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Biochemical Pharmacology 62 (2001) 1059–1070

Biochemical Pharmacology

Catalytic inhibition of human DNA topoisomerase IIα by hypericin, a naphthodianthrone from St. John's wort (*Hypericum perforatum*)

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Received 23 October 2000; accepted 8 March 2001

Abstract

St. John's wort (*Hypericum perforatum*) is the most widely used herbal medicine for the treatment of depression. However, concerns have arisen about the potential of its interaction with other drugs due to the induction of cytochrome P450 isozymes 1A2 and 3A4 by the components hypericin and hyperforin, respectively. Structurally similar natural products are often employed as antitumor agents due to their action as inhibitors of DNA topoisomerases, nuclear enzymes that modify DNA during cellular proliferation. Preliminary findings that hypericin inhibited the DNA relaxation activity of topoisomerase IIα (topo II; EC 5.99.1.3) led us to investigate the mechanism of enzyme inhibition. Rather than stabilizing the enzyme in covalent complexes with DNA (cleavage complexes), hypericin inhibited the enzyme prior to DNA cleavage. *In vitro* assays indicate that hypericin is a potent antagonist of cleavage complex stabilization by the chemotherapeutics etoposide and amsacrine. This antagonism appears to be due to the ability of hypericin to intercalate or distort DNA structure, thereby precluding topo II binding and/or DNA cleavage. Supporting its non-DNA damaging, catalytic inhibition of topo II, hypericin was shown to be equitoxic to both wild-type and amsacrine-resistant HL-60 leukemia cell lines. Moreover, hypericin was incapable of stimulating DNA damage-responsive gene promoters that are activated by etoposide. As with the *in vitro* topo II assay, antagonism of DNA damage stimulated by 30 μM etoposide was evident in leukemia cells pretreated with 5 μM hypericin. Since many cancer patients experience clinical depression and concomitantly self-medicate with herbal remedies, extracts of St. John's wort should be investigated further for their potential to antagonize topo II-directed chemotherapy regimens. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: DNA topoisomerase II; Etoposide; *Hypericum*; Drug interactions; Herbal medicine; Adverse reactions

1. Introduction

In the United States, under the Dietary Supplement Health and Education Act of 1994, herbal medicines are regulated as a special class of dietary products, not as drugs. Even in Europe, India, and Asia, where herbal medicines are more widely used in conventional medicine, manufacturer evaluation of these supplements for preclinical and clinical pharmacology and toxicology is not compulsory. However, given that many prescription and over-the-counter drugs are either natural products or semi-synthetic derivatives, it is reasonable to expect that botanical medicines are capable of pharmacodynamic and pharmacokinetic interactions with other drugs. SJW (*Hypericum perforatum*) is one of the most popular dietary supplements in the United States and Europe as a result of convincing clinical data for its efficacy as an antidepressant [1–4], as well as preclinical evidence for it reducing alcohol intake in alcohol-preferring rodents [5]. However, several important drug–herb interactions

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Abbreviations: SJW, St. John's wort; topo II, topoisomerase IIα; CAT, chloramphenicol acetyltransferase; and XRE, xenobiotic response element.

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PII: S0006-2952(01)00759-6

have become apparent recently due to the ability of components of the herbal extract to act both as serotonin-selective reuptake inhibitors and as inducers of CYP1A2 and CYP3A4 hepatic monooxygenases [6–9].

The antidepressant component of SJW, originally believed to be hypericin, has been suggested to be hyperforin [2]. However, disagreement exists since *Hypericum* extracts have effects on multiple neurotransmitter systems and at least one low-concentration hyperforin extract possesses clinical antidepressant efficacy comparable to that of fluoxetine [4]; hence, its antidepressant mechanism remains the subject of continued study [10–13]. Nonetheless, most herbal manufacturers in the United States standardize their products to 0.3% hypericin. The naphthodianthrone structure of hypericin is reminiscent of the anthraquinone ring structure associated with some antitumor drugs, including mitoxantrone (Novantrone®). Mitoxantrone acts, in part, on the essential nuclear enzyme, DNA topoisomerase IIα (topo II; EC 5.99.1.3), to stabilize the enzyme in covalent complexes with DNA during its catalytic cycle [reviewed in Ref. 14]. These cytotoxic lesions are commonly referred to as "cleavable complexes" or "cleavage complexes." As with other topo II "poisons," such as etoposide (VP-16; VePesid®) or amsacrine (*m*-AMSA), stabilization of cleavage complexes can interfere with replication fork progression, stimulate recombination, and trigger tumor cell apoptosis.

Many of the antitumor topo II poisons act as a result of interactions with both the enzyme and with DNA. Consistent with such action, Miskovsky *et al.* [15,16] have demonstrated that hypericin accumulates in the nuclei of cultured cells as quickly as 3.5 hr after treatment and also directly interacts with DNA at the N7 sites of purine residues. Stopper and coworkers [17] have also shown that related natural product dihydroxyanthraquinones interact with, and inhibit, topo II. Specifically, three compounds (emodin, danthron, and aloe-emodin) were determined to inhibit topo II DNA decatenating activity, albeit at millimolar concentrations. Emodin also induced mutations in the Ames test.

In preliminary work from our laboratory, it was determined that hypericin inhibits the DNA relaxation activity of the purified α form of human topo II [18]. Since some topo II poisons are known human leukemogens [19], we sought to determine the precise nature of topo II inhibition by hypericin since SJW is so widely available and is increasing in use and acceptance across North America, Europe, and Australia. However, in this report, it is demonstrated that hypericin does not stabilize topo II in covalent complexes with DNA. Instead, hypericin appears to be a member of the class of catalytic topo II inhibitors: agents that act at a step upstream of DNA cleavage and covalent binding to DNA. Hypericin itself was unable to mediate the stabilization of topo II–DNA cleavage complexes, was equally effective in killing amsacrine-resistant leukemia cells and their parental counterpart, and failed to activate any of the DNA damage-responsive genes known to be activated by etoposide. In a DNA unwinding assay, hypericin appeared to interact with DNA to create torsional strain that precluded topo II DNA binding activity. Of greatest note, however, was that as a result of catalytic topo II inhibition, hypericin could antagonize the formation of topo II-covalent cleavage complexes mediated by the topo II poisons, etoposide or amsacrine. Most strikingly, complete antagonism of drug-stabilized cleavage complexes could be achieved at hypericin concentrations one-tenth to one-third those of each topo II poison. Moreover, hypericin attenuated etoposide-mediated DNA damage in HL-60 leukemia cells, as quantified by a modified alkaline comet assay. Therefore, the data in this report suggest that hypericin should be investigated further for its potential to antagonize topo II-directed chemotherapy *in vivo.*

2. Materials and methods

2.1. Drugs

Etoposide was obtained from the Sigma Chemical Co. Amsacrine was a gift from Bristol-Myers Squibb. Both topo II poisons were prepared as 100 mM stock solutions in anhydrous DMSO and stored at –20°. Hypericin was obtained from ICN Biochemicals and Calbiochem, and 20 mM stock solutions were prepared freshly in anhydrous DMSO. Stock solutions were protected from light, and cell or enzyme assays involving hypericin were performed in the dark wherever possible to minimize photoactivation of the compound.

2.2. Cell lines

HL-60 human promyelocytic leukemia cells (ATCC CCL 240) were obtained from the American Type Culture Collection. HL-60/AMSA cells, and their parental counterpart HL-60/Beran, were donated by Drs. Leonard Zwelling and Cynthia Herzog, University of Texas M.D. Anderson Cancer Center. HL-60/AMSA is a cell line derived from HL-60/Beran cells and contains a mutation in its topo IIα protein (R486K) that confers cross-resistance to several topo II poisons [20]. All cell lines were maintained at 37° in a humidified atmosphere containing 5% CO_2. ATCC HL-60 cells were maintained in log growth by subculturing at 5×10^5 cells/mL every 2–3 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/mL of penicillin G, and 50 μg/mL of streptomycin. The M.D. Anderson HL-60 clone (HL-60/Beran) and HL-60/AMSA cells were cultured in Iscove's medium containing identical supplements.

