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Selection of cancer chemopreventive agents based on inhibition of topoisomerase II activity

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Abstract

The present study was undertaken to determine if *in vitro* inhibition of one or both of the two most dominant mammalian DNA topoisomerases (topos) is common among chemopreventive agents. To determine if an agent was a topo I inhibitor, we employed the DNA relaxation and nicking assays. For potential topo II inhibitors, we used the DNA unknotting and linearisation assays. 14 of 30 agents (47%) were ineffective in all four assays (IC_{50} > 100 μg/ml), and 11 (37%) inhibited topo II catalytic activity. The sensitivity of the topo II assay was 63%, selectivity 93%, positive predictive value 91%, and total accuracy 77%. For chemopreventive efficacy, the positive predictive value of the unknotting assay was 92%, and the total accuracy was 60%. These data suggest that reduced topo II activity is a desirable property of many known chemopreventive agents. We conclude that the unknotting assay could be a valuable addition to the *in vitro* tests presently used to select chemopreventive agents.

Keywords: Topoisomerase I; Topoisomerase II; Chemoprevention; Unknotting assay; Relaxation assay; Organoselenium; Carcinogenesis; Selectivity; Specificity; Predictive value

1. Introduction

Cancer chemoprevention involves the arrest, reversal, or delay of carcinogenesis using active pharmacological intervention of non-toxic quantities of chemical agents [1]. Candidate agents for chemoprevention studies are initially selected based on findings from basic research or from bioassay-guided fractionation of plant materials [2]. Cell and organ culture assays further refine the selection process before testing the most promising agents in more expensive and time-consuming animal models of carcinogenesis. Animal models were also developed to identify agents that are effective against cancers involved in specific target organs [3]. Preclinical models can distinguish between agents that inhibit the initiation phase (known as blocking agents) from those that inhibit the promotion/progression phases (known as suppressing agents). Further testing is done in human clinical phase I, II and III clinical studies, as outlined by Kelloff and colleagues [4]. A few agents have been tested in phase III clinical studies, wherein the activity of tamoxifen against breast cancer in women is a notable example [5].

In the strategy of chemopreventive agent development, *in vitro* testing is a critical initial component [4]. In the past, chemopreventive agents were selected and developed empirically, but recent advances in molecular biology of carcinogenesis allow a more mechanistic approach in the selection process [1]. For instance, carcinogenesis is viewed as an aberrance of normal cell differentiation. It is generally accepted that abnormal differentiation giving rise to dysplasia can be reversed with non-cytotoxic concentrations of agents that may act like hormones. An example of a successful application of this approach is the use of 13-*cis*-retinoic acid in the prevention of human leucoplakia [6]. The tumour-promoting effect of oestrogen during mammary carcinogenesis has long been realised. The success of

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tamoxifen, an oestrogen antagonist, in reducing the incidence of breast tumours in high-risk women provides evidence for the value of understanding the mechanism of action for the development of effective chemopreventive agents (for a more complete discussion of this subject, see [7]).

Based on these mechanistic considerations, we developed an approach for the discovery of natural product cancer chemopreventive agents [2]. This approach evaluates the effects of test agents on enzymes and processes that induce or promote cell proliferation, promote carcinogen binding to DNA, and inhibit drug detoxification by preventing phase II metabolic enzyme activation. Furthermore, this approach also evaluates the effects of test agents on enzymes and processes that modulate cell differentiation and proliferation.

In the present study, we examined the value of adding four new bioassays to an existing battery of *in vitro* assays. Our objective was to identify additional bioassays that could provide a high predictive value, to make the chemopreventive agent selection process more inclusive, and to reduce the possibility of missing important agents. The new assays could also provide additional information on the *in vivo* mode of action of the selected agents. We chose DNA topoisomerases (topos) I and II as the enzymatic targets of the new assays for the following reasons. Topo II is involved in cell proliferation; it is essential for chromosomal segregation and cell division; and it is regulated during cellular growth and differentiation, with proliferating cells containing up to 25 times higher levels than terminally differentiated or quiescent cells [8]. Topo II is the enzymatic target of clinically useful chemotherapeutic drugs, including etoposide (VP-16) and anthracyclines [9]. These cytotoxic drugs cause enzyme-mediated DNA breaks and collectively are classified as topo II poisons. Another class of topo II inhibitors, known as catalytic inhibitors, are generally inhibitors of tumour cell proliferation and inducers of cell differentiation, which are essential qualities for chemopreventive agents.

Topo I also serves as the target of clinically useful cancer therapeutic drugs such as camptothecin and its analogues. Topo I or topo II poisons prevent completion of the topoisomerisation reaction by stabilising the enzyme–DNA complex and producing what is known as the 'cleavable complex'. Topo II poisons can be identified by the linearisation assay, which reveals the cleavable complex. Topo I poisons are identified by the nicking assay which reveals nicks in plasmid DNA caused by the stabilisation of the topo I–DNA complex. Catalytic inhibitors prevent the binding of the test agent to DNA or enzymatic turnover and regeneration. Catalytic inhibitors of topo I are typically identified by the relaxation assay (in the absence of ATP), while catalytic inhibitors of topo II are assessed by the unknotting assay (in the presence of ATP).

The objective of the present study was to determine if chemopreventive agents inhibit topo I or topo II, if their mode of inhibition differs from that of chemotherapeutic drugs, and if topo assays are useful in identifying chemopreventive agents.

