Alteration in the choice of DNA repair pathway with increasing sequence selective DNA alkylation in the minor groove

Natalie Brooks [1], Peter J McHugh [1], Moses Lee [2] and John A Hartley [1]

Background: Many conventional DNA alkylating anticancer drugs form adducts in the major groove of DNA. These are known to be chiefly repaired by both nucleotide (NER) and base (BER) excision repair in eukaryotic cells. Much less is known about the repair pathways acting on sequence specific minor groove purine adducts, which result from a promising new class of anti-tumour agents.

Results: Benzoic acid mustards (BAMs) tethering 1–3 pyrrole units (compounds **1**, **2** and **3**) show increasing DNA sequence selectivity for alkylation from BAM and **1**, alkylating primarily at guanine-N7 in the major groove, to **3** which is selective for alkylation in the minor groove at purine-N3 in the sequence 5′-TTTTGPu (Pu = guanine or adenine). This increasing sequence selectivity is reflected in increased toxicity in human cells. In the yeast *Saccharomyces cerevisiae*, the repair of untargeted DNA adducts produced by BAM, **1** and **2** depends upon both the NER and BER pathways. In contrast, the repair of the sequence specific minor groove adducts of **3** does not involve known BER or NER activities. In addition, neither recombination nor mismatch repair are involved. Two disruptants from the *RAD6* mutagenesis defective epistasis group (*rad6* and *rad18*), however, showed increased sensitivity to **3**. In particular, the *rad18* mutant was over three orders of magnitude more sensitive to **3** compared to its isogenic parent, and **3** was highly mutagenic in the absence of *RAD18*. Elimination of the sequence specific DNA adducts formed by **3** was observed in the wild type strain, but these lesions persisted in the *rad18* mutant.

Conclusions: We have demonstrated that the repair of DNA adducts produced by the highly sequence specific minor groove alkylating agent **3** involves an error free adduct elimination pathway dependent on the Rad18 protein. This represents the first systematic analysis of the cellular pathways which modulate sensitivity to this new class of DNA sequence specific drugs, and indicates that the enhanced cytotoxicity of certain sequence specific minor groove adducts in DNA is the result of evasion of the common excision repair pathways.

[1]CRC Drug-DNA Interactions Research Group, Department of Oncology, Royal Free and University College Medical School, University College London, 91 Riding House Street, London W1P 8BT, UK
[2]Department of Chemistry, Furman University, Greenville, SC 29613, USA

Correspondence: John A Hartley
E-mail: john.hartley@ucl.ac.uk

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Introduction

An important class of anticancer agents that bind and alkylate in the minor groove of DNA are currently under investigation. These include analogues of naturally occurring compounds such as CC-1065 [1], calicheamycin [2], the pyrrolo[2,1-*c*][1,4]benzodiazepines [3] and distamycin [4] (Figure 1). We have previously evaluated a series of benzoic acid mustard (BAM)-tethered oligopyrrole analogues of distamycin for their cytotoxicity, cross-linking ability and sequence specificity [5,6]. They consist of one (**1**), two (**2**) and three (**3**) pyrrole-amide units tethered to BAM (Figure 1). These analogues were shown to be more cytotoxic than BAM itself, with cytotoxicity increasing as the number of pyrrole units increased. Compound **1** produced guanine-N7 alkylations in the major groove of DNA comparable to the pattern shown by BAM itself, as well as alkylations at some adenine-N3 sites in the minor groove. However, **2** and **3** did not produce detectable guanine-N7 alkylations but alkylated solely within AT tracts. Furthermore, **3** preferentially alkylated at only two sequences, 5′-TTTTGG and 5′-TTTTGA (confirmed as guanine-N3 and adenine-N3 lesions at the underlined bases, respectively), in preference to other sites, including 5′-TTTTAA. Hence, the most cytotoxic drug of the series possessed the most enhanced sequence specificity for alkylation [6]. Tallimustine, a compound of very similar structure to **3** and an identical pattern of sequence specific alkylation, has recently shown activity as an anticancer agent in both phase I and II clinical trials [7,8].

Organisms possess numerous and complex mechanisms that function in maintaining the integrity of their genetic material. These pathways are involved in the removal and tolerance of DNA damage caused by both endogenous and exogenous sources, including the damage produced by a variety of clinically used anticancer drugs. The budding

