Natural-product inhibitors of human DNA ligase I

Ghee T. TAN*, Sangkook LEE*, Ik-Soo LEE*§, Jingwen CHEN†, Pete LEITNER†, Jeffrey M. BESTERMAN†, A. Douglas KINGHORN* and John M. PEZZUTO*‡‖

*Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, IL 60612, †Glaxo Wellcome, Inc., Research Triangle Park, NC 27709, and ‡Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, U.S.A.

Enzymic activity mediated by recombinant human DNA ligase I (hLI), in conjunction with tannin removal procedures, has been applied to a natural-product screen involving ~1000 plant extracts and various pure compounds. The primary hLI activity assay involved the measurement of the amount of radiolabelled phosphate in a synthetic nucleic acid hybrid that becomes resistant to alkaline phosphatase as a result of ligation. A bioactivity-guided fractionation scheme resulted in the isolation of ursolic [IC_{50} = 100 μg/ml (216 μM)] and oleanolic [IC_{50} = 100 μg/ml (216 μM)] acids from *Tricalysia niamniamensis* Hiern (Rubiaceae), which demonstrated similar DNA ligase inhibition profiles to other triterpenes such as aleuritolic acid. Protolichesterinic acid [IC_{50} = 6 μg/ml (20 μM)], swertifrancheside [IC_{50} = 8 μg/ml (11 μM)] and fulvoplumierin [IC_{50} = 87 μg/ml (357 μM)] represent three additional natural-product structural classes that inhibit hLI. Fagaronine chloride [IC_{50} = 10 μg/ml (27 μM)] and certain flavonoids are also among the pure natural products that were found to disrupt the activity of the enzyme, consistent with their nucleic acid intercalative properties. Further analyses revealed that some of the hLI-inhibitory compounds interfered with the initial adenylation step of the ligation reaction, indicating a direct interaction with the enzyme protein. However, in all cases, this enzyme–inhibitor interaction did not disrupt the DNA relaxation activity mediated by hLI. These results indicate that, although the same enzyme active site may be involved in both enzyme adenylation and DNA relaxation, inhibitors may exert allosteric effects by inducing conformational changes that disrupt only one of these activities. Studies with inhibitors are important for the assignment of specific cellular functions to these enzymes, as well as for their development into clinically useful antitumour agents.

INTRODUCTION

DNA ligase catalyses the covalent joining of single-stranded breaks in double-stranded DNA. These enzymes are essential for DNA replication and are involved in DNA repair and genetic recombination [1,2]. Although micro-organisms appear to have only one species of DNA ligase, four distinct ligases, designated DNA ligases I, II, III and IV, have been detected in mammalian cells [3–5]. A human cDNA encoding the 102 kDa DNA ligase I has been cloned and sequenced [6], and the biochemical properties of mammalian DNA ligase I have been extensively characterized [3,7–9].

The identification of four distinct DNA ligases in mammalian cell nuclei and the occurrence of DNA ligase deficiency [10] and DNA-joining defects [11] in certain individuals have stimulated research on these enzymes over the past decade. DNA ligase I is the major DNA replicative activity in proliferating cells; recent studies also suggest its possible involvement in constitutive excision–repair [3,6]. Although implicated in DNA recombination and repair [3,5,12,13], the function of DNA ligases II, III and IV remains unclear. The cloning and functional expression of human DNA ligase I (hLI) cDNA in yeast [6,14] has resulted in the availability of the recombinant enzyme for biochemical studies, and has made feasible the large-scale evaluation of chemical entities for DNA ligase inhibitory activity.

The predominant strategy in cancer chemotherapy to date focuses on substances that interfere with DNA metabolism [15]. Among the enzymes that are involved in DNA replication and repair, DNA ligases have received relatively little attention as possible targets for antitumour agents. In addition, resistance to chemotherapy and/or radiation therapy represents a major problem in the clinical management of tumour proliferation, and is the primary cause of treatment failure in cancer patients. Many biochemical mechanisms, including those affecting DNA repair processes, mediate the resistance phenotype [16]. Studies with cisplatin, an intrastrand cross-linking agent, have indicated that the DNA-repair capacity of cells is a significant factor in the acquired resistance of tumours to the drug [17]. Moreover, DNA repair is the critical factor in cellular survival following X-irradiation. In view of the preceding arguments, the direct antiproliferative effects of DNA ligase inhibitors, and the advantage of compromising ligase-mediated repair mechanisms in cells that bear radiation-induced or chemically induced DNA strand breaks are obvious. In support of the latter approach, aphidicolin, an inhibitor of DNA polymerases α and δ, was found to overcome resistance in the A2780 human ovarian cancer cell line [18]. Abnormalities of DNA repair mechanisms, such as those present in the human genetic disorder ataxia telangiectasia, are also associated with drug and radiation hypersensitivity.

Since hLI plays an obligatory role during replication and repair, it constitutes a potential target for compounds that affect

Abbreviations used: hLI, human DNA ligase I; RT, reverse transcriptase.

§ Present address: College of Pharmacy, Chonnam National University, Kwangju 500-757, Korea.

‖ To whom correspondence should be addressed, at the Program for Collaborative Research in the Pharmaceutical Sciences (M/C 877), Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612, U.S.A.

genome stability and DNA processing [19]. The present study examines the hLI-inhibitory potential of compounds from natural sources, and their modes of action in terms of the proposed two-stage ligation reaction mechanism involving the enzyme–adenylate intermediate. The overall proposed sequence of reactions catalysed by mammalian DNA ligase is as follows [20]:

$$\text{Ligase} + \text{ATP} \rightleftharpoons \text{ligase–AMP} + \text{PP}_i$$

$$\text{Ligase–AMP} + \text{nicked DNA (or other substrates)} \rightleftharpoons \text{ligase–AMP–DNA} \rightleftharpoons \text{sealed DNA} + \text{AMP} + \text{ligase}$$

In addition to the covalent joining of nicks and blunt-ended DNA molecules, mammalian DNA ligase is also able to act at low efficiency as a topoisomerase I according to the general model of DNA relaxation catalysed by all known DNA topoisomerases [21]. The relaxation of supercoiled DNA occurs in a gradual and stepwise fashion via the Mg^{2+}- and AMP-dependent reversal of the last step of the ligation reaction, followed by a religation.