2.3. Topo II-mediated DNA cleavage assay

The ability of a drug to stabilize topo II–DNA complexes was evaluated using the method of Gantchev and Hunting

[21]. Reactions contained 6 units (~13 nM) of human topo IIα (Topogen) and 300 ng of pBR322 DNA per 20 μL sample volume. [Reaction buffer: 10 mM Tris–HCl (pH 7.7), 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, and 5 mM ATP]. Hypericin, etoposide, and/or amsacrine were added to the reaction buffer containing DNA substrate, and the reactions were then initiated by the addition of enzyme. Following a 10-min incubation at 37°, cleavage products were trapped by the addition of 2 μL of 10% SDS. EDTA and NaCl were then added to final concentrations of 10 and 20 mM, respectively, and the reactions were incubated for an additional 5 min at 37°. Enzyme trapped in cleavable complexes was removed by proteolysis to reveal linearized plasmid by adding proteinase K (Roche Molecular Biochemicals) to 0.8 mg/mL and incubating at 55° for 2 hr. Samples were then subjected to electrophoresis at 2 V/cm for 18 hr on 1.3% agarose gels containing 0.7 μg/mL of ethidium bromide. Using this system, all DNA forms were separated as follows: relaxed (RLX; greatest mobility), supercoiled (SC), linear (LIN), and nicked open circular (NC; least mobility).

2.4. Electrophoretic mobility shift assay

The effect of hypericin on topo II DNA binding activity from mammalian cell nuclear extracts was performed exactly as described previously [22]. Oligonucleotides corresponding to residues 87–126 of pBR322, a strong topo II binding site [23], were annealed and end-labeled with [α-^{32}P]dCTP. Nuclear protein extracts (500 mM NaCl extraction) were prepared from logarithmically growing HeLa cells as previously described [24], using Complete™ protease inhibitor (Roche Molecular Biochemicals). Incubations of the binding site with nuclear protein extract were carried out on ice alone or with increasing concentrations of hypericin in a 25-μL reaction volume containing 50 mM KCl, 20 mM Tris (pH 7.6), 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 2.5 μg BSA, and 0.1 μg poly(dI:dC)·(dI:dC) (Amersham Pharmacia Biotech). Free and bound oligonucleotide were separated by electrophoresis through a 4% non-denaturing polyacrylamide gel in 0.25× Tris–borate–EDTA buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8.0). Antibody supershift and specific unlabeled oligonucleotide competition experiments have already been published [22] to document the identity of the topo II: oligonucleotide complex.

2.5. Plasmid DNA unwinding assay

The potential for hypericin to cause DNA unwinding by intercalation was investigated by a method [25] modified from Lee and Morgan [26]. Briefly, 500 ng of pBR322 was incubated on ice for 15 min with various concentrations of either ethidium bromide or hypericin in a reaction buffer consisting of 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 30 μg/mL of BSA. This mixture was then incubated for 45 min at 37° with topo I (10 units; Gibco/Life Technologies) to allow complete relaxation of the plasmid. The reaction was terminated by the addition of 1.5 μL of 10% SDS and 1.2 μL of 0.5 M EDTA. All proteins were then hydrolyzed with proteinase K (0.75 mg/mL) for 90 min at 50°. To extract the intercalating agents, the DNA was isolated by phenol:chloroform (1:1) extraction, followed by a chloroform extraction and ethanol precipitation. The pellet was resuspended in agarose loading buffer and run on a 1% agarose gel for 20 hr at 1 V/cm, and then stained with 0.05 μg/mL of ethidium bromide *after* electrophoresis. In this system, used only for Fig. 4, relaxed plasmid has the least mobility and supercoiled plasmid has the greatest mobility; intermediate topoisomers possess intermediate mobility.

2.6. Protein-linked DNA assay

The potential for hypericin to stabilize topo II–DNA cleavable complexes was assessed using a modification [27] of a K-SDS precipitation assay. Briefly, exponentially growing HL-60 cells (5×10^5/mL) were incubated for 24 hr with [^{14}C]leucine (0.2 μCi/mL; 325 mCi/mmol) and [*methyl*-^{3}H]thymidine (0.6 μCi/mL; 6.7 Ci/mmol). During the final 1 hr, cells received 20–200 μM hypericin, 100 μM etoposide (positive control), or an equivalent amount of DMSO vehicle, in triplicate. Cell suspensions were then pelleted by centrifugation at 1000 *g* for 10 min at 4° and lysed with 500 μL of a solution containing 2.5% (w/v) SDS, 10 mM EDTA, and 0.8 mg/mL of salmon sperm DNA. The lysate was sheared through a 23-gauge needle 15 times, and then incubated at 65° for 15 min. Protein and protein-associated DNA were then precipitated by the addition of 110 μL of 1 M KCl. Precipitates were recovered by centrifugation at 5000 *g* for 10 min at 4° and washed three times with a solution containing 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, and 0.1 mg/mL of salmon sperm DNA. Precipitable [^{14}C] and [^{3}H] were assessed after dissolving the pellets in 500 μL of water followed by liquid scintillation counting. The data are expressed as the ratio of [^{3}H] cpm/[^{14}C] cpm and displayed as means ± SEM.

2.7. Internucleosomal DNA fragmentation

The DNA laddering assay was performed as described by Moran *et al.* [28]. Briefly, 1.5×10^6 logarithmically growing HL-60 cells were treated for 4 hr with either 30 μM etoposide (positive control) or hypericin (1–30 μM). Cells were collected by centrifugation, lysed in sterile water, and subsequently incubated for 1 hr at 25° with 10 mg/mL of RNase A. To resolve the digested nuclear DNA, a 1.8% agarose gel was cast. When this gel had solidified, the area above the wells was removed and replaced with a 0.8% agarose gel containing 0.5× TBE, 1% SDS, and 1 mg/mL of proteinase K. Samples were loaded and run at 20

V for 1 hr, and then at 100 V for 1.5 hr. *Hae*III-digested ϕX174 DNA was used for the molecular size markers.

2.8. Cytotoxicity assay

HL-60/Beran and HL-60/AMSA cells were seeded in U-bottom 96-well plates at 5×10^4 cells/well. Cells were then incubated for 2 hr at 37° with various concentrations of etoposide (positive control) or hypericin. Cells were pelleted by centrifugation of the 96-well plate at 600 *g* for 10 min, and drug-containing medium was aspirated carefully. Cells were resuspended in 100 μL of fresh medium, pelleted again, and resuspended in 200 μL of fresh medium. After 72 hr, cells were assayed for mitochondrial viability via an MTS (CellTiter 96AQ) assay (Promega). To quantify conversion of the MTS substrate into its formazan product, plates were read at 490 nm absorbance on a Molecular Dynamics microplate reader. Data are expressed as the mean percentage of OD_{490} for each respective control group $\pm$ SEM (N = 8 per group).

2.9. Reporter gene assays

Effects of hypericin on a variety of gene promoter constructs were performed using a survey of a screening panel marketed as CAT-Tox(L) [29]. CAT reporter constructs were made separately to contain copies of the NF-κB response element (NF-κBRE), the retinoic acid response element (RARE), and the p53 response element (p53RE), or to contain authentic promoter fragments of human metallothionein IIA (HMTIIA), human c-fos (FOS), or hamster GADD153 (GADD153). Each construct was stably transfected in HepG2 human hepatoma cells as described [29]. It should be noted that HepG2 cells are far less sensitive to many drugs due to their metabolic capacity; therefore, the high concentrations of test compounds used necessitate that this assay be viewed as a *qualitative* evaluator of the ability of a compound to activate various DNA damage-responsive promoters. Various concentrations of hypericin or etoposide (positive control) were incubated with each modified HepG2 cell line (5×10^4 cells/well of a 96-well plate) for 24–48 hr. Cells were then lysed in 5 mM MOPS, 2 mM NaCl, 38 μM $MgCl_2$, 0.25% Triton X-100, pH 6.5, and an ELISA was performed for CAT (5 Prime-3 Prime). Data are expressed as fold-increase in CAT immunoreactivity over the basal activity of each construct in the absence of drug.

