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Differential induction of *Leishmania donovani* bi-subunit topoisomerase I–DNA cleavage complex by selected flavones and camptothecin: activity of flavones against camptothecin-resistant topoisomerase I

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ABSTRACT

Emergence of the bi-subunit topoisomerase I in the kinetoplastid family (*Trypanosoma* and *Leishmania*) has brought a new twist in topoisomerase research related to evolution, functional conservation and preferential sensitivities to the specific inhibitors of type IB topoisomerase family. In the present study, we describe that naturally occurring flavones baicalein, luteolin and quercetin are potent inhibitors of the recombinant *Leishmania donovani* topoisomerase I. These compounds bind to the free enzyme and also intercalate into the DNA at a very high concentration (300 μM) without binding to the minor grove. Here, we show that inhibition of topoisomerase I by these flavones is due to stabilization of topoisomerase I–DNA cleavage complexes, which subsequently inhibit the religation step. Their ability to stabilize the covalent topoisomerase I–DNA complex *in vitro* and in living cells is similar to that of the known topoisomerase I inhibitor camptothecin (CPT). However, in contrast to CPT, baicalein and luteolin failed to inhibit the religation step when the drugs were added to pre-formed enzyme substrate binary complex. This differential mechanism to induce the stabilization of cleavable complex with topoisomerase I and DNA by these selected flavones and CPT led us to investigate the effect of baicalein and luteolin on CPT-resistant mutant enzyme LdTOP1Δ39LS lacking 1–39 amino acids of the large subunit [B. B. Das, N. Sen, S. B. Dasgupta, A. Ganguly and H. K. Majumder (2005) *J. Biol. Chem.* 280, 16335–16344]. Baicalein and luteolin stabilize duplex oligonucleotide cleavage with LdTOP1Δ39LS. This observation was further supported by the stabilization of *in vivo* cleavable complex by baicalein and luteolin with highly CPT-resistant *L.donovani* strain. Taken together, our data suggest that the interacting amino acid residues of topoisomerase I may be partially overlapping or different for flavones and CPT. This study illuminates new properties of the flavones and provide additional insights into the ligand binding properties of *L.donovani* topoisomerase I.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes that govern the topology of DNA inside cells and are involved in vital cellular processes (1). All eukaryotic type IB topoisomerases are monomers and consist of four domains (2). Cleavage occurs by a *trans*-esterification reaction involving nucleophilic attack by an active tyrosine (Tyr^{723} in human Topo I) on a DNA phosphodiester bond resulting in the formation of a covalent DNA 3′-phosphotyrosyl linkage. In the religation phase a similar *trans*-esterification reaction involves attack by the free DNA 5′-hydroxyl that releases the enzyme from the DNA (2,3).

In contrast, *Leishmania donovani* topoisomerase I is an unusual bi-subunit enzyme where the core DNA binding domain and the catalytic domain harboring the consensus SKXXY motif lie in separate subunits. The two subunits are synthesized by two different genes, which associate

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with each other through protein–protein interaction to form an active heterodimeric topoisomerase I within the parasite. This unusual structure of DNA topoisomerase I in the kinetoplastid family may provide a missing link in the evolution of type IB enzyme (4–6).

Camptothecin (CPT), an important class of antitumor agent (7), represents the best characterized topoisomerase IB inhibitor. CPT binds reversibly to the covalent intermediate DNA-enzyme, stabilizing the cleavable complex and reducing the rate of religation (8,9). The stalled topoisomerase I collides with the progression of the replication fork producing lethal double-strand DNA breaks and causing cell death (10). Recently, important contribution toward the understanding of the interaction of CPT with topoisomerase I and DNA has been provided by the crystal 3D structure of the ternary complex between human topoisomerase I covalently linked to DNA and the CPT derivative topotecan (11). The structure shows that the drug intercalates into DNA duplex and moves the 5′-hydroxyl end of the DNA away from the scissile phosphate. This misalignment of the two ends probably slows the religation reaction (11).

We have previously demonstrated the *in vitro* reconstitution of bi-subunit topoisomerase I (LdTOP1LS) of *L.donovani* (5). Our recent findings reveal that 1–39 amino acid residues of the large subunit have a modulating role in non-covalent interaction with DNA and sensitivity to CPT, while the residues within 40 and 99 amino acid region of LdTOP1L appear to be important in relation to interaction with LdTOP1S (6). CPT enhances the formation of 'cleavable complex' at low salt (5). It induces cellular dysfunction in *L.donovani* promastigotes and amastigotes with features that are well characterized by several cytoplasmic and nuclear events of apoptosis (12,13). In *Trypanosoma brucei*, *Trypanosoma cruzi* and *L.donovani*, CPT promotes protein–DNA complex formation with nuclear as well as kinetoplast DNA (14). Recently, highly CPT-resistant *L.donovani* strain (LdRCPT.160) was developed by stepwise exposure to CPT that induces point mutations (Gly 185 Arg and Asp325 Glu) in the large subunit (LdTOP1L) of the bi-subunit topoisomerase I (15).

Flavonoids are a diverse group of naturally occurring polyphenolic compounds with profound pharmacological properties (16). They are reported to have anti-viral (17), anti-cancer (18) and anti-parasitic (19) activities. Among naturally occurring flavonoids, quercetin and luteolin are most widely studied *in vitro* and *in vivo*. (19). Baicalein (5, 6, 7 trihydroxy flavone), another important flavonoid, has multiple biological activities (20–23) and is also reported as topoisomerase II inhibitor (22).

We have previously established that luteolin and quercetin inhibit topoisomerase II and induce apoptotic death of *L.donovani* promastigotes (19). While much attention has been placed on the study of topoisomerase II inhibition by polyphenolic compounds, less attention has centered on topoisomerase I inhibition. Thus, further studies are needed to understand the molecular mechanism of interaction of the selected flavonoids with DNA topoisomerase I.

In the present study, we have dissected the mechanism of action of flavones and CPT. Though the selected flavones, baicalein, luteolin and quercetin stabilize topoisomerase I–DNA cleavable complex like CPT, the interaction of the flavones and CPT appears to be different during the formation of the cleavable complex. This interpretation is well supported by our study with CPT-resistant mutant enzyme and CPT-resistant *L.donovani* promastigotes cells. Thus, our studies provide insights into the flavone-mediated inhibition of *L.donovani* topoisomerase I.

MATERIALS AND METHODS

Materials

Baicalein (5,6,7 trihydroxyflavone) $C_{15}H_{10}O_5$, melting point 260°C, used in the study is isolated from the stem-bark of *Oroxylum indicum*. The structure of the compound was ascertained by spectroscopic analysis and by super imposable infrared (IR) spectra and undepressed mixed melting point with authentic samples (19). Dihydrobetulinic acid (DHBA) was purified as described previously (24). CPT, luteolin and quercetin were purchased from Sigma Chemicals (St Louis, MO). The drugs were dissolved in 100% dimethyl sulfoxide (DMSO) at 20 mM concentration and stored at −20°C.

Purification of recombinant proteins and reconstitution of topoisomerase I activity

Escherichia coli BL21(DE3)pLysS cells harboring *pET16bLdTOP1L*, *pET16bLdTOP1Δ39L* and *pET16bLdTOP1S* described previously (6) were separately induced at $OD_{600} = 0.6$ with 0.5 mM Isopropyl-β-D-thiogalactopyranoside at 22°C for 12 h. Cells harvested from 1 L of culture were separately lysed by lysozyme/sonication and the proteins were purified through Ni^{+2}-NTA agarose column (Qiagen) followed by phosphocellulose column (P11 cellulose, Whatman) as described previously (14). Finally, the purified proteins LdTOP1L, LdTOP1Δ39L and LdTOP1S were stored at −70°C. *E.coli* BL21 (DE3) cells harboring *pGEX-GST-LdTOP1S* were over-expressed, solubilized and purified through glutathione *S*-transferase (GST)–Sepharose column as described previously (14). The concentrations of purified proteins were quantified by Bradford reaction using a Bio-Rad Protein Estimation Kit according to the manufacturer's protocol.

Purified LdTOP1L or LdTOP1Δ39L were mixed with purified LdTOP1S separately at a molar ratio of 1:1 and at a total protein concentration of 0.5 mg/ml in reconstitution buffer (50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM phenylmethlysulfonyl fluoride and 10% glycerol). The mixtures were dialyzed overnight at 4°C and the dialyzed fractions were used for plasmid relaxation activity (5,6).

Plasmid relaxation assay

The type I DNA topoisomerase was assayed by decreased mobility of the relaxed isomers of supercoiled pBluescript (SK^+) DNA in an agarose gel. Relaxation assay was carried out as described previously (5,6) with LdTOP1LS, and LdTOP1Δ39LS serially diluted in the relaxation buffer (25 mMTris–HCl, pH 7.5, 5% glycerol, 0.5 mM DTT, 10 mM $MgCl_2$, 2.5 mM EDTA and 150 μg/ml BSA), supercoiled pBluscript (SK^+) DNA and 50 mM KCl. The amount of supercoiled monomer DNA band florescence after ethidium bromide (EtBr) (0.5 μg/ml) staining was quantitated by using

Gel Doc 2000 under UV illumination (Bio Rad-Quality one software).

