Topoisomerase Enzymes as Therapeutic Targets for Cancer Chemotherapy

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Abstract: The topoisomerase enzymes are essential for DNA metabolism, where they act to adjust the number of supercoils in DNA, a key requirement in the cellular processes of transcription and replication. Their enzymatic mechanism creates transient nicks (type I) or breaks (type II) in the double stranded DNA polymer, allowing DNA to be converted between topological isomers. Humans possess both types of topoisomerase enzymes, however the two types utilize very different enzymatic mechanisms. Both type I and type II topoisomerases have been identified as clinically important targets for cancer chemotherapy and their inhibitors are central components in many therapeutic regimes. Over the course of the last 30 years inhibitors with extensive structural diversity have been developed through a combination of drug screening and rational design programs. Simultaneously much emphasis has been placed upon establishing the mechanisms of action of both classes of topoisomerase enzyme. Crucial structural insights have come from the crystal structure of topoisomerase I, while modelling comparisons are beginning to map out a possible framework for topoisomerase II action. This review discusses these recent advances in the fields of enzyme mechanism and inhibitor design. We also address the development of drug resistance and dose-limiting side effects as well as cover alternative methods in drug delivery.

Key Words: Topoisomerase, inhibitor, camptothecin, topotecan, anthracycline, anthracenedione, drug design.

1. TOPOISOMERASES: A KEY TARGET IN CANCER CHEMOTHERAPY

DNA Topology and the Linking Number

DNA primary structure consists of a right handed double helix formed by two anti-parallel polynucleotide chains. Each nucleotide in the polymer is formed from a deoxy-ribose sugar unit substituted at the 1' position by one of four bases, the purines adenine and guanine or the pyrimidines cytosine and thymine. The nucleotides are linked together by phosphodiester bonds between the 3' and 5' position on contiguous sugars to form a chain. The sugar phosphate backbone runs along the outside of the double helical structure and the bases associate by hydrogen bonding interactions with an opposite partner on the complementary DNA strand to form the inner region of the double helix [1]. The intermolecular forces holding the two chains together consist of hydrogen bonds between each base and its complimentary partner and π-orbital overlap between adjacent base pairs, termed base-pair stacking. These intermolecular forces result in each complimentary strand winding around the other to form the helical primary structure of DNA. Structural variations in the DNA polymer are due to changes in the nucleotide sequence and arise as a result of interactions between adjacent base pairs. This gives rise to the helical repeat, or number of base pairs per turn [2]. As each chromosome is about 2 meters in length, in order to package the DNA inside the nucleus (6 μm diameter) several additional layers of higher order structure are required. The coiling of the DNA polymer about itself, a process termed supercoiling to differentiate it from the coiling of the individual complimentary strands about the helical axis, is therefore essential. However, for DNA metabolism to occur local changes in the level of DNA supercoiling are required to allow access to the stored genetic information [3].

A convergence of biochemistry and the mathematical study of topology has greatly enhanced our understanding of the biological processes behind these DNA transformations. Topology can be defined as the study of the properties of objects which are unchanged by manipulations such as bending, knotting and twisting. The number of times each single strand of DNA crosses its complimentary partner to form the double helix (helical repeat) and the number of coils of the double helix about the helical axis to form supercoils is considered to be topologically equivalent. This implies that the extent to which each single DNA strand can cross over its complimentary partner is fixed. The addition of supercoils to a length of DNA results in the formation of topological isomers (topomers), with the change in the number of supercoils corresponding to a change in the helical repeat [4]. This relationship is defined by the linking number (L), a measurement of the number of crossings or linkages between two strands of DNA. As crossings due to supercoiling (writhing number W) and crossings due to helical turns (twisting number T) are equivalent, the linking number is simply the sum of the two types of crossing, as given by equation 1 [4].

For a constrained system such as DNA *in vivo* the linking number is fixed, which has important biological ramifications. The processes of transcription and mitosis both introduce

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$$L = T + W$$

Equation 1. Topological relationship between supercoils and helical repeats in a constrained segment of DNA. L Linking Number (half the total number of strand crossings), **T** Twisting Number (the number of double helical turns), **W** Writhing Number (the number of supercoil crossings).

torsional strain on the helical structure resulting in the introduction of supercoils [5-6]. The cellular machinery cannot remove these supercoils from the chromosome by physically unwinding the DNA as this will increase the helical repeat, indicating that a compensatory mechanism is required. Nature has evolved an elegant solution to this problem by using a family of enzymes, the topoisomerases, to change the linking number of DNA during DNA metabolism and so regulate the levels of supercoiling within the chromosomal structure [7]. Topoisomerases are therefore essential for cell viability and cellular replication, with abnormal enzyme DNA interactions resulting in either a halt in the cell cycle or apoptosis (programmed cell death) [8].

Mechanism of Action of Topoisomerases

A topoisomerase allows the removal of DNA supercoils without adjusting the helical repeat, apparently contravening the relationship established in equation 1. This alteration in the linking number of the DNA can be accomplished by transiently breaking either one (a type I topoisomerase) or two (type II topoisomerase) of the DNA strands (Fig. **1**). Once a strand has been broken, the DNA is no longer held in a topologically constrained domain and the relationship between supercoils and helical repeat no longer applies. The number of supercoils in the structure can therefore be changed with no impact on the helical repeat. The enzyme then reseals the break, creating a new topologically constrained domain with a new value for the linking number [9]. A type I topoisomerase changes W in steps of one whereas a type II topoisomerase acts in steps of two.

Mammals possess one type I topoisomerase (topo I), a 100 kDa monomeric protein capable of relaxing positive and negative supercoils without the presence of an energy cofactor [10]. Topo I is expressed in cells at a constant level regardless of the stage of the cell cycle [11]. In keeping with its role as a regulatory protein, the activity of the enzyme is sensitive to post-translational modifications. Separate reports have shown both reduction and stimulation of activity *in vitro* when topo I is phosphorylated by tyrosine protein kinases and casein kinase II respectively [12-13]. Poly (ADP) ribosylation of topo I has also been shown to decrease the activity of the enzyme [14-15]. The enzyme functions *via* a covalent intermediate, termed the "cleavable complex,"