2.10. Drug-induced nuclear damage

The single cell microgel electrophoresis assay (comet assay) was performed as described by Gieseler *et al.* [30]: HL-60 cells were pretreated with DMSO vehicle or 5 μM hypericin for 21 hr, and then cultured for another 3 hr with DMSO vehicle or 30 μM etoposide. Samples were prepared for electrophoresis by layering microscope slides with agarose as follows: 85 μL of 0.5% NEEO agarose (Roth) was coated onto the slide. After this had solidified, 5×10^4 cells in 75 μL of 1% low melting point agarose (Sigma) was coated on top of the first layer. Following solidification of the second layer, a final coat of 75 μL of 1% low melting point agarose was added. After solidification of the final layer, the cells imbedded in the agarose slides were then lysed in 1% Triton X-100, 10% DMSO, and 89% lysis buffer, pH 10 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% *N*-laurylsarcosine, sodium salt) for 1 hr. The agarose slides were equilibrated for 20 min in electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH $\geq$ 12.5), and electrophoresis was initiated at 25 V and 300 mA for 20 min. Following electrophoresis, the slides were neutralized with three washes of 0.4 M Tris–HCl, pH 7.5, and DNA was then stained with 50 μL of 20 μg/mL of ethidium bromide solution. The cells were cataloged using a fluorescence microscope, and the evaluator was blinded as to the treatment groups.

Absolute tail length or tail moment measurements were not amenable to the HL-60 system, so a qualitative evaluation system was employed. Class I DNA damage was defined by nuclei that appeared condensed relative to controls with some DNA fragmentation, but without visible "tails." Class II DNA damage was ascribed to nuclei exhibiting a tail length less than the length of one normal (control) nucleus. Class III DNA damage was ascribed to nuclei displaying a tail length greater than the length of one control nucleus. Finally, Class IV DNA damage was ascribed to nuclei that had incurred such great damage that more than 90% of the fragmented DNA had migrated away from the residual nuclear electrophoretic origin.

3. Results

In preliminary experiments, hypericin at 1–100 μM appeared to inhibit the DNA relaxation activity of purified, human DNA topo II using pBR322 plasmid as the substrate [18]. However, inhibition of overall catalytic activity sheds little light on the mechanism of enzyme inhibition and the potential for positive or negative interactions with other topo II-directed agents. Broadly, topo II inhibitors can be classified as two major types: catalytic inhibitors and topo II poisons [14]. Catalytic inhibitors are subdivided as (a) "simple" catalytic inhibitors that inhibit either topo II DNA binding or DNA cleavage activities (like ethidium bromide or merbarone) [31,32], or (b) "closed clamp" catalytic inhibitors (like ICRF-187 and other bisdioxopiperazines) that trap the enzyme on DNA in an intermediate that does not cleave DNA, but is incapable of dissociating from DNA. In contrast, "topo II poisons" permit DNA binding and cleavage, but trap the enzyme in covalent cleavable complexes with DNA through a phosphotyrosine linkage with the 5′-phosphate of each cleaved strand [33,34]. Topo II poisons have been subclassified further into those that inhibit DNA

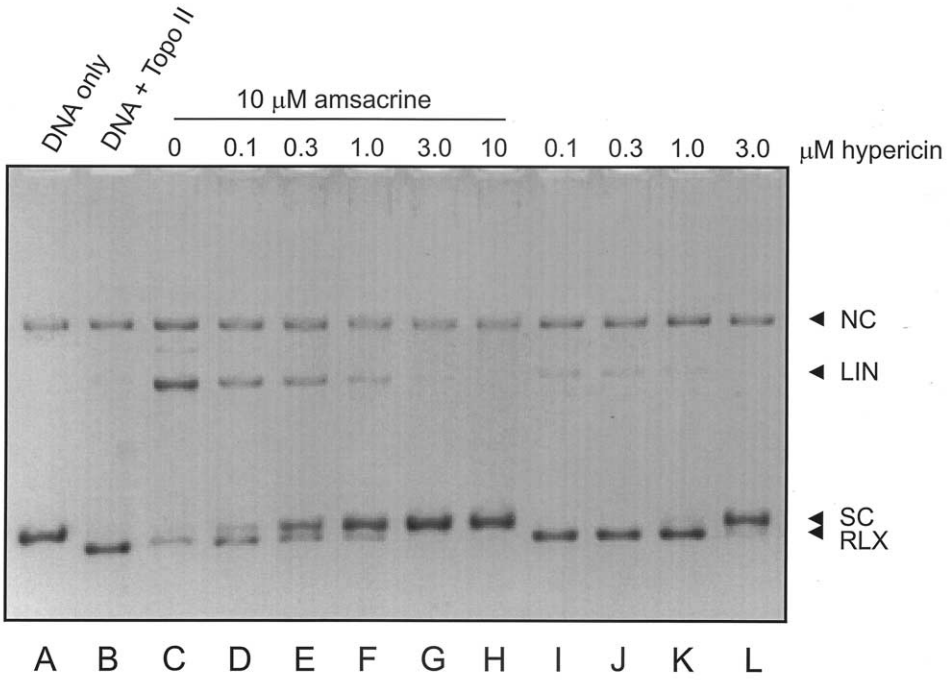

Fig. 1. Effect of hypericin on topo II DNA relaxation activity and amsacrine-stabilized cleavable complexes *in vitro*. The topo II DNA cleavage/relaxation method of Gantchev and Hunting [21] was used to classify the nature of enzyme inhibition by hypericin. Human topo II (6 U) and pBR322 (300 ng) were incubated for 10 min at 37° alone (lane B) or with increasing concentrations of hypericin in the presence or absence of 10 μM amsacrine. Reactions were terminated by the addition of SDS (1%) and proteolyzed with proteinase K (800 μg/mL). Reaction products were run for 18 hr at 2 V/cm on a 1.3% agarose gel containing 0.7 μg/mL of ethidium bromide. Using this system, relaxed open circular plasmid (RLX) runs slightly faster than supercoiled (SC) plasmid, while nicked open circular (NC) plasmid has the least mobility. Stabilized cleavable complexes are evidenced by the linear DNA band (LIN), which results from the enzyme denaturation/proteolysis step. This experiment was repeated three times, and a representative gel is shown here.

religation activity and those that enhance DNA cleavage activity.

Therefore, the effect of hypericin was examined by using a plasmid DNA assay capable of measuring both cleavable complex stabilization and overall catalytic activity [21]. The inclusion of ethidium bromide (0.7 μg/mL) in the gel permits spatial differentiation between nicked open circular DNA (NC) and relaxed open-circular DNA (RLX) that would otherwise co-migrate on a gel lacking ethidium bromide. In this system, the unreacted supercoiled DNA (SC) runs with slightly slower mobility than relaxed plasmid, and covalent, topo II–DNA cleavage complexes migrate as an intermediate linear band (LIN) following the proteinase K digestion in the reaction processing method. A positive control topo II poison, amsacrine, was run in a reaction with purified human topo II and plasmid substrate to reveal: (a) the substantial appearance of linear DNA indicative of cleavage complexes (Fig. 1, lane C), and (b) inhibition of overall DNA relaxation activity (compare lanes B and C of Fig. 1).

The data in Figs. 1 and 2 indicate that hypericin acts in a manner distinct from that of the two topo II poisons. First, the addition of 0.1 to 3 μM hypericin alone (lanes I-L of Fig. 1) causes little appearance of linear DNA above that observed in enzyme controls (lane B), and confirms our previous description of relaxation inhibition (lane L). This finding suggested that hypericin was acting as a catalytic inhibitor. To test this hypothesis, increasing concentrations of hypericin were co-incubated with 10 μM amsacrine and shown to reverse cleavage complex stabilization resulting from amsacrine alone in a concentration-dependent fashion (Fig. 1; compare lanes D-H with lane C). In fact, hypericin was a very potent antagonist of the effect of amsacrine with nearly complete loss of linear DNA at 1–3 μM. A very similar effect of hypericin was observed when etoposide was employed as the topo II poison (Fig. 2), except that hypericin appeared to be even more potent at reversing etoposide-stabilized cleavage complexes.

To clarify whether hypericin was a simple or closed-clamp topo II inhibitor, its effect on topo II DNA binding activity was observed in an electrophoretic mobility gel shift assay exactly as described by Kurz *et al.* [22]. As shown in Fig. 3, the binding of topo II to a strong DNA cleavage site from pBR322 was lost at 3–10 μM hypericin. The selectivity of this effect is emphasized by the fact that HeLa cell nuclear extracts were used as the source of topo II, rather than the pure enzyme itself as used in Figs. 1 and 2.