2. Materials and methods

2.1. Chemicals and reagents

All the test agents were purchased from Sigma Chemical Co, (St Louis, MO, USA). Topo I and Topo II were purchased from Topogen, Inc. (Columbus, OH, USA). Stock solutions of all test agents were dissolved in dimethyl sulphoxide (DMSO). Bacteriophage P4 *Vir1 del10* DNA and plasmid pUC8 DNA were purified as previously described [10].

2.2. Relaxation assay

For the determination of topo I catalytic activity, pUC8 DNA was used as the substrate in a reaction volume of 20 μl containing the following: 10 mM Tris–HCl, pH 7.9, 1 mM ethylene diamine tetraacetic acid (EDTA), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol and 2 units of purified human topo I. The appropriate inhibitor was added as indicated, and the reaction was initiated by the addition of the enzyme. Reactions were carried out at 37°C for 30 min. Gel electrophoresis was performed at 4 V/cm for 5 h in Tris–borate–EDTA buffer. For the quantitative determination of topo I activity, photographic negatives were scanned. The area representing supercoiled DNA, migrating as a single band at the bottom of the gel, was determined. The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

2.3. P4 unknotting assay

To determine topo II catalytic activity, knotted DNA that had been isolated from the tailess capsids of the bacteriophage P4 *Vir1 del1O* was used as the substrate. Reaction mixtures contained 50 mM Tris–HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM adenosine triphosophate (ATP), and 0.5 mM dithiothreitol. The test agent was added, as indicated, followed by the addition of 2 units of human topo II. Reactions (20 μl final volume) were initiated by adding 0.6 μg of knotted DNA and carried out at 37°C for 30 min. Reactions were terminated by the addition of 5 ml of a stop solution containing 5% sodium dodecyl sulphate (SDS), 50 mM EDTA, 25% ficoll, and 0.05 mg/ml bromophenol

blue. Samples were loaded on 0.8% agarose gels, and electrophoresis was performed at 4 V/cm for 5 h in Tris–borate–EDTA buffer. Gels were stained with ethidium bromide, destained, and photographed over an ultraviolet (UV) light source. For the quantitative determination of topo II activity, photographic negatives were densitometrically scanned. Unknotted DNA, migrating as a single band at the top of the gel, was measured in this manner. The concentration of the inhibitor preventing 50% of the substrate (knotted DNA) from being converted into the reaction product (unknotted DNA) was determined from a standard curve. By averaging three to four such experiments, the IC_{50} values were determined.

2.4. Plasmid linearisation assay

Topo II-targeting agents having the ability to enhance topo II-mediated DNA cleavage were screened using the linearisation assay under the reaction conditions provided by the supplier of the enzyme (Topogen, Inc., Columbus, OH, USA). Briefly, 20-μl reaction mixtures contained 30 mM Tris–HCl, pH 7.6, 3 mM ATP, 15 mM β-mercaptoethanol, 8 mM $MgCl_2$, 60 mM NaCl, 1 μl of the test agent (or solvent), 0.3 μg of pUC8, and 10 units of human topo II (added last). After a 15-min incubation at 37°C, SDS-proteinase K was added, and following a 15 min incubation at 37°C, samples were extracted with $CHCl_3$-isopropanol and electrophoresis was performed on a 1% agarose gel containing ethidium bromide. Gels were photographed, and photographic negatives were scanned.

2.5. Nicking assay

Topo I-targeting agents with the ability to enhance topo I-mediated DNA cleavage were screened using pUC8 DNA under the reaction conditions provided by the supplier of the enzyme (Topogen, Inc.). Briefly, 20 μl of reaction mixtures contained 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 μl of the test agent (or solvent), 0.5 μg of pUC8, and 10 units of human topo I (added last). After a 30-min incubation at 37°C, SDS-proteinase K was added, and, following a 30-min incubation at 37°C, samples were extracted with $CHCl_3$-isopropanol and electrophoresed on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were photographed, and photographic negatives were scanned. After integration of the three bands, the reaction product (nicked DNA) was expressed as a percentage of total DNA.

2.6. Animal efficacy studies

The animal models referenced in Table 4 have been described in detail elsewhere ([11], see additional references in Table 4). A brief description is given below. The abbreviations used in Table 4 are given in parenthesis. In most models, the chemopreventive agent is administered in the diet 1 week before carcinogen administration. Efficacy is measured as per cent reduction of tumour incidence or multiplicity compared with carcinogen controls.

2.6.1. N-methyl-N-nitrosourea (MNU)-induced carcinomas in Syrian hamsters (LM)

The carcinogen is administered with a specially designed catheter in the trachea once a week for 15 weeks. Tracheal squamous cell carcinoma are produced in 40–50% of treated animals within 6 months.

2.6.2. Diethylnitrosamine (DEN)-induced hamster carcinomas in male Syrian hamsters (LD)

The carcinogen is injected subcutaneously (s.c.) twice weekly for 20 weeks. Tracheal tumours are produced in 90–100% of the animals and lung tumours in 40–50% of animals.

2.6.3. Mouse colon (CM)

Female CF1 mice are injected intraperitoneally (i.p.) with methylazoxymethanol (MAM) acetate once a week for 4–6 weeks. Colon tumours appear within 38 weeks.

2.6.4. Rat colon (CR)

A single s.c. dose of 30 mg azoxymethane (AOM) per kg body weight is given to 7-week-old F344 male rats, producing colon adenocarcinomas and adenomas within 40 weeks.