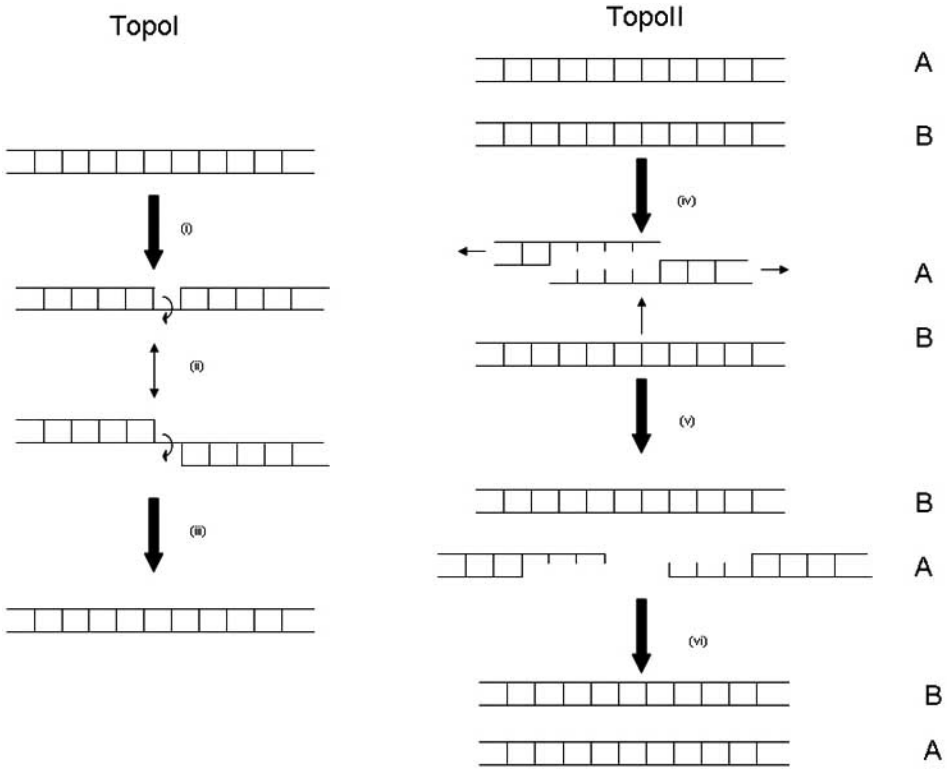

Fig. (1). Schematic Diagram of Topoisomerase Action.

Type I topoisomerases create a single stranded nick in one DNA strand (I). The DNA can then unwind by revolving about the remaining phosphodiester bond to remove supercoils (II). The enzyme then religates the break to restore the DNA with an altered linking number (III). Type II topoisomerases change the linking number by passing one DNA segment through a break in another segment. Initially the enzyme creates a double stranded break four base pairs apart in segment A (IV). The two halves of segment A are then pulled apart and segment B passed through the break (V). The enzyme then religates the double stranded break in segment A (VI).

where the enzyme had severed one strand of the phosphate backbone of the DNA by the nucleophilic attack of a tyrosine residue (Tyr^{723}), forming a covalent bond on the 3' side of the break and leaving a hydroxyl group on the 5' side [16].

The crystal structure of reconstituted eukaryotic topo I with an oligonucleotide substrate has been solved (Fig. **2**) [17], allowing a model for the enzyme's mechanism to be established. Topo I consists of four major regions: the N-terminus from residues 0-210, the core from 210-635, a linker domain between 636 and 712 and the C-terminus 713-765. The core and C-terminus are essential for activity and were included in the reconstitution [18]. Topo I features a positively charged central channel approximately 20 angstroms wide through which the DNA is threaded. The upper region of the enzyme consists of core subdomains I and II forming a "cap" over the DNA. The bottom region of the molecule consists of subdomain III of the core and the C-terminus. The two regions are connected covalently *via* two α helices on one side of the DNA and by electrostatic interactions between three residues on each of subdomains I and III on the opposite side, which together with a salt bridge form the "lips" of the enzyme (Fig. **2**).

To rationalize the enzymes activity with this structure it was hypothesized that the α helices connecting core subdomains II and III act as a hinge, allowing the enzyme to adopt either an open or closed state. In solution the enzyme is in an open state, with the "lips" no longer in contact with each other. The enzyme is then driven by charge interactions to clamp around the DNA, the two lips meeting to encircle the DNA and form the closed state. One strand of the phosphate backbone of the DNA is then broken by the attack of Tyr^{723} to form the covalent intermediate. The DNA is then only held together by a single strand and at the cleavage site is free to rotate about the remaining phosphodiester bond. Driven by the torsional strain inherent in the supercoiled state the DNA then unwinds, removing a supercoil with each cycle and so decreasing the free energy of the conformation [19]. At each unwinding stage the enzyme is able to religate the DNA by attack of the hydroxyl group on the 5' side of the break on the phosphate group covalently attached to Tyr^{723} to restore the phosphate backbone and regenerate the catalytic Tyr^{723}, bringing the unwinding action to a halt. The enzyme can then dissociate from the substrate, resulting in a change to the linking number of the DNA [20].

There are two isoforms of mammalian type II toposiomerase (topo IIα and topo IIβ), both of which are ATP dependent. Topo IIα is ubiquitous in all cell types, where it is essential for the unlinking of intertwined daughter duplexes during DNA replication and for DNA relaxation during transcription, whereas topo IIβ is non-essential and its role is at present poorly defined [21]. Type II topoisomerases function by making a double stranded break in one DNA duplex, passing another DNA duplex through the break and then religating the break (Fig. **1**). Topo IIα is known to be active as a homodimer, with three catalytic regions per subunit. Analogously to topo I the enzyme uses two tyrosine residues, one on each monomer, to make the double stranded break and form a cleavable complex. However unlike topo I the tyrosine residues attach at the 5' side of the break, leaving the free hydroxyl group at the 3' side [21].

The enzyme mechanism of eukaryotic type II topoisomerases is not as well established as type I due to the lack of a crystal structure. However a hypothetical mechanism has been suggested based on topo IIα's homology to the known structures of yeast topo II and the ATPase domain of *E. coli* GyrB [22-25]. The enzyme can be considered to consist of three domains that act as a series of linked gates. The enzyme is conformationally restricted so that at any stage of the catalytic mechanism only one gate may be open. In the resting state the N-gate (formed from the ATP binding domain) is open and the remaining two gates, the DNA and C-gates, are closed. The DNA segment that is going to be

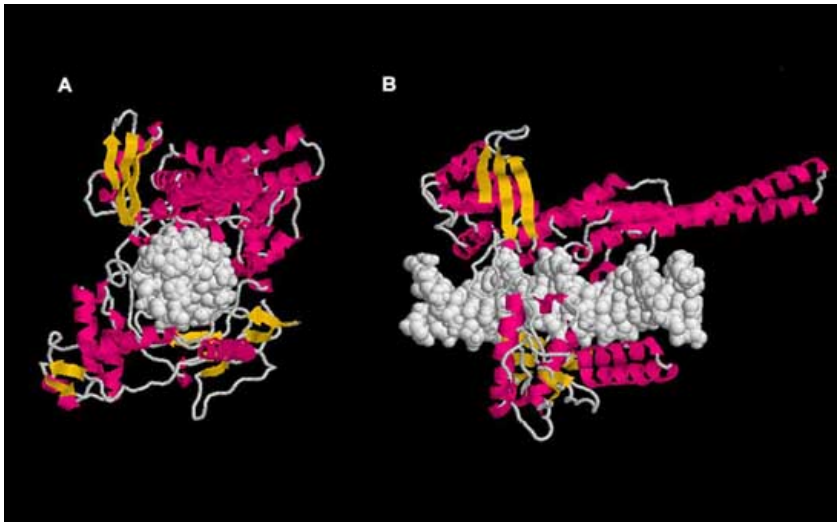

Fig. (2). The Structure of the Eukaryotic Reconstituted Topo I: DNA Complex.

