globally, NER operates by incisions on both sides of the lesion to removes oligonucleotides, 24-32 nucleotides long, followed by gap filling synthesis and ligation.[93] The mechanism of global genome repair (GGR)-NER starts with damage recognition by the XPC/HHR23B complex, after which repair and transcription factor, TFIIH, is recruited and induces the unwinding of the helix. Replication Factor A (RPA)

and XPA are recruited to the factor, for lesion verification. Next, endonucleases XPG and XPF/ERCC1 are recruited to incise the DNA damage on both sides. To complete the repair the gap is filled by DNA synthesis by Polymerases δ, ε, or κ and finally, the DNA is sealed by ligase 1.[94]

Mismatch repair (MMR) primary function is the repair of base mismatches and nucleotide deletions missed by polymerase selectivity and proofreading, improving overall replication fidelity.[95] MMR defects increases the mutation rate and can lead to a carcinogenic mutator phenotype.[96,97] The mismatch repair pathway starts with damage recognition by the heteroduplex MSH2-MSH6 (MutSα). Followed by recruitment of MLH1/PMS2 (MutLα) and PCNA to form a loop formation. Exonucleases and helicases then remove the damaged part after which the gap is filled by DNA synthesis, before DNA ligase I seals the nick.[98]

The Fanconi anemia pathway (FAP) is associated with the genetic disorder known as Fanconi anemia (FA), which significantly increases the risk of developing cancer. FA patients have a 50-fold higher probability of developing cancer, [99] and approximately 76% of them develop solid tumors by the age of 45.[100] One of the underlying causes is the inability of FA patients to repair DNA interstrand crosslinks (ICLs) due to genetic mutations in specific genes within the FAP. Consequently, these patients exhibit higher sensitivity to interstrand crosslinking agents like nitrogen mustards.[101] FAP is complex and enlists elements of NER, homologous recombination (HR), and mutagenic translesion synthesis[102] and is excellently reviewed elsewhere.[103] Although ICLs are mainly repaired by the FAP, cases have been documented in non-replicating cells where ICLs are repaired through Translesion Synthesis and NER, independent of the FA DNA repair pathway[104,105]

Single-Strand Break Repair (SSBR) mends the most common type of DNA damage, i.e., the Single-Strand Breaks. Indirect SSB arise and are repaired during normal functioning of the BER pathway,[106] where APE1 induces a nick and thus a SSB, which is promptly repaired by BER as previously described. In general, the BER and the SSBR pathway share a key group of proteins and show a great degree of mechanistic overlap. Direct SSB generated by ROS are repaired by SSBR in three steps with a key role for proteins Poly [ADP-Ribose] Polymerase, PARP1.[107] The first step is damage recognition as PARP1 rapidly binds to a SSB, which is followed by DNA end-processing repairing defective 3'- and/or 5'-termini by APE1, scaffold protein XRCC1 and a variety of enzymes covering the extent of damage divergence, which is reviewed in the literature.[108] The third step of DNA gap filling and ligation is similar to the BER pathway and consists of long-patch and short-patch pathways. Even though the pathways are similar, the presence of PARP1 is important for SSBR, but might not be necessary for BER where its role remains unclear.[109,110] The short-patch pathway in SSBR repairs a single nucleotide gap by polymerase Polβ and ligation by DNA ligase IIIα (LIG3). In the long-patch pathway the DNA gap is filled by Pol β or in its absence by Pol δ and or Pol ε, where the damaged

5'-terminus flap is removed by endonuclease 1 (FEN1) induced by interactions with PARP1 and proliferating cell nuclear antigen (PCNA)[111] before being ligated by DNA ligase 1 (LIG1) to complete the repair.[112]

Double-strand breaks (DSBs) are the most cytotoxic type of DNA lesion and jeopardizes genome stability.[113] DSBs occur naturally upon replication fork collapse and programmed genome rearrangements or are induced artificially by genotoxic chemicals, PDT, or ionizing radiation.[114] DSBs are mainly repaired by two pathways, Non Homologous End Joining (NHEJ) and Homologous Recombination (HR). HR is a high-precision repair mechanism which requires a DNA template and is therefore leading during meiosis and during late S and G2 cell cycle stages when sister chromatids are present.[115] NHEJ occurs without a template by the relegation of two ends, where missing sequences at the break site result in a mutation or deletion. In the presence of multiple DSBs, arbitrary DNA strand ends may be indiscriminately joined resulting in gross chromosomal rearrangements.[116]