Analysis of duplex oligonucleotides cleavage assay

The 25mer duplex of oligonucleotide 1 (5′-GAAAAAA-GACTT↓AGAAAAATTTTTA-3′) and oligonucleotide 2 (5′-TAAAAATTTTTCTAAGTCTTTTTTC-3′) containing a topoisomerase I binding motif was labeled and annealed as described previously (6). Cleavage was carried out using 20-fold molar excess of the wild-type (LdTOP1LS) and mutant enzymes (LdTOP1Δ39LS) over duplex 25mer DNA (enzymes, 0.2 μM; DNA, 10 nM). The reactions were carried out in standard assay mixture containing 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EDTA and 50 mM KCl in the presence or absence of drugs (60 μM) at 37°C for 30 min or at indicated time periods. All the reactions were stopped by addition of SDS to a final concentration of 2% (w/v). Samples were precipitated with ethanol, digested with 5 μl of 1 mg/ml trypsin and analyzed by 12% denaturing polyacrylamide gel followed by autoradiography. The amount of strand cleavage in the presence of drugs for the wild-type and mutant enzymes were determined by film densitometry as described previously (6).

Fluorescence binding assay

Fluorescence titrations were performed using a F-3010 spectrofluorometer (HITACHI/JAPAN). The intrinsic binding of the flavones to DNA and the enzyme (LdTOP1LS) was performed in different experiments. The fluorescence measurements were performed separately at an excitation wavelength of 364 nm for baicalein, 380 nm for luteolin and 370 nm for quercetin and emission range of 450–600 nm. Excitation and emission slit widths were 10 and 15 nm, respectively.Background emission (<2%) was corrected by subtraction of spectra of blank buffer and enzyme from DNA plus sample buffer and flavones plus enzyme samples respectively. Spectral titration was performed with flavones at 25°C in fluorescence buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl and 10 mM $MgCl_2$). DNA or topoisomerase I was added in increasing concentrations as indicated in the legend. All the assays were performed in duplicate; titration points are corrected as described above; and binding constants for flavones-LdTOP1LS interaction were determined according to following equation (25):

$$1/\Delta F = 1/\Delta F_{max} + (1/K_a.S_t)(1/\Delta F_{max}) \quad \mathbf{1}$$

where $\Delta F = F_x - F_o$, F_x and F_o representing the florescence intensity of the selected flavones in the presence or absence of the added total LdTOP1LS (S_t), respectively. ΔF_{max} is the maximum change in the fluorescence intensity. The intercept of the plots on the $1/F$ axis measures the $1/F_{max}$, while the slope gives the estimation of K_a. Dissociation constant $K_d = 1/K_a$.

Analysis of flavones–DNA intercalation

The intercalation between baicalein, luteolin and quercetin was assessed by two independent techniques. First, the ability of the drug to intercalate into plasmid DNA was determined by topoisomerase I unwinding assay (26,27). Assays were performed with 50 fmol of pBluscript (SK^+) DNA in the presence or absence of baicalein, luteolin, quercetin, *m*-AMSA and etoposide in a 50 μl of reaction mixture as described above. Relaxed DNA was prepared by treatment of the supercoiled plasmid DNA with excess of topoisomerase I, followed by proteinase K digestion at 37°C, phenol/chloroform extraction and ethanol precipitation. After incubation at 37°C for 15 min, reactions were terminated by the addition of prewarmed stop solution [5% SDS, 15% (w/v) Ficoll and 0.25% Bromophenol Blue] and electrophoresed on to 1% agarose gel as described above. The DNA band was stained with 0.5 μg/ml of EtBr and visualized by UV light as described above.

Second, an ethidium-displacement fluorescence assay (28) was employed to determine whether the selected flavone binds in the minor groove of DNA. Fluorescence emission spectra (λ_{max} =590 nm, excitation wavelength 510 nm) were obtained at 25°C. The assays contained 1 μM EtBr, 0–300 μM of the selected flavones and 5 nM calf thymus (CT) DNA in 2 ml of fluorescence buffer.

Single turnover cleavage and religation experiment

A 14mer (5′-GAAAAAAGACTT↓AG-3′) oligonucleotide containing topoisomerase IB specific cleavage site was 5′-^{32}P-end labeled and annealed to 25mer (3′-CTTTTTTCTGAATCTTTTTAAAAAT *p*-5′) oligonucleotide as described previously (6). The suicidal cleavage reaction was carried out with 5 nM of DNA substrate and 0.15 μM of enzyme (LdTOP1LS) in 20 μl reaction mixture under standard assay condition (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EDTA and 50 mM KCl) at 23°C for 4 h in the presence or absence of drugs as described previously (6).

For religation experiments, covalent complexes generated by incubating suicide DNA substrate with LdTOP1LS in the presence or absence of drugs were transferred to 37°C and pre-incubated for 2 min. Religation was initiated by the addition of 300-fold molar excess of the 11mer religation acceptor oligonucleotides (5′-OH-AGAAAAATTTT-3′) in the same reaction mixture and incubated for indicated time periods. Finally, all the reactions were stopped by adding SDS, and DNAs were subsequently precipitated by ethanol. Samples were digested with 5 μl of 1mg/ml trypsin, electrophoresed in 12% denaturing polyacrylamide gel and autoradiographed as described previously (6).

Parasite, culture condition and development of CPT-resistant *L.donovani* strain

L.donovani AG83 promastigotes were grown at 22°C in Rays modified media as described previously (19,24) and M199 liquid media supplemented with 10% fetal calf serum (FCS).

A highly resistant *L.donovani* strain Ld160CPT^R was selected by stepwise exposure to CPT (5, 10, 40, 80, 100 and 160 μM) as described previously (15). The CPT-resistant strain reached a resistance level of 32-fold over the wild-type *L.donovani* AG83 strain.

Cytotoxicity of the drugs was estimated by microscopic counting of viable parasites by trypan blue exclusion method after treatment of *L.donovani* promastigotes with drugs as described previously (24).

Formation of covalent DNA–protein complexes in *Leishmania* promastigotes

DNA–protein complexes can be trapped within cells and quantified by the KCl-SDS coprecipitation assay (29). Exponentially growing *L.donovani* promastigotes (5×10^6 cells/ml) or CPT-resistant *L.donovani* strain Ld160CPT^R were radiolabeled by adding [methyl-^{3}H]thymidine (Amersham) into the medium to a final concentration of 5 μCi/ml for 24 h at 22°C. Cells were then pelleted, washed twice and resuspended in fresh M199 liquid media supplemented with 10% FCS for 3 h. Cells were then exposed to various concentrations of baicalein, luteolin, quercetin, CPT and DHBA at 22°C for indicated time periods. Finally, the cells were lysed in 200 μl of SDS-containing solution [1.25% SDS, 5 mM EDTA (pH 8) and CT DNA (0.4 mg/ml)] prewarmed at 65°C. The lysates were transferred into 1.5 ml microfuge tubes containing 250 μl of 325 mM KCl. After vigorous mixing, the samples were cooled on ice for 10 min and centrifuged. The pellets were resuspended in 500 μl of wash solution [10 mM Tris–HCL, pH 8, 100 mM KCl, 1 mM EDTA and CT DNA (0.1 mg/ml)] and warmed at 65°C for 10 min with occasional shaking. The suspensions were cooled on ice for 10 min and re-centrifuged. The pellets were washed as above, mixed with 4 ml of scintillation liquifluor (Spectrochem, India) and radioactivity was determined in the liquid scintillation counter.

RESULTS

Effect of flavones on the DNA relaxation activity of reconstituted LdTOP1LS

Purification of recombinant subunits (LdTOP1L and LdTOP1S) and reconstitution of enzyme (LdTOP1LS) activity was examined by plasmid DNA relaxation assay as described previously (5,6). We have shown earlier that the parasite-reconstituted enzyme is active at low salt and the optimum activity is at 50 mM KCl and 10 mM Mg^{+2} concentrations (5). Thus, the subsequent relaxation experiments were performed at the above-mentioned conditions.

To investigate the effect of the selected flavones (Figure 1A) on *in vitro* plasmid DNA relaxation activity of the recombinant *Leishmania* topoisomerase I, relaxation experiments were performed in a standard assay mixture where the plasmid DNA and the enzyme (LdTOP1LS) were mixed at a molar ratio of 3:1. Luteolin, quercetin and baicalein inhibit LdTOP1LS relaxation activity compared with the enzyme control (Figure 1B). Under same condition LdTOP1LS is also inhibited by CPT (Figure 1B) as observed previously (6). Thus, in the light of our experiment flavones are also potential inhibitors of topoisomerase I like CPT.

The selected flavones (baicalein, luteolin and quercetin) by themselves do not relax DNA. To test this, supercoiled pBluescript (SK$^+$) DNA was incubated separately with 50 μM each of the flavones. No relaxation and no apparent DNA conformational changes took place because of the unwinding of DNA (data not shown).