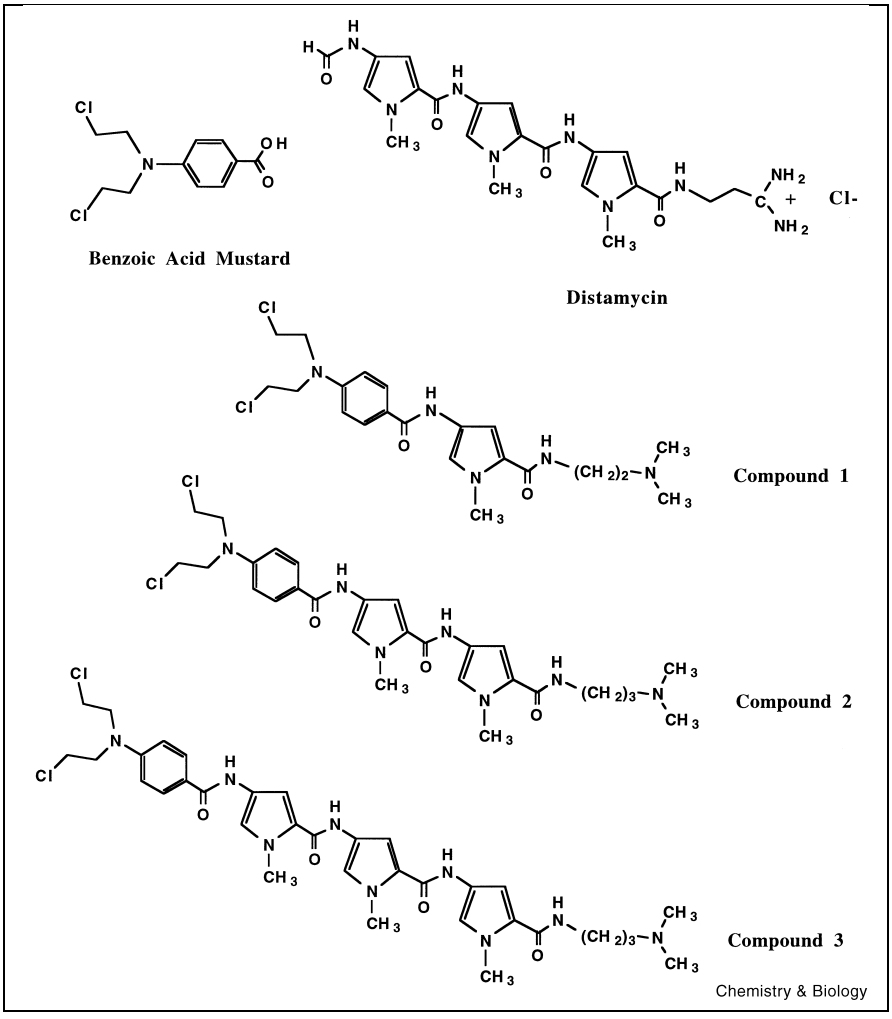

Figure 1. Structures of distamycin and oligopyrrole analogues of distamycin tethered to BAM (**1**, **2** and **3**).

yeast, *Saccharomyces cerevisiae*, has been shown to be a robust model system for repair in human cells [9,10].

Two major systems of excision repair, base (BER) and nucleotide (NER), have been identified [10]. BER facilitates the repair of damaged bases through a sequence of enzyme reactions, the first of which involves cleavage of the *N*-glycosyl bond of the damaged base by specific DNA glycosylases [11]. The apurinic/apyrimidinic site created is excised by a separate apurinic endonuclease or by an apurinic lyase function inherent to the glycosylase and the gap filled. NER involves the ATP-dependent excision of bulky DNA damage, for example, UV pyrimidine dimers and cisplatin-induced intrastrand cross-links, in the form of a lesion-containing oligonucleotide by a multi-enzyme complex [12]. The yeast *RAD3* epistasis group includes *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, *RAD14*, *RAD25*, *RAD7*, *RAD16* and *RAD23* genes. The first seven genes are essential for NER, and loss of function mutants are totally defective in the incision step of NER.

Double-strand breaks (DSBs) and single-strand gaps play a critical role in the initiation of homologous recombination [13] and DSBs may also be repaired by non-homologous end-joining (NHEJ; [14]) in both yeast and mammalian cells. Homologous recombination at DSBs and gaps absolutely requires the activity of the Rad52 protein [13,15]. In yeast, the Ku proteins (Yku70 and Yku80) direct DSBs into NHEJ which is independent of terminal DNA sequence homology [16].

In addition to an important role in maintaining genomic stability, the mismatch repair (MMR) system is known to influence the capacity of cells to repair DNA–anticancer drug adducts. Mutations in the MMR genes *MLH1*, *MLH2*, *MSH2*, *MSH3* and *MSH6* have been shown to result in resistance to cisplatin, carboplatin and doxorubicin [17,18], and a mutation in the yeast *MSH5* gene resistance to the DNA methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine [19].

Finally, the *RAD6* DNA repair epistasis group is the most complex, because it represents several repair sub-pathways: an error free tolerance pathway and a mutagenic pathway [20], as well as proteins involved in cell cycle checkpoints [21]. Previous studies have shown that mutations in the *RAD6* and *RAD18* genes of this group have prominent effects on sensitivity to a diverse variety of DNA damaging agents, such as UV, γ-rays and alkylating agents [9]. It has been demonstrated that whereas Rad6 has no affinity for DNA, Rad18 binds single-stranded DNA. Therefore, it has been suggested that association of Rad6 with Rad18 provides a means of targeting Rad6 to DNA regions containing damage, at which point the ubiquitination activity of Rad6 modulates downstream DNA repair and tolerance mechanisms [22].

Our previous studies have shown that the repair of conventional nitrogen mustard-induced *N*-alkyl purines in the major groove of DNA (at guanine-N7) involves BER and NER in *S. cerevisiae* [23]. However, very little is known about the repair mechanisms for complex agents that bind in the DNA minor groove. In order to gain a better understanding of the repair of damage produced by minor groove alkylating agents, we have investigated the role of BER, NER, MMR, recombination, NHEJ and mutagenesis prone activities in the repair of DNA adducts formed by **1**, **2** and **3** in the model eukaryote *S. cerevisiae* and demonstrated a pivotal role for the *RAD18* product in the repair of targeted minor groove adducts.

Results

NER, BER and recombination mutants are hypersensitive to compounds 1 and 2, but not 3

We employed *S. cerevisiae* strains totally deficient in specific DNA repair pathways (Table 1) in order to delineate the mechanisms involved in the elimination of DNA adducts produced by compounds **1**, **2** and **3** (Figure 1). Survival studies were used as a screen to determine the involvement of specific pathways in the elimination of DNA adducts. Survival curves were constructed and LD_{50} values (the dose of compound required to reduce survival by 50%) were calculated (Figure 2, Table 2). Reduction in survival by 50% was not observed with any of the compounds in the parental repair proficient strains, DBY747, W303-1A and W303-1B, at the highest dose employed (1 mM). The BER mutant, *mag1*, defective in 3-methyladenine DNA glycosylase activity, only showed increased sensitivity to **1**, whereas the *ogg1* strain, defective in formimidopyrimidine DNA glycosylase (Fapy DNA glycosylase) and 8-oxoguanine glycosylase, possessed increased sensitivity to both **1** and **2**. None of the BER mutants showed any sensitivity to **3** at concentrations up to 1 mM.

The *rad4* (NER) mutant showed comparable sensitivity to **1** and **2**, however, another NER disruptant *rad14* was only sensitive to **2** at concentrations up to 1 mM. The *rad4 mag1* double mutant, deficient in NER and 3-methyladenine DNA glycosylase activity, possessed comparable sensitivity to the *mag1* single mutant for **1**. A synergistic effect on sensitivity in this double mutant was however observed for **2** compared to the single mutants alone. Again, no sensitivity to **3** was shown in any BER or NER repair deficient single or double mutants up to 1 mM.

