PHARMACOLOGY OF OXALIPLATIN AND THE USE OF PHARMACOGENOMICS to individualize therapy

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SUMMARY

Oxaliplatin is a relatively new platinum analogue that is currently used in pharmacotherapy of metastatic colorectal cancer. Its dose-limiting toxicity is sensory neuropathy, which can be modulated by infusion of calcium and magnesium. Oxaliplatin exerts its anti-tumour effects by platinum-adduct formation, binding to cellular proteins and possibly interfering with RNA synthesis as well. If they are not removed from DNA, oxaliplatin adducts are lethal. Cellular defense mechanisms prevent adduct formation (e.g., glutathione-S-transferase) or remove DNA adducts (e.g., nucleotide excision repair). Depending on the activity of necessary enzymes in these cellular defense pathways, oxaliplatin induced damage varies from one individual to another.

There is growing evidence that polymorphisms in genes coding for DNA repair enzymes and metabolic inactivation routes contribute to the interindividual differences in anti-tumour efficacy and toxicity of oxaliplatin. Single nucleotide polymorphisms (SNPs) may yield inactive enzymes, or increased gene transcription and hence increased enzyme production. This review covers findings of recent investigations on the associations of SNPs and clinical outcome after oxaliplatin chemotherapy in metastatic colorectal cancer.
INTRODUCTION

DEVELOPMENT OF PLATINUM ANALOGUES

Oxaliplatin (Eloxatin®) is a relatively new platinum analogue that has been licensed in the European Union since 1999, and in the United States since 2002. The drug is currently applied in new promising chemotherapeutic regimens in the treatment of advanced colorectal cancer.

The first platinum analogue cisplatin (Platinol®) was described in 1844 as Peyrone’s chloride. Its cytotoxic properties were unrecognized until 1965 when Rosenberg and his colleagues observed inhibition of bacterial growth by an electric current. Later it appeared that this phenomenon was due to the formation of cytotoxic compounds, e.g., cisplatin in an ammonia buffer around the platinum electrode and not attributable to direct effects of the electric current itself.

Cisplatin was investigated in several clinical trials in the early 1970s and became available for clinical use in 1978. Cisplatin has little effect on colorectal cancer but it changed the prognosis for ovarian and especially for testicular cancer patients to a great extent. However, cisplatin causes severe side effects of which renal toxicity and peripheral neuropathy are dose-limiting. Renal toxicity caused by cisplatin is clinically manifested by elevation of blood urea nitrogen, serum creatinine and electrolyte disturbances (e.g., hypomagnesemia). Adequate intravenous hydration, slow cisplatin infusion rates and simultaneous administration of mannitol are applied to circumvent renal toxicity. Cisplatin has a high emetogenic potential but its gastrointestinal side effects such as nausea and vomiting can be effectively controlled by administration of dopamine agonists, corticosteroids and especially serotonin antagonists, alone or in combination. In contrast, ototoxicity and neurotoxicity by cisplatin are difficult to control and quite often a reason to stop treatment or reduce dose. Ototoxicity is characterized by tinnitus (often reversible) and hearing loss (irreversible), especially in the high-frequency range. Its severity is usually related to the cumulative dose received in subsequent therapeutic courses. Neurotoxicity involves peripheral neuropathy of the upper and lower limbs, including paresthesias, weakness, tremors and loss of taste.

OXALIPLATIN

In an attempt to overcome the renal and gastrointestinal side effects of cisplatin, less toxic platinum analogues were developed and as a result, carboplatin has replaced cisplatin in many chemotherapeutic regimens. Carboplatin has a different spectrum of toxicity, as its primary toxic effects are haematological. Novel platinum compounds are still being tested (e.g., the orally effective satraplatin) and others, such as nedaplatin and oxaliplatin, have recently been approved. Interestingly, oxaliplatin shows no inherent cross resistance with both cisplatin and carboplatin. This is especially relevant for the treatment of colorectal cancer, a disease that is known to be extremely insensitive to the earlier platinum analogues. At the same time the toxicity profile of oxaliplatin is favourable, with frequencies for ototoxicity of <1% and for renal toxicity <3%, except for unusual toxicity with regard to peripheral sensory nerves. Sensory neuropathy usually arises during infusion, affects hands,
feet and the perioral area and is enhanced by cold. These effects appear to be cumulative and they generally reverse within 4–6 months after treatment discontinuation.

Sensory neuropathy affects about 85–95% of all patients. An explanation for this unusual high incidence is proposed by Grolleau et al. Chelation of calcium ions by the oxalate group was suggested to block the voltage-gated sodium channels of sensory neurons, producing acute toxicity to the neuron, and in the long term inducing neuropathy. Indeed, infusion with calcium and magnesium salts significantly reduced the incidence of sensory neurotoxicity in patients with advanced colorectal cancer without affecting tumour response. Only five of 69 patients (7%) receiving calcium/magnesium infusions experienced distal paresthesias, whereas these adverse events were reported for 17 of 65 patients (26%) in the control group. McKeage suggests an alternative mechanism for oxaliplatin induced neurotoxicity assuming that oxaliplatin inhibits rRNA synthesis in ganglionic sensory nerves, causing damage to sensory nerve nucleoli. Peripheral neuropathy, diarrhoea and leucopenia can be modulated by using a dose schedule based on circadian rhythm (chronopharmacology or chronomodulation). Time-dependent dosage is effective in limiting gastrointestinal and neurological toxicities, and at the same time anti-tumour activity is increased compared to continuous infusion of oxaliplatin. Research indicates that, although scarcely used, chronomodulation is not a unique feature of oxaliplatin, and that cisplatin and carboplatin adverse effects can be modulated in the same manner. However, due to its complexity and for practical reasons chronopharmacology of oxaliplatin has only been used by a selected number of specialized medical facilities.