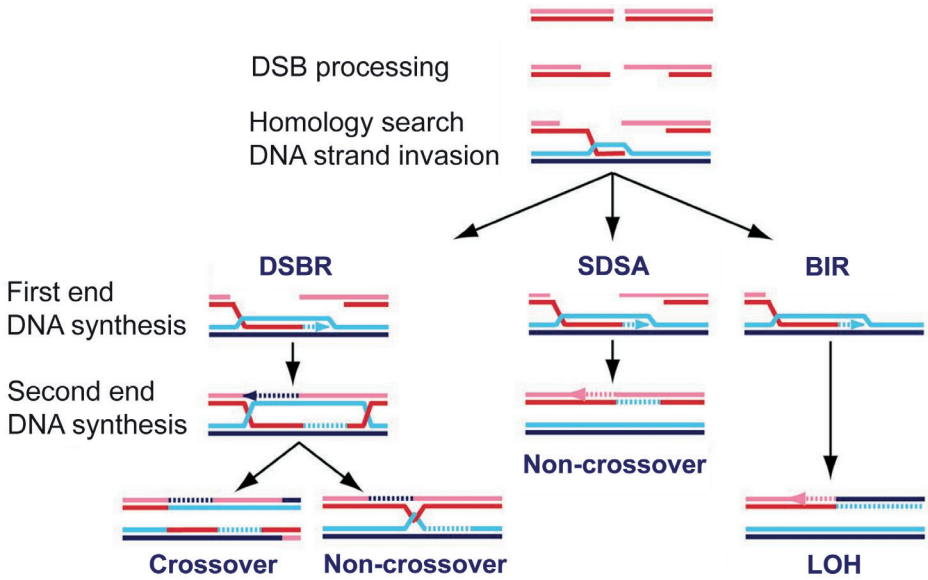

Figure 10. Schematic overview of the homologous recombination and the three pathways resulting in cross-over, non-crossover and in loss of heterozygosity (LOH). Figure taken from Sneeden 2013.[122]

The NHEJ mechanism is relatively straightforward and starts with the binding of the broken DNA ends to the Ku70/80 heterodimer, which recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs).[117,118] The ends are then ligated by the DNA ligase IV (Lig4) and XRCC4 complex.[119] Potentially active end-processing enzymes, when required, are DNA pol μ, pol λ, Artemis and polynucleotide kinase 3'-phosphatase (PNKP), to name a few; the complete mechanism is reviewed elsewhere.[120,121]

Homologous recombination is a convoluted but high-fidelity DNA repair pathway and is split in a minimal of three downstream pathways, that all use DNA strand invasion and template-directed DNA repair synthesis to repair the DSB (figure 10). All three pathways start identically with the recognition of the DSB ends which is followed by the resection to a 3'-OH ending single-stranded tail and the search for a homologous template, with a crucial role for RAD51. This is followed by strand invasion into the homologous sequence generating a D-loop.[122] From this point, the three pathways differ and the double-strand break repair (DSBR) pathway continues with extension of the 3' end by DNA synthesis, the other end of the DSB is captured in an event called second end capture. Second-end DNA synthesis of the non-invading strand and ligation lead to the formation of a double Holliday junction, where four DNA strands are joined together forming a cruciform structure (four-way junction). The final step is the resolution of the Holliday junctions yielding crossover or non-crossover products.[123] The second pathway is synthesis-dependent strand annealing (SDSA), where a single end or both strands invade the homologous template and initiate DNA synthesis. In SDSA the invaded strand is elongated by DNA synthesis and subsequently displaced, allowing annealing with the other end of the break through their complementary base pairing. Further DNA synthesis and ligation results in only non-crossover repaired products.[124] The third pathway is break-induced replication (BIR),[125] and occurs when a broken chromosome only has one end available. The end invades a homologous sequence initiating DNA and can copy the sequence until the end of the chromosome, thereby resulting in a loss of heterozygosity (LOH).[126]

