Nucleotide excision repair and anti-cancer chemotherapy

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Abstract

DNA repair is an important effector of anti-cancer drug resistance. In recent years, it has become apparent that DNA repair is an extremely complex process. Processes within DNA repair that may contribute to one or more drug resistance phenotypes include; O-6-alkyltransferase activity, base excision repair, mismatch repair, nucleotide excision repair, and gene specific repair. Clearly, several of these processes may show increased activity within any single cell, or tumor, at any one time. This review attempts to touch briefly upon the question of the distinctions between each of these specific pathways; and then seeks to expand on nucleotide excision repair as a possible effector of cellular and clinical resistance to platinum-based anticancer therapy.

DNA Repair and Anticancer Drug Resistance

The role(s) of DNA repair in the development of cellular and clinical resistance to anti-cancer chemotherapy is an evolving science. We have moved substantially from the days of using simple assays to assess various types of DNA damage resulting from platinum compounds, alkylating agents, and/or intercalating agents [1–3]. The development of molecular biology methods to assess the repair of DNA damage of various types, have led to the realization that DNA repair is a multi-faceted process, with specific types of DNA damage being repaired through pathways specific for that damage, with some degree of ‘cross over’ [4].

The role of O-6-alkyltransferase has been reviewed extensively elsewhere [5,6]. This DNA repair activity is relatively specific for one particular type of damage; O-6 alkylation. Other DNA repair pathways tend to be more general, in that any specific pathway appears to repair selected types of DNA damage that may occur from exposure to a variety of different agents. In this regard, most of the work done to date involves base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). First, we shall review briefly the types of general DNA repair pathways

Table 1. Proteins essential to base excision repair

Single base repair – or – ‘Short patch’ base excision repair

ONLY ONE NUCLEOTIDE IS AFFECTED

Uracil-DNA glycosylase (UDG)

AP Endonuclease (HAP1)

DNA polymerase β

XRCC1

DNA Ligase I or DNA ligase III

‘Short patch’ repair – or – ‘Long patch’ base excision repair

SPANS TWO TO SIX NUCLEOTIDES

The five proteins noted above; and

Dnase IV (FEN1) (counterpart to E coli DNA polymerase I)

PCNA stimulates the reaction, but is not essential

? Role of other polymerases

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that may be important in anti-cancer chemotherapy. Unfortunately, terminology can get very confusing.

Base excision repair (BER) may be performed in the ‘short patch’ fashion, or in the ‘long patch’ fashion [7]. BER is the process by which one, or, a small number of bases are repaired/replaced, associated with

Table 2. Summary of 'essential' nucleotide excision repair genes

Old name	Current nomenclature	Function
ERCC1	ERCC1	DNA damage recognition. Possibly, 5' incision into DNA strand.
ERCC2	XPD	Part of basal TFII complex–helicase.
ERCC3	XPB	Part of basal TFII complex–helicase. Links DNA repair with DNA transcription.
ERCC4	ERCC4 (possibly XPF)	Complexes with ERCC1, and then XPA, to perform DNA damage recognition. ERCC4 may perform the 5' incision into the DNA strand.
ERCC5	XPG	3' Incision into DNA strand.
ERCC6	CSB	Essential for gene specific repair.
XPAC	XPA	Complexes with ERCC1 and ERCC4, to enhance DNA damage recognition.
HSSB (RPA)	same	HSSB = human single stranded DNA binding protein; RPA = replication protein A. HSSB = RPA. This protein has three subunits.
PCNA	same	PCNA = proliferating cell nuclear antigen
TFII complex	same	Has XPB, XPD, and six other subunits; p62, p50, p44, p41, p38, and p34.

the appearance of an apurinic or apyrimidinic (or, AP) site in cellular DNA. The frequency with which anticancer chemotherapeutic agents cause this type of DNA damage is unclear, but it is probably quite common. Simple alkylating agents and agents that cause oxidative DNA damage can, in theory, readily cause such changes in the DNA. Oxidative agents may include the anthracyclines and anthracenediones [8].

Lindahl's group reconstituted the BER system with purified proteins, and defined what proteins/activities are necessary for the repair of a single AP site, or a more complex site involving up to six DNA bases [9]. This distinction defines 'short patch BER', versus 'long patch BER'. The differences in the two systems are noted in Table I. For short patch BER, five proteins are necessary to effect repair, including UDG, HAP1, DNA polymerase beta, XRCC1, and DNA ligase I or DNA ligase III. For long patch BER, only one additional protein is essential (Dnase IV), and PCNA appears to be particularly useful as well. To this author's knowledge, no studies have been performed to carefully assess the relative importance of this pathway in the determination of cellular sensitivity and/or resistance to any class of chemotherapeutic agents.

