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The Molecular Pharmacology of Doxorubicin *in vivo*

DOXORUBICIN is one of the most valuable anticancer drugs in present clinical use. Its effectiveness, however, remains restricted on two fronts: dose intensity due to acute bone marrow toxicity which has only partly been ameliorated by the introduction of bone marrow stem cell colony stimulating factors and dose frequency because of the development of drug resistance and cardiotoxicity. In this article we will focus on the drug's *in vivo* molecular pharmacology from the point of view of antitumour activity. Drug resistance has been comprehensively treated in recent reviews [1] and there is now good agreement that cardiotoxicity is mediated via the formation of drug and oxygen derived free radicals generated in the heart which results in peroxidation of lipid biomembranes and disruption of cellular organelle structure and function [2]. A single explanation for antitumour activity (if indeed one exists) has proved elusive despite over 20 years of intensive research. Four mechanisms which could produce tumour cell cytotoxicity have emerged to the fore: which of these have *in vivo* or clinical relevance and operates at clinically achievable drug concentrations is the subject of the remainder of this commentary.

DNA INTERCALATION AND TOPOISOMERASE II

Doxorubicin binds spontaneously with high efficiency to DNA (Kbapp 2.2×10^{-6M}; binding sites per nucleotide $n = 0.28$) by a mechanism termed intercalation where the planar drug molecule inserts between and binds in parallel to the base pairs of DNA non-covalently [3]. Intercalation is believed to cause the observed inhibition of DNA replication and RNA transcription that follows treatment of cells with the drug. Drug binding is kinetically a multi-stage process which whilst appearing to be a non-specific event shows some sequence specificity for CpG steps and transcriptionally active regions of DNA [4]. Drug complexes are stabilised by at least three binding forces: hydrophobic interactions, hydrogen bonding to the phosphate backbone of DNA through a molecule of water and insertion of the drug amino sugar group into the minor grove of DNA. This gives rise to a long residence time which appears to be the critical factor between an intercalator with antitumour activity and one without. In living cell nuclei more than 99.8% of doxorubicin is present in a DNA-bound form [5] and in human tumour biopsies after a dose of 25 mg/m^2 administered peroperatively this value is over 80% [6] implicating strongly DNA as the major locus for the clinical activity of the drug.

Doxorubicin induces specific single strand and double strand protein associated breaks in DNA that occur at drug concentrations in the region of 0.5–2.5 μmol/l, well within the clinically relevant range [6]. The protein associated with these breaks has subsequently been shown to be topoisomerase II and the damage to be catalysed by the enzyme itself [7]. Topoisomerase II is now known to be a common target for many families of DNA intercalating anticancer drugs such as acridines and ellipticines, as well as the anthracyclines, the chemical class to which doxorubicin belongs, including members such as AD32 which does not even bind to DNA, and 5-iminodaunorubicin, the non-free radical generator (for review see [8]).

The main biological function of topoisomerase II is its adenosine triphosphate (ATP) dependent DNA strand passing activity important for replication fork movement and segregation of daughter cell chromosomes, although it also performs a range of other DNA transactions such as supercoiling. The enzyme (170 kD homodimer) binds to both strands of DNA and breaks open each by forming a transient covalent bond with the 5′-phosphoryl end of the broken strand and a tyrosine residue of each subunit of the protein (known as the cleavage complex), thus allowing another DNA molecule to pass through. Doxorubicin at cytotoxic and clinically relevant concentrations stabilises the cleavage complex. At higher concentrations doxorubicin inhibits completely strand passing activity but once the drug has been removed from DNA, the inhibition of topoisomerase II is reversed. Protein binding appears to be a prerequisite for topoisomerase II inhibition; ethidium bromide, although being an intercalator, does not inhibit the enzyme nor does it display antitumour activity. When cells enter division topoisomerase II levels increase rapidly throughout the genome and its altered regulation in tumour cells provides a rationale for the tumour selectivity of topoisomerase II poisons. Three factors inhibit the enzyme: high salt concentration, low temperature and high drug concentrations, and all are known to modulate the cytotoxicity of doxorubicin. Although there has been considerable debate whether anthracycline cytotoxicity correlates with DNA–protein associated damage, evidence is increasing for the involvement of topoisomerase II. Stabilisation of the cleavage complex alone is probably not sufficient to produce cell death and other as yet unidentified factors must also be involved.

