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Determination of DNA topoisomerase Ⅱ activity from L1210 cells — a target for screening antitumor agents

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ABSTRACT DNA topoisomerase Ⅱ was isolated from mouse leukemia L1210 cells and the activity was determined by using P4 phage knotted DNA and pBR 322 DNA as the substrates. Based on these results, a method for screening antitumor agents by using DNA topoisomerase Ⅱ as a target was established.

The experiments showed that DNA topoisomerase Ⅱ catalyzed pBR 322 DNA breaking and relaxing which were revesible and dependent on ATP. The activity was increased 2 – 4 times in the presence of ATP 1 mmol · L^{-1}. In contrast with type Ⅱ enzyme, the activity of DNA topoisomerase Ⅰ was completely inhibited in the presence of ATP 1 mmol · L^{-1} and had full activity in the absence of ATP. Type Ⅱ enzyme also showed the unknotting activity by using p4 phage knotted DNA as a substrate. DNA cleavage and relaxing reaction induced by type Ⅱ enzyme increased 5-fold in the presence of Doxorubicin (Dox) 1 $\mu g \cdot ml^{-1}$ or daunorubicin (Dau). Etoposide (Eto) and aclarubicin B (Acl B) also stimulated the reaction at 100 $\mu g \cdot ml^{-1}$. The cleavage reaction resulted from topoisomerase Ⅱ was inhibited by other agents, such as frankincense extracts, terpenic compounds (BC series).

KEY WORDS DNA untwisting proteins; DNA gyrase; leukemia L1210; antitumor drug screening assays

DNA topoisomerase Ⅱ is a nuclear enzyme which modifies DNA topology by its ability to break and reseal one or both strands. This enzyme plays very important roles in DNA replication, and other genetic processes[1]. DNA topoisomerases Ⅰ and Ⅱ are fruitful targets for both antimicrobial and oncologic drug development[2,3]. Many intercalative antitumor drugs have been shown to induce reversible protein-linked DNA breaks. DNA topoisomerases Ⅰ and / or Ⅱ are responsible for this action[4]. A high degree of correlation was found between cytotoxicity and potency of inducing cleavable complex formation *in vivo* and *in vitro*[5]. In the present work an attempt was made to use DNA topoisomerase Ⅱ as a target for screening antitumor agents.

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MATERIALS AND METHODS

Materials Phenylmethyl-sulfonyl fluoride (PMSF), Sigma Chemical Company product, was kindly provided by Professor MA Bender, New York. The working solution in propyl alcohol was made before use. P4 phage DNA was kindly provided by Professor L F Liu, Baltimore, USA. Topoisomerase Ⅰ was purchased from Promega. ATP was obtained from BDH Chemicals. 1,4-Dithiothreitol (DTT) and proteinase K were products of Merck Company. Sarkosyl, doxorubicin (Dox) and daunorubicin (Dau) were purchased from Sigma Chemical Company and Farmitalia. Aclarubicin B (Acl-B) was a gift from Dr Zhuang Zeng-Hua, Eto was produced by Lian-yun-gang Pharmacentical Factory. Meisoindigo (Mei), ranuculin (Ran) and terpenic compounds (BC series) were synthesized or extracted by our institute. Acetylshikonin (Ace) was provided by Institute of Botany, the Chinese Academy of Sciences. [^{3}H] TdR (925 GBq · $mmol^{-1} \cdot L^{-1}$) was purchased from Chinese Academy of Atomic Energy Sciences.

Cells Mouse leukemia L1210 cells were obtained from the DBA / 2 mouse 6 or 7 d

after transplantation, washed with phosphate buffer solution (PBS) for several times and counted. KB cells were grown in RPMI 1640 medium (Gibco) with penicillin 40 IU · ml^{-1}, streptomycin 50 $\mu g \cdot ml^{-1}$ and 10% fetal calf serum, and incubated at 37℃ in the presence of 5% CO_2. The cells were seeded for 24 h before the experiment.