Nucleotide excision repair (NER) is important in the repair of bulky covalent lesions within the DNA, such as UV dimers, polycyclic aromatic hydrocarbons, platinum-DNA lesions [10,11], and probably other heavy metals as well [12]. A large number of proteins are known to be involved in the NER pathway. However, recent studies suggest that only 16 proteins are

essential to perform the DNA damage recognition and excision functions [13,14]. These proteins are listed in Table II, and can be arbitrarily subdivided into at least two groups. One group of proteins clearly fall into the 'DNA damage recognition/excision' group (ERCC1, XPA, etc.); and one group of proteins clearly fall into the 'helicase' type of group (ERCC2, ERCC3, etc.). The complexity of NER has been the focus of study in a number of laboratories. One group has identified the 'rate-limiting' step within NER, which they concluded is the damage recognition/excision step [15]. It should be noted that this rate-limiting step does not include the filling in of the gap within the DNA, after release of the damaged segment. Although not clearly distinguished in the seminal report [15], one could readily suggest that the recognition and excision functions are separable – although they may be performed by the same complex of ERCC1, XPA, ERCC4, and possibly other proteins [16,17]. Based on analysis of the DNA sequence, it is possible that ERCC1 may have both a DNA damage recognition function and a DNA damage excision function.

Certain characteristics of the rate limiting step of NER are worth noting, as listed in Table III. It is unclear what may be the 'lead' gene within this group, but a strong candidate gene for this role is ERCC1, as will be detailed below. Available data suggests that genes within this step may be under the influence of coordinate regulation in normal tissues, and to some extent, even in malignant tissues [18–20]. The molecular mechanism of this coordinate regulation is not

Table 4. Characteristics of ERCC1

Genomic location	19q13.2
Has homology with	RAD10 (yeast) uvrA and uvrC (E. coli)
Genomic characteristics	~15 Kb; 10 exons Has a helix-turn-helix motif for DNA binding. Has specific binding sites for XPA, etc. Exon VIII possibly responsible for DNA incision.
Promoter	~170 bp upstream of start site. Classical elements not present: i.e., CAAT, TATA, and GC boxes.
Size of RNA transcript(s)	– Differential polyadenylation gives two transcripts; 3.4 and 3.8 kb. – Ultimately; a 1.1 kb 'full length' mRNA <i>and</i> one lacking exon VIII.
Key facts:	a) Absence of ERCC1 is probably not compatible with life. b) ERCC1 deficiency in cell lines = most severe DNA repair defect. c) ERCC1 appears to be the 'lead' gene for the NER process in human ovarian cancer tissues (other tissues as well?). d) ERCC1 may have critical functions that are yet undiscovered. e) Alternative splicing of ERCC1 appears to impact on DNA repair efficiency. f) Cisplatin-DNA adduct repair does not occur with a defective ERCC1. g) Relative ERCC1 mRNA levels may reflect overall DNA repair activity, when the NER system is intact.

Table 3. The rate-limiting step of NER

– DNA damage recognition and excision, is considered 'one step'.
– This step has a minimal requirement of sixteen (16) proteins; which includes all proteins listed in Table II (each subunit of HSSB and of TFII, counts as one protein), except ERCC6 (CSB).
– 'Release' of the damaged DNA segment is effected by proteins that are different from the excision proteins.
– ERCC1 appears to be the 'lead gene' in this group, based on mRNA expression patterns in fresh human tissues.

currently clear. This concept of a rate-limiting step has great utility, in the development of treatment strategies utilizing known DNA damaging medicinals. Because of the relative importance of ERCC1, it is noteworthy to examine what is currently known about the function(s) of this critical NER gene. This information is summarized in Table IV.

ERCC1 is located on chromosome 19q, band 13.2. It has homology with DNA repair genes found in yeast and in E. Coli, as well as every species that has yet been studied in this regard. Based on data presented

below, this laboratory now believes that ERCC1 may be the 'lead' gene within the NER process. This may allow the study of NER in the intact human host, by the use of ERCC1 as a surrogate marker for the process.

Mismatch repair (MMR) has received a great deal of attention with respect to the possible role of DNA repair and anticancer drug resistance. This is curious, because the consensus has clearly developed that MMR probably plays a small role in the direct repair of most chemotherapy induced DNA damage; although it may play a role in the repair of some types of alkylator-DNA damage [21,22]. The primary role(s) of MMR is to repair mispairs within the DNA, as well as slipped intermediates [21–24]. Major proteins that form the MMR complex(es) are listed in Table V, and include hMSH2, hMLH1, hMutS alpha, and hMutL alpha. Modrich has put forward the idea that mismatch repair may be necessary for the linkage to occur between the induction of cellular DNA damage, and the initiation of apoptosis [25]. The precise relative roles of NER and MMR in this linkage, and how they interact, are not clear at this time. NER appears to play the primary role in the repair of cisplatin-DNA

Table 5. Proteins involved in the mismatch repair pathway

Sometime referred to as 'Long patch' repair; up to ~1000 bases		
Necessary proteins include:		
hMSH2	–	All mismatches require this for repair
hMLH1	–	All mismatches require this for repair
GTBP/p160		
hMSH2:GTBP/p160 heterodimer = hMutS alpha		
hMutS alpha	–	Repairs single base mispairs, and
hMutS alpha	–	Repairs slipped intermediates in single-base runs
hMutL alpha	–	Recruited by the hMSH2:DNA complex.
hPMS2		

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lesions [11,16]. Since cisplatin is an agent that is highly studied, most of the discussion below will focus on cisplatin-DNA damage, and the NER DNA repair pathway which may be primarily relevant to cellular and clinical resistance to platinum compounds.