RAD51 is a key protein in the homology search at the start of homologous recombination repair. Its function was first elucidated in *Saccharomyces cerevisiae* (figure 11). After recognition of the DSB, the 3'-OH ending single-stranded tail is bound by replication protein A (RPA) which coats the ssDNA. RPA removes secondary structures formed on the ssDNA facilitating Rad51 filament formation.[128] Furthermore RPA has a higher affinity for binding ssDNA than Rad51 and thus prevents Rad51 from binding to the DNA.[129] The inhibitory effect of RPA is overcome by several mediator proteins such as Rad52 and the heterodimer consisting out of Rad55 and Rad57.[130] Both interact with Rad51 and allow its loading onto RPA-coated ssDNA. RPA is then replaced by Rad51 forming extended helical filaments on the DNA.[131] DsDNA Rad51 filaments are composed of approximately 6 Rad51 proteins per helical turn, which correspond to approximately 18.6 base pairs of DNA per turn. Furthermore, the DNA is stretched from 3.4 Å per basepair in B-DNA to 5.1 Å per basepair in the Rad51 filament. Interestingly, Rad51 filaments associated with ssDNA have demonstrated both extension and compression behaviors. [127,132] The Rad51 filament searches for homology by probing dsDNA for a sequence of at least 8 nucleotides that form a microhomology and dismiss sequences of 7 and lower.[133,134] The search for homology is faster in A-T rich sequences

and is attributed to the ability of A:T base pairs to readily flip out of the helix, serving as nucleation sites for homologous pairing.[135] The combination of Rad54 and Rdh54/Tid1, from the Snf2/Swi2 family of DNA-dependent ATPases, stimulates filament strand invasion and promotes D-loop branch point migration.[136] The proteins of the HR pathway are largely conserved between humans and *S. cerevisiae*, although some proteins are not present in yeast, such as tumor suppressors BRCA1 and BRCA2.[137] Interestingly, BRCA2 mediates the RAD51 filament formation in humans, despite the presence of RAD52.[138,139]