2.6.5. Colon crypts (CY)

Male F344 rats receive two injections of 15 mg AOM per kg body weight 1 week apart. After sacrifice, the frequency of aberrant crypt foci is determined by histopathological evaluation. Two protocols are used: one is designed to evaluate agents that inhibit initiation of colon crypts and another to identify agents that inhibit the postinitiation phase.

2.6.6. Rat mammary gland tumours induced with MNU (MM)

A single intravenous (i.v.) injection of 50 mg/kg body weight is given to Sprague–Dawley rats at 50 days of age. Mammary tumours appear 60–80 days post-carcinogen treatment. MNU-induced tumours are invasive, predominantly adenocarcinomas and mostly hormone-dependent.

2.6.7. Dimethylbenz(a)anthracene (DMBA)-induced rat mammary gland tumours (MD)

The carcinogen is given in a single intragastric injection at 12 mg. DMBA is activated in the liver so agents that inhibit cytochrome P-450 can be tested in this

model. Tumours are encapsulated with a high percentage of adenomas and fibroadenomas. DMBA-induced tumours are also mostly hormone-dependent.

2.6.8. Mouse skin (MS)

SENCAR mice are administered a single dose of 2.5 μg DMBA in 0.2 ml acetone. Beginning 1 week later, the tumour promoter 2-O-tetradecanoylphorbol-13-acetate (TPA) is applied twice weekly. Skin papillomas begin to appear after 6–7 weeks of treatment with TPA.

2.6.9. Mouse bladder (MB)

Male BDF mice are given eight weekly doses of N-butyl-N-(4-hydroxybutyl)nitrosamine (OH-BBN) by intragastric instillation beginning at 50 days of age. The carcinogen produces urinary bladder invasive transitional cell carcinomas in approximately 40% of the animals.

2.6.10. Lung hamster model (LB)

Benzo(a)pyrene (B(a)P) silicone pellets are implanted in the right bronchus, releasing carcinogen and mimicking chronic human exposure. Epidermoid carcinomas similar to non-small cell lung cancer occur at implant site. Histological progression is evaluated 150 days post-carcinogen administration.

2.6.11. A/J mouse lung nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) model (LN)

The A/J mouse strain is sensitive to both spontaneous and carcinogen-induced formation of lung tumours. The tobacco-specific NNK, produces a progression characterised by alveolar hyperplasis, solid and papillary adenomas, and carcinomas.

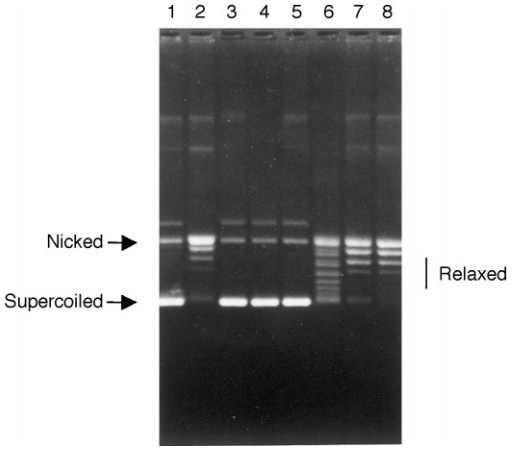

Fig. 1. Representative relaxation assay for determining the effect of 1,4-phenylene bis(methylene) selenocyanate (pXSC) on topo I catalytic activity. Substrate supercoiled pUC8 DNA was incubated with two units of purified topo I (lanes 2–7) plus the concentrations of pXSC shown below: lane 1, substrate supercoiled pUC8 DNA in the absence of test agent; lane 2, relaxed pUC8 DNA without test agent; lane 3, 100 μg/ml pXSC; lane 4, 50 μg/ml pXSC; lane 5, 25 μg/ml pXSC; lane 6, 15 μg/ml pXSC; lane 7, 5 μg/ml pXSC; lane 8, 1.0 μg/ml pXSC. The IC_{50} was determined by averaging the data from at least three independent experiments.

2.6.12. Epidermal lesions in transgenic mice (MO)

The transgenic mice used in these studies are from K14-HPV16, containing the wild-type version of the human papilloma virus (HPV) early region cloned behind the human keratin-14 enhancer promoter. Chemoprevention of mice treated with the agent, for example (α-Difluoromethylornithine) DFMO administered in drinking water [12], is evaluated by serial sacrifice at 4–32 weeks of age. The incidence of visible and microscopic epidermal cancers is compared between the untreated control animals and those treated with the carcinogen.

2.7. Additional bioassays

Bioassays that are used for identification or characterisation of chemopreventive agents have been previously described in detail [13].