One common misconception is that p53 is a DNA repair gene. p53 Does not play a role in the excision of DNA damage from the DNA strand, nor does it play a role in the release of the damaged strand from the complimentary sequence [13,14]. Nor does p53 play a role in the polymerase activity of repair [16]. p53 May complex with DNA repair proteins and modulate their activity [26,27]. p53 Is clearly a very important cell cycle regulator, that appears to modulate cellular response to DNA damage [28]. Such responses include induction of cell cycle arrest in response to DNA strand breaks [28,29], and the induction of the p53 dependent apoptotic pathway(s) [30].

A Brief Review of Nucleotide Excision Repair and Cellular Resistance of Cisplatin

A large body of evidence clearly shows that the nucleotide excision repair pathway (NER) is the pathway that repairs platinum-DNA adduct in cellular DNA [11,16]. Pertinent points regarding NER include the following. NER requires at least two protein activities in order to effect DNA repair; the helicase activity/complex, and the excision activity/complex. It is unclear whether or not there are only two, or more than two, complexes involved. The helicase activity is effected by the basal transcription factor TFII, which includes the genes ERCC2 (XPD) and ERCC3 (XPB). It has been suggested that XPD has a 3'→5' helicase function; and that XPB has a 5'→3' helicase

function [17,31]. An additional function of XPB is to 'link' DNA repair with DNA transcription [17,31]. The excision complex (or at least one of the excision complexes) is composed of ERCC1, along with other genes including XPA, ERCC4 (XPF), and possibly other proteins as well [16,17,32,33]. It is unclear where ERCC5 (XPG) complexes with other proteins, but ERCC5 is also an excision nuclease which performs the 3' endonuclease function [16]. ERCC6 (CSB) is another important NER protein, and appears to be essential to effect gene-specific repair [34]. However, its precise position within one or more complexes is also not clear.

Current knowledge suggests that DNA damage is recognized by the complex that contains ERCC1. Covalent DNA damage is excised through cuts into the DNA strand 5' and 3' to the site of the lesion, at defined distances [16,17]. The 5' cut is effected by ERCC1 [35] or ERCC4 [16,17]. The 3' cut is effected by ERCC5 [11,16]. The helicase complex TFII releases the DNA damage, and ERCC3 (XPB) effects the linkage between the excision and repair of the damage, and transcription of the repaired DNA. In cell free protein extract systems, it has been shown that 16 proteins appear to be necessary for the DNA damage recognition, excision, and release reactions to occur, as discussed above. Those 16 proteins are listed in Table II.

Cellular resistance to cisplatin is multifactorial, and has been reviewed in detail elsewhere [36,37]. Several lines of evidence suggest that at low levels of cellular resistance, DNA repair appears to be the most important factor. Eastman and colleagues were the first to address the relative importance of different molecular mechanisms in effecting cisplatin resistance, in

a murine leukemia L1210 system [38]. He concluded that at low levels of resistance (<20-fold over baseline), DNA repair appeared to be the most important factor. Factors studied by Eastman included DNA repair, cytosolic inactivation of drug, and alterations in intracellular drug accumulation. Reed and colleagues have studied this question in human ovarian cancer cell lines [39], and in human T lymphocyte cell lines [40,41]. In both situations studied by Reed, DNA repair appeared to be the most important contributor to cellular resistance to cisplatin, at low levels of resistance. Ozols and Hamilton have studied this question as well, in a large number of human ovarian cancer cell lines [42,43]. Their data show that at levels of resistance of >40-fold over baseline, glutathione metabolism appears to take on primary importance [42]. However, at low levels of resistance, the data of Ozols and Hamilton reiterates the importance of DNA repair.

Bohr and colleagues studied the contribution of gene specific repair to the cisplatin resistance phenotype in Chinese hamster ovary cells [44], and in human ovarian cancer cells [45]. In both situations, the contribution of gene specific repair appeared to be very substantial. In human ovarian cancer cells for example, the difference in gene specific repair between cisplatin-sensitive and cisplatin-resistant cells was 6 to >8 fold, in a setting where differences in overall genomic repair was only 2–4 fold [45]. By demonstrating the importance of gene specific repair in this setting, Bohr firmly placed NER at the heart of the cisplatin-DNA adduct repair question. Lee et al addressed the issue of which gene was more important, ERCC1 or ERCC3/XPB [46]. In Chinese hamster ovary cells with defined deficits of ERCC1 or ERCC3/XPB, the data showed total absence of cisplatin-DNA adduct repair in ERCC1 deficient cells; whereas repair could be readily measured in ERCC3 deficient cells. This was interpreted to show that ERCC1 was essential to effecting cisplatin-DNA adduct repair, whereas XPB was probably not essential.

