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Rationale for the use of aliphatic N-oxides of cytotoxic anthraquinones as prodrug DNA binding agents: a new class of bioreductive agent

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Summary

NAD(P)H dependent cytochrome P450's and other haemoproteins under hypoxia, mediate two-electron reduction of a wide range of structurally dissimilar N-oxides to their respective tertiary amines. Metabolic reduction can be utilised, in acute and chronic hypoxia, to convert N-oxides of DNA affinic agents to potent and persistent cytotoxins. In this respect a knowledge of N-oxide bioreduction and the importance of the cationic nature of agents that bind to DNA by intercalation can be combined to rationalise N-oxides as prodrugs of DNA binding agents. The concept is illustrated using the alkylaminoanthraquinones which are a group of cytotoxic agents with DNA binding affinity that is dependent on the cationic nature of these compounds. The actions of the alkylaminoanthraquinones involve drug intercalation into DNA (and double stranded RNA) and inhibition of both DNA and RNA polymerases and topoisomerase Type I and II. A di-N-oxide analogue of mitoxantrone, 1,4-bis{[2-(dimethylamino-N-oxide)ethyl]amino}5,8-dihydroxyanthracene-9,10-dione (AQ4N) has been shown to possess no intrinsic binding affinity for DNA and has low toxicity. Yet in the absence of air AQ4N can be reduced *in vitro* to a DNA affinic agent with up to 1000-fold increase in cytotoxic potency. Importantly the reduction product, AQ4, is stable under oxic conditions. Studies *in vivo* indicate that antitumour activity of AQ4N is manifest under conditions that promote transient hypoxia and/or diminish the oxic tumour fraction. The advantage of utilising the reductive environment of hypoxic tumours to reduce N-oxides is that, unlike conventional bioreductive agents, the resulting products will remain active even if the hypoxia that led to bioactivation is transient or the active compounds, once formed, diffuse away from the hypoxic tumour regions. Furthermore, the DNA affinic nature of the active compounds should ensure their localisation in tumour tissue.

Nomenclature and properties of N-oxides

The term N-oxide is a functionality used to describe the oxide of a tertiary amine created by addition of atomic oxygen to the lone pair electrons of the nitrogen atom. Aromatic, arylaliphatic and aliphatic tertiary amines can all form N-oxides. The resulting dative covalent nitrogen-oxygen bond is semi-polar and can be described formally as N^{+}-O^{-}. N-Oxides are weaker bases than the respective parent tertiary amines generally by about 2 pKa units. However such N-oxides are still sufficiently basic to protonate at acid pH and form addition salts e.g. hydrochloride or acetate.

1. Chronic and acute tumour hypoxia and limitations of conventional bioreductive drugs

The evidence for hypoxic regions in human tumours is generally accepted [1, 2] and has been well characterised in rodent tumour models [3–5]. The blood flow and specific diffusion rates in tumours is not uniform and not necessarily influenced by tumour size [6, 7]. When blood perfusion is limited in an area of tumour then chronic hypoxia results because the consumption of oxygen, for metabolic purposes, by cells close to the tumour blood supply limits the amount of oxygen available for diffusion to more distant cells. In effect, cell greater than 120–150 micron distance from a blood vessel are starved of oxygen, die and ultimately constitute necrotic tumour regions. The boundary between fully oxygenated and necrotic tumour regions is composed of cells experiencing degrees of hypoxia varying from 20 mm Hg to radiobiological hypoxia (1 mm Hg) [8]. There is evidence that viable hypoxic cells may contribute up to 20% of tumour mass [1]. Chronic hypoxia is considered to give rise to a resistant subpopulation of potentially clonogenic cells which are held in G_0/G_1 and as such are not undergoing the normal growth cycle. Diffusion limited or acute hypoxia has also been described which results from temporary closing of the vasculature in a particular area of a tumour [3, 8]. This can be explained by a time dependent compression of the expanding tumour on thin walled capillaries, high interstitial fluid pressure and/or by microcirculatory disturbances including arteriovenous shunt perfusion [8] and physical blockage [3].

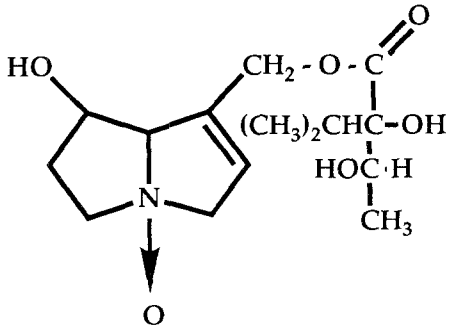
Molecular oxygen is utilised in cells principally as a terminal electron acceptor in oxidative phosphorylation but also for a variety of dioxygen requiring processes including steroidogenesis and haem degradation. Flavoprotein containing dehydrogenases and oxidoreductases mediate the flow of electrons from reducing equivalents in the form of the nicotinamide dinucleotide coenzymes, NADH and NADPH, to dioxygen and other endogenous acceptor molecules. Various chemotherapeutic agents are also substrates for certain flavoprotein enzymes that ordinarily participate in intermediary metabolism. In particular NADH dehydrogenase, cytochrome P450 and cytochrome P450 reductase, xanthine oxidase, aldehyde oxidase [9] and DT-diaphorase [10] have been identified.

On the basis that bioreduction is facilitated under hypoxic conditions agents including the nitroimidazoles [11], benzotriazine di-N-oxide (SR4233), mytomycin c and EO9 [12] have been developed and are either in clinical use or entering phase I trials [12]. Such compounds are designed to undergo reductive activation in hypoxic tumours to potent cytotoxins which inhibit cellular activity in a way that is not cell cycle specific. However, there are major problems in the use of these 'conventional' bioreductively activated agents. Firstly, such agents are likely to be dependent on chronic hypoxia to maintain activity since diffusion of the reactive species or parent compound away from the hypoxic tissue, or tissue reoxygenation will result in inactivity. Secondly, the intracellular target for most bioreductive agents is considered to be DNA and yet none of these agents accumulate in the nucleus since they do not possess specific, non-covalent DNA binding affinity.