To investigate the interaction of the selected flavones with the enzyme in the relaxation experiment, LdTOP1LS was pre-incubated separately with luteolin, quercetin and baicalein at different concentrations for 5 min at 37°C before the addition of DNA. The inhibition of the enzyme by pre-incubation with the compounds was compared with the inhibitory effects of the compounds incubated simultaneously with enzyme (LdTOP1LS) and supercoiled DNA in the relaxation assay. Interestingly, under pre-incubation condition luteolin, quercetin and baicalein showed ~85–90% of enzyme inhibition at 5, 15 and 3 μM concentrations, respectively (Figure 1C) with IC_{50} values of 2.5, 5.5 and 1.5 μM, respectively (Figure 1E), whereas under simultaneous condition similar inhibition was achieved at 20, 30 and 15 μM of luteolin, quercetin and baicalein, respectively (Figure 1B), with IC_{50} values of 14, 17 and 9 μM, respectively (Figure 1D). But CPT does not show any enhanced rate of inhibition when pre-incubated with the enzyme (compare lane 20 of Figure 1C with lane 18 of Figure 1B). Thus the flavones probably interact with the enzyme (LdTOP1LS) and enhance the inhibitory effect in relaxation reaction.

Flavones stabilize topoisomerase I–DNA complexes

In order to investigate the mechanism of inhibition of topoisomerase I of *L.donovani* by these flavones, *trans*-esterification was examined under equilibrium condition by reacting LdTOP1LS with 25mer duplex oligonucleotide in the presence of 60 μM baicalein, luteolin and quercetin. The cleavage experiment was carried out with 5′-^{32}P-end labeled 25mer duplex oligonucleotide containing a topoisomerase IB specific binding motif as described in Materials and Methods.

Baicalein, luteolin and quercetin enhanced cleavage by ~30–36% with respect to the extent of cleavable complex formed without the drug (Figure 2A, compare lane 2 with lane 5). These results indicate that these selected flavones stabilize the covalent complex formed between 25mer duplex DNA and LdTOP1LS, and correlate with the reduction of relaxation activity in the presence of the flavones.

To investigate the rate of stabilization of cleavable complexes by baicalein, luteolin and quercetin we compared it with that of CPT. Time course cleavage experiments were performed in a standard assay mixture where the 5′-^{32}P-end labeled 25mer duplex oligonucleotide and the enzyme (LdTOP1LS) were mixed at a molar ratio of 1:20 in the presence of the drugs. The stabilization of cleavable complexes was determined by the percentage of substrate converted to products and was plotted as a function of time (6). With baicalein and luteolin, ~60% of the input DNA was cleaved and become covalently bound to the protein. The reaction reached its cleavage plateau after 60 min of incubation, whereas CPT completed the reaction after 35 min of incubation producing 70% of the cleavage product. Quercetin shows a reduced rate of stabilization of cleavable complex and reached the cleavage plateau approximately after 90 min (Figure 2B). This observation indicates that the rates of stabilization of cleavable complex by the flavones were ~2- to 3-fold reduced compared with that of CPT.

Flavones stabilize *in vivo* DNA–protein complexes

In *T.brucei*, *T.cruzi* and *L.donovani*, CPT promotes protein–DNA complex formation with nuclear as well as kinetoplast DNA (14). The ability of baicalein and other related flavones to induce covalent complexes of topoisomerases and DNA in the *L.donovani* promastigotes were quantified by KCl-SDS precipitation assay (29). Experiments were performed with

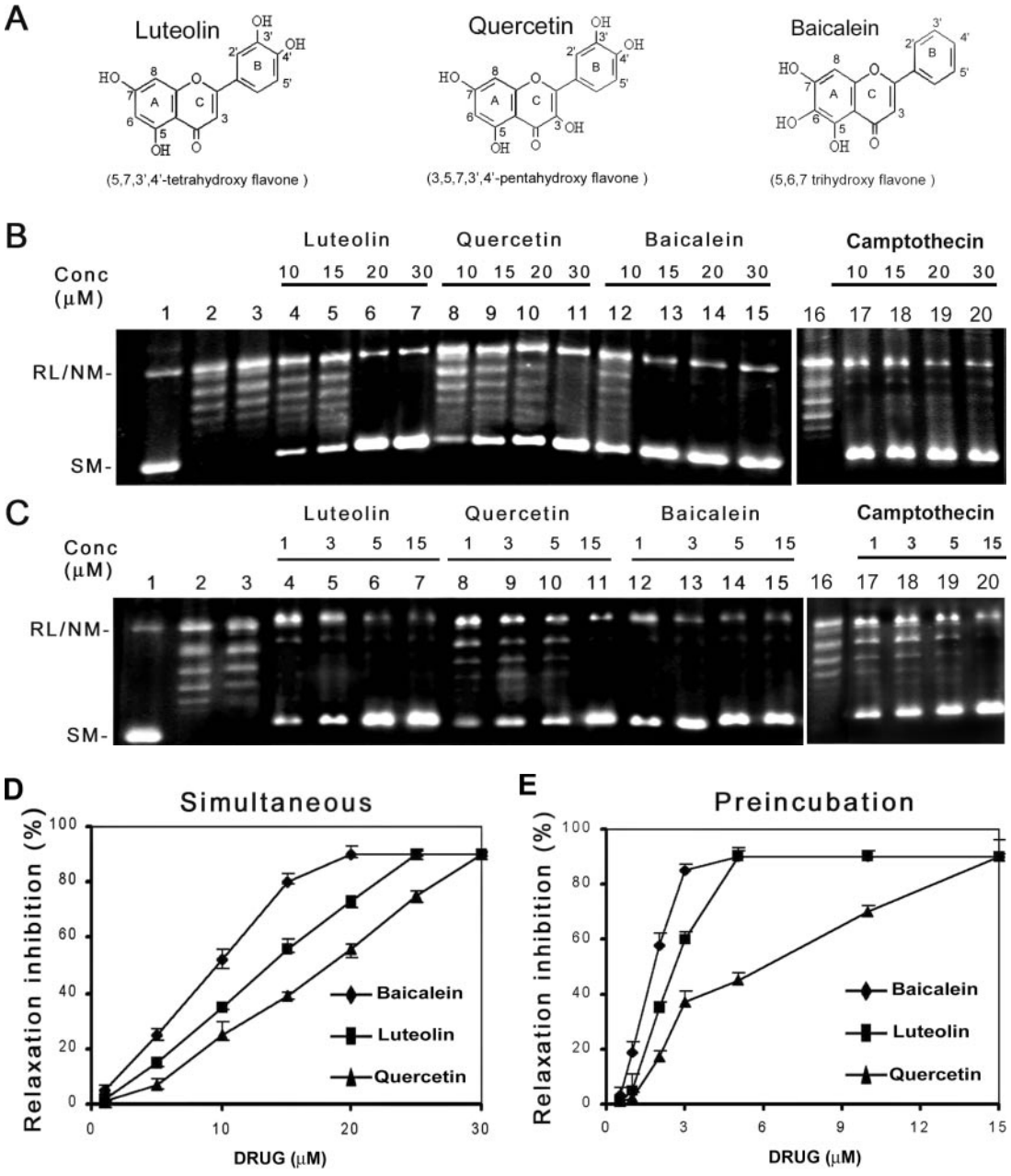

Figure 1. Inhibition of *L.donovani* topoisomerase I activity by flavones. (**A**) Structure of the flavones. (**B**) Relaxation of supercoiled pBS (SK^+) DNA with reconstituted LdTOP1LS at a molar ratio of 3:1. Lane 1, 90 fmol of pBS (SK^+) DNA; lanes 2 and 16, same as lane 1, but simultaneously incubated with 30 fmol of LdTOP1LS; lane 3, same as lane 2, but with 4% DMSO, respectively. Lanes 4–15 and 17–20, same as lane 2 and 16, but in the presence of 10, 15, 20 and 30 μM of luteolin, quercetin, baicalein and CPT simultaneously incubated with pBS (SK^+) DNA and LdTOP1LS at 37°C for 15 min. (**C**) Pre-incubation of LdTOP1LS with the selected flavones and CPT followed by addition of DNA. Lane 1, 90 fmol of pBS (SK^+) DNA; lanes 2, 3 and 16, same as lane 1 but DNA was added after pre-incubation of 30 fmol LdTOP1LS with reaction buffer and with 4% DMSO, respectively, for 5 min at 37°C. Lanes 4–15 and 17–20 same as lane 2 and 16, but the enzyme was pre-incubated with 1, 3, 5 and 15 μM of luteolin, quercetin, baicalein and CPT, respectively, as indicated. Reactions were stopped by addition of SDS to a final concentration of 0.5% (w/v) and were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM), relaxed and nicked monomer (RL/NM) are indicated. (**D**) Quantitative representation of enzyme inhibition in the presence of flavones in relaxation experiments. LdTOP1LS (100 fmol) was incubated with 1, 5, 10, 15, 20, 25, 30 μM concentrations of baicalein, luteolin and quercetin simultaneously with pBS (SK^+) DNA for 15 min at 37°C. (**E**) LdTOP1LS was pre-incubated separately with 0.5, 1, 2, 3, 5, 10, 15 μM concentrations of baicalein, luteolin and quercetin for 5 min at 37°C in relaxation buffer followed by addition of pBS (SK^+) DNA and further incubated for 15 min at 37°C. Reactions were stopped and electrophoresed as described above. The percentage of relaxation inhibition is plotted as a function of drug concentrations as indicated. All the experiments were performed three times and representative data from one set of these experiments are expressed as means ± SD. Variations among different set of experiments were <5%.