3. Results

3.1. Evaluation of test agents as topo I or topo II inhibitors

The effect of various test agents on the catalytic activity of topo I was evaluated using the relaxation assay. Initially, all compounds were evaluated at a concentration of 100 μg/ml. Agents not showing an effect at 100 μg/ml were considered ineffective and were not tested further. Agents showing inhibition at 100 μg/ml were tested in the 1–100 μg/ml range, until the minimum concentration that was necessary to inhibit 50% of the topo I or topo II catalytic activity (IC_{50}) was determined. An example of the approach for determining the inhibitory effects of 1, 4-phenylene bis(methylene) selenocyanate (pXSC) on topo I catalytic activity is shown in Fig. 1. In the initial test, pXSC inhibited topo I relaxation at 100 μg/ml. This compound was further tested at concentrations ranging from 1 to 100 μg/ml (Fig. 1). The IC_{50} (the agent concentration that prevents topo I from converting 50% of supercoiled DNA to its relaxed form), was calculated by averaging three independent experiments and found to be 15 μg/ml (see lane 6). Only three additional agents were found effective in inhibiting the catalytic activity of topo I: protamine sulphate (25 μg/ml), quercetin (12.8 μg/ml), and ellagic acid (0.6 μg/ml). These four agents were further tested using the nicking assay to determine if they stabilised the topo I cleavable complex (data not shown). As none was found to be effective, these agents were classified as topo I catalytic inhibitors. However, these compounds also inhibited topo II catalytic activity (see below).

A similar approach was used to identify topo II catalytic inhibitors and determine their mean IC_{50} values. Although the relaxation assay can also measure topo II activity if ATP is included in the reaction mixture, the preferred test for this purpose is the unknotting assay. The unknotting assay is specific for measuring topo II activity because it is based on the conversion of knotted DNA to its unknotted form, which requires DNA double-strand breakage followed by strand rotation and ligation-activities uniquely performed by topo II. The removal of these knots by the enzyme can be visualised in agarose gels. Following electrophoresis, under low voltage, P4-knotted DNA migrates as a smear, while unknotted DNA migrates as a single band (see Fig. 2). When we tested the 30 agents shown in Table 1 at an initial concentration of 100 μg/ml, 16 were found to inhibit the conversion of knotted P4 DNA to the unknotted form. These agents were further tested at a lower range of concentrations to define IC_{50} values (see Table 1). The agent concentration required to inhibit topo II from converting 50% of the unknotted DNA to its knotted form is defined as the IC_{50}. The mid-point (the lane in which equal amounts of unknotted and knotted DNA are present) was determined by scanning the photographic negatives of gels similar to the one shown in Fig. 2. In this gel, pXSC, which completely inhibited topo II activity in the test range of 1–100 μg/ml, was retested in the 0.0625–2.5 μg/ml range, and the IC_{50} was determined as 0.125 μg/ml. Fig. 2 shows the results of one of three experiments used to determine the mean IC_{50} of pXSC and the other 16 positive agents. Of these agents, 11 had mean $IC_{50} \leq 25$ μg/ml, and they were considered positive (Table 1). These agents were further tested using the linearisation assay to determine if they stabilised the topo II cleavable complex (data not shown). Only genistein was found effective in this assay, as anticipated from previous studies [14]. With the exception of genistein (which also functions as a topo II poison), the remaining 10 agents listed in Table 1 were classified as topo II catalytic inhibitors.

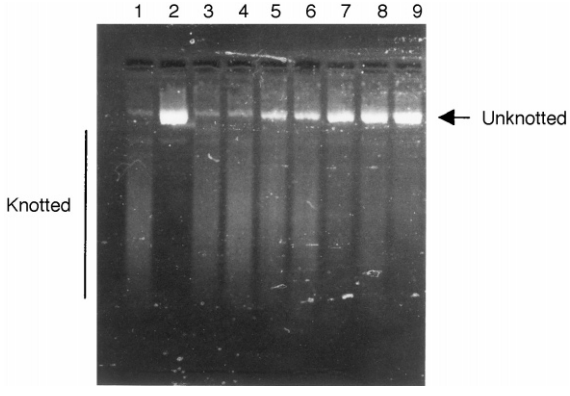

Fig. 2. Representative unknotting assay for determining the effect of pXSC on topo II catalytic activity. Substrate P4 DNA was incubated with two units of purified topo II (lanes 2–9) with the following concentrations of the test agent. Lane 1, control knotted substrate P4 DNA; lane 2, unknotted P4 DNA without test agent; lane 3, 100 μg/ml teniposide (positive control); lane 4, 2.5 μg/ml pXSC; lane 5, 1.5 μg/ml pXSC; lane 6, 0.5 μg/ml pXSC; lane 7, 0.25 μg/ml pXSC; lane 8, 0.125 μg/ml pXSC; lane 9, 0.0625 μg/ml pXSC. The IC_{50} was determined by averaging the data from at least three independent experiments.

The most potent topo II catalytic inhibitors (in order of potency with the IC_{50} values in μg/ml shown in parentheses) were: pXSC (0.125), protamine sulphate (0.25), ellagic acid (0.7), caffeic acid phenethyl ester (CAPE) (2.5), 1,2-dithiole-3-thione (5), sulphoraphane (5), quercetin (6.9), indole-3-carbinol (12.5), resveratrol (15), genistein (24) and anethole trithione (25). Four other agents inhibited topo II catalytic activity in the first screen at 100 μg/ml, but were ineffective at lower concentrations (Table 1). The chemical structures of these agents are shown in Fig. 3. Obvious structural similarities can be seen in the two thiones (Group A) and the phenols and catechols (Group B). The miscellaneous compounds (Group C) do not fit any obvious structural pattern, and they may act by different mechanisms. The inhibitory mechanism of action for all of the compounds shown in Fig. 3 is not known; however, the phenols and catechols, in particular, probably act through their aromatic hydroxyl groups either directly or indirectly. Future work will involve exploring the inhibitory mechanisms further in order to make predictions about the relative efficacy of other structurally similar candidates.

