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Review paper

Topoisomerases IIα and β as therapy targets in breast cancer

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Topoisomerase II enzymes play an essential role in human DNA metabolism. They are also recognized as primary targets of a number of anti-cancer drugs used in the treatment of breast cancer, which remains a leading cause of cancer-related death in women. While topoisomerase inhibitors have produced significant response rates in this disease, their use has been limited both by toxicity and by the development of resistance. In this article we review the extensive work which has not only increased our understanding of the biochemistry and molecular biology of type II topoisomerases but also enabled more rational drug design. Such knowledge should translate into increased clinical efficacy in the treatment of breast cancer and other malignancies.

Key words: Breast cancer, chemotherapy, drug resistance, topoisomerase.

Introduction

Topoisomerase II is a key target for a number of common anticancer drugs used in the treatment of breast cancer and other malignancies.

Over recent years much has been discovered both of how type II topoisomerases function at the molecular level and of how inhibitors affect this function. In this article we describe ways in which molecular biology and clinical medicine, working together, offer the possibility of significant improvements in the outcome for the many patients afflicted by breast cancer (hitherto incurable once metastatic disease appears). While this discussion focuses on breast cancer, many of the principles apply to other malignancies as well.

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After reviewing the molecular biology of the enzymes in normal and malignant cells, we will discuss the use of topoisomerase inhibitors at both molecular and clinical levels, and the mechanisms underlying the development of resistance. In conclusion we consider how this information is being used to develop new drugs and how existing drugs may be used more effectively.

Structure and function of topoisomerases

During normal DNA metabolism, the repeated unwinding and duplication of the double-helix produces twisting, knotting and interlinking of the helical strands. Topoisomerases are a class of enzymes which resolve these topological problems and allow efficient nuclear function.

On the basis of their catalytic function, topoisomerases are classified as either type I or type II enzymes. Type I topoisomerases produce transient single-stranded breaks in DNA, allow the other DNA strand of the helix to pass through the break and then the primary strand is religated. In human cells, their main functions are in transcription and DNA elongation.[1,2]

In contrast, type II topoisomerases produce transient double-stranded breaks, enabling the passage of a complete DNA double-helix through the break before it reseals (reviewed by Watt and Hickson[3]). In eukaryotic cells, the enzyme is a homodimer which binds with relatively low specificity to segments of DNA where topological problems exist. On binding, topoisomerase II forms a staggered double-strand break which enables a separate double-stranded segment of DNA to pass through the 'gate' formed by the enzyme dimer bound to the 'cleaved' DNA. This is followed by religation of the original strands. Hydrolysis of ATP reverses binding of the

enzyme to DNA, enabling it to initiate subsequent catalytic cycles. This function of type II topoisomerases makes them essential in a number of aspects of DNA metabolism including replication, transcription, recombination suppression, chromosome segregation and condensation during mitosis and probably meiosis.[3–8] The remainder of this discussion will focus on the type II enzymes.

Analyses of eukaryotic topoisomerase II have indicated that the protein consists of three structural domains. The N-terminal region contains the ATP-binding domain[9] essential for reversal of enzyme binding to DNA. The central domain of the enzyme contains the active site tyrosine responsible for DNA cleavage and religation. The amino acid motif **PLRGK** has been implicated in the covalent binding of drugs to the DNA–enzyme complex,[10] referred to as the 'cleavable complex'. These complexes accumulate in cells as the drug permits DNA cleavage, but inhibits religation.[7] The build up of these DNA 'adducts' initiates a series of events which culminate in cell death.[11]

The C-terminal domain of human topoisomerase II is non-catalytic but probably plays an essential role in modifying enzyme function. It contains nuclear localization signals[12,13] which enable transport of the enzyme to the nucleus where it is functionally active. This region may also be a site for protein–protein interactions which modify enzyme function.[3,14]

Type II topoisomerases are phosphoproteins *in vivo*, with a number of potential phosphorylation sites in the C terminal domain.[8,15,16] Phosphorylation at these sites may allow differential control of isozyme activity, direct nuclear localization, influence enzyme stability, and control the extent of topoisomerase II interactions with DNA and other nuclear proteins.[8,15] Topoisomerase II from lower eukaryotes is phosphorylated by many kinases *in vitro*, including casein kinase II (CKII), protein kinase C, $p34^{cdc2}$ kinase and Ca^{2+}/calmodulin-dependent protein kinase (reviewed by Gasser *et al.*[8]). Such phosphorylation reactions increase topoisomerase activity.[17,18] Cell cycle specific phosphorylation also occurs, with the enzyme being both highly phosphorylated and more active, at the G_2/M phase boundary where topoisomerase II has a key role.[19–21] Phosphorylation may thus modulate topoisomerase II activity to permit chromosome condensation and segregation during mitosis. Phosphorylation of topoisomerase IIα by CKII is likely to be of physiological importance in humans. Both enzymes associate *in vivo* in a catalytically active molecular complex.[22,23] Topoisomerase IIα from this complex is inactivated on dephosphorylation and so far CKII is the only enzyme reported to reactivate the enzyme by phosphorylation.[22] Wells *et al.*[16] have also mapped two *in vivo* phosphorylation sites within the C-terminal domain of human topoisomerase IIα, at Ser1524 and Ser1376, that are phosphorylated *in vitro* by CKII.