On the other hand acute hypoxia in tumours could be a useful aspect of tumour physiology with regard drug targeting if a bioreductively activated agent can be developed that maintains activity even after oxygen reperfusion takes place. Ideally, such agents should be non-toxic in oxic tissue until irreversibly converted to a persistent, oxygen insensitive active drug specifically under hypoxic conditions. N-oxides of DNA affinic agents that undergo a concerted two-electron reduction have the potential to fulfill such requirements.

2. Chemotherapeutic N-oxides – intrinsically active cytotoxins or bioreductive prodrugs?

Several N-oxides as cytotoxic agents have been described. Some N-oxides are believed to be intrinsically cytotoxic. These include the pyrrolizidine alkaloids N-oxides, as exemplified by indicine N-oxide **1** [13] its derivatives [14, 15]. Indicine N-oxide underwent intensive preclinical evaluation and was the subject of three Phase II clinical trials until evidence of severe hepatotoxicity halted its progress



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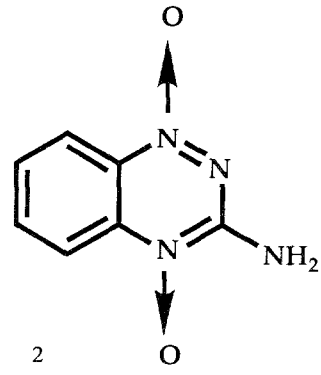

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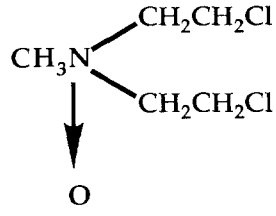
(reviewed by [13]). The mechanism of action of indicine N-oxide was thought to involve metabolic allylic oxidation and dehydration into alkylating species but only after its reduction to the indicine free base. The observation that indicine N-oxide is cytotoxic *in vivo* and that the majority of the drug is excreted as unchanged N-oxide was at variance with this. However, some indicine is observed as a reduction product of indicine-N-oxide in animals and humans and this may account for the antitumour activity observe in rodent tumours and human xenografts [13]. Possibly indicine N-oxide is a prodrug that undergoes bioreductive activation to indicine in small but sufficient quantities in tumour tissue to form indicine-derived alkylating species. The benzotriazene di-N-oxide, SR4322 (**2**) is also believed to be a prodrug that is one electron reduced to a nitroxide radical intermediate which is suggested to H-atom abstract from DNA resulting in lethal cell damage [16]. However nitroxide radicals in the absence of air disproportionate quantitatively [17] with an exact stoichiometry of 2 moles of parent SR4233 consumed to produce 1 mole of benzotriazene-mono-N-oxide [18] making H-atom abstraction from DNA an unlikely event. It is clear, however, that benzotriazene-di-N-oxide is associated with formation of an active compound since the mono-N-oxide (two electron reduction product) and amine derivative (four electron reduction product) are both inactive [19]. The N-oxide of nitrogen mustard (nitromin, **3**) is 30–40 times less toxic than nitrogen mustard itself and was synthesised in order to increase the half life of the chloroethylamino alkylating groups [20]. Coincidently, nitromin was probably the first example of an N-oxide to be prepared that could be selectively reduced in hypoxic tumours. Nitromin is active against solid tumours in rats. This aspect appears to have resurrected interest in nitromin and a one electron reduction intermediate, the nitrogen anion radical has been proposed to explain the DNA strand breakage observed in addition to DNA cross linking in cells treated with nitromin [21]. However no evidence is presented and given the propensity for two electron reduction of aliphatic teriary amine N-oxides (see later) such an intermediate is unlikely. Cytosine arabinoside 3-N-oxide [22] and 3′-deoxyadenosine N^1-oxide [23] are both heteroaromatic N-oxides that inhibit DNA synthesis but only after reduction to their respective purine bases and hence can be viewed as true prodrugs. However, tamoxifen N-oxide, **4** has been described but is no less cytotoxic than tamoxifen [24]. This was taken to indicate, with other evidence, that the amine functionality of tamoxifen did not contribute to its anti-oestrogenicity or influence the intrinsic antitumour activity of this molecule.

3. Enzymes involved in N-oxide reduction

The metabolic reduction of amine-N-oxides to their respective tertiary amine bases involves donation of 2-electrons to the N-oxide. The enzymology of this has not been investigated in detail for many aliphatic N-oxides, presumably because until recently the significance of this reduction process was not apparent. Several reports suggest that N-oxide reduction can be an enzymic [25–28, 33] or non en-



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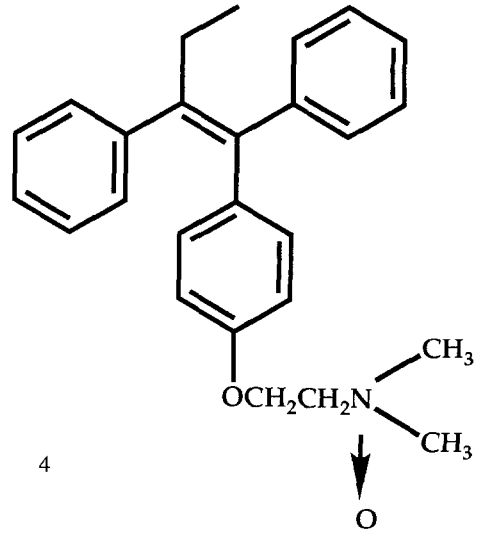
zymic [25–28] mechanism or both [27]. However in early studies, the non-enzymic reduction of N-oxides [25–28] may have arisen from denatured products generated during work-up procedures prior to or during analysis. Consistent with this Fe^{II} mediates reduction of N-oxides [29]. In cellular systems several NADH and/or NADPH requiring flavoproteins and cytochrome P450 are implicated in heteroaromatic-N-oxide and aliphatic N-oxide reduction. Flavoprotein mediated reduction occurs by sequential transfer of one and/or two electrons as occurs with cytochrome P450 reductase [18], and aldehyde oxidase [30, 31], xanthine oxidase [34–36] whilst cytochrome P450 acts as an 'obligate' two electron donor to the N-oxide substrate even though it accepts electrons sequentially. The mechanism(s) of cytochrome P450 mediated electron transfer will be described later (section 3.1).

