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Review paper

DNA repair mechanisms associated with cellular resistance to antitumor drugs: potential novel targets

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The 1990s have already heralded an enormous expansion of our knowledge of DNA repair. Gene by gene, protein by protein, each partner in the molecular processes of DNA repair is being identified and characterized, not only in bacteria and yeast, but also in mammalian cellular systems. Several distinctive mechanisms are now explained at a molecular level, even if certain specific parts still remain to be elucidated fully. The techniques used to study DNA repair have also profited from this progress with a plethora of novel *in vitro* assays, specific antibodies, together with DNA or RNA probes becoming available. The increased use of these tools has permitted a multiplicity of studies on DNA repair which are now not exclusively mechanistically based. Thus, certain studies have now implicated DNA repair processes as likely to be involved in the multifactorial phenomenon of drug resistance to anticancer drugs. Under these circumstances, DNA repair mechanisms should provide useful pharmacological targets to attack with novel inhibitors, with the aim of reducing and/or sensitizing tumor cells to anticancer drugs which damage DNA. Our increased knowledge of the molecular mechanisms associated with DNA repair permits us now to consider such new pharmacological targeting. In this article, we review the present status of these DNA-repair-related pharmacological studies, and discuss both the likely and possible approaches which might have potential therapeutic applications. [© 1998 Rapid Science Ltd.]

Key words: Antitumor drug, cellular resistance, DNA repair, novel targets.

Introduction

During the treatment of cancer with chemotherapy, tumor cells frequently develop resistance to the anticancer drugs used, thereby limiting their long-term efficacy.[1] The emergence of resistance is known to be multifactorial,[1,2] with the importance of each resistance mechanism varying and most probably depending not only on the actual drugs used, but also on the type of tumor cells being treated. Amongst these mechanisms, certain DNA repair processes have now been implicated in cases of resistance to DNA-damaging agents.[3–5] Consequently, DNA repair processes represent potential new targets for combating cellular resistance to certain classes of anticancer drugs. In the case of cisplatin, for example, a change of 2-fold or less in sensitivity was shown to account for treatment failure in human tumor xenografts.[6] Thus, even very low levels of resistance could be sufficient to cause a lack of clinical responsiveness. Under these circumstances, it has been difficult to identify whether certain mechanisms are of greater clinical significance than others. Consequently, it remains necessary today to devise strategies successfully to combat or neutralize all known resistance mechanisms in our efforts to ensure clinical therapeutic benefit.

The mechanisms associated with the known repair processes have been comprehensively reviewed recently.[7–9] In this article, we will merely summarize each mechanism briefly, to aid the reader in understanding the molecular and pharmacological implications of their inhibition, then we will discuss their established or potential involvement in drug resistance, and finally consider propects for their inhibition.

O^6-alkylguanine-DNA alkyltransferase (AGAT)

The AGAT is certainly the repair process which has received the most attention in terms of inhibition studies.[10,11] This 'enzyme' repairs DNA via a suicide mechanism without any cofactor requirements or any interaction with other proteins.[3] The 'simplicity' of this process has, therefore, provided the main impetus for investigators to understand and to describe it, as well as to determine the potential molecular target which has proved to be unique.

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AGAT is a primary defense against O^6-alkylguanine, generated as a result of the use of alkylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), dacarbazine, triazene or triazine.[12,13] This enzyme, with a molecular weight of approximately 25 kDa, exerts its activity by transfering stochiometrically small alkyl adducts from the O^6 atom of guanine in DNA to an internal cysteine residue.[14] This reaction returns the DNA base to an unmodified state and permanently inactivates the AGAT enzyme (Figure 1).

The level of expression of AGAT varies throughout different cell types,[15] including tumor tissues,[16] and such expression is well correlated with sensitivity to alkylating agents which produce O^6-alkylguanine. Transgenic mice expressing increased levels of AGAT in different organs have also been produced and the resultant mice were protected from alkylating agent treatment with a specificity shown for tissues in which the AGAT transgene was expressed.[17] In contrast, a group of human tumor cell lines that lack alkyltransferase activity, corresponding to the Mer⁻ phenotype or methylation repair minus, was found to be hypersensitive to drugs that produced O^6-alkylguanine.[18] The transgenic mice technology was also used to show hypersensitivity to alkylating agents of mice deficient in the AGAT gene.[19] Finally, the relationship between AGAT expression levels and sensitivity of brain tumors to BCNU has been confirmed recently in a retrospective clinical study using biopsies from 167 patients.[20] Moreover, this increased AGAT expression could result from an amplification of the corresponding gene.[21] These observations indicate that AGAT should interest not only the DNA repair specialists, but also practicing clinicians. At present, an induction of AGAT cannot be excluded as another explanation for the emergence of resistance to these alkylating agents since, using certain cellular models, an induction of AGAT has been shown in response to methylating agents [*N*-methyl-*N*,*N*'-nitro-*N*-nitroguanidine (MNNG) and methyl methanesulfonate (MMS)], UV light, γ radiation or hydrogen peroxide.[22,23] In spite of this, the previous studies emphazing a relationship between AGAT expression and resistance to anticancer alkylating agents, like BCNU, have provided evidence for considering AGAT as a major site to target in order to sensitize tumor cells to the chloroethylnitrosoureas (CENUs).

With this idea in mind, several derivatives of alkylguanine have been tested with the aim of inactivating the AGAT enzyme.[10] Thus, it has been shown that exposing cells to O^6-benzylguanine results in inactivation of AGAT and potentiation of CENUs, like BCNU. O^6-benzylguanine inactivates AGAT by acting as a substrate for alkyl transferase and by forming *S*-benzylcysteine at the acceptor site of the protein.[24] This activity and the potentiation of BCNU have been demonstrated not only with cultured cells,[25] but also using a number of human cancers xenografted onto nude mice[26–29] or onto athymic rats.[30] More recently, the potentiation of temozolomide, a methylating imidazotetrazinone, by O^6-benzylguanine was also reported.[31] Now, the strategy of

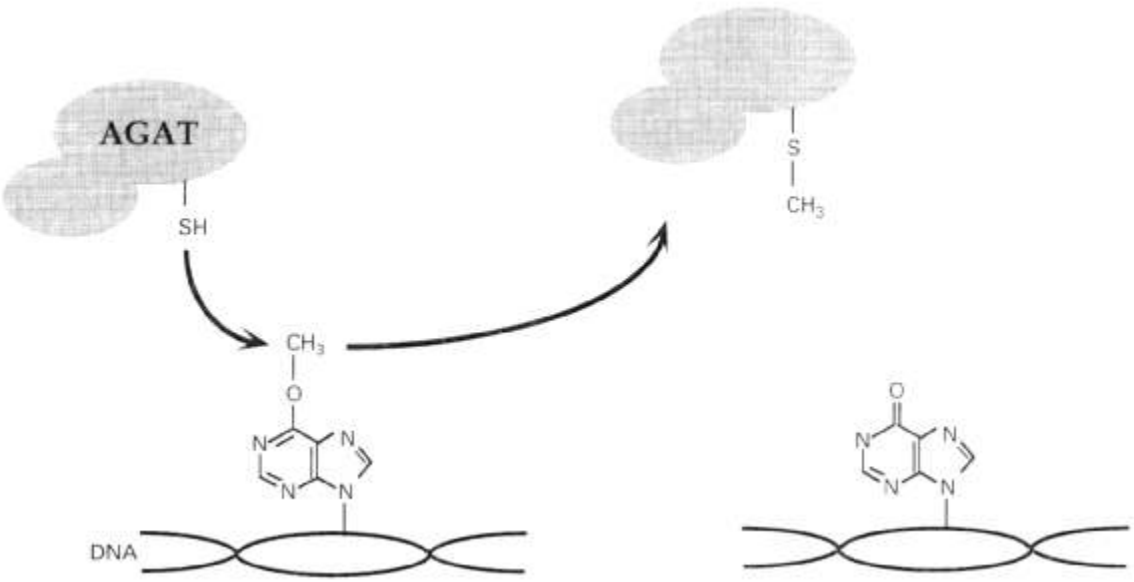

Figure 1. Mode of action of human O^6-alkylguanine-DNA alkyltransferase.