The enzyme is depicted in cartoon format using the Rasmol graphics package (PDB ID 1A36). Structural elements are indicated by color: α helices (red); β sheets (yellow); regions of undefined structure and DNA (white). A: View parallel to the helical axis, B: View perpendicular to the helical axis [17,20].

cleaved (known as the gate or G segment) enters the open N-gate and is initially bound at a high affinity site just above the closed DNA gate. Upon ATP binding dramatic conformational change occurs, with the N-gate closing and the DNA gate opening. Simultaneously the G segment is cleaved by the nucleophilic attack of two tyrosine residues (Tyr^{804} for topo IIα [26]), forming 5'-phosphotyrosyl enzyme linkages on opposite strands of the G segment. The two fragments of the G segment then move apart to form a double stranded break. Additionally as the N-gate closes it forms a DNA binding site which can entrap a second DNA segment (the transfer or T segment). If the T segment is entrapped by the closing N-gate, it is now passed through both the break in the G segment and the open DNA gate to a high affinity DNA binding site in the enzyme's central cavity. The binding of the T segment to the central cavity again causes extensive conformational change, closing the DNA gate and religating the G segment while opening the C-gate. The T segment is then expelled at the opposite side of the enzyme to which it entered. Hydrolysis of ATP then opens the N-gate, closing the C-gate and allowing the G segment to dissociate and so complete the enzyme's catalytic cycle. If the T and G segments are part of the same molecule then this strand passing activity functions to remove supercoils, while if the segments belong to separate DNA strands then the action of type II topoisomerases can disentangle intertwined chromosomes.

2. STRUCTURE AND FUNCTION OF TOPOISOMERASE INHIBITORS

Topoisomerase inhibitors can be divided into two categories. Type I inhibitors are the most clinically utilized in cancer chemotherapy and act to stabilize the enzyme-DNA intermediate at the point when the enzyme has formed a break in the DNA. This intermediate is known as the cleavable complex and agents that stabilize the cleavable complex can be considered topoisomerase poisons, as they convert the enzymes' mechanistic cycle into a cytotoxic event. Formation of the cleavable complex is a reversible process as the enzyme's activity can be fully restored by dilution. A common structural motif for type I inhibitors is a planar aromatic ring system, allowing the inhibitor to insert and stack between the base pairs of DNA, a binding mode termed intercalation (Fig. **3**). Type II inhibitors are known as catalytic inhibitors and act at other points in the enzymatic cycle, for example by blocking the ATP binding site of topo II.

Type I Cleavable Complex Inhibitors

The only topo I inhibitors to have undergone clinical trials as antineoplastic drugs are Camptothecin (CPT) and its structural derivatives such as Topotecan (Fig. **3**) [27]. Originally isolated in the 1960's from *Camptotheca Acuminata* as part of a natural product screen, CPT demonstrated activity against L1210 and P388 leukaemia [28]. Initial clinical trials showed the drug to have severe side effects, myleosuppression and haemorrhagic cystitis being the dose limiting factors. Additionally the drug's water solubility is poor, and it has therefore not been extensively utilized in clinical practice. Development of CPT has focused on hydrophilic modifications, resulting in greater potency and improved water solubility. The synthetic derivatives of CPT Irinotecan (Camptosar) and Topotecan are FDA approved (Fig. **3**) [29]. It has proven technically

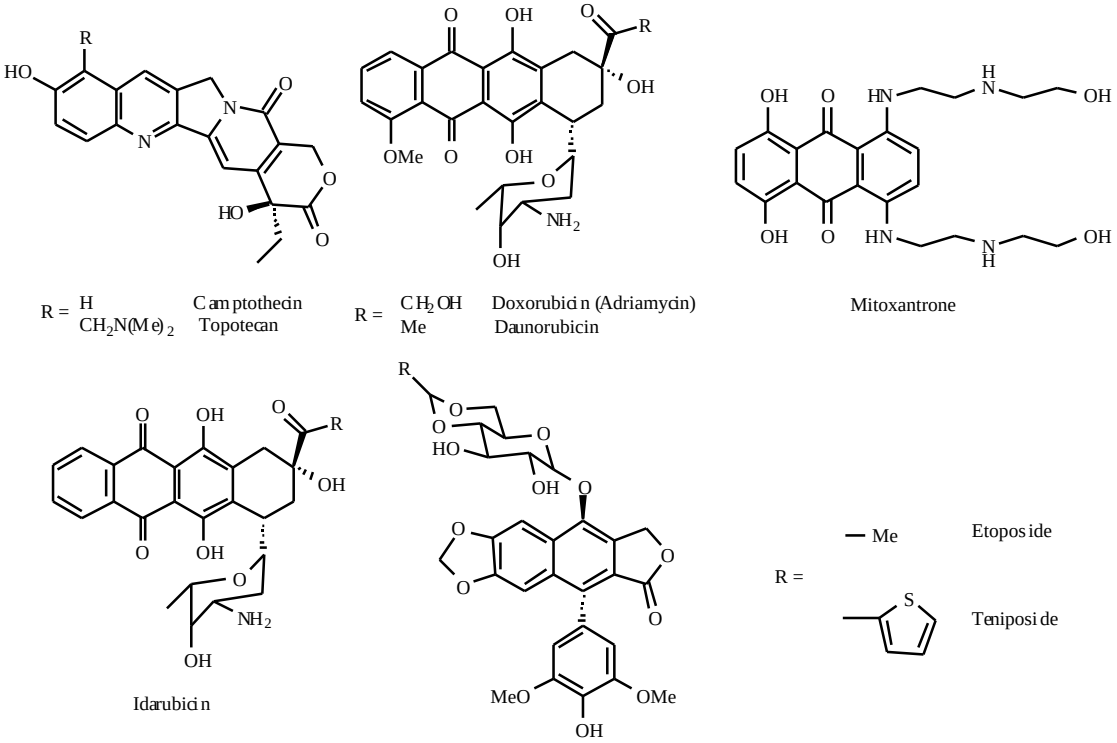

Fig. (3). Structures of Type I Topoisomerase Inhibitors.

challenging to model the mechanism by which CPT stabilizes the cleavable complex, as it is an uncompetitive inhibitor that only binds a transient enzyme-DNA complex. However recent crystallographic studies with Topotecan have demonstrated that the drug intercalates the nicked DNA substrate directly at the cleavage site after the attack of Tyr^{723} (Fig. **4**). Topotecan's planar aromatic ring system occupies a DNA base-pair site and is stabilized by base-pair stacking with both adjacent base-pairs. This intercalation displaces the downstream base-pair and moves the 5'-OH group away from the phosphorylated Tyr^{723} residue, preventing religation of the nicked DNA [30]. This binding mode also explains the drug's specificity for the cleavable complex, as Topotecan's binding site is only present after the initial DNA cleavage step of topo I's catalytic cycle.