Modrich and colleagues [25] and Howell and colleagues [47], have investigated the possible role of mismatch repair in effecting cisplatin resistance in human ovarian cancer cells [25], or in colon cancer cells [47]. Modrich studied cells where major differences have already been shown for NER [39,45], whereas Howell studied cells that appear to be NER intact [47]. In both set of studies, data showed that an intact mismatch repair system appeared to be es-

sential for the linkage of DNA damage/repair with the initiation of apoptosis. In the Howell studies, the difference between cell lines in cisplatin resistance was only 2-fold. Because of the previously reported NER differences in cell lines used in the Modrich studies (>8-fold differences in gene specific repair, as an example), the true contribution of mismatch repair in his studies is not clear. Taken together, these reports suggest that mismatch repair is a comparatively small contributor to the cisplatin resistance phenotype, at least in ovarian cancer and in colon cancer.

Another question of note, is whether cisplatin resistance is a function of 'baseline' cellular activity; or, is it a function of the cell's ability to induce DNA repair activity to higher or lower levels? In human T cell lines, when platinum-DNA adduct repair was measured by AAS, the differences between cell lines could be most dramatically demonstrated when cells were 'pushed' to repair increasing levels of DNA damage, associated with >80% cell kill in the respective cell line [40]. In A2780/CP70 cells, the level of resistance of these cells as compared to the A2780 parental cell line, appears to be related to the degree to which ERCC1 – and presumably other NER genes – can be induced after a single exposure to cisplatin [48]. In human ovarian cancer cells, the greater the extent to which the cells can induce the NER system, the greater the level of resistance to cisplatin. The molecular mechanism for this is under study in our laboratory.

Since ERCC1 appears to be critical to NER, we have investigated the complexity of ERCC1 mRNA expression and protein expression in human tissues of malignant and non-malignant origin [49–53]. ERCC1 appears to exist in at least two forms in each tissue and each cell line examined to date, regardless of origin. One form is the full length mRNA and full length protein. The second form is an alternatively spliced form which lacks exon VIII. The alternatively spliced form exists as mRNA and as protein [52]. Exon VIII is that portion of the gene that has strong homology with uvrC of *E. Coli* [35,54], and has been suggested to possibly effect the 5' cut into the DNA strand [35,54]. In the oncogene literature, the function of alternative splicing appears to be that of natural inhibition of the full length version of the respective gene [55]. For ERCC1, indirect data suggests that alternative splicing may serve the function of a naturally occurring inhibitor of the NER process in human ovarian cancer cell lines [52]. Figure 1 shows results from RT-PCR in a cohort of human ovarian cancer tissue specimens.

Figure 1. Autoradiographs showing RT-PCR results for full length ERCC1 mRNA (the 268 bp signal), and alternatively spliced ERCC1 mRNA (the 196 bp signal) which lacks exon VIII. As shown, each sample has some degree of alternative splicing. In this grouping, one sample has it's stronger signal as the alternatively spliced variant (sample #22).

In each specimen, the full length ERCC1 mRNA and the alternatively spliced variant can be demonstrated. Figure 2, shows the relationship between the percentage of alternative splicing of ERCC1, and actual DNA repair activity for cisplatin-DNA adduct at the IC₅₀ doses for the respective cell lines. Figure 1 shows that alternative splicing of ERCC1 is a common event; and Figure 2 suggests that the role of that alternative splicing may be as a natural inhibitor of NER.

Also, in human tissues, ERCC1 has a polymorphism within exon IV which codes for the same amino acid within the protein [51]. This polymorphism appears in approximately 50% of tissues examined. However, this polymorphic codon has a rate of codon usage that is ~50% that of the wild type codon. What possible influence this lowered codon usage may have

on the ability to up-regulate ERCC1 in response to DNA damage, is under study.

Although the details of the molecular processes are the focus of active study, several facts appear clear at this time. In human tissues, DNA repair appears to be the primary determinant of cellular resistance to platinum compounds, and possibly, the primary determinant for other agents that covalently bind DNA. Within DNA repair, nucleotide excision repair appears to be pathway of major importance. Within NER, ERCC1 appears to be the critical gene; and ERCC1 appears to be influenced by many factors within the cell, including alternative splicing and polymorphic variants.

Figure 2. Alternatively spliced ERCC1 mRNA/protein, as a percentage of total ERCC1 mRNA/protein, is plotted against the total amount of cisplatin-DNA adduct repaired in the same cell lines. Each cell line was treated at its respective IC50, and DNA repair was measured at that dose level. As shown, as the percentage of alternative splicing increases, DNA repair activity goes down.

Evidence that DNA Damage is Important in the Clinic

Starting in the mid-1980's, platinum-DNA adduct levels were assessed in clinical samples in several settings, in an effort to relate DNA damage levels with clinical endpoints. Poirier and colleagues used a polyclonal antisera elicited in rabbits [56]; Fitchinger-Schepman utilized HPLC assays [57]; and Reed and colleagues utilized atomic absorbance spectroscopy with Zeeman background correction [58]. Perera and colleagues assessed adduct levels, along with a number of other molecular endpoints, in a cohort of patients with germ cell tumors [59].