ENZYME CATALYSED AND IRON MEDIATED FREE RADICAL FORMATION

Handa and Sato first showed in 1975 *in vitro* that doxorubicin/daunorubicin could be converted into a semiquinone drug free radical by NADPH dependent one electron reduction and that this or a similar process occurred in intact Ehrlich ascites cells [9]. The semiquinone can transfer its free electron to molecular oxygen, returning the drug back to native doxorubicin and producing a cascade of oxygen derived free radicals by a continual process termed redox cycling. Reactive oxygen species are potentially harmful to cells through peroxidation of lipids and chemical degradation of DNA. Since these early observations, a large body of at times seemingly contradictory data has been published on the free radical chemistry of doxorubicin and its biological relevance. A detailed account of these data is beyond the scope of this commentary but the interested reader is referred to excellent reviews [2, 10, 11]. Briefly, once formed the semiquinone will immediately and preferentially react with molecular oxygen (O_2) (rate constant $k = 10^8$ mol/l/s). The rate limiting step here is the relatively slow formation of the semiquinone

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through bioreduction, as a result of an unfavourable reduction potential of −300 mV. Hence, the inherent bioreductive capability of a cell line or tissue will determine whether free radicals are produced in biologically relevant amounts. In the absence of oxygen, two semiquinone molecules will rearrange by disproportionation to form one molecule of native doxorubicin and one molecule of the fully reduced hydroquinone form of doxorubicin with an equally high rate constant (10^7–10^9 mol/l/s). These properties confer upon the doxorubicin semiquinone free radical a probable biological half life of 10^{-7} s and a maximum ability to diffuse 0.6 μm within the cell. Oxygen is reduced to the superoxide radical $O_2^{\cdot-}$, which in its own right is non-toxic but can generate toxic species by first reacting with two protons ($2H^+$) to form hydrogen peroxide (H_2O_2). Hydrogen peroxide can then be reduced to the hydroxyl radical ($OH^{\cdot}$) either by the superoxide radical itself in the presence of catalytic amounts of iron (the Haber Wise reaction), by reduced iron Fe^{2+} (the Fenton reaction), or under low oxygen tension similar to that found in tumour cells by the doxorubicin semiquinone ($k = 10^4$ mol/l/s). The hydroxyl radical is a short lived reactive species which can damage DNA through proton abstraction and can lead to the peroxidation of lipids but must be produced in a site-specific manner (because of its high reactivity) to be effective. It is, more than likely, the ultimate damaging species in doxorubicin free radical formation. The deleterious effects of oxygen radicals are abrogated by an array of cellular defense mechanisms: superoxide dismutase which converts $O_2^{\cdot-}$ to hydrogen peroxide; catalase and glutathione peroxidases which convert H_2O_2 to water and molecular oxygen as well as a series of endogenous free radical scavengers such as vitamin E. Along with reductive capability, cellular defenses will dictate the chemosensitivity of a cell line or tissue to free radicals. No specific enzyme has been discovered which acts as a doxorubicin quinone reductase, however several accept the drug as a substrate for one electron reduction: NADPH cytochrome P-450 reductase in endoplasmic and sarcoplasmic reticulum; NADH dehydrogenase in mitochondria; xanthine oxidase in the cytoplasm and an unidentified component in nucleii.

For the free radical mechanism to have clinical relevance, at clinically and biologically relevant drug concentrations the following set of criteria would have to be satisfied *in vivo*: (a) identification of hydroxyl radicals being produced in a site specific manner, (b) detection of damage consistent with free radical formation and (c) antagonism of cytotoxicity by free radical scavengers and detoxification enzymes. Topoisomerase II has satisfied an analogous set of criteria; however, with free radicals the evidence is much less convincing. Intercalated doxorubicin cannot undergo enzyme catalysed quinone reduction due to inaccessibility, thus ruling out site specific free radical generation close to DNA although an alternative may be open to the drug by complexing iron. Although $OH^{\cdot}$ has been detected in sensitive MCF-7 cells by electron spin resonance (ESR), a doxorubicin concentration of 330 μmol/l (1000 higher than the IC_{50} and two orders of magnitude higher than clinically achievable concentrations) was required and here the majority of radicals were detected outside cells [12]. Since ESR is capable of measuring down to 10^{-8} mol/l free radicals it is clear that only a very small fraction of doxorubicin may have been converted to the semiquinone. More recent studies have failed to detect $OH^{\cdot}$ in MCF-7 cells and showed that $O_2^{\cdot-}$ is produced in the extracellular space after diffusion of the semiquinone form inside cells [13]. Non-protein associated DNA breaks, consistent with radical damage were only observed at concentrations above 2.8 μmol/l in L1210, well in excess of the IC_{50} [14]. At drug concentrations below 2.8 μmol/l only protein associated breaks of the type produced by topoisomerase II were evident. Results from studies with free radical scavengers are more equivocal. In certain cell lines (MCF-7 and Ehrlich ascites) they antagonise cytotoxicity consistent with a free radical mechanism being important; in other cell systems (A2780) they do not. It has been argued that MCF-7 and Ehrlich ascites are not representative of the *in vivo* situation in that Ehrlich ascites express low levels of catalase and glutathione dependent enzymes which protect against radicals and MCF-7 expresses unusually high levels of the reducing enzymes which produce the free radicals in the first place [2]. Taken overall, a minor role is anticipated for free radicals in the *in vivo* mechanism of action of doxorubicin.