Depending on the mechanism enzymic N-oxide reduction can be sensitive to oxygen. This is because oxygen is a competing substrate with the N-oxide as happens with xanthine oxidase and cytochrome P450 or because the formation of a one electron reduction product (nitroxide radical) is able to reduce oxygen in a redox cycling process. There are several examples of oxygen sensitive N-oxide reductions described. For example, nicotinamide N-oxide is reduced by xanthine oxidase [34, 35]. It would appear that reduction can be mediated by direct transfer of the oxygen atom from the N-oxide [36]. The reduction of nicotine 1′ N-oxide has also been linked, in part, to xanthine oxidase [37]. Reduction of benzotriazene-di-N-oxide has been extensively investigated and shown to undergo one electron reduction by cytochrome P450 reductase generating an oxygen sensitive nitroxide radical [17]. There is evidence for cytochrome P450 haemo-



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protein involvement in reduction of benzotriazine di-N-oxide to yield mono-N-oxide by a process inhibited by oxygen [18] which presumably involves direct two electron reduction without formation of a nitroxide radical intermediate. Reduction of indicine N-oxide to indicine by NAD(P)H cytochrome P450 is also inhibited by oxygen [38].

3.1. Cytochrome P450 and other haemoprotein based reduction of N-oxides

The information in the proceeding sections indicates NAD(P)H dependent cytochrome P450 is enzymatically involved in the reduction of N-oxides by an oxygen sensitive mechanism. In addition to this enzyme, haemoglobin [25, 26] and denatured haem [27] also reduce N-oxides in the presence of NAD(P)H. There is also an oxygen insensitive mechanism that does not require reducing equivalents (see section 3.3).

3.2. NAD(P)H dependent cytochrome P450 as an N-oxide reductase

Most information on the nature of aliphatic N-oxide reductase(s) has resulted from an investigation of the reduction of imipramine-N-oxide, tiaramide-

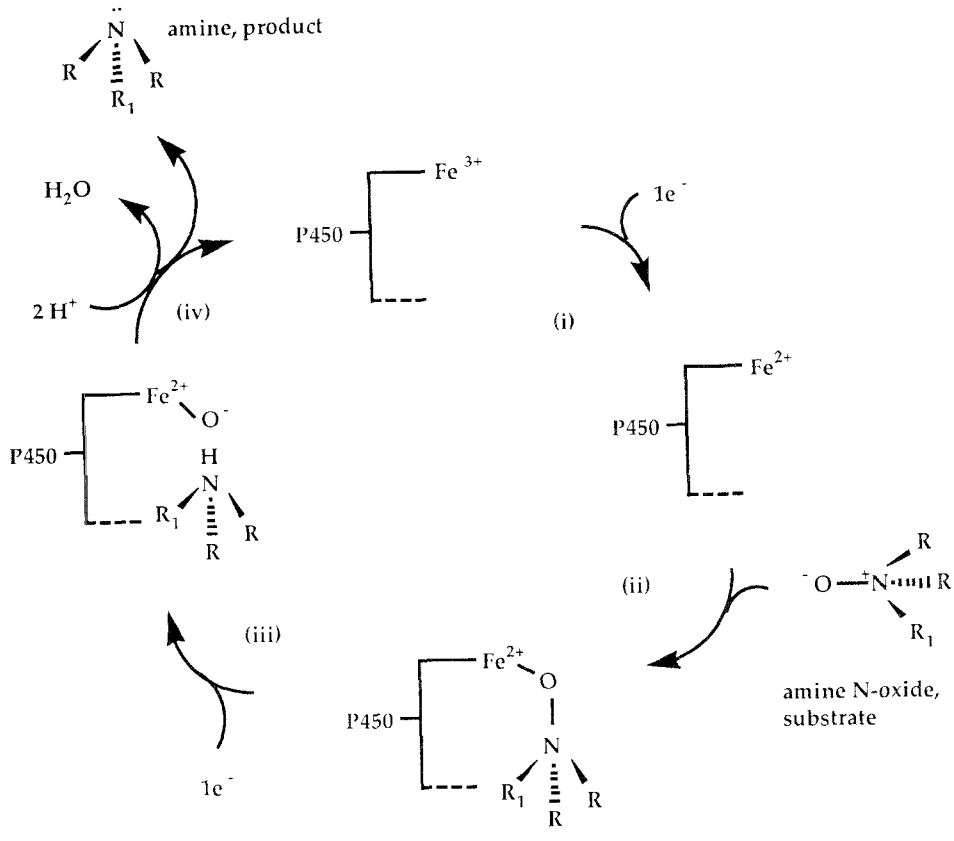


Fig. 1. Proposed mechanism of NAD(P)H dependent cytochrome P450 mediated N-oxide reduction. The scheme shows the reduction of amine oxide by cyt. P450 in a catalytic cycle requiring NAD(P)H but independent of dioxygen as follows: (i) cyt. P450 is one-electron reduced by NADPH via cyt. P450 reductase a process that is prerequisite for binding of the N-oxide to the haem centre (ii) formation of a cyt. P450/N-oxide substrate complex with the substrate hydrophobic moiety binding to the cyt P450 substrate binding domain (depicted as -----) and the N-oxide moiety binding to the haem iron (Fe^{2+}) (iii) further reduction by NADPH cyt P450 reductase or NADH b_5 reductase and formation of an amine: iron-oxy pair (perhaps as shown) (iv) release of the amine and formation of water.