**Efficacy and Tolerability**

The US Food and Drug Administration (FDA) have now approved oxaliplatin for treatment of metastatic colorectal cancer in combination with 5-fluorouracil (5-FU) and leucovorin (LV). Patients receiving oxaliplatin should have experienced recurrence or progression of metastatic disease within 6 months of completion of first-line 5-FU/ LV + irinotecan combined therapy. Although grade 3 and 4 haematopoietic and gastrointestinal toxicity is limited for 5-FU monotherapy, combined administration with oxaliplatin significantly increases the incidence of thrombocytopenia, neutropenia, diarrhoea and nausea. Oxaliplatin combination therapy (85 mg/m² every 2 weeks or 130 mg/m² every 3 weeks) has a twofold higher response rate compared to 5-FU/LV therapies and also improves progression free survival (PFS) in chemotherapy-naïve patients (Table 1). In addition, patients who underwent curative resection of stage II or III colon tumours had 23% less chance of relapse within 3 years if they had received 5-FU/LV + oxaliplatin compared to 5-FU/LV alone as adjuvant treatment.

The efficacy of oxaliplatin in combination with 5-FU/LV was compared to 5-FU/LV or oxaliplatin monotherapy in metastatic colorectal cancer patients who experienced relapse or progression within 6 months of first-line therapy. At interim analysis, patients with the combination regimen had a significantly higher response rate and longer median time to progression (9.9%, 4.6 months) compared to 5-FU/LV (0%, p= 0.0002, 2.7 months) and oxaliplatin alone (1%, 1.6 months). The most frequent grade 3 and 4 side effects for patients receiving combination therapy were neutropenia (44%), neuropathy (7%), nausea (11%), vomiting (9%) and diarrhoea (11%). A randomized multicenter trial demonstrated that a
A regimen consisting of 5-FU/LV and oxaliplatin was more effective and better tolerated than the topo-isomerase I inhibitor irinotecan in combination with oxaliplatin, or than irinotecan with 5-FU/LV. Survival rate was significantly lower for the 5-FU/LV/irinotecan regimen and response rate was significantly higher for 5-FU/LV/oxaliplatin (45%) compared to oxaliplatin/irinotecan (35%, p = 0.03) and 5-FU/LV/irinotecan (31%, p = 0.002) combinations.13

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Table 1
Comparison of different chemotherapeutic regimens in metastatic colorectal cancer.
Response was defined as complete or partial response based on WHO criteria11 or RECIST criteria.10

INDIVIDUALIZATION OF THERAPY
Although response rates shown in various clinical trials vary depending on patient selection and concomitant therapy, it is clear that oxaliplatin is a promising new agent in the scarce armamentarium of drugs available for the treatment of metastatic colorectal cancer. However, the clinical efficacy and toxicity for the individual patient are still largely unpredictable, and at the start of chemotherapy it is unclear which chemotherapeutic regimen the individual patient will benefit most from. Objective response rates for various chemotherapeutic regimens range from 10% to 50%. For some other drugs, such as antiepileptics and antibiotics, patient outcome can be described by pharmacokinetic and/or pharmacodynamic modelling, based on measurements of the drug in blood or other body fluids. For example, phenytoin serum levels are monitored in epileptic patients, as they are useful for predicting efficacy and toxicity in the individual patient. Unfortunately, for anti-tumour therapy there are generally no such simple concentration–effect relationships 14 and clinical efficacy depends upon a diversity of factors, including inherited and acquired drug resistance of tumour tissue or host body.
In addition to classical therapeutic drug monitoring the newly evolving field of pharmacogenetics advocates drug choice taking into account genetic differences among patients and/or tumours. Pharmacogenetics gives us more insight into clinically relevant issues of response diversity, and will therefore most likely change the management of cancer therapy in the future.

In this manuscript the pharmacogenetics relevant to oxaliplatin chemotherapy are reviewed. After a brief introduction on the pharmacology of oxaliplatin, genetic polymorphisms in genes coding for DNA-repair, biotransformation and colorectal tumour response are discussed and their impact on clinical outcome is reviewed. This information gives detailed insight into (experimental) approaches on how to individualize oxaliplatin-based chemotherapy in colorectal cancer aiming at increasing drug efficacy while minimizing chemotherapy-induced toxicity.

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PHARMACOLOGY AND PHARMACOGENOMICS OF OXALIPLATIN

PHARMACOKINETICS

The oxaliplatin molecular structure consists of a central platinum atom (Pt), surrounded by a 1,2-diaminocyclohexane group (DACH) and a bidentate oxalate ligand (Fig. 1). Due to its DACH ligand, stereochemical isomers of the oxalate–Pt–DACH complex exist, of which the trans-1-(R,R)–DACH–Pt isomer (oxaliplatin) was shown to be the most cytotoxic.7

Oxaliplatin shows similar chemical behaviour and has a similar mechanism of action as compared to the other platinum derivatives. First, the pro-drug oxaliplatin is activated by conversion to monochloro, dichloro and diaquo compounds by non-enzymatic hydrolysis and displacement of the oxalate group (Fig. 2). The kinetics of hydrolysis differ among platinum compounds, being slower for oxaliplatin than for cisplatin.17 The highly reactive monochloro, dichloro and diaquo intermediates react with sulphur- and amino groups in proteins, RNA and DNA. Its anti-tumour effects are thought to be related to the formation of Pt–DNA adducts. Other reactions include irreversible binding to biomolecules such as albumin, cysteine (Cys), methionine (Met) and reduced glutathione (GSH), which are in fact the first steps of in vivo biotransformation and cellular detoxification7 (Fig. 2).