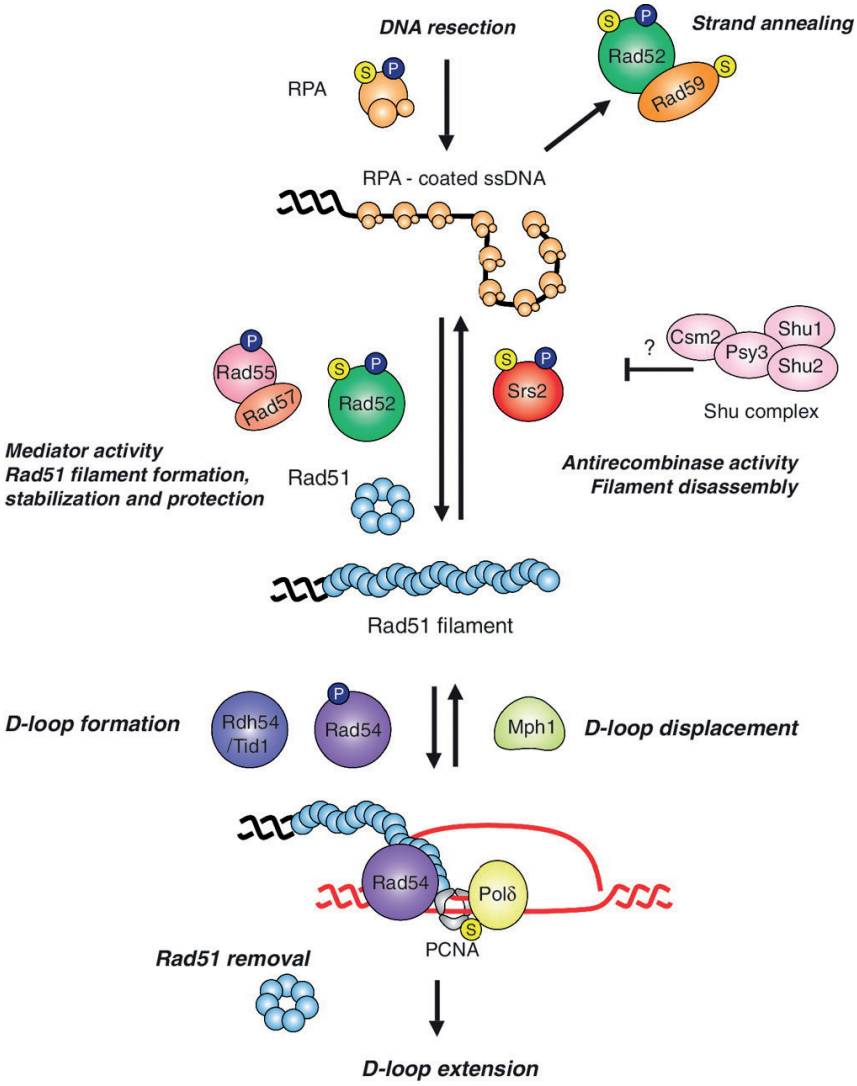

Figure 11. Schematic overview of the role of RAD51 during homology search and strand invasion. Figure taken from Krejci 2012.[127]

1.5 Chemotherapy and DNA repair

A wide range of chemotherapy drugs today demonstrates some selectivity in killing cancerous cells over normal cells, which is often achieved by targeting DNA directly or indirectly. Replication of damaged DNA increases cell-cycle arrest and cell death, increasing toxicity for fast-replicating cells over slow replicating cells. This principle is the foundation of various chemotherapy drugs today, such as the previously discussed platinum- and nitrogen mustard-based drugs. The downside of these treatments is that the human body is inherently resistant to these types of treatments, as it has developed intricate and detailed DNA repair pathways. Figure 12 provides a detailed overview of chemotherapies that (in)directly target DNA and their corresponding DNA repair pathways. DNA lesions, caused by alkylating agents during chemotherapy, may be repaired by: 1) DRR reversing the damage,[140] if the lesion is one of the three types detailed above (named Alkyl transferases in Figure 12), 2) BER or NER,[141] which eliminate the DNA damage by excising a base/nucleotide sequence, or 3) Translesion synthesis allowing replication to proceed despite the presence of DNA damage.[142] Intrastrand crosslinks are repaired by NER[143] and interstrand crosslinks are repaired by FA or FA in combination with HR and NER.[144] If these damages persist and interfere with replication fork progression, they may result in the formation of replication fork-associated DSBs, which are mostly repaired by HR,[145,146] while direct DSBs are repaired by NHEJ.[147] The repair of DNA lesions induced by alkylating agents before interacting with the replication machinery, reduces the overall effectiveness of the treatment. However, treatment with inhibitors of specific DNA repair pathways could be used in combination with DNA-damaging agents to enhance treatment efficacy and selectivity.[80]

The combination of DNA-damaging agents and their linked DNA repair pathways in Figure 12 allows identification of potential combination therapies, where inhibitors sensitize cells for specific DNA-damaging agents. For instance, inhibition of DNA-PK, a key protein in NHEJ, sensitized cells (*in vitro*) and tumor xenografts (*in vivo*) to DSB inducers, such as radiotherapy and doxorubicin.[148–150] Furthermore, inhibition of PARP, a key protein in SSB repair, also resulted in increased tumour radiosensitivity.[151,152] Cisplatin is one of the most widely studied cross-linking agents and a myriad of studies have shown enhanced results in combination with DNA repair inhibitors. For example, treatment with the RAD51 inhibitor B02 reduced HR activity and sensitized triple-negative breast cancer cells to cisplatin.[153] Similar effects were reported for RAD51-knockdown cells, [154] while cell lines that overexpress RAD51 showed resistance to DNA-damaging agents like cisplatin.[155] Impaired NER activity resulted likewise in cisplatin sensitization, which was achieved by targeting ERCC1[156,157] or the XPA-ERCC1 interaction.[158] The FA pathway, mainly repairs ICL and inhibition of the FA related proteins FANCD2[159] or USP28[160] enhanced cisplatin treatment. A large-scale