associating O^6-benzylguanine and BCNU treatments is currently being evaluated in clinical trials and results should be very informative,[11] especially in terms of the toxicity of such treatments, since it has already been demonstrated that O^6-benzylguanine inhibits human and not murine AGAT (rendering the *in vivo* animal studies uninformative vis-à-vis this point).[17]

Until recently, O^6-benzylguanine was considered the best compound to inactivate AGAT, since testing this strategy with other compounds failed to yield such positive results. Methylating agents like streptozotocin or dacarbazine had initially been proposed for depletion of AGAT.[11] Unfortunately, these combinations were found to be less active and more toxic *in vivo*.[28] Moreover, it was shown in the clinic that whilst streptozotocin decreased active AGAT, depletion was not complete and the residual AGAT was sufficient to maintain clinical resistance to BCNU.[32] Nevertheless, irrespective of the future clinical results with O^6-benzylguanine, second generation compounds must be actively sought since (i) despite efforts to better solubilize O^6-benzylguanine,[33] its limited water solubility and rapid clearance remain a problem,[34] and (ii) it has already been shown that certain mutations of the AGAT gene can result in resistance to inactivation by O^6-benzylguanine.[32] Second-generation compounds could include 5-nitrosobenzylloxypyrimidine or O^6-fluorobenzylguanine, since initial studies have shown encouraging results concerning their activities as inactivators of AGAT and as sensitizers of CENUs.[34–37] On the other hand, the fact that some mutated forms of AGAT resist inactivation by O^6-benzylguanine should not be considered as a therapeutic disadvantage. It might also be exploited positively since transfection of hematopoietic cells with these mutated genes should serve to protect them from the DNA damaging effects of BCNU, as recently proposed.[11,38] Under these circumstances, therefore, a presumed disadvantage could be transformed into an advantage if it can be demonstrated that such transfection can protect patients from myelosuppression, the major dose-limiting toxicity of O^6-alkylating agents.[39]

Base excision repair (BER) and single-strand break (SSB) repair

BER is the second process, together with AGAT, used by cells to repair damaged bases in DNA. The inhibition of such a mechanism has generally been less studied than that of AGAT for several reasons. Firstly, BER is a multienzymatic process which is more difficult to investigate than the repair system using AGAT, a process implicating a single enzyme. The BER process has been reviewed recently[9,40] and this mechanism has recently been reconstituted *in vitro*.[41,42] A summary of present knowledge concerning this pathway is presented in Figure 2. Initially, the damaged base is recognized and removed from the DNA by a DNA glycosylase. There are several mammalian DNA glycosylases that initiate BER of DNA damage. The diversity of these enzymes is related in terms to the diversity of base damage.[43] Most DNA glycosylases recognize and remove several structurally different damaged bases, while a few have very narrow substrate specificity. At the second step, an apurinic/apyrimidic lyase (AP lyase) incises 3′ to the abasic site. This AP lyase is usually the AP endonuclease (APE) since it accounts for almost 99% of incisions at AP sites into DNA present in human cell extracts.[44] The release of the deoxyribose moiety by hydrolysis 5′ to the abasic site could also occur by APE, but several other proteins have this same activity, one of which is polymerase β (pol-β).[45] Finally, the resulting gap is filled and religated by a complex containing a DNA polymerase and a DNA ligase. Recently, several studies have concluded that the repair synthesis of BER is performed essentially by DNA pol-β[45] and to a lesser degree by the pol-δ and -ε (Figure 2).[46] The implication of pol-β in BER was recently confirmed *in vivo* using cultured cells deficient in pol-β.[47] These cells were found to be hypersensitive to mono-alkylating agents of which DNA lesions are supposed to be repaired by BER. Moreover, complementation of deficiency in pol-β resulted in a lost of this hypersensitivity.

The enzyme pol-β has been found not only to interact with APE,[48] but also with XRCC1,[41,49] which also interacts with DNA ligase III[49] and thus reconstitutes an *in vitro* active BER complex.[41] The function of XRCC1 is not yet known, but it might stabilize the repair complex. At the next step, the involvement of ligase I, interacting with pol-β, has also been suggested and observed *in vitro*.[50] These two ways of religating the neo-synthesized DNA are not exclusive, but ligase I is largely involved in DNA replication and nucleotide excision repair, which are most probably its main functions.

As indicated previously there is an alternative pathway for synthesis and ligation of DNA, involving DNA pol-δ and/or -ε.[40,51] In this case, BER is associated with generation of longer repair patches (2–10 nucleotides). The resulting overhanging 5′-terminal single-stranded region of DNA can be removed by a flap endonuclease, FEN-1.[42] This longer patch BER requires proliferating cell nuclear antigen (PCNA), suggesting that the longer patches are generated by the pol-δ and/or -ε but FEN-1 is also stimulated by the

presence of PCNA[42] and could be the reason for this dependance on PCNA.[51] Under these circumstances, the real involvement of pol-δ and -ε, in addition to pol-β, remains questionable.

Other factors that have tended to lead to a certain lack of interest from pharmacologists in this pathway include the fact that it appears to be rarely induced by DNA damaging agents, and only a weak correlation has