Alkaline Elution The alkaline elution technique for assaying DNA single-strand breaks (SSB) has been described previously[6]. Briefly, 2.5×10^5–5.0×10^5 of KB cells were labeled with [^{3}H]TdR for 16 h. The medium containing radioactivity was removed and fresh medium was added to the culture and the cells were incubated for another 12 h. The labeled cells were treated with Eto, Dox, Mei or Ran for 1 h. Washed twice with ice-cold PBS (pH 7.4), harvested by rubber policeman, loaded on polyvinyl chloride filter (1.2 μm) and lysed with a solution of 2% SDS, EDTA 20 mmol · L^{-1} (pH 10.0). The DNA was eluted from the filter with NaOH at pH 12.0. The rate of elution was 1.5 ml · h^{-1} with a fraction interval of 1 h. And a total elution time of 10 h.

Extraction of topoisomerase Ⅱ from mouse leukemia L1210 cells The mouse leukemia L1210 cells were drawn from mouse 6 or 7 d after transplantation and washed twice with ice-cold PBS. 1×10^8 Cells were suspended in 10 ml of TMN (Tris 10 mmol · L^{-1}, pH 7.5, $MgCl_2$ 1.5 mmol · L^{-1} and NaCl 10 mmol · L^{-1}) and allowed to set at 0℃ for 10 min. One ml of 10% Sarkosyl was added to the cell suspension and the mixture gently triturated and finally left at 0℃ for 15 min. The cells were then centrifuged at 3000 $\times g$ for 10 min, and the pellet was resuspended in 2 ml of ice-cold buffer A (Tris 50 mmol · L^{-1}, pH 7.5, KCl 25 mmol · L^{-1}, $CaCl_2$ mmol · L^{-1}, $MgCl_2$ 3 mmol · L^{-1}, sucrose 0.25 mol · L^{-1}), and fresh PMSF in propanol was added to final concentration of 1 mmol · L^{-1} and kept in ice for 2 min, the 0.6 ml of buffer B (buffer A with 0.6 mol · L^{-1} sucrose) and sedimented at 5000 × g for 10 min. The pellet was resuspended in 2 ml buffer C (buffer A without $CaCl_2$ and with $MgCl_2$ 5 mmol · L^{-1}), centrifuged at 5000 × g for 10 min and finally the pellet was resuspended in 0.15 ml of buffer D (same as buffer C but without sucrose), 15 μl of EDTA 0.2 mol · L^{-1} (pH 8.0) and 0.33 ml of buffer E [Tris 80 mmol · L^{-1}, pH 7.5, EDTA2 mmol · L^{-1}, DTT 1 mmol · L^{-1}, NaCl 0.53 molL^{-1}, and 20% glycerol, (vol · vol^{-1})]. This mixture was gently triturated, left at 0 ℃ for 30 min, and centrifuged at 40000 × g for 20 min. The protein concentration of the supernatant containing topoisomerase Ⅱ was determined by colorimetric method[7]. The enzyme solution was diluted with an equal volume of glycerol, and BSA and PMSF were added to get final concentrations of 1 mg · ml^{-1} and 0.5 mmol · L^{-1}, respectively. The enzyme is stable at −20 ℃ for 3 wk.

Cleavage of plasmid pBR 322 DNA Supercoiled pBR 322 DNA was isolated accocding to procedures of "large scale preparation of plasmid DNA"[8] and treated with 200 $\mu g \cdot ml^{-1}$ of RNase at 37 ℃ for 1 h. Three bands (form Ⅰ: supercoiled; form Ⅱ: nicked circular and form Ⅲ: linear) should appear on 0.8% agarose gel after electrophoresis. A 20-μl assay contained 5 μl of cleavage buffer (Tris 200 mmol · L^{-1}, pH 7.5, KCl 340 mmol · L^{-1}, $MgCl_2$ 40 mmol · L^{-1}, DTT 20 mmol · L^{-1}, BSA 120 $\mu g \cdot ml^{-1}$ and ATP 4 mmol · L^{-1}), 1 μg of pBR 322 DNA, and various amounts of topoisomearse Ⅱ. After incubation at 37℃ for 30 min, the reaction was terminated by adding 2 μl of 10% SDS and 2 μl of proteinase K (10 mg · ml^{-1}). The mixture was then incubated at 37 ℃ for another 30 min. Two μl of loading buffer (bromophenol blue 0.42% , xylene cyanol 0.42% and glucerol 50% in H_2O) were added, the DNA was subjected to electrophoresis on 0.8% agarose gel at 80 V for 2 h, and the gel was stained with 1 $\mu g \cdot ml^{-1}$ of EB solution for 30 min.

