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DNA Repair and Resistance to Cancer Therapy

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1. Introduction

Humans are constantly exposed to diverse chemical and physical agents that have the potential to damage DNA, such as reactive oxygen species (ROS), ionizing radiation (IR), UV light, and various environmental, dietary or pollutant chemical agents. The integrity and survival of a cell is critically dependent on genome stability, and cells possess multiple pathways to repair these DNA lesions. These pathways are diverse and target different types of lesions.

The critical role played by DNA repair in the maintenance of genome stability is highlighted by the fact that many enzymes involved have been conserved through evolution [1-4]. Very rarely germ line mutations occur in several of the DNA repair genes and are the cause of cancer predisposing syndromes, such as *Xeroderma pigmentosum* (XP), [5], Fanconi anemia (FA) and ataxia telangiectasia (AT) and are associated with inherent chromosome instability [2]. One of the most well-known examples of a defect in DNA repair leading to cancer is the association of germ-line *BRCA1/2* mutations with breast, ovarian and peritoneal malignancies [6]. These rare human DNA repair syndromes have been invaluable in providing mechanistic explanations for the involvement of DNA repair system in cancer. They have also been instrumental in the translation of these findings to the clinic.

On the other hand, recent studies have shown that defective DNA damage repair is present in virtually all sporadic tumours [7]. Mutations in DNA repair genes could be either responsible for the occurrence of tumours or could arise due to random accumulation of mutations during cycling of cancer cells. The presence of incorrect DNA repair in tumour cells predisposes them to accumulate even more genetic alterations. For example, colorectal and endometrial cancers with defective DNA mismatch repair (MMR) due to

mutations in the *MLH1* and *MSH2* genes exhibit increased rates of acquisition of single nucleotide changes and small insertions/deletions [8]. Thus, the presence of a "mutator phenotype" [9] could increase the evolutionary acquisition of alterations that ultimately could lead to enhanced drug resistance.

A further reminder on the importance of DNA repair is the observation that mutations in specific genes can lead not to an increase in cancer but to accelerated aging syndromes [7]. An example of this is Cockayne's syndrome (CS), which causes severe progeroid syndromes [10]. Mutations in the genes that encode two proteins in a nucleotide excision repair (NER) sub-pathway called transcription coupled repair (TCR) cause global premature cell death through apoptosis. In this case apoptosis ensures that DNA mutations are not transmitted to daughter cells, albeit at the expense of cell viability, and highlights the importance of maintaining DNA integrity.

One major problem in cancer therapy is the fact that of the 7.6 million cancer deaths that occur every year worldwide (2008 data; http://www.who.int/cancer/en/), many are due to failure of cancer therapy associated with acquired and intrinsic resistance mechanisms. These mechanisms of resistance can be classified in different ways, but the most characterized are altered cellular drug transport, increased survival or decreased cell death, altered DNA repair, and alterations in drug targets [11, 12]. Over the last years the importance of DNA repair pathways in resistance to chemotherapy has been increasingly recognized, but translation to the clinic is still scarce. Since many classical cancer therapies target DNA, the influence of DNA repair systems in response to DNA damage which primarily result from chemotherapy and radiotherapy is critical to cell survival. The use of inhibitors of DNA repair or DNA damage signalling pathways provides an interesting opportunity to target the genetic differences that exist between normal and tumour tissue [13, 14].

The rationale underlying the use of DNA damaging agents in therapeutic strategies is to kill cancer cells while sparing normal tissues, due to increased cell cycling of cancer cells. Unfortunately highly cycling normal cells (e.g. bone marrow, hair follicles and gastrointestinal epithelia) are also targeted by DNA damaging therapeutic agents, giving rise to the secondary effects normally seen after cancer therapy (e.g. diarrhoea, mouth ulcers, hair loss, anaemia and susceptibility to infections). Nevertheless, DNA-damaging chemotherapeutic agents are effective and prolong survival of cancer patients [15]. Chemotherapeutic agents commonly used in cancer treatment produce a plethora of lesions that can be targets for cellular responses. For example, DNA double strand breaks (DSBs), single-strand breaks (SSBs), and oxidized bases are induced by ionizing radiation (IR), anthracyclines, platinum compounds and taxanes. Anthracyclines are topoisomerase II inhibitors and DNA intercalating agents, which when used can lead to DSBs. Platinum compounds are bifunctional alkylating agents that induce predominantly intra- and interstrand crosslinks (ICLs) and taxanes are mitotic inhibitors. All these lesions induce cellular responses that cover a multitude of pathways, including DNA repair pathways, DNA tolerance mechanisms, coordination networks that link repair and cell cycle progression, as well as apoptotic and other cell death pathways when DNA damage is irreparable [16-19].

The DNA repair pathways that respond to these lesions include: direct repair of alkyl adducts by O6-alkylguanine DNA alkyltransferase (MGMT); repair of base damage and SSBs by base excision repair (BER); repair of bulky DNA adducts by nucleotide excision repair (NER); repair of cross-links by DNA interstrand cross-link repair and repair of mismatches and insertion/deletion loops by DNA mismatch repair (MMR); repair of DSBs by homologous recombination (HR) and non-homologous end joining (NHEJ). Detailed description of the biochemical pathways of DNA repair is beyond the scope of this chapter as several reviews on the subject have been published [1, 17, 20-23].

The observation that a variety of tumours frequently present deregulated expression of DNA repair genes (e.g. *MGMT*, *PARP1*) rapidly lead to the notion that DNA repair pathways could be targeted in cancer treatment and lead to personalized therapy [24, 25]. Tumours with specific DNA repair defects could be completely dependent on back-up DNA repair pathways for their survival. This dependence could be exploited therapeutically to induce cell death and apoptosis in tumour cells [26, 27]. The genetic state in which simultaneous inactivation of 2 genes (or pathways) is lethal, while loss of one or the other alone is viable is called synthetic lethality (also known as conditional genetics). The rationale for inducing synthetic lethality in cancer is that certain cancer cells lack one pathway to repair their DNA (e.g. HR) but have alternative pathways (base excision or single-strand repair) that allow them to survive. Inhibition of these alternative pathways would then impair DNA repair and induce cell death [26, 27]. Therefore it predicts that genotoxic agents leading to a particular type of DNA damage will kill cancer cells with genetic deficits in repair of that type of damage. Recently, this specific anticancer strategy has been the focus of intense investigations [28, 29].

In the case of the hereditary *BRCA1/2*-deficient breast and ovarian cancer syndromes, mentioned earlier, this strategy has been translated into the clinic, in the form of PARP inhibitors. These *BRCA1/2⁻* tumours are defective in the repair of DSBs by HR. When a replication fork in one of these tumours encounters a DNA SSB, it converts that into a DSB, but the presence of a DSB prevents progression of the replication apparatus. Since *BRCA1/2* are both required for DSB repair, the tumour cells with those mutated genes will depend on repair of SSBs to prevent DSBs from occurring. The DNA repair protein PARP1 is required for repair of SSBs, and small molecular inhibitors of PARP1 will prevent repair of SSBs, more specifically in cells that are deficient in *BRCA1/2*. Since normal cells have the ability to repair the DSBs generated at the replication fork, because they have at least one normal allele of *BRCA1/2*, the use of PARP inhibitors has the potential of targeting only tumour cells. This proof of concept proven clinically, where the PARP1 inhibitor olaparib improves the progression-free survival of familial breast cancer [30]. Following this lead several small molecule DNA repair inhibitors are being developed worldwide.

However, not all *BRCA1/2* defective tumours respond equally well to this type of therapy. Thus, in the past years evidence has accumulated that drug resistance is also linked to alterations in these pathways [31-33]. Thus, tumour cells may also acquire resistance by invoking biochemical mechanisms that reduce drug action or by acquiring additional alterations in

DNA damage response pathways [34]. Therefore, the focus has also been directed on DNA repair pathways that could be responsible for cancer drug resistance.

Resistance to chemotherapy limits the effectiveness of anti-cancer drug treatment. Tumours may be intrinsically drug-resistant or develop resistance to chemotherapy during treatment. Acquired resistance is a particular problem, as tumours not only become resistant to the drugs originally used to treat them, but may also become cross-resistant to other drugs with different mechanisms of action. Resistance to chemotherapy is believed to cause treatment failure in over 90% of patients with metastatic cancer [35]. Thus, drug resistance is clearly a major clinical problem.