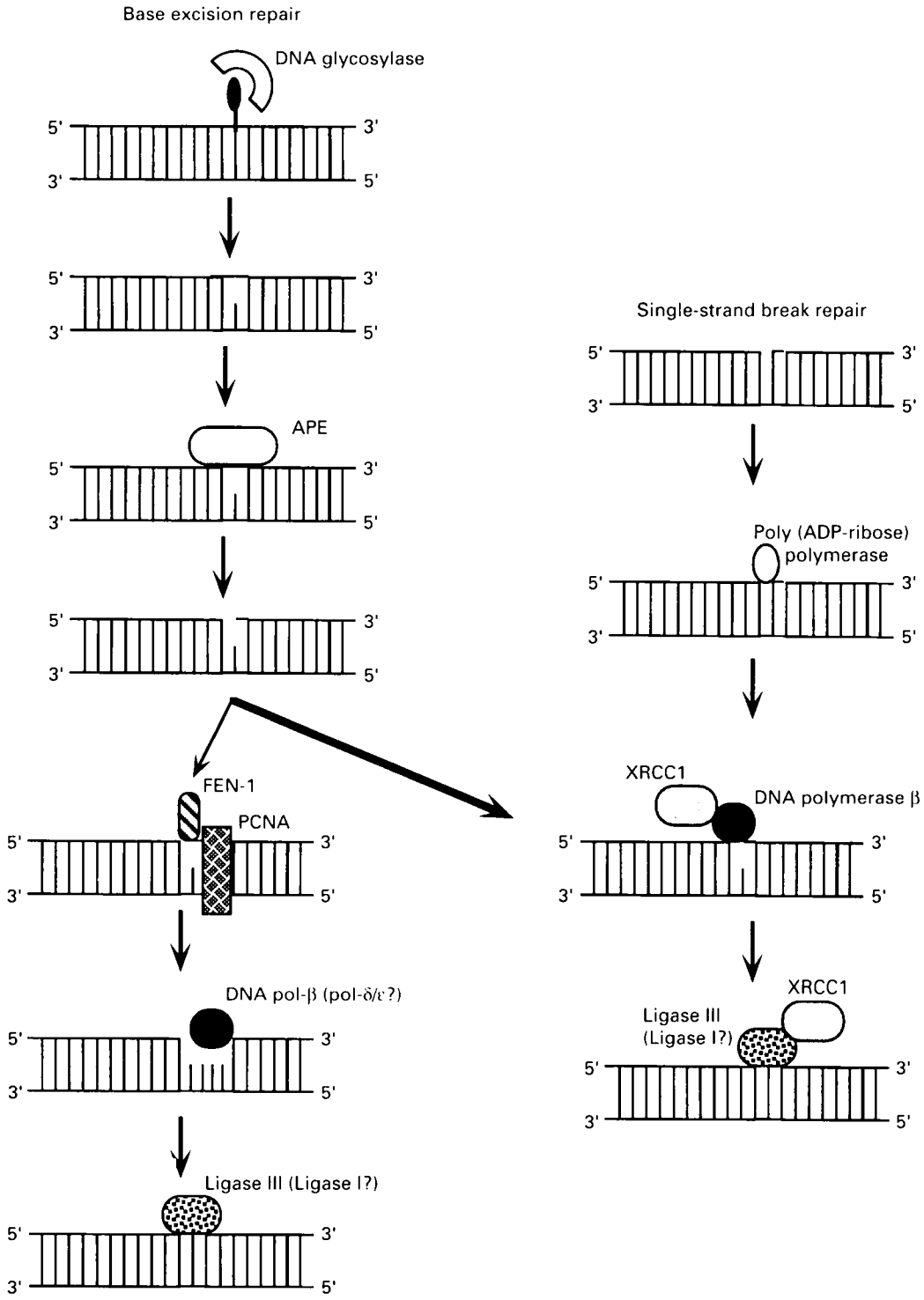

Figure 2. Model for BER and SSB repair in human cells.

been found between resistance to alkylating agents and overexpression of BER proteins. In a few cases, it has been suggested that drug resistance may be due to elevated levels of AP endonucleases and of methyl purine glycosylase (MPG), a DNA glycosylase.[52] These increases can be shown after an induction by differents types of DNA-damaging agents such as cisplatin or γ radiation.[53] Nevertheless, experiments based on gene transfection and cellular overexpression of MPG protein have never permitted the establishment of any relationship between the level of MPG expression or activity and the degree of cellular resistance to alkylating agents.[54] Moreover, a selection of clones from mammalian cells resistant to mono-alkylating agents did not reveal any overexpression of MPG.[21] However, a recent study has shown that cells from mice deficient in 3-methyl DNA glycosylase, another DNA glycosylase, have increased sensitivity to alkylating drugs like BCNU and mitomycin C.[55] Nevertheless, all these studies were still related to only one protein of the BER process and, in order to clarify certain discordent results, an overall detailed analysis of all the proteins involved in BER should be made. Results obtained with pol-β have been less controversial, since it was found that pol-β was overexpressed in response to DNA-damaging agents,[56] and an overexpression of pol-β has been observed in cells resistant to both mono- and bis-alkylating agents.[57–59]

The pharmacological inhibition of BER has not been extensively studied because of the complexity of this pathway which, at this time, remains incompletely understood. The low level of pharmacological interest in such investigations is also probably due to the fact that BER is known to repair only certain damage induced by mono-alkylating agents, therefore limiting the benefit of such a pharmacological intervention. However, the reaction of bis-alkylating agents with DNA produces intermediate mono-adducts, certain of which may be repaired by the BER process.[60] Thus, a deficit in BER could result in a cellular hypersensitivity to bis-alkylating agents, as observed by Engelward *et al.* with BCNU and mitomycin C.[55] For this reason targeting BER inhibition could be, finally, more interesting from a pharmacological point of view than initially anticipated.

However, all the proteins in the BER pathway cannot be considered as targets for BER inhibitors. For example, some proteins, like FEN-1 or XRCC1, are actually not well enough described structurally or functionally to be considered. The DNA glycosylases should also not be targeted, in view of the complicated fact that they are several of the enzymes known with different specificities and possible cross-selectivities to certain DNA damage.[43,61] However, inhibition of the DNA glycosylases is conceptually possible. Thus, pyrrolidine-based inhibitors have been successfully tested *in vitro* on bacterial (AlkA) and human (ANPG) DNA glycosylases.[62]

On the other hand, the involment of pol-β in BER and its overexpression frequently found in resistant tumor cells provide good reasons for using this enzyme as a target for BER inhibition. 2′,3′ dideoxycytidine (ddC) and 3′-azido-3′deoxythymidine (AZT) were found to inhibit DNA pol-β.[59,63] However, ddC only modestly enhanced the antitumor activity of melphalan against melphalan-resistant tumor cells s.c. xenografted onto nude mice.[59] Moreover, these nucleotide analogs could cross-react with other polymerases, and thus provoke non-specific and unexpected side effects. With the aim of reducing this problem, recent crystallographic studies of pol-β should be informative in terms of conceiving original chemical structures of new inhibitors.[64]

Another promising target should be APE, which is a crucial enzyme in BER, since it is nearly the sole AP endonuclease interacting in this process. Moreover, recent studies have shown that its AP endonuclease activity and its regulatory function of proto-oncogenes by redox activity were not coupled.[65] Thus, an inhibition of the endonuclease activity of APE should neither modify its other cellular role(s) nor induce corresponding secondary effects. To date, no BER inhibitors which interact and inhibit APE have been described. However, a new concept of inhibition in relation to the AP site has emerged in the last few years: artificial endonucleases.[66,67] These compounds are constituted by three parts: a nucleic base for abasic site recognition, an intercalating agent (9-amino-acridine) to re-enforce and stabilize the interaction with DNA, and a polyamino-chain linking the two moieties, and can cleave DNA by a β-elimination process.[68] These compounds could mask the abasic site to APE and thus result in its inhibition. Moreover, the DNA cleavage produced by these compounds should drastically increase the cytotoxicity of DNA-damaging agents whose adducts are repaired by BER. For example, in the presence of an alkylating agent, the more active the BER, the more DNA cleavage should be produced. This hypothesis is currently under investigation. Such an activity has been already described for compounds with simpler structures: 9-amino-ellipticine,[69] 3-aminocarbazole,[70] a tripeptide Lys-Trp-Lys[71] and suggested for DMP-840, a bis-naphtalimide anticancer agent in phase II anticancer clinical trials.[72] Such compounds could provide novel leads for the synthesis of new sensitizers of DNA-damaging agents.