Human topoisomerase IIα and β

There are two type II enzymes in humans—topoisomerase IIα (170 kDa) and topoisomerase IIβ (180 kDa). These are encoded by distinct genes on separate chromosomes.[24,25] Initial studies of type II topoisomerases in humans did not distinguish between the two isoforms, but it appears that these analyses were measuring predominantly the α form. Drake[26] separated the two forms and a number of important functional differences between the two isozymes have emerged. While apparently sharing the same catalytic function *in vitro*, the two show distinct patterns of expression during the cell cycle and following malignant transformation. They also show different nuclear localization and tissue-specific expression patterns, indicating different, but as yet incompletely defined, roles for each isozyme. The amino acid sequence of the two isoforms differs most markedly in the C-terminal region suggesting differing forms of regulation and protein interaction.

Expression of topoisomerase IIα fluctuates during the cell cycle with maximal protein levels and activity in G_2/M phases.[27] Characterization of the topoisomerase IIα promoter has revealed a number of motifs potentially involved in cell cycle regulation.[28] Levels of the α isozyme increase with proliferation while, in quiescent cells, there is relatively higher expression of the β isozyme.[29] In most cell lines, levels of β remain relatively constant throughout the cell cycle. However, recent studies of normal[30] and malignant hemopoietic cells[31] have shown a rise in topoisomerase IIβ levels on mitogenic stimulation. These findings may reflect a tissue-specific difference or different effects in cell lines compared with tumor samples, but must be qualified by the observation that characterization of topoisomerase IIβ has been with antibodies that do not detect the full length 180 kDa form. Cloning of the topoisomerase IIβ promoter and further studies of the specific control mechanisms for this isozyme are essential to resolve its regulation.

The subcellular localization of the two isoforms may also differ, with α present in the nucleoplasm,

and β present in both nucleoplasm and nucleoli.[32]

The expression of both type II topoisomerases varies in different tissues. Holden *et al.*[33] found differences in enzyme activity between different normal tissues with highest levels in proliferating lymphoid tissues such as the spleen and thymus. This evidence was supported by the findings of Capranico *et al.*[34] who found highest topoisomerase IIα RNA levels in bone marrow and spleen. Levels of β were highest in non-proliferating tissues. These findings support a potential use for topoisomerase IIα as a proliferation marker, and may explain some of the bone marrow toxicity and immune impairment that occurs with some topoisomerase inhibitors which seem to predominantly target the α form.

In tumor samples, the highest levels of activity are seen in those tumors with a high proliferation index and aggressive clinical behavior.[33] More recently, Hasegawa *et al.*[35] showed high expression of topoisomerase IIα in lung tumors with undetectable levels in normal lung tissue from the same patients. Kim *et al.*[36] also showed higher topoisomerase IIα mRNA levels in a variety of tumors relative to adjacent normal tissue, offering the potential for selective targeting. In other tumors there appears to be higher expression of topoisomerase IIβ.[37]

Type II topoisomerase inhibitors in breast cancer

Cytotoxic agents such as doxorubicin had been in routine clinical use for over 10 years when it was recognized that type II topoisomerases were a primary target of such drugs in the mid-1980s.[38]

A wide range of drugs used to treat breast cancer have now been shown to inhibit type II topoisomerases (see Table I). Most of these agents, e.g. anthracyclines, amsacrines, ellipticines and anthracenediones, act by intercalating DNA close to the active site of topoisomerases, stabilizing the transient DNA–enzyme complex and preventing the enzyme from religating the cleaved DNA strands. Other agents, e.g. epipodophyllotoxins, bind only weakly to DNA without intercalation, but still stabilize the cleavable complex to produce cell toxicity.[39] Like many DNA-damaging agents, these type II topoisomerase inhibitors also induce sister chromatid exchange, chromosomal recombination and chromosome aberrations,[40,41] and are associated with a significant risk of secondary leukemia.[42–44]

Another group of compounds, e.g. ICRF-159, ICRF-193 and suramin, exert their effects by inhibiting enzyme function without forming cleavable complexes.

It should be emphasized that type II topoisomerase inhibitors often have additional modes of action which enhance their cytotoxicity but may also contribute to their toxicity. The challenge in drug development and molecular oncology at present is to identify agents with greater tumor specificity.

While isozymes may vary in relative drug sensitivity,[26,45] as yet there is no direct evidence that different drugs solely target specific topoisomerase II isoforms. If isozyme-specific agents are developed it may then be possible to target tumors according to their expression of the two enzymes. Another possibility is that one of the isoforms may be primarily responsible for normal tissue toxicity, so that avoiding this isoform could improve tumor selectivity.

Table 1. Major effects of various chemotherapy agents on topoisomerase II

Drug	Intercalates	Stabilizes cleavable complexes	Inhibits catalytic activity
Anthracyclines	✓	✓	
Amsacrines	✓	✓	
Makulavamines	✓	✓	
Anthracenediones	✓	✓	
Epipodophyllotoxins		✓	✓
Ellipticines		✓	✓
Merbarone			✓
Novobiocin			✓
ICRF-159/193			✓
Suramin			✓

Mechanisms of drug action

Intercalating agents

Though type II topoisomerase inhibitors are structurally diverse, most share a common mode of action in binding the DNA–enzyme complex in a stable 'cleavable complex' where the broken strands are misaligned, preventing religation.[46] Different drugs bind to DNA at different sites in the same region,[38,47] lying in the groove between the DNA strands (intercalation). The binding is to specific bases on the immediate flank of the cleavage site which are believed to stack inside the cleavage site, trapping the cleavable complex and preventing religation (reviewed by Pommier[48]).