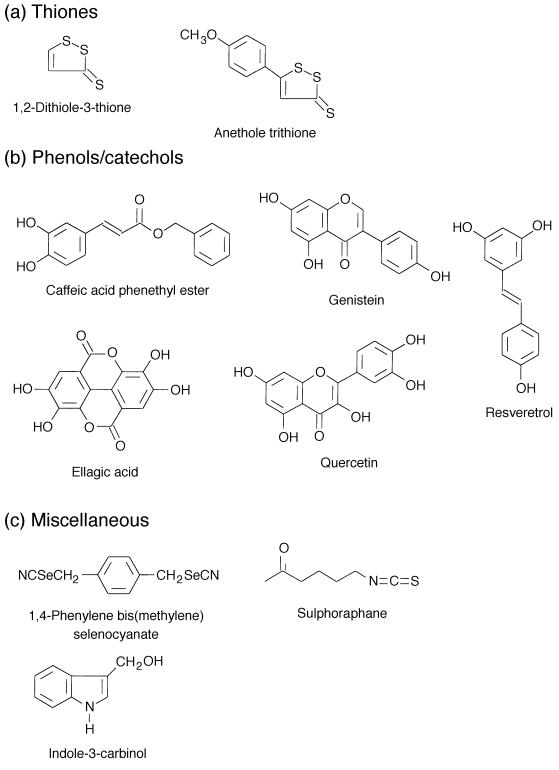

Fig. 3. Chemical structures of effective topo II catalytic inhibitors that are also effective chemopreventive agents.

Table 1
The effect of potential chemopreventive agents on topo I and topo II activities

Agent name[a]	Relaxation assay[b] (IC_{50} μg/ml)	Nicking assay[c] (IC_{50} μg/ml)	Unknotting assay[d] (IC_{50} μg/ml)	Linearisation assay[e] (IC_{50} μg/ml)
S-Allyl-L-cysteine	NE	NE	NE	NE
γ-Amino-N-butyric acid (GABA)	NE	NE	NE	NE
Anethole trithione	NE	NE	25	NE
Ascorbigen	NE	NE	NE	NE
Astaxanthin	NE	NE	NE	NE
Caffeic acid phenethyl ester (CAPE)	NE	NE	2.5	NE
trans-Chalcone	NE	NE	100	NE
Diflunisal	NE	NE	100	NE
1,2-Dithiole-3-thione	NE	NE	5	NE
Ellagic acid	0.6	NE	0.7	NE
Galanin	NE	NE	NE	NE
Gallic acid	NE	NE	NE	NE
Genistein	NE	NE	24	10
Hederagenin	NE	NE	100	NE
4-Hydroxy-2-ethyl-5-methyl-3[2H]-furanone (HEMF)	NE	NE	NE	NE
Indole-3-carbinol	NE	NE	12.5	NE
Madecassoside	NE	NE	NE	NE
Maltol	NE	NE	NE	NE
Nifedipine	NE	NE	50	NE
Oleanolic acid	NE	NE	100	NE
1,4-Phenylene bis(methylene) selenocyanate (pXSC)	15.0	NE	0.125	NE
Piroxicam	NE	NE	NE	NE
Protamine sulphate	25.0	NE	0.25	NE
Protocatechuic acid	NE	NE	NE	NE
Quercetin	12.8	NE	6.9	NE
Resveratrol	NE	NE	15.0	NE
Sphingomyelin	NE	NE	NE	NE
Sulphoraphane	NE	NE	5	NE
Thiaproline	NE	NE	NE	NE
Ursolic acid	NE	NE	NE	NE

NE, No effect at up to 100 μg/ml concentration; NBT, nitrobluetetrazolium; NSE, non-specific esterase activity; SE, specific esterase activity; GST, glutathione S-transferase activity; QR, quinone reductase activity.

[a] Selection of these agents was based on their chemical structures and/or literature studies, which contained characteristics that may provide chemopreventive qualities.

[b] The relaxation assay in the absence of adenosine triphosphate (ATP) measures topo I catalytic activity.

[c] The nicking assay measures topo I poisons.

[d] The unknotting assay measures topo II catalytic inhibitors.

[e] The linearisation assay identifies topo II poisons.

3.2. *Comparison of relaxation and unknotting assays to other* in vitro *bioassays*

In Table 2, the results of additional *in vitro* assays are compared with the two new assays: topo I relaxation and topo II unknotting. For comparative purposes, we adapted the same definitions that were used by Steele and colleagues in a previous study [15]. We calculated the concordance (selectivity, specificity and accuracy) of each of the two new assays and compared these with the data obtained from the panel of 12 assays (Table 3). Sensitivity was defined as the number of agents positive in at least one assay of the panel of 12 and positive in the topo assay (relaxation or unknotting) divided by the total number positive in one of the assays alone (see Table 3). Selectivity was defined as the number of agents negative in the panel of 12 and also negative in the topo assay divided by the total number negative in the bioassays. Accuracy was defined as the fraction of correct positive and negative comparisons divided by the total number of comparisons. The unknotting assay had the highest sensitivity, at 63%. Both the relaxation and unknotting assays had a high selectivity, 93% each. The overall accuracy was defined as the total number of correct responses in a particular assay compared with the panel of 12, divided by the total agents considered. The overall accuracy of the unknotting assay was highest, at 77%, compared with 53% for the relaxation assay (Table 3).