screening for potential FA pathway inhibitors showed that most chemicals inhibiting the FA pathway also inhibited homologous recombination.[161] Moreover, suppressing DNA repair pathways enhances the effectiveness of the topoisomerase inhibitor and double-strand break (DSB) inducer, doxorubicin. Reducing NHEJ capabilities by DNA-PK[150,162] inhibitors or by DNA ligase IV inhibitor SCR7[163] directly led to increased cytotoxicity of doxorubicin. Similar to cisplatin, doxorubicin showed enhanced effect when combined with reduced HR repair through RAD51 inhibitors.[164,165] Although a vast array of synergetic combinations between agents are reported in the literature for *in vitro* cellular assays, where concentrations are easily controlled, the actual clinical success is limited due to dissimilarities in pharmacokinetics and tissue distribution between each agent in the combination.[166]

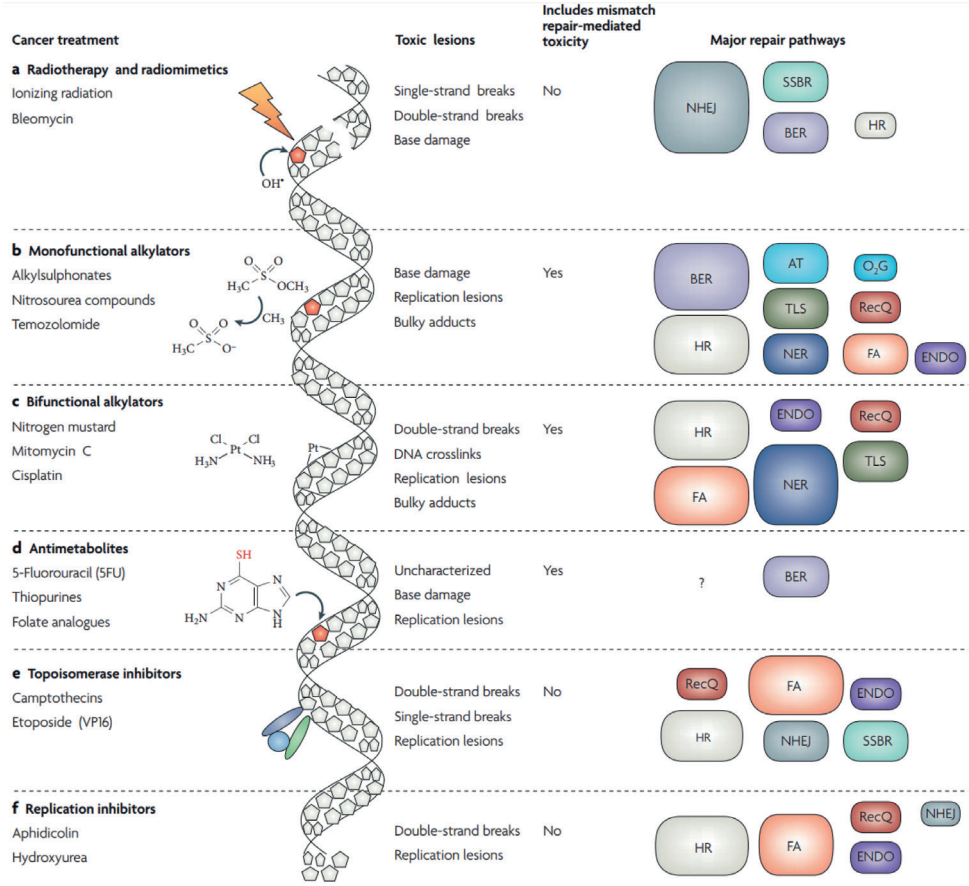

Figure 12. Overview of commonly used chemotherapy agents that impair DNA (in)directly and corresponding DNA repair pathways ronsponsible for the repair of the induced damage. Figure taken from Helleday 2008.[80]