Poirier and colleagues demonstrated that platinum-DNA adduct levels, as measured in her ELISA assay, could be measured in a variety of tissues taken from human patients, but her primary focus was on nucleated peripheral blood cells [60–64]. Her group showed that adduct levels in peripheral blood cells correlated with disease response in advanced stage ovarian cancer [60,61,63], testicular cancer [60,62], and breast and colon cancers [64]. Poirier also assessed adduct levels at autopsy in various settings. She showed that adduct levels in bone marrow approximated adduct

levels measured in tumor tissues, in autopsy cases of patients who received platinum-based therapy antemortem [61,65]. Shortly after Poirier's initial investigations, it became apparent that the antisera she developed did not measure all platinum-DNA lesions that may exist within a specimen. Poirier then investigated the numerical relationships between adduct measured by her ELISA assay, and adduct levels measured by more precise techniques, such as atomic absorbance spectroscopy (AAS). In experiments using rat tissues and human tissues, she showed that the ELISA assay detected about 0.2% of total platinum-DNA covalent damage, as measured by AAS [66]. The importance of this observation will be discussed further, below.

Fitchinger-Schepman and colleagues performed several studies to examine the human biology of platinum-DNA adduct formation, using HPLC technology [57,67]. Fitchinger-Schepman also investigated the kinetics of platinum-DNA adduct formation in peripheral blood, after an IV infusion of platinum-based chemotherapy. She showed that the disappearance of adduct from peripheral blood cells was biphasic; with a rapid phase of removal during the first 12–16 hr, followed by a relatively flat plateau of undetermined duration [68]. Based on the character of

the second phase of the removal curve, adduct levels appeared to be relatively stable between 16 hr after the infusion and 48 hr. This has been interpreted to suggest that adduct formation may occur in the bone marrow, as well as in the peripheral circulation, of patients receiving platinum-based therapy. This also suggests that the rapid phase of adduct removal (the first 12–16 hr after platinum infusion) is composed of at least two activities – cell loss and DNA repair. Fitchinger-Schepman also showed that one can accurately predict the level of platinum-DNA adduct formation within a specific patient, by taking a sample of blood and incubating that sample with a defined cisplatin exposure *in vitro* [69].

Perera and colleagues assessed a number of measures of DNA damage in germ cell tumor patients, after the receipt of a complex platinum-based regimen for the treatment of their disease [59]. Measurements taken included; platinum-DNA adducts, platinum-protein adducts, sister chromatid exchange, micronuclei, somatic gene mutation at the HPRT locus, and at the glycophorin A loci NO and NN. Agents used in the treatment of these patients included cisplatin or carboplatin, etoposide or vinblastine, cyclophosphamide, dactinomycin, and bleomycin. Although platinum-DNA adduct levels did not directly correlate with clinical response in Perera's studies [70], total DNA damage levels (as measured by sister chromatid exchange assays) did correlate [71]. A patient's long term prognosis was more favorable in that group which experienced the greatest number of SCE's in peripheral blood, immediately after the administration of chemotherapy [71]. Since this treatment regimen was very complex, and contained $\geq$ four separate cytotoxic agents, this suggests that the summed leukocyte DNA damage level is directly related to clinical outcome, whether or not the damage is directly caused by cisplatin.

This aspect of the Perera studies is extremely important. If clinical response to DNA damaging regimens is a function of total DNA damage, then to examine platinum-DNA adduct alone (when there are multiple agents involved) may be to examine only a portion of the relevant information. This suggests that for simplistic regimens such as cisplatin-cyclophosphamide or carboplatin-paclitaxel, platinum-DNA adduct levels may suffice. However, for more complex regimens – and especially those that contain non-platinum DNA damaging agents – more global measures of DNA damage may be needed to assess the true biology of the drug exposure(s).

Reed's group has studied platinum-DNA adduct formation in clinical settings, using AAS. The two most important observations from Reed's group relate to the broadening of the principles of the original observations by Poirier; and relating the observations of all the major groups to one another on the molecular level. In a blinded study of the combination of cisplatin and carboplatin, Reed studied a cohort of 49 patients, which included 24 different tumor types [72,73]. This was a phase I study of this platinum combination, and clinical responses and non-responses were seen at each dose level. In this cohort, platinum-DNA adduct level after the first dose of therapy was directly related to disease response, regardless of the tumor type. The 2-sided p value for this relationship after the first dose of therapy was 0.007; and after the first two cycles of therapy, the summary p value for the relationship was 0.0004 [73].

This observation by Reed has been confirmed by several independent groups. The most convincing was a recent report from Schellens et al [74]. They studied a group of 45 patients with a variety of tumor types who received cisplatin-based therapy. They assessed platinum-DNA adduct in several ways, including an area-under-the-curve (AUC) type of analysis. In their work, platinum-DNA adduct AUC was directly related to a favorable clinical response – regardless of tumor type – with a two-sided p value of <0.0001 [74].