The attempt to develop more targeted therapeutics has been a major objective in cancer research in last years, and more and more molecular targets are being identified (e.g. tyrosine kinase inhibitors, monoclonal antibodies targeting membrane receptor kinases). Some of these targeted therapies are in clinical use, while others are being evaluated in clinical trials to validate their efficacy. More recently, the quest for targeted therapies has also focused on DNA repair pathways. Unfortunately, resistance to these therapies is also likely to appear, as has occurred with other targeted therapies, such as the tyrosine kinase inhibitors of the fusion *BCR-ABL1* gene, responsible for most cases of chronic myeloid leukaemia (e.g. imatinib, dasatinib, nilotinib). The application of DNA repair inhibitors in the clinic has also shown to be fraught with difficulty, since they also target DNA repair pathways in normal cells. The early clinical trial with MGMT inhibitors in combination with temozolomide (TMZ) was stopped early because the combined treatments harmed bone marrow as well as cancer tissue, whereas the clinical success of PARP inhibitors transpired since PARP is not critical to cell survival. Hence, unlike past visions of a "magic bullet" towards cancer, future research on cancer therapy should more reasonably envisage cancer therapy as a "never ending story", in which novel targeted therapeutics are constantly being overcome by the evolutionary processes present in cancerous cells [36].

2. Targeting DNA repair pathways

As mentioned, DNA repair pathways include the direct reversal of lesions, essentially de-alkylation of alkylated bases by *MGMT*, NER, BER, MMR and the double strand break repair by HR and NHEJ. Alterations in all these pathways have been observed in drug resistant tumour cells; however, the clinical significance of the alterations is not completely understood. Numerous genes involved in each of these pathways have been shown to be up- or down-regulated in diverse types of tumours and constitute a potential source of biomarkers to evaluate drug resistance to cancer chemotherapeutics [25, 32, 33].

3. MGMT and drug resistance

Alkylating agents are widely used to treat cancers, and one of the major DNA lesions formed occurs essentially by the alkylation of DNA at the O^6-position of guanine, which

subsequently can generate DNA breaks and cell death. TMZ, streptozotocin, procarbazine and dacarbazine are examples of cancer chemotherapeutics that methylate DNA [37].

Direct repair of alkylated guanine residues proceeds through the removal of the alkyl moiety by MGMT. MGMT is a conserved protein from prokaryotes through eukaryotes. The MGMT protein removes the alkyl group from O^6-alkylguanine by direct transfer to a cysteine residue in its active site to which the alkyl group becomes covalently attached, resulting in the inactivation of the protein. The MGMT protein is subsequently ubiquitinated and degraded by the proteasome [38, 39]. The O^6-alkylguanine adduct accounts for about 10% of total alkylations, but displays a strong mutagenic and cytotoxic potential, because O^6-alkylguanines exhibit distorted base pairing characteristics in pairing with thymine, thereby, resulting in G:C to A:T transitions upon DNA replication [40]. Hence the unique DNA repair mechanism which depends on the suicidal degradation of the MGMT protein.

Tumour expression of *MGMT* varies and correlates with therapeutic response to alkylating agents. Numerous studies have found a strong correlation between MGMT activity and drug resistance in primary tumours and established human tumour cell lines [16, 41, 42]. High levels of expression have also been noted in melanoma [43], pancreatic carcinoma [16] besides glioblastomas [44]. Resistance to alkylating agents such as TMZ has been linked to over-expression of *MGMT* [43]. Therefore MGMT levels are being studied as biomarkers of intrinsic chemosensitivity to alkylating agents, such as TMZ or BCNU (carmustine).

Conversely, reduced MGMT activity in cultured tumour cells and human tumours is often the result of epigenetic silencing by promoter methylation of CpG islands, which leads to the formation of inactive chromatin that limits transcription, and therefore higher chemosensitivity to alkylation. Hegi *et al*. reported that of 206 patients with glioblastoma that were treated with TMZ and radiotherapy, those with a methylated *MGMT* promoter (45%) had a significantly better survival [45]. Hence, *MGMT* promoter methylation status is emerging as a prognostic factor for tumour therapy and is currently being assessed for selecting glioblastoma chemosensitivity towards TMZ [46-48]. The mechanisms underlying increased *MGMT* promoter methylation are complex and not completely known, although it is one of the most studied DNA repair genes [38]. In normal cells *MGMT* promoter methylation is uncommon, but occurs frequently in tumours. Approximately 25% of tumours of many different types, including non-small-cell carcinoma of the lung, lymphoma, head and neck cancers, and up to 40% of glioma and colorectal tumours were found to present CpG island promoter methylation [49].

Since high *MGMT* expression results in drug resistance to alkylating agents, one strategy to overcome resistance and improve efficacy is to use pseudo substrates of MGMT, such as O^6-benzylguanine (O^6-BG) or O^6-(4-bromothenyl) guanine (O^6-BTG or lomeguatrib or PaTrin-2) which inactivate the enzyme and enhance cell death [50]. O^6-BG is a specific, potent, and nontoxic inhibitor and leads to sensitization of cancer cells to cisplatin, chloroethylating and methylating agents [51, 52]. Clinical trials are underway to test combinations of O^6-BG with carmustine or TMZ for the treatment of glioma, anaplastic glioma, lymphoma, myeloma, colon cancer, melanoma and sarcoma, among others [53]. O^6-BTG presents higher bioavailability than O^6-BG, but also presents higher haematological toxicity when co-administered with

TMZ compared to TMZ alone. Therefore full use of this inhibitor may be more distant [54, 55]. Haematological toxicity was also observed with O^6-BTG co-administered with dacarbazine in patients with advanced melanoma and other solid tumours [56]. The combination of O^6-BTG and TMZ was also evaluated in a phase I clinical trial for advanced solid tumours [57], and in a pilot study for refractory acute leukaemia [58]. A phase I clinical trial was also conducted associating O^6-BTG with Irinotecan for colorectal cancer [59]. A phase II clinical trial of O^6-BTG plus TMZ for stage IV metastatic colorectal cancer is already completed. The trial was considered completed after the recruitment of 19 patients due to the absence of responses and also because evidences from other studies suggest that the O^6-BTG dosing regimen was inappropriate [55]. These studies showed a consistent depletion of MGMT and provided non-toxic doses of O^6-BG or O^6-BTG to be used in further studies. The haematological toxicity observed with the combination of MGMT inhibitors and chemotherapeutic agents might be attributed to an effective depletion of MGMT in off-target cells [60]. Additionally, the administration of a sub-optimal dose of the MGMT inhibitor, a therapeutic dosing schedule that allows the recovery of the MGMT activity or the choice of an inadequate treatment for the type of cancer could explain the lack of effects in clinical trials. In view of this, tumour-targeted delivery of MGMT inhibitors by the development of specific formulations or local administration [61] could be adopted to improve the therapeutic efficacy of the chemotherapeutic drugs and to translate into the clinic the results obtained in preclinical studies. Nonetheless, it is not clear if clinical application of MGMT inhibitors is a viable therapy in all settings.

4. Targeting MMR in cancer drug resistance

MMR is involved in the detection and repair of base-base mispairs during DNA replication, small insertion/deletion mutations at repetitive microsatellite regions and also in the regulation of homologous recombination [62]. MMR proteins are also involved in the repair of DNA damage caused by ROS and alkylating agents. MMR proteins interact with components of other repair pathways, including NER, BER, and HR, thus signalling with other pathways in response to DNA damage.

The MMR system consists of various proteins. MSH2 heterodimerizes with MSH6 or MSH3 to form MutSα or MutSβ, respectively, both of which are ATPases that play a critical role in mismatch recognition and initiation of repair. This induces a conformational change in MutS, resulting in a clamp that translocates on DNA in a ATP dependent manner, recruits the MutL complex, which in humans is a heterodimer consisting of the MLH1 and PMS2 proteins, and displaces DNA polymerase and PCNA, thereafter recruiting an exonuclease (EXO1) that degrades the newly synthesized DNA strand [63]. Other MMR genes (*MLH1*, *MLH3*, *PMS1*, and *PMS2*) are involved in MMR. MLH1 also heterodimerizes with PMS2, PMS1, or MLH3 to form MutLα, MutLβ, or MutLγ, respectively [63]. Polymerase δ (pol δ) then polymerizes the DNA stretch and DNA Ligase I performs ligation.