Other factors influence binding and action of these drugs. Chen and Liu[49] postulate that different drug classes interact with the enzyme in an open- or closed-gate configuration depending on ATP binding. ATP-stimulated drugs such as epipodophyllotoxins and doxorubicin(adriamycin) may interact in the closed-gate conformation while ATP-independent drugs, e.g. menadiones, interact with the open gate conformation.

Epipodophyllotoxins

These drugs have been most frequently used in lung cancer. Although initial studies showed little benefit in breast cancer,[50] an increased understanding of their pharmacokinetics is stimulating their reintroduction into breast cancer trials.

Epipodophyllotoxins not only increase cleavable complex formation, but also directly inhibit the catalytic function of topoisomerase II.[51] A further potential antineoplastic effect has been suggested by Jing *et al.*[52] who showed etoposide to have a synergistic effect with retinoids, inducing differentiation of leukemic cells as well as inhibiting their growth.

Enzyme inhibitors

Depending on their site of action on the enzyme, these fall into three groups.

Catalytic Inhibitors, e.g. ICRF-159 and ICRF-193. Bis(2,6-dioxopiperazine) derivatives, such as razoxane (also known as ICRF-159), were synthesized as antitumor drugs over 20 years ago.[53] Based on chelating agents, these compounds were described as antiproliferative drugs that block cells in the G_2 phase of the cell cycle.[54] More recently, Ishida *et al.*[55] have demonstrated that ICRF-193 inhibits chromosome condensation and segregation in mitosis but does not inhibit cell cycle transition. Drug exposure causes cells to traverse an unusual M phase with an 'absence of chromosome segregation' (ACS-M phase), leading to polyploidy and loss of viability. Inactivation of the topoisomerase II by ICRF-193 uncouples chromosome dynamics from other processes of the cell cycle which normally proceed in a coordinated manner.

The limited clinical application of these compounds, due partly to their low solubilities and short plasma half-life, has been improved by the synthesis of various masked compounds which show increased bioavailability and antitumor activity over the parental compounds.[56] One of these compounds (MST-1) is effective against a range of cancer cell lines including: P388, L1210 leukemias, B-16 melanomas and colon 26.[57]

Unlike the cleavable-complex forming topoisomerase II poisons, the dioxopiperazines inactivate DNA topoisomerase II by trapping the closed-clamp conformation of the enzyme[58] in the presence of ATP and subsequently cause some inhibition of the ATPase enzyme. Ishida *et al.*[59] have demonstrated direct targeting of topoisomerase II in cell lines by ICRF-193 in competition studies with etoposide.

ATPase inhibitors. Other drugs interfere more directly with the ATPase domain of topoisomerase II isozymes and are in clinical use as antibiotics, e.g. Novobiocin. More recently they have been investigated in mammalian cell lines and been found to produce super-additive cytotoxicity when combined with epipodophyllotoxins.[60] These drugs are being assessed as potential drug resistance-modifying agents in clinical studies.

Suramin. Suramin is a hexasulfated naphthylurea that has been used in the treatment of trypanosomiasis and filariasis for many years. Recently an understanding of its molecular action has expanded its applications. It is a non-specific growth factor antagonist, interfering with the binding of growth factors to their receptors. It also inhibits a number of enzymes including DNA and RNA polymerases, reverse transcriptase, protein kinase C and topoisomerase II.[61]

It inhibits topoisomerase II by reducing phosphorylation mediated by protein kinase C without stabilizing cleavable-complex formation. Its use is

limited by neurological toxicity, but analogs may offer increased therapeutic potential.

The multiple actions of suramin mean that while it inhibits the growth of breast cancer cell lines, this activity is critically dependent on dose[62] and duration of exposure[63] to the drug. These factors must be optimized before its true potential in therapy can be determined.

Cytotoxicity mechanisms

Although it is accepted that the initial cytotoxic event following treatment with topoisomerase inhibitors is the formation of cleavable complexes, a number of other processes are required for cell death.[64,65] While the cleavable complexes are present only transiently, they may be processed rapidly during DNA synthesis into lethal DNA damage. This is supported by the fact that during DNA synthesis, sensitivity to topoisomerase II inhibitors increases, an effect which can be blocked by inhibitors of DNA synthesis.[11,19,66,67]

In addition, RNA synthesis may also be affected by cleavable complex formation.[11] The significance of cleavable complexes in cytotoxicity is supported by evidence that the cytokine, tumor necrosis factor, potentiates both cleavable complex formation and cytotoxicity in cell line experiments.[68] Within different classes of topoisomerase II inhibitors a reasonable correlation exists between the number of cleavable complexes formed and cell lethality.[69,70]

Cell killing is a multistep process. Topoisomerase interaction is essential but other processes, e.g. protein synthesis,[71] nucleic acid synthesis[11] and calcium,[72] also appear to have a role. The effects of topoisomerase inhibition on DNA repair, on cell cycle regulation and on apoptosis also need to be explained as such effects may be altered in tumor cells.

It should be emphasized that many topoisomerase II inhibitors have additional cytotoxic mechanisms, e.g. doxorubicin also produces free radicals which are associated both with its cytotoxicity in breast cancer and with the development of cardiomyopathy.[73]

Drug resistance to topoisomerase II inhibitors[43,74,75]

Mechanisms that underlie the resistance of cancer cells to topoisomerase II inhibitors have recently been reviewed elsewhere.[64,74,75] Identifying determinants of sensitivity is essential to optimize drug efficacy and minimize toxicity as both normal and malignant cells express type II topoisomerases. There are two major forms of resistance to these drugs.

