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MECHANISMS OF ACTION OF QUINONE-CONTAINING ALKYLATING AGENTS: DNA ALKYLATION BY AZIRIDINYLQUINONES

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1. ABSTRACT

Aziridinyl quinones can be activated by cellular reductases eg. DT-diaphorase and cytochrome P450 reductase to form highly reactive DNA alkylating agents. The mechanisms by which this activation and alkylation take place are many and varied. Using clinically relevant and experimental agents this review will describe many of these mechanisms. The agents discussed are Mitomycin C, EO9 and analogues, diaziridinylbenzoquinones and the pyrrolo[1,2-alpha]benzimidazolequinones.

2. INTRODUCTION

The most characteristic feature of quinones is their ease of reduction. Most naturally occurring quinones have this common ability and indeed, the quinones involved in photosynthesis and aerobic/anaerobic glycolysis rely on this redox ability in order to transfer electrons within the biochemical pathways. However, redox cycling is also utilised by toxic quinones in fungi, plants, bacteria and insects as part of elaborate defence and killing strategies.

Redox cycling is a process wherein a quinone is reduced by cellular reductases to form semiquinone radicals or hydroquinones. These species can also react with oxygen to form superoxide radicals and/or hydrogen peroxide, which result in the production of several other active oxygen species (1). The semiquinones of the anti-cancer agents Doxorubicin, Mitomycin C and EO9 react rapidly with oxygen to form superoxide radicals and there is substantial evidence to show that these radical processes can at least contribute to their cytotoxicities and, in the case of Adriamycin and EO9, contribute to their dose limiting toxicities.

Simple quinones can also be toxic by virtue of their ability to undergo Michael addition reactions with cellular thiols (eg. the –SH groups of cysteine) and amines (eg. the -NH_2 groups of lysine). It has been proposed that some of the quinones that are formed from the oxidation of certain polycyclic aromatic hydrocarbons may be able to undergo Michael additions with the bases of DNA. However, this has yet to be proven.

The activity of simple alkylating agents can also be enhanced by the inclusion of a quinone moiety. For example, in a series of experiments comparing quinone mustards with aniline mustards it was shown that a quinone mustard can be more than 600 times more active against L5178Y lymphoblasts than an aniline mustard (2). The enhanced cytocidal activity could be correlated with the formation of strand breaks and DNA-DNA cross links. It was suggested that the inclusion of the quinone moiety assists the compounds binding to DNA, presumably through hydrogen bonding (3).

This review concentrates on the more complex quinones whose biological activity is, or has been synthetically enhanced by the addition of specific alkylating functions capable of reacting with DNA. The main quinones discussed are Mitomycin C, indoloquinones, aziridinylquinones and the pyrrolo[1,2-alpha]benzimidazolequinones (Figure 1).

3. DNA ALKYLATION

Despite the major advances that have occurred in cancer research over the last ten years, the major mechanism by which most of the clinically relevant anti-cancer agents kill cells involves interfering with replication.

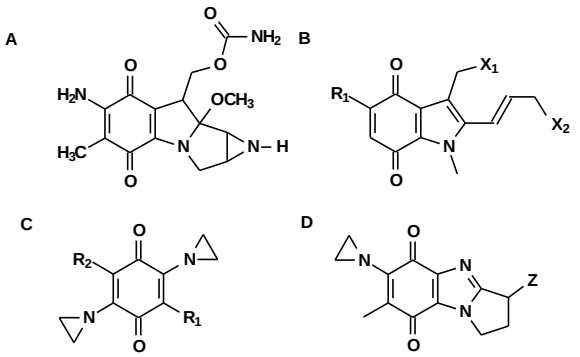

Figure 1. Structures of aziridinylquinone anti-tumour agents. Mitomycin C (A), EO indoloquinone analogues (B), Diaziridinylquinones (C) and, Pyrrolo[1,2-alpha] benzimidazolequinones (D).

One of the simplest methods of bringing this about is via alkylation of DNA. Essentially, alkylation is brought about as the alkylating agent acts as an electrophilic trap for the nucleophilic sites in DNA. Alkylation invariably occurs on guanines or adenosines as these are the most easily oxidised bases and are the best nucleophiles. For most simple alkylating agents, the N-7 position of guanine in the major groove is the preferred site of alkylation. This is the most negative site anywhere within the bases of DNA. However, with more complex agents, the sites of alkylation can be controlled by non-covalent DNA interactions.