MMR deficiency leads to a wide range of tumour types. Germline deficiency in MMR accounts for the Lynch syndrome (hereditary non-polyposis colorectal cancer -HNPCC), in

which a large increase in frequency of insertion and deletion mutations in simple repeat (microsatellite) sequences, a phenomenon known as microsatellite instability (MSI), is observed [64]. DNA mismatch repair deficiency in sporadic tumours is seen in colonic, gastric, endometrial, and other solid tumours. MSI is also associated with a wide variety of non-HNPCC and non-colonic tumours, including endometrial, ovarian, gastric, cervical, breast, skin, lung, prostate, and bladder tumours as well as glioma, leukaemia, and lymphoma [65].

Defects in MMR are also associated with resistance to certain chemotherapeutic agents [66]. Resistance to alkylating agents such as TMZ and procarbazine occurs with inactivation of MMR in tumour cells [63]. MMR-deficient cells are relatively resistant to methylating agents (up to 100 fold), whereas cells with a functioning MMR system enter either G2 arrest or apoptosis, depending on the severity of the DNA damage [67]. Down regulation of proteins of the MMR pathway is associated with resistance to clinically important drugs including platinum-containing compounds, anthracyclines, alkylating agents, antimetabolites and epipodophyllotoxins [68].

For example, MSH2 protein deficiency by enhancing MSH2 degradation leads to substantial reduction in DNA mismatch repair and increased resistance to thiopurines. Somatic deletions of genes regulating MSH2 degradation result in undetectable levels of MSH2 protein in leukaemia cells, MMR deficiency and drug resistance [69].

Another agent, etoposide, is a topoisomerase II alpha (*TOP2A*) inhibitor, which is used in the treatment of breast cancer. Alterations in the expression of drug targets or DNA repair genes are among the important resistance mechanisms against TOP2A inhibitors. Decrease in the expression levels of *TOP2A*, and the MMR genes *MSH2* and *MLH1* may play significant roles in the development of chemotherapeutic resistance to etoposide in breast cancer. These genes may be considered for further development of new strategies to overcome resistance against topoisomerase II inhibitors [70].

MMR is also involved in repair of cross-linking agents such as platinum based chemotherapeutics. Increased tolerance to platinum-induced DNA damage can occur through loss of function of the MMR pathway. During MMR, cisplatin-induced DNA adducts are recognized by the MMR pathway, but are not repaired, giving rise to successive repair cycles, ultimately triggering apoptosis. Thus in MMR deficient cells, cell death is not as efficient, promoting tolerance to platinum agents [71].

MMR-deficient cells are also more tolerant to 6-thioguanine treatment, used to treat leukaemias, than MMR-proficient cells. The anti-metabolite 6-thioguanine is incorporated into DNA, where it can be methylated by *S*-adenosylmethionine to 6-methylthioguanine (Me6-thioguanine), which has similar miscoding properties as methylguanine [68].

Nevertheless, although many preclinical studies suggest MMR-deficient cells are resistant to alkylating agents, few clinical studies have been published regarding MMR deficiency and response to alkylating agents. On the contrary, for example, Maxwell *et al.*, [72] found that MMR deficiency does not seem to be responsible for mediating TMZ resistance in adult malignant glioma. Coupled with the lack of substantial data linking polymorphisms within the MMR genes and resistance to chemotherapy or radiotherapy, published work suggests that

the MMR pathway has low priority in the quest for new cancer therapies. However, ongoing research on the role of microRNAs and cancer drug resistance could increase interest in this pathway. Published work has suggested that for example miR-21 targets *MSH2* and consequently induces resistance to 5-Fluorouracil (5-FU) in colorectal cancer [73] (see the section of microRNAs and drug resistance).

5. Targeting BER in cancer drug resistance

BER is the main pathway for removing small, non-helix-distorting base lesions from the genome. Thus, BER targets predominantly base lesions that arise due to oxidative, alkylation, deamination, and depurination/depyrimidination damage. Some examples of chemotherapeutic agents that generate lesions that are targeted by BER include TMZ, melphalan, dacarbazine/procarbazine, and streptozotocin [33]. Some chemotherapeutic agents also generate ROS as a "by-product" such as platinum-based drugs (*i.e.* oxaliplatin and cisplatin), anthracyclines, (*i.e.* epirubicin, daunorubicin, doxorubicin) and paclitaxel [31, 33]. ROS induce DNA lesions that are also repaired by the BER pathway. Additionally, IR produces a number of DNA lesions that are repaired by the BER pathway. Endogenous production of ROS also gives rise to several lesions, which are variable in number and consequence. For instance the highly mutagenic 8-hydroxyguanine (8-oxoG) is formed in large quantities as a consequence of the high oxidation potential of this base, and has a miscoding effect, due to DNA polymerase activity which inserts adenine opposite to 8-oxoG, resulting in G:C to A:T transition mutations.

The BER pathway is initiated by one of many DNA glycosylases, which recognize and catalyze the removal of different damaged bases. After recognition of the damaged base by the appropriate DNA glycosylase, it catalyzes the cleavage of an *N*-glycosidic bond, thus removing the damaged base and creating an apurinic or apyrimidinic site (AP site). The DNA backbone is cleaved by either a DNA AP endonuclease or a DNA AP lyase, activity present in some glycosylases. This creates a single-stranded DNA nick 5' to the AP site. The newly created nick is processed by the AP endonuclease, creating a single-nucleotide gap in the DNA. At this point BER can proceed through a short-patch BER, where polymerase β (pol β) introduces a single nucleotide past the abasic site and Ligase IIIα seals the DNA nick, or through a long-patch BER, where Polymerase δ/ε introduces two to eight nucleotides past the abasic site. The resulting overhang DNA is excised by FEN1 endonuclease and the nick sealed by DNA ligase I [74]. In addition to these enzymes, a number of accessory proteins are involved in BER, including the X-ray cross-complementation group 1 protein (XRCC1), PARP1, the proliferating cell nuclear antigen (PCNA), and the heterotrimer termed 9-1-1, which function in scaffolds for the core BER enzymes [75].

Preclinical evidences have implied the BER pathway in the repair of DNA lesions induced by antimetabolites, monofunctional alkylating drugs, radiotherapy and radiomimetic agents. Moreover, BER modulation may also sensitize cancer cells to the effect of chemotherapeutic drugs that are able to generate ROS [31, 33]. Therefore, targeting BER with inhibitors

of the multifunctional AP Endonuclease 1 and DNA pol β is an attractive field to the development of novel therapeutic compounds.

Some studies have found deregulation of BER genes in tumours. For example pol β has been shown to be overexpressed in a variety of tumour cells [76]. N-methylpurine DNA glycosylase (MPG) overexpression, together with inhibition of BER, sensitizes glioma cells to the alkylating agent TMZ in a DNA pol β - dependent manner, suggesting that the expression level of both MPG and pol β might be used to predict the effectiveness of BER inhibition and PARP-mediated potentiation of TMZ in cancer treatment [77]. We recently observed an increase in expression of the BER genes *MDB4* and *NTHL1* in Imatinib resistant K562 leukaemia cells, and knockdown of their expression in resistant cells using siRNA decreased cell survival after treatment with doxorubicin [78]. Nevertheless, the involvement of deregulated BER components in chemotherapy resistance is not completely evident at present, except for PARP, and the AP endonucleases. The following text shall describe ongoing research targeting these components of the BER pathway.

The major AP endonuclease in mammalian cells is apurinic/apyrimidinic endonuclease 1/ redox-factor-1 (APE1/Ref-1, also called APEX1), and has been found to be elevated in a number of cancers such as ovarian [79], prostate [80], osteosarcoma [81] and testicular cancer [82]. Over-expression of APE1 *in vitro* led to increased protection against bleomycin [82]. Thus elevated levels of APE1 in cancer cells have been postulated to be a reason for chemotherapeutic resistance [81, 83, 84]. Inhibition of APE1 has been shown to increase cell killing and apoptosis and also to sensitize cancer cells to chemotherapeutic agents, and thus APE1 is considered as a molecular target in therapeutics [85, 86].