Membrane transporter genes

These include the multidrug resistance gene (MDR-1), involving the membrane associated chloride pump, P-glycoprotein. This pump causes drug efflux from cells and resistance to a number of topoisomerase inhibitor classes as well as microtubule poisons and antibiotics (reviewed by Morrow[76]). MDR-1 is expressed in over 50% of primary breast cancers,[77,78] but the level of expression is variable. Such expression has been associated with poor prognosis.[79,80]

A separate membrane transporter gene is MDR-related protein (MRP) which is amplified in some resistant cell lines.[81,82] Its relative contribution to clinical drug sensitivity is being investigated.

Atypical MDR

This form of resistance involves alterations in topoisomerase II. It involves all drugs that target topoisomerase II but its relevance to clinical drug resistance varies with different drugs. This may be due to different resistance mechanisms developing in response to different drugs or to changes in expression of the different isozymes.[83]

Topoisomerase II levels. Atypical MDR may occur due to reduced levels of topoisomerase II arising from selective mutation, or to down-regulation induced in cells with a low proliferation potential or nutritional deprivation.[67,84,85]

Cell line studies have shown some correlation between levels of topoisomerase II isoforms and chemosensitivity. High levels of α confer topoisomerase II-targeting drug sensitivity[86] and low levels confer resistance. Webb *et al.*[87] and Fry *et al.*[88] showed a similar correlation in testis and bladder cancer cell lines but emphasized that other factors contributed to sensitivity. Such factors include the influence of associated proto-oncogene expression, e.g. cells that over-express the c-*myc* oncogene are hypersensitive to the topoisomerase inhibitor m-AMSA which also cleaves the c-*myc* gene promoter.[48,89] Also, the topoisomerase IIα gene is located close to that for c-*erb*-B2, a transmembrane

glycoprotein and a poor prognostic marker in women presenting with primary breast cancer.[90] A study of breast cancer tumors and cell lines showed co-amplification of topoisomerase IIα with c-*erb*-B2 in 12% of cases, with no sign of isolated amplification of topoisomerase IIα.[91] The cell line showing co-amplification in this study was also the most sensitive to topoisomerase inhibitors, suggesting that c-*erb*-B2 may alter drug sensitivity and that patients with this pattern of expression should be targeted with topoisomerase inhibitors.

There are differences in the sensitivity profile of topoisomerase II isoforms to different drugs.[26] While the precise role of topoisomerase IIβ in drug resistance is uncertain, levels have been reduced in some cell lines with atypical MDR features suggesting a role at least with certain drugs.[45,92] It is possible that relative expression of the two isozymes could change on exposure to topoisomerase II inhibitors with predominant expression of the non-targeted form, allowing functional resistance.

A modest number of clinical studies show no clear correlation between topoisomerase II level and drug sensitivity. Kim *et al.*[36] found levels of topoisomerase IIα mRNA to be increased in responding breast cancer patients, while in studies of other tumors this has not been found,[31,93] possibly reflecting tissue specific differences in level and regulation. This is supported by the fact that drugs show differing toxicity in cell lines from different tissues.[94] Further studies are required to confirm a correlation in breast cancer as this has important implications for therapy.

Phosphorylation. There are often other changes present in topoisomerase II, e.g. altered phosphorylation and methylation, which contribute to enzyme activity and sensitivity to drugs (reviewed by Hochhauser and Harris[75]).

The state of topoisomerase II phorphorylation appears to influence drug sensitivity. *In vitro* phosphorylation of *Drosophila* topoisomerase II, by both protein kinase C and casein kinase II, attenuates the ability of m-AMSA or etoposide to stabilize the cytotoxic enzyme–DNA cleavage complexes.[7,95] Some drug resistant cell lines have both decreased topoisomerase II expression and increased phosphorylation,[96] which may both induce resistance and enable cells to maintain normal DNA metabolism despite low enzyme levels. In contrast to these findings, Ritke *et al.*[97] have reported a cell line resistant to etoposide with reduced phosphorylation together with reduced topoisomerase II expression and activity. The authors postulated that in this situation drug resistance may have been due to hypophosphorylation inducing the instability of the cleavable complex seen in these cells relative to the sensitive parental cell line.

North *et al.*[98] showed that overexpression of the R1α subunit of protein kinase A conferred hypersensitivity to both topoisomerase II inhibitors and 8-chloro-cyclic AMP (a site-selective cyclic AMP analog) without changes in cellular topoisomerase II level or activity, suggesting involvement of this kinase in downstream processing of topoisomerase II- mediated events. These findings emphasize both the significance of phosphorylation events in topoisomerase II metabolism and the need for their further investigation to identify new therapies.

Other factors inducing resistance. A recent study by Gudkov *etal.*[99] reported a new mechanism of inducing topoisomerase II resistance by expression of genetic suppressor elements which lower the cellular levels of functional topoisomerase II.

Since topoisomerase II poisons cause DNA damage, it is not surprising that exposure to such drugs has led to point mutations which in a number of studies have been associated with drug resistance (reviewed by Chen[49]). Others have reported in some resistant cell lines a lengthening of cell cycle time,[100] which may function by exposing the cell to drug in the sensitive S phase for a smaller fraction of the cell cycle.