Since hypericin has been reported to interact with DNA *in vitro* and in intact cells [15,16], a classic DNA unwinding assay was employed to determine whether hypericin binding to DNA could cause unwinding of a supercoiled plas-

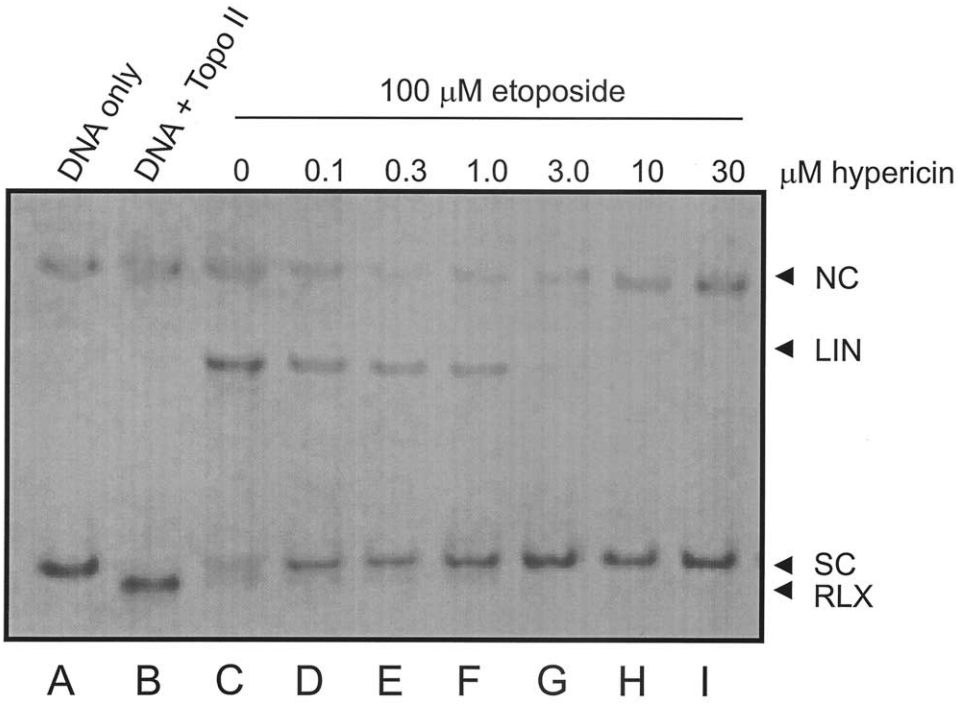

Fig. 2. Effect of hypericin on etoposide-stabilized cleavable complexes *in vitro.* The topo II DNA cleavage/relaxation method of Gantchev and Hunting [21] was used to confirm cleavable complex antagonism by hypericin as in Fig. 1, except that 100 μM etoposide was used as the topo II poison. This experiment was repeated three times, and a representative gel is shown here.

mid. This assay has been used to demonstrate DNA unwinding as a result of binding of ethidium bromide, an intercalating agent [26]. The assay is based on the use of an excess of type I topoisomerase to cause $>$ 95% relaxation of the DNA molecules in the absence of DNA interactive xenobiotics (Fig. 4, lane B). (Note that this gel is run in the absence of ethidium bromide and then stained after electrophoresis, producing the more classic impaired co-migration of nicked circular and relaxed DNA relative to supercoiled plasmid.) With ethidium bromide, progressive intercalation of each molecule causes a 26° unwinding of the helix, thereby presenting to the enzyme DNA molecules that are apparently progressively relaxed. The excess topo I is then incubated with the DNA and relaxes each molecule to apparent completion (the assay depends on the demonstrated assumption that the agent tested does not directly inhibit topo I activity at the concentrations used). Following proteolysis and organic extraction of the reaction mixture, torsional strain is reintroduced into the plasmids in direct proportion to the relaxation caused by the intercalating agent. Hence, lanes D through F of Fig. 4 demonstrate how increasing concentrations of the positive control, ethidium bromide, progressively unwound the plasmid DNA. Ethidium bromide at 1 μg/mL essentially presented topo I with a plasmid molecule that appeared to already be completely relaxed; consequently, removal of this concentration of ethidium bromide caused the reintroduction of torsional strain into the DNA such that its degree of supercoiling was indistinguishable from that of unreacted DNA (compare lanes G and A). In a similar fashion, hypericin at 2–6 μM (lanes J–L) produced a similar phenomenon, suggesting that its interaction with DNA had introduced significant torsional strain at these concentrations. Taken together with the data from Fig. 3, it is clear that hypericin interacts with DNA and that this interaction can interfere with the binding of topo II.

Cellular experiments were also conducted to confirm that the *in vitro* effects of hypericin as a catalytic inhibitor of topo II were also evident in intact tumor cells. HL-60 cells were investigated for the appearance of protein-linked DNA using the assay of Zwelling as modified by Ritke and Yalowich [27] (Fig. 5). While the positive control topo II poisoning antitumor drug etoposide produced a substantial increase in protein-linked DNA (as indicated by a [^{3}H]/[^{14}C] ratio nearly three times that of control cells), hypericin was without effect in this assay even at concentrations up to 200 μM. Figure 6A shows, nonetheless, that hypericin could indeed induce HL-60 apoptosis at 1–30 μM as evidenced by the appearance of internucleosomal DNA laddering of genomic DNA.

However, the mechanism of cell killing by hypericin was unrelated to the stabilization of topo II cleavage complexes but more likely, and as suggested by the earlier *in vitro* experiments, to the catalytic inhibition of topo II. HL-60/AMSA is a cell line derived from HL-60 for resistance to the cleavable complex stabilizing the topo II drug amsacrine [20]. This line displays a 50- to 100-fold greater IC_{50} value for amsacrine, with respect to the parental line, due to its

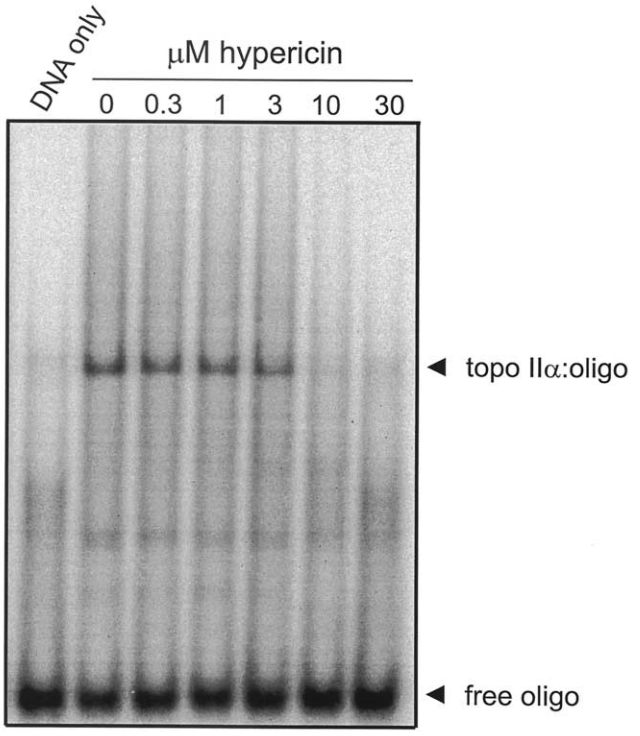

Fig. 3. Effect of hypericin on topo II DNA binding in an electrophoretic mobility shift assay. A strong, topo II binding/cleavage oligonucleotide from pBR322 was [^{32}P] end-labeled and incubated with a HeLa cell nuclear extract as the topo II source in the presence or absence of increasing concentrations of hypericin using the conditions of Kurz *et al.* [22]. Specific competition of the shifted band with unlabeled oligonucleotide and antibody supershifting with topo II antiserum have been used previously to confirm the identity of topo IIα:oligonucleotide complexes [22]. This experiment was repeated once, and a representative autoradiogram is shown here.