Since none of the BER, NER or excision repair double mutants was found to be sensitive to **3** at the highest concentration employed, we explored possible roles for other mechanisms of DNA repair and damage tolerance such as recombination, NHEJ, MMR and post-replicative repair. The homologous recombination mutant, *rad52*, showed

Table 1
***S. cerevisiae* strains used in this study.**

Strain	Genotype	Source	Reference
DBY747	*MATα his3-Δ1 leu2-3, 112 trp1-289 ura3-52*	W. Xiao	[26]
WXY 9394	DBY747 with *rad4Δ::hisG-URA3-hisG*	W. Xiao	[26]
LP14Δ	DBY747 with *rad14Δ::URA3*	R. Waters	[37]
WXY 9345	DBY747 with *rad4Δ::hisG-URA3-hisG* and *mag1Δ::hisG*	W. Xiao	[26]
JC 8901	DBY747 with *mag1::hisG-URA3-hisG*	L. Samson	[36]
WXY 9387	DBY747 with *rad52Δ::LEU2*	W. Xiao	[26]
WXY 9382	DBY747 with *rev3Δ::LEU2*	W. Xiao	[26]
WXY 9326	DBY747 with *rad18Δ::TRP1*	W. Xiao	[26]
WXY 9376	DBY747 with *rad6Δ::LEU2*	W. Xiao	[26]
rev3 rad18	DBY747 with *rev3Δ::LEU2 rad18Δ::TRP1*	W. Xiao	–
W303-1A	*MATα ade2-1 his3-11 leu2-15 trp1-1 ura3-3 can1-100*	S. Boiteux	[38]
W303-1B	*MATα ade2-1 his3-11 leu2-15 trp1-1 ura3-3 can1-100*	S. Boiteux	[38]
CD132	W303-1A with *ogg1Δ::TRP1*	S. Boiteux	[38]
yku70	W303-1B with *yku70::URA3*	S. Jackson	–
JDY 2	W303-1B with *yku70::leu2* and *rad52::TRP1*	S. Jackson	–
WT-B	*MATα trp1-h3 his4-cla leu2-r met13-4 lys2-d ade1 ura3 cyhr canS*	R. Brown	[17]
RBT 311	WT-B with *pms1::LEU2*	R. Brown	[17]
RBT 289	WT-B with *msh2::LEU2*	R. Brown	[17]

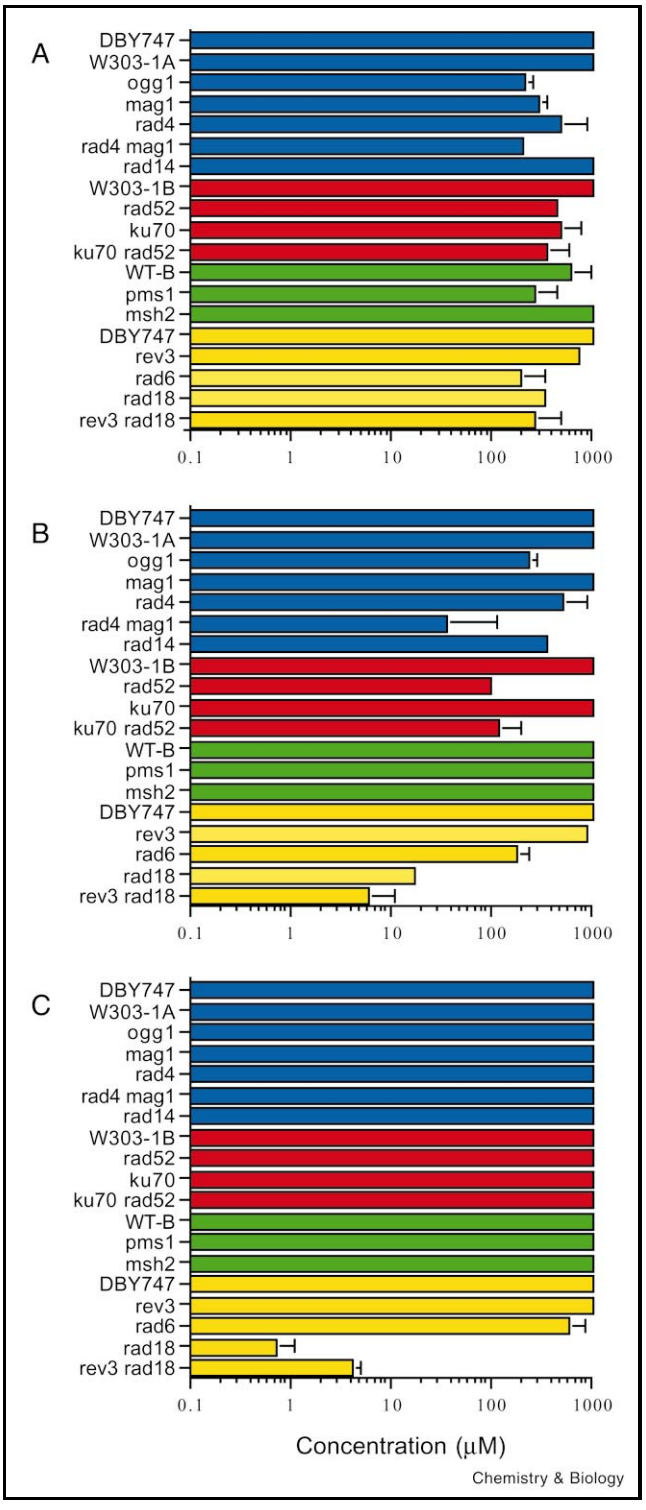

Figure 2. Sensitivity of BER, NER, recombination, MMR, post-replication repair and mutagenesis mutants to **(A)** compound **1**, **(B)** compound **2** and **(C)** compound **3**. Bars are colour-coded: blue represents NER and BER mutants and their repair proficient parental strains (DBY747, and W303-1A for *ogg1*), red recombination and NHEJ mutants (parent W303-1B), green mismatch mutants (parent WT-B) and yellow members of the *RAD6* epistasis group (parent DBY747). Results are the mean LD_{50} of at least three independent experiments, error bars show standard error of the mean.
←

some increased sensitivity to **1** and greater sensitivity to **2**, whereas the mutant defective in NHEJ, *yku70*, only showed increased sensitivity to **1** at up to 1 mM. A double mutant, *yku70 rad52*, was sensitive to **1** and **2**, but the level of sensitivity of the double mutant was comparable to the *rad52* mutant alone for both **1** and **2**. No increased sensitivity was observed to **3** in any of the recombination and NHEJ deficient mutants at the highest concentration used.