Effects of antitumor agents on the enzyme activity The cleavage reaction induced by DNA topoisomerase Ⅱ can be affected by some antitumor agents in 2 ways, ie, stimulation and inhibition. For the former 0.2 U of the enzyme in the cleavage reaction was used in which supercoiled pBR 322 DNA (form Ⅰ) did not become nicked in circles (form Ⅱ) or linear (form Ⅲ) significantly. For the latter 1 U of the enzyme was used so that the form Ⅰ DNA band disappear completely in the absence of drug. Then the reaction was performed as above except that an antitumor agent was added to the reaction system.

RESULTS

Catalytic activities of topoisomerases The catalytic activity of topoisomerase Ⅱ was estimated by cleavage reaction. One μg of pBR 322 DNA was incubated with 0.1, 0.3 and 0.5 μg of the proteins extracted from L1210 cells in the reaction mixtures for 30 min as described above and 1 U of the enzyme is defined as the amount of proteins which caused 1 μg of supercoiled pBR 322 DNA to become form Ⅱ or form Ⅲ DNA at 37℃ in 30 min. As shown in Fig 1a (lane B to D), the catalytic reaction showed a function of the amount of enzyme proteins in the presence of ATP 1 mmol · L^{-1}. About 0.5 μg of the proteins were needed to complete the cleavage reaction. The specific activity of the enzyme was found to be about 2000 U · mg^{-1}. However, the activity decreased (1.5 μg of the enzyme proteins were needed for full reaction) when the reaction was performed in the absence of ATP. In contrast with topoisomerase Ⅱ, the activity of topoisomerase Ⅰ was inhibited by ATP 1 mmol · L^{-1} (Fig 1, lane I to J) and the enzyme showed a full activity in the absence of ATP (Fig 1b, lane K to L).

Effects of NaCl on topoisomerase Ⅱ activity One of the characters of topoisomerase Ⅱ is that the cleavage of supercoiled DNAby the enzyme can be inhibited by high concentrations of NaCl. In our reaction system pBR 322 DNA 2.5 μg, emzyme proteins 2.5 U and different amount of NaCl were added to cleavage buffer to a final vloume of 50 μl. Fig 2 indicated that the inhibition of NaCl on catalytic activity of topoisomerase Ⅱ was a function of the concentration of the salt. Addition of high concentrations of NaCl (0.25 mol · L^{-1}, lane E and 0.5 mol · L^{-1}, lane F) to the reaction mixture the cleavage was completely blocked. This result is consistent with the previous report[9].