Topo II inhibitors display great structural diversity and are widely used as antineoplastic agents. The topo II is a major target for antineoplastic agents used in the treatment of a wide range of malignancies including breast, lung and prostate cancer. Six topo II cleavable complex inhibitors: Etoposide, Teniposide, Doxorubicin (DOX), Daunorubicin, Idarubicin and Mitoxantrone have been approved for clinical use in the US by the FDA [29]. The first identified topo II cleavable complex inhibitor was Etoposide (VP-16), which gained FDA approval in 1983 [31]. Etoposide and its analogues Teniposide and Etoposide phosphate (a prodrug) are based upon structural studies of the podophyllotoxins, a family of natural products used in traditional medications to treat cancer [32]. The anthracyclines Daunorubicin (Daunomycin) and DOX (Adriamycin) (Fig. **3**) were originally isolated as antibiotics from *Streptomyces peucetius* but were shown to have potent antitumour activity in the 1960's [33-34] They have been shown to act as DNA intercalators and stabilize a topo II ternary complex [35-36]. In contrast Mitoxantrone (MTX) (Fig. **3**) was originally designed as a DNA intercalator as part of a rational drug development program to create DOX analogues [37-38]. Pharmacological studies confirmed that MTX binds to DNA *via* intercalation [39] and stabilizes the topo II cleavable complex. It is also worth noting that the acridine m-AMSA (4'-(9-acridinylamino)-methanesulfon-m-anisidide) although not in clinical use was one of the compounds originally utilized to link DNA intercalation with antitumour activity [40]. Due to the lack of a crystal structure for the enzyme, the information available regarding the potential binding sites of topo II inhibitors is mainly hypothetical. Additionally the extensive conformational change associated with the enzyme's catalytic mechanism makes computer modelling of the potential drug binding sites problematical. Nonetheless it has been shown for Etoposide that the drug must act at each of the two DNA cleavage sites for effective formation of double stranded breaks [41]. This has led to the proposal that DOX may act in a similar manner to Topotecan, intercalating between the base pairs at the cleavage site [42].

These structural developments have prompted a renewed interest in the rational design of topo inhibitors. Several strategies to design topo I inhibitors based on the anthraquinone scaffold have been examined. A range of anthraquinones *mono*- substituted with amino acids and dipeptides have been shown to act as topo I inhibitors (Fig. **5**) [43-45], with no evidence of topo II inhibition. These compounds showed moderate activity against tumour cell lines and a tyrosine analogue showed antitumour activity *in vivo* against HT-29 (colon cancer) and NXOO2 (non small-cell lung cancer) xenografts in mice [45]. We have followed up on these studies by demonstrating a solid phase approach to synthesizing anthraquinone conjugated peptides that has

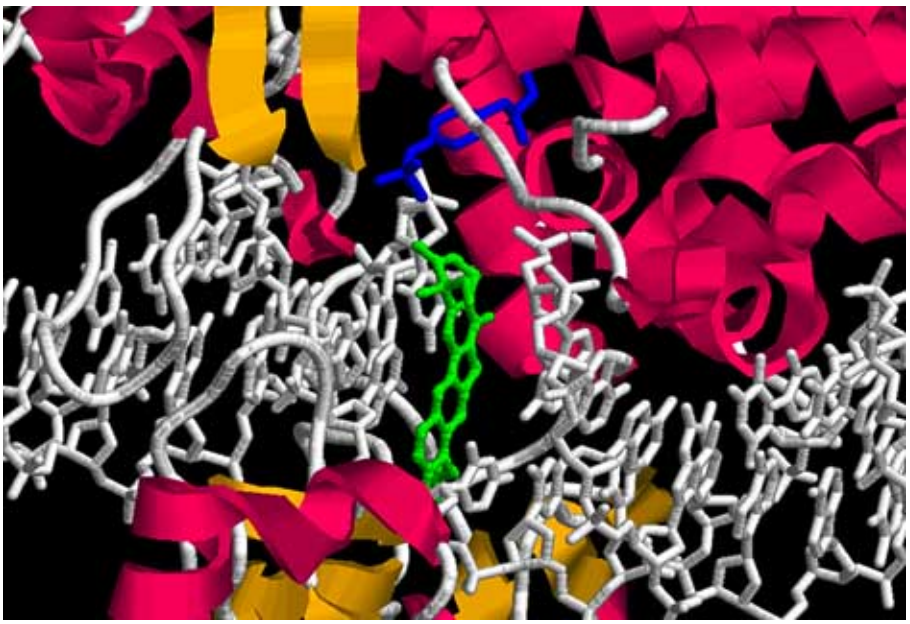

Fig. (4). Structure of the Active Site of the TopoI : DNA : Topotecan Ternary Complex.

The active site of topoisomerase I [color coded as for Fig. (**2**)] with DNA and the type I inhibitor Topotecan (green) to form the cleavable complex (PDB ID 1K4T). The catalytic residue Tyr^{723} is highlighted (blue). Topotecan intercalates with the DNA base pairs adjacent to the nicked bond, moving the nicked strand containing the free hydroxyl group away from Tyr^{723} and so preventing the religation step [30].