The parent (WT-B) and *pms1* MMR mutants were slightly sensitive to **1**, however, decreased sensitivity to **1** was seen in the *msh2* repair mutant. These MMR mutants showed sensitivity equivalent to the parent for **2** and **3**.

The *RAD6* and *RAD18* genes are important determinants of cellular sensitivity to 3

Of the *RAD6* epistasis group, involved in post-replication repair and mutagenesis, a *rev3* strain, deficient in a subunit of the mutagenic DNA polymerase ζ, possessed a small increased sensitivity to **1** and **2**, but not to **3** at up to 1 mM. The *rad6* mutant was equally sensitive to **1** and **2** and was one of only two tested mutant strains that showed any increased sensitivity to **3**, although the level of sensitivity was less than that observed for **1** and **2**. In contrast,

Table 2
Sensitivity of parental strains and repair mutants to 1, 2 and 3.

Strain	LD_{50} (μM)		
	1	**2**	**3**
DBY747	>1000	>1000	>1000
W303-1A	>1000	>1000	>1000
ogg1	211±55	237±54	>1000
mag1	287±71	>1000	>1000
rad4	492±444	503±432	>1000
rad4 mag1	203±82	35±2.3	>1000
rad14	>1000	350±44	>1000
W303-1B	>1000	>1000	>1000
rad52	440±14.1	97±17	>1000
yku70	491±313	>1000	>1000
yku70 rad52	348±266	116±84	>1000
WT-B	616±380	>1000	>1000
pms1	270±200	>1000	>1000
msh2	>1000	>1000	>1000
rev3	717±25	900±173	>1000
rad6	189±155	181±60	600±300
rad18	335±23	17±1	0.7±0.4
rev3 rad18	263±240	6±5	4±1.2

Results are the mean of at least three experiments. Errors are the standard error of the mean.

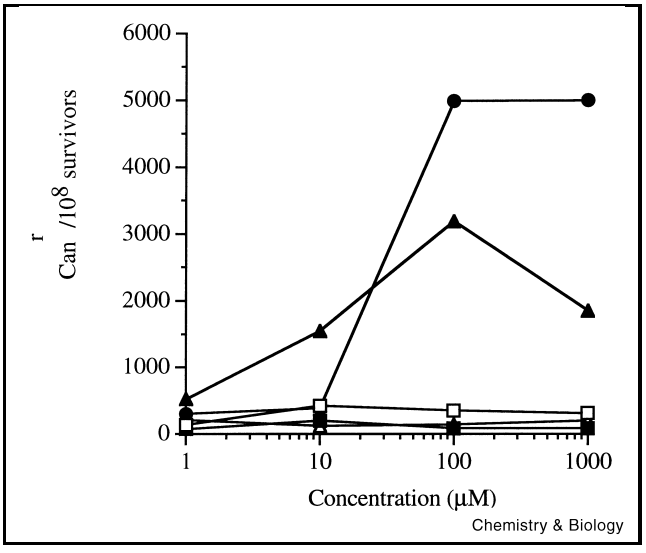

Figure 3. Compound **3**-induced forward mutation frequency in the *ARG1* gene. Parental (DBY747), *rev3*, *rad18* and *rev3 rad18* mutants were treated with stated doses of compound **3** for 5 h at 28°C. Aliquots containing 2×10^6 cells were plated out onto SC-arg plates supplemented with L-canavanine and scored after 5–6 days growth at 28°C. Values calculated by scoring the fraction of canavanine resistant colonies arising per 10^8 survivors where survival was simultaneously measured. Open squares, DBY747; closed square, *rev3* mutant; closed circle, *rad18* mutant; open triangle, *rev3 rad18*; and closed triangle, DBY747 treated with HN2. Results are the mean of two independent experiments.

the *rad18* mutant was extremely sensitive to compound **3** by over three orders of magnitude. This strain was also the most sensitive to **2** of any of the strains tested but at least 20-fold less than for **3**. Only a moderate increased sensitivity was observed for **1** in the *rad18* strain, which was of a level seen with several other mutants. The order of sensitivity in the *rad18* mutant to **1**, **2** and **3** was the same as that observed previously in human tumour cells [5]. The *rev3 rad18* double mutant demonstrated equivalent sensitivity to the *rad18* single mutant for **1**, **2** and **3**.

Compound 3 is mutagenic in a *rad18* mutant

The mutagenic potential of **3** was assessed in the *rad18*, *rev3*, *rev3 rad18* and isogenic parent (DBY747) strains using the L-canavanine resistance forward mutation assay. The conventional non-targeted nitrogen mustard mechlorethamine (HN2) was also included in this experiment as a positive control since it is an established mutagen in this test [23]. These data show (Figure 3) that **3** was not mutagenic in the repair proficient, parental yeast strain DBY747 or, as expected, a *rev3* strain. However, **3** was highly mutagenic in the *rad18* strain (Figure 3). This mutagenesis was eliminated in the *rev3 rad18* double mutant. The mutagenic potential of HN2 was confirmed in the DBY747 (Figure 3).