Simple mono-alkylation of DNA results in many types of damage including mismatching of DNA base pairs, the production of apurinic sites and the formation of single strand breaks. Bisalkylation of the DNA helix can lead to inter-strand, intra-strand, inter-helix or DNA-protein cross links. It is generally recognised that the inter-strand cross-links are the most toxic as they can prevent strand separation during DNA replication and are difficult to repair due to the involvement of both DNA strands.

Some of these concepts can be illustrated with the clinically relevant quinone anti-tumour agents.

3.1. Mitomycin C.

Mitomycin C and other mitomycins are isolated from various strains of *Streptomyces* and in particular, *Streptomyces caespitosus*. Mitomycin C is the best known of all the aziridinylquinones and, at the present time, is the most clinically relevant. Essentially, this drug has three potentially active constituents. These are the quinone, an unusual aziridine, and a C-10 carbamate group. Several studies have shown that all three constituents are utilised in the cytotoxic action of Mitomycin C, although the mechanisms of activation are complex and are still being debated.

Mitomycin C is very stable a physiological pH but becomes unstable on reduction by chemical reducing agents or reductive enzymes. Although it is widely accepted that Mitomycin C has to undergo reduction in order to interact with DNA, there has been some controversy as to which enzymes are responsible for this process. Until recently, it was believed that the cytotoxicity of Mitomycin C was solely due to the formation of reactive semiquinones produced by the one-electron reducing enzymes. Several different groups also produced evidence to show that Mitomycin C is not a substrate for the obligatory two-electron reducing enzyme, DT-diaphorase. It was therefore proposed that the hydroquinone plays no part in the activation of the quinone (4-6). Similarly, earlier studies had shown that a reactive species can be produced by "selective one-electron reduction" by chemical agents or different one-electron reducing enzymes. Again, the implication from these studies was that the hydroquinone played no part in the cytotoxic mechanisms (7,8).

However, the precise roles of the one- or two-electron reducing enzymes in the activation of Mitomycin C became more confused when it was shown that the hydroquinone, formed from the dismutation of the semiquinones, yields similar products to those produced from the different one-electron reducing systems (9). Furthermore, it has now been proven that Mitomycin C is a substrate for DT-diaphorase (10) and indeed, the cytotoxicity of Mitomycin C in different cell lines can be correlated with the intracellular levels of this enzyme (11).

Several types of Mitomycin-base adducts have been isolated and the main site of attack is at guanines with about 90% of the adducts at the N-2 position. A guanine N2-/guanine N-2 cross linked adduct has been isolated from mouse mammary tumours following Mitomycin C treatment (12, 13).

Figure 2 shows a reaction scheme that summarises an accepted mechanism for the activation of Mitomycin C (adapted from 14). In this scheme, the hydroquinone (B) can be formed either by disproportionation of the semiquinones or by direct reduction by DT-diaphorase. This hydroquinone forms reactive intermediates (C) and (D). The quinone methide (D) reacts with DNA to produce mainly adducts at the N-2 position of guanine in the minor groove. In the absence of DNA, the main products produced are the *cis*- and *trans*-forms of 2,7 diamino-1-hydroxymitosene (E) and 2,7-diaminomitosene (F). More recent studies have shown that at pH 5.8, where the rate of reduction by DT-diaphorase is much faster than at pH 7.4, the yield of cross links increases 6-20 fold. Furthermore, major groove 2,7-diaminomitosene adducts are produced (15). These adducts have also been detected after Mitomycin C treatment *in vivo* (16). The monofunctional and bifunctional activation pathways are controlled by the state of protonation of the leuco-aziridinomitosene (B).