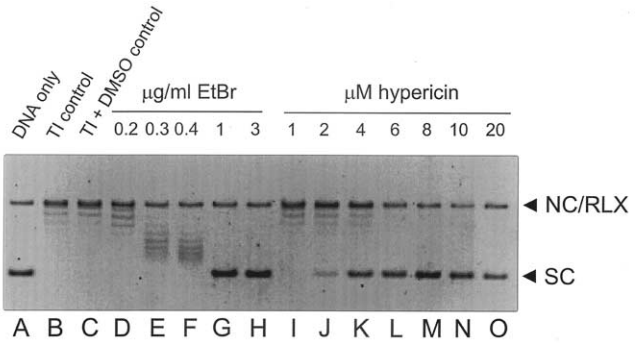

Fig. 4. Effect of hypericin in a plasmid DNA unwinding assay. Supercoiled pBR322 DNA (500 ng) was incubated with the indicated concentrations of ethidium bromide (positive control) or hypericin for 15 min on ice. Purified type I topoisomerase (10 U) was added in excess, and this mixture was incubated for 45 min at 37° in order to relax the DNA. Reactions were terminated by the addition of SDS and EDTA to 0.5% and 20 mM, respectively. Following proteinase K digestion, ethidium and hypericin were removed from the DNA by extraction with phenol:chloroform (1:1) and chloroform followed by ethanol precipitation. This extraction permits reintroduction of a degree of torsional strain into the DNA that had been relieved by binding of the DNA interactive agent. The resulting DNA pellet was resuspended in agarose loading buffer and run on a native 1% agarose gel without ethidium bromide at 1 V/cm for 20 hr. DNA bands were visualized after electrophoresis by staining with 0.05 μg/mL of ethidium bromide. The rank order of plasmid mobility in this native system is SC > RLX = NC with progressively relaxed topoisomers migrating with intermediate mobility. This experiment was repeated twice, and a representative gel is shown here.

inability to efficiently form drug-stabilized cleavable complexes as a result of a single amino acid substitution in the enzyme. As would be expected, HL-60/AMSA is also cross-resistant to other cleavable complex-stabilizing drugs such as etoposide. But, as shown in Fig. 6B, hypericin was equitoxic to HL-60 and HL-60/AMSA cells, with an IC_{50} value of 5.5 to 7 μM in both lines. Had hypericin been acting in cells to stabilize cleavable complexes, a much greater IC_{50} value would have been observed in the HL-60/AMSA cells. Taken together with previous data, there is no evidence *in vitro* or in intact cells that hypericin can act as a cleavable complex-stabilizing topo II poison. Additionally, its IC_{50} value in HL-60 cells makes it tempting to speculate that catalytic inhibition of topo II may be a contributing mechanism to its cytotoxic action in some cell types. Studies are in progress to examine changes in topo II function in hypericin-resistant cells, as well as to examine the effect of hypericin in cell lines resistant to topo II catalytic inhibitors.

We next sought to extend the *in vitro* observations of Figs. 1 and 2 to determine whether hypericin could antagonize the DNA damage of topo II-stabilizing cleavable complex drugs in intact tumor cells. DNA damage was assessed in the single cell microgel electrophoresis assay, or comet assay [30], following exposure of HL-60 cells to etoposide, hypericin, or a combination of the two. The DNA damage in individual nuclei (100 nuclei/treatment group) was then classified by a blinded observer and denoted by qualitative nomenclature as outlined in "Materials and methods." A concentration of etoposide (30 μM) was selected to result in 100% of cells exhibiting extensive, class IV DNA damage. A pretreatment concentration of hypericin alone (5 μM) was selected to cause minimal DNA damage (likely due to reactive oxygen species). Etoposide-mediated DNA damage was then compared with and without hypericin pretreatment. Similar to the *in vitro* results of Fig. 2, hypericin pretreatment of HL-60 cells could substantially antagonize the extent of etoposide-mediated DNA damage, reducing class IV DNA damage from 100 to 24% and shifting most of the damage to the lesser classes II and III (Fig. 7). Therefore, hypericin was capable of antagonizing etoposide-mediated DNA damage in HL-60 cells as well as *in vitro.*

However, it was of note that hypericin treatment alone led to some degree of DNA damage in the comet assay. Hypericin was therefore compared with etoposide for their respective abilities to induce known DNA damage response genes in HepG2 human hepatoma lines individually selected for stable integration of a DNA damage response gene element upstream of a CAT reporter gene [29]. (Since

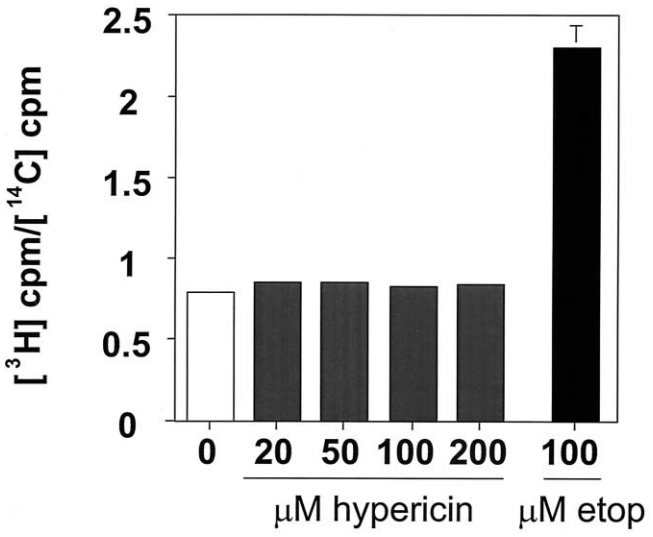

Fig. 5. Effect of hypericin on the formation of protein-linked DNA breaks in HL-60 cells. A standard K-SDS precipitation assay [27] was employed to detect the potential of hypericin to cause an increase in protein-linked DNA in cells similar to the topo II poison etoposide. HL-60 cells (N = 3 per group, experiment repeated once) were labeled for 24 hr with [^{14}C]leucine (0.2 μCi/mL) and [^{3}H]thymidine (0.6 μCi/mL). Cells were then treated with DMSO control vehicle or the indicated concentrations of hypericin or etoposide for 1 hr, and lysed, and protein–DNA complexes were precipitated as described in "Materials and methods." Protein-linked DNA was quantitated by liquid scintillation counting, and the mean values are expressed as a ratio of [^{3}H] cpm/[^{14}C] cpm ± SEM.

these cells have far more metabolic capacity than HL-60 cells, much higher concentrations of both drugs were used to examine these effects. The IC_{50} value for hypericin in HepG2 cells is roughly 50-fold greater than in HL-60 cells.) From Table 1, it is readily apparent that etoposide was capable of substantially activating such DNA damage-responsive enhancers such as those from p53, GADD153, NF-κB, and c-Fos. However, hypericin was without effect on any of these classical DNA damage-responsive genes, suggesting that whatever the DNA damage caused by hypericin in Fig. 7, it was distinct in nature from that elicited by etoposide. Interestingly, the only gene enhancer element activated by hypericin in this screen was that of the XRE of the cytochrome P450 1A gene family. This observation is consistent with the polyaromatic nature of hypericin's structure and the well-known action of polyaromatic hydrocarbons as CYP1A inducers.

Again, it should be mentioned that these concentrations of hypericin are likely to be supraphysiologic, especially with regard to the HepG2 assay. However, it should also be appreciated that a closely related naphthodianthrone, pseudohypericin, is normally present in *Hypericum* extracts at 2- to 5-fold higher concentrations than hypericin. Therefore, further study of the effects of hypericin in the context of commercially available *Hypericum* products on topo II action and topo II-targeted antitumor drugs is clearly warranted.

4. Discussion

The naphthodianthrone structure of hypericin vaguely resembles a molecular dimer of the topo II poison mitoxantrone. This observation, combined with reports demonstrating cellular nuclear accumulation and DNA binding of hypericin [15,16], as well as the genotoxicity of related natural product dihydroxyanthraquinones [17], led to the investigation of hypericin as a potential inhibitor of the DNA modifying enzyme topo II. Our initial concern was that hypericin in SJW supplements that are freely available in pharmacies and health food stores might act as a topo II poison. Since several topo II poisons have been linked with the occurrence of secondary, therapy-related leukemias [19], there was concern regarding the leukemogenic potential of one of the most popular herbal supplements on the market. Instead, hypericin was shown to inhibit topo II by a mechanism distinct and upstream from the step at which topo II poisons act. Several independent assays indicated that hypericin, even at high concentrations, was incapable of stabilizing covalent, topo II–DNA cleavage complexes or otherwise significantly activating the DNA damage response. However, its inhibition of topo II DNA-binding activity was shown *in vitro* to antagonize the cytotoxic lesion produced by the topo II poisoning antitumor drugs etoposide and amsacrine. Finally, in one cellular measure of DNA damage (the alkaline comet assay), pretreatment with hypericin partially protected HL-60 cells from etoposide-mediated DNA damage. These results are reminiscent of the pioneering work of Rowe *et al.* [32] and Drake *et al.* [31] who demonstrated independently that other DNA-distorting agents like ethidium bromide and merbarone could effectively antagonize topo II-poisoning antitumor drugs via a similar mechanism. There also exists another class of topo II catalytic inhibitors (e.g. razoxane; ICRF-187) that act upstream of topo II–DNA cleavage by trapping the enzyme in a non-covalent, closed clamp intermediate on DNA and also antagonize cleavable-complex stabilizing agents.