After a 2-h infusion of oxaliplatin 70% of the drug is bound to plasma proteins (mostly albumin) thereby losing its anti-tumour potential, and 5 days after a single infusion this fraction increases to about 95%.18 The estimated volume of distribution is 35L for total oxaliplatin and 440L if only the unbound fraction is considered.19,20 Plasma obtained by filtration (PUF) or centrifugation (PUC) is not totally identical but adequately reflects the so-called ‘free’ platinum fraction (which represents the active drug). Both PUF and PUC give an overestimate of the free platinum concentration because they both include inactive oxaliplatin bound to amino acids and other small biomolecules present in blood. A significant correlation exists between PUF and total platinum, and between the free fraction and total platinum (Fig. 3). The measurement of platinum in PUF, PUC or total plasma can give us valuable insight into the pharmacological behaviour of the active platinum species, though measured indirectly. The main compound in PUF after a 2-h infusion period was found to be monochloro-DACH–Pt.7,21

Oxaliplatin species are widely distributed among various tissue sites and free platinum is eliminated from the body, mainly by renal clearance. Up to 50% of the dose is eliminated within the first day, depending on renal function. In contrast to carboplatin there is no simple relationship between renal function and platinum exposure (AUC).22 At least 21 platinum species are found in urine, but the main constituents are glutathione (GSH)–DACH–Pt and several creatinine derivatives.21 Only 2% of the dose is excreted by faeces. Substantial uptake of platinum takes place in erythrocytes, where it is trapped by reaction with small intracellular biomolecules. The maximal Pt concentration in erythrocytes is reached 3 h after infusion, followed by a slow decline with a mean half-life of 29–50 days, close to the half-life of the erythrocytes themselves, indicating that once inside, oxaliplatin is trapped in the erythrocyte and does not substantially diffuse into plasma.7
Figure 2
Non-enzymatic hydrolysis reactions of oxaliplatin in vivo and in vitro.

The aquated derivatives of oxaliplatin are considered to be the biologically active species, capable of adduct formation with various sulphide- and amino groups. Such groups are abundant in cellular DNA and biomolecules. Cellular detoxification processes competing with DNA-adduct formation include conjugation of the aquated compound to glutathione (GSH), methionine (Met) and cysteine (Cys). The conjugation products are subsequently excreted from the cell and eliminated from the body.
Figure 3
Platinum concentrations during a 120-h oxaliplatin constant rate infusion (20 mg/m²/day for 5 days) with combined continuous administration of fluorouracil and leucovorin.

A correlation between platinum in plasma and ultrafiltrate (free) is evident at day 1. During days 3–5, the ultrafilterable (‘free’) platinum concentration remains relatively stable whereas the amount of bound oxaliplatin (attached to biomolecules) varies considerably during the day [adapted from Levi 7].

PHARMACODYNAMICS

The cytotoxic activity of oxaliplatin is initiated by formation of a DNA adduct between the aquated oxaliplatin derivative and a DNA base. Initially, only monoadducts are formed but eventually oxaliplatin attaches simultaneously to two different nucleotide bases, resulting in DNA cross-links. Compared to cisplatin, this conversion takes more time, but in vitro the two-step process is generally completed in about 15 min.3 The adducts are formed with the N-7 positions of guanine and adenine preferentially and in most cases these reactions result in intrastrand cross-links. In the cell approximately one of every 100,000 bases can be cross-linked by a platinum atom, resulting in 10,000 platinum atoms per cell.17 About 60% of intrastrand platinum adducts are formed between two guanine bases and 30% are formed between an adenine and a guanine base. Only 1% of all platinum adducts is of the interstrand type or the result of a reaction binding platinum to a biomolecule and a DNA base (DNA–protein cross-link). The precise cytotoxic efficacy of the different types of DNA adducts are as yet unknown.7

In general, the cytotoxic efficacy of platinum compounds in cancer cells can be related to inhibition of DNA synthesis or to saturation of the cellular capacity to repair Pt–DNA adducts. Platinum atoms modify the three-dimensional DNA structure, which inhibits the normal DNA synthesis and repair processes. A direct relationship between the cytotoxic effect and the number of platinum atoms was shown for nedaplatin, while upregulation of DNA repair decreases sensitivity to, e.g., cisplatin.17 Platinum trans adducts are 30 times less toxic than
cis adducts due to more effective repair. Despite the fact that oxaliplatin only forms trans adducts, it induces more efficient or different damage since a 10-fold lower adduct ratio was found relative to cisplatin at an equitoxic dose of oxaliplatin. In addition, more DNA strand breaks are formed with oxaliplatin compared to cisplatin. There is some evidence suggesting that the formation of Pt adducts with oxaliplatin is not the only mechanism explaining the drug’s anti-tumour activity but interference with RNA and proteins may add to this effect. It was found that in mouse leukaemia cell cultures oxaliplatin interferes with RNA synthesis whereas cisplatin did not. There is some evidence that oxaliplatin conjugates with sulphhydryl groups in hydrophobic pockets of different cellular proteins that may be poorly reactive with the hydrophilic cisplatin. This conjugation will result in inactive proteins that will interfere with cell function, increasing cellular toxicity.

Compared to the amino ligands of both cisplatin and carboplatin, the DACH ligand of oxaliplatin is remarkably bulkier and more hydrophobic. In contrast to other DACH complexes however, oxaliplatin shows relatively good water solubility. Together, these properties result in a greater deformation of tumour DNA by steric hindrance of adduct formation and this may explain the more effective inhibition of DNA synthesis by oxaliplatin as compared to, e.g., cisplatin. Interestingly, cellular DNA repair mechanisms seem to differ in their response to Pt or Pt-DACH complexes. The mismatch repair complex (MMR), e.g., is unable to bind to a DACH-Pt adduct site because of its great steric distortion of the DNA structure. After DNA-adduct formation by oxaliplatin, (tumour) cells will activate cellular repair mechanisms. In general, DNA repair is carried out by enzymes that consist of several aminoand sulphur groups. Therefore, oxaliplatin can be covalently bound to these repair enzymes as well, impairing their function. In order to obtain new repair proteins DNA transcription is activated, but this transcription may be hindered because of the existing DNA damage. If substantial DNA damage persists this may ultimately lead to the activation of apoptotic pathways and cell death.