3.2. EO9

EO9 [3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-indione)-prop-2-en-1-ol] (Figure 1b R_1 = aziridinyl $X_1 = X_2 = OH$) was originally synthesised as part of a large study which involved the synthesis of Mitomycin C analogues based around indoloquinones (17). EO9 was selected for clinical study because of its activity against hypoxic cells and its apparent lack of bone marrow toxicity in animal studies (18). This quinone was also shown to be a

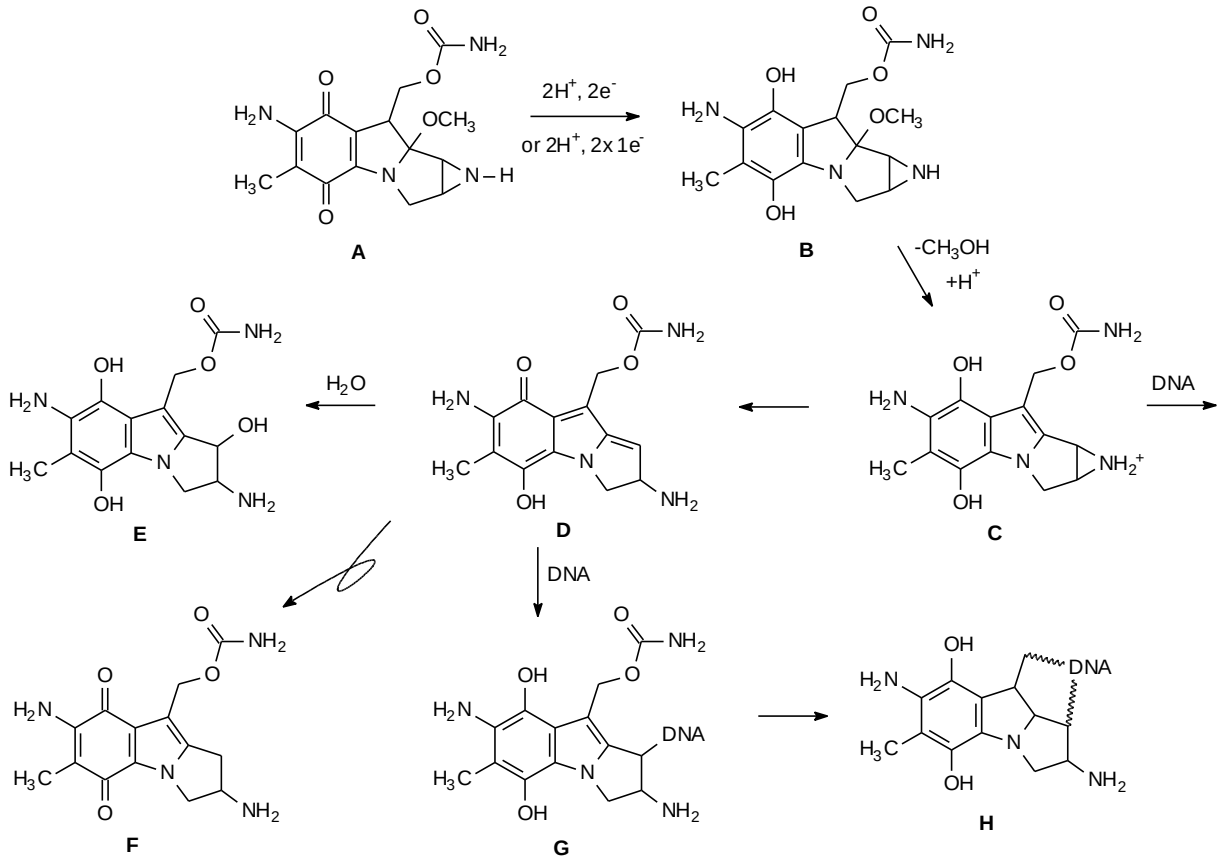

Figure 2. Some of the proposed mechanisms for activation and DNA cross-linking of Mitomycin C.

good substrate for DT-diaphorase and the reduced products damaged DNA (19).

The indoloquinones were initially designed to alkylate DNA after reduction via the formation of a reactive methide species (Figure 3). These methide species are formed by an inter-molecular rearrangement of the hydroquinone form resulting in the elimination of the two leaving groups, X (C). Mechanistic studies have shown that the C-10 substituted hydroxymethyl group forms alkylation products more readily than the substituted propenol group (17). EO4 (Figure 1b, R_1 = aziridinyl X_1 = X_2 = $OC(O)CH_3$) has been shown to readily cross-link DNA after reduction (20) and it is likely that this cross link is formed by the aziridine and C-10 methyl groups. In the same study, reduced EO9 did not form such cross links. This could be consistent with the fact that EO9 has no obvious leaving groups to facilitate methide formation and hence would be expected to only form mono-adducts via the aziridine group. However, another study has demonstrated that reduced EO9 forms cross links in plasmid DNA and in intact cells (21).