The DNA adducts formed by compound 3 are not eliminated in the *rad18* strain

Since compound **3** gave no significant cytotoxicity in the majority of the yeast strains tested, the possibility that this compound did not reach its cellular target had to be eliminated. The single-strand ligation polymerase chain reaction (sslig-PCR) method was used to follow the induction and repair of the DNA damage at the nucleotide level in cells, in order to clearly establish that **3** is able to enter the yeast cells, that it hits the same sequence specific DNA target in vivo and in vitro, and to investigate whether the Rad18-mediated pathway involves elimination or only tolerance of the DNA adducts. The repair capacity of the highly sensitive *rad18* mutant compared to its isogenic parent DBY747 was assessed using primers specific for the

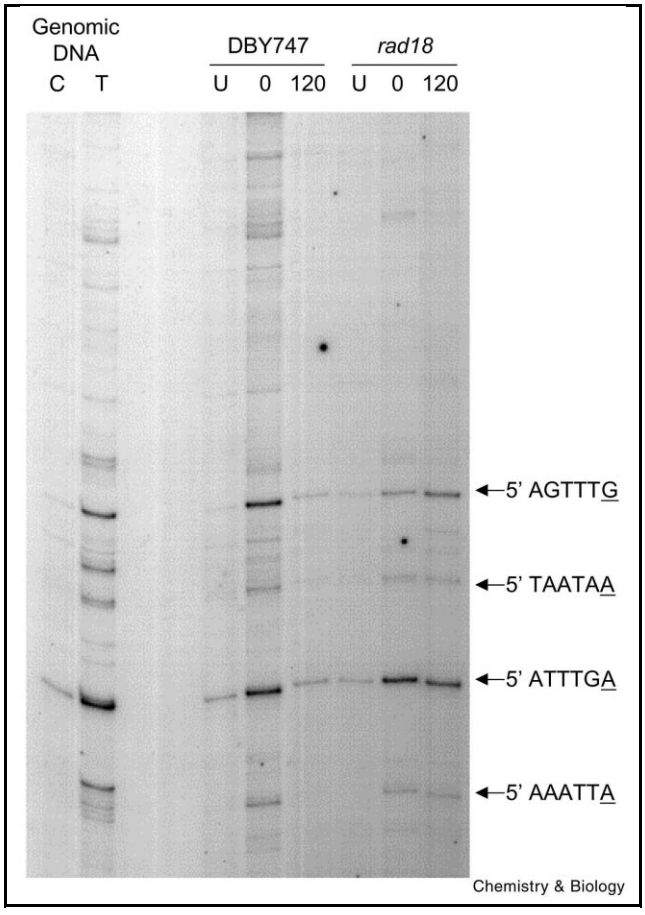

Figure 4. sslig-PCR of DNA extracted from a parental (DBY747) strain and a *rad18* disruptant. Lane C, control untreated genomic DNA; lane T, genomic DNA treated with **3** (10 μM) for 5 h at 28°C; lane U, DNA from control untreated DBY747 or *rad18* mutant cells; lanes represented by 0 min, DNA extracted from DBY747 or *rad18* mutant cells after 5 h treatment at 28°C with **3** (250 μM); lanes represented by 120 min, DBY747 or *rad18* cells resuspended in YEPD medium and DNA extracted following 120 min post-treatment incubation.

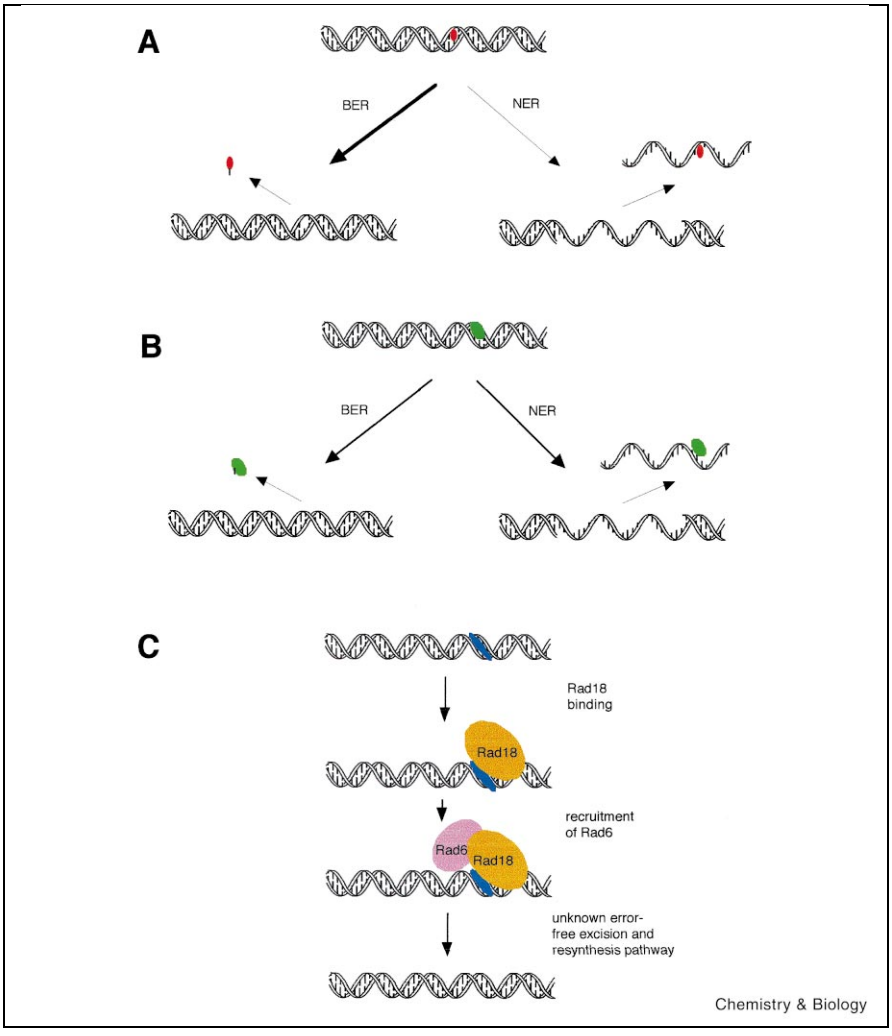

Figure 5. Model for the repair pathways acting on the DNA adducts produced by **1 (A)**, **2 (B)** and **3 (C)**. The adducts produced by **1** at purines in the major groove are repaired by both NER and BER, and the bold arrow indicates that BER dominates over NER. The adducts produced by **2** in the minor groove with limited sequence selectivity are good substrates for both BER and NER. In contrast, the adducts produced by **3** with strong sequence specificity at purine-N3 in the minor groove are not repaired by NER or known BER activities, but an error free process requiring Rad18 and involving Rad6 is instead employed. Note that the damaged DNA may have to be single-stranded prior to Rad18 binding.