PHARMACOGENETICS

Several mechanisms are described that confer decreased sensitivity or resistance to oxaliplatin, including diminished cellular drug accumulation, increased intracellular drug detoxification and increased Pt–DNA adduct repair (Table 2).

The uptake of platinum by cells is not completely understood but there is evidence that decreased accumulation is the most common mechanism of resistance to cisplatin. Comparable observations are described for oxaliplatin resistant cell lines. Platinum uptake by cells is an energy requiring process, but it is not saturable and possibly involves transport by a yet unidentified efflux pump. Once inside the cell, conjugation to glutathione (catalyzed by the enzyme glutathione-S-transferase, GST) effectively inactivates platinum compounds before DNA damage is induced. This conjugation reaction is followed by cellular excretion and is therefore related to cellular drug resistance as well. A number of studies indicate an important role of GST in oxaliplatin resistance. Platinum-induced DNA adducts can be repaired by several enzyme systems: base excision repair (BER), nucleotide excision repair (NER), post-replication repair and mismatch repair (MMR). Induction of the enzymes
involved in these systems results in increased DNA repair activity, more efficient adduct removal and hence decreased sensitivity to platinum drugs. However, the overall sensitivity of a cell is multifactorial and the relative importance of each process on ultimate drug sensitivity is difficult to predict (Fig. 4).

There is growing evidence that common gene variants (polymorphisms) affect the activity of cellular DNA repair and platinum conjugation. Such polymorphisms in genes coding for enzymes involved in oxaliplatin accumulation, detoxification and Pt-DNA repair may influence cellular response to oxaliplatin. An overview of several pharmacogenomic studies on the effects of polymorphisms in DNA repair and detoxification of oxaliplatin will be given in the following sections. Polymorphic distributions of these genes in colorectal cancer patients as well as therapeutic consequences are summarized in Table 3.

Figure 4
Schematic view of cellular defence mechanisms involved in oxaliplatin resistance.

Cellular uptake and efflux determine the level of oxaliplatin in intracellular fluid. In plasma, extracellular conjugation of oxaliplatin to plasma proteins (mainly albumin) results in renal excretion of inactive drug species. Once inside the cell, the oxaliplatin prodrug is hydrolyzed to monochloro-, dichloro- and diaquo- active species which form DNA adducts. Intracellular conjugation to glutathione effectively inactivates these highly reactive oxaliplatin-species before DNA damage occurs, followed by cellular excretion into plasma. DNA damage is repaired by nucleotide excision repair (NER), base excision repair (BER) and replicative bypass.
Table 2
Mechanisms involved in loss of sensitivity to oxaliplatin (inherited or acquired resistance).
NER: nucleotide excision repair; BER: base excision repair; MMR: mismatch repair; n.a.: not applicable.

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Table 3
Polymorphisms associated with clinical outcome in oxaliplatin combination therapies of advanced colorectal cancer.
Notation: amino acid substitution (first column; wt-codon#-mt), nucleotide change (second column; wt-nucleotide position-mt allele). Rs-numbers and nucleotide position according to NCBI database. Symbols: wt = wildtype, mt = mutant, gastrintest = solid tumours, 75% gastrointestinal, mrcr = metastatic colorectal cancer (undefined), 1mrcr = first line, 2mrcr = second-line etc.,* for EGFR wt/wt is both alleles 16 CA repeats, wt/mt one 16 CA and one 18 CA repeat allele, mt/mt is one 16 CA and one 20 CA allele. Race distribution of populations; (A) not described, probably predominantly caucasian, (B) caucasian-hispanic-blacks-asian (respectively), (C) 75% caucasian, ≥13% hispanic, (D) 97% caucasian.81
DNA REPAIR POLYMORPHISMS

Since the primary anti-tumour mechanism of oxaliplatin is the formation of Pt–DNA adducts (ultimately leading to cell cycle arrest and apoptosis), polymorphisms in genes involved in the repair of these adducts, such as nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and other post-replicative repair pathways (Table 2), may affect oxaliplatin efficacy.

MISMATCH REPAIR

Mismatch repair (MMR) is a DNA repair pathway that corrects base mispairs and small strand loops that occur during replication. Loss of MMR function results in an increased spontaneous mutation rate. The MMR system consists of six different proteins, originating from the hMLH1, hMLH2, hPMS2, hMSH2, hMSH3 and hMSH6 genes. The gene products form complexes that exert their effects during DNA repair and are designated as hMutSα (hMSH2 + hMSH6) and hMutSβ (hMSH2 + hMSH3). Together, these complexes recruit hMutLα (hMLH1 + hPMS2). Decreased sensitivity to cisplatin has been correlated with defects in one or more of these complexes, of which hMLH1 appears to be the most important. After recognition of a damaged DNA site, replicative bypass is initiated as an attempt to resynthesize undamaged DNA. However, once replicative bypass occurs in a normal cell, the hMutSα/hMutLα complex will bind to the Pt–DNA adduct, actually increasing its cytotoxicity. This apparent contradiction is explained by ‘futile cycling’ originally proposed by Goldmacher in 1986 and supported by recent findings.

Following the futile cycling model, MMR proteins bind to the platinum cross-link during replication bypass of the adduct. The MMR proteins select for the parental DNA strand and remove the newly synthesized DNA (Fig. 5). The Pt–DNA adduct is thus retained and a new attempt by replication bypass enzymes fails likewise.