The DNA cross linking studies on EO9 in intact DT-diaphorase rich cells were carried out using the alkaline elution technique. However, this same study showed that these cross links were almost completely hidden by the presence of extensive DNA strand breaks (21). This is consistent with another study which showed that the hydroquinone of EO9 is unstable in the presence of oxygen and readily autoxidises producing hydrogen peroxide (22). This simple redox cycling can therefore explain the DNA strand breaks and may explain why EO9 shows an excellent correlation between its cytotoxicity and the intracellular levels of DT-diaphorase in different cell lines (11). EO9 has undergone Phase I/II clinical trials but has shown no response. The major toxicity was reversible proteinuria (23).

3.3. Diaziridinylbenzoquinones.

The diaziridinylbenzoquinones are structurally the simplest of all the aziridinylquinones but have given rise to some of the more interesting studies on quinone-DNA interactions. There have been four major studies carried out on aziridinylbenzoquinones, each involving extensive synthesis followed by biological evaluation and all producing a candidate for clinical evaluation. 2,5-Diaziridinyl-1,4-benzoquinone (Figure 1C; R_1 = R_2 = H) and many of the simpler analogues were originally synthesised by Petersen and co-workers in the early fifties (24). The most extensively studied analogue was Trenimon (Figure 1C; R_1 = H R_2 = aziridinyl) which underwent clinical trials in Poland, Italy and Germany for the treatment of leukaemias, breast cancers, Hodgkins disease and carcinomas of the cervix. However, Trenimon was removed from the clinic due to its tendency to cause myelosuppresion (25).

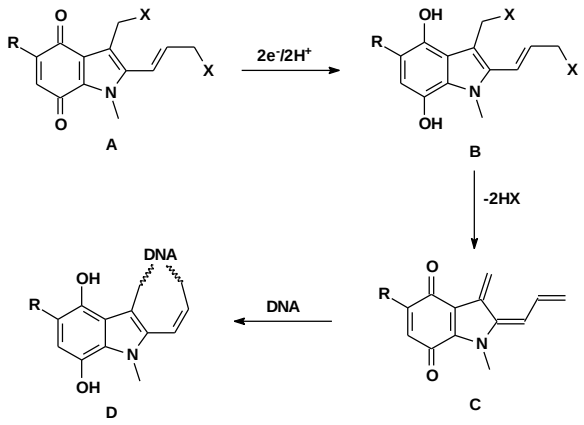

Figure 3. Proposed mechanism for DNA cross-link formation of EO analogues via a reactive quinone methide intermediate (C).

In the early seventies, Nakao and co-workers synthesised a series of benzoquinones in order to mimic the alkylating ability of the carbamate side chain of Mitomycin C. It was found that the diaziridinyl analogues of these quinones were very active against mice bearing L1210 tumours. More than forty diaziridinylbenzoquinones were then synthesised by this group (26). These quinones included both carbamate and alkyl- substituted analogues. This resulted in the identification of Carboquone (Figure 1C; R_1 = Me R_2 = $CH(OCH_3)CH_2OCONH_2$) as a potential clinical candidate. This quinone is still used today in combination therapy in Japan for the treatment of prostate cancer (27) and in Finland for the treatment of ovarian cancer (28). Once again, the major side effect is myelosuppresion (29).

In the late 1970s, Driscoll and co-workers designed a series of diaziridinylquinones as potential central nervous system anti-tumour agents. These were designed to be able to cross the blood brain barrier due to their high lipid solubility and low ionisation. The most active compound identified was AZQ (Figure 1C; R_1 = R_2 = $NHCO_2Et$) which was effective against both inter-peritoneally and inter-cerebrally implanted tumours (30). A more water soluble analogue, BZQ (Figure 1C, R_1 = R_2 = NHC_2H_4OH), was also identified from these studies (31). Both AZQ and BZQ entered clinical trials although BZQ is not reduced by the one-electron reducing enzymes and is not a substrate for DT-diaphorase (32). AZQ has undergone phase II trials against recurrent primary brain tumour (33) and advanced large bowel cancer (34). BZQ did not proceed past phase I trials.