transcribed strand of the *MFA2* gene (Figure 4). Lanes C and T represent untreated purified genomic DNA and the same DNA treated in vitro with **3** (10 μM) for 5 h, respectively. There are several prominent sites of DNA adduct formation along the length of the *MFA2* gene, shown by the arrows, corresponding to adducts at 5′-TAATAA, 5′-AAATTA with two major sites at 5′-AGTTTG and 5′-ATTTGA (Figure 4). Note that the DNA sequence analysed does not contain the most preferred 5′-TTTTGG or 5′-TTTTGA sequences [6]. In DBY747 and *rad18* cells treated with 250 μM **3** for 5 h (lanes 0, Figure 4), the DNA damage sites in both strains are comparable to those seen in genomic DNA treated with **3** (lane T, Figure 4). Following removal of drug and further incubation of cells in rich media for 120 min, near-complete removal of the damage is observed in the parental DBY747 strain. Indeed additional experiments (not shown) indicate that significant repair occurs within the first 30 min of post-treatment incubation in this strain. In contrast, in the *rad18* mutant (Figure 4) or the *rad18 rev3* strain (not shown), the damage is still clearly evident after 120 min post-treatment incubation. Since it is possible that the decrease in adducts observed is the result of cell growth in DBY747, rather than elimination of adducts, it was necessary to be certain that no cell division occurred during the drug treatment, or subsequent post-treatment incubation. No growth was observed in either DBY747 or the *rad18* strain during this period (data not shown), since it appears that following 5 h incubation in phosphate-buffered saline (PBS), even in the absence of drug, the cells are arrested for at least a further 120 min.

Discussion

We have determined the DNA repair mechanisms involved in the elimination of the DNA adducts produced by the oligopyrrole compounds **1**, **2** and **3**, and a model summarising our findings is presented in Figure 5 and discussed below.

The yeast strains deficient in BER, NER, MMR and recombination possessed no increased sensitivity over their isogenic parent to **3** at the highest dose used, even though the expected sequence specific minor groove adducts are readily formed in the DNA of yeast cells as demonstrated by sslig-PCR. This suggests that the cytotoxic DNA adducts formed by **3** are not processed by these repair pathways. In contrast, the increased sensitivity to **1** in the 3-methyladenine DNA glycosylase mutant, *mag1*, over wild type suggests that the DNA adducts formed by **1** are recognised and removed by this enzyme in vivo. These data are consistent with *Escherichia coli* BER (AlkA protein) data both in vitro and in vivo [24]. Several studies have recently revealed the structural basis of the activity of 3-methyladenine glycosylase proteins, which are enzymes with a extraordinarily broad substrate specificity for damaged bases [25].

Compound **1** was previously shown to alkylate guanine bases at the N7 position with a pattern comparable to that shown by BAM [6]. These adducts may also be removed by the Fapy DNA glycosylase enzyme since an increase in sensitivity is observed in the Fapy DNA glycosylase mutant, *ogg1*, over its isogenic parent W303-1A. Although **2** does not alkylate at guanine-N7, it does alkylate at adenine-N3 in the minor groove [6]. These observations are consistent with previous work that has found that some adenine-N3 and guanine-N3 adducts are substrates for the Fapy DNA glycosylase [11]. The present study suggests that 3-methyladenine and Fapy DNA glycosylases can act on adducts from **1**, which are primarily purine-N7, only Fapy DNA glycosylase can act on adducts from **2**, which are exclusively purine-N3, and neither glycosylases act on the highly sequence specific but bulkier minor groove purine-N3 adducts produced by **3**.

We have recently examined the excision repair of untargeted nitrogen mustard *N*-alkyl purine adducts in *S. cerevisiae* [23] in detail and have confirmed the involvement of both NER and BER (specifically the 3-methyladenine DNA glycosylase). In the present study, the sensitivity of the *rad4 mag1* strain was primarily the result of the Mag1 defect since the sensitivity of this double mutant was not significantly greater than that of the *mag1* single mutant, and the *rad4* single mutant was rather less sensitive. Further, the *ogg1* strain was even more sensitive than the *rad4 mag1* strain, indicating that Fapy glycosylase activity is also important in eliminating the adducts formed by **1**. Taken together, this indicates that BER is more important than NER for the elimination of these untargeted major groove adducts, which significantly differs from observations made with nitrogen mustard where NER clearly dominates over BER [23].

In contrast, a synergistic effect is observed in the *rad4 mag1* double mutant, with an increased sensitivity over the single mutants for **2** of over 29-fold (*mag1*) and 14-fold (*rad4*). Therefore, both BER and NER appear to be involved as overlapping repair pathways for the adducts formed by **2**. Along with bulky adduct removal, it is known that bases modified by monofunctional alkylating agents are substrates for NER [26,27]. These agents produce adducts which cause only minor helical distortions, for example, methyl methanesulfonate (MMS). The synergistic sensitivity seen in the double repair mutant, *rad4 mag1*, suggests that NER (*rad4*) and BER (*mag1*) pathways compete for the same lesions caused by **2**. In addition, we have ruled out any functional redundancy between the NER and Fapy glycosylase activities in the repair of adducts produced by **3** since the sensitivity of a *rad14 ogg1* double disruptant is the same as its isogenic parent W303-1A (results not shown).

Mutations in *RAD52* and *yku* genes result in sensitivity to agents that produce DSB and are only minimally sensitive to agents that generate other types of DNA lesions. The data obtained in this study suggest that adducts formed by **2** are more readily repaired by homologous recombination compared to compound **1** adducts. Furthermore, both homologous recombination and NHEJ may play a prominent role in the repair of adducts produced by **1** but not **2**. This is demonstrated by the comparable sensitivity observed for **2** in the double repair mutant, *yku70 rad52*, compared to *rad52* alone. Eliminating homologous recombination may result in accumulation of DSB and single-strand gaps, particularly in replicating cells during the repair of **1** and **2**.