APE1 endonuclease activity is indirectly inhibited by blocked AP sites that result from the binding of the small molecule methoxyamine (MX) to the DNA. With the APE1's substrate unavailable, BER cannot proceed and the cytotoxic abasic sites accumulate in the cell, eventually leading to cell death. The promising results from *in vitro* and *in vivo* experiments showing MX sensitization to the cytotoxic effect of TMZ [87-90], carmustine [91], pemetrexed [92] and 5-iodo-2'-deoxyuridine (IdUrd) as well as a potentiation of IdUrd-mediated radiosensitization [93, 94], in multiple solid tumours models, provided the proof-of-concept to conduct clinical trials with MX as adjuvant therapy of anticancer agents. A Phase I clinical trial of pemetrexed and oral methoxyamine hydrochloride (TRC102) in patients with advanced refractory cancer is already completed [95]. According to the authors, this drug is well tolerated after daily oral administration and potentiates the activity of chemotherapy. Safety, pharmacokinetic and pharmacodynamic profile of MX was also evaluated in combination with TMZ in a Phase I clinical trial for patients with advanced solid tumours [96]. Currently, two clinical trials (Phase I) are recruiting patients to study the side effects and the best dose of MX to be administered in combination with TMZ and fludarabine phosphate in patients with advanced solid tumours and relapsed or refractory hematologic malignancies, respectively.

In view of the emerging roles of APE1, many efforts have been made to develop small molecule inhibitors that can be translated to the clinic. *In silico* based approaches with design of pharmacophore models [97, 98] and high-throughput screening of several commercially

available libraries of compounds have been performed to identify a pharmacologically active inhibitor for APE1 [86, 99-102]. Lucanthone acts as a direct inhibitor of APE1 but also interacts with other cellular targets and the associated toxicity hinders their therapeutic use [103, 104]. CRT0044876 was identified by a fluorescence-based high-throughput assay and showed promising results in *in vitro* studies [105]. However, some authors were not able to reproduce the reported effects of this compound [85].

Hypersensitivity of DNA pol β-null cells to methyl methanesulfonate (MMS), a DNA-methylating agent, displayed another potential target in BER [106]. Several small-molecule inhibitors of DNA pol β have been identified and many of these compounds are natural products, such as koetjapic acid (KJA), a triterpenoid. Pamoic acid was one of the first synthetic small molecule inhibitors of DNA pol β to be characterized and is more active than the former compound [107]. Nevertheless, the actually known inhibitors of DNA pol β have low potency and specificity that make them weak candidates to drug development (for a comprehensive review see [108]). In view of the preclinical data that suggest an important role of DNA Pol β in the repair of chemotherapeutic-induced DNA damage, the design of effective DNA Pol β inhibitors is an attractive research area.

In what concerns PARP1, this enzyme is a DNA damage sensor that binds to DNA breaks to activate the repair pathways. PARP1 is not directly involved in the repair of the lesions but is essential to signal the damage and to coordinate the functions of several BER and DSB repair proteins. PARP inhibitors have been thoroughly developed and several reviews papers published under this topic. For a recent comprehensive review on PARP inhibitors see Javle *et al* [109]. PARP inhibitors were first evaluated in clinical trials as chemosensitizers. After AG014699 combination with TMZ [110], other PARP inhibitors, specifically INO-1001, ABT-888 and AZD2281 were also tested as adjuvant therapy of multiple anticancer agents such as gemcitabine, carboplatin, TMZ or chemotherapeutic combinations (e.g. cisplatin plus gemcitabine) [111]. Currently, several PARP inhibitors are being evaluated in clinical trials, either in combination with chemotherapeutic drugs or in monotherapy [28, 109, 112-117].

Some of these chemicals showed an enhancement of the toxicity in normal tissues that required dose adjustments and optimization of the therapeutic schedule. Interestingly, preclinical and clinical data revealed that PARP inhibitors as single agents could be less toxic to the normal cells and are more effective in killing *BRCA1-* and *BRCA2*-mutated cancer cells since these cells are defective in HR, the backup pathway responsible for the repair of DSBs generated after PARP chemical inhibition. Similarly, mutations in other proteins related to the DNA damage response, such as ATM and PTEN have also been associated to defects in DSB repair and may be involved in an increased sensitivity to PARP inhibitors [118-120]. These findings led to a novel potential therapeutic indication of the DNA repair inhibitors as single agents in cancer therapy which is currently being evaluated in clinical trials [121]. This synthetic lethal approach was also reported in an *in vitro* study with APE1 inhibitors in BRCA and ATM deficient cells [116, 122].

Recently, negative results from the first phase III clinical trial in breast cancer patients with a combination of iniparib (BSI-201) and gemcitabine/carboplatin were reported [123]. The

mechanism of action of this inhibitor is not fully understood, an issue that should be further clarified. Nonetheless, promising positive outcomes have already been suggested with other PARP inhibitors [124, 125]. A further understanding of the complex PARP interactome, the discovery of PARP1 specific small molecule inhibitors and an accurate selection of the best candidates to the treatment is still needed to improve the quality of information obtained from preclinical and clinical trials and to promote the development of currently known PARP inhibitors as well to discover novel compounds.

6. Targeting NER in drug resistance

NER repairs DNA lesions which alter the helical structure of the DNA molecule and interfere with DNA replication and transcription, such as bulky adducts and cross-linking agents [2]. Briefly, NER consists of the recognition of DNA damage and demarcation of the specific area affected, followed by the formation of a complex to unwind the damaged portion and excise a 24-32 oligonucleotide section that contains the lesion. Finally, the excised nucleotides are resynthesized and ligated. Two NER sub-pathways exist with partly distinct substrate specificity: global genome nucleotide excision repair (GGR) surveys the entire genome for distorting lesions and transcription-coupled repair (TCR) focuses specifically in the transcribed strand of expressed genes, by targeting damage that blocks elongating RNA polymerases. In total more than 30 proteins participate in NER [126]. The genes involved in GGR are DNA damage recognition by XPC-HR23B complex, lesion demarcation and verification by a TFIIH complex, assembly of a pre-incision complex (RPA, XPA and XPG), DNA opening by XPB and XPD helicases, dual incision by ERCC1-XPF and XPG endonucleases, release of the excised oligomer, repair synthesis to fill in the resulting gap, and ligation by ligase I. Defects in the proteins involved in NER result in three autosomal recessive disorders XP, CS, and TTD.

The most relevant class of chemotherapeutics associated with NER is the platinum-based group of agents. Platinum-based chemotherapy has been used for the treatment of a wide variety of solid tumours including lung, head and neck, ovarian, cervical, and testicular cancer for many years [127]. These agents interact with DNA to form predominantly intra-strand cross-link DNA adducts that trigger a series of intracellular events that ultimately result in cell death. The most studied platinum based cancer therapeutics are cisplatin and the less toxic carboplatin and oxaliplatin, but there has been a resurgence in the development of platinum based drugs, and more platinum based chemotherapeutics are in clinical trials [128].

The basic mechanism of action of cisplatin (and carboplatin) involves covalent binding to purine DNA bases: platinum binding to the N7 position of the imidazole ring of the purine bases of DNA — guanine (G) and adenine (A) — to form either monofunctional or bifunctional adducts. In the case of cisplatin, most occur on the same DNA strand and involve bases adjacent to one another, and are therefore known as intra-strand adducts or crosslinks, namely GpG 1,2 intra-strand (60–65% of all adducts) and ApG 1,2 intra-

strand (20–25%) which primarily leads to cellular apoptosis [128]. These DNA lesions are repaired by the NER pathway.

Cisplatin has been used successfully as therapy to treat metastatic testicular cancer with >90 % cure rate. The high sensitivity of testicular tumour cells is attributed to reduced DNA-repair capacity in response to platinum–DNA adducts [129]. Extracts from testicular cancer cells had low constitutive NER capacity and, in particular, low levels of the protein XPA [130]. Further studies have shown low levels of XPA and other NER proteins (XPF and ERCC1), in testicular cancers. This suggested that reducing NER capacity in a cancer holds the potential to sensitize the cancer to cisplatin. Parallel studies revealed that increased DNA repair capacity was a common function in cancers that were inherently resistant to cisplatin or that acquired resistance following treatment [130].