The interest in diaziridinylquinones was further increased after it was recognised that DT-diaphorase is over expressed in several different types of tumours (35) and some diaziridinylquinones are excellent substrates for this enzyme (32, 36). These studies identified MeDZQ (Figure 1C; R_1 = R_2 = CH_3) as an excellent substrate for the enzyme and the hydroquinone is very efficient at cross linking DNA. More recently, a water soluble analogue, RH1 (Figure 1C; R_1= -CH_3, R_2 = -CH_2OH), has been developed (37) which compared to MeDZQ, is a better substrate for DT-diaphorase, a more efficient DNA cross-linking agent and shows high cytotoxicity differentials in DT-diaphorase-rich and –deficient cell lines. On the basis of these and other results, RH1 has been accepted for Phase I/II clinical trials.

The selectivity of a quinone for specifically targeting DT-diaphorase rich cells depends on a number of factors and in particular, on the relative rates of reduction of the quinone by the one-electron reducing enzymes. However, fortunately, the rates of reduction by the one-electron reducing enzymes tend to be strongly dependent on the one-electron reduction potentials of the quinone (eg. 38). Hence, if a quinone is thermodynamically more easy to reduce (ie. the value of $E[Q/Q^{\cdot-}]$ is more positive), it will be easily reduced by the enzyme. This means that the rates at physiologically relevant concentrations are not dependent on the enzyme-substrate binding. Recent studies in our laboratory have so far shown that this is not true for the reductions by DT-diaphorase. It would appear that binding of the quinone to the enzyme is necessary for the two-electron transfer and the K_m values for many quinones are very low. Thus for example, when a series of alkyl substituted diaziridinylquinones were tested as substrates for the purified enzyme, no simple correlations with rates could be observed, despite the fact that their two electron reduction potentials (Q/Q^{2-}) were similar (36). Similarly, AZQ has a relatively positive two-electron reduction potential but is a very poor substrate for DT-diaphorase compared to MeDZQ, which has a much more negative two-electron potential.

All aziridinyl compounds, can be activated to alkylate DNA by protonation of the aziridine groups followed by nucleophilic attack. Indeed, it was demonstrated several years ago, that simple aziridinylbenzoquinones in aqueous solution can cross-link DNA in the absence of reduction and this process is pH dependant (39). This “acid assisted” activation process is probably the main mechanism for DNA alkyation by the aziridinyl compounds which are easily protonated such as Triethylenemelamine, thioTEPA and BZQ.

The quinones which can undergo bioreduction have the added advantage in that in many cases, although the pK for the aziridines in the quinone are around 3-4, the pK increases to around 5-6 when the quinone is reduced. Thus for example, the pK of Mitomycin C is 3.1 whereas the pK of the hydroquinone is around 5 (9). This means that DNA alkylation will only significantly occur after the quinone has been reduced and hence allows selectivity.

The known mechanisms whereby the diaziridinylquinones can damage DNA are shown in Figure 4. The efficiency of DNA cross linking by the diaziridinylquinones depends on the reactivity of the second aziridine group after the first aziridine has alkylated at one site on the DNA. This should be a very favourable process simply because as the first aziridine will form an aliphatic amine on alkylation. The resulting electron donation from this amine into the hydroquinone/