Cytosolic factors may also contribute to drug resistance. Hypoxia and glucose-related stress responses in cell lines have been shown to induce resistance to a number of topoisomerase inhibitors.[101–103] This is of particular relevance to the development of topoisomerase II inhibitor resistance in large necrotic tumors. Estrogen has been shown to potentiate response to etoposide in a human breast cancer cell line and appears to increase topoisomerase II levels at transcription level.[104]

Many drugs, e.g. doxorubicin and etoposide, are affected by a combination of MDR, MRP and atypical MDR mechanisms. While *m*-AMSA has low activity against solid tumors, it is a poor substrate for P-glycoprotein.[48] This raises the possibility of designing new agents combining the features of potency against solid tumors, such as breast cancer, and maintaining activity despite MDR.

Differences in the spectrum of activity of different type II topoisomerase inhibitors may be due to differences in specific gene damage as well as interaction with specific oncogenes.[48] Also, deletion of a presumed nuclear localization signal producing al-

tered subcellular distribution of topoisomerase IIα has recently been shown to induce resistance to a number of topoisomerase II inhibitors.[13]

Clinical use of topoisomerase II inhibitors in breast cancer

Topoisomerase II inhibitors have an established role in the management of breast cancer. Doxorubicin remains the most clinically active drug in this disease,[105] while other anthracyclines, especially epirubicin, and anthracenediones are commonly used in breast cancer chemotherapy. Recently, etoposide has also been increasingly advocated for use in both high dose regimes and in relapsed patients with resistance to anthracyclines. The use of these agents will be considered in three settings: adjuvant chemotherapy of localized disease, neoadjuvant therapy of locally advanced disease and in the treatment of metastatic breast cancer.

Adjuvant therapy

The use of combination chemotherapy began in the late 1960s in an attempt to improve the outcome for the large numbers of women who relapsed with metastatic breast cancer despite aggressive local surgical treatment of their primary tumor with or without radiotherapy. The combination of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) has been used extensively in those with involved axillary nodes at primary surgery, and has produced a 20–25% increase in disease-free survival in premenopausal women, 5 years after completing primary treatment.[106]

The initial use of doxorubicin showed no clear advantage over standard CMF, particularly in those with less than four involved nodes.[107,108] A recent trial, however, looking at women at high risk of relapse (with four or more involved nodes), showed a regime scheduling four initial courses of doxorubicin followed by eight courses of CMF to possess a clear advantage over an alternating schedule of the two treatments.[109] The survival benefit for this form of scheduling has been maintained at 6 years and, if confirmed, this use of doxorubicin would appear most appropriate where patients are at particularly high risk of relapse following standard CMF treatment. The situation should be further clarified by the current ECOG trial of CMF versus CAF (cyclophosphamide, doxorubicin and 5-fluorouracil) regimens.

The oncogene profile of breast tumors has also recently been identified as being clinically significant. Muss *et al.*[90] demonstrated a dose–response relationship with doxorubicin-containing chemotherapy only in high expressors of c-*erb*-B2, suggesting resistance proportional to the degree of expression. This contrasts with the increased chemosensitivity seen in those cell lines who co-amplify topoisomerase IIα and c-*erb*- B2.[91] This co-amplification has also been reported using *in situ* hybridization studies of breast tumors.[110]

Epirubicin is the 4′ epimer analog of doxorubicin. It has equivalent cytotoxicity to doxorubicin but a lower tendency to produce both myelotoxicity and particularly cardiotoxicity (reviewed by Plosker and Foulds[111]). In view of these advantages it is being used in adjuvant treatment regimes both in standard dose comparison with CMF and in the setting of high-dose chemotherapy with stem cell rescue for women at high risk of relapse. Wils *et al.*[112] found a non-significant trend towards better outcome with the epirubicin combination but, as with doxorubicin, it may be that clear benefits are not seen unless the optimal scheduling is used and only patients at higher risk of relapse are treated.

Mitoxantrone is a dihydroxy-anthrocenedione which lacks the amino sugar of doxorubicin and, as indicated earlier, has reduced cardiotoxicity with retained cytotoxicity.[113] It has been shown to be effective in metastatic breast cancer but in the adjuvant setting only one small study has been reported, showing no greater efficacy than CMF but with increased toxicity.[114]

The place of high dose adjuvant chemotherapy in women with a high risk of relapse is unclear. Several groups are undertaking these studies often using topoisomerase II inhibitors such as etoposide—with or without an anthracycline—in combination with alkylating agents and a platinum drug.[115] Results from high-dose treatments are awaited with interest, but many of these trials are non-randomized with small numbers or have very short follow-up at this stage.

Neoadjuvant (primary) therapy

This approach involves giving chemotherapy, usually with an anthracycline-containing regime, prior to definitive surgery. It was introduced in an attempt to 'down-stage' advanced local disease and has been shown to make conservative surgery possible in the vast majority of cases.[105] This chemotherapy approach is now also being explored in selected patients with operable disease.

In the first study of primary chemotherapy for large breast tumors, four different drug combinations were compared in women with tumors larger than 3 cm.[116] The drugs were cyclophosphamide and 5-fluorouracil combined with methotrexate (CMF), doxorubicin (FAC), epirubicin (FEC) or mitoxantrone (FNC). High response rates were found in all treatment groups and conservative surgery was subsequently possible in 87% of those with tumors up to 5 cm at presentation. Interestingly, in a separate subgroup, doxorubicin as a single agent gave equivalent response rates to the combination regimes.