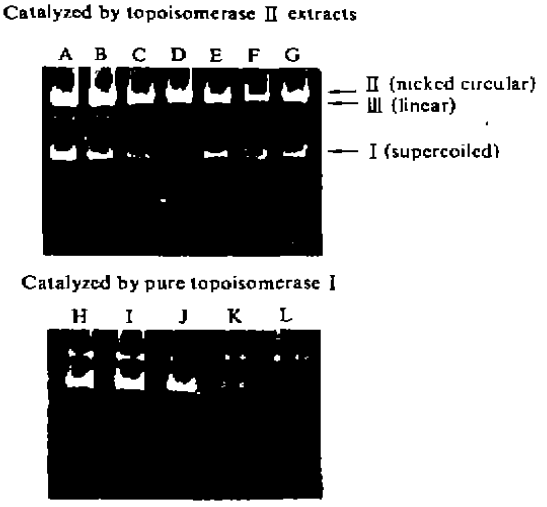

Fig 1. Cleavage and relaxing of pBR 322 DNA by topoisomerases with or without ATP. Lane A: pBR 322 DNA only; lane B to D: DNA and 0.1, 0.3, 0.5 μg of the extracts with ATP 1 mmol · L^{-1}; lane E to G:same as the "B to D", but without ATP; lane H: DNA only; lane I to J: DNA and 0.5, 1.0 U topoisomerase Ⅰ with ATP 1 mmol · L^{-1}; lane K to L: same as the "I to J", but without ATP.

Reversibility of the topoisomerase Ⅱ cleavage reaction Cleavage reaction induced by topoisomerase Ⅱ is reversible. After completion of the reaction, the mixture was set on ice bath or highconcentration of NaCl was added to the mixture (final concentration of NaCl 0.5 mol · L^{-1}) and incubated at 37℃ for 30 min. As shown in Fig 3, the cleavage reaction was reversed by decreasing temperature from 37 ℃ to 0 ℃ (lane C) or high concentra—

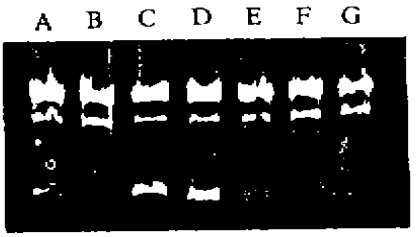

Fig 2. Inhibition of NaCl on cleavage of DNA by mouse leukemia L1210 cell topoisomerase II. A reaction mixture (30 μl) contained 7.5 μl CB, 3.0 μg pBR 322 DNA, 3 U topoisomerase II and different concentrations of NaCl. Lane A is the control (no enzyme). Final NaCl concentrations in lane B to G were 0, 500, 250, 125, 62.5 and 31 mmol · L^{-1}, respectively.

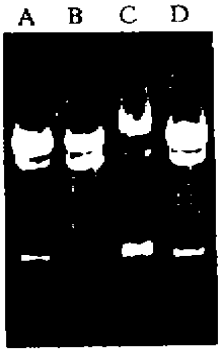

Fig 3. Reversible cleavage of pBR 322 DNA by mouse leukemia L1210 cell topoisomerase II. A 30-μl assay contained the same buffer as above, 3.0 μg pBR 322 DNA and 3.0 U DNA topoisomerase II. Lane A (without the enzyme) and B (with the enzyme) are the controls. Lane C: after incubation at 37℃ for 30 min, the reaction mixture was kept on ice-bath for 30 min. Lane D : 500 mmol · L^{-1} of NaCl was added to the mixture and incubated at 37℃ for another 30 min. The reactions both in lane C and D were terminated.

tion of sodium chloride (lane D) compared with control (lane A). This result is also in agreement with the previous report[9].

Unknotting activity The unknotting of knotted P4 phage DNA is shown in Fig 4. As the reaction procedes, the faster migrating smear of multiple knotted DNA was converted to the slower moving heavy band of unknotted circular molecules. This reaction was inhibited by ADM (comparing lane C with lane D).

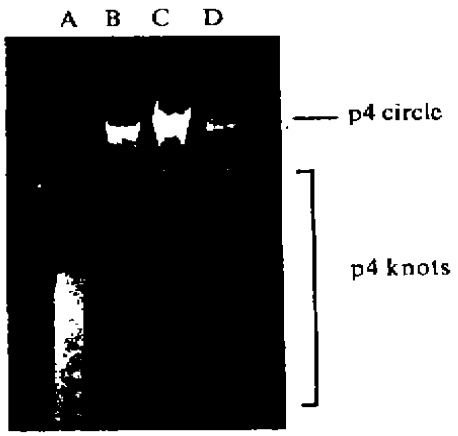

Fig 4. Assay for unknotting activity of topoisomerase II from L1210 cells. Reactions (20 μl each) were conducted under the same conditions as those decribed for the cleavage assay except that P4 knotted DNA (1 μg) was used. Lane A: P4 knotted DNA only; lane B, C: P4 DNA with 1.5 and 3.0 μg enzyme extracts; lane D: P4 DNA with 3 μg enzyme extracts and 10 μg Dox.