The inhibitory activity of several classes of natural-product antileukaemic drugs, including the anthracyclines, the *Catharanthus* (*Vinca*) alkaloids, and podophyllotoxin, against purified DNA ligase from both normal and leukaemic lymphocytes has been described previously [22]. However, the mechanisms of many of these inhibitory effects have not been elucidated further, and, thus far, very few compounds have been shown to inhibit hLI. Natural products represent a rich and largely untapped source of biologically active and structurally novel chemicals which are worth investigating as specific inhibitors of DNA ligases. To the best of our knowledge, this is the first report of a systematic effort in the search for inhibitors of hLI. Active compounds, if not eventually advocated for clinical development, may serve as new leads for the synthetic development of novel antitumour agents.

MATERIALS AND METHODS

Test and reference compounds

Test compounds were either isolated or obtained from the natural-product compound repository of the Program for Collaborative Research in the Pharmaceutical Sciences at the College of Pharmacy, University of Illinois at Chicago. The identity and purity of all compounds were reported previously [23]. A complete list of all plant extracts tested is available from the authors.

Reagents and enzymes

Synthetic oligo(dT)$_{30}$ was obtained from Boehringer Mannheim Corp. (Indianapolis, IN, U.S.A.). This material was 5′-dephosphorylated by treatment with alkaline phosphatase and subsequently gel-purified. Polynucleotide kinase and covalently closed pBR322 were purchased from Promega Corp. (Madison, WI, U.S.A.), and alkaline phosphatase and AMP were obtained from Boehringer Mannheim Corp. [γ-^{32}P]ATP (6000 Ci/mmol) and [2,5′,8-^{3}H]ATP (35 Ci/mmol) were obtained from Amersham Inc. (Arlington Heights, IL, U.S.A.).

DEAE-81 anion-exchange paper or DE-81 filter discs were obtained from Whatman Labsales (Hillsboro, OR, U.S.A.) or VWR Scientific (Batavia, IL, U.S.A.). Prepacked spin columns containing DNA-grade Sephadex G-50 (fine) (NICK Spin columns) and poly(dA) were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). ATP, dithiothreitol, BSA and all other reagents of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

hLI

The hLI cDNA [6] was subcloned into a baculovirus expression vector, pVL1392 (PharMingen) (J. Chen, unpublished work). Homogeneous recombinant hLI (125 kDa) from baculoviral-infected cells was purified by the procedure of Tomkinson et al. [9], with modifications. The recombinant hLI preparation used in all experiments had a protein concentration of 5 mg/ml, and a specific activity of 4 units/mg of protein in the hLI ligation assay used. One unit of DNA ligase activity is defined as that which catalyses the conversion of 1 nmol of terminal phosphate residues into a phosphatase-resistant form in 1 h at 30 °C.

Preparation of poly(dA)·oligo(dT)$_{30}$ (DNA ligase I substrate)

5′-Dephosphorylated oligo(dT)$_{30}$ was labelled at the 5′ end with [γ-^{32}P]ATP in a reaction mixture (final volume 30 μl) containing the following: 70 mM Tris/HCl (pH 7.6), 10 mM $MgCl_2$,6H_2O, 5 mM dithiothreitol, 1.7 μM oligo(dT)$_{30}$ and 0.83 μM [γ-^{32}P]ATP. The reaction was initiated by the addition of 16 units (2 μl) of polynucleotide kinase and incubated at 37 °C for 40 min. Incubation at this temperature was extended for 5 min after the reaction was quenched with ATP (2 μl) at a final concentration of 15.6 μM. Polynucleotide kinase was inactivated by heating the labelling mixture at 65 °C for 10 min. The free unincorporated [γ-^{32}P]ATP and unlabelled ATP were removed by column chromatography over Sephadex G-50 (fine grade) by using commercially available NICK Spin columns. Oligonucleotide recovery was observed to be approx. 80 %, whereas more than 50 % of the [γ-^{32}P]ATP was incorporated. The approximate concentration of the recovered end-labelled oligonucleotide was 0.8 μM.

The recovered end-labelled oligo(dT)$_{30}$ was hybridized to poly(dA) at a ratio of 4:1 in buffer (final volume 200 μl) consisting of the following: 7.3 mM Tris/HCl (pH 8.0), 0.73 mM EDTA, 0.2 μM oligo(dT)$_{30}$ and 0.05 μM poly(dA). The hybridization mixture was heated at 65 °C for 5 min, and then allowed to cool slowly to room temperature.

hLI assay

The DNA ligase assay utilized for the screening of natural products as inhibitors of the enzyme was developed in accordance with the procedures of Tomkinson et al. [3], with modifications. The assay was carried out in 96-well microtitre plates. DMSO (10 %, v/v, in water; 10 μl) was dispensed into microtitre wells containing previously dried methanolic plant extracts (approx. 8 μg). Alternatively, 10 μl of DMSO (20 %, v/v, in water) was used as solvent for pure natural products. DNA ligase activity was unaffected by final DMSO concentrations of up to 10 % (v/v).

The hLI reaction mixture (final volume 40 μl) contained the following: 50 mM Tris/HCl (pH 8.0), 8 mM $MgCl_2$.6H_2O, 4 mM dithiothreitol, 40 μg/ml BSA, 0.125 mM ATP, 3 nM labelled substrate, 20 nM unlabelled substrate [with respect to oligo(dT)$_{30}$] and 55 ng of hLI. The wells were sealed with Parafilm to prevent evaporation of water during incubation. The reaction was carried out in a heating block at 30 °C for 20 min, and terminated at 65 °C for 20 min.

The Parafilm was removed and 30 μl (2 units) of alkaline phosphatase in buffer consisting of 50 mM Tris/HCl (pH 8.5) and 0.1 mM EDTA was added to each well. An excess of alkaline phosphatase was used to preclude effects resulting from its inhibition. The wells were resealed and the plate was maintained at 65 °C for an additional 60 min. The contents of each well were spotted on to DE-81 (Whatman) filters, and washed three times

with aq. 5 % Na_2HPO_4 and once with water (15 min each wash). Finally, the filters were dried and subjected to scintillation counting in order to determine the conversion of 5′-^{32}P-labelled phosphomonoesters into alkaline-phosphatase-resistant diesters.