1.6 Aim and outline of this thesis

The goal of the research described in this thesis was the development of novel metal complexes as potential chemotherapy drugs, and to characterize their interactions with DNA or the DNA repair pathways. This goal was achieved by two main research lines; (I) A structural study of d^8 metal compounds based on the $[M(H_2bapbpy)]^x$ scaffold, and the characterization of their interactions with B-DNA; (II) The development of photoactivated ruthenium compounds for PACT based on the same tetrapyridyl ligand scaffold, but bearing in axial position a RAD51 inhibitor, to test the possible synergy between the photoactivated ruthenium moiety inducing DNA damage, and HR inhibition by the RAD51 inhibitor.

In Chapter 2, the report details the synthesis of a library of racemic platinum(II) and palladium(II) compounds based on the $[Pt(H_2bapbpy)]^{2+}$ scaffold is reported. The terminal pyridyl groups have been functionalized with a methyl, methoxy, chloride, or trifluoromethyl substituent, to study the influence of the electron-withdrawing or -donating properties of the substituent on DNA interaction of the complex, while the amine bridges have been replaced by thioether, ether, methylene, or carbonyl bridges, to study the role of the hydrogen-bonding properties of the bridge on DNA interaction. Single crystal X-ray structures were obtained for 23 compounds, which allowed discussing a structure-activity relationship for the interaction of these compounds with duplex DNA. From these studies, the pK_a and protonation state of the metal complex proved crucial for intercalation.

In chapter 3 is the study of the helical chirality generated by the coordination of the H_2bapbpy or H_2biqbpy (H_2biqbpy= bis(aminoquinoline)bipyridine) ligands to a ruthenium(II) center reported. The helical chirality is imposed by the ligands: it cannot adopt a flat planar conformation once coordinated because of the steric clash between terminal pyridines or quinolines. For ruthenium(II) complexes, the additional presence of two different axial ligands generates diastereotopic aromatic protons that can be distinguished by ^{1}H NMR and variable-temperature ^{1}H NMR. This effect allowed for determining the coalescence energies corresponding to the chirality interconversion of the H_2bapbpy-based metal compounds. Increasing the steric strain further by introducing methoxy groups *ortho* to the nitrogen atoms of the terminal pyridyl groups in H_2bapbpy, resulted in the serendipitous discovery of a ring-closing macrocycle reaction not reported before.

In chapter 4 is the synthesis and chemical characterization of a series of photocaged RAD51 inhibitors obtained by the conjugation of B0Cl, an improved analog of B02, by several ruthenium photocages reported. Their photophysical properties were determined and the induced cytotoxicity was assessed in two uveal melanoma cell

lines, OMM2.5 and MM66. The combination therapy of photocaged RAD51 with the double-strand breaking drug doxorubicin was studied by immunofluorescence imaging. Our preliminary results demonstrate that the B0Cl inhibitor was successfully photocaged as compound [**1**]$(Cl)_2$ and can be activated by light in cells to reduce homologous recombination activity.

In chapter 5 is the possibility of a concept called “synthetic lethality” explored. In genetics, this principle is based upon the idea that a genetic mutation in a tumor may, on its own, not impact cell viability, but when combined with a second gene disruption, it may result in cell death. Here we developed a chemical genetics approach to this principle: a preparative route to bind two different DNA repair inhibitors to a single ruthenium photocage, thus affording a prodrug that can release two inhibitors by local photoactivation. This method induces synthetic lethality without requiring any genetic mutation.
The RAD51 inhibitor BOCl was combined with STF31, a NAMPT inhibitor, or PIK75, a DNA-PK inhibitor. The photocaged compounds remained inert in the dark but were photoactivated to release both inhibitors upon visible light irradiation. The photosubstitutions quantum yields were determined for both the first and second photosubstitution reactions. Interestingly, the B0Cl inhibitor substitution occurred at a faster rate than STF31, although both inhibitors were coordinated to ruthenium via a similar pyridine moiety. The structural and photochemical characterization of these compounds proved chemically the concept of the photocaging of two distinct inhibitors by one ruthenium scaffold for dual PACT tumor treatment.

1.7 Literature

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