However, repeated attempts to test whether hypericin could interfere with etoposide- or amsacrine-mediated HL-60 cytotoxicity in intact cells were hampered by the steep dose–response curve of the naphthodianthrone. Unlike ethidium bromide, merbarone, or other topo II catalytic inhibitors, an adequate hypericin concentration that either had no effect on etoposide action or did not result in 100% cytotoxicity could not be identified. Our choice of this model cell system may have been sub-optimal in that HL-60 cells possess relatively weak antioxidant defenses and are precariously primed toward the apoptotic program. This shortcoming has led us to continue studies with other tumor cell lines *in vitro* and *in vivo* to determine the relevance of hypericin's *in vitro* antagonism of topo II-poisoning antitumor drugs.

Individual SJW components and whole *Hypericum* extracts have been linked recently to the induction of CYP1A2 and CYP3A4 phase I drug-metabolizing enzymes. CYP1A2

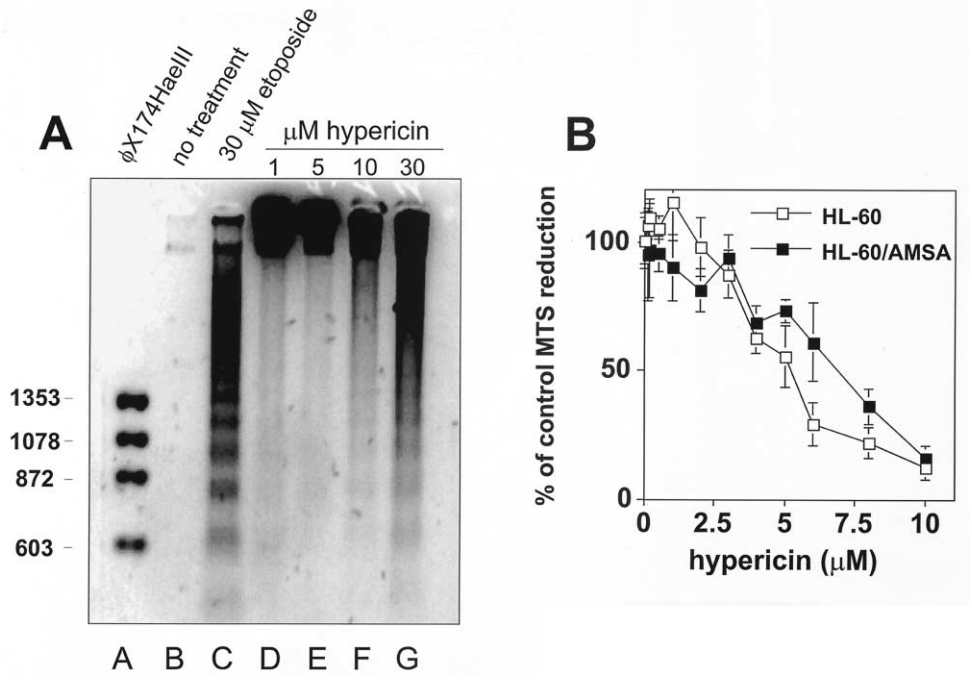

Fig. 6. Effect of hypericin (A) on intranucleosomal DNA fragmentation and (B) in drug-sensitive parental (HL-60) and amsacrine-resistant (HL-60/AMSA) cells. (A) DNA laddering assay. To confirm that hypericin-induced HL-60 cell death was mediated by apoptosis, the parental line was subjected to an assay to measure internucleosomal DNA fragmentation following hypericin treatment. Approximately 1.5×10^6 logarithmically growing HL-60 cells were exposed for 4 hr to either 30 μM etoposide (positive control), 1–30 μM hypericin, or DMSO control vehicle. Internucleosomal DNA laddering was detected using the method of Moran *et al.* [28]. (B) Cell survival assay. HL-60/AMSA is a human leukemia cell line that is 50- to 100-fold resistant to amsacrine and is cross-resistant to other topo II-cleavable complex poisons due to a point mutation in topo IIα (R→K at amino acid 486; [20]). To provide support for this hypothesis that hypericin does not act as a cleavable complex poison in cells, the wild-type and mutant cell line (50,000 cells/well of a 96-well dish) were exposed to various concentrations of hypericin for 2 hr, plates were centrifuged, and cells were washed one time with drug-free medium, then finally resuspended in drug-free Iscove's and incubated for 72 hr. Cells were then incubated for 2–4 hr with MTS (CellTiter 96AQ), a chromogen whose mitochondrial reduction product is quantified by spectrophotometry at 490 nm. No significant differences were observed between the two cell lines at any hypericin concentration tested. Data are expressed relative to the MTS reduction activity in each respective control group (means ± SEM, N = 8).

induction may be mediated by hypericin, as suggested by our observation of a concentration-dependent increase in XRE-mediated transcription in the HepG2 reporter assay in Table 1. Associated with this study, our group has presented a case report implicating SJW in the increased clearance of the anti-asthmatic agent and CYP1A2 substrate, theophylline, in a 42-year-old woman [7]. While this patient was also on other agents with the potential to induce CYP1A2, cessation of SJW supplementation led to a restoration of therapeutic theophylline levels. In work to be presented elsewhere, it has now been confirmed that hypericin can indeed induce CYP1A2 enzyme activity in cultured human hepatocytes[3]. Clinically, CYP1A2 induction also has the potential for increasing the clearance of *R*-warfarin, the tricyclic antidepressants imipramine and clomipramine, and the newer antipsychotic drugs clozapine and olanzapine. Nonetheless, caution should be exercised in extrapolating these data to the clinical situation, as no prospective study to date has directly demonstrated CYP1A2 induction in human volunteers given either hypericin or *Hypericum* extracts.

In contrast, CYP3A4 induction by SJW is mediated instead by hyperforin via a mechanism involving the binding and activation of the PXR transcription factor [35]. These effects appear to be clinically relevant. In fact, hyperforin had far more potent effects on PXR than the benchmark CYP3A4 inducer rifampicin. Since CYP3A4 metabolizes roughly 50% of prescription drugs used today, enzyme induction in patients taking SJW extracts surprisingly has not caused documented drug–herb interactions. In prospective and case report studies, respectively, SJW greatly reduces plasma levels of the HIV protease inhibitor indinavir, as well as the immunosuppressant drug cyclosporine [8,9]. The latter interaction resulted in two Swiss patients exhibiting signs of acute transplant rejection symptoms following a heart transplant due to subtherapeutic levels of the immunosuppressant [9]. With regard to our current results, it should be noted that the topo II poison etoposide is also a CYP3A4 substrate [36]. Therefore, in addition to the pharmacodynamic antagonism demonstrated in this report, it is also theoretically likely that SJW can stimulate the hepatic metabolism of etoposide. However, this postulated effect of SJW may be more far-reaching than simply causing increased etoposide clearance. Of related concern is that the

[3] LeCluyse EL, Jolley SL, Hamilton GA, Kroll DJ, manuscript in preparation.

Table 1
Effect of hypericin on selected DNA damage response and drug-metabolizing gene promoters

Enhancer element	Fold-increase in CAT immunoreactivity				
	Etoposide	Hypericin			
	720 μM	10 μM	25 μM	50 μM	125 μM
p53RE	7.0 ± 2.9	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
GADD153	4.2 ± 1.7	0.7 ± 0.2	1.0 ± 0.1	0.8 ± 0.3	1.2 ± 0.3
NF-κBRE	10.4 ± 5.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
FOS	3.9 ± 1.3	0.6 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	1.0 ± 0.0
RARE	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
XRE	2.7 ± 0.7	1.8 ± 0.3	3.0 ± 0.3	4.9 ± 0.7	5.8 ± 1.1
HMTIIA	1.1 ± 0.0	0.7 ± 0.3	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

CAT reporter constructs were made separately to contain copies of the xenobiotic response element (XRE), NF-κB response element (NF-κBRE), retinoic acid response element (RARE), and p53 response element (p53RE), or to contain authentic promoter fragments for human metallothionein IIA (HMTIIA), human c-Fos (FOS), or hamster GADD153 (GADD153). Each construct was stably transfected in HepG2 human hepatoma cells. Various concentrations of hypericin or etoposide (positive control) were incubated with each modified HepG2 cell line (5×10^4 cells well of a 96-well plate) for 24–48 hr. Cells were then lysed in 5 mM MOPS, 2 mM NaCl, 38 μM $MgCl_2$, 0.25% Triton X-100, pH 6.5, and an ELISA was performed for CAT (5 Prime-3 Prime). Data are expressed as fold-increase in CAT immunoreactivity over the respective basal activity of each construct in the absence of drug. The means (±SEM) of data from three experiments are shown.