Effects of antitumor agents on topoisomerase II activity

1 Stimulation of DNA cleavage After incubation of pBR 322 DNA 1 μg with 0.2 U of topoisomerase II extracted from mouse leukemia L1210 cells in the presence (lane E to P in Fig 5A) or absence (Fig 5A, lane B) of Eto, Dox, Dau and Acl-B 1, 10, and 100 μg · ml^{-1}, the reaction products were treated with SDS, proteinase K and then electrophorised on 0.8 % agarose gel. The presence of Dox and Dau 1 μg · ml^{-1} reproducibly stimulated DNA cleavage by 5-fold in the presence of the enzyme (only 0.2 U of the enzyme was needed for complete cleavage). Eto and Acl-B also enhanced the cleavage ability of topoisomerase II but with higher concentrations in our experimental conditions (Tab 1). The high concentrations of Dox and Dau (100 μg · ml^{-1}) induced alteration of mobility of the DNA on the agarose gel electrophoresis. This might result from the intercalation of the drugs to DNA. However, the stimulations of DNA

cleavage by topoisomerase Ⅱ in the presence of Mei, Ace, Ara-c, and Ran were not seen (Fig 5B).

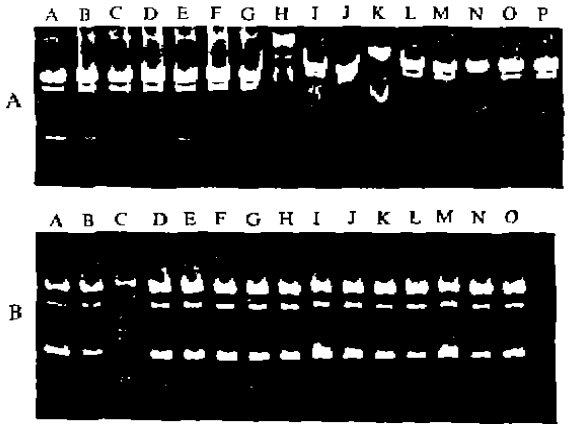

Fig 5. Effect of antitumor drugs on pBR 322 DNA cleavage reaction by topoisomerase Ⅱ. A: Lane A: DNA only; lane B to D: DNA with the enzyme (0.1, 0.3 and 0.5 μg); lane E to G: 0.2 U enzyme and treatment with 1, 10, 100 μg · ml^{-1} of Eto; lane H to J: 100, 10 and 1 μg · ml^{-1} of Dox; lane K to M: 100, 10 and 1 μg · ml^{-1} of Dau; lane N to P: 100, 10 and 1 μg · ml^{-1} of Acl-B. B: Lane A: DNA only; lane B: DNA with 0.2 U enzyme; lane C: 10 μg · ml^{-1} Dox; lane D to F: 100, 10 and 1 μg · ml^{-1} of Mei; lane G to I: 100, 10 and 1 μg · ml^{-1} of Ace lane J to L: 100, 10 and 1 μg · ml^{-1} of Ran; lane M to O: 100, 10 and 1 μg · ml^{-1} of Arc-C.

2 Inhibition on the catalytic activity

The cleavage reaction catalysed by topoisomerase Ⅱ can be inhibited by some chemicals. As shown in Fig 6 and Tab 1, when 2.5 μg of pBR 322 DNA reacted with 2.5 U of topoisimerase Ⅱ in the absence of drugs band I completely disappeared. The reactions were stopped when terpenic compound BC-1, BC-4, BC-9 100 μmol or BC-M 200 μmol were added to the reaction system. These agents also showed cytotoxicity (data not shown).