N-oxide and N,N-dimethylaniline N-oxide [33, 39, 40]. Microsomal cytochrome P450 was the enzyme shown to predominantly metabolise these N-oxides by a process that was oxygen sensitive. Enzymes in mitochondria, lysosomes and cytosol were also suggested to contribute to reduction. Evidence for cytochrome P450 involvement was based on N-oxide inhibitor studies, specifically carbon monoxide and type II inhibitors (see later) and NADH and NADPH were equally important in the rate of N-oxide reduction [33, 39]. Reconstituted systems using purified cytochromes P450 and P448 from rabbit in combination with cytochrome P450 reductase reduced tiaramide N-oxide at comparable rates [39]. The involvement of cytochrome P450 reductase in the overall reduction of N-oxides appears to be as 2-electron donor since reduced methyl viologen could substitute as an electron donor [40] Cytochrome P450 has been implicated in reduction of indicine-N-oxide, an aliphatic amine N-oxide pyrrolizidine alkaloid. Induction and inhibitor studies suggest a unique isozyme of P450 which could utilise NADH to effect indicine-N-oxide reduction but which would not contribute to oxidative drug metabolism [38].

A number of N-oxides, including tiaramide N-oxide and N,N dimethylaniline have been shown to undergo type II spectral interactions with cyto-

chrome P450 in the absence of oxygen (reviewed by [41]). Such spectra are considered to be a result of direct coordination of the N-oxide (acting as a lone pair electron donor) with haem iron (Fe^{II}) of cytochrome P450. This binding is presumably prerequisite for cytochrome P450 mediated reduction of N-oxides. It is not clear as to how electron transfer is related to nitrogen-oxygen bond cleavage in the reduction of N-oxides but given the requirement for reducing equivalents and haem binding of the N-oxide moiety the scheme shown in Fig. 1 is proposed.

3.3. Other mechanisms of haem-based N-oxide reduction

A mechanism of N-oxide reduction by cytochrome P450 that does not require reducing equivalents has been studied using N,N-dimethylaniline-N-oxide (an arylaliphatic amine). The N-oxide is, in effect, considered as an alternative source of 'activated' oxygen. A Fe-O-N complex is suggested and reduction to the amine requires heterolytic cleavage of this complex to form the $Fe^{IV} = O$ amine cation radical pair and subsequently $Fe^{V} = O$ and amine pair. Decomposition of the cytochrome P450 haem bound perferryl species back to the Fe^{III} state is suggested to occur by oxidation of alternative substrates [42]. This pathway of N-oxide reduction is in competition with amine oxide N-dealkylation, a route that is kinetically much more favourable. N-Oxide N-dealkylation of can also occur non-enzymatically in the presence of denatured haem as was shown with certain N,N-dialkylanilines [25, 28]. This involves transfer of the oxygen from nitrogen to an alpha carbon with subsequent non-enzymic N-C bond breakage. The end result is the same as for metabolic N-dealkylation i.e. formation of a secondary amine and an aldehyde. Further support for N-oxide induced hypervalency iron involvement in N-oxide reduction comes from the use of Fe^{II} tetraphenylporphinato as a haem model system. A reaction scheme has been proposed to describe the reduction of aliphatic and heteroaromatic N-oxides. Essentially formation of Fe^{IV} tetraphenylporphinato is proposed which can be reduced by triphenylphosphine to produce a catalytic cycle [43]. Biologically this oxygen insensitive mechanism is considered of minor importance because (a) amine-N-oxides have low oxidation potentials (b) amine-N-oxide reduction is in competition with N-oxide N-dealkylation a route that is kinetically more favourable.

Oxygen inhibits NAD(P)H dependent cytochrome P450 mediated reduction of all the aliphatic and heteroaromatic N-oxides investigated to date. However Fe^{III} chelated with EDTA or DETAPAC in NADPH supplemented microsomes greatly stimulates reduction of indicine N-oxide. Such chelation serves to decrease the reduction potential of the FeIII/FeII couple there by facilitating electron transfer from NAD(P)H cytochrome P450 reductase and hence reduction of indicine-N-oxide [44]. This process is air insensitive and concomitantly appears to result in the formation of hydroxyl radicals as detected by electron spin resonance spectroscopy using the technique of spin trapping [44]. Hemoglobin and denatured haem *in vitro* can also mediate indicine N-oxide [26] and arylaliphatic amine-N-oxide reduction [27].

Investigations into the fate of N-oxide administration to animals show that, in the main, aliphatic N-oxides are stable *in vivo* and are recovered quantitatively following intravenous dosing [25, 13]. Hence it would appear that aliphatic N-oxides are not metabolised in oxygenated tissue to any significant extent.

N-oxides of DNA affinic agents as bioreductive prodrugs

It is clear from the preceeding discussion that cytochrome P450 and possibly other haemoproteins have biological relevance in the two electron reduction of N-oxides to their respective amines. As will be discussed later this has potential in generation of stable and persistent reduction products of N-oxide prodrugs that can be activated in chronic (diffusion limited) and acute (perfusion limited) hypoxic tumour tissue. For this reason cytotoxic DNA affinic agents have an intrinsic advantage as antitumour agents in that binding to DNA results in accumu-

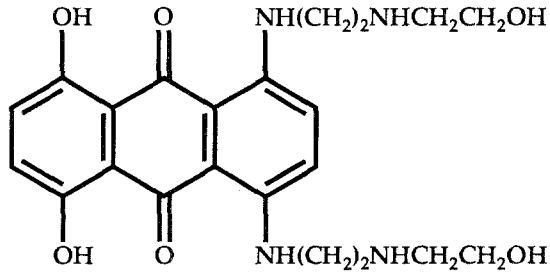
lation of drug in the nucleus i.e. the site of action. Furthermore such binding slows removal of drug by passive diffusion from the target cells. For this reason DNA binding agents in clinical use are extremely potent. Unfortunately the severe side effects of these agents, in particular myelosuppression, often limits the dose administered which therefore may not be sufficient to overcome tumour resistance. This situation could be improved using prodrug DNA affinic agents which, in principle, should possess low systemic toxicity but undergo site-specific conversion within hypoxic tumour regions to generate, locally, the active compound. In this respect a knowledge of N-oxide functionality bioreduction and the importance of the cationic nature of all agents that bind to DNA by intercalation can be combined to rationalise N-oxides as prodrugs of DNA binding agents. Studies with an aliphatic nitroacridine N-oxide have been described. This compound is required to undergo both N-oxide reduction and nitro reduction to generate a DNA binding derivative with potential as a 'conventional' bioreductive agent [45] (as discussed in section 1).