Clinical studies in ovarian cancer patients have correlated increased excision repair cross-complementation group 1 – (ERCC1) mRNA levels with clinical resistance to platinum based chemotherapy [131, 132]. In metastatic colorectal cancer patients, higher ERCC1 expression levels were considered as predictive for lower survival rates when treated with oxaliplatin in combination with 5-fluorouracil, suggesting that enhanced DNA repair decreases the efficacy of platinum-based treatment [133]. In another study a subgroup of 761 patients with metastatic lung cancer treated with a platinum based compound were retrospectively evaluated by immunohistochemical analysis of ERCC1. This study showed a statistically significant survival benefit in patients with low levels of ERCC1 who had received platinum based chemotherapy, compared to patients with low levels of ERCC1 who did not receive chemotherapy and patients with high levels of ERCC1 who received cisplatin chemotherapy [134]. Also, low ERCC1 expression correlated with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer (NSCLC) [135].

Hence, it is hypothesized that high expression of the ERCC1 gene might be a positive prognostic factor, and could predict decreased sensitivity to platinum-based chemotherapy. Expression of ERCC1 has been used to stratify patients treated with platinum based chemotherapeutics with some success, and also to predict improved survival in platinum treated patients [136]. Nonetheless, results from the published data are inconsistent. To derive a more precise estimation of the relationship between ERCC1 and the prognosis and predictive response to chemotherapy of NSCLC, a meta-analysis was performed and results indicated that high ERCC1 expression might indeed be a favourable prognostic and a drug resistance predictive factor for NSCLC [137].

Other studies with different tumour/chemotherapy associations have shown that ERCC1 mRNA expression in tumours may be a predictive marker of survival for Irinotecan-resistant metastatic colorectal cancer receiving 5-FU and Oxaliplatin combination chemotherapy [133]. In this study patients whose tumours had low ERCC1 mRNA expression had a significantly longer median survival than those with high ERCC1 expression.

Other genes involved in NER have been shown to influence drug resistance. For example, increased expression of excision repair cross-complementation group 4 (ERCC4 or XPF) was observed in hydroxycamptothecin (HCPT) treated bladder cancer tissue compared to un-

treated samples. Complementary *in vitro* studies showed that enhanced *ERCC4* expression decreased the sensitivity of bladder T24 cells and 5637 cells to HCPT, whereas after gene silencing of *ERCC4* the chemotherapeutic resistance of bladder cancer cells to HCPT was significantly decreased [138].

Since the NER pathway is crucial for the repair of bulky adducts and cross-linking agents in normal cells, the development and application of NER inhibitors in clinical settings is scarce, although preclinical data show that the manipulation of this pathway could be a relevant strategy in cancer chemotherapy. For example, preclinical studies have demonstrated that the chemotherapeutic action of the platinum agent oxaliplatin is improved when combined with cetuximab, a chimeric IgG1 monoclonal antibody targeting the epidermal growth factor receptor. This antibody has been shown to reduce the expression of *ERCC4* and *ERCC1*. A concomitant increase in the accumulation of platinum and apurinic/apyrimidinic sites on DNA during oxaliplatin treatment was observed, thus leading to an increase in apoptosis [139, 140]. These interesting results are suggestive that targeting other pathways that regulate expression of DNA repair genes could be a promising strategy.

7. HR and drug resistance

HR repairs DSBs, which occur through exposure to various chemotherapeutic agents, including IR, topoisomerase inhibitors and DNA crosslinking agents (e.g. mitomycin, camptothecins, etoposide, doxorubicin, daunorubicin and bleomycin). HR is also recruited to restart stalled replication forks and to repair ICL, the repair of which also involves the FA protein complex. HR ensures the accurate repair of DSBs by using a homologous undamaged DNA strand from an intact sister chromatid as a template for DNA polymerase to extend past the break, and is thus restricted to late S and G2 of the cell cycle. Components of HR include the RAD group of proteins (including RAD50, RAD51, RAD52, and RAD54), RPA, XRCC2, XRCC3, and the BRCA proteins. Briefly, HR occurs through pre-synapsis, preparation of a recombination proficient DNA end; synapsis, formation of a joint molecule between the recombination proficient DNA end and a double-stranded homologous template DNA; post-synapsis and resolution, repair of DNA strands and separation of the recombined DNA molecules [19]. DSBs can also be repaired by NHEJ that do not utilize significant homology at the broken ends. In NHEJ, DSBs are recognized by the Ku protein that then binds and activates the protein kinase DNA-PKcs, leading to recruitment and activation of end-processing enzymes, polymerases and DNA ligase IV. Whereas HR is restricted to late S and G2, NHEJ functions in all phases of the cell cycle and ligates broken DNA ends without the need of an undamaged template.

Following DNA lesions initial checkpoint signalling is performed by the kinases ATR and ATM, two phosphatidylinositol 3-kinase family members. Activation of these kinases leads to activation of the effector kinases, checkpoint kinases 1 and 2 (Chk1 and Chk2; serine/threonine kinases). The activated effector kinases are then able to transiently delay cell cycle progression through the G1, S, or the G2 phases so that DNA can be efficiently repaired. The

ATM/Chk2 pathway predominantly regulates the G1 checkpoint and the ATR/Chk1 pathway the S and G2 checkpoints. However, there is cross-talk between the pathways implying a role for both ATR and ATM pathways in all cell cycle checkpoints. In addition to directly regulating the cell cycle, the pathways also affect DNA repair, transcription, chromatin regulation, and cell death. Many details of these pathways are not fully known.

One consequence of DSBs is the localized alteration of chromatin adjacent to DSBs in order to facilitate recruitment of repair proteins. For examples, ATM not only phosphorylates DNA repair proteins recruited to DNA ends but also the histone variant H2AX in nucleosomes adjacent to DSBs, which is also phosphorylated by DNA-dependent protein kinase (DNA-PK), another protein kinase activated by DSBs. Phosphorylated H2AX (known as γ-H2AX) around DSBs facilitates the recruitment of a number of DNA repair proteins and chromatin modulating factors. The presence of large patches of γ-H2AX around a DSB has made its detection by fluorescent tagged antibodies a biomarker for DSBs [141, 142].

There is accumulating evidence for the existence of HR defects not only in familial cancers but also in sporadic cancers. Mutations or epigenetic alterations have been observed in several genes known to be involved in HR regulation and repair, such as *BRCA1* and *BRCA2*. Functional analysis of human cancer tissues and cancer cell lines has revealed HR deficiency, chromatid-type chromosomal aberrations, severe ICL hypersensitivity, and impaired formation of damage-induced RAD51 foci. For example, although genetic mutations in *BRCA1* or *BRCA2* are only rarely found in sporadic tumors, in contrast to familial breast and ovarian cancers, epigenetic gene inactivation of the *BRCA1* promoter is a fairly common event in sporadic breast cancers, with aberrant methylation being detected in 11 to 14% of cases [143]. Non-triple-negative sporadic breast cancers may also harbor HR defects. It has been suggested that ~20% of these cancers are defective in HR as measured by an impaired ability to mount RAD51 foci in response to chemotherapy [144]. There is emerging evidence that approximately up to one fifth of non-familial breast cancers harbour HR defects that may be useful targets for therapy.

The BRCA1 and BRCA2 proteins are involved in HR, in association with FA proteins, forming a complex DNA damage response network [145]. *BRCA1* expression levels have been demonstrated to be a biomarker of survival following cisplatin-based chemotherapy for NSCLC and ovarian cancer, suggesting that this gene could be involved in response to platinum therapy [146, 147]. *In vitro* studies indicate that loss of *BRCA1* or *BRCA2* increases sensitivity to agents that cause DSBs such as bleomycin and/or ICLs including platinum agents. Conversely, loss of *BRCA1* or *BRCA2* may increase resistance to microtubule interfering agents such as taxanes and vincristine [148, 149]. In contrast, *BRCA1* may increase sensitivity to spindle poisons by activating the mitotic spindle checkpoint and signalling through a proapoptotic pathway. This dual role of increasing apoptosis and therefore sensitivity to spindle poisons and also promoting DNA repair and cell survival after treatment with DNA-damaging drugs may influence the response of breast and ovarian cancer cells to treatment [150]. Chemotherapy in breast and ovarian cancers is attained by treatment with platinum based compounds and anthracyclines and also taxanes, all of which induce both

SSBs and DSBs. Efforts are underway to use *BCRA1* as a predictive marker for chemotherapy customization and response [151].