Figure 5

The futile cycling model proposed by Goldmacher.
A nucleotide adduct in a DNA strand results in helical distortion, which is recognized by the hMutLα/hMutSα MMR complex (1). The strands are separated (2) and mismatch repair is attempted (3), but the newly synthesized strand is removed (4A). The continued action of these ‘futile cycles’ results in the persistence of adducts or induction of strand breaks, causing cell death. In MMR-deficient cells, however, the newly synthesized strand is retained, discarding the damaged nucleotide (4B). In the short term, MMR deficiency therefore protects against apoptosis caused by DNA-adduct formation.
Moreover, binding of MMR proteins shields the DNA adducts from repair enzymes, prolonging their lifetime and retaining DNA damage. The continuous operations of these ‘futile cycles’ would lead to cell death caused by gaps and strand breaks.\textsuperscript{28} As a result of these observations, MLH1 functions as a damage recognition unit like high mobility group protein (HMG1), which is in concordance with its observed function in cell cycle regulation and apoptosis.\textsuperscript{13}

\textit{In vitro} studies showed that MMR is not involved in oxaliplatin induced DNA-damage repair, whereas it serves as an important mechanism in cisplatin and carboplatin adduct repair. The conformational distortion of the oxaliplatin DNA complex is different from the cisplatin and carboplatin adduct and this, together with the less polar properties of the DACH–ligand, contributes to a recognition failure of MMR proteins to detect oxaliplatin adducts. Indeed, the \textit{Escherichia coli} derived MMR enzyme MutS binds cisplatin adducts with a twofold greater affinity compared to oxaliplatin. This difference is even greater after addition of adenosine diphosphate (ADP).\textsuperscript{29} These observations favour the use of oxaliplatin over cisplatin in MMR deficient tumours. Cisplatin adducts are preserved in MMR proficient cells by shielding or ‘futile cycling’, whereas they are effectively removed in MMR-deficient cells. This fundamental difference is particularly of importance for the treatment of colorectal cancer, since colon tumour cells are frequently MMR deficient as a result of promoter hypermethylation. About 5–15\% of sporadic colorectal tumours is MMR defective and this defect is frequently associated with loss of hMLH1 expression.\textsuperscript{24} To date, no polymorphisms in the MMR pathway genes are known that influence the anti-tumour effects of oxaliplatin.

**REPLICATIVE BYPASS**

Post-replication repair is a replicative bypass of damaged DNA-strands without the introduction of gaps or discontinuities into the newly synthesized strand. DNA repair enzymes involved in replicative bypass are able to carry on DNA synthesis despite the presence of adducts like Pt on the leading strand. Adducts therefore do not represent an absolute block of DNA replication, but the helical distortions associated with adducts do affect enzyme action and accuracy. This type of repair is essential since the persistent presence of gaps or faults can be lethal to cells. Normally, replicative bypass takes place primarily during cell replication, but in cisplatin-resistant cell lines this process appears to be active as well. Some investigators distinguish between replicative bypass and translesion synthesis, according to the type of enzyme that is responsible for replication, with the latter involving only DNA polymerases and the first involving any replication enzyme. Replicative bypass is either error free or error prone, depending on the type of polymerase and replication mechanism involved. DNA repair involving template switching is generally seen as error free replication, whereas direct translesion synthesis is considered error prone.\textsuperscript{28} Replicative bypass of DNA lesions may determine tolerance to platinum adducts.\textsuperscript{3}

DNA polymerases have a number of similar functional domains: a palm domain containing three carboxylate groups that is involved in the nucleotide transferase reaction, a finger region and a thumb region which are important for positioning on the DNA strand. Four polymerase families are distinguished, based on sequential differences.\textsuperscript{28} Functional absence of polymerase \(\eta\) is associated with reduced ability to repair UV-damaged DNA
and a high incidence of tumours. DNA polymerases $\beta$, $\gamma$ and $\eta$ are known to discriminate in vitro between Pt adducts with different carrier ligands such as cisplatin and oxaliplatin. This distinction is probably related to difference in MMR and HMG1 recognition of both adducts. It is still unclear which polymerases are involved in translesion synthesis in vivo, and if resistance to platinum drugs can be caused by induction of polymerase activity. However, a HCT-8 human colon tumour cell line was recently reported to express high levels of polymerase $\beta$ in concordance with cellular resistance to oxaliplatin. Polymorphisms in polymerase enzymes that influence replicative bypass near oxaliplatin adducts are yet unidentified.

**BASE EXCISION REPAIR**

Single-strand breaks resulting from exposure to endogenously produced active oxygen, ionizing radiation or alkylating agents are repaired by the base excision repair system (BER). X-ray repair cross-complementing group 1 enzyme (XRCC1) contains a domain which functions as a protein–protein interface that interacts with poly (ADP-ribose) polymerase (PARP). PARP is a zinc finger-containing enzyme that detects strand breaks, subsequently removes proteins from the DNA helix, which in turn becomes more accessible for repair enzymes.