Pre-screens were carried out with a single concentration of each pure compound or plant extract at 200 μg/ml. Control assays, performed with an equivalent volume of solvent alone, showed no effect in every case. The antileukaemic alkaloid fagaronine chloride [24] was used as the positive-control substance. The IC_{50} (median inhibitory concentration) values of all active entities were then determined from linearly regressed dose–response plots of percentage of control activity versus concentration or log concentration of compound. Each data point represents the average of duplicate tests. Adopting previously established thresholds for the enzyme-inhibitory activity of natural products [23], pure compounds demonstrating IC_{50} values < 200 μg/ml were deemed active. Plant extracts that showed 100 % inhibition at 400 μg/ml were considered for further phytochemical evaluation.

DNA ligase adenylation assay

Recombinant hLI (0.5 μg) was incubated at 37 °C for 20 min in a reaction mixture (10 μl) identical with that for ligation, except that 1 mM EDTA and 1.9 μM [2,5′,8-^{3}H]ATP (1 μCi) were included. Compounds (2 μl) were introduced in 20 % (v/v) DMSO. Reactions were terminated by addition of 20 μl of a solution containing 10 mM EDTA, and placed on ice. A portion of each reaction mixture (25 μl) was spotted on to DE-81 anion-exchange filters, and processed by washing as described for the ligation assay. Adenylated hLI was determined by scintillation counting. It was previously determined by a 5 % trichloroacetic acid-precipitation and a filtration (GF/C filters) protocol performed at 4 °C that the DNA ligase–AMP intermediate is stable under the DE-81 washing conditions employed. Trichloroacetic acid precipitation was not adopted, due to inaccuracies generated by an inherently high background count.

Assay for DNA topoisomerase I activity of hLI

The reaction mixture (20 μl) used was identical with that for hLI adenylation. In addition, each reaction also contained naturally supercoiled plasmid pBR322 (400 ng), hLI (1 μg) and AMP (1 mM). Test compounds (4 μl) were added to reaction mixtures before addition of hLI. After 30 min at 37 °C, reactions were quenched by addition of 2 μl of a loading solution containing 1 mg/ml Bromophenol Blue, 50 % (v/v) glycerol and 5 mg/ml SDS, and analysed on a 1.2 %-agarose gel with 40 mM Tris/acetate/2 mM EDTA as running buffer. The electrophoretic run was at 4 V/cm of gel until Bromophenol Blue had migrated 8 cm (approx. 5 h). The gel was then stained with ethidium bromide (1 μg/ml) for 30 min, and subsequently destained with water for 15 min. DNA was revealed and the gel photographed with the aid of an UV illuminator. In a separate experiment, reaction mixtures containing compounds known to interact with nucleic acids were extracted with phenol and chloroform before addition of the loading solution in order to prevent interference with DNA migration.

RESULTS AND DISCUSSION

The complexity of the reactions mediated by hLI furnishes several mechanistically distinct steps for the action of inhibitors, including those from natural sources. Recombinant hLI, in conjunction with bioassay-directed fractionation and tannin removal procedures, has been applied to a natural-product screen involving both pure compounds and plant extracts. In order to characterize better the nature of the inhibition mediated by natural products, mechanistic studies were performed on all active isolates. In general, compounds that interact with the enzyme protein (at the active site or elsewhere) are of interest from the viewpoint of selectivity; this may translate either to the inhibition of the initial adenylation of hLI, or to the subsequent transfer of the adenylyl group to the 5′-phosphoryl terminus of the single-stranded break in double-stranded DNA. In an effort to explore these possibilities, DNA ligase–[^{3}H]AMP adducts were formed in the absence and presence of the polynucleotide substrate and compounds, respectively. Additionally, the effect of natural-product inhibitors on the DNA relaxation activity of hLI was also assessed. This activity of hLI, which requires the participation of the enzyme active site, isolates the second and final stage of the overall ligation process, and involves a nicking reaction followed by a re-ligation.

Before the search for inhibitors, preliminary evaluations were carried out to standardize the hLI assay parameters. This constitutes an important prerequisite for the valid comparison of enzyme inhibitors, since the concentrations of reagents used (especially those of the enzyme and substrate) determine the catalytic efficiency of the enzyme, and ultimately its susceptibility to inhibition by agents with varied mechanisms of action. Studies were performed to assess effects of experimental variables such as reaction time, and polynucleotide substrate and hLI concentrations, on phosphodiester formation. The ligation assay was subsequently established by utilizing sub-saturation conditions with respect to all of these parameters (results not shown). Under these conditions, hLI should demonstrate optimum sensitivity to inhibitors acting by diverse mechanisms of action.

Similar experiments were performed to establish an assay to monitor the formation of the hLI–adenylate complex (Figure 1). Kinetic studies revealed a steady ^{3}H incorporation even after 20 min (Figure 1A), with decreased adenylation after 30 min, probably indicating decreased stability of the adenylated enzyme. The reaction was dependent on hLI and ATP concentrations (Figures 1B and 1C respectively), although a higher concentration of hLI was required to generate a detectable response in the adenylation assay regardless of whether [2,5′,8-^{3}H]ATP or [α-^{32}P]ATP was used. However, both the ligation and adenylation assays were carried out with an excess of ATP, to preclude any effects that inhibitors might have on this substrate. The development of the ligation protocol for the large-scale evaluation of inhibitors, and strategies for the general screening of pure compounds and plant extracts, were carried out as reported previously for HIV reverse transcriptase (RT) [23].