The repair of SSBs can be considered in this section since the enzymatic system proposed is similar to the next step of BER (Figure 2).[73] The only difference is the presence of poly-(ADP-ribose)-polymerase (PARP), which is supposed to be a constituent, together with XRCC1, pol-β and ligase III, of a cellular 'nick-sensor' which should detect and repair SSBs.[49]

PARP is a paradox since its roles are not clearly defined, but it is certainly the most studied enzyme in terms of inhibition aimed at sensitizing cells to DNA-damaging agents. Two functions, corresponding to two protein domains, have been clearly demonstrated: a DNA binding domain and a poly-(ADP-ribosyl)ation activity.[74] The cellular roles of this protein are much more uncertain and involvement of PARP has already been suggested in many processes: cellular energetic homeostasis,[75] regulation of chromatin structure,[76] regulation of the activity of other proteins,[76–78] apoptosis,[79] necrosis,[80] BER,[81] SSB repair,[73] as well as recombination.[73,82] None of these actions are mutually exclusive,[83] but remain the subject of several controversies and even techniques like knockout mice have not yet succeeded in providing clear cut answers.[84,85]

The only facts which are not contested are the recognition of SSBs and, to a lesser extent, of double-strand breaks (DSBs), by the DNA binding domain of PARP[86] and the increase of intracellular poly(ADP-ribosyl)ation in response to treatment of cells by several DNA damaging agents: γ radiation, UV radiation, cisplatin, etoposide, etc.[85,87] However, certain studies have shown that cells which do not show this increase in response to DNA-damaging agents were hypersensitive to these agents.[88,89] It has been confirmed that this induction resulted from an increase of PARP activity and not from an over-expression of the PARP protein.[90] An increase of PARP activity has also been proposed in order to explain certain cellular resistance to drugs such as cisplatin or bleomycin.[91,92] Interestingly, an artificial (by transfection) overexpression of PARP leads to a sensitization of cells transfected vis-à-vis the DNA-damaging agents.[93,94] Such sensitization has also been observed when only the DNA-binding domain of PARP was overexpressed, suggesting that this domain reacts as a *trans*-dominant inhibitor of poly(ADP-ribosyl)ation.[95] Accordingly, inhibition of the catalytic activity of PARP should be preferred to an inhibition of its DNA binding activity in order to sensitize cells to DNA-damaging agents.

With the perspective of inhibiting the activity of PARP, sensitization of cells to a large spectrum of DNA-damaging agents should be considered, since SSBs and DSBs are found temporally during almost all DNA repair processes. As initially discussed, inhibition of the catalytic activity of PARP has been widely documented and has been the subject of several publications concerning nicotinamide or derivatives of aminobenzamide, thymidine or, the most active, isoquinoline.[96–99] As with all the research relating to DNA repair inhibitors, the question about the specificity of these particular inhibitors is also very important. Derivatives of benzamide potentially could interact with several others enzymes which use NAD^+ as a substracte or co-factor.[100] This problem need to be solved, since the crystal structure of the catalytic fragment of PARP has recently been elucidated and could clearly be useful in elaborating new series of inhibitors.[74] Nevertheless, several attempts at sensitization of cells with PARP inhibitors, already described, have been made with some success. For example, sensitization of cells by PARP inhibitors to temozolomide, BCNU, cisplatin, bleomycin or γ radiation has been shown *in vitro*.[96,101,102] Similar results have also been obtained using an antisense strategy.[103] Such results are promising and have, in part, been reinforced by certain results *in vivo*.[101] However, as observed by Olsson *et al.*[104] with nicotinamide, the lack of specificity of such inhibitors and the doses necessary to reveal a clear sensitization *in vivo* could cause considerable complications with normal tissues. Thus, more specific and more potent compounds will certainly be needed before clinical studies can be initiated.

Nucleotide excision repair (NER)

Most DNA damage is repaired by the NER system which recognizes DNA adducts induced by numerous chemical treatments.[9] This mechanism, now fairly well characterized, is known to consist of two distinct major steps: (i) the incision reaction involving damage recognition and excision of the damaged oligonucleotide or base, and (ii) repair synthesis of new DNA using the complementary strand as a template and its subsequent ligation to restore strand continuity.[9,105,106] At a molecular level, major advances have been made in recent years in our understanding of this repair pathway in mammalian cells.[9,105,106] Human genetic defects in NER have been found in association with the severe disorders, e.g. Xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and Trichothiodystrophy (TTD).[105,106] In each of these diseases, several genetic complementation groups have been defined from XPA to XPG, from TTD1 to TTD3, and CSA and CSB. These have been assigned to 11 complementation groups and so far have allowed the