Besides PARP, XRCC1 interacts with DNA ligase III and polymerase $\beta$. Although BER differs significantly from nucleotide excision repair (NER, following section), the XPG protein has been shown to have overlapping functions in both systems. Shen et al. identified three polymorphisms in the XRCC1 gene. One of these, located in exon 10 of this gene, causes the amino acid change Arg399 $\rightarrow$ Gln resulting in an amino acid substitution in the PARP binding domain (amino acids 301–402). The polymorphic enzyme is supposed to be less capable of initiating DNA repair due to altered binding characteristics. Chinese hamster ovary (CHO) cells with non-conservative XRCC1 polymorphisms in this particular domain show reduced repair of single strand breaks and hypersensitivity to ionizing radiation. In individuals with the mutant Arg399 $\rightarrow$ Gln codon increased DNA damage marker levels are found due to inadequate repair or increased damage tolerance. Patients with at least one of the mutant alleles have a more than fivefold risk of combined oxaliplatin/5-FU chemotherapy failure compared to patients with two wild type alleles. Other common non-conservative polymorphisms in the coding region of the XRCC1 gene include Arg194 $\rightarrow$ Trp and Arg280 $\rightarrow$ His. However, these polymorphisms appear to be non-functional and do not show correlation with increased levels of DNA damage in vivo. Polymorphisms of other enzymes of the BER pathway include XRCC3 Thr241 $\rightarrow$ Met and a XRCC5 A $\rightarrow$ G substitution in the 3'UTR (untranslated region); expression of the latter was shown to be correlated with ERCC2 expression (next section) in head/neck cancer patients.
NUCLEOTIDE EXCISION REPAIR

Nucleotide excision repair (NER) is a pathway involved in the recognition and repair of damaged or inappropriate nucleotides. A wide variety of DNA-damage is repaired by NER, including UV-induced photoproducts, helix-distorting monoadducts, cross-links and endogenous oxidative damage. At least six proteins are essential for damage recognition and removal by this repair pathway. The first step in this process is recognition of a damaged or inappropriate base by XPA (xeroderma pigmentosum complementation group A protein) and RPA (replication protein A). The adhesion of XPA and RPA to a DNA strand attracts other repair factors to the site followed by enzymatic unwinding of the helix lesion area by XPD. The XPD gene, also known as ERCC2 (excision repair cross complementing group 2), encodes an ATP-dependent helicase that is a component of transcription factor TFIIH. Moreover, XPD has functions in the basal transcription process and is required for transcriptional activity by RNA polymerase II. After partial unwinding of the helix, the XPA.RPA complex recruits endonucleases (XPG and the XPF.ERCC1 complex) by which a double incision is made, respectively, in the 3' and 5' sites flanking the lesion. A portion of 22–32 nucleotides is excised from the strand and this is followed by DNA resynthesis and ligation of the new strand to fill in the resulting gap.

Several distinct genetic disorders are caused by defects in the enzymes of the NER pathway such as xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). These syndromes are clinical distinct disorders that are characterized by defects in excision repair of UV-induced DNA damage. Most of the XP cases are caused by ERCC2 (XPD) defects. XP group D patients are extremely sensitive to UV light and have a 1000-fold risk to develop cancer. A number of causative mutations in the ERCC2 gene have been identified (Fig. 6), nearly all involving alteration of seven highly conserved protein elements necessary for unwinding of the DNA helix. In the majority of patients (72%) described by Taylor et al., parts of the gene coding for helicase domain V or VI were altered.

![Figure 6](image_url)

Mutations of the XPD gene found in XP patients.
Most mutations involve alteration of helicase domains I–VI. Upper part: mutations described by Taylor et al. 46; lower part: described by Kobayashi 47 and Broughton 48.
Apart from the deleterious mutations in XP patients, a number of common non-conservative polymorphisms are described in healthy humans at nucleotide positions 156, 312 and 751 of the ERCC2 gene and having a linked prevalence. A significant relationship with clinical response to platinum-based chemotherapy was found for the Lys751→Gln polymorphism only. This SNP causes an amino acid change in exon 23 about 50 base pairs upstream from the poly(A) signal, apparently affecting protein function but not resulting in an alteration of any of the seven helicase domains. In one study, patients with two mutated alleles were found 6–12 times more likely to have progression of advanced colorectal cancer compared to the other genotypes. Metastatic colorectal cancer patients treated with oxaliplatin/5-FU showed different tumour response for the various genotypes; 24% responders in the Lys/Lys group, versus 10% in the Lys/Gln and 10% in the Gln/Gln groups, respectively. Similar findings have recently been reported for lung cancer patients receiving various platinum agents: Gln/Gln individuals had significantly more risk of death during follow-up (OR 4.5) compared to the other genotypes. Conversely, a significant association was found for the presence of two Gln alleles and low NER repair capacity for benzo(a)pyrene adducts in a cohort of lung cancer patients.

It remains uncertain whether the XPD Lys751→Gln (A751C) polymorphism causes an increase or a decrease in NER activity since, depending on the assay used to test DNA repair capacity, conflicting results are reported on this issue. However, the enzyme seems to play an important role in colorectal cancer since it was found that ERCC2 (and ERCC1) expression in tumour tissue were high compared to adjacent normal mucosa. Besides the possibility of modulating enzyme activity, the amino acid change Lys > Gln may alter intracellular ERCC2 levels by affecting post-transcriptional and/or post-translational stability. However, Dabholkar et al. could not demonstrate a relationship between ERCC2 protein levels and tumour response to platinum analogues in ovarian cancer patients. Therefore, it seems more likely that the amino acid change influences the interaction of the ERCC2 protein with other NER enzymes, or that the A751C polymorphism is linked to nucleotide changes in nearby-located genes, like ERCC1 and XRCC1.

It was shown by the host cell reactivation assay that both the A751C and the G312A genotypes are associated with less optimal DNA repair capacity. However, both the G312A and the conservative C156A single nucleotide polymorphisms were not found to be individually associated with response or survival after 5-FU/oxaliplatin chemotherapy. The C156A polymorphism transforms a high usage codon (CGC) into a low usage codon (CGA) and might have yet unknown consequences for cellular processes. The interrelationship between the three ERCC2 polymorphisms and the net effect on tumour response or survival is as yet unclear and remains to be determined.