In an effort to reconcile the different methods of measurement of platinum-DNA adduct in clinical studies, Reed assessed the reported platinum-DNA adduct levels in a series of reports over an 11 year period [75,56]. The conversion factor of 0.2% (or, 500-fold) was used as discussed above [66], to convert adduct levels assayed by ELISA to adduct levels assayed by AAS. When the data of Poirier is converted to 'AAS equivalents', a striking pattern becomes apparent, as shown in Table VI. Very high levels of DNA damage (more than 0.060 lesions per kb) are very difficult to obtain with clinically tolerable doses of cisplatin or carboplatin [72,73]. Patients who experience a clinical complete remission tend to have leukocyte DNA adduct levels ≥ 0.030 platinum lesions per kb of cellular DNA. Those who have a partial remission appear to have at least ≥ 0.010 lesions per kb; and those who do not respond consistently have lower levels of DNA damage, with the exception of testes cancer [60,62]. These DNA damage measurements were taken, for the most part, in nucleated human peripheral blood cells. However, as indicated above, available data would suggest that these levels are in the

Table 6. Association between defined DNA damage levels and biological endpoints

DNA damage level	Biological association: Lab and clinic
0.060 lesions/kb	Very difficult to obtain clinically. Rarely occurs. Necessary to get >80% cell kill of resistant cells: i.e., mesothelioma, resistant ovarian cancer cells, etc.
>0.030 lesions/kb	Readily obtainable with good platinum doses. Associated with >90% cell kill in sensitive cells. Associated with CR's and PR's in clinical situations.
<0.010 lesions/kb	Relatively easy to obtain clinically. Associated with clinical response in testes cancer; clinically, and in tissue culture situations. Rarely associated with response in other tumor types.

range of what has also been observed in tumor tissues taken at autopsy [61,65]. These levels of DNA damage appear to be relevant in the evaluation of platinum-resistance/sensitivity in tissue culture, as well (Reed, unpublished observations).

Evidence that DNA Repair is Important in the Clinic

It has been consistently quite difficult to assess DNA repair in a direct manner, in human cancer patients. Whereas the study of fibroblasts grown in vitro may be quite informative, this is possibly very different from direct measurements of the removal of cellular DNA damage within an intact host. One study has attempted to assess the removal of platinum-DNA damage from cells within intact hosts, during the course of anti-cancer treatment [77]. Sixteen patients with cancer of the head and neck, were studied for platinum-DNA adduct levels in peripheral blood cell DNA, during the first and second cycles of platinum-based chemotherapy. Each treatment cycle was separated by 21 days. As discussed above, when platinum-based therapy is administered, platinum-DNA damage occurs in many tissues throughout the body. Specifically, DNA adduct occurs in bone marrow as well as in peripheral blood cells. In this 16 patient cohort, DNA adduct measurements were made 24 hr after cycle 1 of chemotherapy, and immediately before cycle 2 of chemotherapy, 20 days later. This gives an assessment of the ability of bone marrow tissue to remove adduct over an approximate 3 week period of time.

In patients with head and neck cancer that *did not* respond to therapy, the median rate of adduct removal

over this time period was 3-fold higher than the rate seen in patients whose tumors *did* respond; 62.2, versus 20.6 picograms per ug DNA [77]. Also, the nadir white blood cell count (WBC) was inversely related to the amount of DNA adduct that was repaired; e.g., the more adduct removed from cellular DNA over this time frame, the less severe the wbc nadir. A curious trend in the data, showed the possible existence of at least two subgroups within this cohort. One subgroup had a comparatively high level of DNA repair activity, and one subgroup had a comparatively lower level of DNA repair activity. This is consistent with reports on mRNA expression levels of the NER genes ERCC1 and XPA in human tumor tissues, where there was also the suggestion of at least two subgroups of individuals [53].

The first gene that was cloned and sequenced within the NER pathway was ERCC1 [54]. Cells with an ERCC1 deficit appear to have the most severe DNA repair deficit [35,54], and mice with the ERCC1 knockout genotype consistently die before weening [78]. Because of these facts, and the fact that there is no known human disease with an ERCC1 deficit, it has been suggested that ERCC1 is essential for life. We know that ERCC1 may exist in an alternatively spliced form, and in a polymorphic form, in human tissues (see above). These issues beg the question as to whether the study of ERCC1, as it may relate to drug resistance, may offer insight into the NER process in human clinical tissues.

Reed and colleagues were the first to study NER gene expression in fresh tumor tissues of patients who received platinum-based therapy [53,79]. These studies included ERCC1, ERCC2 (XPD), and XPA. In human ovarian cancer patients, high tumor tissue

Figure 3. Coordinate expression of three NER genes in human ovarian cancer tissues; XPA, ERCC3/XPB, and ERCC6/CSB. In panel A, tumor tissues that were platinum-sensitive appear to have concurrent low levels of mRNA of each gene. In panel B, tumor tissues that were platinum-resistant appear to have concurrent high levels of mRNA for each gene.