Approx. 1000 crude methanolic extracts representing species from a diverse group of plant families were included in this study; 15 % of these plant extracts were found to be active. However, tannins and other polyphenolic compounds, which appear to be ubiquitous in the plant kingdom, have been reported to mediate a general enzyme-inhibitory response [23]. Thus the tannin removal procedure utilizing insoluble polyvinylpyrrolidone [23] was applied as an effective method for polyphenol removal before the testing of plant extracts. Additionally, the hLI assay when applied at various stages of the fractionation scheme of *Tricalysia niamniamensis* Hiern (Rubiaceae) directed the isolation procedure to yield two triterpenoids, namely ursolic [$IC_{50} = 100$ μg/ml (216 μM)] and oleanolic [$IC_{50} = 100$ μg/ml (216 μM)] acids as the hLI-inhibitory constituents (Figures 2 and 3). Aleuritolic acid [25], a triterpenoid of related structure, was found to induce a similar response. As indicated in Table 1, similar IC_{50} values were noted in the adenylation and ligation assays for this class of compounds, suggesting an interaction

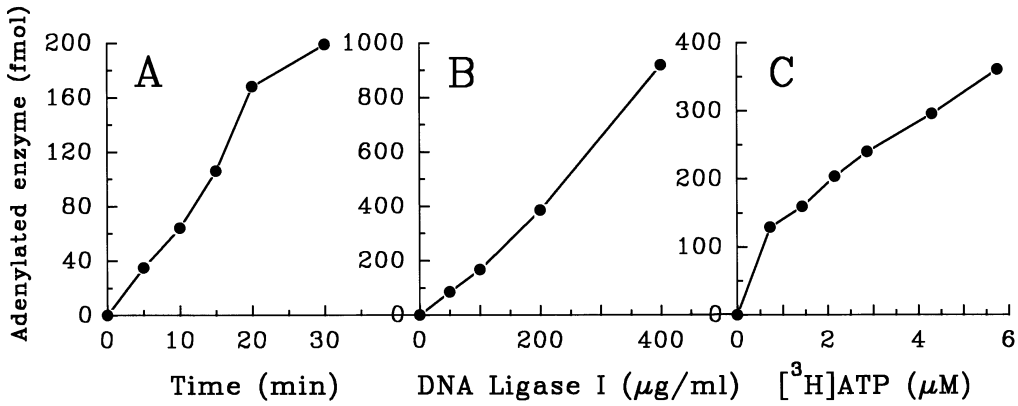

Figure 1 Dependence of hLI adenylation on time (A), enzyme concentration (B) and [^{3}H]ATP concentration (C)

Recombinant hLI (0.5 μg) was incubated with 1.9 μM [2,5′,8-^{3}H]ATP (1 μCi) at 37 °C for 20 min. Enzyme adenylation was monitored by measuring the amount of hLI–[2,5′,8-^{3}H]AMP complex that bound to anion-exchange filters (DE-81) after the removal of unincorporated label with aq. 5% Na_2HPO_4. The reaction time, hLI and [2,5′,8-^{3}H]ATP concentrations were varied in (**A**), (**B**) and (**C**) respectively.

between the inhibitor and the enzyme protein. More detailed experiments revealed that the hLI-inhibitory activity of triterpenes was affected by the BSA concentration in the reaction mixture; inhibition of hLI was completely negated by the presence of BSA at a concentration of 200 μg/ml through a process of competitive binding. The structural and conformational features of protein molecules targeted by these triterpenoid compounds, and the nature of the interaction involved, are at present unclear. Nonetheless, the response of various biological systems to the inhibitory effect of triterpenoids is undoubtedly protein (enzyme)-specific. In the case of hLI, the inability of a further triterpenoid, betulinic acid (Figure 2), to generate an inhibitory response would also indicate specific structural requirements for the action of these inhibitory molecules.

Pure compounds representing many natural-product classes (Figure 2) were also tested. The hLI-inhibitory profiles of selected compounds are illustrated in Figures 3 and 4, and summarized in Table 1. A number of benzophenanthridine alkaloids such as fagaronine chloride and nitidine chloride have demonstrated significant cytotoxic activity against P-388 leukaemia in mice [24]. These alkaloids have also been reported to inhibit various RNA- and DNA-polymerizing enzymes via interaction with nucleic acid template–primers [26]. It has been suggested that the inhibitory property of benzophenanthridine alkaloids is in accordance with the postulated active site (the iminium ion, $-C=N^+-CH_3$) for antitumour and other biological activities [27]; this functionality represents the key structural requirement for the intercalative ability of these alkaloids. Thus far, it is not known if these molecules interact with the enzyme protein as well. The most potent ligation-inhibitory activity was demonstrated by fagaronine chloride and nitidine chloride, which exhibited IC_{50} values of 10 μg/ml (27 μM) and 25 μg/ml (69 μM) respectively (Figure 3 and Table 1). Chelidonine and *N*-demethylfagaronine were inactive up to a concentration of 200 μg/ml, presumably due to the absence of the quaternary nitrogen atom in these molecules [26]. The median inhibitory concentrations obtained for fagaronine chloride and nitidine chloride were insignificantly different from those previously obtained with HIV-1 RT [28]. In addition, hLI adenylation was affected by concentrations of nitidine chloride and chelerythrine chloride many times in excess of that which affected the ligation reaction (Figure 4 and Table 1), indicating the insignificant role of hLI binding in the inhibition of the overall ligation reaction. This effect was even more clearly demonstrated in the case of fagaronine chloride, which was totally inactive in the adenylation assay (Table 1). Additional ligation and adenylation experiments verified that the level of inhibition mediated by benzophenanthridine alkaloids was unaffected by the concentration of BSA, hLI or ATP present in the system, and directly implicated the nucleic acid substrate as the primary target in the inhibition. Nevertheless, the inhibitory effect on mammalian DNA ligase may contribute to the cytotoxic and antitumour activity of this class of compounds.

The protoberberine alkaloids such as berberine chloride (Figure 2), which are structurally related to the benzophenanthridine alkaloids, have been shown to possess a wide variety of biological properties such as antimicrobial and antitumour activities [29]. Despite the ability of protoberberine molecules bearing a quaternary nitrogen atom (e.g. berberine chloride and coptisine chloride) to interact with nucleic acids [30], these alkaloids were inactive in the hLI system (Table 1), up to a concentration of 200 μg/ml.

Flavonoids comprise a large group of low-molecular-mass phenolic compounds which have a wide spectrum of pharmacological properties [31], including interference with a variety of mammalian enzyme systems. Although the enzyme-inhibitory properties of flavonoids have been observed to show stringent conformational requirements, the mechanisms underlying the inhibitory effect on non-nucleic acid-dependent enzymes remain to a large extent unknown. The molecular planarity of flavones, exemplified by myricetin and morin, is thought to facilitate the intercalation of these molecules into double-stranded nucleic acids [32]. Furthermore, the number of phenolic hydroxy groups on flavonoids bears a striking correlation with the enzyme-inhibitory potential of these compounds [33]. Consistent with these observations, myricetin and morin were found to be more potent hLI inhibitors as compared with apigenin (Figure 3 and Table 1), whereas (−)-catechin was inactive up to a concentration of 200 μg/ml. The greater IC_{50} values obtained in adenylation assays (Table 1) suggest that interaction with the nucleic acid substrate constitutes the predominant mechanism in the inhibition of the overall ligation reaction. Although ligation was restored by elevated concentrations of poly(dA)·oligo$(dT)_{30}$, flavonoid-inhibited ligation and adenylation systems were insensitive to variable concentrations of BSA, hLI or ATP.