Regarding other types of cancer, *BRCA1* promoter hypermethylation is also found in approximately 5-30% of sporadic ovarian cancers. Also, mutations in *BRCA1* and *BRCA2* have recently been found in up to 20% of unselected ovarian cancers [152]. Thus, these HR deficient cancers are viable targets for synthetic lethality approaches with PARP inhibitors. Defects in the FA/BRCA pathway as well as ATM defects have been described in a variety of other malignancies, such as prostatic adenocarcinoma, colorectal cancer, leukaemia, lymphoma, and medulloblastoma [153, 154]. However, it remains to be seen whether these defects can be targeted effectively in the clinic.

Single-agent chemotherapy with a nitrogen mustard, usually Chlorambucil, is the standard initial therapy for Chronic lymphocytic leukaemia (CLL) and at least 60–80% of patients respond but eventually all patients become resistant to these agents. XRCC3 protein levels and DNA-damage induced RAD51 foci correlates with chlorambucil drug resistance in lymphocytes from CLL patients and with melphalan and cisplatin resistance in epithelial tumor cell lines, indicating that increased HR can be involved in drug resistance to these agents [155].

Another component of the HR pathway, *RAD51*, has been found to be increased in expression in a wide range of human tumors, most likely contributing to drug resistance of these tumors. Over-expression of *RAD51* in different cell types leads to increased homologous recombination and increased resistance to DNA damaging agents to disruption of the cell cycle and apoptotic cell death. *RAD51* expression is increased in p53-negative cells, and since *TP53* is often mutated in tumor cells, there is a tendency for *RAD51* to be overexpressed in tumor cells, leading to increased resistance to DNA damage and drugs used in chemotherapies [156].

Chronic myeloid leukaemia (CML) cell lines expressing the fusion protein BCR-ABL1 utilize an alternative non-homologous end-joining pathway (ALT NHEJ) to repair DSBs. The expression levels of PARP1 and DNA ligase IIIα served as biomarkers to identify a subgroup of CML patients who may be candidates for therapies that target the ALT NHEJ pathway when treatment with TKIs has failed [157]. Tamoxifen- and aromatase-resistant derivatives of MCF7 cells and Estrogen Receptor-/Progesterone Receptor- (ER-/PR-) cells have higher steady-state levels of DNA ligase IIIα and increased levels of PARP1, another ALT NHEJ component. Notably, therapy-resistant derivatives of MCF7 cells and ER-/PR- cells exhibited significantly increased sensitivity to a combination of PARP and DNA ligase III inhibitors that increased the number of DSBs. Thus, ALT NHEJ may be a novel therapeutic target in breast cancers that are resistant to frontline therapies and changes in NHEJ protein levels may serve as biomarkers to identify tumors that are candidates for this therapeutic approach [158].

Another interesting approach in this field is to target components of the DNA damage response, namely DNA damage signalling and cell-cycle checkpoints [34]. The members of the phosphatidylinositol (PI) 3-kinase-like (PIKK) family perform crucial roles in the activation of DSB repair pathways, namely in HR and NHEJ. ATM, a PIKK family mem-

ber, is a DSB signalling protein mainly implicated in the phosphorylation of effector proteins from HR. ATM has been also involved in the regulation of NHEJ. KU55933, 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one is a specific and potent small-molecule inhibitor of ATM identified by screening of a combinatorial library. Preclinical studies have shown an increase in the cytotoxicity of multiple chemotherapeutic drugs as doxorubicin, etoposide, camptothecin and ionizing radiation [159, 160] while the UV-induced cellular effects were not modified. More recently, KU60019, an improved analogue of KU55933, was developed. Besides its radiosensitizing properties, *in vitro* studies revealed that KU60019 may also impair the migration and invasion of tumor cells by inhibiting ATM-mediated AKT phosphorylation [161].

DNA-PK is also a target to the development of chemo- and radiosensitizers [162]. In fact, the identification of specific small molecule modulators of DNA-PK [163-165], namely NU7441 and NU7026, was shown to potentiate the effects of ionizing radiation as well as chemotherapeutic agents in human tumor cell lines and in *in vivo* xenograft models.

Another example is the development of AZD7762, which potently inhibits Chk1 and Chk2, abrogates DNA damage-induced S and G2 checkpoints, enhances the efficacy of gemcitabine and topotecan, and modulates downstream checkpoint pathway proteins [166]. This agent has been evaluated in clinical trials, however due to an inadequate response the drug has been discontinued in 2011 (http://www.astrazenecaclinicaltrials.com).

8. MicroRNAs and chemotherapy resistance

MicroRNAs (miRs) are small non-coding RNAs (19 to 25 nucleotides) that regulate gene expression by binding to 3' untranslated region (UTR) of several mRNAs, thus blocking translation. Recently, it was also shown that miRs can act by binding to open reading frames or 5'UTR of mRNAs, as revised by Iorio and Croce [167]. Due to small size and incomplete complementarity to mRNA, one miR can have a widespread effect on the transcriptome of a cell, acting as a hallmark of several diseases, including cancer. Numerous studies have been performed regarding biogenesis and function of miRs, being revised elsewhere [168-170]. *In vitro* and *in vivo* studies have suggested that miRNAs might be useful as diagnostic and prognostic markers, and recent data suggest that miRNA profiling can be used for tumor typing.

Although it is well established that miRs have an important role in cancer, the complexity of their action remains to be understood and questions regarding their use as cancer therapy need further investigation. The strong pleiotropy of miRs in deregulating normal cellular homeostasis due to misexpression, has led investigators to believe that they are valuable targets for cancer therapy and consequently for drug resistance. Two major approaches for using miRs as therapeutics can be described. First, miRs can be used as single molecules or combined in order to target one or multiple transcripts. In this approach, a miR or a set of miRs are antagonized or mimicked to alter miR levels and consequently change the protein

outcome in a cancer cell. Second, miRs can act as modulators of cell sensitivity for cancer therapy [167, 171]. This second approach will be our focus.

Many studies regarding miRs expression patterns in cancer cells have been performed. These studies not only allow investigators to determine novel biomarkers for a better and easily prognostication of several types of cancer but also the functional role of the same miRs. These can give us the knowledge if the loss or gain of miR function interferes with the original balance of protein levels which may be important, but not only, in drug response and consequently lead to drug resistance. Since miRs expression seems to be tissue, grade and stage specific, the ectopic expression or repression of miRs in conjugation with cancer therapy seems promising. For that reason, recent studies that evaluate miR expression profiles of sensitive and resistant cell lines have been made in order to find the key miR signatures related to drug response, which not only promote further analysis of the mechanisms of cancer drug resistance, but also allow the discovery of new drug targets and individualized medicine.

Although the study of the therapeutic potential of miRs is still recent, several studies have been published and compiled. For example, Tian *et al*. [172] and Kutanzi *et al*. [173], published compilations of several studies reporting influence of miRs in mechanisms of drug resistance and how they can modulate drug response in breast cancer.

With regard to miRs and modulation of drug resistance through regulation of DNA damage and repair genes, studies are scarce. It is known that miRs have an important role in DNA damage response, which includes DNA repair [174, 175]. One example how miRs can influence drug resistance through DNA repair is demonstrated by Valerie et al. [73]. The authors showed that miR-21 targets *MSH2* and consequently induces resistance to 5-FU in colorectal cancer. Since miR-21 has a pleiotropic effect, it is possible that it could regulate other genes associated with drug resistance. However, the impact of *MSH2* seems to be of extreme importance on acquired 5-FU resistance since when knocked out cells for *MSH2* are transfected with miR-21, cell-cycle arrest or apoptosis is not altered. These results show that the inhibition of miR-21 action might represent an important treatment to overcome 5-FU resistance. A correlation between miR-21 and *MSH2* in breast cancer was also found [176]. It is recognized that TGF-β is a promoter of miR-21 processing through the interaction with the SMAD and DROSHA complex. On the other hand, *MSH2* is a proven target of miR-21. Thus, TGF-β inhibits *MSH2* gene expression and consequently increases drug resistance. Indeed, to find out if TGF-β contributes to drug resistance through *MSH2*, the authors tested the response of breast cancer MDA-MB-231 cell line to cisplatin, methyl methanesulfonate (MMS) and doxorubicin in the presence and absence of TGF-β. Exposure to TGF-β for 24 h increased cell viability upon treatment with these DNA damaging agents and knock down of *MSH2* induced resistance to both cisplatin and doxorubicin. In contrast, transfection of the anti-miR-21 enhanced the effect of cisplatin in MDA-MB-231 cells.