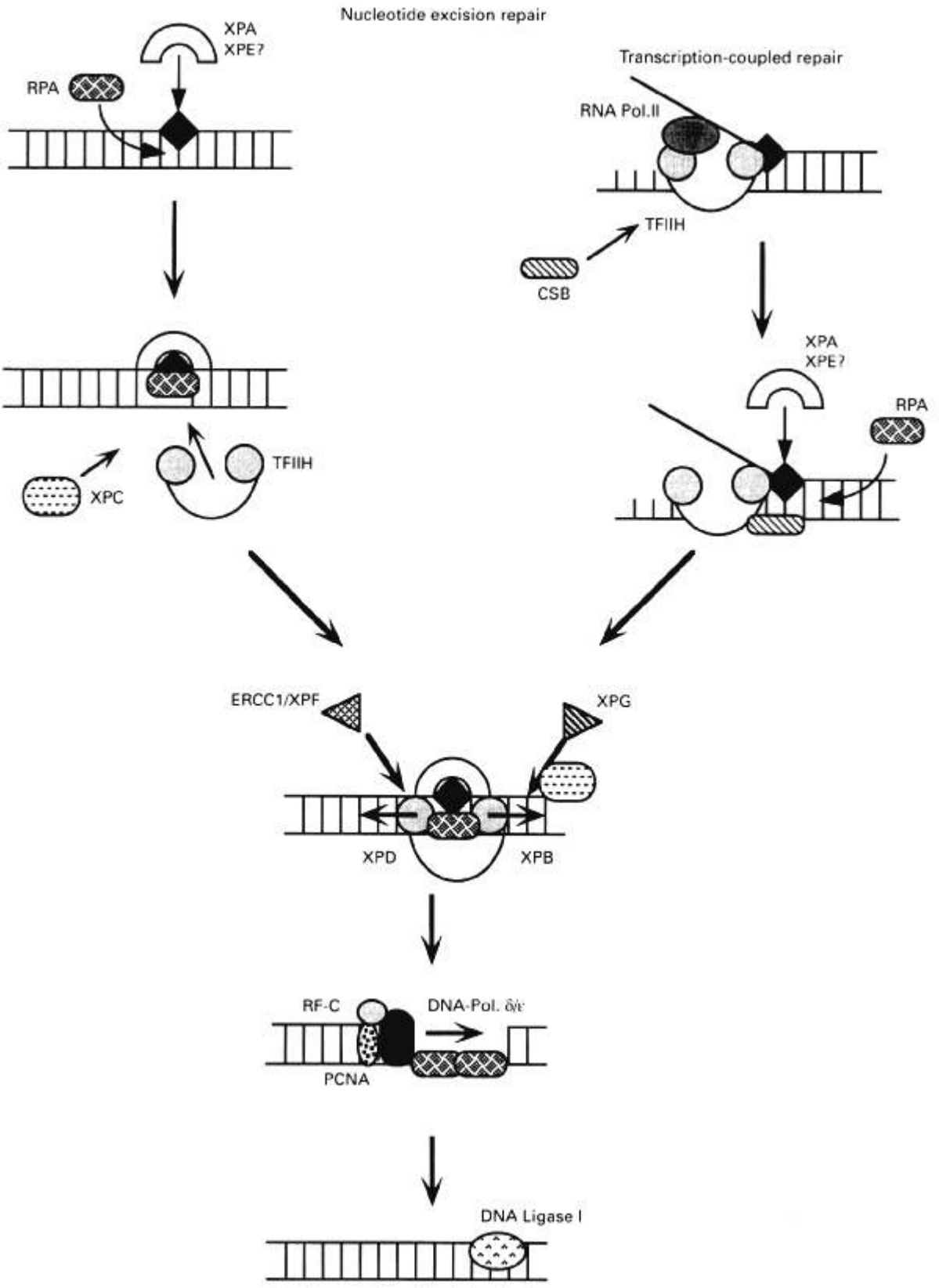

Figure 3. Model for human NER.

cloning of six human NER genes. Another class of laboratory-induced mammalian UV-sensitive NER-deficient mutants has been obtained from cultured rodent cells.[107] These have been designated as excision repair cross-complementing genes (ERCC). Each of these genes and their corresponding proteins have been the subject of numerous studies aimed at describing NER at the molecular level.[105,108] Thus, the enormous amount of research by many groups during the past 10 years has led to a comprehensive description of the NER pathway, which is summarized as follows (Figure 3): the NER mechanism is initiated by XPA which binds to the damaged site. XPE was also proposed as a protein involved in the recognition step.[109] However, several studies have shown that XPE is not essential for this step.[105] The binding of XPA facilitates entry of replication protein A (RPA) and forms a complex with it. This complex then recruits TFIIH and ERCC1/XPF complexes. The helicase activity of XPB and XPD contained in TFIIH locally unwinds DNA. The structural modification(s) of DNA induced by this unwinding facilitates the 5′ incision made by ERCC1/XPF. Similarly, XPA/TFIIH recruits XPG which makes the 3′ incision. Then, the repair synthesis is carried out by pol-δ and/or -ε which requires PCNA and replication factor C (RFC) to form a DNA synthesis complex as in DNA replication. Finally, the repair patch is sealed by a ligase, most likely ligase I.

As shown in Figure 3, another pathway has been found preferentially to repair the transcribed strand of DNA. This pathway has also been reviewed recently.[8,110] This pathway is relatively similar to the basic repair mechanism. A modification is located at the recognition step, where probably a blockage of the transcription machinery leads to the recruitment of XPA. The second difference is the presence of CSB in the repair complex, instead of XPC.

The mechanism used by cells to repair interstrand cross-links (ICL) is not clearly understood. However, several studies have suggested that ERCC1 and XPF, probably formed by a complex containing ERCC4 and ERCC11, are probably involved in this mechanism.[111] As shown for classical NER, the ERCC1/XPF complex might interact also with XPA to facilitate the repair of ICL. The mechanism might involve cooperation between NER and recombination, certainly outside of the classical context of NER.[112]

The levels of NER and ICL repair have been shown to be increased in cells resistant to several DNA-damaging agents such as cisplatin, BCNU, cyclophosphamide, L-phenylalanine mustard and mechlorethamine.[4,113–116] The cell lines used in these investigations were of diverse origins, including lymphoid leukemia, non-small cell lung cancer, ovarian cancer, medulloblastoma and liver. These data imply that these mechanisms of resistance (NER and ICL repair) could be used by cells more frequently than initially supposed. However, most of the proteins involved in NER have often not been found to be overexpressed in cells resistant to DNA-damaging agents, as was the case with XPB and XPD.[115] Generally, ERCC1 or XPA are overexpressed in tumor cells resistant to cisplatin.[117,118] On the other hand, cells deficient in the ERCC1 or ERCC4 genes were found to be hypersensitive to cisplatin, cyclophosphamide, melphalan or mitomycin C.[119–121] Although again, in similar studies, it was also shown that cells deficient in ERCC2 (XPD) or ERCC3 (XPB) were not hypersensitive to drugs like cisplatin, suggesting a marginal role for these proteins in resistance.[119,121,122] These results are somewhat surprising since XPB and XPD are essential to the NER process. One explanation of these results could be that NER utilizing XPB and XPD, as described in Figure 3, may not participate in the repair of certain damage, resulting from the drugs tested, like ICL. Therefore, this particular damage could lead to an increased relative cytotoxicity. With the aim of clarifying our knowledge of this pathway it will be very interesting, although an enormous amount of work, to investigate at the same time the expression of all the proteins found to be involved in NER, with the aim of reaching a more constructive and definitive conclusion.

Identification of the several types of DNA damage recognized and repaired by NER has very readily led groups to propose research of inhibitors of this mechanism, even before a detailed molecular description of this pathway has been provided. Some investigators have studied drugs acting against NER indirectly, to point out the co-involvement of certain proteins. Thus, inhibitors of topoisomerases were tested *in vitro* or on cultured cells with the aim of establishing any involvement (or not) of topoisomerases in NER.[123,124] The conclusions of these studies remain contradictory, depending of the drug tested, the DNA damage and the techniques used. The influence of topoisomerases on NER could be indirect, via their effects on the overall topology of DNA[125] and the inhibition observed with certain inhibitors could be due to their general lack of specificity, especially for the intercalating drugs or minor-groove binders, rather than their topoisomerase inhibition *per se*.[124,126] Thus, certain compounds, like actinomycin D, doxorubicin or distamycin A, may inhibit several possible targets involved in NER such as the pol-δ or -ε, the endonucleases XPF or XPG, and, more particularly, the helicases XPB and XPD, since several helicases have already been found to be inhibited by such com-

pounds.[127,128] Inhibition of the helicase activity of XPD and/or XPB was also proposed recently to explain the synergy of activity observed with a combination of pyrazoloacridine and cisplatin.[129] Moreover, it was shown that the helicase activity of XPD might be necessary in DNA repair, but not essential for the transcription process.[106] Thus, it would be possible to inhibit DNA repair without having side effects on transcription. However, the question, asked above, of the possible involvement of these proteins in resistance to certain DNA-damaging agents, remains to be elucidated before any major interest in their ability to inhibit such proteins can be justified.

XPA and ERCC1/XPF should definitively be considered as interesting targets, since these proteins are often found to be overexpressed in tumor cells resistant to DNA-damaging agents.[117,118] Furthermore, it has been suggested that the incision step, which involves XPA and ERCC1/XPF, is the rate-limiting step of NER.[130] So, any inhibition of XPA or ERCC1/XPF should have the most efficient impact on NER. Another reason could also be that these proteins were found to be important, not only in classical NER, but also in ICL repair.[120] Thus, inhibitors of these proteins would have many applications, with the prospect of potentiating a larger spectrum of DNA-damaging agents. In spite of this, however, no inhibitors of either XPA or ERCC1/XPF have yet been described in the literature.