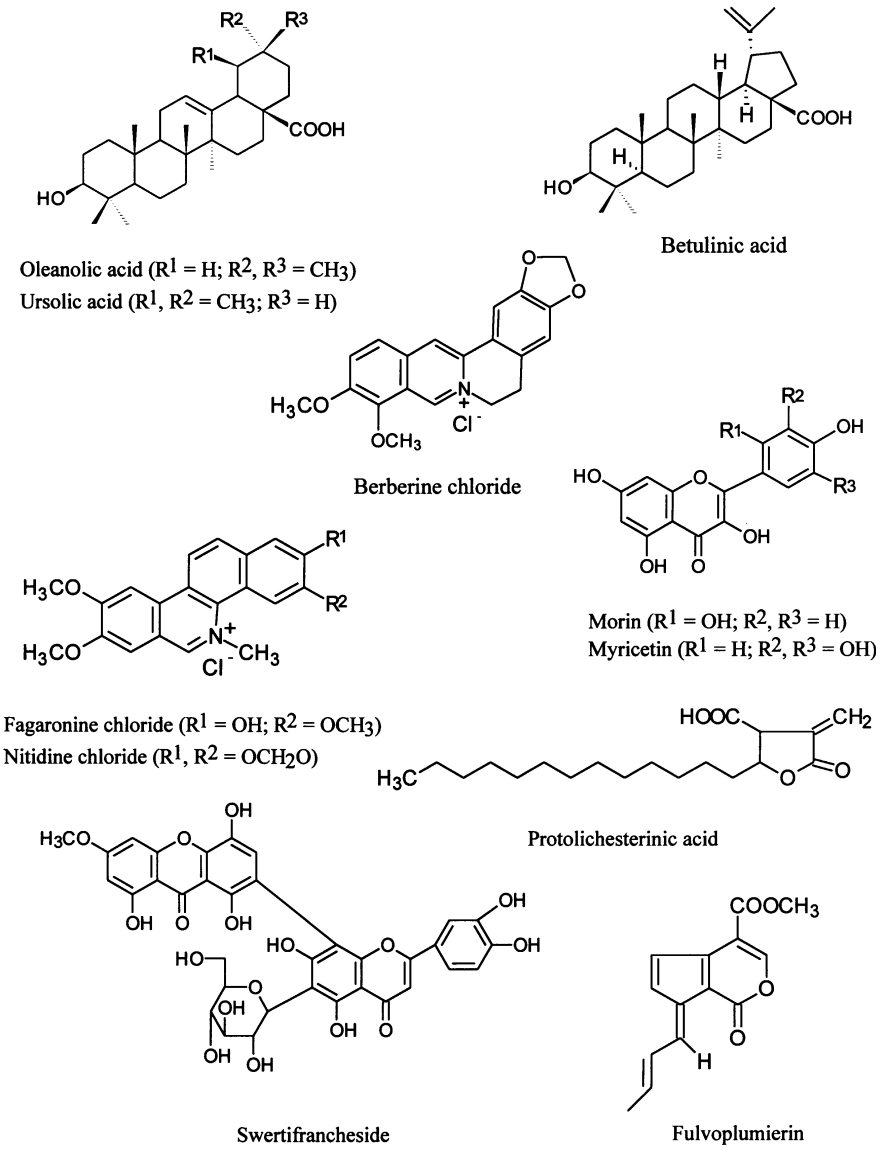

Figure 2 Structures of selected natural-product inhibitors of hLI

Swertifrancheside is a flavonoxanthone glucoside previously isolated from *Swertia franchetiana* [34]. The disruption of the ligation activity of hLI [IC_{50} = 8 μg/ml (11 μM)] (Figure 3) correlated with the ability of the compound to interact with nucleic acids [35]. The decreased formation of DNA ligase–adenylate complexes in the presence of swertifrancheside [IC_{50} = 76 μg/ml (105 μM)] (Figure 4) also suggests a possible inhibitory role of hLI binding, albeit of much less significance than nucleic acid interactions in the interference with the overall ligation. Mechanistic studies aimed at elucidating the kinetics of this process are in progress.

Fulvoplumierin, an iridoid obtained as a constituent of *Plumeria rubra* L. [36], has previously been shown to inhibit HIV-1 [IC_{50} = 98 μg/ml (400 μM)] and HIV-2 [IC_{50} = 87 μg/ml (357 μM)] RTs to similar extents [37]. Interestingly, both hLI adenylation and the ligation of nicked poly(dA)·oligo$(dT)_{30}$ were also affected, with similar IC_{50} values of 87 μg/ml (357 μM) and 88 μg/ml (361 μM), respectively (Table 1). The identical IC_{50} values obtained in the last two hLI assay systems, and the activity against the unrelated HIV RTs, imply that fulvoplumierin interacted with structural features common to both enzyme proteins. These structural and/or conformational attributes remain to be identified. Increasing concentrations of BSA exerted minimal effects on the hLI-inhibitory potency of fulvoplumierin.