Another example of miR influence in DNA repair and consequent drug response is miR-182 that targets *BRCA1*. Moskwa and colleagues showed that ectopic expression of miR-182 represses BRCA1 protein expression and sensitizes breast cancer cells to PARP inhibitors [177]. However, PARP inhibitors are mostly used in patients with BRCA1 inherited muta-

tions. Therefore, the question if PARP inhibitors are useful therapeutic drugs in sporadic breast cancer rises. Theoretically, if administered with BRCA1 repressors such as miR-182, PARP inhibitors can have the same effect as in inherited breast cancer. Further studies need to be done in order to clarify this issue.

As described previously, MGMT has DNA repair activity insofar as it can remove mutagenic O^6-alkylguanine induced by alkylating agents. Although TMZ has been widely used in glioblastoma multiforme (GBM), many patients become or are resistant to this chemotherapy agent, since MGMT can repair the DNA damage induced by TMZ. Epigenetic regulation mechanisms, such as methylation of the *MGMT* gene promoter can sensitize cancer cells to alkylating chemotherapeutic drugs. Glioblastoma patients with positive methylation status of *MGMT* gene promoter have been reported to present a better response to TMZ treatment [44], but these results have not been confirmed by other studies, and therefore results are ambiguous [178]. Indeed, some patients with unmethylated status of *MGMT* promoter gene also have good response to TMZ, which points out to other regulatory mechanisms of *MGMT* expression [179]. Thus, miRs appear as good alternative regulation candidates of *MGMT* expression levels. Recent evidence also suggests that the miR-181 family might be associated to drug response [180]. The authors found that glioblastoma patients with low expression of miR-181b and miR-181c have a better response to TMZ. On the contrary, miR-181d seems to post-transcriptionally regulate *MGMT* since both directly interact and inversely correlate in relation to expression levels [181]. This fact is important because it could be a predictive biomarker for chemotherapy response in GBM. Lakomy and collaborators found that high expression of miR-195 and miR-196b is significantly associated with longer survival of GBM patients and miR-21 and miR-181c with high risk GBM patients [182]. However none of these miRs were associated with *MGMT* gene promoter status.

Altogether the potential for use of miRs in cancer therapy is high, so are the challenges, since each miR can target up to hundreds of mRNA targets. The rapid elucidation of the role they play in cancer suggest that translation of this knowledge will rapidly reach the clinic.

9. Phytochemicals as alternative therapies against drug resistance

As discussed previously, frequently novel therapeutics that show promising results in pre-clinical assays reveal unacceptable toxicity in clinical trials. Since cancer cells frequently present deregulation of multiple cellular pathways, targeting multiple pathways seems more promising than using single agents that target single pathways. In recent years natural dietary compounds such as curcumin, resveratrol and soy isoflavones such as genistein, have received attention due to the fact that they frequently target multiple cell signalling pathways, including the cell cycle, apoptosis, proliferation, survival, invasion, angiogenesis, metastasis and inflammation. Thus their use in chemoprevention has gained attention [183, 184]. Additionally, since most of the cancer drugs developed have been deliberately directed toward specific molecular targets that are involved in one way or another in enabling particular cellular functions, in response to monotherapy cancer cells may reduce their depend-

ence on a particular proficiency (e.g. a single repair pathway), becoming more dependent on another, thus contributing to acquire drug resistance. Thus, as an alternative approach, selective co-targeting of multiple core and emerging hallmark proficiencies in mechanism-guided combinations could result in more effective and durable therapies for human cancer [185]. Phytochemicals can be highly pleiotropic, modulating numerous targets, including the activation of transcription factors, receptors, kinases, cytokines, enzymes, and growth factors [186]. Therefore current efforts are highly engaged in discovering natural plant-based chemicals that could assist in the fight against drug resistance.

For example soy isoflavones inhibited APE1 expression in prostate cancer cells in a time- and dose-dependent manner, whereas IR up-regulated expression of this BER gene, in response to DNA damage [187-190]. Pretreatment of cancer cells with soy isoflavones inhibited the increase in expression of APE1, and enhanced the efficacy of chemotherapy and radiation therapy of multiple cancers models *in vitro* and *in vivo*, possibly through down-regulation of this DNA repair gene [188]. Another phytochemical, resveratrol, was also shown to inhibit APE1 endonuclease activity and render melanoma cells more sensitive to treatment with the alkylating agent dacarbazine [191]. Thus both resveratrol and isoflavones such as genistein can have therapeutic potential as an APE inhibitor. A series of analogs of resveratrol have been generated in recent years, which exhibit increased potency and/or a range of selective activities compared to the parental compound resveratrol, and possibly improved pharmacokinetic properties [192]. A clinical trial of resveratrol in colon cancer has recently been completed (http://www.clinicaltrials.gov).

Resveratrol can also increase *BRCA1* and *BRCA2* expression, although no effect is seen at the protein level [193]. An increase in BRAC1 expression can lead to increased arrest of cells in the G2 phase, thus making them much more sensitive to conventional therapy. One common chemotherapeutic drug is doxorubicin, which predominantly induces DNA damage in G2 phase cells [194]. Resveratrol, curcumin and the naturally occurring flavolignan deoxypodophyllotoxin [195] can induce G2/M cell cycle arrest, and alter the expression of cell cycle regulatory proteins, thus allowing doxorubicin to induce lesions and as a consequence enhance the apoptotic effect [186, 196, 197]. Le Corre *et al.*, also demonstrated that resveratrol has an effect on the expression of genes implicated in the regulation of BRCA1 protein functions and in multiple nuclear processes modulated by BRCA1 in human breast cancer and fibrocystic breast cells [198]. One of the mechanisms by which resveratrol can enhance *BRAC1* expression is by association with BRCA1, repressing the aromatic hydrocarbon receptor (AhR). AhR binds many natural dietary bioactive compounds therefore combination diets with AhR antagonists may offer the advantage of higher cancer prevention efficacies [199]. In HR-deficient tumours, patients with heterozygous mutations in the HR genes *BRCA1* and *BRCA2* develop breast and ovarian tumours with functional loss of HR activity, and deficiency in this pathway may dictate the sensitivity of tumours to certain DNA-damaging agents and this may be another possible approach to test natural compounds to overcome resistance, and once more enhance combinatory strategies to optimize treatment outcome [32].

Recently an extract of neem leaves was characterized and a significant up-regulation of genes associated with metabolism, inflammation and angiogenesis, such as *HMOX1* and

AKR was observed. However genes associated with cell cycle, DNA replication, recombination, and repair functions were down-regulated [200]. One study analysed 531 compounds derived from plants and found no correlation with genes involved in NER (*ERCC1, XPA, XPC, DDB2, ERCC4, ERCC5*) or BER (*MPG, APE1, OGG1, XRCC1, LIG3, POLB*). It is possible that natural compounds may target different molecular pathways from those of standard anti-tumor drugs, hence if DNA repair is involved in the development of resistance to established anticancer drugs, natural compounds may be attractive sources of novel drugs suitable to treat drug resistant tumours, with the advantage of having reduced side effects [201].

Likewise, most plant derivatives can act as antioxidants and some of them can increase human *MGMT* expression (e.g. curcumin, silymarin, sulforaphane and resveratrol) beyond its steady-state levels, having a role in cancer chemoprevention [202]. Additionally, both *BRCA1* and *MGMT* genes are susceptible to hypermethylation, and green tea polyphenols and bioflavonoids have been shown to reverse the effects of DNA hypermethylation [203].These results suggest that some dietary compounds may have a potential demethylating effect, and could be promising adjuvants to chemotherapy in drug resistant settings.