The fact that the final step of NER is DNA resynthesis has resulted in attempts to inhibit NER with antimetabolic drugs. Historically, this was the first step attempted at a molecular level and it was the basis of the first pharmacological approach with a real rationale.[131] Studies involving such drugs have indeed indicated that they can be used to potentiate the toxicity of certain DNA damaging agents, such as chlorambucil, cisplatin, etc. Aphidicolin was one of the first of these compounds with which such a potentiation activity was demonstrated on cultured cells,[132] *in vivo*,[133] using fresh tumor cells from patients[134,135] and which is now under clinical evaluation. Hydroxyurea, Ara-C, and, more recently, fludarabine and gemcitabine have generally shown similar potentiation activity.[134,136–140] Their potentiation effects on cultured cells have also already been confirmed, at least for certain of these compounds, by *in vivo* evaluations,[138] studies involving fresh tumor cells taken directly from patients[134,140] and susquently in clinical trials.[141] For example, recently, a combination of gemcitabine and cisplatin induced a high response rate in both stage IIIB and IV non-small cell lung cancer, with only modest side effects.[141] Such results are clearly promising and encouraging, and serve to highlight the potential pharmacological interest of such an approach. However, detailed mechanistic studies must follow to confirm any definite validity for this approach. It is widely appreciated that any combination of two cytotoxic drugs is relatively difficult to manage and to optimize since the inter-dependence of the effects of drugs have to be considered.[140] This fact together with the need to define the sequence of drug administration, their effects on cell cycle, their variable incorporation by the different polymerases, the sensitivities of tumor cells to them individually and their cellular NER activities are just some of the numerous phenomena which can be evoked in explaining of any variability of responses to such combinations.[136,140] Thus, potentiation of alkylating agents with non-cytotoxic drugs should be easier to manage. To select such novel types of compound, the technical progress made recently in screening, easily and rapidly, *in vitro* DNA repair inhibitors should be useful.[126,142]

DSB repair

A series of different non-exclusive models have been proposed to explain DSB repair in mammalian cells, by analogy with those described in bacteria and yeast.[143] Nevertheless, the DSB repair processes are probably the most complex since, irrespective of the organism studied, the recombination mechanisms used to repair DSB require at least 20 gene products.[8,144,145]

The better characterized component of DSB repair so far is DNA-PK.[146] DNA-PK is a nuclear protein serine/threonine kinase that has the property of being active only when bound to DNA ends. DNA-PK is a multiprotein complex including a large catalytic subunit (DNA-PKcs). The binding to DNA ends involves another subunit, named Ku, and is constituted by two polypeptides, Ku70 and Ku80. Recently, it has been suggested that Ku may play a role in physically orientating DNA for ligation by binding the ends of adjacent DNA molecules.[147] Ku has also been described as an ATP-dependent helicase that may be involved in the DSB repair processes.[148] Thus, the DNA-PKcs subunit should bind to Ku, inducing its activation observed *in vitro* by the phosphorylation of several proteins such as p53, RNA polymerase II, transcription factors or indeed by autophosphorylation of Ku70, Ku80 and DNA-PKcs subunits.[145,146] However, none of these phosphorylations has been demonstrated definitively *in vivo* and so their roles remain hypothetical.

Nevertheless, several studies have shown that DNA-PK is involved in DSB repair and is certainly a central

component of both illegitimate recombination for repairing DSB and V(D)J recombination.[149,150] Nevertheless, illegitimate recombination is known as the dominant mode of DSB repair in cells of multicellular eucaryotes.[151] This type of recombination is not yet precisely described and only hypothetical models have been proposed to explain the role of DNA-PK in this mechanism.[146,151] Recently, it was suggested that XRCC4, KAP-1 and ligase IV may collaborate with DNA-PK in this process, but mechanistic evidence remains to be provided.[151-153] The integrity of each subunit of DNA-PK is essential to DSB repair, since an inactivating mutation in the gene of DNA-PKcs or Ku resulted in a deficiency in DSB repair.[154] Unfortunately, the only published study of which we are aware, involving cell lines derived from biopsies obtained from patients with glioblastoma, failed to find a correlation between DNA-PK activity and tumor cell radiosensitivity.[155] However, this type of result is not too surprising since cellular sensitivity usually results from multifactorial phenomena and DNA-PK is not the only protein implicated in DSB repair. Moreover, this DSB repair mechanism might be associated with other mechanism(s), independent of DNA-PK,[145,149] like homologous recombination.[151]

On the basis of the relationship between DNA-PK inactivity and deficiency in DSB repair, it has been suggested recently that inhibition of DNA-PK could be useful in sensitizing cells to ionizing radiation.[156] The advantages of such inhibitors would not, however, be restricted to radiation, since such compounds should also potentiate the activity of cytotoxic compounds which generate DSB, like bleomycin, or those topoisomerase II inhibitors, like etoposide, which stabilize cleavable complexes. These perspectives are proposed since cells deficient in DSB repair and particularly in DNA-PK activity were found to be hypersensitive to several inhibitors of topoisomerase II which stabilize cleavable complexes: teniposide, amsacrine, ellipticine, mitoxantrone, doxorubicin and daunorubicin,[157] while displaying similar levels of topoisomerase II activities.[158,159] In contrast, transfection of cells deficient in Ku restored resistance to etoposide.[160] Thus, although induction of DNA-PK by these drugs has already been found, the fact that inhibitors of DNA-PK could increase the efficacy of such anticancer drugs remains a most interesting and potentially exploitable pharmacological possibility. The absence of several other proteins corresponding to the other groups of complementation of the X-ray sensitive cells has provided similar hypersensitivity to others drugs, suggesting that DNA-PK is not the only potential target. However, biochemical characterization of such proteins has not yet received the degree of interest already shown vis-à-vis DNA-PK.

To elaborate on the potential for an inhibitor of DNA-PK, one could envisage specific inhibitors of the ATPase or DNA-binding activities of Ku or its helicase activity as proposed for NER. This approach was investigated recently using chemotherapeutic agents but revealed marked specificity for Ku.[161] To date, only inhibition of the phosphorylation activity of DNA-PKcs has been investigated at a large scale. One such DNA-PK inhibitor could be the fungal metabolite wortmannin.[162] This compound, identified initially as an inhibitor of the myosin light chain kinase, was shown strongly to inhibit PI-3 kinase.[162] Therefore, whilst lipid phosphorylation activity has not been ascribed to DNA-PKcs, on the basis of its sequence this protein has been classified as a member of the PI-3 kinase superfamily.[163] Moreover, wortmannin has already been used to sensitize cells to radiation, suggesting that it might inhibit DNA-PK activity.[164] This inhibitory activity is specific for DSB repair and does not interact with SSB repair.[165] Nevertheless, the action of wortmannin was recently found to be non-specific for DNA-PK, since cells deficient in DNA-PK have also been partially sensitized to radiation by wortmannin.[166] Similar results have also been found with cells deficient in the product of the ATM gene, another PI-3 kinase probably involved in the response to DNA damage.[163,166] Thus, several PI-3 kinases could be implicated in cellular responses to DSBs.[164,166] The instability of wortmannin in serum-containing media was also demonstrated, which has restricted its use to very high concentrations.[164] Indeed, such concentrations could explain its non-specific activity reported in previous studies. Accordingly, synthesis of more stable and more specific derivatives of wortmannin should be useful in identifying new sensitizers of DSB inducers. Recently, 2-(4-morpholinyl)-8-phenyl-4H-1benzopyran-4-one (or LY294002), a chemically-unrelated PI-3 kinase inhibitor, has resulted in similar effects to those found with wortmannin,[166] i.e. inhibition of *in vitro* DNA-PK activity and sensitization of cells to radiation. Thus, this compound could well provide the lead for a new series of DNA-PK inhibitors. In 1995, results of screening for inhibitors of DNA-PK revealed that 3-cyano-5-(4-pyridyl)-6-hydrazonomethyl-2-pyridone, also named OK-1035, was a potent inhibitor *in vitro*.[167] Its chemical structure is completely different from that of wortmannin and its high order of selectivity for DNA-PK has been demonstrated, since it was found not to inhibit several other kinases: PKC, cdk2, MAP kinase, EGF-receptor, and creatinine kinase I and II.[167] Nevertheless, no results of sensitization by DNA-PK inhibition with this compound have yet been published. However, OK-1035 was used in fundamental research