Although ERCC2 is indisputably indispensable for NER function, it was found to be less crucial for the repair of interstrand cross-links compared to ERCC1 or ERCC4. This might be reflected by the fact that a number of patients with ERCC2 defects are known, whereas to date no case reports have been published identifying live human subjects suffering from an ERCC1 disorder. Most information considering the importance of the ERCC1 enzyme is therefore based on observations in cell lines equipped with artificial NER defects and mouse knockout models. For example, in human ovarian cancer cells, the C200 variant was found to be cisplatin resistant due to enhanced NER activity and ERCC1 expression.
Furthermore, cisplatin cross-link removal by UV-20 CHO cells lacking ERCC1 was small compared to its wild-type (AA8) counterpart. Since the overall rate and efficacy of the NER process is comparable for cisplatin and oxaliplatin, the lack of ERCC1 is considered to influence oxaliplatin sensitivity as well. Indeed, decreased sensitivity to oxaliplatin was associated with high ERCC1 expression levels in HCT116 colorectal cancer cells. Similar observations were made by Arnould et al. in a panel of colon cancer cell lines; ERCC1 and XPA expression were found to be predictive of oxaliplatin sensitivity. ERCC1 null mice are extremely small at birth and die within 3 weeks of age. Death is related to liver failure, most likely by elevated levels of oxidative DNA damage, but the brain and kidneys are affected as well.

The ERCC1 enzyme consists of 297 amino acids and appears to harbour a nuclear localization signal and a domain characteristic of a DNA-binding protein. In normal individuals, a common polymorphism at exon 4 can be found at codon 118, changing AAC into the less used AAT codon. This is a conservative nucleotide change, as both codons result in the same amino acid (Asn) being incorporated into the protein. However silent, this polymorphism was found to be associated with decreased ERCC1 expression in ovarian cancer cells but, contrasting, with increased intratumor levels of ERCC1 in 32 patients with metastatic colorectal cancer treated with 5-FU/oxaliplatin chemotherapy. Shirota et al. reported a significant association between intratumoural ERCC1 mRNA levels and survival after oxaliplatin based chemotherapy in 5-FU resistant colorectal tumors. Patients with intratumoural mRNA ≥4.9x10⁻³ (relative to the expression of the house-keeping gene β-actin) had a median survival of 1.9 months, compared to 10.2 months for patients with mRNA expression below 4.9x10⁻³. When 7.4x10⁻³ was chosen as the expression cutoff value for non-response, median survival for the high expression group was 150 days compared to 292 days in the low expression group. An inverse association between ERCC1 expression and survival of gastric cancer patients treated with platinum agents was found in another study. These findings indicate that the Asn118ÆAsn (CÆT), however silent, may be related to outcome and survival by changing ERCC1 expression.

Since ERCC1 combines with ERCC4 (XPF) to form the endonuclease complex XPF.ERCC1, it may not be surprising that cells lacking ERCC4 show similar sensitivity to the cross-linking agent mitomycin compared to ERCC1 deficient cells. ERCC4 upregulation in human KCP-4 carcinoma cells was associated with cisplatin and oxaliplatin resistance. In XP group F patients numerous non-synonymous polymorphisms can be found, predominantly in exons 8 and 11. Polymorphisms in exon 11 are known to interfere with the formation of the ERCC1 complex, which may result in rapid degradation of ERCC1. The ERCC4 Arg415ÆGln substitution, located near some XPF disease related mutations in exon 8, was found to increase breast cancer risk. This mutation is found in a highly conserved gene region and protein function may therefore be affected, as predicted by the sorting intolerant from tolerant substitutions (SIFT) database tool.
OTHER POLYMORPHISMS

Glutathione-S-transferase
Polymorphisms associated with cellular platinum drug clearance affect oxaliplatin efficacy by lowering intracellular concentrations of the drug. This so-called cellular detoxification is the result of conjugation of Pt drug to biomolecules such as methionine, cysteine and glutathione (Fig. 2). The latter conjugation is catalyzed by the glutathione-S-transferase enzyme (GST). The conjugation of toxic and carcinogenic electrophilic molecules with glutathione by GST is followed by cellular excretion of the conjugate, thereby protecting DNA and other macromolecules from damage. Several subclasses of the GST enzyme are distinguished: the α, π, μ, θ and ζ subclasses (alpha, pi, mu, theta and zeta). A single nucleotide polymorphism (SNP) in exon 5 at position 313 (A → G) in the GSTP1 (π) gene results in a valine being incorporated into this enzyme at site 105 instead of the usual isoleucine (Ile105 → Val). The mutant GSTP1 (π) enzyme is less potent in detoxification of carcinogens and individuals with two mutant alleles have shown a significant survival benefit from combined oxaliplatin/5-FU treatment, with a median survival of 24.9 months compared to only 7.9 months for metastatic colorectal cancer patients with two wild-type alleles. A similar result was found in a retrospective study among 240 breast cancer patients treated with radiotherapy or various alkylating agents (inducing DNA adducts). Individuals with the 105 Val allele had superior survival (hazard ratios for death were 0.8 and 0.3 for Ile/Val and Val/Val genotypes, respectively) compared to the 105 Ile allele. Apparently, the 105 Val allele is an independent prognostic factor in breast cancer because the observed survival benefit was shown to be irrespective of the treatment used.

Other common polymorphisms in the GSTT1 (θ) and GSTM1 (μ) genes include deletions that result in complete loss of enzyme activity in homozygous individuals. However, no association with altered survival or clinical response in patients with advanced colorectal cancer treated with oxaliplatin/5-FU was observed for the GSTT1 and GSTM1 genotypes. It seems therefore likely that the θ and μ subclass enzymes play a less important role in colorectal tissue (cancer) cells as compared to the π subclass. Recent findings confirm that π subclass enzymes are over expressed in colorectal cancer tissues relative to normal mucosa.