Doxorubicin can also generate oxygen radicals by complexation of iron at C11 and C12 to form a doxorubicin$_3$-Fe^{3+} chelate. Redox cycling analogous to enzyme linked quinone reduction occurs but with the complexed iron acting as the catalyst. Fe^{3+} can be reduced both enzymatically, and non-enzymatically by either cellular thiols or by intramolecular autoxidation of the doxorubicin B ring hydroquinone. The stability of the complex has been reported to be as high as $10^{33.4}$ [15] explaining why doxorubicin has the ability to sequester iron from protein bound sites. Doxorubicin$_3$-Fe^{3+} binds tightly to DNA but unlike native doxorubicin not only retains the ability to generate free radicals actually has its ability to produce H_2O_2, $OH^{\cdot}$ and DNA damage enhanced [16]. Chelation of iron by doxorubicin results in a characteristic spectral shift in its chromophore making the complex easily detectable spectrophotometrically. Nevertheless, no evidence has been reported of the complex being present in clinical specimens. Also, autoxidation produces a 9-COOH metabolite which again has never been reported *in vivo* despite many clinical pharmacokinetic studies. Recently the effective binding constant K_{eff} has been shown to be strongly pH dependent with a value of $10^{16.2}$ mol/l at pH 7.4 [17]. This means that at clinically achievable doxorubicin concentrations the drug will not bind adventitious iron and a preformed doxorubicin$_3$-Fe^{3+} chelate will dissociate upon drug administration.

COVALENT BINDING TO DNA

Three different routes have been postulated for doxorubicin covalent binding to DNA: aerobic quinone reduction (as above) and direct addition of the semiquinone free radical (or an unidentified species) to form possible DNA crosslinks; anaerobic quinone reduction to a quinone methide aglycone or a C-7 centered radical aglycone reactive intermediate (classic bioreductive alkylation); and chelation of iron followed by binding of the ternary complex to DNA. Low levels of covalent binding (picomoles/100 μg DNA) occur with both proteins and DNA under aerobic conditions but non-physiological concentrations of doxorubicin are required: 50 μmol/l for DNA binding in MCF-7 cells; > 10 μmol/l for crosslinking in Hela S_3 cells (EC_{50} 0.044 μmol/l; 1 mmol/l with rat hepatic nuclei and 100 μmol/l for covalent binding to proteins. That doxorubicin could possibly function as an anaerobic bioreductive alkylating agent was first proposed by Moore on theoretical chemical grounds [18]. In a series of papers published in the early 1980s experimental verification was provided for this hypothesis by Sinha [19]. Either chemical reducing agents or NADPH enriched liver microsomes produced under anaerobic conditions, and only under anaerobic conditions, covalent binding as high as 1 adduct per 12 nucleotides to DNA and 1 adduct per 60–65 nucleotides

to RNA. Covalent binding occurred with highest frequency with deoxyguanosine containing nucleotides but the chemical nature of specific adducts was never elucidated. These values are several orders of magnitude higher than all other similar studies which only detected covalent binding under aerobic conditions. Using quantitation of stable transcriptional blockage sites to measure DNA covalent adducts these earlier studies of Sinha have been recently corroborated [20]. However, important differences exist between the two sets of experimental conditions. In the later studies incubations were performed under oxic conditions and adduct formation was dependent on the presence of high concentrations of Fe^{3+} (75 μmol/l). The contribution of oxygen mediated damage was not addressed and this clearly complicates the interpretation of these results.