Protolichesterinic acid is an aliphatic α-methylene-γ-lactone obtained from the lichen *Cetraria islandica* (L.) Ach. [38]. Previous experiments from our laboratory failed to detect any interaction between protolichesterinic acid and calf thymus DNA by spectrophotometric methods [35]. However, kinetic experiments revealed that protolichesterinic acid bound to HIV-1 RT at non-substrate-binding sites [35]. Presumably, such non-specific binding also applies to hLI. The protolichesterinic acid-mediated interference with ligation [IC_{50} = 6 μg/ml (20 μM)] and hLI adenylation [IC_{50} = 116 μg/ml (387 μM)] (Table 1) were observed to be much more sensitive to the concentration of BSA in the reaction mixture than was the inhibitory effect induced by any other compounds tested in the present study. The binding of multiple molecules of protolichesterinic acid per molecule of

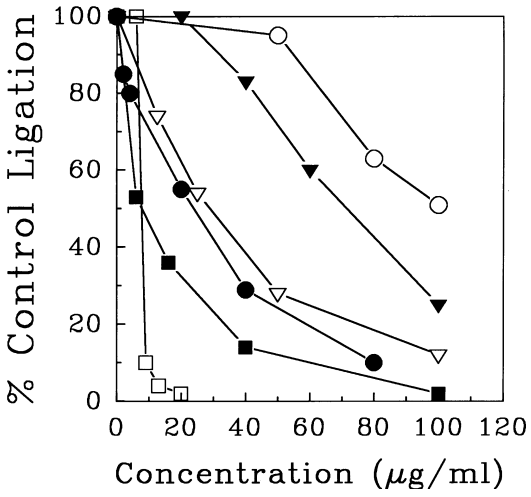

Figure 3 Effect of natural products on the ligation activity of hLI

The ligation activity of hLI was monitored by measuring the amount of radiolabelled phosphate in poly(dA)·oligo(dT)$_{30}$ that became resistant to alkaline phosphatase as a result of nick closure. hLI (1.375 μg/ml) was incubated with 3 or 20 nM of labelled or unlabelled substrate, respectively, in the presence of 0.125 mM ATP for 20 min at 30 °C. Upon heat inactivation of hLI, hydrolysis of unreacted phosphate was performed with an excess of alkaline phosphatase at 65 °C for an additional 60 min. Radioactivity associated with the ligated product remained on DE-81 anion-exchange filters after a washing procedure with aq. 5% Na_2HPO_4. Standard assays were performed in the presence of the indicated concentrations of compounds: oleanolic acid (○), nitidine chloride (●), myricetin (▽), fulvoplumierin (▼), protolichesterinic acid (□) and swertifrancheside (■).

BSA most likely accounts for this observation. The greater IC_{50} value obtained for the inhibition of adenylation will then be a direct reflection of the higher hLI concentration used in this assay.

Preliminary observations on the effect of selected inhibitors on the DNA relaxation activity of hLI are presented in Figure 5, which is designed to illustrate a qualitative all-or-none inhibitory response. Concentrations tested were equivalent to IC_{50} values in the ligation assay, or higher when solubility permits. AMP-dependent DNA topoisomerization involves the stepwise relaxation of the supercoiled DNA substrate [21]. The distributive mode of hLI action results in a progressive decrease in the number of superhelical turns, thus generating a population of partially relaxed forms, as exemplified in lanes 11 and 14. However, in order to produce a system with maximal sensitivity to the inhibitory effects of compounds, assay conditions were adopted such that in most cases all supercoiled pBR322 DNA molecules (Form I) were converted to the fully relaxed form (Form II: nicked circles) in 30 min at 37 °C (lane 2), even though some nicked circular DNA was present as a contaminant in the original pBR322 preparation (lane 1). The benzophenanthridine alkaloids (lanes 3–6) were found to interfere with DNA migration, presumably through an intercalative effect. Upon compound and protein removal by organic-solvent extraction, a gel pattern identical with that in lane 1 was observed, indicating the presence of the original supercoiled plasmid and the complete inhibition of DNA relaxation (results not shown). Since topoisomerase inhibitors can act by one of two mechanisms, i.e. by preventing the formation of the covalent hLI–AMP–DNA complex (topoisomerase antagonists) or by stabilizing the complex such that the re-ligation step is prevented (topoisomerase poisons), the absence of nicked circular DNA upon removal of the enzyme by solvent extraction would support the earlier mech-

Table 1 Evaluation of the potential of natural products to inhibit the ligation and adenylation reactions catalysed by hLI

Standard assay conditions as described in the Materials and methods section were used. Results are given as IC_{50} values [μg/ml (μM)]: IA, inactive ($IC_{50} > 200$ μg/ml).

	Ligation	Adenylation
Triterpenes		
Ursolic acid	100 (216)	120 (259)
Oleanolic acid	100 (216)	85 (184)
Aleuritolic acid	95 (205)	90 (194)
Betulinic acid	IA	IA
Benzophenanthridine alkaloids		
Fagaronine chloride	10 (27)	IA
Nitidine chloride	25 (69)	136 (374)
Chelerythrine chloride	75 (226)	150 (452)
Sanguinarine nitrate	127 (322)	190 (482)
Chelidonine	IA	IA
N-Demethylfagaronine	IA	IA
Protoberberine alkaloids		
Berberine chloride	IA	IA
Coptisine chloride	IA	IA
Tetrahydroberberine	IA	IA
Tetrahydropalmatine	IA	IA
Flavonoids		
Myricetin	28 (91)	169 (549)
Morin	68 (236)	180 (625)
(−)-Catechin	IA	IA
Apigenin	IA	IA
Flavanoxanthone		
Swertifrancheside	8 (11)	76 (105)
Iridoid		
Fulvoplumierin	87 (357)	88 (361)
Aliphatic γ-lactone		
Protolichesterinic acid	6 (20)	116 (387)

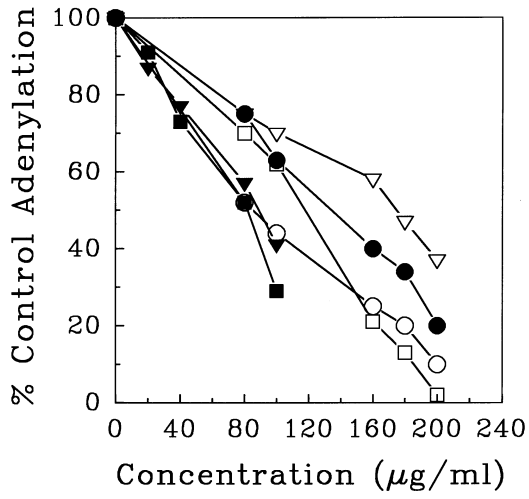

Figure 4 Effect of natural products on the adenylation of hLI

Recombinant hLI (0.5 μg) was incubated with 1.9 μM [2,5′,8-^{3}H]ATP (1 μCi) and test compound at 37 °C for 20 min. Radioactivity associated with the enzyme–adenylate complex was measured by using anion-exchange filters (DE-81) and a washing protocol with aq. 5% Na_2HPO_4. The indicated concentrations of compounds were added: oleanolic acid (○), nitidine chloride (●), myricetin (▽), fulvoplumierin (▼), protolichesterinic acid (□) and swertifrancheside (■).