[^{3}H]thymidine labeled promastigotes, treated with different concentrations of baicalein, luteolin, quercetin and camptothecin. The data are summarized in Figure 3. Treatment of the cells with 10, 50, 100, 150 and 200 μM of baicalein, luteolin and quercetin for 5 h significantly increase the SDS-K^+ precipitable complex compared with the untreated control cells (Figure 3). The extent of SDS-K^+ precipitable complex was similar to that obtained by 150 μM of CPT for 5 h

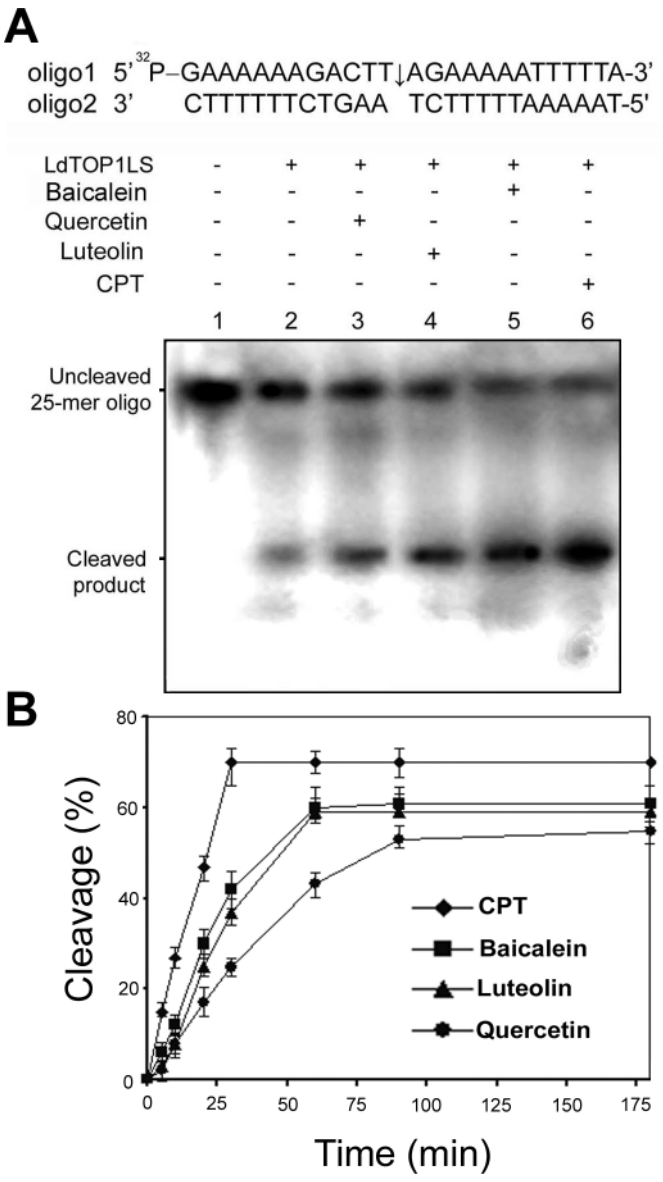

Figure 2. Comparisons of topoisomerase I-mediated DNA cleavage induced by flavones and CPT. (**A**) Cleavage reactions and electrophoresis in a denaturating polyacrylamide gel were performed as described in Materials and Methods. Lane 1, 10 nM of 5′-^{32}P-end labeled 25mer duplex oligonucleotides as indicated above. Lane 2, same as lane 1, but incubated with 0.2 μM of LdTOP1LS in the absence of inhibitors. Lanes 3–6, same as lane 2, but incubated with 60 μM of quercetin, luteolin, baicalein and CPT, respectively, for 60 min at 23°C. Positions of uncleaved oligonucleotide (25mer) and the cleavage product (12mer oligonucleotide complexed with residual topo I) are indicated. (**B**) Percentage of cleavable complex with LdTOP1LS and 5′-^{32}P-25mer duplex oligonucleotide DNA substrate, incubated for 5, 10, 20, 30, 60, 90, 180 min at 23°C in cleavage buffer containing 60 μM of baicalein, luteolin, quercetin and CPT separately. All the reactions were stopped by addition of SDS to a final concentration of 2% (w/v). Samples were precipitated with ethanol, digested with trypsin and analyzed by 12% denaturing polyacrylamide gel as described in Materials and Methods. The percentage of cleaved DNA substrate was plotted as a function of time. The results depicted were performed three times and representative data from one set of these experiments are expressed as means ± SD. Variations among different set of experiments were <6%.

(Figure 3). On the contrary, DHBA, a pentacyclic triterpenoid and a catalytic inhibitor of both topoisomerases I and II that antagonizes CPT-induced cleavage (24), did not induce the

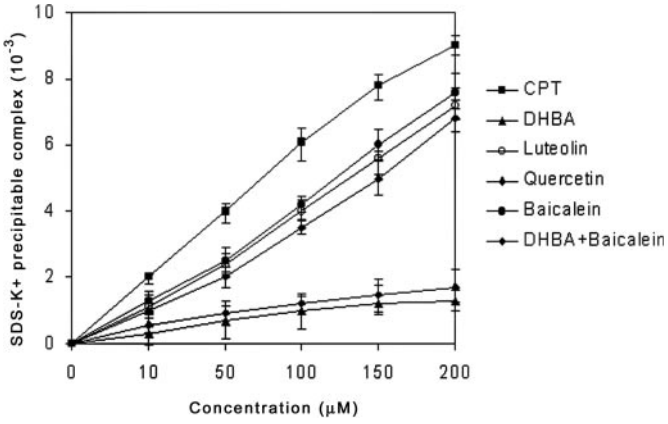

Figure 3. Analysis of drug induced covalent topoisomerase I–DNA complex formation in *L.donovani* promastigotes by KCl-SDS precipitation assay. Exponentially growing *L.donovani* promastigotes (5×10^6 cells /ml) were labeled with [^{3}H]thymidine at 22°C for 24 h and then treated with different concentrations of drugs as indicated. Parts of the labeled cells were treated with DHBA (150 μM) for 10 min before the addition of different concentration of baicalein as indicated. SDS-K$^+$ precipitable complex were measured as described in Materials and Methods. Experiments were performed three times and representative data from one set of experiments are expressed as means ± SD. Variations among different set of experiments were <6%.

formation of SDS-K$^+$ precipitable complex, when *L.donovani* cells were incubated for 3 h with the compound (Figure 3). To prove that the SDS-K$^+$ precipitable complexes induced by the selected flavones are due to topoisomerase–DNA links, the cells were pretreated with DHBA before incubation with baicalein. The SDS-K$^+$ precipitable complex formation induced by baicalein was clearly decreased when the cells were pretreated with 150 μM DHBA. These results establish that the SDS-K$^+$ precipitable complex is due to the formation of covalent complexes between topoisomerases and DNA and not due to any other protein cross-linked to DNA. Thus, we can surmise that stabilization of LdTOP1LS-mediated duplex oligonucleotide DNA cleavage with baicalein, luteolin and quercetin correlates with the protein-linked DNA breaks in *L.donovani* promastigotes and drug-induced cytotoxicity.

Selected flavones are acting reversibly against the enzyme

The selected flavones (baicalein, luteolin and quercetin) are potent inhibitors of LdTOP1LS. Pre-incubation experiments suggest that the compounds interact with the enzyme (Figure 1C), but it is not clear whether these compounds are acting reversibly or irreversibly against the enzyme. This critical matter has been sorted out by dilution experiment. Reconstituted LdTOP1LS was pre-incubated with 3, 5 and 15 μM of baicalein, luteolin and quercetin, respectively (Figure 4A, lanes 4–6), the concentration at which 85–90% inhibitions is achieved, before the addition of DNA. The reaction mixtures were subsequently diluted 10-fold so the final drugs concentrations become 0.3, 0.5 and 1.5 μM of baicalein, luteolin and quercetin, respectively. Dilution study shows complete relief of inhibition (Figure 4A, lanes 7–9). Drug-control reaction, i.e. inhibition study with 0.3, 0.5 and 1.5 μM of the respective drugs, showed the expected pattern of inhibition (Figure 4A, lanes 10–12). Thus, the relief of inhibition on dilution suggests that the selected flavones are acting reversibly against topoisomerase I.