4. Anthraquinone antitumour agents as candidates for N-oxide prodrugs

Detailed cell free and studies *in vitro* have provided convincing evidence that the cytotoxicity of alkylaminoanthraquinones is as a result of their high affinity for DNA, a process that results in distortion of the DNA double helix and is associated with chromatin aggregation and compaction. These distortions give rise to inhibition of DNA and RNA polymerases and stabilisation of topoisomerase I- and II-DNA cleavable complexes (see section 4.2). In particular, the slow dissociation from DNA of dihydroxyanthraquinones, as exemplified by mitoxantrone, is an important factor in the potent cytotoxicity of these compounds (see section 4.4).

4.1. Interaction of cytotoxic anthraquinones with DNA

Since the introduction of the anthracyclines into the clinic there has been intense efforts to develop other agents that possess the broad spectrum of activity of doxorubicin, but with less systemic toxicity and lack of cross resistance. It was soon apparent from mechanistic studies that DNA binding was a major component of the cytotoxicity of the anthracyclines and hence this was the focus of attention in the design of new agents. This heralded a new area of cancer chemotherapeutic agent design since rational development of wholly synthetic agents was shown to be possible.



5. Mitoxantrone

Early studies with synthetic 2-substituted-aminoalkylaminoanthraquinones showed these agent were excellent DNA binding agents [46, 47]. Subsequent work identified cytotoxic activity with 1,4-bis substituted aminoalkylaminoanthraquinones including mitoxantrone [48, 49]. These agents bind reversibly to DNA by intercalation, a process that involves insertion of the drug between DNA base pairs. This was first proposed from studies demonstrating anthraquinone mediated stabilisation of DNA to thermal denaturation [50]. Subsequently it has been established by DNA helix unwinding studies. These include drug induced elongation of linear DNA as observed by electron microscopy [51] decreased electrophoretic mobility of covalently closed DNA, [51], influence of topoisomerase I on drug-induced covalently closed DNA unwinding [52] and increased plasmid DNA viscosity [51, 52]. Computer graphic modelling indicates that there are two possible modes of intercalative binding for 1,4 substituted alkylaminoanthraquinones. Such studies show the chromophore either parallel to the DNA long axis with the 1,4 bis alkylamino side chains lying in each groove straddling the intercala-

tion site and/or the chromophore perpendicular to the DNA long axis with the two side chains in the major groove [53]. There is some evidence from computer modelling that mitoxantrone intercalation exhibits GC base pair preference [54] which is supported by some studies using DNA homopolymers showing preferential binding of mitoxantrone to GC rich sequences of DNA [55, 56] and DNA ase I footprinting studies [57]. It has been proposed from nmr studies on $d(CpGpCpG)_2$ oligonucleotide/mitoxantrone complexes that mitoxantrone favours the perpendicular mode of intercalation with its alkylamino side chains resting in the major groove [55]. In conclusion studies to date generally indicate that the planar, electron deficient anthraquinone chromophore is inserted perpendicular (preferably) or parallel to the electron rich base pairs in the hydrophobic interior of the DNA helix whilst the alkylamino cationic side chains stabilise the drug/DNA complex by electrostatic interactions with the deoxyribose phosphate backbone which has been described as a 'tunnel' of negative charge [56].

DNA/drug equilibrium binding constants for mitoxantrone and its analogues of around 10^6 M^{-1} [53, 58] demonstrate that these agents possess a high affinity for DNA and the rate of drug/DNA dissociation is of importance in rationalising the cytotoxic activity of the alkylaminoanthraquinones [56, 59, 60]. In this respect studies have shown that the 5,8 hydroxy groups in the mitoxantrone chromophore contribute a 3–4 fold slowing of the dissociation of this drug from DNA, especially GC-rich sites, compared to ametantrone, where the chromophore hydroxy groups are absent [56]. This apparent GC preference is likely to be as a result of slow dissociation from GC sites, due to a slower rate of opening of GC sites, compared to AT sites [61]. It is suggested that the bulkiness of the hydroxy groups hindered disengagement of mitoxantrone hence slowing the dissociation process [56]. In addition 5,8 dihydroxy groups undergo intramolecular hydrogen bonding with the central carbonyl oxygens which increases the lipophilicity of mitoxantrone compared to ametantrone. This will favour retention of the mitoxantrone chromophore in the hydrophobic interior of the DNA helix. In contrast, alteration in the distance between chromophore and the terminal cationic amino functionality has little effect on the dissociation rate of a series of 1,4 alkylaminoanthraquinones, including mitoxantrone, from DNA [56]. Bis-substitution of the anthraquinone is also essential since monosubstituted anthraquinones are poor DNA intercalators [53] and possess rapid dissociation rates from DNA [59].

4.2. Inhibition of topoisomerases and polymerases by cytotoxic anthraquinones

The intercalation process effectively provides a mechanism for targeting double stranded DNA and RNA. It follows that prevention of DNA and RNA synthesis, presumably by inhibiting RNA and DNA polymerase(s) activity would be a likely mechanism of anthraquinone cytotoxicity. This has been substantiated for mitoxantrone using cellular systems to monitor inhibition of nucleic acid incorporation [48, 62] and flow cytometry of total DNA and RNA studies in whole cells [63] and purified *E. coli* RNA polymerase [64]. Mitoxantrone treated cells accumulate in G2 phase consistent with its inhibition of DNA synthesis [63] and promotes condensation and compaction of chromatin [65]. It is also observed that cellular distribution of mitoxantrone favours the nucleolus, which is rich in HnRNA [58]. Furthermore, mitoxantrone can cause the accumulation of cellular RNA despite a block on RNA synthesis. This is suggested to be as a result of mitoxantrone preventing cellular processing of HnRNA and rRNA [58].