A second comparison measured the predictive value of a positive or negative result with the new assay and with the panel of 12 assays. Thus, the predictive value of an assay is the ratio of true test results to the total number of results. The predictive value of a positive topo assay would be the number of results that were positive both in the topo assay and in at least one of the

Table 2
Performance of potential chemopreventive agents in the standard panel of *in vitro* bioassays compared with their ability to inhibit topo I or topo II catalytic activities as determined by the relaxation and unknotting assays, respectively

Agent name	Relaxation assay	Unknotting assay	Standard panel of *in vitro* bioassays[a]
γ-Amino n-butyric acid (GABA)	–	–	–
Anethole trithione	–	+	AO
Ascorbigen	–	–	–
Astaxanthin	–	–	–
Caffeic acid phenethyl ester (CAPE)	–	+	BP, MET, ER, AO, QR, HL
Diflunisal		–	–
1,2-Dithiole-3-thione	–	+	BP, MET, QR, ODC, HL, QR, GST, GSH
Ellagic acid	+	+	QR
Galanin	–		–
Gallic acid	–	–	AO
Genistein	–	+	BP, MET, QR, HL, QR
Hederagenin	–	–	–
4-Hydroxy-2-ethyl-5-methyl-3[2H]-furanone (HEMF)	–	–	–
Indole-3-carbinol	–	+	QR
Madecassoside	–	–	–
Maltol	–	–	BP, AO, ODC
Nifedipine	–	–	–
Oleanolic acid	–	–	ODC
1,4-Phenylene bis(methylene)selenocyanate (pXSC)	+	+	ODC, HL, CYT
Piroxicam	–	–	AO
Protamine sulphate	+	+	–
Protocatechuic acid	–	–	AO
Quercetin	+	+	CO, AO, QR
Resveratrol	–	+	AM, CO
S-allyl-L-cysteine	–	–	–
Sphingomyelin	–	–	–
Sulphoraphane	–	+	BP, MET, QR, ODC, HL, QR, GSH, CYT
Thiaproline	–	–	–
trans-Chalcone	–	–	–
Ursolic acid	–	–	QR, CYT

–, Indicates that the agent was not effective at the highest concentration tested; +, indicates that the agent was strongly effective with $IC_{50} \leqslant 25$ μg/ml.

[a] The panel of *in vitro* bioassays includes the following 12 bioassays which have been described previously [13] and the following abbreviations apply: BP, inhibition of carcinogen [benzo(a)pyrene]-DNA binding in human bronchial epithelial (BEAS-2) cells; AO, antioxidant potential through free radical scavenging activity or inhibition of TPA-free radical formation in HL-60 cells; ODC, inhibition of TPA–ODC activity in mouse epidermal ME 308 cells; HL, induction of HL-60 cell differentiation, analysed by NBT reduction, enzymatic activity (NSE/SE) and ^{3}[H] thymidine incorporation; QR, induction of quinone reductase activity in murine hepatoma Hepa 1c1c7 cells; GSH, induction of glutathione (GSH) content in cultured rat hepatoma H4IIE cells; GST, induction of GST activity in H4IIE cells; MET, modulation of benzo(a)pyrene metabolism in cultured BEAS-2B cells; ER, oestrogen receptor competitive assay with ^{3}[H]-oestradiol; CYT, cell cytotoxicity; CO, cyclo-oxygenase inhibition; TPA, 2-O-tetradecanoylphorbol-13-acetate; AM, antimutagenicity assay.

Table 3
Sensitivity, selectivity, accuracy and predictive value of the relaxation and unknotting assays versus assays currently used to screen potential chemopreventive agents

	Result	Panel of bioassays		Predictive value	% Total (accuracy)
		Sensitivity	Selectivity		
		+	–		
Relaxation assay	+	3	1	3/4	75
	–	13	13	13/26	50
	Total	3/16 (19%)	13/14 (93%)	16/30	53
Unknotting assay	+	10	1	10/11	91
	–	6	13	13/19	68
	Total	10/16 (63%)	13/14 (93%)	23/30	77

Table 4
Topo II inhibitory effect of potential chemopreventive agents compared with their chemopreventive efficacy in animal studies

Agent[a]	Unknotting assay (summary)	*In vivo* studies		[Ref.]
		Positive test results	Negative test results	
α-Difluoromethylornithine (DFMO)	–	CR, CY, MD, MM, MO, MB, MS		11
1,2-Dithiole-3-thione	+	CR, MD		16
1,4-Phenylene bis(methylene)selenocyanate (pXSC)	+	CR, MD, LN		17,18
Anethole trithione	+	CR,CY,MD		19–21
Caffeic acid phenethyl ester (CAPE)	+	CR		22
Catechin	–	MD		11
Diflunisal	–		CY	11
Ellagic acid	+	CR	MD, MM, MB	15,16,23
Genistein	+	CY	CR	24
Ibuprofen	–	CR, CY, MM, MB		11
Indole-3-carbinol	+	CM, CY, MD, MM		25,26
Ketoprofen	–	CR, CY,MB		11
Morin	–	CR		11
Oltipraz	–	LD, LM, CM, CR, CY, MD, MM, MB,MS		11
Phloretin	+		CR	11
Piroxicam	–	CR, CY, MB, MS		11
Quercetin	+	CM, CY, MD		27
Resveratrol	+	CY		28
S-allyl-L-cysteine	–	CY, CR		11
Selenite sodium	+	LD, CY, MD		11
Sphingomyelin	–		CY	11
Sulphoraphane	+	MD		29
Suramin	+	CY		11
Ursolic acid	–		CY	11
Vitamin E	–		LM, MM, MB	11

–, no effect at an agent concentration of 25 μg/ml or less.