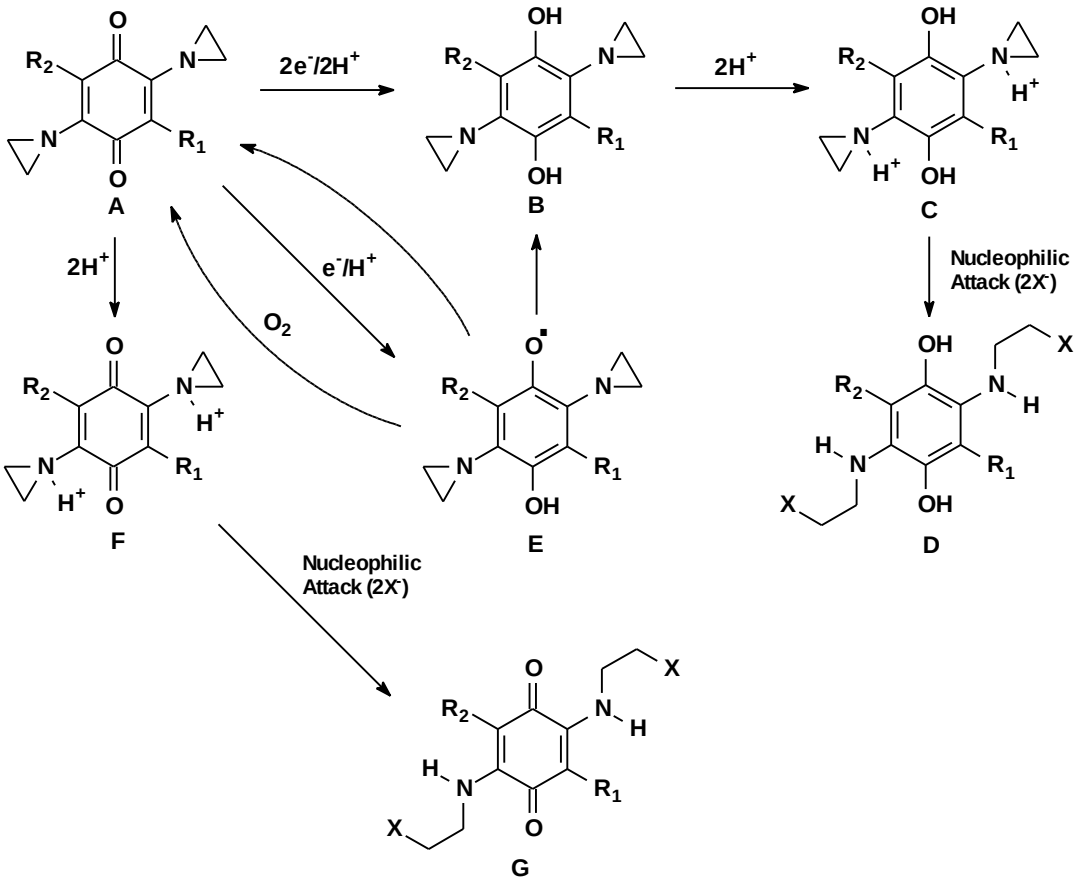

Figure 4. Proposed cytotoxic pathways for the diaziridinylquinones. The disproportionation reaction is 2E →A + B. The semiquinone can react with oxygen; $E + O_2 \rightarrow A + O_2^{\cdot -}$

semiquinone aromatic ring system should then lower the pK of the remaining aziridine. In this manner, this aziridine will have a lower pK than the first aziridine and hence should more readily react with the bases. The efficiency of cross linking also depends on the relative positions of these aziridines.

Although the diaziridinylquinones are relatively simple compounds, their interactions with the bases of DNA can be quite complex and are strongly dependent on the structure of the quinone. For example, alkylation by many diaziridinylquinones is primarily at the guanine N-7 positions with a preference for guanines within runs of continuous guanines (40). This sequence selectivity is similar to that seen with several other types of bifunctional alkylating agents such as the nitrogen mustards (41). In contrast, DZQ following reduction, alkylates at N-7 primarily at 5'-GC sequences, and in particular at 5'-TGC sites (42,43). Without reduction, this sequence selectivity is not observed. The reduced DZQ was shown to preferentially cross link DNA at these 5'-TGC-3' sequences with the cross link spanning two base pairs. In contrast, reduced MeDZQ cross linked preferentially at 5'-GNC-3' sequences with the cross link between guanine N-7 positions spanning three base pairs (43).

In the case of DZQ, the hydroquinone can initially intercalate between the bases of a 5'-GC-3' sequence to form hydrogen bonds with the O-2 and the C4-NH_2 groups of the cytosine. This positioning also allows the protonated aziridine to associate with the N-7 of guanine (44). The 5'-TGC-3' selectivity can be explained by the crystallographic results from Dickerson and colleagues (45,46). Essentially, a 5'-GC-3' sequence as opposed to a 5'-CG-3' is preferred as the GC step has a high twist profile. This means that the sequence has a high twist, a low rise, a positive cup and a negative roll. The positive cup ensures that there is more room in the centre space between the base pairs and the negative roll means that these bases are tilted open towards the major groove. Thus the GC step is a more open site of attack (Figure 5A). A CG step has a low twist profile with a negative cup and a positive roll and is thus more compact. The extension of the sequence to 5'-TGC-3', and hence 5'-GCA-3' on the complimentary strand, can be explained by the occurrence of three centre hydrogen bonds between adjacent base pairs in the major groove. These were found to frequently occur at CA steps (45). The occurrence of one of these bonds in a 5'-TGC-3' sequence causes the middle cytosine on the complementary strand to be pulled further up making the space between the GC step even larger (see Figure 5A). Following these initial interactions, the aziridines can alkylation at the two guanine N-7 positions and therefore cross link the DNA. From comparisons with other diaziridinylquinones, it was shown that the 5'-TGC-3' selectivity only occurs when there is a simple –H in position-6 of the quinones. This was