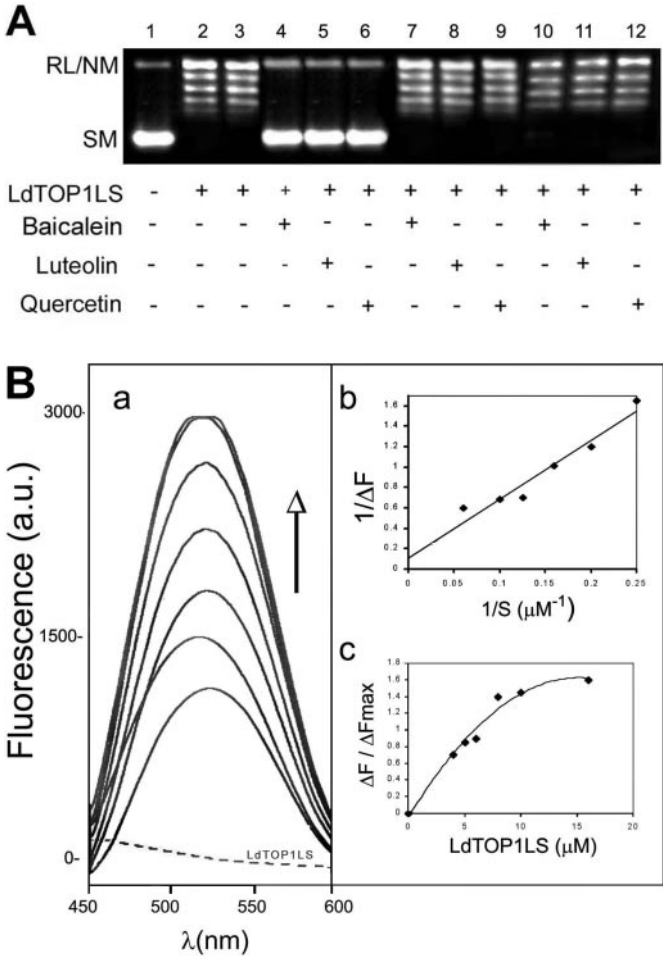

Figure 4. Analysis of flavones-LdTOP1LS interaction (**A**) Lane 1, 50 fmol of pBS (SK^+) DNA. Lane 2, LdTOP1LS (100 fmol) incubated at 37°C for 5 min in the reaction mixture before addition of pBS (SK^+) DNA; lane 3, same as lane 2. Lanes 4–6, same as lane 2 but in the presence of 3, 5 and 15 μM of baicalein, luteolin and quercetin, respectively, pre-incubated with LdTOP1LS for 5 min at 37°C in relaxation buffer followed by addition of 50 fmol of pBS (SK^+) DNA and further incubated for 10 min at 37°C. Lanes 7–9, same as lanes 4–6 but diluted 10-fold so that the final drug concentrations became 0.3, 0.5 and 1.5 μM of baicalein, luteolin and quercetin, respectively. These were followed by addition of pBS (SK^+) DNA and further incubated for 10 min at 37°C. Lanes 10–12, same as lane 2 but the enzyme was pre-incubated with 0.3, 0.5 and 1.5 μM of baicalein, luteolin and quercetin before addition of DNA. (**B**) Fluorescence study of luteolin–enzyme interaction. (a) Fluorescence emission spectra ($\lambda_{excitation} = 380$ nm) of 50 μM luteolin were determined as a function of topoisomerase I (LdTOP1LS) concentration in the range of 0–20 μM. Control fluorescence spectrum of LdTOP1LS is shown. Arrow indicates the change in the emission spectra of luteolin on addition of LdTOP1LS. (b) Shows a plot of the fractional fluorescence intensities of luteolin as a function of inverse topoisomerase I concentration. (c) A plot of $\Delta F/\Delta F_{max}$ versus increasing concentration of LdTOP1LS.

Fluorescence spectroscopy was used to check directly the binding of luteolin, baicalein and quercetin to the enzyme (LdTOP1LS). Addition of enzyme to luteolin led to an increase in the fluorescence intensity with slight shift in the fluorescence maxima (λ_{max}) from 525 to 520 nm (Figure 4B, panel a). A plot of fractional fluorescence intensity as a function of inverse topoisomerase concentrations is shown in Figure 4B (panel b). A plot of $\Delta F/\Delta F_{max}$ versus concentration of LdTOP1LS was found to be hyperbolic, indicating the formation of a drug–protein complex (Figure 4B, panel c). The dissociation constant (K_d) calculated from the double reciprocal plot using Equation 1 for luteolin-LdTOP1LS is 4.6×10^{-5} M. The dissociation constant for baicalein and quercetin were also calculated and were 6.5×10^{-5} M and 5.2×10^{-5} M, respectively. These results provide that the binding site of luteolin is located in the hydrophobic region of the protein, because the shift of the λ_{max} to the shot wave length means the fluoropohore was located in the hydrophobic environment (30). Thus, the fluorescence titration data reveal that these selected flavones interact with free enzyme and perhaps explain for stronger inhibition in the enzyme–drug pre-incubation experiments.

Analysis of flavones–DNA interaction

In order to investigate the interaction of baicalein and quercetin with DNA we used florescence titration with CT DNA. Free baicalein when excited at 364 nm has a fluorescence emission maximum at 540 nm. Addition of CT DNA causes slight shift of maximum peak from 540 to 537.6 nm (Figure 5A). A progressive change in the fluorescence spectra of baicalein on addition of different concentrations of DNA indicates an association between them. The dissociation constant (K_d) calculated from the values using Scatchard analysis (31) for baicalein is 3×10^{-5} M. The K_d of quercetin–DNA complex was calculated as described above and is 4.8×10^{-5} M.

Many chemical compounds that alter the gross structure of DNA either by intercalation or by minor groove binding have dramatic effect on the activity of topoisomerase I (32). Our fluorescence titration data reveal that flavones interact with the substrate DNA. Therefore, it is possible that flavones inhibit topoisomerase I by one of the two mechanisms. Two approaches were followed to answer this question.

First, the ability of the flavones to intercalate into DNA was determined by topoisomerase I-catalyzed unwinding assay (Figure 5B), which is based on the ability of intercalating compounds to unwind the DNA duplex and thereby change the DNA twist (22,26,27). The relaxed DNA was prepared as described in Materials and Methods. Briefly, the supercoiled pBluescript (SK^+) DNA was treated with excess of topoisomerase I so that no supercoiled DNA was left in the reaction mixture. The DNA substrate was purified topological isomers of pBluescript (SK^+) DNA and used as substrate for unwinding assay. In the presence of strong intercalative drug such as *m*-AMSA, a net negative supercoiling of the relaxed substrate DNA was induced at 50 and 300 μM concentration (Figure 5B, lanes 3 and 4). Conversely, no unwinding was observed with non-intercalative drug etoposide at 50 and 300 μM concentrations (Figure 5B, lanes 5 and 6). Baicalein, luteolin and quercetin at 50 and 100 μM concentrations had no effect on the topological state of the DNA (Figure 5B, lanes 7, 8, 10, 11 and lanes 13 and 14, respectively). However, at 300 μM concentration luteolin, quercetin and baicalein induce negative supercoiling like *m*-AMSA (Figure 5B, lanes 9, 12 and 15, respectively). Thus, the selected flavones intercalate into DNA at very high concentration, which is way beyond the inhibitory concentration for the recombinant *L.donovani* topoisomerase I.

Second, the ability of the selected flavones to displace EtBr from DNA was determined by a fluorescence emission assay. The DNA-bound form of ethidium has a significantly stronger fluorescence emission than does ethidium; thus, displacement