Table 7. Suggested ‘Order’ of selected NER genes that are essential in the rate-limiting step of the process

mRNA expression patterns in human ovarian cancer tissues suggest that:			
ERCC1	appears before	ERCC3, XPA, and ERCC6	
ERCC3	appears before	XPA and ERCC6	
XPA	appears before	ERCC6	
The suggested order is:			
ERCC1 (DNA damage recognition and excision)	before		
ERCC3 (linkage of DNA repair and transcription)	before		
XPA (fine tunes DNA damage recognition)	before		
ERCC6 (essential for gene specific repair)			

Table 8. Types of DNA repair currently associated with drug resistance

Type of repair	Comments
O-6-Alkyltransferase	Effects repair of O-6-alkyl-purine lesions.
Base excision repair	Effects repair of damage from ionizing radiation, simple alkylating agents, hydrolytic and oxidative processes.
Nucleotide excision repair	Repairs bulky DNA adducts, caused by covalent damage to the DNA, such as cisplatin, UV, PAH's.
Mismatch repair	Repairs mispairs and slipped intermediates within the DNA. May link NER with apoptosis.
Gene specific repair	A subset within nucleotide excision repair.

levels of ERCC1 mRNA were associated with clinical resistance to platinum, whereas low mRNA levels were associated with clinical sensitivity. The mRNA expression of ERCC2 showed no such association [79]. This was consistent with *in vitro* findings which showed that the excision nuclease activity appeared to be a critical factor in cisplatin resistance, whereas helicase activity appeared to be much less of a factor – if at all [46]. A subsequent analysis of this cohort showed that a substantial fraction of the ERCC1 mRNA was alternatively spliced in each tumor tissue sample examined [53]. In platinum-sensitive tumors and in platinum-resistant tumors, the alternatively spliced species of ERCC1 could be a low percentage (<10%), or a high percentage (>50%), of the total ERCC1 mRNA within the sample. There was no clear association with alternative splicing and clinical resistance to platinum, in this small cohort.

A subsequent study showed that the mRNA's of ERCC1, and XPA, and p53, tended to be highly expressed in concert, or, expressed to low levels in concert, within a specific ovarian cancer tumor specimen [80]. This suggested that, in general, this 'coordinate' expression pattern may be a common feature for genes involved in NER [80,81]. This would make biological sense, because all of the 16 essential genes involved within NER are needed in order to successfully complete the DNA repair process [13,14]. If the process is to show increased activity, it would be necessary to have increased activity of each contributor to the process. Figure 3 illustrates coordinate mRNA expression of 3 of the 16 essential NER genes. Assessment of the genomic status of XPA in human ovarian cancer tissues show that neither gene amplification nor gene mutations were necessary, to observe increased mRNA expression of XPA within a specimen [82].

Recently, an important confirmatory study of ERCC1 was performed by a group using cisplatin and 5-fluorouracil in the treatment of gastric cancer [83]. A series of 38 patients was assessed for tumor mRNA levels of ERCC1 and thymidylate synthase, prior to receiving this combination chemotherapy for their disease. mRNA Levels were assessed by RT-PCR, using a modification of the method reported by Reed and colleagues [53,79]. Median mRNA levels of ERCC1 in tumors that showed objective response to this platinum-based regimen, were two-fold lower than levels seen in tumors that were not responsive to the regimen. This magnitude of difference between responders and non-responders, is very similar to what was seen in ovarian cancer patients [53,79]. In the gas-

tric cancer study, patient survival was also inversely related to ERCC1 mRNA levels in tumor tissues [83]. This appears to be clinical confirmatory evidence to suggest that: a) ERCC1 may be a useful gene to follow with respect to clinical sensitivity/resistance to platinum-based chemotherapy, regardless of tumor type; b) ERCC1 mRNA levels may be reflecting the overall capability of the tumor to repair cisplatin-DNA damage; and, c) When ERCC1 levels are high, this may reflect the coordinate high expression of all of the critical genes within the NER process. Alternative splicing of ERCC1 was not assessed in this study.

The next corollary question is whether this knowledge can be used for clinical benefit. At least one clinical study strongly suggests that the answer may be, yes. Paclitaxel is a recently approved anticancer agent, and is a natural product extracted from the bark of the Pacific yew tree [84]. Paclitaxel synergizes with DNA damaging agents *in vitro*, and greatly inhibits the repair of cisplatin-DNA adduct when the two agents are properly sequenced [85,86]. This information was used to design a novel clinical trial, the basis for which was the modulation of DNA damage and repair on a clinical level [87,88]. DNA damage was induced by the agents cisplatin and cyclophosphamide, and paclitaxel was used to modulate the repair of such damage. Clinical studies had shown that cisplatin and paclitaxel were useful together [89], and that cyclophosphamide and paclitaxel were useful together [90]. The novel clinical trial was to use cisplatin, cyclophosphamide, and paclitaxel – sequenced in a specific fashion – in the treatment of advanced stage epithelial human ovarian cancer [87,88]. Data from the first 36 patients studied, showed that in patients with poor prognosis disease, there was 2–4 fold improvement in: a) the expected pathological disease response rate; b) the progression free survival time; and, c) the overall survival time [88,91]. Therefore, the knowledge obtained with respect to NER modulation *in vitro*, appears to be somewhat transferable to the clinical situation in human ovarian cancer.