MMR is not involved in the repair of adducts produced by **2** or **3**. However, the data indicate that inactivation of the MMR gene, *msh2* (but not the *pms1* gene), results in decreased sensitivity to **1**. Decreased sensitivity in the MMR mutants treated with the anticancer drugs cisplatin, carboplatin and doxorubicin was also observed previously by Durant et al. [17] and was of similar magnitude. The authors have postulated that MMR proteins, in addition to their role in mismatch recognition, decrease adduct tolerance during DNA replication by modulating levels of recombination-dependent bypass and NER [17]. Tolerance is, therefore, enhanced upon removal of MMR proteins resulting in decreased sensitivity to anticancer agents [18].

Sensitivity to **3**, at less than 1 mM, was only observed in the *RAD6* epistasis group involved in error free and error prone tolerance pathways. The *rad6* and *rad18* mutants are defective in both error free and error prone tolerance pathways and mutagenesis, the *rev3* mutant affects only the mutagenesis pathway, and the *mms2* mutant was recently shown to display defective error free post-replication repair parallel to the *rev3* mutagenesis pathway [20]. In particular the *rad18* mutant was highly sensitive to compound **3**. The function of the Rad18 protein has not yet been clearly defined, but significantly it appears to bind to single-

stranded DNA at runs of poly dT [28]. The sites of preferred alkylation for **3**, i.e. at 5′-TTTTGG and 5′-TTTTGA, contain poly dT stretches [6]. We can speculate that the Rad18 protein upon binding to the single-stranded DNA may provide access to the DNA adduct and perhaps recruit the Rad6 protein, thus allowing further proteins to repair or tolerate adducts formed by **3**. However, these results are novel since the literature indicates that isogenic *rad6* and *rad18* strains normally demonstrate very similar (and epistatic) hypersensitivity to DNA damaging agents [29]. Indeed the *rad6* and *rad18* strains used here have been shown to exhibit equivalent levels of hypersensitivity to cisplatin, nitrogen mustard and MMS (P.J.M., unpublished results, and [26]). In addition, since *rad6* strains are known to readily accumulate suppressor mutations in the *SRS2* gene [30] which might obscure the real drug sensitivity of the strain, care was taken to avoid the accumulation of these mutations and only slow-growing *rad6* cultures were used in experiments, and the strain was regularly independently shown to demonstrate the expected level of hypersensitivity to nitrogen mustard and cisplatin. While *rad6* null mutants display phenotypic defects separate from those involved in the DNA damage response, for example defects in telomere-associated gene silencing [31] and N-end rule protein degradation [32], we are not aware of any studies where a *rad18* strain demonstrates a much more severe repair phenotype than its isogenic *rad6* relative. This suggests that the Rad18 protein might be playing a role additional to the Rad6-mediated pathway outlined above; perhaps Rad18 recruits other repair factors to the site of the adducts produced by **3** in addition to Rad6. We would further postulate that human cells, which are much more sensitive to **3** than repair proficient yeast cells [6], are perhaps deficient either in an equivalent Rad18 activity or in the factors acting downstream from this, assuming that the level of DNA damage incurred is similar.

Previous studies have shown that mutagenesis by both mono- and bifunctional alkylating agents is responsible for the carcinogenic side effects of treatment with these agents [33]. Therefore, the mutation frequency of **3** was determined using the canavanine forward mutation assay. Measurements of forward mutation frequency (can^s to can^r) indicate that mutagenic events are substantially induced by **3** in *rad18* cells, but not in the parental strain DBY747 or the *rev3* strain. This demonstrates that error free Rad18-dependent processes are involved in the removal or tolerance of adducts produced by **3**. By performing sslig-PCR, we have established that adducts formed by **3** are excised from the DNA and not simply tolerated. Since excision of the adducts produced by **3** is absent in the *rad18* mutant, the adducts must be tolerated via an error prone pathway accounting for the high level of induced mutagenesis in this strain. This error prone pathway was clearly shown to be that controlled by Rev3 (polymerase ζ) since the strong mutagenic effect of **3** on the *rad18* strain was eliminated in the *rad18 rev3* double mutant.

Significance

There is currently considerable interest in the use of sequence specific DNA binding molecules as anti-tumour drugs. It appears that greater sequence specificity, combined with a preference for the minor groove, enhances the cytotoxicity of such agents. One possible explanation for this is that the DNA adducts produced evade those DNA excision repair pathways which efficiently act on the bulky drug adducts produced in the major groove by conventional nitrogen mustards and cisplatin. We have systematically compared the DNA repair pathways acting on a family of non-targeted major groove binding and targeted minor groove binding oligopyrrole analogues of distamycin tethering BAM. The yeast *S. cerevisiae* was chosen as the experimental model since strains with disruptions of all the known major eukaryotic DNA repair pathways are available. Screening a library of strains for drug sensitivity indicated that the non-targeted major groove binding adducts are repaired by a combination of NER and BER, as we have previously demonstrated for nitrogen mustard. In sharp contrast, the sequence specific minor groove adducts produced by a targeted molecule are not substrates for these pathways. Using PCR-based techniques to follow the induction and repair of the minor groove DNA adducts at nucleotide resolution in intact cells, we demonstrated that they are rapidly eliminated in repair competent cells. A *rad18* disruptant, however, is extraordinarily sensitive to this drug, and is unable to eliminate the adducts. We propose that the Rad18 protein is able to recognise the minor groove adducts, and then recruit further repair factors required to eliminate the adducts.

These observations support the idea that the enhanced cytotoxicity of some minor groove sequence specific DNA adducts is the result of evasion of the common excision repair pathways, and repair is instead dependent on Rad18 as a damage sensor prior to adduct elimination. Clearly an appreciation of the repair pathways which modulate the sensitivity of cells, both normal and tumour, to such drugs is central to their development as novel therapies. In addition, there is the exciting possibility of developing dual therapies consisting of agents which interfere with relevant repair pathways and potentiate the efficacy of specific DNA damaging drugs.

Materials and methods

Materials

The synthesis and characterisation of the BAM conjugates (Figure 1) has been reported previously [5]. Compounds **1**, **2** and **3** were prepared as 100 mM stocks in DMSO/1% HCl.