More information about the animals models of cancer chemoprevention is given in the Materials and methods section.

LD, lung DEN; LM, lung MNU; LN, lung NNK; LB, lung B(a)P; CM, mouse colon; CR rat colon; CY, colon crypts; MD, mammary DMBA; MM, mammary MNU; MO, skin transgenic; MB mouse bladder; MS skin DMBA; DEN, diethylnitrosamine; MNU, N-methyl-N-nitrosourea; NNK, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; B(a)P, benzo(a)pyrene.

[a] Only agents that were tested in animal studies are included in this table.

panel of 12 assays. The positive predictive value was determined as 75% for the relaxation assay and 91% for the unknotting assay. The predictive value of a negative topo assay would be the number of negative results both in the topo assay and in the entire panel of 12 assays. The negative predictive value was determined at 50% for the relaxation assay and 68% for the unknotting assay (Table 3).

3.3. Comparison of unknotting assays to the animal efficacy results

Table 4 lists the agents that have been tested in at least one animal model and for which we have topo II inhibitory data. The sensitivity of the unknotting assay, defined as the number of agents positive in animal efficacy studies which are positive in inhibiting topo II divided by the total number positive in one of the assays, was found to be 60% (Table 5). Selectivity of the unknotting assay (the number of agents negative in animal efficacy studies which were negative in inhibiting topo II divided by the total number that were negative) was 80%. A positive predictive value of 92%, a negative predictive value of 33%, and an overall accuracy of 64% were calculated for the unknotting assay when compared with the animal efficacy tests.

Table 5
Sensitivity, selectivity, accuracy and predictive value of the unknotting assay versus chemopreventive efficacy in animal studies[a]

	Result	Animal studies		Predictive value	% Total (accuracy)
		Sensitivity	Selectivity		
		+	–		
Unknotting assay	+	12	1	12/13	92
	–	8	4	4/12	33
	Total	12/20 (60%)	4/5 (80%)	16/25	60.0

[a] Values were calculated based on data presented in Table 4. Agents that are positive in at least one animal model are considered effective.

4. Discussion

For the initial discovery and characterisation of cancer chemopreventive compounds, it is imperative to employ *in vitro* bioassay procedures. Based on known or suspected mechanisms of carcinogenesis, a broad range of assay procedures can be devised. We recently reported the evaluation of various chemopreventive agents employing a panel of 12 assay procedures [2]. These assays were selected based on the modulation of activities or processes considered critical in carcinogenesis. The data presented here strongly support the hypothesis that inhibitors of the catalytic activity of topo II (as determined by the unknotting assay) are often effective chemopreventive agents. Out of 11 agents that inhibited topo II catalytically with an $IC_{50} \leqslant 25$ μg/ml, 10 were effective chemopreventive agents in preclinical studies (see below), giving a positive predictive value of 91%. This value is greater than the predictive value of 76% reported for agents that were positive in at least one of the cell and tissue culture assays that are routinely used by the National Cancer Institute (NCI) for this purpose [15].

Five *in vitro* assays are currently used by the NCI before testing potential chemopreventive agents in animals: inhibition of (a) transformation in rat tracheal epithelial cells; (b) anchorage independence in human lung tumour cells; (c) hyperplastic alveolar nodule formation in mouse mammary organ cultures; (d) anchorage independence in mouse epidermal cells; and (e) calcium tolerance in human foreskin epithelial cells [15]. These assays are not only more time-consuming and expensive than the unknotting assay, but they also give lower positive predictive values (individually or combined). More importantly, three of the agents identified with the unknotting assay (genistein, indole-3-carbinol and pXSC) are among 16 agents selected for clinical development by the Cancer Chemoprevention Branch of the NCI. The selection was based on evidence for safety and efficacy in preclinical and clinical studies [19].

Chemopreventive agents that are potent topo II inhibitors fall into three categories: thiones, phenols/catechols and miscellaneous compounds (Fig. 3). Among the thiones, anethole trithione inhibited DMBA-induced mammary cancer multiplicity but not cancer incidence in rats [20]. However, it inhibited both the incidence and multiplicity of colon adenocarcinomas in male F344 rats [21]. 1,2-Dithiole-3-thione was also effective against rat colon and DMBA-induced rat mammary tumours, and it also inhibited aflatoxin-induced hepatic lesions in rats [16].

From the group of phenols/catechols, CAPE inhibited TPA-promoted skin papillomas in mice [22]. This agent arrested the growth of HL-60 cells and inhibited DNA, RNA, and protein synthesis in these cells with IC_{50} of 1–5 μM [30]. Ellagic acid inhibited carcinogen-induced tongue carcinogenesis in male F344 rats, as indicated by reduced numbers and areas of nucleolar organiser region proteins, a cell proliferation marker [23]. In other studies, ellagic acid has proven an effective chemopreventive agent against colon, skin, lung, liver and oesophageal carcinogenesis [15].