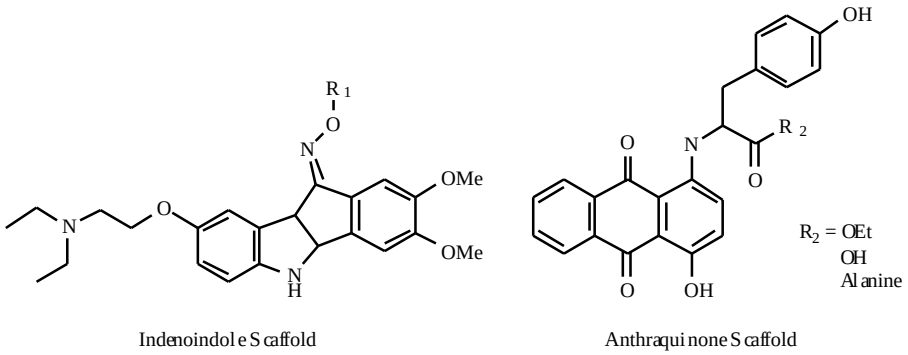

Fig. (5). New Structural Motifs for Topoisomerase Inhibition.

the potential to generate greater structural diversity [46]. The indenoindole scaffold has also attracted attention as a potential topo II inhibitor. Promising results from this new class of topo II inhibitor have shown these compounds function as DNA intercalators and can effectively stabilize the topo II-DNA cleavable complex with a similar potency to Etoposide [47].

Type II Catalytic Inhibitors

This drug category consists of a range of compounds with little structural similarity that act to inhibit topoisomerase action at points in the enzymes catalytic cycle rather than stabilize the cleavable complex. This can be broken down into compounds that block the binding of topoisomerases to DNA, compounds that interact with the ATP binding site of topo II or compounds that prevent topoisomerases from dissociating from DNA. DNA intercalators change the helical repeat of DNA *via* intercalation between the base pairs. From equation 1 this necessarily changes the levels of supercoiling and can prevent topoisomerases recognizing the DNA. This mode of action is necessarily antagonistic to the type I topoisomerase inhibitors. Such interactions have been shown to be clinically useful in the case of the anthracycline Aclarubicin, which inhibits the action of both topo I and topo II (Fig. **6**) [48-49]. Other compounds affect the ATP binding domain by preventing ATP binding such as Novobiocin (Fig. **6**) [50]. The bisdioxopiperazine derivatives ICRF-154, ICRF-187 and ICRF-193 (Fig. **6**) appear to have multiple binding sites

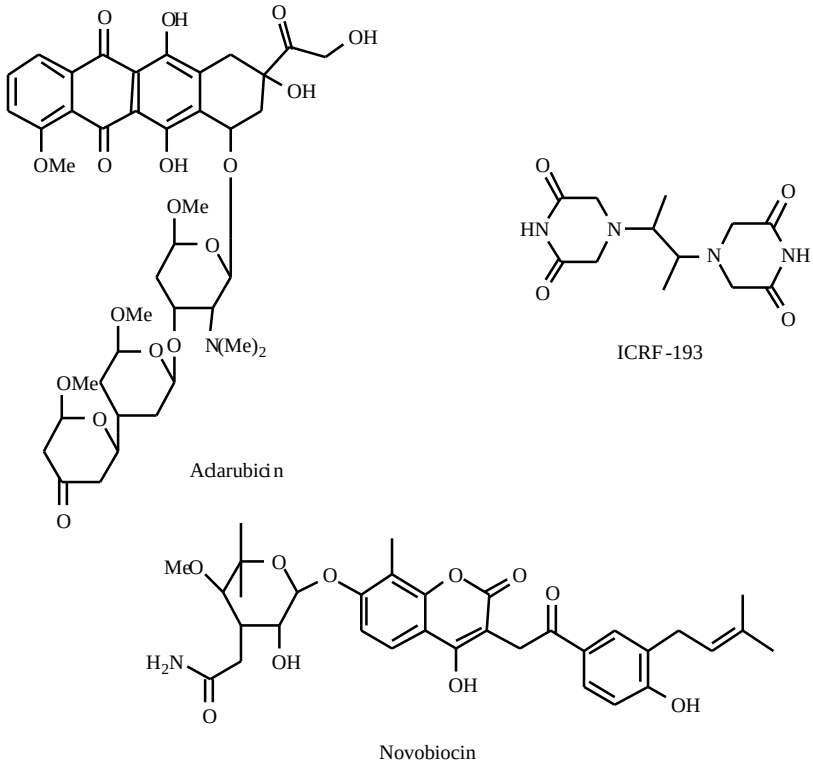

Fig. (6). Structures of Type II Topoisomerase Inhibitors.

on topoisomerases and stabilize a non-covalent complex between enzyme and DNA [51-52]. A caveat to these conclusions is that they are mainly drawn from cell free enzyme assays and recent reports have shown that ICRF-193 appears to cause DNA strand breaks in fibroblasts through as yet unidentified mechanisms [53]. The *in vivo* mechanisms of action of type II inhibitors are therefore currently the subject of considerable conjecture.

3. PHARMACOLOGY OF TOPOISOMERASE INHIBITORS

Cytotoxic Mechanism: The Replication Fork Collision Model

Initial studies on the mechanism of action of type I topoisomerase inhibitors demonstrated that administration of the drug resulted in fragmentation of chromosomal DNA, with the DNA fragments being linked to the topoisomerase enzymes [54-56]. In the case of the topo I inhibitor CPT it was demonstrated that the drug resistance of the lympho-blastic leukaemia cell line RPMI 8402 was associated with a mutated topo I enzyme [57]. Additionally L1210 cells treated with CPT showed a reduction in cell survival to 55%, indicating that the cytotoxic effect of topo I inhibition was specific to cells growing in S phase. Co-administration of CPT and aphidicolin (a known DNA polymerase inhibitor) resulted in the restoration of cell survival to the pre-treatment level in a dose dependent fashion. These results suggested that the cytotoxic effect of CPT was associated with both topoisomerase inhibition and DNA replication. In particular the role of topo I in relaxing supercoils ahead of the replication fork provided a possible rationale to link the experimentally observed dependence of cytotoxicity on the stage of the cell cycle and the enzyme inhibitory properties of CPT [58]. These results led to the conclusion that it was the collision of the topo I cleavable complex with the replication fork that was the cytotoxic event leading to cell death in tumor cells exposed to CPT. CPT is not cytotoxic to non- replicating cells, where it would be expected to form the nicked species due to intracellular levels of topo I. Therefore the collision with the replication fork must lead to DNA strand breaks, resulting in the formation of DNA lesions.