Another issue in cancer chemotherapy is the use of monotherapy *vs* combined therapy, and several studies have been performed regarding possible combinatory chemotherapy with natural compounds (less aggressive than the majority of chemotherapeutic drugs), albeit in pre-clinical settings, *e.g.* silibinin extract [204], ixabepilone [205] and curcumin [206]. Some of these agents are being evaluated in clinical trials. Silibinin strongly synergized the growth-inhibitory effect of doxorubicin in prostate carcinoma cells, which was associated with a strong G2-M arrest followed by apoptosis [204]. Ixabepilone, an analogue of the natural product epothilone B, is already indicated for the treatment of locally advanced or metastatic breast cancer in the US. In a phase III trial in women with locally advanced or metastatic breast cancer that were pretreated with, or resistant to, anthracyclines (e.g. doxorubicin) and resistant to taxanes, progression-free survival was significantly longer in ixabepilone plus capecitabine recipients compared with recipients of capecitabine monotherapy [205]. Combination therapy using curcumin with gemcitabine-based chemotherapy, in a phase I/II study, in patients with pancreatic cancer warrants further investigation into its efficacy [206].

Finally, an interesting recent development concerns the observation that miRs could be regulated by natural agents, leading to the inhibition of cancer cell growth, epithelial to mesenchymal transition (EMT), drug resistance, and metastasis [207]. For most epithelial tumors, progression toward malignancy is accompanied by a loss of epithelial differentiation and a shift toward mesenchymal phenotype [185]. During the acquisition of EMT characteristics, cancer cells lose the expression of proteins that promote cell-cell contact, such as E-cadherin and γ-catenin, and gain the expression of mesenchymal markers, such as vimentin, fibronectin, and N-cadherin, leading to enhanced cancer cell migration and invasion. It has been shown that down-regulation or the loss in the expression of the miR-200 family is associated with EMT. Gemcitabine-resistant pancreatic cells having EMT characteristics showed low expression of the miR-200 family and miR-200 is lost in invasive breast cancer cell lines with mesenchymal phenotype. Hence the interesting observation that isoflavone could induce miR-200 expression in gemcitabine-resistant pancreatic cells, resulting in altered cellular morphology

from mesenchymal-to-epithelial appearance and induced E-cadherin distribution that is more similar to epithelial-like cells. Likewise, let-7 has been found to regulate cell proliferation and differentiation, and inhibit the expression of multiple oncogenes, including ras and myc, and again it was observed that isoflavone could significantly up-regulate the expression of let-7 family, suggesting that this phytochemical could reverse EMT characteristics in part due to the up-regulation of let-7 [207]. Other reports have shown that curcumin, isoflavone, indole-3-car-binol (I3C), 3,3′-diindolylmethane (DIM), (−)-epigallocatechin-3-gallate (EGCG) or resveratrol, can alter miRNA expression profiles, leading to the inhibition of cancer growth, induction of apoptosis, reversal of EMT phenotype, and increasing drug sensitivity [208].

It remains to be seen if phytochemicals can affect miRs that regulate DNA repair pathways, but since any given miR can target several transcripts, this regulation is highly likely. Over-all, natural compounds, may have an important role in chemoprevention and in combined therapy, and may prevent resistance to chemotherapy [188, 189, 208-210].

10. Conclusion and future directions

As discussed in this chapter, the ultimate target of chemotherapy and radiotherapy is the cancer cell, and use of DNA damaging agents is justifiable since most of these cells are high-ly cycling cells. The targeting of DNA repair pathways is but one of the many strategies de-veloped in the fight against cancer. Cancer cells frequently possess altered DNA repair capacities, and this can be put to use in the clinic. Thus the quest for specific therapies that target DNA repair has produced many potentially useful agents (Table 1). Using such agents can theoretically increase the efficacy of existing chemotherapy and/or radiotherapy. Nevertheless, the same difficulties encountered by all other alternative strategies are also arising when we disrupt DNA repair processes.

The success of these agents ultimately will depend on our basic knowledge of the various DNA repair processes present in a given cell type or tissue. Not all DNA repair pathways are present in all tissues, as evidenced by the fact that mutations in specific pathways give rise preferentially to certain tumour types and not others. Secondly, the success will also de-pend on the specific genomic and genetic landscape of each tumour, implying that different combinations of inhibitors and chemical agents shall have to be tailored to each tumour. We are still far from achieving this goal, but great strides have been taken in the past years. Thirdly, we shall have to redirect the strategy to discover a "cure for cancer" and instead follow strategies that allow us to accompany the inevitable and inexorable evolution of the cancer cell and consistently find and implement more and more targeted therapies, even if these strategies lead us to return to abandoned therapies. The resurgence of drug holidays, in which a therapy is abandoned temporarily to be taken up after a certain period, not un-like what can be adopted with antibiotics, is one such strategy. In this case the absence of a selective pressure imposed by a specific agent may lead cancer cells to lose resistance to this agent, making them again vulnerable to the same agent. This strategy has been followed in certain cancers and could be adapted in others, with the advantage of offering reduced time on chemotherapy, reduced cumulative toxic effects, and improved quality of life [211, 212].

Target	Drug	Condition or tumor	Combination therapy agent(s)	Phase of clinical trial planned, ongoing or recently completed*	Reference
MGMT	O⁶-Benzylguanine	Multiple Myeloma and Plasma Cell Neoplasm	Carmustine	Phase II completed	www.cancer.gov
		Glioblastoma, Gliosarcoma	Temodar	Phase II completed	
		Melanoma	Carmustine	Phase II completed	
		Colorectal Cancer	Carmustine	Phase II completed	
PARP1	AZD-2281/ KU59436 (Olaparib)	Triple Negative Breast Cancer	Cisplatin	Phase I/II active	www.astrazeneca.com
		Triple Negative Metastatic Breast Cancer	Paclitaxel	Phase I/II completed	
		Known BRCA Ovarian Cancer or Known BRCA/ Triple Neg. Breast Cancer			
	AG014699/ PF-01367338 (Rucaparib)	Solid tumors	Temozolomide	Phase I completed	www.pfizer.com
		Melanoma	Various agents	Phase II ongoing	
	INO-1001	Melanoma	Temozolomide	Phase I terminated	www.inotekcorp.com
	BSI-201/ (Iniparib)	Uterine Carcinosarcoma	Carboplatin, Paclitaxel,	Phase II active	www.biparsciences.com www.sanofi.com
		Breast Cancer	Gemcitabine/ Carboplatin	Phase II completed Phase III active	
	ABT-888/ (Veliparib)	Breast cancer	Carboplatin Temozolomide	Phase II active	www.abbott.com
		Prostate Cancer	Temozolomide	Phase I active	
		Melanoma	Temozolomide	Phase II active	
		Various cancers	Various agents	Phase I/II active	
	MK4827	Solid BRCA Ovarian	Single agent Various agents	Phase I ongoing	www.merck.com
	CEP-9722	Solid tumours	TMZ Various agents	Phase I	www.cephalon.com www.tevapharm.com
	GPI 1016/ E7016	Solid tumours	TMZ Various agents	Phase I	www.eisai.com
	LT673	Hematological cancers Solid tumours	Various agents	Phase I ongoing	www.bmrn.com
	NMS-P118			Preclinical; highly selective against PARP-5 (tankyrase)	www.nervianoms.com
BER	Methoxyamine/ TRC-102	Advanced refractory solid cancers	Pemetrexed	Phase I active	www.traconpharma.com
		Hematological cancers	TMZ Fludarabine	Phase I ongoing	
ATM Kinase	KU55933			Preclinical	www.astrazeneca.com
CHK1	AZD7762				www.astrazeneca.com
	PF-00477736				www.pfizer.com
	XL844				www.exelixis.com
FA	Curcumin	Gastrointestinal cancers		Phase II	

Target	Drug	Condition or tumor	Combination therapy agent(s)	Phase of clinical trial planned, ongoing or recently completed*	Reference
Pathway					
c-ABL	Imatinib	Various solid tumours		Phase III	www.novartis.com
EGFR	Erlotinib	NSCLC	Monotherapy or combination	Phase II/III	www.gene.com
	Gefinitib				www.astrazeneca.com

* As of 10 September 2012, http://clinicaltrials.gov

Table 1. Targeted therapeutics in development, in clinical use or in clinical trials*.

This leads to the final and perhaps most challenging problem in the development of agents that modulate DNA repair, which is toxicity to normal cells, in particular to the hematopoietic system and the gastrointestinal epithelia. Various strategies are being followed to minimize toxicity, which include the intermittent administration during therapy, mentioned above, alternating with other therapies, using highly localized radiotherapy together with inhibitors to minimize collateral damage, and using inhibitors as single agents [213, 214]. Altogether, the combined use of the various weapons at our disposal in a coordinated, comprehensive fashion could effectively lead to improved patient treatment.

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