From early studies on the actions of mitoxantrone it was apparent that in addition to its effect on RNA and DNA processing, this compound also elicits protein associated DNA single and double strand breaks [62, 66, 67]. The origin of these breaks was not clear initially but it became apparent that they were associated with inhibition of DNA topoisomerase Type II [68] and later Type I [69]. This strand breakage, which is only revealed in cellular DNA after treatment with strong protein denaturation, is often interpreted as the end event associated with trapping of a topoisomerase-DNA cleavable complex [70]. In essence, the presence of mitoxan-

trone causes an inhibition of topoisomerase catalysed DNA strand passing by stabilising the topoisomeras-DNA pre- and/or post strand -passing complex. How this is related to mitoxantrone (and other intercalator) mediated lethal events in tumour cells is not clear but is likely to involve a DNA-intercalated drug induced DNA-enzyme covalent complex. This gives rise to a disturbance of topoisomerase function which is the essential factor and not trapping of the cleavable complex *per se*. With respect to disturbance of function, Topoisomerase II is more highly expressed in actively dividing cells and is generally believed to be involved in decatenation, unknotting and unwinding of DNA during the replication process. Topoisomerase I is found tightly complexed to RNA polymerase I where it is likely to facilitate supercoil relaxation during transcription (reviewed by [71, 72]). Mitoxantrone has been shown to bind persistently to nucleic acids (and cytoplasmic proteins) [73, 74] and following removal of cells from exposure to mitoxantrone, continued depression of DNA synthesis and the persistence of mitoxantrone induced topoisomerase II DNA complexes is observed [75, 76]. It has been proposed that the potent cytotoxicity of mitoxantrone is as a result of the persistent binding of mitoxantrone to DNA, which favours long-term trapping of topoisomerase complexes [76], and cytoplasmic sequestration [73, 77]. Consistent with this, mitoxantrone-resistant P388 cells contain a reduced level of tightly bound or non-exchangeable drug [78].

4.3. Free radical generation and anthraquinone cytotoxicity

The activation of the anthraquinones in biological systems is as a consequence of electron transfer processes via an anthraquinsemiquinone free radical a process that is related to the one electron reduction potential at pH 7.0 (E^{1}_{7}) of these compounds. This process is catalysed by the same one-electron donating reductases described for reduction of certain N-oxides (section 3), principly cytochrome P450 reductase and NADH dehydrogenase [79]. The process involves (i) one-electron transfer to generate the semianthraquinone directly [eq. 1] or via a dismutation/comproportionation equilibrium between semianthraquinone ($AQH^{\cdot -}$) and hydroanthraquinone (AQH_2) as illustrated by [eq. 1, 2, & 3] or (ii) direct two-electron transfer to generate the hydroanthraquinone [eq. 3] with concomitant comproportionation to the semianthraquinone [eq. 3]. Protonation of the semianthraquinones generated in these reactions will depend on their respective pKa values and the pH of the reaction medium.

$$AQ + e^{-} + H^{+} \rightarrow AQH^{\cdot -} \quad [1]$$

$$AQH^{\cdot -} + e^{-} + H^{+} \rightarrow AQH_2 \quad [2]$$

$$AQ + AQH_2 \rightarrow 2AQH^{\cdot -} \quad [3]$$

The generation of free radicals by anthraquinones has received considerable attention and, by analogy with the anthracyclines, is suggested to rank alongside effects on DNA and RNA processing as a mechanism of action. Studies have shown that certain alkylaminoanthraquinones do undergo redox cycling in MCF-7 cells, a cell line well characterised for its propensity for anthracycline free radical formation [82]. This results in consumption of NADPH and concomitant formation of superoxide anions and hydroxyl radicals [80]. Importantly, neither mitoxantrone or ametantrone generate detectable free radicals in MCF-7 cells but are more cytotoxic than doxorubicin in this cell line and is suggested that the 1,4 substitution pattern of mitoxantrone and ametantrone agents renders them poor substrates for one-electron reductases [81]. This is in contrast to other alkylaminoanthraquinones with identical side arms in the 1 substituted, 1,5 or 1,8-positions which do undergo redox cycling [81, 83]. Furthermore, although mitoxantrone and ametantrone are also substrates for purified DT-diaphorase, an obligate 2-electron donating enzyme [84], there is no evidence that this results in toxicity in MCF-7 cells.

It can be concluded that reduction of the clinically relevant anthraquinones, mitoxantrone and ametantrone, does not contribute to their cytotoxic potential. Although the one electron reduction of mitoxantrone and ametantrone is not likely to con-

tribute to the actions of these anthraquinones, oxidative activation cannot be ruled out. Activation of mitoxantrone by horseradish peroxidase has been shown to result in one- and two- electron oxidation products [85] which are sufficiently reactive to covalently bind [86] and damage DNA [87]. Although not proven, it is possible that cellular peroxidases such as myeloperoxidase and prostaglandin endoperoxidase could oxidatively activate mitoxantrone to cell damaging species.