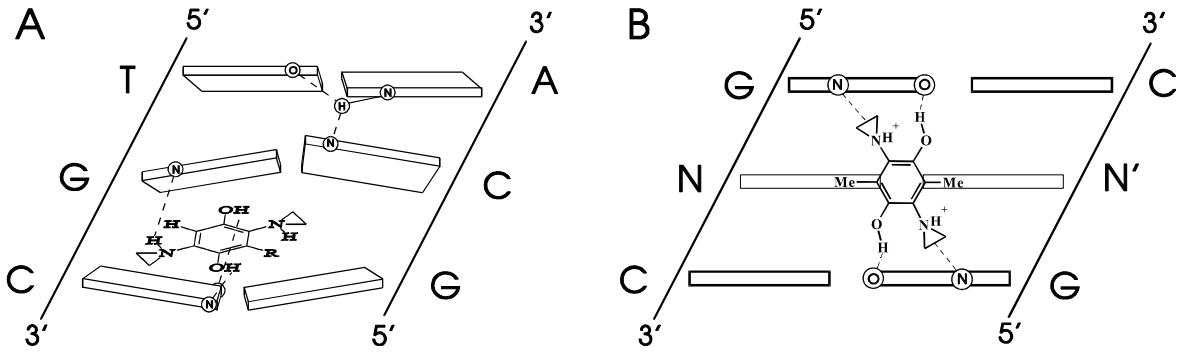

Figure 5. Schematic representations of the 5'-TGC-3' and 5'-GNC-3' sequence selectivities of cross linking by DZQ and MeDZQ. A) The open nature of the GC step and the three centre hydrogen bond allows the hydroquinone form of DZQ to enter and form hydrogen bonds between the OH groups, and the O-2 and the C4-NH_2 groups of cytosine. The protonated aziridine associates with the N-7 of guanine on the same strand. B) The initial interactions between MeDZQ hydroquinone and DNA. The dotted lines indicate the hydrogen bonds between the hydroquinone OHs and the guanine O-6 and the interactions between the protonated aziridines and the guanine N-7s.

explained by the fact that a simple –H group is small and can allow the hydroquinone to enter and intercalate between the GC bases (44).

It has also been shown that when one of the aziridinyl groups in diaziridinylquinones is replaced by a methyl or another small alkyl group, cross linking DNA still takes place at the 5'-TGC-3' sequences (47). Hence for example, reduced 2-aziridinyl-5-methyl-1,4-benzoquinone cross links DNA at these sequences. Furthermore, the cross-linking ability is decreased when the methyl group is replaced with an ethyl or propyl group. It has been proposed that the cross linking occurs as a consequence of the formation of a quinone methide species. Essentially, the hydroquinones initially intercalate at the 5'-TGC-3' sequence, in a similar way to the hydroquinone of DZQ. The aziridine then alkylates at one guanine N-7. As there is no other aziridine to alkylate the opposite guanine, the hydroquinone can then slowly autoxidise back to a quinone. This quinone then tautomerizes to form the reactive quinone methide species which alkylates this guanine. The loss of activity when the methyl group is changed to an ethyl or propyl is attributed to steric hindrance in the initial intercalation (47).

The mechanism for the 5'-GNC-3' sequence selective cross-linking of MeDZQ is relatively simple (36). As MeDZQ does not have a hydrogen at position-6 of the quinone, it is unable to initially intercalate into DNA. The hydroquinone of MeDZQ therefore interacts face on with DNA to form hydrogen bonds between the two hydroquinone OH groups and the two guanine O-6s and the protonated aziridines associate with the two guanine N-7 positions. These hydrogen bonds hold the drug in place while the aziridines react to form a cross-link between the two guanines (Figure 5B). Once again, if both of the methyl groups are replaced by ethyl or propyl, the cross linking efficiency decrease as these larger alkyl groups sterically hinder the initial hydrogen bonding interactions.