Other studies have reported similar response rates[117] which are often higher than those reported for metastatic disease using the same drug combinations.[118] The rationale behind such tumor sensitivity may be related to exposure of drugs to a 'cytotoxic naive' tumor population which has not expressed the various resistance mechanisms discussed previously. In addition, it has been reported that non-curative surgery and radiotherapy induce the expression and release of a serum growth factor which stimulates residual tumor cell growth. This effect can be blocked by prior chemotherapy or hormone therapy.[119]

Topoisomerase II inhibitors in advanced breast cancer

At present chemotherapy for metastatic breast cancer is not curative but rather is given to improve quality of life and prolong survival. Topoisomerase II inhibitors are amongst the most active drugs in this setting and it is in this group of patients that most potential lies for increasing the potency and specificity of treatment with new and modified topoisomerase II inhibiting drugs to meaningfully improve outcome.

Of existing drugs, doxorubicin remains the most active as a single agent and when used in combination has a 10–20% higher response rate than other polydrug regimes.[105] Epirubicin has similar potency and is commonly used in salvage therapy as a single agent or in combination. The drugs in CMF have different cytotoxic mechanisms to anthracyclines and drug sensitivity to topoisomerase II inhibitors is often maintained when anthracyclines are used on relapse. Such patients who have previously had CMF as adjuvant therapy have response rates to anthracyclines of 25–40%.

Mitoxantrone is being increasingly used in metastatic disease, often in combination with mitomycin C and methotrexate (MMM). This regime has been shown to be equivalent to CMF in advanced disease and is well tolerated.[120]

Etoposide was initially used in intermittent bolus schedules in breast cancer in the 1970s with disappointing results.[50] It was reintroduced when it became apparent that the cytotoxic effects of etoposide are highly schedule-dependent with low dose, prolonged daily dosing increasing both bioavailability and response rates in lung cancer when compared with bolus regimes.[51] By using low-dose oral etoposide for a 14–21 day period, different groups have reported significant activity in pretreated patients with advanced breast cancer.[121,122] The activity of etoposide in this setting underlies the importance of optimising scheduling for drugs which are likely to work in a cell cycle dependent way and suggest that, by further optimizing existing drug design and scheduling, further improvements in drug efficacy can be obtained.

Toxicity of topoisomerase II inhibitors

These aspects have been extensively reviewed elsewhere.[105,123] The toxicities of primary concern, particularly in the context of potentially curative therapies, are secondary leukemia and myelotoxicity.

Acute myeloid leukemia occurring after topoisomerase II inhibitors occurs with short latency (1–2.5 years) and is associated with chromosome 11q23 rearrangements. This leukemia has mainly been reported in the treatment of childhood hematological malignancy and particularly with epipodophyllotoxins. There is a clear dose–response phenomenon with an incidence of 6–12% at 6 years in patients who received etoposide at 300 mg/m^2 1–2/week (total dose 9.9 g/m^2) during treatment of primary leukemia.[123] There is also a risk from other topoisomerase II inhibitors and in solid tumors although this is difficult to quantify due to the frequent concurrent use of alkylating agents and the difficulty in routinely distinguishing the cause.[42]

Cardiotoxicity has been described with anthracyclines with a 1–10% risk of clinically significant cardiomyopathy when doxorubicin is given in a cumulative total dose greater than 550 mg/m^2.[73] Analogs of doxorubicin, e.g. epirubicin, have been found to be less cardiotoxic while maintaining cytotoxicity. Another approach has been to give concurrent cardioprotective agents, e.g. ICRF-187, which enable optimal dose intensity with minimal

cardiotoxicity. ICRF-187 is a bisdioxopiperazine that is hydrolyzed *in vivo* to form a bidentate chelator similar to EDTA. It minimizes cardiac toxicity by preventing formation of a doxorubicin–iron complex which generates free radicals.[124] Its use has been reported to allow increased dose intensity without concurrent cardiotoxicity.[125]

Modification of topoisomerase II inhibitor function

The activity and level of expression of topoisomerase II isozymes can be influenced at many levels and by many agents as outlined in Figure 1. The areas with most potential for therapeutic gain are discussed below.

Hormonal modification

Epstein and Smith[104] showed that estrogen increases the number of cleavable complexes and cytotoxicity of doxorubicin and m-AMSA. This suggests that anti-estrogens, if given concurrently, may reduce the efficacy of topoisomerase inhibitors. In addition, tamoxifen, which is cytostatic, reduces the proportion of cancer cells in the drug-sensitive S phase. These facts may account in part for the failure of combined chemo-endocrine therapy to show a consistent benefit in young women (reviewed in Harris *et al.*[105]). However, neither has the opposite approach of combining estrogens and chemotherapy shown a consistent enhancement of cytotoxicity,[126,127] perhaps due to sub-optimal scheduling or because only women with estrogen-inducible tumors, with low topoisomerase II levels, will benefit from such a combination.