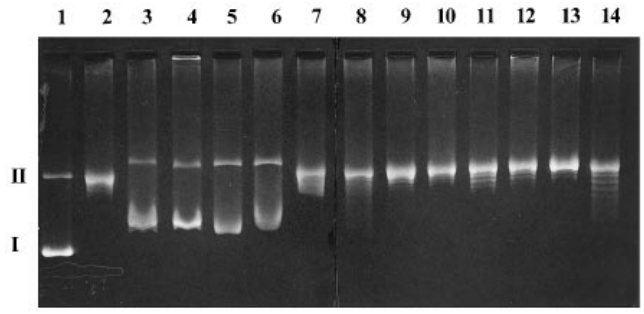

Figure 5 Effect of selected compounds on the DNA relaxation activity of hLI

Reaction mixtures (20 μl) were identical with those for DNA ligation and hLI adenylation, except that naturally supercoiled plasmid pBR322 (400 ng), AMP (1 mM) and hLI (1 μg) were also included. Test compounds (4 μl) were added to reaction mixtures before the addition of hLI. After 30 min at 37 °C, reactions were quenched by addition of 2 μl of a loading solution containing 1 mg/ml Bromophenol Blue, 50% (v/v) glycerol and 5 mg/ml SDS, and analysed on a 1.2%-agarose gel with 40 mM Tris/acetate/2 mM EDTA as running buffer. Gels were stained with ethidium bromide to reveal bands. Lane 1, pBR322 alone; lane 2, pBR322 incubated with hLI and AMP in the absence of added compounds; lanes 3–14, compounds (100 μg/ml) were added in the following respective order: fagaronine chloride, nitidine chloride, sanguinarine nitrate, chelerythrine chloride, berberine chloride, fulvoplumierin, myricetin, ursolic acid, oleanolic acid, aleuritolic acid, protolichesterinic acid and swertifrancheside. No solvent extraction was performed before loading. I, supercoiled substrate; II, nicked circular product.

anism for fagaronine chloride and nitidine chloride at the concentration tested. Neither the integrity of pBR322 nor its hLI-catalysed relaxation (Figure 5) was visibly affected by all the other compounds assayed. These results indicate that, although the same enzyme active site may be involved in both enzyme adenylation and DNA relaxation, inhibitors may exert allosteric effects by inducing conformational changes that disrupt only one of these activities. It is also noteworthy that, even though the protoberberine alkaloids (e.g. berberine chloride, lane 7) and swertifrancheside (lane 14) were reported to interact with nucleic acids and cause changes in the UV spectrum of DNA [30,35], no interference with pBR322 migration was observed with these compounds. In addition, it was reported that myricetin did not intercalate with pBR322 DNA, even at concentrations as high as 250 μM [39], although its ability to produce nicked circular DNA from covalently closed pGEM-1 has been described [32]. Myricetin has been reported to inhibit both DNA topoisomerase I and DNA topoisomerase II without stabilizing cleavage complexes [40]. Therefore, it is unclear whether the apparent resistance of the DNA relaxation activity of hLI to myricetin is a legitimate property of the enzyme or merely an artifact arising from the choice of plasmid used.

Adjacent 3′-OH and 5′-PO_4 functionalities in double-stranded DNA are required for the generation of a new phosphodiester bond. DNA–ligand interactions result in the distortion of the DNA duplex, thus preventing the proper positioning of the nick in the active site of the DNA ligase. This results in the inhibition of DNA ligase–AMP–Mg^{2+}–DNA complex-formation or the displacement reaction involving the attack of the 3′-OH terminus of the nick on the 5′-terminus bearing the AMP group. Although preincubation of the nucleic acid substrate with the DNA-binding molecule before the addition of hLI caused significant inhibition, it is not known whether assembled or active complexes are spared from the effects of drug binding to the substrate. Conceivably, the active-site conformation of the substrate-bound enzyme might physically hinder the approach of inhibitor molecules to the critical portion of the nucleic acid substrate. The fact that T4 DNA ligase is resistant to several intercalating anthracyclines lacking a free amino group on the 3′-position of the sugar [41], and the present finding that the activity of hLI is unaffected by protoberberine alkaloids, do not support an obligatory cause–effect relationship of nucleic acid interaction and the disruption of ligation activity. Currently, the understanding of the structure and function of hLI is not at a level that would permit the rationalization of this observation. Differences might also exist between poly(dA)·oligo$(dT)_{30}$ and DNA recognition. Therefore, substrate sequence may exert a profound effect on the inhibitory potential of these compounds.

In summary, as an attempt to discover novel inhibitors of hLI and to expand our knowledge on the structural and catalytic properties of this enzyme, we assayed the inhibitory activity of pure compounds derived from a diverse group of natural-product classes. Consequently, a number of natural-product hLI inhibitors were uncovered. Several unique mechanisms of action are represented, which serve to accentuate the magnitude of the task aimed at identifying pertinent structural features of both inhibitor and enzyme. Henceforth, enzyme-kinetic studies are warranted in order to probe deeper into the mode of inhibition mediated by these compounds. At present, it is unclear whether the observed hLI-inhibitory properties of some of these compounds are relevant to the modulation of their cytotoxic effect in cell culture. A similar approach to the study of T4 and *Escherichia coli* DNA ligases, in addition to all known mammalian DNA ligases from diverse sources, will provide further insight into the biochemistry and functional relationships within this class of enzymes.

We thank Dr. Kristín Ingolfsdóttir of the Department of Pharmacy, University of Iceland, Reykjavik, Iceland, for a sample of protolichesterinic acid, and Professor Yong-Long Liu of the Department of Phytochemistry, Institute of Medicinal Plant Development, Beijing, People's Republic of China, for a sample of swertifrancheside. This work was sponsored, in part, by grant U01-CA-52956 from the National Cancer Institute, NIH, Bethesda, MD, U.S.A.