DNA single strand breaks induced by antitumor agents DNA single strand breaks (SSB) induced by antitumor agents was estimated by alkaline elution technique. As shown in Fig 7 SSB in KB cells were observed after the treatment with Eto and Dox, and thenumbers of breaks were increased with increasing drug concentrations. At higher concentrations, Eto

Tab 1. Stimulations of Eto, Dox, Dau and Acl-B and inhibitions of BC series on pBR 322 DNA cleavage reactionscatalyzed by topoisomerase Ⅱ. −: no activity; +: half activity and ++: full activity.

Topoisomerase Ⅱ (units)	Drug	Stimulation or inhibition activity 1.0	10.0	100.0	200.0
0.2	Eto, μg · ml^{-1}	−	+	++	
	Dox, μg · ml^{-1}	++	++	++	
	Dau, μg · ml^{-1}	++	++	++	
	Acl-B, μg · ml^{-1}	−	−	+	
	Arc-C, μg · ml^{-1}	−	−	−	
	Ace, μg · ml^{-1}	−	−	−	
	M-GAG, μg · ml^{-1}	−	−	−	
	CMT, μmol · L^{-1}	−	−	−	
	Mei, μmol · L^{-1}	−	−	−	
	Ran, μmol · L^{-1}	−	−	−	
	VCR, μmol · L^{-1}	−	−	−	
1.0	BC-1, μmol · L^{-1}	−	+	++	
	BC-3, μmol · L^{-1}			−	
	BC-4, μmol · L^{-1}			−	
	BC-4, μmol · L^{-1}			+	
	BC-6, μmol · L^{-1}			−	
	BC-8, μmol · L^{-1}			−	
	BC-9, μmol · L^{-1}	−	+	++	
	BC-M, μmol · L^{-1}			−	+

Fig 6. Inhibition of topoisomerase Ⅱ activity by antitumor agents. Lane A: DNA only; lane B: DNA with 1 U of the enzyme; lane C: treated with 10 μg · ml^{-1} of Dox; lane D to F: treated with 1, 10, 100 μmol · L^{-1} of BC-1; lane G to I: treated with 1, 10, 100 μmmol · L^{-1} of BC-1; lane J to K: treated with 200 and 100 μmol · L^{-1} of BC-4M.

produced greater numbers of SSB, although the curves tended to level off, as if approaching saturation (Fig 7). But DNA single strand breaks were not obtained when KB cells were exposed to Ran and Mei under the same experimental conditions (Fig 7).

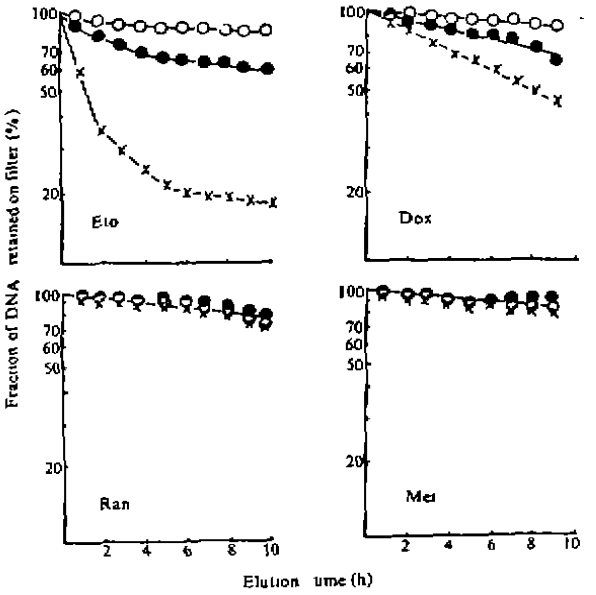

Fig 7. Analysis of DNA single strand breaks induced by antitumor drugs in KB cells by means of alkaline elution. The cells were exposed to 10 and 30 $\mu g \cdot ml^{-1}$ of Eto, Dox, or 10 to 30 $\mu mol \cdot L^{-1}$ of Ran and Mei for 1 h, washed twice with cold PBS and eluted with NaOH solution (pH 12.00). (○) : control; (●) : 10 $\mu g \cdot ml^{-1}$ or $\mu mol \cdot L^{-1}$; (×) : 30 $\mu g \cdot ml^{-1}$ or $\mu mol \cdot L^{-1}$.