Yeast strains

The *S. cerevisiae* haploid strains employed, their genotypes and source are listed in Table 1.

Yeast survival curves

For *S. cerevisiae* cytotoxicity curves, a single colony picked from a YEPD plate from the appropriate yeast strain was grown overnight to exponential phase (12–16 h) in YEPD [34] medium at 28°C. The density of the yeast cultures was determined by counting with a haemocytometer. Cells were pelleted at 500×*g*, 10 min, 20°C and adjusted to a final density of 2×10^7 cells/ml in cold PBS. Cells were then treated with test agent for 5 h at 28°C in a orbital incubator. After drug treatment, 2×10^7 cells were washed twice in PBS solution and resuspended in a final volume of 1 ml. Subsequent dilutions of the stock cell suspension were made and plated onto YEPD/agar plates at a density of 200 colonies per plate. The number of yeast colonies per plate was scored after 3 days incubation at 28°C.

L-Canavanine resistance forward mutation assay

A single colony from the appropriate yeast strain was grown overnight (as described above) and resuspended at a final concentration of 2×10^7 cells/ml in PBS. Cells were then treated with test agent for 5 h at 28°C. After drug treatment, the cells were washed twice and 2×10^7 cells resuspended in a final volume of 1 ml in PBS. From this stock suspension of cells, 100 μl cells (2×10^6 cells) were plated onto CSM −arg+can plates (1.7% agar, 0.67% yeast nitrogen base without amino acids, 2% glucose; Sigma, UK, and 0.074% complete supplement mixture minus arginine (Bio 101, UK) supplemented with 60 μg/ml L-canavanine, pH 6.5). The number of yeast colonies per plate was scored after 5–6 days incubation at 28°C.

Oligonucleotide primers

For the *MFA2* gene transcribed strand: 2.5B 5′-biotin CAT TGA CAT CAC TAG-3′; 2.5C 5′-AGA CAC CAG CGA GCT ATC AT-3′; 2.5D 5′-AGC GAG CTA TCA TCT TCA TAC AA-3′. Ligation oligonucleotide: 5′-ATC GTA GAT CAT GCA TAG TCA TA-3′. Ligation primer: 5′-TAT GAC TAT GCA TGA TCT ACG AT-3′.

sslig-PCR [35]

A single colony from the appropriate yeast strain was grown overnight, as described above. Cells were counted, centrifuged and resuspended at a concentration of 3×10^7 cells/ml in PBS. Cells were treated with test agent for 5 h at 28°C. After drug treatment, cells were washed twice, 10 ml of cells was removed for time 0 min sample and the remaining 20 ml was resuspended in YEPD medium and incubated at 28°C for 60 and 120 min. After this post-treatment incubation, the cells were centrifuged at 500×*g* for 10 min at 20°C. Genomic DNA was purified using a Nucleon kit for yeast DNA extraction (Nucleon Biosciences, UK). The genomic DNA was then cut with 10 U of *Rsa*I overnight in 1×One-Phor-All buffer (Pharmacia, UK). The DNA was precipitated and the DNA concentration determined fluorometrically.

First round primer extension was carried out, in a volume of 40 μl, using 0.02 μg digested DNA. The PCR was carried out using 0.6 pmol of 5′-biotinylated primer B and the reaction mixture composed of 20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl pH 9.0, 0.01% (w/v) Tween 20, 4 mM $MgCl_2$, 0.2% gelatin, 250 μM each dNTP and 1 U *Taq* polymerase. The DNA was initially denatured at 94°C for 4 min and then subjected to 30 cycles of 94°C, 1 min, 51°C, 1 min, 72°C, 1 min+1 s extension/cycle on a PT-100 thermal cycler with a hot bonnet (MJ Research, USA). The mixture was subjected to a final extension at 72°C for 5 min and then cooled to 4°C. To capture and purify the products of biotinylated primer extension the PCR mixture, 10 μl of 5×washing and binding buffer (5 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 M NaCl) was added, followed by 5 μl washed streptavidin M-280 Dynabeads (Dynal, UK). The suspension was incubated for 30 min at 37°C with occasional agitation. The beads were sedimented in a magnetic rack and washed three times with 200 μl 10 mM TE (pH 7.6). The beads were resuspended in 10 μl ligation mixture containing 10×T4 RNA ligase buffer (including 1 mM hexammine cobalt III chloride), 50% PEG 8000, 20 pmol ligation oligonucleotide and 20 U T4 RNA ligase, and ligated overnight at 22°C. The ligation oligonucleotide was supplied with a 5′-phosphate, essential for ligation, and a 3′-terminal amine which blocks its self-ligation. After ligation, the beads were washed three times with 200 μl TE pH 7.6 and resuspended in 40 μl ddH_2O for the second round PCR. The second round PCR mixture, the volume of which was 100 μl, contained 10 pmol of each primer C and ligation primer that was complementary to the ligated oligonucleotide. The buffer composition was as for the first round PCR except that 2.5 U of *Taq* polymerase was used. The cycling conditions were: an initial denaturation at 94°C for 4 min, then 18 cycles of 94°C, 1 min, 58°C, 1 min, 72°C, 1 min+1 s extension/cycle. The mixture was finally incubated at 72°C for 5 min and cooled to 4°C for 1 h. The third round was carried out by adding 10 μl of ^{32}P 5′-end-labelled primer D (5 pmol) and 1 U *Taq* polymerase in PCR reaction buffer. The mixture was subjected to four further cycles of 94°C, 1 min, 61°C, 1 min and 72°C, 1 min, 72°C, 5 min and 4°C, 1 h. The beads were removed by spinning briefly and rinsed with 100 μl ddH_2O. The supernatant was removed, precipitated with ethanol. Samples were resuspended in 5 μl formamide loading buffer, denatured at 95°C for 3 min, cooled on ice and electrophoresed at 1500 V for 3 h in a 50 cm×21 cm×0.4 mm, 6% polyacrylamide sequencing gel (SequaGel 6; National Diagnostics, UK). The gel was dried and autoradiographed (Kodak Hyper film, Amersham. UK).

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