The sequence selectivity of all these diaziridinylquinones position the aziridines in such a way that they can hydrogen bond to guanines on opposite strands of the helix. This merely increases the efficiency of cross linking and this can be related to their enhanced cytotoxicities in different cell lines. This sequence selectivity is very short and is certainly not long enough to target unique sequences in the human genome. However, recent advances have shown that the sequence selective of alkylating diaziridinylquinones can be extended by conjugation of the quinone to an oligonucleotide (48). In this study a 21 base pair triplex was formed using a MeDZQ analogue. This large analogue was able to selectively cross link a designed target DNA at a 24 base pair sequence.

3.4. Pyrollo[1,2-alpha]benzimidazolequinones

The pyrollo[1,2-alpha]benzimidazolequinones (PBIs) are the most recently designed group of aziridinylquinones. These types of quinones interact with the major groove of DNA, like the diaziridinylquinones, but they target the alkylation to the phosphate backbone rather than the bases. The reduced forms of the PBIs hydrogen bond to the DNA bases and hold the drug in the major groove. These bonds fix the aziridine group in the correct location to allow alkylation of the phosphate backbone and result in the formation of a phosphotriester (49). These phosphotriesters are hydrolytically labile and produce DNA strand breaks (Figure 6).

The base pair specificity of PBIs can be altered through small structural changes at the 3 position of the molecule (Figure 1D). These structural changes can vastly alter the hydrogen bond donating/accepting ability of the PBIs and thus affect the interactions with the bases in DNA. For example, when position 3 is occupied by a carbamate (Figure 1D; Z = $OC(O)NH_2$) an amine (Z = NH_2) or a ureido (Z = $NHC(O)NH_2$) group, the alkylation occurs at A-T bases. Alternatively, a carbonyl group at position 3 (Z = O) results in an alkylation at G-C bases whereas alkylation at both A-T and G-C sites can be

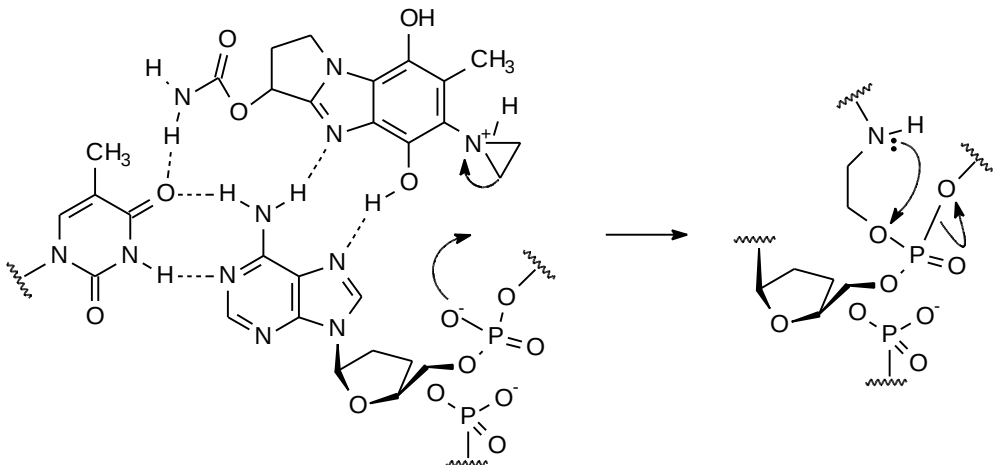

Figure 6. The interaction of a PBI with an A-T base pair and the formation of a phosphotriester leading to the hydrolytic cleavage of the phosphate backbone.

observed for acetyl group (Z= $OC(O)CH_3$). Interestingly, the analogues wherein the aziridine is replaced by an acetamido group have been found to be potent topoisomerase inhibitors. The PBIs are therefore extremely interesting compounds and several clinical candidates have been put forward (50).

4. PERSPECTIVE

There are a number of varied mechanisms by which aziridinyl quinones can interact with and alkylate DNA. Increased understanding of these mechanisms allows the development of new agents and also allows the redevelopment and improvement of previously utilised agents. This review has outlined many of these mechanisms and illustrates the advances made in this area of drug development.

5. ACKNOWLEDGEMENTS

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