DISCUSSION

In recent years, many methods have been reported for the isolation and purification of topoisomerase II [10,11], but most of them are complicated and time-consuming. In the present study, we utilized the procedures reported by Sullivan *et al*[12], with some modifications. The major amendment was that at the last step of centrifugation higher speed was used to remove nucleic acid which may interfere with the cleavage reaction. The procedures for extraction of topoisomerase II reported here have an advantage of easiness and time-saving. All extractions can be finished in a few hours. The characters of topoisomerase II we detected, such as unknotting activity, cleavage reaction, effects of ATP and NaCl on cleavage reaction and the reversibility of the cleavage reaction by topoisomerase II, are consistent with the previous reports[9,13,14]. The findings that cleavage reaction catalyzed by topoisomerase I can be inhibited by ATP 1 $mmol \cdot L^{-1}$ indicate that interference of topoisomerase I which may occur in the extracts can be removed by adding ATP 1 $mmol \cdot L^{-1}$ to the reaction system.

Some workers[4,15] reported the effects of DNA intercalating agents on topoisomerase II induced DNA SSB and breakage-reunion. In our experiments, stimulations of Dox, Dau, Eto and Acl-B and inhibitions of BC-1, BC-4, BC-9 and BC-M on DNA cleavage reaction by topoisomerase II were observed. SSB induced by Eto and Dox was also obtained by means of alkaline elution technique. Nevertheless the stimulations of cleavage reaction resulted from Mei, Ace, Ran and Ara-c and DNA SSB induced by Ran and Mei in the presence of DNA topoisomerase II have not been found. There is a good correlation between the stimulation of DNA cleavage by topoisomerase II and formation of DNA SSB in the presence of some antitumor agents.

A number of concepts regarding topoisomerases as drug targets are becoming better defined. Lock *et al*[2,3] suggested that for inhibitors of both topoisomerases, a major effort must be made to elucidate the physicochemical nature of the drug-enzyme DNA interaction. Synthetic and natural product screening efforts will hopefully generate new inhibitors for both enzymes which, upon systematic analysis, may lead to a unifying model to account for drug effects at the molecular level. Based on this idea, we estab-

lished a method for screening anticancer drugs by using topoisomerase Ⅱ as a target. Some natural products have been screened by using the method we reported here and interesting results have been obtained. However, as with the development of other drugs there are limitations for using this method to screen antitumor drugs. In fact this method is only suitable to the agents which may interfere with topoisomerase Ⅱ.

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细胞 L1210 拓扑异构酶Ⅱ活性的测定—筛选抗癌药的靶点

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提要 以小鼠 L1210 白血病细胞为材料提取了 DNA 拓扑异构酶Ⅱ，并以 P-4 打结 DNA 和 pBR 322 DNA 为底物测定了该酶活性，在此基础上初步建立了以拓扑异构酶Ⅱ为靶点筛选抗癌药的方法. 实验表明拓扑异构酶Ⅱ能使 pBR 322 DNA 断裂和解旋，该反应是可逆的，ATP 依赖性的. 在 ATP 1 mmol·L^{-1} 时，酶活性可增加 2-4 倍. 相反 ATP 1 mmol·L^{-1} 能抑制拓扑异构酶 I 的活性. 实验还表明，1 μg·ml^{-1} 阿霉素、柔红霉素能使拓扑异构酶Ⅱ催化的 DNA 断裂反应增强 5 倍. 足叶草甙、阿克拉霉素 B 在 100 μg·ml^{-1} 时也有同样作用. 相反，植物提取物 BC 系列对该酶介导的 DNA 断裂则有抑制作用.

关键词 DNA 脱氧核糖核酸非扭曲蛋白类；脱氧核糖核酸解旋酶类；白血病 L1210；抗肿瘤药；筛选试验