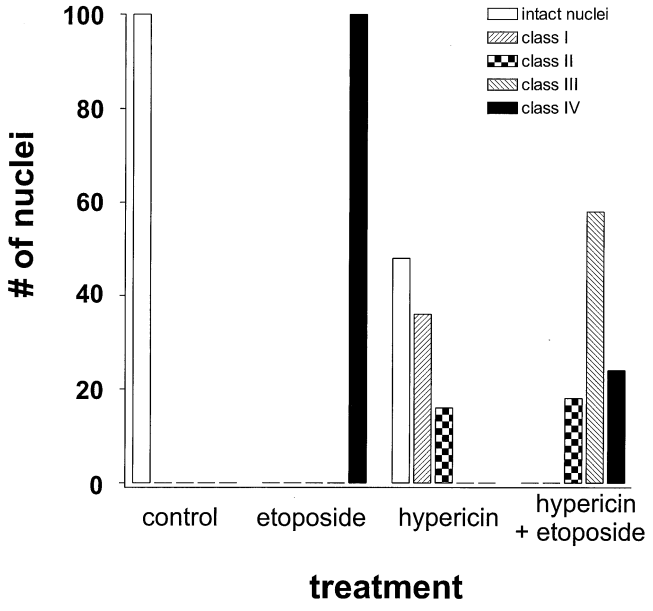

Fig. 7. Effect of hypericin on etoposide-mediated DNA damage in HL-60 cells assessed by an alkaline comet assay. Logarithmically growing HL-60 cells were subjected to a 21-hr pretreatment with 5 μM hypericin or DMSO vehicle, and then exposed for 3 hr to 30 μM etoposide or DMSO vehicle. Cells were then processed for qualitative examination of DNA damage using the modified single cell alkaline comet assay of Gieseler *et al.* [30] as described in "Materials and methods." One hundred nuclei were examined in each treatment group by a blinded observer using the following classifications: intact nuclei, no DNA damage; Class I DNA damage, nuclei that appeared condensed with some DNA fragmentation, but without visible "tails"; Class II DNA damage, nuclei exhibiting a tail length less than the length of one normal (control) nucleus; Class III DNA damage, nuclei displaying a tail length greater than the length of one control nucleus; Class IV DNA damage, nuclei that had incurred such great damage that more than 90% of the fragmented DNA had migrated away from the residual nuclear electrophoretic origin. This experiment was repeated twice with qualitatively equivalent results.

action of CYP3A4 on etoposide can also generate etoposide catechol [37], permitting peroxidase-mediated production of etoposide *ortho*-quinone, and etoposide phenoxy radicals [38]. These latter species are currently under investigation as the leukemogenic metabolites of etoposide, and the question is raised of whether CYP3A4 induction by SJW might increase the leukemogenic potential of this chemotherapeutic agent. This point is of particular importance since some commercially available SJW products are now being enriched for hyperforin content (WS5572, Movana®, Perika MMSPro™). Therefore, clinicians should be especially vigilant about potential CYP3A4-mediated drug–herb interactions with high hyperforin-containing SJW preparations.

Finally, these studies deserve comment regarding the potential relevance of chemotherapeutic antagonism of etoposide and amsacrine by hypericin in human tumor cells, particularly when one examines only a single purified component of a complex botanical mixture. First, and as mentioned earlier, specific antagonism studies in leukemia cells were hindered by the steep cytotoxicity curve of hypericin (with 2.5 μM being almost non-toxic and $>$ 5 μM causing $>$ 3-log cell kill) and, therefore, will require further investigation in other systems less prone to hypericin-mediated apoptosis. In addition, concentrations of hypericin in human volunteers given commercial *Hypericum* extracts generally range from 0.05 to 0.2 μM. Peak plasma levels of etoposide in patients given intravenous chemotherapy range from 10 to 30 μM, but it is likely that the antitumor effects of this agent are aided by the long duration of etoposide remaining during its clearance (for example, the related epipodophyllotoxin VM-26 (teniposide) is cytostatic to leukemia cells in culture at 15 nM, equivalent roughly to 0.15 μM etoposide [39]). Given that hypericin antagonizes the topo II-poisoning antitumor drugs at one-tenth to one-third their concentration, it is reasonable to speculate that both classes of agents could be present at physiologically rele-

vant concentrations in patient plasma to cause antagonism. Finally, it should be recognized that hypericin is not the predominant naphthodianthrone in *Hypericum* extracts; instead, pseudohypericin is also usually present at 2- to 5-fold greater concentrations [40]. Therefore, it is essential that future studies be broadened to include investigation of high-quality, commercially available *Hypericum* products, and that studies with various cell lines of tumors normally treated with topo II-directed agents be investigated in culture and in human tumor xenograft models in immunocompromised mice.

It has been estimated that 25% of cancer patients develop clinical signs of depression that require antidepressant medication [41]. Several North American studies have uniformly revealed that at least two-thirds of cancer patients also pursue alternative therapies, including self-medication with herbal medicines, while receiving conventional chemotherapy [42–44]. These patients should be carefully counseled to be made aware that there exist a number of clinically useful antidepressants to choose from that do not interfere with chemotherapeutic efficacy. Our data raise a cautionary flag that depressed cancer patients, especially those receiving topo II-poisoning chemotherapy regimens, are best advised to seek treatment with standard prescription antidepressant therapy until the antagonistic effects of hypericin (and *Hypericum* extracts) can be evaluated in systems more relevant to *in vivo* cancer chemotherapy.

Acknowledgments

The authors are grateful to Brante P. Sampey and Dr. Stavros Valsamas for assistance with the topo II DNA cleavage and comet assays, respectively. The authors also wish to thank Drs. Len Zwelling and Cynthia Herzog for their gift of the HL-60/Beran and HL-60/AMSA cell lines and Dr. Heather S. Shaw (Duke Comprehensive Cancer Center) for the suggestion to test intercalative topo II poisons. This work was supported by grants from the American Cancer Society (RPG-97–084-01-CDD) and the National Institutes of Health (RO1 CA76201) to D.J.K., and a grant from the Cancer League of Colorado to E.U.K.

Note added in proof

On August 16, 2001, The authors were notified of the death of Dr. C. Norman Gillis, Professor Emeritus of Pharmacology at the Yale University School of Medicine. Dr. Gillis authored a highly-cited ginseng pharmacology review in this journal (Biochem Pharmacol 1997;54:1–8), founded the herbal pharmacology interest group of The American Society of Pharmacology and Experimental Therapeutics (ASPET), and personally encouraged the authors in preparing this report. His scientific rigor and support of junior investigators will be greatly missed.