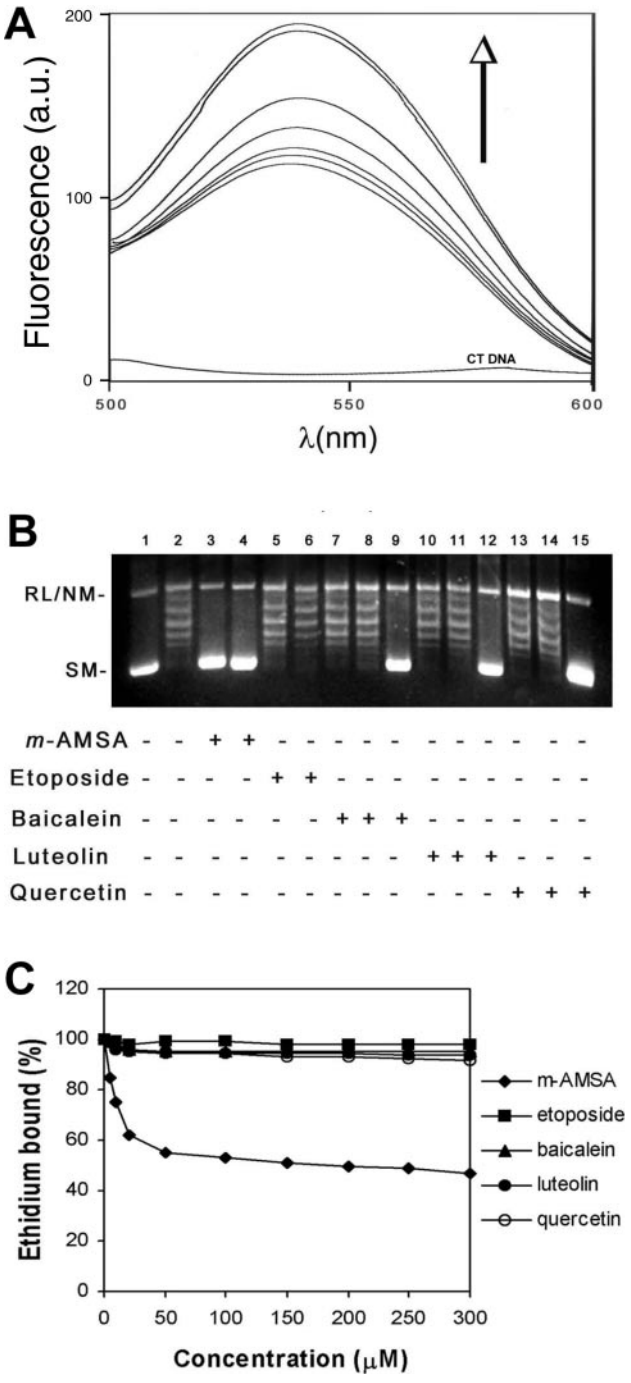

Figure 5. Study of flavone–DNA interaction. (**A**) A change in the emission spectra ($\lambda_{excitation}$ = 364 nm) of 50 μM baicalein was determined as a function of CT DNA concentration in the range of 0–0.22 mM. Arrow indicates the change in the emission spectra of baicalein on addition of CT DNA. Control fluorescence spectrum of CT DNA is indicated. (**B**) Flavones–DNA interaction as studied by agarose gel electrophoresis. Lane 2, relaxed pBS (SK^+) DNA generated by treatment of pBS (SK^+) DNA (lane 1) with excess of topoisomerase I, followed by phenol–chloroform extraction and ethanol precipitation. Lanes 3–6, same as lane 2, but incubated with 50 and 300 μM of *m*-AMSA and etoposide, respectively, as indicated. Lanes 7–15, same as lane 2, but incubated with 50, 100, 300 μM of baicalein, luteolin and quercetin as indicated. (**C**) Fluorescence-based ethidium bromide displacement assay. Samples contained 1 μM of EtBr and 5 nM CT DNA. Increasing concentration (0–300 μM) of baicalein, luteolin, quercetin, *m*-AMSA and etoposide were added as indicated. EtBr fluorescence was monitored with excitation wavelength at 510 nm and emission at 590 nm.

of ethidium from DNA can be monitored by a decrease in fluorescence signal (28). Moreover, since EtBr intercalates to minor groove of DNA, the assay is capable of detecting drugs that either intercalate or bind in the minor groove of the DNA.

As shown in Figure 5C, the intercalative drug *m*-AMSA at 50 μM concentration readily dislodged the bound fluorophore, whereas non-intercalative drug etoposide was unable to do so. Quercetin and luteolin, the weak intercalators, cause ~6% displacements at a concentration of 300 μM. Similarly, baicalein displaces 4.6% at that concentration (Figure 5C). Taken together, these data indicate that though the selected flavones intercalate into DNA at high concentrations, they do not bind to the minor groove of DNA. Moreover, this high concentration (300 μM) compared with the very low IC_{50} (2.5, 5.5, 1.5 μM for luteolin, quercetin and baicalein) of topoisomerase I inhibition for the selected flavones suggests that the mode of inhibition is something different from flavone–DNA interaction.

Effect of flavones on single turnover cleavage and religation activity

Catalytic assays do not allow a precise chronological dissection of the inhibitory mechanism in relation to the catalytic cycle. Thus, the step at which an inhibitor needs to enter the catalytic cycle, and the step at which it becomes effective in trapping or inhibiting the enzyme cannot be differentiated. In order to overcome this problem, we have made the oligonucleotide suicide substrate of topoisomerase IB, which restricts the enzyme to a single round of cleavage and religation and allows for addressing the two half-reactions separately as we have described previously (6).

The substrate consisted of 5′-^{32}P-labeled 14 bp duplex with a 11 base 3′ tail (6). Upon cleavage and formation of covalent protein–DNA complex the AG dinucleotide at the 3′ end of the scissile strand is released. Suicidal cleavage assay was performed in the presence and absence of baicalein, luteolin, quercetin and CPT at 23°C for indicated time periods as described in Materials and Methods. Religation was studied under single turnover conditions by assaying the ability of the covalent intermediate to attach a 5′-hydroxyl-terminated 11mer to the covalently cleaved 12mer to form a 23mer product (6). CPT stabilizes the suicide cleavage complex with topoisomerase I of *L.donovani* and inhibits the religation step (Figure 6A, lane 3) which are in keeping with that of monomeric type IB topoisomerase (8.9). Similarly, baicalein, luteolin and quercetin also inhibit the religation step when added simultaneously with the enzyme and DNA in the suicidal cleavage assay (Figure 6A, lanes 4–6) like CPT. The rate of religation activity by LdTOP1LS in the presence of CPT, baicalein, luteolin and quercetin was plotted as a function of time (Figure 6B). The results indicated that religation kinetics for LdTOP1LS was relatively faster in the absence of the drugs while in the presence of flavones and CPT the time required to complete religation for LdTOP1LS is increased ~15- to 20 fold, indicating that the flavones stabilize the suicide enzyme–DNA covalent complex and inhibit the religation reaction. On the contrary, DHBA, a catalytic inhibitor of topoisomerase I, inhibits the suicidal cleavage reaction (Figure 6A, lane 7). However, when DHBA was pre-incubated for 5 min with the enzyme

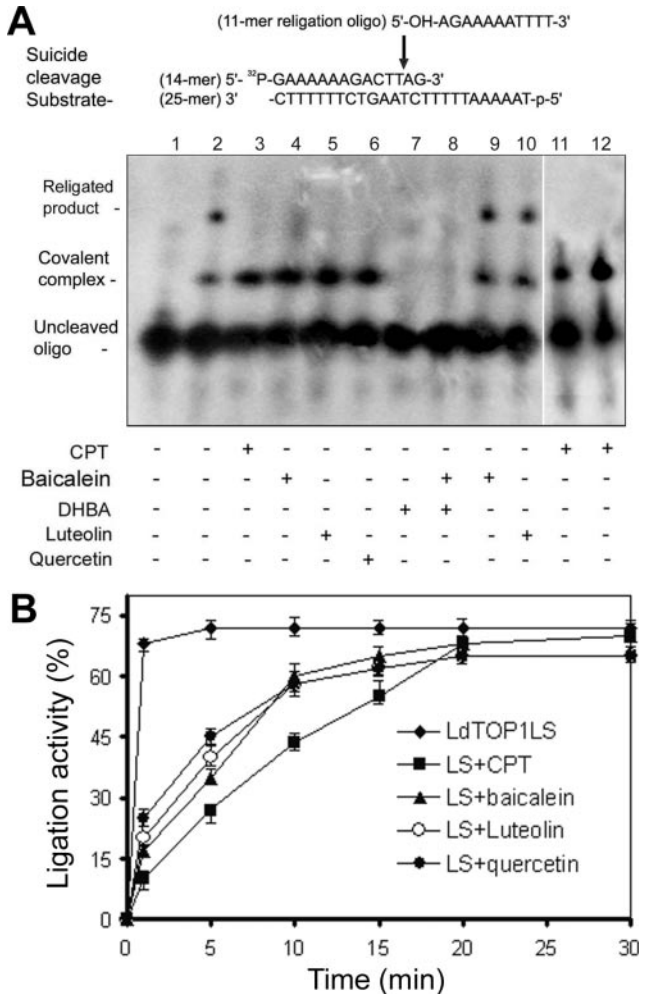

Figure 6. Effect of flavones on single turnover cleavage and religation. (**A**) Suicidal cleavage assay was performed with the 5′-^{32}P-end labeled suicide DNA substrate (14mer/25mer) as indicated. The DNA substrate was incubated with LdTOP1LS in the presence or absence of inhibitors at 23°C as described in Materials and Methods. Active cleavage complexes containing LdTOP1LS attached to the covalently cleaved 12mer of the suicide substrate were reacted with 5′-hydroxyl-terminated 11mer to form a 23mer product for 10 min at 37°C and the products were analyzed in a denaturating polyacrylamide gel. Lane 1, suicide DNA substrate only; lane 2 same as lane 1, but in the presence of LdTOP1LS and 5′-hydroxyl-terminated 11mer religation oligonucleotides. Lanes 3 and 12, same as lane 2, but incubated with 50 μM CPT. Lanes 4–6, same as lane 2, but with 50 μM baicalein, luteolin and quercetin added simultaneously with the enzyme and DNA in the suicidal cleavage assay; lane 7, same as lane 2, but with 50 μM DHBA pre-incubated for 2 min at 23°C with the enzyme before the addition of DNA in the suicidal cleavage assay. Lane 8, same as lane 7, but followed by addition of 50 μM baicalein simultaneously in the cleavage assay. Lanes 9–11, same as lane 2, but 50 μM baicalein, luteolin and CPT were added after suicidal cleavage reaction (on enzyme–substrate complex) together with 11mer religation oligo. All the reactions were stopped by addition of SDS to a final concentration of 2% (w/v). Samples were precipitated with ethanol, digested with trypsin and analyzed by denaturating polyacrylamide sequencing gel electrophoresis. The uncleaved suicidal oligonucleotide, covalent complex and the religation products are indicated. (**B**) Religation activity of LdTOP1LS in the presence of inhibitors as indicated in the figure. Active cleavage complexes containing LdTOP1LS covalently attached to the cleaved 12mer of the suicide substrate were reacted with 5′-hydroxyl-terminated 11mer to form a 23mer product. The reactions were carried out for 1, 5, 10, 15, 20 and 30 min at 37°C and the products were analyzed as above. The relative amount of cleavage products converted to ligation products in each sample were plotted as function of time. The results depicted were performed three times and representative data from one set of these experiments are expressed as means ± SD. Variations among different set of experiments were <5%.