to provide evidence of the consequences of an inhibition of DNA-PK, and particularly to determine whether any relationship(s) existed *in vivo* between DNA-PK and other proteins having an important role in the regulation of DNA repair and cell cycle events, like p53 and p21.[168] Thus, the first results of sensitization with OK-1035 are eagerly awaited in order to decide whether or not such compounds have definite pharmacological interest in this respect.

Mismatch repair (MMR)

The mismatched base is removed by a particular repair mechanism designed to recognize and elimininate mismatches from DNA.[169] The damage considered here is due to an abnormal base mispairing, while the bases constituting it are normal. The techniques used to study this process have been similar to those described for studying the other repair mechanisms.[170,171] Thus, although the human MMR system has not been reconstituted with purified protein, genetic studies with mutants and biochemical experiments using cell-free extracts have led to the following model being proposed (Figure 4). The mismatch is recognized, depending on its structure, by the hMutSα heterodimer, constituted by hMSH2 and GTBP proteins, or by the MutSβ heterodimer, constitued by hMSH2 and hMSH3.[40] This complex is then recognized by hMutLα, another heterodimer constituted by hMLH1 and hPMS2 proteins. Then, an exonucleolytic degradation of DNA is initiated from a nick to the mismatched base, probably by the FEN-1 nuclease stimulated by PCNA. The stretch of DNA containing the mismatch is removed, resynthesized, maybe by pol-δ,[172] and religated to complete repair. Such a tie between replication and MMR and the fact that this process concerns exclusively base mispairing implies that MMR is certainly the major defense against point mutations. To reinforce this idea, it was found that a large fraction of non-polyposis colon cancer cases and a subset of sporadic cancers are caused by a mutation of the MSH2, MLH1 or PMS2 genes.[171,173]

Actual research on MMR systems has generally been associated with describing in detail the mechanism(s) of the processes involved and the relationships between deficiency in MMR and tumorigenesis.[171,174] However, recently, the involvement of MMR in resistance to anticancer drugs has also been investigated and the first studies have demonstrated a relationship between MMR deficiency (in hMSH2 or hMLH1 function) and resistance to an antimetabolite, 6-thioguanine,[175,176] to two DNA cross-linking agents, cisplatin and carboplatin,[177,178] to a mono-alkylating agent[179] and to an intercalating drugs, doxorubicin.[175] Moreover, complementation of MMR-deficient cells with chromosomes 2 or 3 containing the hMSH2 or hML1 genes, respectively, reversed the resistant phenotype.[177] These results suggest that loss of MMR should contribute to anticancer drug resistance. The molecular mechanism of this tolerance of lesions on DNA remains unclear,[180] but an absence of a particular mismatch binding function could lead to an absence of signals inducing DNA repair and/or cell death and so to tolerance of such a mutagenic lesion.

In conclusion, if future investigations confirm the hypothesis of a relationship between deficiency in MMR and drug resistance, then MMR should not be considered as a pharmacological target since its inhibition would lead to a resistant phenotype, so achieving the exact opposite of the intended goal. Nevertheless, preliminary studies have also suggested that resistance due to loss of MMR was specific to only certain platinum analogs, i.e. to cisplatin and carboplatin, and not to others, such as oxaliplatinum, tetraplatin, transplatin, JM335 and JM216.[178] These studies on the involvement of MMR with anticancer drug resistance have only been initiated in the past few years and more detailed evaluation is required in order to determine which drug might best be used against MMR-proficient as opposed to MMR-deficient tumor cells. Moreover, MMR activity or inactivity must be considered in addition to the other DNA repair processes. Thus, certain results concerning the incidence of DNA repair in drug resistance, actually defined as 'illogical', might possibly be explained if consideration were given at the same time to the activities of several DNA repair processes, rather than considering each of these activities independently. For example, Liu *et al.*[181] recently showed that deficiency in MMR could override AGAT in conferring resistance to temozolomide, since cancer cells deficient in MMR, yet containing AGAT, were found to be insensitive to O^6-benzylguanine. In addition, this phenomenon should be specific for certain DNA damage, since it has not been observed in similar experiments with BCNU.[181] Thus, determination of the DNA damage recognition (or not) by the MMR system should be another factor to consider in selecting the 'ideal DNA-damaging agents' for future use.

Discussion

It is apparent that numerous questions still remain to be answered before DNA repair mechanisms can be *completely* understood. However, some pharmacological perspectives have already emerged. Our knowl-

edge relative to MMR appears to exclude this process from the list of promising targets, but this could be reversed by future discoveries. On the other hand, several potential targets are now clearly defined involving the other four known mechanisms. The 'lead' target for a pharmacological approach is AGAT, with results of phase I studies in the USA of combinations with BCNU being eagerly awaited. Any