Anaerobic bioreduction of doxorubicin leads ultimately to a 7-deoxyaglycone metabolite by a process which was originally referred to as reductive glycosidic cleavage [21]. The chemical pathways of reductive deglycoslation although much better understood remain controversial. It has been proposed that the semiquinone degrades directly to a 7-deoxyaglycone via a C7 centred aglycone radical based on ESR detection of an immobilised signal consistent with a non-water soluble aglycone species [22]. Abdella and Fisher have argued convincingly on chemical grounds that a C-7 radical is not sufficiently reactive to alkylate DNA [10]. The majority of evidence and the mechanism favoured by the authors is that anaerobic bioreductive deglycoslation proceeds through a fully reduced hydroquinone intermediate formed either by 2 electron quinone reduction or more likely by disproportionation of the semiquinone and produces a quinone methide aglycone as an intermediate. The quinone methide has a half-life of several seconds (15 s) and is sufficiently long-lived to diffuse through the cell and alkylate DNA [23]. It exhibits both weak nucleophilic and weak electrophilic characteristics and preferentially reacts with a solvent proton to form the 7-deoxyglycone metabolite. However, it only has limited capability to react with sulphydryl groups on proteins and it is probably not sufficiently reactive to bind covalently to DNA, unless favourable site specific conditions were available. Thus, anaerobic bioreduction primarily results in drug inactivation to a 7-deoxyaglycone without the evolution of DNA covalent binding intermediates.

Whole animal experiments with protein microspheres incorporating anthracyclines lend support to this conclusion. In a system where anaerobic bioreduction of 4′-deoxydoxorubicin is stimulated by a large factor in the tumour itself, no enhancement of antitumour activity is seen (against a rat mammary carcinoma); in fact activity is actually reduced [24]. With doxorubicin-loaded albumin microspheres it has been reported that increased antitumour potency is associated with a stimulation of quinone anaerobic bioreduction [25]. Using a series of different protein matrices it is now clear that increased activity is a consequence of alterations in parent drug pharmacokinetics and not bioreduction which correlates with decreased drug potency [26]. In human tumours no evidence of quinone bioreduction has ever been detected [6] but clinical activity correlated well with the levels of parent drug. These data indicate that *in vivo* native doxorubicin is the most important determinant of antitumour activity and strongly suggest a mechanism of action not involving free radicals or DNA covalent binding species.

THE CELL MEMBRANE

The cell membrane and signal transduction are receiving increasing attention as targets in cancer chemotherapy. Native doxorubicin interacts with biomembranes due to an affinity for negatively charged phospholipids especially cardiolipin, the major constituent of mitochondrial membranes. As a result of this interaction many normal biochemical functions of membranes are altered. Indeed doxorubicin has been shown to be cytotoxic to L1210 cells without entering the cell when it was immobilised covalently on to aragose beads of a diameter too large to be internalised [27]. This mechanism proposes a specific interaction of the native drug with the bilayer not involving either free radicals or DNA. In an elaboration of this hypothesis Tritton has shown that subcytotoxic levels of doxorubicin promote growth in murine and human cancer cells and he concludes that at higher drug concentrations growth inhibition results as an over stimulation of the same cell membrane mediated signalling mechanisms normally responsible for growth promotion [28]. This notion may be over simplistic. In human ovarian cell lines, whilst subcytotoxic doses of doxorubicin did increase the production of IP_3 (a second messenger normally released in response to receptor mediated extracellular mitogenic factors) consistent with growth promotion, high drug concentrations did not overproduce IP_3 but actually decreased its release [29]. Presentation of cells with doxorubicin covalently bound to agarose beads may not be relevant to the normal cytotoxic processes produced by the native drug. 7R,9R-4-demethoxydaunorubicin is a completely inactive stereomer of doxorubicin, when coupled to polymeric supports it becomes a potent cytotoxic drug [30]. Subcytotoxic levels of doxorubicin are in the range of 10^{-8} to 10^{-10} mol/l which are actually several orders of magnitude lower than the concentrations of the drug seen by human tumours (10^{-6} to 10^{-7} mol/l) and here doxorubicin is not immobilised at the cell membrane but is bound to DNA [6]. In conclusion, present evidence still suggests, as originally thought, that DNA is the main intracellular target for doxorubicin with antitumour (cytotoxicity) being mediated via stabilisation of a topoisomerase II cleavage complex. Whilst doxorubicin can clearly generate free radicals in biological systems by several different mechanisms, concentrations well in excess of those clinically achievable are a prerequisite. So also for covalent binding to DNA. Finally, the cell membrane appears not to be the primary locus of action of doxorubicin, however, since second messengers can modulate the cytotoxicity of the drug they may play a role perhaps by phosphorylation of a critical intracellular target (topoisomerase II).

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