4.4. Structure activity considerations for selection of anthraquinones for use as N-oxide prodrugs

Mitoxantrone is 10-fold more cytotoxic than ametantrone *in vitro* and has a broader spectrum of activity *in vivo* [62, 88]. The presence of 5,8 hydroxy groups on mitoxantrone but not ametantrone is responsible for this increase in activity and is consistent with the slow dissociation rate of mitoxantrone from DNA [56] and long term trapping of DNA-topoisomerase complexes by this agent [76]. The rigorous structural requirements for maintaining optimal antitumour activity in this class of compound is further illustrated by structure activity studies which have shown clearly that the 1,4-bis substituted $HN(CH_2)_nNR'R$ side chains are more active than any other functionality *in vitro* including $HNCO(CH_2)_nNR'R$ [89] and $HN(CH_2)_2O(CH_2)_2OH$ [90]. Specifically the $HN(CH_2)_2NR'R$ spacer group has maximum activity compared to compounds where the methylene bridge spacer group is greater than 2 [50, 62, 88]. Furthermore whilst the terminal nitrogen retains some activity as a primary (NH_2) or tertiary nitrogen ($N,N(CH_3)_2$; $N,N(CH_2CH_3)_2$ optimal activity is often observed with a secondary ethanolamine ($NH(CH_2)_2OH$) [50, 62, 88] as is found in mitoxantrone. Since a tertiary amine functionality is prerequisite for N-oxide formation, unfortunately mitoxantrone cannot be converted to an N-oxide based prodrug. However, 1,4-bis{[2-(dimethylamino)ethyl]amino}5,8-dihydroxyanthracene-9,10-dione (AQ4) (see section 5) is a tertiary amine which has activity *in vitro* similar to mitoxantrone [91] and is also active *in vivo* against P388 ascites fluid implants in mice [88]. AQ4 also has similar DNA binding characteristics and topoisomerase II inhibition to mitoxantrone (see Table 1).

5. Activity of 1,4-bis{[2-(dimethylamino-N-oxide) ethyl]amino} 5,8-dihydroxyanthracene-9,10-dione (AQ4N), a bioreductively activated di-N-oxide prodrug DNA binding agent

The lead compound synthesised as an aliphatic N-oxide prodrug analogue of mitoxantrone was 1,4-bis{[2-(dimethylamino-N-oxide)ethyl]amino} 5,8-dihydroxyanthracene-9,10-dione (**6**) (AQ4N) [92]. The rational for preparing an N-oxide of **7**, a dihydroxyanthraquinone, is given in section 4.4. AQ4N is inactive aerobically (EC90 > 10 μM, Fig. 2) against V79 cells *in vitro* consistent with lack of measurable binding to DNA and is 25–50 fold less active as a topoisomerase inhibitor (Table 1). The activity of AQ4N against V79 cells is significantly increased in the absence of air (up to 1000 fold in the presence of a rich source of cytochrome P450). AQ4N is metab-

Table 1. DNA binding, topoisomerase II inhibition and cytotoxicity of the aliphatic di-N-oxide cytotoxic anthraquinone, AQ4N and the authentic reduction product, AQ4

compound	$K \times 10^6$ M^{-1} (n)[a]	Δ Tm[b] °C	Topo II inhibition[c] (μM)	EC_{90} V79 cells[d]
AQ4N (N-oxide)	nd	1	>50	>10 μM
AQ4 (reduction product)	3.3 (0.21)	29	2	10 nM

nd = not detected. [a] Drug/calf thymus DNA binding constant. See [53] for method. [b] Difference in calf thymus DNA melting temperature in presence and absence of drug. See [53] for method of determination. [c] Concentration of drug causing total inhibition kDNA decatenation by V79 cell topoisomerase II (nuclear extract). [d] reference [91].

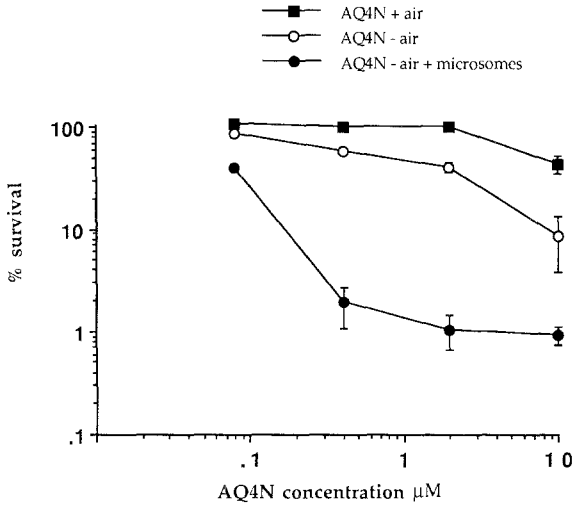


Fig. 2. Effect of AQ4N on V79 cell survival. Cells (5×10^4 ml^{-1}) were grown aerobically for 24 h and then treated with AQ4N for 2.5 h – ■ – aerobically, – ○ – under nitrogen – ● –, under nitrogen in the presence of NADPH (5 mM) supplemented rat liver microsomal fraction (10 mg ml^{-1}). Drug containing medium was then exchanged for fresh medium and the cell grown for a further 4 days aerobically prior to Coulter counting.

olised in rat liver microsomes under nitrogen to the reduction product 1,4-bis{[2-(dimethylamino-ethyl]amino} 5,8-di-hydroxyanthracene-9,10-dione (**7**) (AQ4) (Fig. 3) in a process that is inhibited by air, CO and involves NAD(P)H dependent cytochrome P450 (results not shown). Several studies *in vivo* using rodent hypoxic tumours are encouraging. Against a murine colon adenocarcinoma (MAC26) in NMRI mice, AQ4N (50 mg/kg i.p.) was effective only when administered 5 minutes following hydralazine (10 mg/kg i.v.). This treatment resulted in a tumour growth delay of 7 days at a relative tumour volume (RTV) of 5. Against MAC16, AQ4N (50 mg/kg i.p.) markedly slowed tumour growth over 14 days in combination with thiotepa (10 mg/kg i.p.) (RTV = 1.4± 1.0) compared to untreated tumours (RTV = 7.0± 3.7). These results support the use of AQ4N as a bioreductive prodrug that is effective against tumours with a well-defined hypoxic fraction where AQ4N is anticipated to be bioreduced to AQ4 a persistent, oxygen insensitive DNA binding agent.