followed by simultaneous addition of baicalein, and the suicidal substrate, the suicide-complex was completely inhibited (Figure 6A, lane 8). In contrast to CPT, baicalein and luteolin failed to inhibit the religation reaction, when added on the enzyme substrate covalent complex that had been previously formed in the absence of the drug, i.e. a condition where the drugs and 11mer religation oligo were added together (Figure 6A, lanes 9 and 10) after the suicidal cleavage reaction. On the other hand, CPT could inhibit the religation reaction under the same condition (Figure 6A, lane 11) or when the drug is added simultaneously during suicidal cleavage reaction (Figure 6A, lane 12). Although these experiments do not exclude the existence of a drug ternary complex (topoisomerase–DNA complex with bound flavones), we detected no effect of baicalein and luteolin on the stability or strand transferase activity of the binary protein–DNA complex. Taken together, these data suggest that interaction of these flavones with enzyme during *trans*-esterification reaction with DNA is a pre-requirement for the stabilization of topoisomerase I-cleavable complex and subsequently inhibiting the religation step.

Effect of baicalein and luteolin on the relaxation activity of mutant *L.donovani* topoisomerase I

Our previous observation reveals that residues within 1 and 39 amino acids of the large subunit have significant roles in modulating CPT sensitivity (6). So to understand the possible molecular interaction between the flavones (baicalein and luteolin) and the enzyme, we investigated the inhibitory effect on the mutant enzyme (LdTOP1Δ39LS) in plasmid DNA relaxation assay. Interestingly, we observed that baicalein and luteolin show considerable inhibition of relaxation activity up to 60% at 30 μM concentration (Figure 7A, lanes 8 and 9) compared with that of CPT, where no inhibition of relaxation activity was observed (Figure 4A, lane 5).

Selected flavones induce topoisomerase I-mediated DNA cleavage in the presence of CPT-resistant topoisomerase I enzyme

We investigated the interaction of baicalein and luteolin with CPT-resistant mutant enzyme (LdTOP1Δ39LS) in the equilibrium cleavage assay. We have described earlier that with LdTOP1Δ39LS, CPT-induced stabilization of cleavable complex reduced to 6-fold compared with the wild-type enzyme (6). Figure 7B shows that the DNA cleavage efficiency of LdTOP1Δ39LS in the presence of CPT is same as in the absence of CPT (Figure 7B, compare lanes 2 and 3), while with baicalein and luteolin, cleavage efficiency of the mutant enzyme increased to 35% (Figure 7B, lanes 4 and 5). This result indicates that the residues (1–39 amino acids of the large subunit) although important in modulating bi-subunit topoisomerase I activity *in vitro* are not critical for baicalein-induced topoisomerase I-mediated DNA cleavage.

To further establish the *in vivo* effect of the selected flavones on CPT-resistant topoisomerase I, we selected a single highly resistant *L.donovani* strain (Ld160CPT^{R}) as described by Marquis *et al.* (15). It was previously described that highly resistant *L.donovani* strain (LdRCPT.160) developed by stepwise exposure to CPT induces point mutations (Gly 185 Arg and Asp325 Glu) in the large subunit (LdTOP1L) of the

bi-subunit topoisomerase I (15). Relaxation assays performed with nuclear extract prepared from Ld160CPT^R cells show reduced fold of activity of the mutant enzyme and sensitivity toward CPT compared with the wild-type endogenous enzyme. Moreover, nuclear extract prepared from CPT-resistant cell (Ld160CPT^R) shows inhibition of topoisomerase I activity by baicalein and luteolin (data not shown). In order to find out whether the selected flavones could also stabilize covalent complexes of topoisomerase I and DNA inside the CPT-resistant cell (Ld160CPT^R), we carried out KCl-SDS coprecipitation assay as described above. The KCl-SDS precipitation assays in CPT-resistant cells are summarized in Figure 7C. Baicalein and luteolin were the most effective compounds that significantly increase the SDS-K$^+$ precipitable complex compared with the untreated control cells when applied to the medium at 200 μM concentration for 5 h (Figure 7C). However, treatment of the CPT-resistant cell (Ld160CPT^R) with 200 μM CPT for 5 h did not induce the formation of SDS-K$^+$ precipitable complex (Figure 7C). Thus, our results suggest that baicalein and luteolin can inhibit CPT-resistant mutant enzyme and favor the interpretation that the binding site of CPT may be partially overlapping or different from that of the selected flavones.