Clinical Studies Regarding the Biology of Nucleotide Excision Repair

Whereas it is the goal of translational research to 'translate' basic science questions into matters of practical human benefit, it may be also possible to learn potentially critical pieces of information regarding the 'basic science' of a question from a careful examina-

tion of clinical information. In other words, it may be desirable to go 'backwards', as well as 'forwards', in this process.

Dabholkar and colleagues examined the question of coordinate expression of mRNA's for selected NER genes in malignant and non-malignant brain tissues taken from a series of human patients [19,20,92]. These were individuals taken to surgery to excise the malignant brain lesion. In such individuals, a portion of surrounding normal tissue is also taken at surgery, to ensure a tumor-free surgical margin. This offers the opportunity to assess mRNA expression of such genes in malignant tissues and non-malignant tissues from the same individual(s). In such settings, all malignant tissues can be compared; all non-malignant tissues can be compared; and a number of other comparisons can be made, as well. One can assess 'coordinate' expression of two genes, by plotting the numerical values for the relative levels of mRNA expression against one another, and performing linear regression analysis [19,20]. Coordinate expression is suggested if both genes show the same relative levels of expression in each tissue examined. The degree of coordinate expression can be compared from one situation to another, by the 'goodness of fit' of the respective data sets to linear regression equations.

In their first report, Dabholkar and colleagues studied coordinate expression patterns for the genes ERCC1 (an excision nuclease), ERCC2 (a helicase), and XPA, in malignant and non-malignant brain tissues [19]. What was shown, is that normal tissues show excellent levels of coordinate expression of these genes, concurrent with a situation where malignant tissues show disruption of coordinate expression of these same genes, in tissues from the same individuals. This had not been observed before. The data were taken to suggest that in human tissues, the helicase and excision functions of NER were well coordinated under normal circumstances; and that this coordination was lost in malignant cells. This observation may help to explain why X-irradiation and/or other DNA damaging agents may be effective in reducing the volume of some tumor masses, while surrounding normal tissues may be relatively unaffected by that same specific treatment, in that individual.

In a subsequent report, Dabholkar and colleagues expanded their observations to include mRNA levels of ERCC3 (a helicase) and ERCC6 (essential for gene specific repair) [20]. Further, tissue samples were sub-categorized into normal tissues, low grade malignancy, and high grade malignancy. A detailed evaluation of

mRNA expression patterns showed that normal tissues were characterized by excellent coordinate expression; that high grade malignancy was characterized by very poor coordinate expression; and that low grade malignancy was intermediate between the two. These data were interpreted to suggest that as cells move from the state of normalcy, to low grade malignancy, to high grade malignancy, there is increasing disorder in the NER process. This observation is compatible with the long held clinical observation that high grade brain tumors tend to be more responsive to immediate interventions, such as X-irradiation, than low grade tumors – although low grade tumors are generally more slow growing.

Another 'basic' question that has been addressed using clinical information, has to do with the 'order' of mRNA expression within the NER process. If these genes (ERCC1, XPA, XPB, XPD, etc.) are co-ordinately expressed, are they up- or down-regulated simultaneously, or is there some defined order within the process? We attempted to address this question through the use of human ovarian cancer tissues [93,94]. A series of tumor specimens (n=28) were assessed for mRNA expression of ERCC1, XPA, XPB, and ERCC6/CSB, using RT-PCR. Within this group, if we observed that ERCC1 (as an example) was measurable when XPA was not – but never the reverse – we interpreted this to suggest the possibility that ERCC1 must always precede XPA in an obligate fashion. Using this strategy, one can assess these four genes, in independent pairings. Such an analysis led to the suggested order summarized in Table VII. These data sets strongly suggest that the obligate order of the appearance of mRNA's for these four NER genes in human ovarian cancer tissues is: ERCC1 (essential for life), before ERCC3/XPB (links DNA repair with DNA transcription), before XPA (fine tunes DNA damage recognition), before ERCC6/CSB (essential for gene specific repair). These data suggest a biological order for these genes within the NER process. The biological order suggested, is compatible with current knowledge regarding the respective functions of these specific proteins. Although data suggests a definite order to the up-regulation of these coordinately expressed genes, it should be noted that all genes studied within the process to date, show coordinate expression in human ovarian cancer tissues [81,91].

Table VIII summarizes, in a very simplified fashion, what we currently believe to be true in the human biology of several major DNA repair pathways. Most of the work done to date, focuses on molecular

mechanistic questions, in highly controlled laboratory conditions. Therefore, it is not proven that what we believe to be true based on laboratory observations, is truly applicable to the human situation. Clearly, much work needs to be done.

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