DNA strand breaks arising from the effects of cancer therapy are detected by a variety of pathways, the most studied due to its prevalence in human cancer being the p53 pathway [59]. Kinases such as the DNA dependant protein kinase (DNA-PK) or the ataxia-telangiectasia mutated gene product (ATM) activate p53 by phosphorylation upon DNA strand breakage [60-61]. This results in the translational activation of the bax apoptosis regulatory protein and the family of p53 inducible genes [62-63] initiating a proteolytic cascade leading to cellular apoptosis [64]. The processes of apoptosis and cell cycle traverse have been shown to be mutually regulating [65]. The overexpression of p53 after DNA damage occurs can result in G_1 arrest *via* transcription of the CDK inhibitor p21 [66] as an alternative to the induction of apoptosis. In keeping with the known DNA repair mechanisms, micro injection studies have shown that the damaged DNA substrates have to consist of either a single stranded break of greater than 30 nucleotides in length or alternatively a double stranded break [67]. The pathway leading to cell cycle arrest seems to involve the transcriptional activation of genes such as the growth arrest and DNA damage inducible gene (gadd45); the overexpression of the corresponding gene product results in cell cycle arrest although the precise sequence of events is as yet undetermined. Phosphorylation of p53 influences its DNA sequence binding specificity and the cyclin dependent kinases (CDK's) may modulate which proteins are transcribed depending on the stage of the cell cycle, resulting in either p53 mediated apoptosis or cell cycle arrest [68].

The low apoptotic threshold characteristic of cells overexpressing an oncogene enables a therapeutically managed dose regime to selectively kill malignant cells while merely halting the cell cycle in their non- mutated counterparts. The prerequisite for a drug to act as an effective apoptosis inducer would appear to be the ability to generate either double stranded breaks in the DNA structure or alternatively a bulky DNA lesion, as these types of genetic damage are inefficiently corrected by the cell's DNA repair pathways. Clinically the effectiveness of an apoptosis inducing drug is not necessarily coupled to the replicative rate of the tumour, as fast growing cancers often become resistant to anticancer drugs whilst malignant cells growing at a slow rate are still curable by chemotherapy [69]. This indicates that one of the mechanisms of resistance developed by the malignancy towards these anticancer drugs involves the cells becoming insensitive to apoptosis. Mutation of the p53 regulatory protein in the DNA damage induced apoptotic pathway seems to be the most frequent mechanism by which this resistance occurs in a large number of human cancers. The development of this mutation has been associated with poor prognosis in the clinic, although apoptosis has been shown to occur even in cells lacking functional p53 [69]. In the case of type II topoisomerase inhibitors intercalators such as aclarubicin have also been shown to result in DNA breaks. The damage occurs primarily in the S phase of the cell cycle, suggesting that it is linked to the action of the replication fork [70], however further studies are required before to clarify this cytotoxic mechanism.

Adverse Secondary Effects of Topoisomerase Inhibitors

Clinically the dose limiting factor in the administration of both the anthracyclines and the anthracenediones has been shown to be their chronic cardiotoxicity [71]. The observed cardiomyopathy appears to be a result of a combination of apoptosis and dysfunction in cardiomyocytes leading to deterioration of contractile function of the heart [72]. The cytotoxic mechanisms of drug action are complex and have yet to be fully determined; it has however become apparent that several different routes are involved, with contributions from both the administered drug and its active metabolites. Most of the pharmacological studies have been conducted using DOX as a model compound and this review will focus mainly on recent developments relating to DOX toxicity.

An initial event linking many of the proposed pathways is thought to be the generation of reactive oxygen species (ROS). In the case of the anthracyclines ROS generation has

been shown to occur *via* enzymatic reductase activation of the quinone moiety to a free radical semiquinone intermediate or the fully reduced dihydroquinone, which can occur by the reductase action of enzymes such as the cytochrome P450 [73] and Complex I of the mitochondrial electron transport chain (NADH oxidase) [74]. The reduced quinone species can then redox cycle with molecular oxygen, a process known as autoxidation, to generate the superoxide anion free radical. Action of the cellular antioxidant enzyme superoxide dismutase disproportionates superoxide to hydrogen peroxide, a readily diffusible ROS [75]. Additionally in the presence of ferrous iron (Fe^{+2}) peroxide can form the extremely reactive hydroxyl radical *via* the Fenton reaction [76]. ROS have the potential to cause extensive oxidative damage to proteins, DNA and membranes [77], leading to impaired cellular function and ultimately necrosis or apoptosis. DOX is also known to chelate iron and copper and the disruption of metal homeostasis may be implicated in a non-specific impairment of cellular function [78-79]. Redox cycling reactions are also accelerated if the drug has formed an iron complex, leading to extensive hydroxyl radical formation [80]. Due to the reduction of DOX by Complex I of the mitochondria, the autoxidation reaction is expected to result in an increase in ROS in close proximity to the mitochondria. This may result in an increase in "free" iron due to oxidation of the iron-sulfur clusters of proteins such as aconitase associated with the mitochondria [81]. This would be predicted to both impair the function of the mitochondria and increase the cellular concentration of unbound Fe^{+2}, with potentially deleterious effects due to hydroxyl radical formation.

In addition to toxic non-specific redox reactions there is an increasing body of evidence that suggests that DOX can also initiate specific apoptotic pathways in cardiac myocytes. It has been recently been shown that DOX can trigger mitochondrial-mediated apoptosis *in vivo* by inducing release of cytochrome c from the mitochondria and subsequent activation of caspase-9. The mechanism has yet to be clearly defined but may result from an increase in ROS generation at the mitochondria [82]. It has also been proposed that DOX may induce apoptosis *via* a sarcoplasmic reticulum (SR) pathway. In cardiac myocytes the SR acts as a Ca^{+2} store, with the partitioning of Ca^{+2} between the SR and the cytosol regulating contractility. The SR ATPase pumps Ca^{+2} into the SR, leading to relaxation, while the ryanodine receptor releases Ca^{+2} causing contraction [83]. Recent developments have shown that an abnormal change in Ca^{+2} homeostasis results in apoptosis by activation of caspase-12, a mitochondria independent route [84]. DOX administration has been shown to result in cleavage of caspase-12 *in vivo*, possibly *via* an ROS mediated oxidative modification to the redox sensitive Ca^{+2} transport system [85].

Another possible mechanism for cardiotoxicity which is currently attracting significant interest is the interaction between DOX generated ROS and the signalling molecule nitric oxide (NO). The SR Ca^{+2} transport system appears to be regulated by NO, although the precise sequence of events remains controversial. The reaction with superoxide is an important biological sink for NO as it is essentially diffusion limited ($k \approx 7x10^9\ M^{-1}s^{-1}$) [86] and NO is comparatively unreactive towards other cellular molecules (with the exception of soluble guanylate cyclase). The reaction proceeds *via* a radical-radical combination to form peroxynitrite. Under physiological conditions peroxynitrite is short lived ($t_{1/2} < 2$ sec at pH 7.4) and predominantly reacts with the carbonate anion to form a short lived peroxynitrosocarbonate species, which undergoes homolytic fission to liberate both the carbon dioxide radical and nitrogen dioxide radical [87]. If sufficiently high levels of superoxide are produced by the redox cycling of DOX, this NO scavenging mechanism may affect NO homeostasis in cardiac myocytes, a potentially toxic event due to NO's role in the contractile response [88].