In previous studies, we found that genistein, a soy isoflavone, exerted pronounced antiproliferative effects on both oestrogen receptor-positive and-negative human breast carcinoma cells [31]. Induction of cell differentiation in a variety of tumour cell lines was associated with reduced cell growth and reduced topo II activity [21]. Murrill and colleagues reported the chemopreventive efficacy of genistein against DMBA-induced mammary tumours in prepubertal rats [24]. Quercetin inhibited oral carcinogenesis initiated with 4-nitroquinoline-N-oxide (4-NQO) in rats, and this effect was attributed to the suppression of cell proliferation. Quercetin also inhibited colonic aberrant crypts in rat colon and DMBA-induced mammary tumours in rats [27].

We previously showed that resveratrol, a constituent of grapes, prevents carcinogenesis in murine models. Specifically, resveratrol inhibited the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and inhibited tumorigenesis in a mouse skin cancer model [28]. It acted mechanistically as an antioxidant and antimutagen to induce phase II drug-metabolising enzymes and to induce HL-60 cell differentiation. A recent study suggests that the antiproliferative action of resveratrol in human mammary adenocarcinoma cells is independent of the oestrogen receptor [32].

In the miscellaneous group, indole-3-carbinol reduced aflatoxin B1-induced hepatocarcinogenesis in male Fischer rats when administered either before or after the carcinogen. This agent was effective in a double-blind dose-ranging chemoprevention study involving 57 women, as determined by measuring the ratio of urinary oestrogen metabolite 2-hydroxyoestrone to the surrogate endpoint biomarker 16 alpha-hydroxyoestrone [25]. Indole-3-carbinol was effective against lung carcinogenesis induced by a tobacco-specific carcinogen in A/J mice [26]. Sulphoraphane is also an inducer of phase II detoxification enzymes. It has been isolated from one variety of broccoli and has been shown to block the formation of DMBA-induced mammary tumours in Sprague–Dawley rats [29].

Comparative studies between different organosulphur analogues of selenite identified pXSC as the most effective [17]. This agent was the strongest topo II inhibitor in our study and was effective in many animal models of chemoprevention. It was effective in both high-fat and low-fat diets in reducing incidence and multiplicity of colon tumours in laboratory animals [18]. It significantly inhibited DMBA-induced tumours in the

mammary glands in female CD rats [33]. Several biochemical mechanisms of pXSC action have been proposed. These include apoptosis in colonic tumours, inhibition of oxidation of xenobiotics and procarcinogens by human cytochrome P450 enzyme *in vitro*, and inhibition of DMBA–DNA adduct formation in the mammary glands [17,19]. Our data suggest that the inhibition of topo II may contribute to the antiproliferative effects of pXSC. The effectiveness of pXSC during the postinitiation phase against tongue carcinomas is consistent with an antiproliferative action that might be mediated through topo II. However, it remains to be established if this effect on topo II is taking place in the target tissue of animals that consume pXSC in their diets.

One exception is protamine sulphate, which strongly inhibited topo II and topo I activities, but did not show chemopreventive action. This polycationic peptide is known to bind to and condense DNA [34], which is the enzymatic substrate in our assays. Three other agents were effective in inhibiting both topo I and topo II. These were quercetin, ellagic acid and pXSC. Quercetin intercalates to the DNA weakly [35], ellagic acid binds to DNA, but intercalation is not involved [36] and there is no evidence that pXSC binds to DNA. DNA intercalation, however, is not sufficient to provide inhibition of both topoisomerases. For example, 4-(9-acridinylamino)methanesulphon-m-anisidine) (mAMSA) and 3-nitrobenzothiazolo(3,2-a)quinolinium (NBQ) are DNA intercalators known to inhibit topo II, but do not inhibit topo I [37]. Protamine sulphate is different in this regard because it binds tightly to the major grove of DNA and promotes an efficient and extremely tight packaging of DNA [38]. Thus, the inhibition of topo II and topo I may not be specific, but rather the result of steric interference by the tight binding of another protein (protamine sulphate) that promotes DNA condensation.

The Chemoprevention Branch of the National Cancer Institute uses the results of basic research as initial evidence to identify compounds or classes of compounds that are included in a list of agents that undergo further testing. For example, inducers of phase II metabolic enzymes, such as oltipraz, constitute good candidates for chemoprevention studies because of their capacity to enhance the excretion of carcinogens. Topo II catalytic inhibitors may constitute a new class of chemopreventive agents that can inhibit carcinogenesis via their antiproliferative or cell-differentiating action. The requirement of topo II for the completion of mitosis makes the enzyme essential for cell division and cell proliferation. Differentiated cells express very low levels of topo II while highly proliferative and tumour cells often express 25–300 times the levels of quiescent cells [8,39]. Many topo II inhibitors were shown to induce cell differentiation [10,40]. An inverse association has been demonstrated between topo II activity and tumour cell differentiation [41]. In fact, our previous studies suggested that reduced topo II activity might trigger the differentiation pathway in HL-60 cells [10]. The present investigation suggests that topo II inhibitory effects might be linked to the antiproliferative activity of chemopreventive agents such as pXSC. Our data also show that the unknotting assay is suitable for identifying new chemopreventive agents. In contrast, the linearisation and nicking assays were not suitable for the identification of chemopreventive agents. By their nature, the latter two assays may be more suitable for the selection of chemotherapeutic drugs. The present study reveals a new use of known compounds that have been selected for evaluation in clinical studies. This knowledge may provide a better understanding of structure–activity relationships, and it may also provide a rational approach in the design of combination chemoprevention regimens.

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