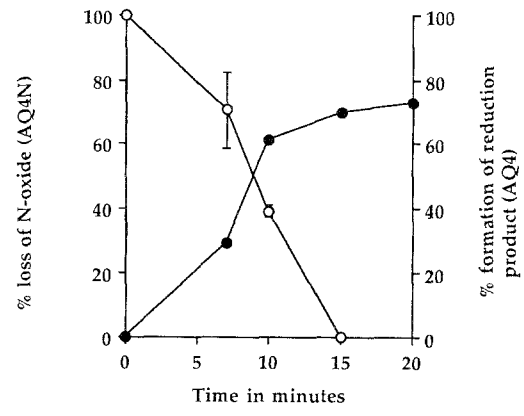
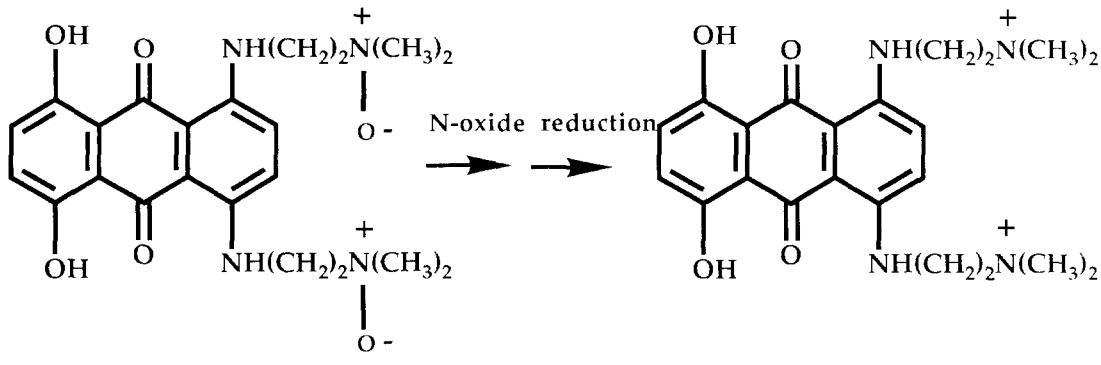


Fig. 3. Reduction of AQ4N in rat liver microsomes. – ○ – Loss of AQ4N (50 μM) and – ● – formation of the reduction product AQ4 in NADPH supplemented rat liver microsomes incubated under nitrogen. No loss of N-oxide was observed in the presence of air. AQ4N and AQ4 determined by high performance liquid chromatography. Less than 100% recovery of AQ4 was observed due to binding of this product to microsomes.

6. Conclusions

The reduction of a variety of N-oxide containing agents including certain cytotoxic compounds can be attributed mainly to NAD(P)H dependent cytochrome P450. This enzyme mediates a two electron reduction of N-oxides that, crucially, is inhibited in air (i.e. is oxygen sensitive) but rapidly turns over in the absence of dioxygen. Importantly, cytochrome P450 (or more likely different P450 isoforms) is able to effect the reduction of structurally dissimilar N-oxides to their respective amines. Other haemoproteins may also mediate this process. N-oxides as prodrugs of cytotoxic agents include benzotriazine di-N-oxide and probably indicine N-oxide and nitromin. However these agents, suffer from a lack of specific targeting to DNA, their anticipated site of action. In addition, the reduction product of benzotriazine N-oxide is oxygen sensitive and hence must be retained in the hypoxic tumour region to maintain a cytotoxic effect.

The alkylaminoanthraquinones are a group of cytotoxic agents with DNA binding affinity and are exemplified in the clinic by mitoxantrone, a 1,4 bis-substituted hydroxyanthraquinone. Mitoxantrone and other anthraquinones intercalate into DNA (and double stranded RNA) and inhibit both DNA and RNA polymerases and topoisomerase Type I



6 AQ4N

7 AQ4

and II. In contrast there is no convincing evidence that reductive activation to generate free radicals is involved in the activity of the 1,4-bis substituted alkylaminoanthraquinones. Considerable attention has been focused on mitoxantrone mediated topoisomerase II inhibition since this event is considered lethal to dividing cells. Intercalation is a crucial part of topoisomerase inhibition by DNA affinic agents and rate of dissociation of the anthraquinones from DNA is considered to most influence cytotoxicity of these agents. Hydroxy groups in the anthraquinone chromophore, exemplified by mitoxantrone, is shown to be more important than subtle changes in the cationic alkylamino side chain. Hence although HN $(CH_2)_2$ NR'R side chain is optimal for activity R can be H, alkyl or ethanol with an absolute preference for R'= H, R= $NH(CH_2)_2OH$ (i.e. mitoxantrone side chain). Although such changes will alter the overall lipophilicity and H-bonding properties of the molecule the cationic nature is preserved.

N-oxides of alkylaminoanthraquinones are formally electrically neutral and therefore are poor DNA binders and topoisomerase II inhibitors. However such N-oxides have promise as prodrugs. A lead compound, 1,4-bis{[2-(dimethylamino-N-oxide)ethyl]-amino}5,8-dihydroxy-anthracene-9,10-dione (AQ4N) has no intrinsic binding affinity to DNA and has low cytotoxicity and yet in the absence of air AQ4N can be reduced quantitatively *in vitro* to AQ4, a DNA affinic agent with up to 1000 fold increase in cytotoxicity. Importantly the reduction product, AQ4, is stable under oxic conditions. Studies *in vivo* indicate that AQ4N is more toxic under conditions that promote transient hypoxia and/or diminish the oxic tumour fraction. The advantage of utilising the reductive environment of hypoxic tumours to reduce N-oxides is that, unlike conventional bioreductive agents, the resulting products will remain active even if the hypoxia that led to bioactivation is transient or the reduced compounds once formed diffuse away from the hypoxic tumour regions. The DNA affinic nature of the reduction product of the N-oxide will ensure localisation in surrounding tumour tissue. This concept, in principle, can be extended to any DNA affinic agent with a tertiary amine functionality. Currently under investigation are N-oxides of other DNA affinic anthraquinones as well as anthrapyrazoles [93] and anthracenes [94].

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