REFERENCES

1 Johnston, L. H. and Nasmyth, K. A. (1978) Nature (London) **247**, 891–893
2 Fabre, F. and Roman, H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4586–4588
3 Tomkinson, A. E., Roberts, E., Daly, G., Totty, N. F. and Lindahl, T. (1991) J. Biol. Chem. **266**, 21728–21733
4 Lindahl, T. and Barnes, D. E. (1992) Annu. Rev. Biochem. **61**, 251–281
5 Wei, Y.-F., Robins, P., Carter, K., Caldecott, K., Pappin, D. J. C., Yu, G.-L., Wang, R.-P., Shell, B. K., Nash, R. A., Schär, P., Barnes, D. E., Haseltine, W. A. and Lindahl, T. (1995) Mol. Cell Biol. **15**, 3206–3216
6 Barnes, D. E., Johnston, L. H., Kodama, K., Tomkinson, A. E., Lasko, D. D. and Lindahl, T. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6679–6683
7 Teraoka, H. and Tsukada, K. (1982) J. Biol. Chem. **257**, 4758–4763
8 Tomkinson, A. E., Lasko, D. D., Daly, G. and Lindahl, T. (1990) J. Biol. Chem. **265**, 12611–12617
9 Tomkinson, A. E., Totty, N. F., Ginsburg, M. and Lindahl, T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 400–404
10 Rusquet, R. M., Feon, S. A. and David, J. C. (1988) Cancer Res. **48**, 4038–4044
11 Chan, J. Y.-H. and Becker, F. F. (1988) J. Biol. Chem. **263**, 18231–18235
12 Creissen, D. and Shall, S. (1982) Nature (London) **296**, 271–272
13 Li, J.-H. and Rossman, T. G. (1989) Mol. Toxicol. **2**, 1–9
14 Petrini, J. H. J., Huwiler, K. G. and Weaver, D. T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7615–7619
15 Black, D. J. and Livingston, R. B. (1990) Drugs **39**, 489–501
16 Carmichael, J. and Hickson, I. D. (1991) Int. J. Radiat. Oncol. Biol. Phys. **20**, 197–202
17 Eastman, A. and Schulte, N. (1988) Biochemistry **27**, 4730–4734
18 Masuda, H., Ozols, R. F., Lai, G.-M., Fojo, A., Rothenberg, M. and Hamilton, T. C. (1988) Cancer Res. **48**, 5713–5716
19 Yang, S.-W., Huang, P., Plunkett, W., Becker, F. F. and Chan, J. Y. H. (1992) J. Biol. Chem. **267**, 2345–2349
20 Söderhäll, S. (1975) Eur. J. Biochem. **51**, 129–136
21 Montecucco, A., Pedrali-Noy, G., Spadari, S. and Ciarrocchi, G. (1988) Biochim. Biophys. Acta **951**, 330–334

22 David, J.-C., Bassez, T., Bonhommet, M. and Rusquet, R. (1985) Cancer Res. **45**, 2177–2183
23 Tan, G. T., Pezzuto, J. M., Kinghorn, A. D. and Hughes, S. H. (1991) J. Nat. Prod. **54**, 143–154
24 Messmer, W. M., Tin-Wa, M., Fong, H. H. S., Bevelle, C., Farnsworth, N. R., Abraham, D. J. and Trojanek, J. (1972) J. Pharm. Sci. **61**, 1858–1859
25 Chauduri, S. K., Fullas, F., Brown, D. M., Wani, M. C., Wall, M. E., Cai, L., Mar, W., Lee, S.-K., Luo, Y., Zaw, K., Fong, H. H. S., Pezzuto, J. M. and Kinghorn, A. D. (1995) J. Nat. Prod. **58**, 1–9
26 Pezzuto, J. M., Antosiak, S. K., Messmer, W. M., Slaytor, M. B. and Honig, G. R. (1983) Chem.-Biol. Interact. **43**, 323–339
27 Sethi, M. L. (1981) Can. J. Pharm. Sci. **16**, 29–34
28 Tan, G. T., Kinghorn, A. D., Hughes, S. H. and Pezzuto, J. M. (1991) J. Biol. Chem. **35**, 23529–23536
29 Kondo, Y. (1976) Heterocycles **4**, 197–219
30 Krey, A. K. and Hahn, F. E. (1969) Science **166**, 755–757
31 Havsteen, B. (1983) Biochem. Pharmacol. **32**, 1141–1148
32 Shinozuka, K., Kikuchi, Y., Nishino, C., Mori, A. and Tawata, S. (1988) Experientia **44**, 882–885
33 Kakiuchi, N., Hattori, M., Namba, T., Nishizawa, M., Yamagishi, T. and Okuda, T. (1985) J. Nat. Prod. **48**, 614–621
34 Wang, J.-N., Hou, C.-Y., Liu, Y.-L., Lin, L.-Z., Gil, R. R. and Cordell, G. A. (1994) J. Nat. Prod. **57**, 211–217
35 Pengsuparp, T., Cai, L., Constant, H., Fong, H. H. S., Lin, L.-Z., Kinghorn, A. D., Pezzuto, J. M., Cordell, G. A., Ingolfsdóttir, K., Wagner, H. and Hughes, S. H. (1995) J. Nat. Prod. **58**, 1024–1031
36 Kardono, L. B. S., Tsauri, S., Padmawinata, K., Pezzuto, J. M. and Kinghorn, A. D. (1990) J. Nat. Prod. **53**, 1447–1455
37 Tan, G. T., Miller, J. F., Kinghorn, A. D., Hughes, S. H. and Pezzuto, J. M. (1992) Biochem. Biophys. Res. Commun. **185**, 370–378
38 Ingolfsdóttir, K., Breu, W., Huneck, S., Gudjonsdóttir, G. A., Müller-Jackic, B. and Wagner, H. (1994) Phytomedicine **1**, 167–170
39 Yamashita, Y., Kawada, S.-Z. and Nakano, H. (1990) Biochem. Pharmacol. **39**, 737–741
40 Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A. and Moon, R. (1995) J. Nat. Prod. **58**, 217–225
41 Montecucco, A., Pedrali-Noy, G., Spadari, S., Zanolin, E. and Ciarrocchi, G. (1988) Nucleic Acids Res. **16**, 3907–3918

Received 21 August 1995/10 November 1995; accepted 21 November 1995