The anthracenediones MTX and ametantrone have been shown to posses more negative redox potentials than those found in the anthracyclines [89] making them poorer substrates for the enzymes involved in reductive metabolism. This results in lower cardiotoxicity and myelosupression becomes the dose limiting factor [90]. The most frequent adverse events associated with CPT and Topotecan-based regimens have been reported as a decrease in immune system function due to lowered levels of neutrophils (neutropenia) [91], although little work has been done on the molecular mechanism.

Development of Drug Resistance

Cell lines resistant to topo II inhibitors have been associated with a decrease in expression of either topo IIα or topo IIβ. Both isoforms are therefore implicated in drug resistance. Cellular resistance to topo II inhibitors can also be gained by mutations to the enzyme. In the case of Etoposide, mutations to the topo IIα isoform convey drug resistance, probably due to reduction in the Etoposide's binding affinity. Topo IIα mutations are most frequently observed in tumours at the dinucleotide binding site (residues 450–495) and the tyrosine active site (Tyr^{805} [92]). A similar resistance profile is observed for CPT resistant cells. Here mutations to topo I around the catalytic Tyr^{723}, and the DNA binding lip domains (residues 361–364 and 533) conveyed drug resistance [93].

Tumor cells exposed to topo inhibitors also develop multi-drug resistance (MDR) [94]. The MDR phenotype is characterized by the overexpression of P170 glycoprotein (PGP), an ATP-dependent drug efflux transporter that acts to lower cytosolic drug levels. PGP is a member of the ABC transporters [95], which is expressed upon increased transcription of the *mdr1* gene. PGP has a broad target recognition and effectively transports most clinically utilized topo inhibitors [96]. A more controversial area is the recognition of topoisomerase inhibitors by the enzyme glutathione-S-transferase (GST). GST ligates the cellular antioxidant thiol glutathione to xenobiotics as an initial detoxification step. Tumours are known to display high levels of GST, but the link between enzyme expression and drug efflux is not well established [97]. GST may however play a cardioprotective role as overexpression of the enzyme has been reported to decrease the levels of DOX induced ROS [98].

Resistance to anticancer drugs also involves alterations to the malignant cell's DNA repair capabilities. In the case of

chemotherapeutic agents that create lesions in DNA, such as alkylating agents, this is generally achieved by increasing the activity of the DNA repair pathways [99]. Contrastingly cancerous cells also acquire resistance to type I topoisomerase inhibitors by a loss of DNA mismatch repair activity, suggesting that the mismatch repair system may also function in apoptotic signalling pathways *via* its function as a DNA damage sensor [100-101].

4. DRUG DEVELOPMENT STRATEGIES

Despite numerous attempts to develop structural analogues of the topoisomerase inhibitors presently in clinical use, slow progress has been made over the last 25 years in bringing new drugs to the clinic. In light of this, many recent research programs have focused on improving delivery strategies for the currently approved drugs. An attractive possibility has been the microencapsulation of topoisomerase inhibitors to construct an improved drug delivery system. Nanoparticles have recently been examined as carriers for DOX, with a positively charged carrier predicted to interact with the negatively charged cell membrane and so increase drug uptake into cells. Due to DOX's cationic charge at neutral pH (pKa = 8.2), the drug would be expected to have a low incorporation into a positively charged particle due to charge repulsion. Chitosan nanoparticles were therefore formulated with a dextran sulfate polyanion to complex with DOX. This enabled the formation of a positively charged carrier with high DOX entrapment. This formulation successfully acted as a delivery system, with an initial burst release of 17% after 2 hrs, presumably due to DOX complexed to the surface of the nanoparticles, followed by a much slower release of 4.5% over 2 days attributed to encapsulated DOX [102]. The cytotoxic activity of DOX was maintained upon release, indicating no degradation of the molecule during the encapsulation process or upon storage. Nanoparticles have also been shown to be effective for intravenous delivery of SN-38, a hydrophobic CPT analogue [103]. Similarly cyclodextrins have been used as an encapsulating agent for CPT, with encapsulation improving both the solubility of CPT and its activity against human derived myeloid THP-1 leukaemia cells [104].

Lipid emulsions (parenteral emulsion) consisting of egg lecithin (a phospholipid mixture) and cholesterol, leading to negatively charged lipid nanospheres, have been investigated as potential Etoposide carriers. This delivery system showed substantial effects on Etoposide pharmacokinetics, with slower plasma clearance and altered tissue distribution. The formulation also displayed improved antitumour activity in Lewis lung carcinoma bearing mice [105]. Liposomes have also been extensively used as a drug delivery vehicle for both Etoposide [106] and DOX [107]. These formulations show a characteristic increase in clearance time and improved activity. In particular the encapsulation of Etoposide in positively charged liposomes lead to significant improvements in activity in an *in vivo* tumor model, possibly through targeting the liposome to the tumor by charge interactions between the cationic liposome and the cell surface of endothelial cells forming the tumor vasculature [108]. These parameters can be fine tuned by adjusting the size of the liposome, changing from a positive, neutral or negative charge and varying charge density. The chemical composition of the liposome is also highly significant when determining liposome stability and ratios of phospholipids to cholesterol must be investigated for each drug and tumor [109].

An alternative strategy has been to investigate means of targeting a pro-drug to the tumor. Prodrugs can be activated by enzymatic conversion to the active form. This can be achieved by utilizing an enzyme that is over-expressed in cancer cells relative to normal tissue [110]. Candidate enzymes include carboxylesterase and β-galactosidase (Fig. **7**), both of which can remove protecting groups and have an added advantage as they also change the drug's solubility, converting a lipophilic prodrug to a hydrophilic active from inside the cell. This decreases the diffusion of the drug through cell membranes and so effectively concentrates the drug inside cells expressing the activating enzyme. Using this approach, analogues of DOX and Daunomycin have been synthesized and found to be 100-10,000 fold more active than the parent compound. [111-112].

In order to improve the specificity of these agents, investigators have examined the possibility of directly introducing the required activating enzyme to the tumor. Gene-directed enzyme prodrug therapy (GDEPT) uses a vector, usually liposomes, to introduce the gene for the foreign enzyme to the tumor. This has been studied as a drug targeting system for the anthracyclines where nitroreductases have been used to convert a nitrobenzyl carbamate prodrug of DOX to the active form [113], whereas a carboxyesterase has been used to activate a CPT prodrug [114]. As an alternative to physical gene entrapment, replication incompetent viruses have also been examined as potential vectors, a variant technique known as virus-directed enzyme prodrug therapy (VDEPT). Adenovirus, retrovirus and Epstein- Barr virus have all received pharmacological attention as carriers for genes encoding both eukaryotic and prokaryotic drug metabolising enzymes. VDEPT can achieve specificity based on the retroviral and adenovirus's selectivity for proliferating cells [115]. In a similar manner to GDEPT, VDEPT has been used to activate CPT prodrugs, where the approach has shown activity *in vivo* against tumours in nude mice [116].