Epidermal growth factor receptor
The epidermal growth factor receptor (EGFR) is a 170 kD transmembrane protein that is composed of an extracellular ligand binding domain, a transmembrane lipophilic region and an intracellular protein tyrosine kinase site. The binding of a ligand results in the dimerization (followed by internalization) of EGFR molecules or heterodimerization with other closely related receptors such as HER2/neu. This in turn activates the tyrosine kinase domain via autophosphorylation, resulting in signal transduction and regulating cellular growth. Endogenous ligands to this receptor include epidermal growth factor and transforming growth factor α (TGFα). The EGFR is essential for normal cellular function and increased levels of EGFR mRNA are associated with metastasis and more aggressive tumour growth. EGFR, alternatively called HER1, is over expressed in many types of cancers, including colorectal cancer. The EGFR gene transcription and enzyme expression
are presumably inversely related to the number of CA repeats in intron 1. It was shown that an individual with two alleles with 16 repeats (16/16) has worse response to second-line oxaliplatin/5-FU treatment compared to the 16/18 and 16/20 genotypes. Median survival rates for patients with metastasized colorectal cancer were 63, 133 and 805 days for the 16/16, 16/18 and 16/20 genotypes, respectively. Conversely, it has been demonstrated that tumour EGFR genotype is non-prognostic of survival in head/neck cancer patients, whereas germline genotype could predict survival adequately. In 33% of cases, tumour DNA had a smaller number of EGFR dinucleotide repeats compared to normal tissue, probably due to microsatellite instability. This favours the use of peripheral blood specimens for genotyping EGFR.

The exact mechanism explaining how EGFR levels relate to oxaliplatin/5-FU efficacy is not known. Probably the EGFR genotype is related to basic mechanisms for cell growth or cell death making it prognostic for the response to chemotherapy and patient outcome in general. This correlation was shown for a number of malignancies including tumours of bladder, breast and lungs. When oxaliplatin is combined with the tyrosine kinase inhibitor ZD1839 (Iressa®), a synergistic effect is observed in colon cancer cell lines. Platinum adduct removal is reduced, with 90% of oxaliplatin induced Pt–DNA adducts remaining unrepaired, and apoptosis is prolonged compared to oxaliplatin administration alone. Combination of oxaliplatin with EGFR antibodies or tyrosine kinase inhibitors seems a promising new direction in colorectal cancer therapy.
DISCUSSION

Pharmacogenetics sheds new light on the classical pharmacological question and understanding why individuals respond differently to various drug treatments. Current anticancer drug treatments are effective in only a minority of patients and it is not yet possible to reliably predict which patient benefits from a certain chemotherapeutic drug treatment or which patient will experience (life-threatening) drug toxicity. Pharmacogenetics is aiming at individuating drug therapy, thereby increasing the potential efficacy of novel anticancer drugs such as oxaliplatin. Several polymorphisms influencing drug sensitivity, e.g., affecting repair of Pt-DNA adducts or cellular detoxification, are described but not yet routinely applied in the pharmacotherapeutic treatment of colorectal cancer. One reason for this is that it is still unclear how the different polymorphisms interrelate. Moreover, patients are frequently treated by multiple drugs at one time, and the efficacy of these drugs will be influenced by several concurrent interindividual genetic differences. Since germline SNPs are polymorphisms that originate from the ovary and sperm parental DNA, these SNPs are theoretically found both in normal and in tumour tissue. In contrast, tumour tissue may have additional non-germline mutations that initially caused neoplastic growth, for example by overexpressing oncogenic proteins. However, analysis on gene mRNA expression profiles has shown that only 0.2–10% of all gene transcripts are differentially expressed between colorectal tumours and non-malignant tissue. Most of the transcripts with abnormal high expression are related to malignancy, and many others have comparable expression to normal cells.78 Germline SNPs therefore give a good representation of the main bulk of tumour DNA, apart from oncogenic SNPs. For this reason, it is rational and preferable to develop pharmacogenetic tests based on germline SNPs in, e.g., lymphocytes, which represent a more accessible tissue compared to tumour material.

This strategy seems to be very promising as suggested by recently published studies.79 Stoehlmacher et al. studied survival and time to progression in a group of refractory colorectal cancer patients treated with 5-FU/oxaliplatin combination therapy. Of the 10 polymorphisms studied in peripheral blood samples (GSTP1, GSTM1, GSTT1, XRCC1, TS 28 base pair repeat and 6 base pair deletion, ERCC2 Lys751Gln and Arg156Arg, XPA and ERCC1 Asn118Asn), only ERCC2 Lys751Gln, GSTP1 Ile105Val, ERCC1 Asn118Asn and TS-3′UTR (a 6 base pair deletion likely associated with 5-FU responsiveness) were associated with overall survival. However, combining these polymorphisms increased predictability of survival; patients homozygote for two or more favourable SNPs had a median survival time of 17.4 months, compared to 10.2 months for one or 5.4 months for none of the favourable allele combinations (p< 0.001).72 In this study no association was found between anti-tumour response and genotype, probably due to the low response rate of <10% observed in the heavily pretreated patients. Different results were reported by McLeod and colleagues, who studied the association between 28 germline SNPs and the response to first-line treatment (5-FU/irinotecan, 5-FU/oxaliplatin and oxaliplatin/irinotecan) of advanced colorectal cancer. Preliminary results of this prospective study indicate that GSTP1 Ile105Val and ERCC2 Lys751Gln polymorphisms are not related to oxaliplatin responsiveness or toxicity.80 In conclusion, (combined) SNP-analysis may help us predict responsiveness and survival benefit in colorectal cancer patients treated with oxaliplatin containing regimens. Hopefully, this type of genetic information may help us to select individual patients that have a high potential for benefit from chemotherapeutic treatment, thereby ‘tailoring’ anti-neoplastic drug therapy in a rational manner.
REFERENCES


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