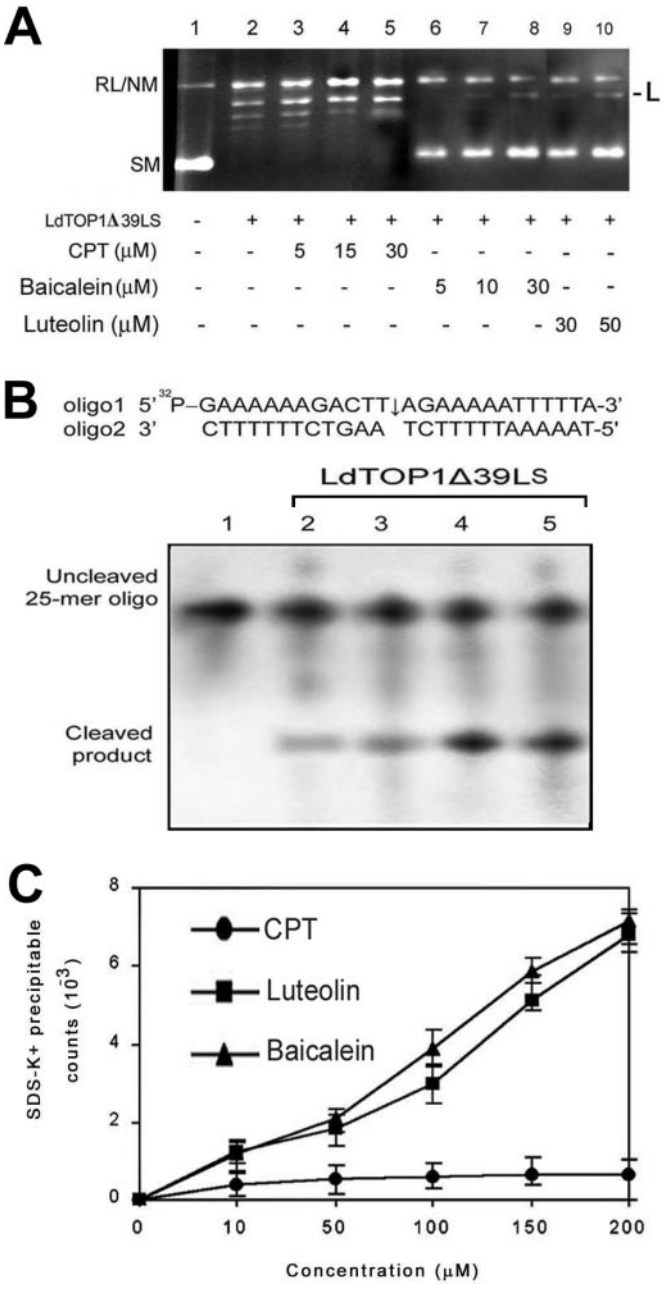

Figure 7. Effect of baicalein and luteolin on CPT-resistant *L.donovani* topoisomerase I. (**A**) Plasmid DNA relaxation experiments were performed with LdTOP1Δ39LS separately in the presence of CPT, baicalein and luteolin as indicated. Plasmid DNA and reconstituted mutant enzyme LdTOP1Δ39LS were mixed at a molar ratio of 1:2. Lane 1, 60 fmol of pBS (SK$^+$) DNA; lane 2, same as lane 1, but incubated with 120 fmol LdTOP1Δ39LS in the absence of inhibitors; lanes 3–10, same as lane 2, but incubated with different concentrations of CPT, baicalein and luteolin at 37°C for 10 min. All reactions were stopped by addition of SDS to a final concentration of 0.5% (w/v) and were electrophoresed in 1% agarose gel. Positions of supercoiled monomer (SM), relaxed and nicked monomer (RL/NM) and linear DNA (L) are indicated. (**B**) Stabilization of cleavable complex with CPT-resistant enzyme (LdTOP1Δ39LS) in the presence of baicalein and luteolin. Lane 1, 10 nM of 5′-^{32}P-end labeled 25mer duplex oligonucleotides as indicated. Lane 2, same as lane 1, but incubated with equal amount (0.2 μM) of LdTOP1Δ39LS; lane 3, same as lane 2, but incubated in the presence of 60 μM CPT; lanes 4 and 5, same as lane 2, but in the presence of 60 μM of baicalein and luteolin, respectively, incubated for 60 min at 23°C. Positions of uncleaved oligonucleotide (25mer) and the cleavage product (12mer oligonucleotide complexed with residual topo I) are indicated. (**C**) Topoisomerase I–DNA linkage analysis in CPT-resistant *L.donovani* promastigotes (Ld160CPT^R) by KCl-SDS precipitation assay. CPT-resistant *L.donovani* promastigotes (5×10^6 cells/ml) were labeled as described in Materials and Methods. The labeled cells were treated with different concentrations of CPT, luteolin and baicalein as indicated. SDS-K$^+$ precipitable complexes were measured as described in Materials and Methods. The experiments were performed three times and representative data from one set of these experiments are expressed as means ± SD. Variations among different set of experiments was <5%.

DISCUSSION

Crystal structure of human topoisomerase I seems compatible with a rotational model for the relief of supercoils during DNA relaxation. Modeling studies indicated that the DNA would likely contact with both the CAP and linker regions of the protein during strand rotation (33). Thus, it appears that the CAP and catalytic domain of human topoisomerase I undergo a conformational change after cleavage to accommodate DNA rotation (33,34).

But interestingly, *L.donovani* topoisomerase I is an unusual bi-subunit enzyme where the core DNA binding domain and the catalytic domain harboring the consensus SKXXY motif lie in separate subunits (4,5). Our recent findings reveal that 1–39 amino acid residues of the large subunit that resemble the CAP region of the monomeric enzymes have a modulating role in non-covalent interaction with DNA and sensitivity toward CPT (6). Thus, the N-terminal of the bi-subunit enzyme has an important role in strand rotation and DNA relaxation. The linker domain, which is poorly conserved and variable in length, links the core and catalytic domain of the monomeric enzyme and is responsible for the processivity of the enzyme (35). However, the functional linker is absent in *L.donovani* enzyme that perhaps account for the reduced processivity and

DNA binding affinity compared with monomeric rat liver topoisomerase I (5).

CPT, an important class of antitumor agent (7), is an uncompetitive inhibitor that directly traps the enzyme–DNA covalent complex and slows the religation step of the nicking closing cycle (8,9). CPT also hinders or blocks DNA rotation, which is evidenced by the crystal structure of the ternary complex between human topoisomerase I (topo 70) covalently linked to the DNA and the CPT derivative topotecan (11). If rotation is severely impeded by the bound CPT such that little or no rotation occurs during some nicking closing cycle, then the rate of DNA relaxation would reduce (35). This hypothesis corresponds well with our observation that CPT inhibits *L.donovani* topoisomerase I activity by ~15-fold at low salt concentration (6). Recent finding reveals that a highly CPT-resistant *L.donovani* strain (LdRCPT.160) developed by stepwise exposure to CPT induces point mutations (Gly 185 Arg and Asp325 Glu) in the large subunit (LdTOP1L) of the bi-subunit topoisomerase I. The mutant enzyme shows reduced activity as well as reduced sensitivity toward CPT (15).

Flavones are also potent inhibitors of topoisomerase II and monomeric topoisomerase I (19,22,27,36–38). Recently, we have described that luteolin inhibits *L.donovani* topoisomerase II by interfering with the ATPase activity (38). Flavones have also been described as inhibitors of hexameric replicative helicases RepA and interfere with the ATPase and double-stranded DNA-unwinding activity of the enzyme (39). In this study, we have tried to gain insight into the molecular mechanism of inhibition of unusual bi-subunit topoisomerase I with luteolin (5,7,3′,4′-tetrahydroxy flavone), quercetin (3,5,7,3′,4′-pentahydroxy flavone) and baicalein (5,6,7 trihydroxy flavone) and compare it with CPT. Baicalein, luteolin and quercetin are potent inhibitors of recombinant *L.donovani* bi-subunit topoisomerase I like CPT in the plasmid DNA relaxation assay, but in contrast to CPT, flavones bind with the free enzyme. Interaction of luteolin, baicalein and quercetin with the free enzyme was indicated by the following two experiments: (i) pre-incubation of the enzyme with the flavones before addition of DNA in relaxation assay (Figure 1C) and (ii) florescence titration of the flavones with increasing concentration of the enzyme (Figure 4). This observation is consistent with the monomeric topoisomerase I and RepA interacting with the flavones (27,38,39). However, the *L.donovani* topoisomerase I–flavone interaction was reversible, as studied by dilution experiments.

Baicalein, luteolin and quercetin are planer aromatic flavones that could bind DNA by intercalation due to the presence of the double bond between the 2 and 3 carbon of the C-ring (37). Direct measurement of this interaction made by fluorescence spectroscopy reveals K_d values of baicalein-DNA of 3×10^{-5} M, quercetin–DNA of 4.8×10^{-5} M and luteolin-DNA of 4.6×10^{-5} M (27). These values are comparable with the K_d values of topoisomerase II poisons *m*-AMSA-DNA of 2.1×10^{-5} M (40) and etoposide-DNA of 5.8×10^{-5} M (41). The flavones–DNA interactions are much weaker than EtBr binding to DNA (4×10^{-7} M) (42). We have addressed this issue by two independent experiments. EtBr displacement assay reveals that the selected flavones intercalate with DNA at high concentrations (300 μM) and do not bind to the minor groove of DNA. This observation is further supported by DNA-unwinding assay. However, baicalein, luteolin and quercetin completely inhibit *L.donovani* topoisomerase I at 3, 5 and 15 μM concentrations, respectively, when the enzyme is pre-incubated with the compounds. Thus, it becomes evident that inhibition of relaxation by flavones is not due to intercalation with DNA. Instead, it is more likely that the compounds exert their effects by specific interactions with the enzyme or with the enzyme–DNA binary complex.

A topoisomerase reaction has three general mechanistic steps, i.e. (i) binding of the enzyme to the substrate DNA, (ii) cleavage by *trans*-esterification reaction and subsequent strand passage through the break leading to the change in the linking number and (iii) strand religation. Investigation on the kinetics of stabilization of cleavage complex (step ii) reveals that the rate of stabilization of cleavable complex by the flavones were ~2- to 3-fold reduced compared with that of CPT. KCl-SDS precipitation assay reveals that the selected flavones stabilize *in vivo* covalent topoisomerase I–DNA complex in *L.donovani* promastigote cells (Figure 3) and is consistent with the observation in mammalian HL-60 cells (36). Thus, with the stabilization of cleavage complex the flavones inhibit the subsequent religation reaction (step iii) like CPT, as we observed in single turnover condition (Figure 6). Flavones inhibit religation reaction up to 15-fold compared with the religation property of the wild-type enzyme. However, in contrast to CPT, baicalein and luteolin failed to inhibit the religation step when the drugs were added to pre-formed enzyme substrate cleavable complex (Figure 6). Thus, we can surmise that although the mechanism of inhibition of topoisomerase I by these selected flavones and CPT are similar, the interaction of flavones with enzyme during *trans*-esterification reaction with DNA is a pre-requisite for stabilization of cleavable complex.

This differential mechanism to induce the stabilization of cleavable complex with topoisomerase I and DNA by CPT and the selected flavones led us to investigate the effect of baicalein and luteolin on CPT-resistant mutant enzyme LdTOP1Δ39LS lacking 1–39 amino acids of the large subunit (6). In duplex oligonucleotide cleavage assay with LdTOP1Δ39LS, the DNA cleavage efficiency of CPT dropped by 6-fold while that of baicalein was reduced by only 1.5-fold. The results indicate that the residues within 1 and 39 amino acid of the large subunit of bi-subunit topoisomerase I although important in modulating topoisomerase I activity and CPT sensitivity are not critical for baicalein-induced topoisomerase I-mediated DNA cleavage. These observations are in keeping with that of the stabilization of cleavable complex formation between topoisomerase I and DNA inside CPT-resistant cells (Ld160CPT^R). Thus, in the light of our experiments one possibility could be that the interacting amino acid residues of topoisomerase I may be partially overlapping or different for the flavones and CPT.

Our understanding of the interaction of *L.donovani* topoisomerase I–DNA and the selected flavones could be enhanced immeasurably by crystal structure of the enzyme bound to DNA and drugs, with the aim to synthesize better inhibitors with higher specificity.

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Conflict of interest statement. None declared.

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