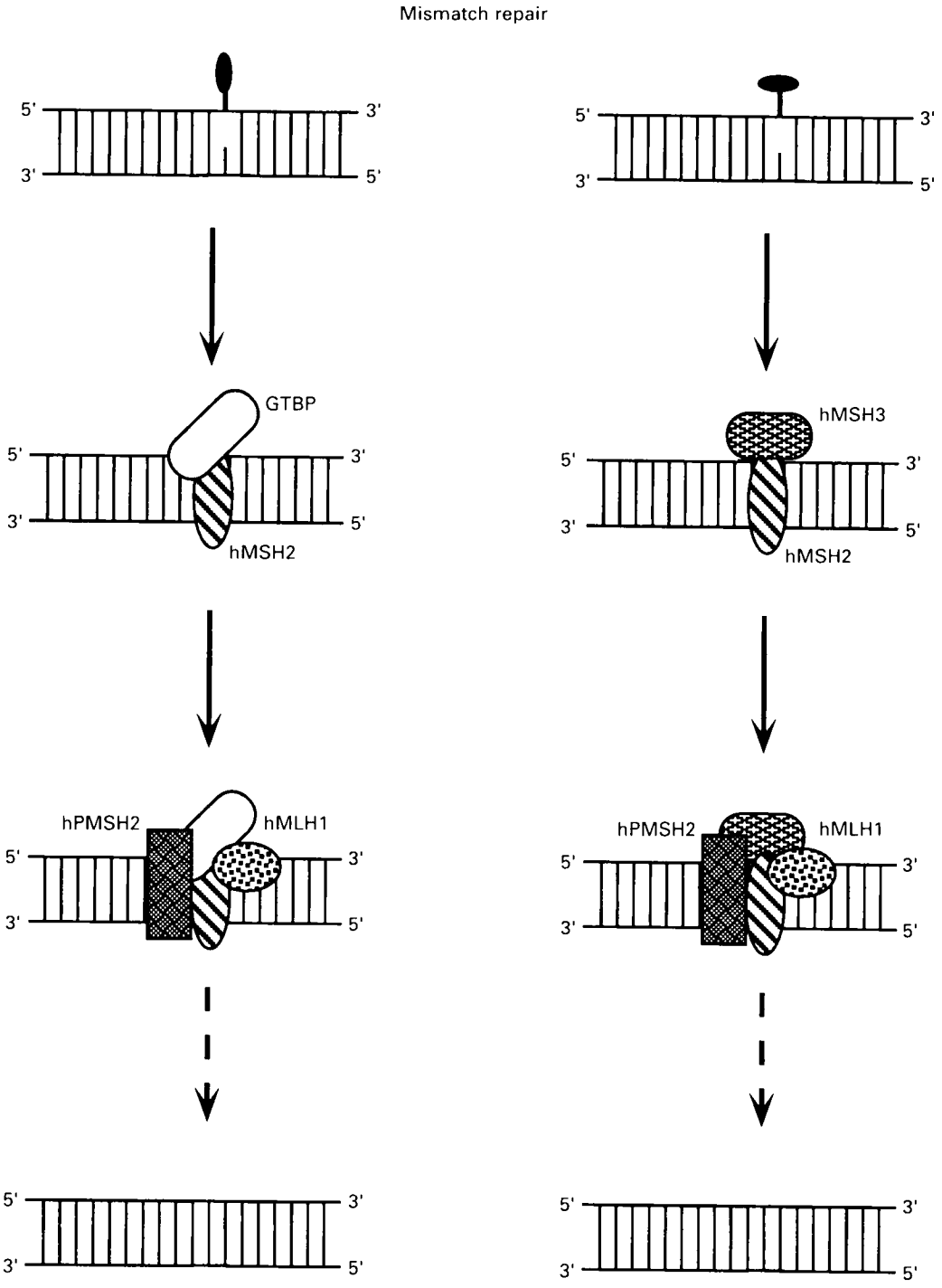

Figure 4. Model for human DNA MMR. Only the early steps are presented since the proteins involved in the other steps remain to be confirmed.

success of such a combination should definitely have an impact on the overall research interest in DNA repair inhibition. In parallel, some encouraging results have already been obtained from studies with cultured cells involving compounds proposed as inhibitors of PARP or DNA-PK, suggesting that approaches exploiting other mechanisms might soon emerge. Moreover, an increased knowledge of the BER and NER systems has already given rise to proposals for possible means of inhibiting these processes by specific inhibition of certain polymerases, helicases or crucial enzymes such as APE, XPA or ERCC1. Nevertheless, future confirmatory results are required and eagerly anticipated in order to confirm the true utility of such approaches.

In considering eventual connections between the different mechanisms of DNA repair mentioned above, it is important to point out certain other aspects of DNA repair. For clarity in this review, we have chosen to present each mechanism separately, and a too rigid interpretation of these individual schemes might serve to suggest a lack of any interaction between them. This is almost certainly not the case since several experimentally derived cell lines containing one deficient protein involved in repair of X-ray-induced DNA damage have also been found to be sensitive to mono- and bis-alkylating agents, as well as to UV irradiation, suggesting that this one protein is probably necessary for repairing all these different types of DNA damage. The XRCC1 protein is a good example since it was supposed originally to be involved in DSB repair and yet now various elegant molecular studies have suggested that this protein interacts with the BER system.[41,49] Another example is the presumed importance of DNA-PK in the regulation of several DNA repair mechanisms, since cells deficient in DNA-PK activity were found also to be hypersensitive to alkylating agents or to UV irradiation.[145] Recently, it has been shown that Ku, critical for DSB repair, may interact physically with NER processes.[182] Under these conditions, certain potential applications of sensitizers should be more extensive than originally envisaged. Therefore, these findings serve to reinforce the potential interest of such compounds, since most DNA-damaging agents used as anticancer drugs induce *per se* different types of DNA damage, repaired by different mechanisms.[5] For example, genetic and biochemical evidence from bacterial systems has implied that recombination systems could be involved, in association with NER and/or BER, in the repair of ICL formed by bis-alkylating agents, while intrastrand cross-links could be repaired essentially by the NER system.[7] As an extension of this, if future studies reveal any real importance in simultaneously blocking several of the DNA repair mechanisms, then possible targets to be considered could include the proteins involved in the DNA damage response, regulating the different DNA repair processes as suggested, e.g. for p53, GADD45 or the product of the AT gene.[156]

The complexity of the approach aimed at interfering with multiple pathways could of course be even further increased if we accept that other repair mechanisms almost certainly exist and remain to be fully explored. For some of these, like post-replication repair, the main reason for our limited knowledge is that the enzymatic mechanism has not yet been fully characterized and this is required to permit a clear definition of the molecular target involved. Recently, several polymerases, like pol-β or -ζ have been shown to be capable of translesion synthesis,[51] suggesting that this characteristic of these enzymes might be one of a number of explanations of the postreplication repair phenomenon.[183] In this case, the possible inhibition of such polymerases, already suggested as forming the basis for interfering with BER or NER, might be considered. In addition, this example serves to reinforce both the idea of interconnections between different DNA repair processes and of that of DNA repair inhibitors which might be far more potent that was initially supposed. Thus, the complexity of DNA repair, frequently cited as a disadvantage in terms of its successful pharmacological manipulation, could turn into an advantage.

Finally, it is essential to point out that DNA repair mechanisms are used initially by cells to protect themselves against mutagens and carcinogens.[8] According to these considerations, the pessimistic conclusion could be that DNA repair inhibitors would essentially increase the mutagenicity and carcinogenicity associated with DNA-damaging activity. However, at this point, it appears to be far more realistic to conclude that the true potential of DNA repair inhibitors in anticancer chemotherapy remains to be defined. We cannot ignore the possibility that any clearly demonstrated increased efficacy of anticancer drug therapy, arising from the addition of such sensitizers, could permit modification of existing protocols. This modifications, involving perhaps an overall reduction of the cumulative dose(s) of cytotoxic drugs administered, could lead to a real clinical benefit in terms of drug response and reduction of associated side effects. The case of cisplatin is a good example since its anticancer activity is essentially due to its binding to DNA, whilst its nephrotoxicity might result from another biochemical effect.[184] Thus, DNA repair inhibitors may potentiate the antitumor activity of cisplatin without any increase of its main toxicity. Meanwhile, the one sure thing is that due consideration of the possibility of pharmacological exploitation

of DNA repair has now been placed on a firm footing. Furthermore, this has provided the impetus to ensure that there is further elucidation of the fundamental processes involved. Thus, irrespective of the therapeutic responses that might be obtained in the future, the challenge raised by these pharmacological studies will certainly serve to augment our knowledge of these intriguing fundamental and potentially exploitable mechanisms.

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