A complimentary approach utilizes an antibody-enzyme conjugate to localize the prodrug metabolising enzyme to the surface of tumor cells. This antibody-directed enzyme prodrug therapy (ADEPT) approach requires both a high affinity monoclonal antibody and the elevated expression of an appropriate antigen on the tumor cell membrane. For CPT prodrugs β-Glucuronidase has been a popular choice as the prodrug activating enzyme, as levels of this enzyme are elevated in some cancer tumors and the glucuronide moiety is highly hydrophilic, which can overcome the dose-limiting insolubility of CPT. Such CPT analogues have shown improved cytotoxicity against some tumour cell lines [117]. By combining DOX prodrug and microencapsulation strategies it has been possible to improve these statistics and achieve antiproliferative activity over several weeks [118]. For all of these vectors selective delivery of the enzyme is a significant clinical drawback, as at most only approximately 50 % of tumor cells can be successfully targeted [119].

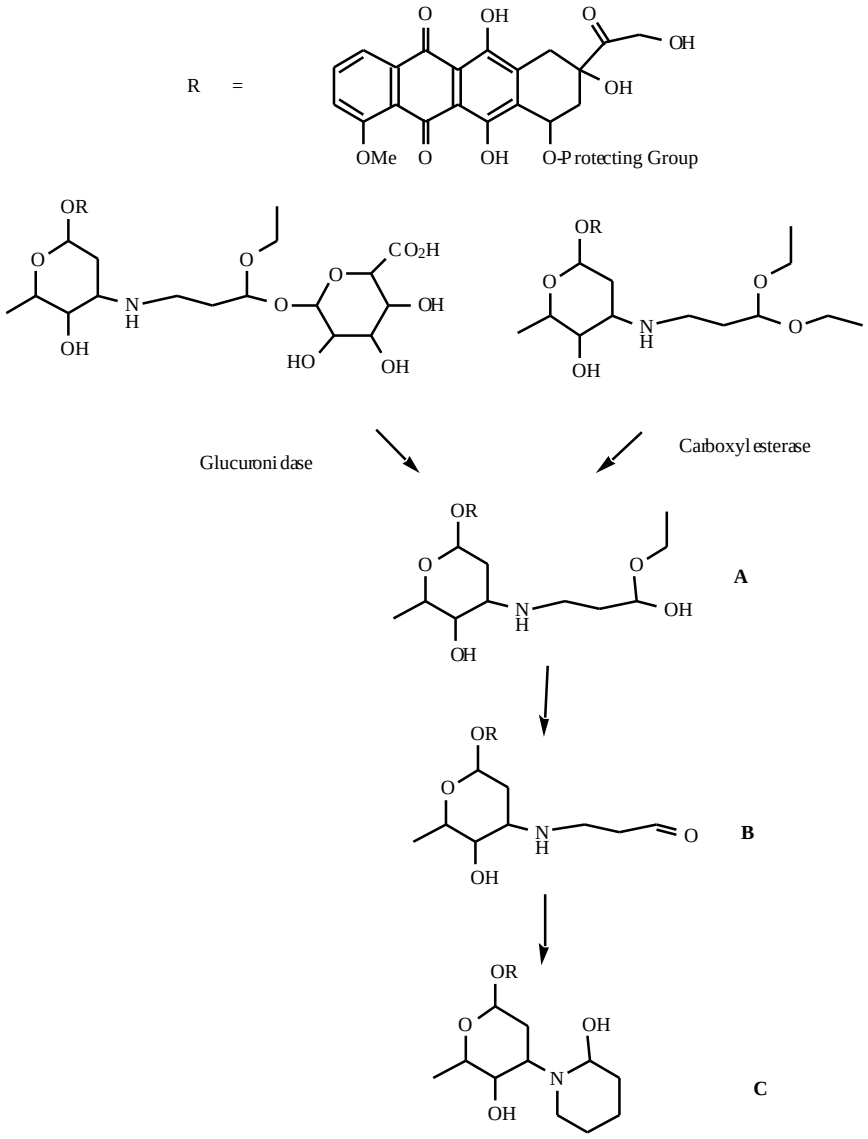

Fig. (7). Drug Activation Strategies for DOX Prodrugs.

DOX prodrugs can be converted to an active hemiacetal metabolite (**A**) by the action of the enzymes β-glucoronidase or carboxylesterase. This then decomposes to aldehyde (**B**), which may then cyclize (**C**).

5. SUMMARY

Topoisomerase inhibitors have been extensively used in cancer chemotherapy over the last 30 years. However despite their long standing use and extensive pharmaceutical research, with the exception of Topotecan, comparatively little progress has been made in the development of superior analogues to the compounds originally identified as "cleavable complex" stabilizing agents in the 1960's and 70's. In part this is attributable to a lack of structural information concerning their binding sites on either the topo I or topo II enzyme-DNA complex. In the case of topo I inhibitors, with the recent identification of a crystal structure for the topo I-Topotecan-DNA ternary complex this bottleneck may have been removed. As a result there is now a renewed interest in the rational design of topo I inhibitors based on computer simulations of inhibitor docking to this putative binding site. In the case of the topo II enzyme a definitive binding site for type I inhibitors remains elusive. Nonetheless structure-based virtual screening techniques are beginning to be applied to identify new inhibitors based on the activity of known pharmacophores [120]. Pharmaceutical effort has also focused on examining alternative drug delivery strategies. In particular combinations of ADEPT and VDEPT approaches with the use of vectors such as liposomes has been shown to enhance the activity and pharmacokinetics of DOX. These developments offer an exciting new avenue of research for cancer chemotherapy, the success of which awaits the results of clinical trials.

ABBREVIATIONS

ADEPT	=	Antibody-directed enzyme prodrug therapy
CPT	=	Camptothecin
DOX	=	Doxorubicin
FDA	=	U.S. Food and Drug Administration
GDEPT	=	Gene-directed enzyme prodrug therapy
GST	=	Glutathione-S-transferase
MTX	=	Mitoxantrone
MDR	=	Multi-drug resistance
NO	=	Nitric oxide
PDB	=	Protein Data Bank
PGP	=	P170 glycoprotein
ROS	=	Reactive oxygen species
SR	=	Sarcoplasmic reticulum
topo I	=	Topoisomerase I
topo IIα	=	Topoisomerase IIα
topo IIβ	=	Topoisomerase IIβ
topo II	=	Topoisomerase II
VDEPT	=	Virus-directed enzyme prodrug therapy

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