References

[1] Brenner R, Azbel V, Madhusoodanan S, Pawlowska M. Comparison of an extract of hypericum (LI 160) and sertraline in the treatment of depression: a double-blind, randomized pilot study. Clin Ther 2000; 22:411–9.
[2] Laakmann G, Schule C, Baghai T, Kieser M. St. John's wort in mild to moderate depression: the relevance of hyperforin for the clinical efficacy. Pharmacopsychiatry 1998;31(Suppl 1):54–9.
[3] Linde K, Ramirez G, Mulrow CD, Pauls A, Weidenhammer W, Melchart D. St John's wort for depression—an overview and meta-analysis of randomised clinical trials. BMJ 1996;313:253–8.
[4] Schrader E. Equivalence of St John's wort extract (Ze 117) and fluoxetine: a randomized, controlled study in mild-moderate depression. Int Clin Psychopharmacol 2000;15:61–8.
[5] Rezvani AH, Overstreet DH, Yang Y, Clark E . Attenuation of alcohol intake by extract of *Hypericum perforatum* (St. John's Wort) in two different strains of alcohol-preferring rats. Alcohol Alcohol 1999;34:699–705.
[6] DeMott K. St. John's wort tied to serotonin syndrome. Clin Psychiatry News 1998;26:28.
[7] Nebel A, Schneider BJ, Baker RK, Kroll DJ. Potential metabolic interaction between St. John's wort and theophylline. Ann Pharmacother 1999;33:502.
[8] Piscitelli SC, Burstein AH, Chaitt D, Alfaro RM, Falloon J. Indinavir concentrations and St John's wort. Lancet 2000;355:547–8.
[9] Ruschitzka F, Meier PJ, Turina M, Luscher TF, Noll G. Acute heart transplant rejection due to Saint John's wort. Lancet 2000;355:548–9.
[10] Baureithel KH, Buter KB, Engesser A, Burkard W, Schaffner W. Inhibition of benzodiazepine binding in vitro by amentoflavone, a constituent of various species of *Hypericum.* Pharm Acta Helv 1997; 72:153–7.
[11] Calapai G, Crupi A, Firenzuoli F, Costantino G, Inferrera G, Campo GM, Caputi AP. Effects of *Hypericum perforatum* on levels of 5-hydroxytryptamine, noradrenaline and dopamine in the cortex, diencephalon and brainstem of the rat. J Pharm Pharmacol 1999;51: 723–8.
[12] Chatterjee SS, Bhattacharya SK, Wonnemann M, Singer A, Muller WE. Hyperforin as a possible antidepressant component of hypericum extracts. Life Sci 1998;63:499–510.
[13] Cott JM. *In vitro* receptor binding and enzyme inhibition by *Hypericum perforatum* extract. Pharmacopsychiatry 1997;30(Suppl): 108–12.
[14] Burden DA, Osheroff N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1998;1400:139–54.
[15] Miskovsky P, Chinsky L, Wheeler GV, Turpin PY. Hypericin site specific interactions within polynucleotides used as DNA model compounds. J Biomol Struct Dyn 1995;13:547–52.
[16] Miskovsky P, Sureau F, Chinsky L, Turpin PY. Subcellular distribution of hypericin in human cancer cells. Photochem Photobiol 1995; 62:546–9.
[17] Muller SO, Eckert I, Lutz WK, Stopper H. Genotoxicity of the laxative drug components emodin, aloe-emodin and danthron in mammalian cells: topoisomerase II mediated? Mutat Res 1996;371: 165–73.
[18] Baker RK, Brandt TL, Kroll DJ. Inhibition of DNA topoisomerase II activity by hypericin. Proc Am Assoc Cancer Res 1998;39:422.
[19] Felix CA. Secondary leukemias induced by topoisomerase-targeted drugs. Biochim Biophys Acta 1998;1400:233–55.
[20] Hinds M, Deisseroth K, Mayes J, Altschuler E, Jansen R, Ledley FD, Zwelling LA. Identification of a point mutation in the topoisomerase

II gene from a human leukemia cell line containing an amsacrine-resistant form of topoisomerase II. Cancer Res 1991;51:4729–31.
[21] Gantchev TG, Hunting DJ. The *ortho*-quinone metabolite of the anticancer drug etoposide (VP-16) is a potent inhibitor of the topoisomerase II/DNA cleavable complex. Mol Pharmacol 1998;53: 422–8.
[22] Kurz EU, Leader KB, Kroll DJ, Clark M, Gieseler F. Modulation of human DNA topoisomerase IIα function by interaction with 14–3-3ϵ. J Biol Chem 2000;275:13948–54.
[23] Thomsen B, Bendixen C, Lund K, Andersen AH, Sorensen BS, Westergaard O. Characterization of the interaction between topoisomerase II and DNA by transcriptional footprinting. J Mol Biol 1990;215:237–44.
[24] Kroll DJ. Homologous and heterologous protein-protein interactions of human DNA topoisomerase IIα. Arch Biochem Biophys 1997;345: 175–84.
[25] Kroll DJ, Sullivan DM, Gutierrez-Hartmann A, Hoeffler JP. Modification of DNA topoisomerase II activity via direct interactions with the cyclic adenosine-3′, 5′-monophosphate response element-binding protein and related transcription factors. Mol Endocrinol 1993;7: 305–18.
[26] Lee JS, Morgan AR. A rapid method for the measurement of the unwinding angle of intercalating agents and the superhelix density of circular DNAs. Nucleic Acids Res 1978;5:2425–39.
[27] Ritke MK, Yalowich JC. Altered gene expression in human leukemia K562 cells selected for resistance to etoposide. Biochem Pharmacol 1993;46:2007–20.
[28] Moran JL, Siegel D, Sun XM, Ross D. Induction of apoptosis by benzene metabolites in HL60 and $CD34^+$ human bone marrow progenitor cells. Mol Pharmacol 1996;50:610–5.
[29] Todd MD, Lee MJ, Williams JL, Nalezny JM, Gee P, Benjamin MB, Farr SB. The CAT-Tox (L) assay: a sensitive and specific measure of stress-induced transcription in transformed human liver cells. Fundam Appl Toxicol 1995;28:118–28.
[30] Gieseler F, Bauer E, Nuessler V, Clark M, Valsamas S. Molecular effects of topoisomerase II inhibitors in AML cell lines: correlation of apoptosis with topoisomerase II activity but not with DNA damage. Leukemia 1999;13:1859–63.
[31] Drake FH, Hofmann GA, Mong SM, Bartus JO, Hertzberg RP, Johnson RK, Mattern MR, Mirabelli CK. *In vitro* and intracellular inhibition of topoisomerase II by the antitumor agent merbarone. Cancer Res 1989;49:2578–83.
[32] Rowe T, Kupfer G, Ross W. Inhibition of epipodophyllotoxin cytotoxicity by interference with topoisomerase-mediated DNA cleavage. Biochem Pharmacol 1985;34:2483–7.
[33] Nelson EM, Tewey KM, Liu LF. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. Proc Natl Acad Sci USA 1984;81:1361–5.
[34] Tewey KM, Chen GL, Nelson EM, Liu LF. Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J Biol Chem 1984; 259:9182–7.
[35] Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, Kliewer SA. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. Proc Natl Acad Sci USA 2000;97:7500–2.
[36] Relling MV, Evans R, Dass C, Desiderio DM, Nemec J. Human cytochrome P450 metabolism of teniposide and etoposide. J Pharmacol Exp Ther 1992;261:491–6.
[37] Felix CA, Walker AH, Lange BJ, Williams TM, Winick NJ, Cheung N-KV, Lovett BD, Nowell PC, Blair IA, Rebbeck TR. Association of *CYP3A4* genotype with treatment-related leukemia. Proc Natl Acad Sci USA 1998;95:13176–81.
[38] Kagan VE, Yalowich JC, Borisenko GG, Tyurina YY, Tyurin VA, Thampatty P, Fabisiak JP. Mechanism-based chemopreventive strategies against etoposide-induced acute myeloid leukemia: free radical/antioxidant approach. Mol Pharmacol 1999;56:494–506.
[39] Tan SK, Tang SJ, Wang MC, Hwang J, Wang FF. Regulation of topoisomerase II expression during the VM-26 induced differentiation of IW32 murine erythroleukemia cells. Leuk Res 1996;20: 249–57.
[40] Upton R, Graff A, Williamson E, Bunting D, Gatherum DM, Walker EB, Butterweck V, Lieflander-Wulf U, Nahrstedt A, Wall A, Winterhoff H, Cott JM, Marquis MC, Shumake RL, Stansbury J, Petrone C. St. John's wort (*Hypericum perforatum*); quality control, analytical and therapeutic monograph. In: Upton R, editor. American herbal pharmacopoeia. Santa Cruz, CA: American Herbal Pharmacopoeia, 1997. p. 1–32.
[41] Pirl WF, Roth AJ. Diagnosis and treatment of depression in cancer patients. Oncology 1999;13:1293–301.
[42] Boon H, Stewart M, Kennard MA, Gray R, Sawka C, Brown JB, McWilliam C, Gavin A, Baron RA, Aaron D, Haines-Kamka T. Use of complementary/alternative medicine by breast cancer survivors in Ontario: prevalence and perceptions. J Clin Oncol 2000;18: 2515–21.
[43] Richardson MA, Sanders T, Palmer JL, Greisinger A, Singletary SE. Complementary/alternative medicine use in a comprehensive cancer center and the implications for oncology. J Clin Oncol 2000;18: 2505–14.
[44] Sparber A, Bauer L, Curt G, Eisenberg D, Levin T, Parks S, Steinberg SM, Wootton J. Use of complementary medicine by adult patients participating in cancer clinical trials. Oncol Nurs Forum 2000;27: 623–30.