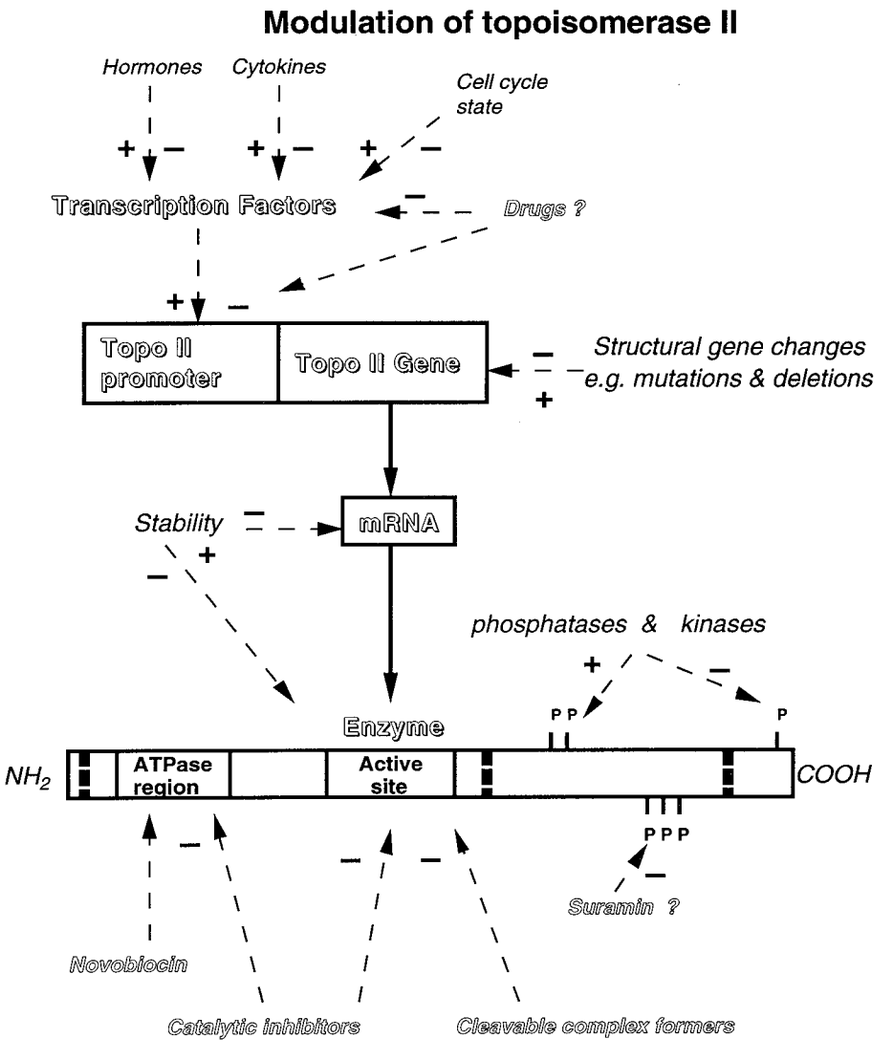

Figure 1. A schematic representation of the levels of potential interaction with topoisomerase II enzymes whereby expression and/or activity of the enzymes may be altered. Key: Topo, topoisomerase; +, stimulate activity or synthesis; –, inhibit activity or synthesis; P, putative phosphorylation sites; ■, possible nuclear localization signals.

Structural modifications of existing drugs

The sugar residues on etoposide and anthracyclines are not essential for their enzyme inhibition, but may act to stabilize DNA binding.[48,128–130] This enhanced stability may increase chromosomal defects. Idarubicin, an analog that lacks these residues, has been shown to be more potent, both as an enzyme inhibitor and as an antitumor agent, than daunorubicin which has them.[131] Anthracyclines have been further modified by removing the charged amine at the 3′ position which may interact both with cardiolipin (potentiating cardiotoxicity) and P-glycoprotein (potentiating multidrug resistance). Such analogs not only maintain potency in cell lines but are much more potent against cell lines expressing MDR.[48]

Amsacrine, a classical intercalating topoisomerase II inhibitor, plays an established role in the treatment of leukemia but has little activity against solid tumors. The tricyclic carboxamides, derivatives that have shown activity against solid tumors, appear to possess a different mechanism for stabilizing the cleavable complex.[132] This may both increase their spectrum of activity and render them less susceptible to resistance mechanisms. Like amsacrine, they are poor substrates for P-glycoprotein. One of these agents, *N*-[2-(dimethylamino) ethyl] acridine-4-carboxamide (DACA), also has activity against amsacrine resistant cell lines.[133]

Scheduling

Some evidence exists that drug treatment should be timed according to circadian rhythms.[134] Such rhythms, in which tumor and normal cells often diverge, have particular relevance to an enzyme which demonstrates cell cycle regulation. Tumor cells may be in S phase at different times of the day to normal cells, offering the potential for selective killing by scheduling alone.

As discussed earlier, the anti-tumor activities of etoposide and amsacrine are schedule dependent, while the timing of administration of anthracyclines also influences their toxic effects.[135] Increased awareness of the optimal schedules for existing drugs may increase the cell kill:toxicity ratio at least as effectively as the development of new drugs.

Cytokines

A number of different cytokines have been shown to interact with topoisomerase II and to affect cytotoxicity. Towatari *et al.*[136] showed that priming with granulocyte colony stimulating factor (G-CSF) increased topoisomerase II mRNA levels and cytotoxicity in leukemic cells with G-CSF receptors. While this does not relate directly to breast cancer, it provides evidence that increasing topoisomerase II expression can potentiate cell kill.

Topoisomerase inhibitors induce apoptosis in certain cell lines[137]—an effect which may occur via tumor necrosis factor (TNF).[138] Others have shown synergism of topoisomerase II inhibitor toxicity by prior administration of TNF.[68] Although the mechanism underlying these effects is currently unclear, the potential for therapeutic benefit remains and requires further investigation.

Delivery systems

Polyalkylcyanoacrylate nanoparticles were introduced as drug carriers with the potential to modify tissue distribution and specificity of anticancer drugs.[139] As carriers of doxorubicin, they have been found to increase efficacy against murine hepatic metastases with less cardiac toxicity.[140] Gibaud,[141] however, showed increased bone marrow toxicity when doxorubicin was delivered in this way. Since this is the current dose-limiting toxicity, further work needs to be done if this delivery system is to prove viable.

The development of 'stealth' liposomes represents such a modification of liposomal delivery systems. These are sterically designed liposomes with a prolonged circulation half-life of 1 day[142] and, in animal models, accumulate in tumors at much higher levels than in normal tissues.[143] This is due to reduced mononuclear phagocyte uptake[144] and increased permeability of the tumor microvasculature. This system, which others have confirmed as showing higher therapeutic effects in animal models, is entering early phase trials in our unit and others.

Lonidamine

Lonidamine, a dichlorinated derivative of indazole-3-carboxylic acid, depletes cellular ATP and may impede DNA repair.[145] It potentiates epirubicin cytotoxicity in breast cancer cell lines,[146] and in two clinical trials has been shown to increase response rates and disease-free survival when combined with anthracyclines.[147,148]

MDR reversal

Anti-estrogens, already an integral part of breast cancer therapies, have been shown to reverse MDR in cell lines when used in high dose.[149,150] A potential role for these compounds in this situation, however, must be balanced against their apparent adverse scheduling effects when used in combination with topoisomerase inhibitors as discussed above. A number of other *in vitro* studies (reviewed in Lum *et al.*[151]) have shown reversal of MDR with unrelated agents such as verapamil, cyclosporine and quinidine. Non-randomized clinical studies have suggested potential therapeutic gain with these agents,[152] but the only prospective, randomized trial in breast cancer, using quinidine in a dose that blocked MDR *in vitro* along with epirubicin and prednisolone, demonstrated no additive benefit to chemotherapy alone.[153] Toxicity has also often limited the use of MDR-blockers when such agents have been used in a dose sufficient to provide MDR blockade *in vitro*.[152,154] Studies continue to identify effective agents and to more clearly define the pharmacokinetic effects of adding these agents to topoisomerase II inhibitors which should enable more effective scheduling and prediction of toxicity. Patient selection is also critical in this approach as only tumors with high MDR expression are likely to be significantly affected by reversal of this resistance mechanism.

Changing the mechanism of topoisomerase inhibition

Possible strategies for circumventing acquired drug resistance entail switching to drugs that target either a different enzyme or the same enzyme by a different mechanism. In contrast to the topoisomerase II cleavable-complex forming inhibitors, which become less cytotoxic as the target topoisomerase II levels are reduced, catalytic inhibitors should be more effective in conditions of reduced nuclear topoisomerase II. In addition, resistance to catalytic inhibitors could be mediated by mechanisms different from those relevant to cleavable complex forming topoisomerase II inhibitors.

In a preliminary study of four doxorubicin resistant/sensitive paired cell lines with different patterns of topoisomerase II isozyme down-regulation, no cross-resistance could be found to the catalytic inhibitor of topoisomerase II ICRF-159 (unpublished observations). Similarly, Chen *et al.*[155] have reported a lack of cross-resistance, to non-cleavable complex forming topoisomerase II inhibitors, in a teniposide resistant CEM cell line. This lack of cross-resistance between the cleavable-complex forming topoisomerase II poisons and catalytic inhibitors gives a new option for second line chemotherapy of breast cancer.

New topoisomerase II inhibitors

Anthrapyrazoles

These are structural analogs of anthracenediones. CI941, for example, has a chromophore modification of the anthracenedione nucleus where an additional pyrazole ring has replaced a carbonyl group. The DNA-intercalating properties are maintained but the added ring prevents reductive metabolism from forming free radicals which are believed to be the basis of the cadiotoxicity of anthracyclines.[156] In a group of patients not previously exposed to topoisomerase II inhibitors this drug had a 63% response rate with leucopenia the limiting toxicity and severe alopecia in only 32%. There was a minor reduction of 6% in left ventricular function but this did not produce symptoms in any patients.[156] A larger phase II study confirmed significant activity in relapsed breast cancer with the same toxicity profile.[157]

Intoplicine

Intoplicine, a recently synthesized anticancer drug, belongs to the 7*H*-benzo[*e*]pyrido[4,3-*b*] indoles and manifests a broad spectrum of antitumor activity in animal models.[158] It has activity against cell lines and tumor samples resistant to other topoisomerase inhibitors.[159,160]

Its action is to unwind DNA and to induce cleavable complex formation and catalytic inhibition of both topoisomerase I and II.[159] Previously, the combination of topoisomerase I and II inhibitors has produced antagonism of cytotoxicity, possibly due to competition for cleavage sites.[161] With intoplicine, on the other hand, cytotoxicity is maximal when both enzymes are targeted, which may be because it binds strongly to DNA at a different cleavage site from those occupied by other topoisomerase II inhibitors and by camptothecan (a topoisomerase I inhibitor). While this drug has significant potential to increase cytotoxicity, its strong DNA binding may induce toxicity and its side effect profile is awaited with interest.

Makaluvamines

Makaluvamines are pyrroloiminoquinones derived from the Fijian sponge *Zyzzya* sp. Early studies have shown them to be intercalating agents which target topoisomerase II with significant activity against a solid human tumor xenograft model.[162] Their further development is awaited with interest.

Conclusions

Our growing understanding of the molecular structure and function of type II topoisomerases promises not only to assist us in using existing drugs more effectively but may also lead to the development of new drugs. More effective use might result from such novel procedures as modifying enzyme phosphorylation and inhibiting catalytic function, as well as improving the tumor specificity and scheduling of existing drugs.

Further research is needed into the significance of expression and selective inhibition of individual isoforms. The ability to modify these factors, to overcome resistance mechanisms and to reduce toxicity more effectively should enable more effective management of the patient with breast cancer, and emphasizes the potential of a measured, molecular approach to improving cancer care.

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