A NOVEL DEOXYRIBONUCLEASE INHIBITOR FROM MICROMONOSPORA

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In the course of screening inhibitors to deoxyribonuclease (DNase) and topoisomerase I, we found that Micromonospora echinospora MG299-fF35 produced a new DNase inhibitor with a low molecular weight. This paper describes isolation and its properties. This compound inhibited enzyme activity of various DNases and, in addition, that of topoisomerase I from adenocarcinoma FM3A cells. As far as we know, this is the first isolation of an inhibitor to topoisomerase I. Coupled with inhibitors to topoisomerase II (gyrase) such as nalidixic acid and novobiocin, this may contribute very much to the further elucidation of DNA replication and other mechanisms. This compound was found to be identical with dotriacolide1) produced by the same strain, a β-lactamase inhibitor.

M. echinospora MG299-fF35 was grown in a medium consisting (%) of glucose 1, glycerol 1, sucrose 1, oatmeal (Quaker White) 0.5, soybean meal 2, granular pressed yeast (Oriental) 1, Casamino acid 0.5, CaCO3 0.1, pH 7.0, for 4 days at 27°C in a rotary shaking machine, and then 5 ml of the culture were transferred to a production medium consisting (%) of potato starch 3, soybean meal 1.5, corn steep liquor 0.5, yeast extract 0.2, NaCl 0.3, MgSO4·7H2O 0.05, CaCO3 0.3, CaCl2·6H2O 0.001, pH 7.0, of 100 ml in a 500 ml flasks and grown at 27°C for 7 days. The maximum potency was obtained under this condition at 7 to 9 days of cultivation, at which time the amount of the inhibitor accumulated was about 130 μg/ml.

The supernatant (2155 ml), obtained by centrifugation of the fermentation broth (2500 ml), was passed through a column of HP-20 (200 ml). After washing the column with water (5 liters), the active substance was eluted with 50% aqueous acetone and obtained as brown powder (833.6mg) after concentration under reduced pressure and lyophilization. The inhibitor was applied to a column of silica gel (150 g) and the column was eluted with tert-butanol and water (7: 2). The active fractions were combined and evaporated to dryness to give 231 mg of pale brown powder.

The final preparation gave only one spot on thin-layer chromatograms with solvent systems of n-butanol - methanol - water (4: 1 : 2), n-propanol - 28 % NH3OH - water (40: 50: 6) and tert-butanol - water (7: 3), which was detected by aqueous sulfuric acid and was coincident with that detected by inhibitory activity against DNase I (Fig. 1). Electrophoresis on a cellulose acetate plate with 1 M acetic acid and pyridine (pH 3.5) as a development solvent gave only one spot which was detected by toluidine blue.

The inhibitory activity was determined against DNase I, DNase II and three kinds of DNases from Streptomyces strains 6016, 4290 and 4098, using methods described in a previous paper1) and of KUNITZ4). In the case of DNase II and KUNITZ method, 65.6 mM acetate buffer of pH 4.8 was used instead of phosphate buffer, and the concentration of MgSO4 was reduced from 10.5 mm to 0.26 mm. Enzyme activity of all the DNases was inhibited by the presence of the inhibitor, IC50 values being 6.1, 9, 9.7, 42 and 47 μg for...
DNase I, DNase II, DNases from *Streptomyces* strains 6016, 4290 and 4098, respectively (Fig. 2).

When DNA concentration in the reaction mixture was changed from 50 to 710 µg/ml without changing DNase I concentration (65.8 ng/ml), almost no change was observed in the magnitude of inhibition. On the other hand when DNase I concentration was changed from 6.58 ng/ml to 65.8 ng/ml with constant DNA concentration (625 µg/ml), the degree of inhibition decreased with increasing DNase I concentration. This indicates that the DNase inhibitor inhibited the enzymatic activity by direct binding to DNase I. That the inhibitor is bound to DNase I was supported by the experiments at various concentrations of substrate and inhibitor (Fig. 3), which indicate that the inhibitor inhibited DNase I by mixed type mechanisms of inhibition with *K*<sub>i</sub> value of about 3 × 10<sup>-6</sup> M.

Inhibitory activity was determined against topoisomerase I<sup>5</sup> derived from FM3A cells using Colicin E1 DNA as a substrate. When Colicin E1 DNA was incubated with topoisomerase I, several bands were observed in agarose gel electrophoresis, depending on the degree of unwinding of the DNA (Fig. 4A, no inhibitor). Addition of the inhibitor at concentrations of 5.2 or 2.6 µg/ml prevented the unwinding of DNA completely. Even at 1.3 µg/ml, partial inhibition of the topoisomerase activity was observed. An analogous
Fig. 4. Inhibition of topoisomerase I by the inhibitors, dotriacolide (A) and izumenolide (B).

Control showed Colicin E1 DNA. The enzyme activity of topoisomerase I from adenocarcinoma was estimated by incubating the reaction mixture (38 µl) comprised of Colicin E1 DNA, 0.87 µg; appropriate amounts of topoisomerase I and inhibitor; sodium potassium phosphate 0.15 M and sucrose 0.05 M, pH 7.5 for 15 minutes at 37°C. After addition of 38 µl of tris·HCl of pH 8.0 40 mM, EDTA 0.4 mM, NaCl 40 mM, sucrose 20% and sodium dodecyl sulfate 2%, the reaction was stopped by incubation for 15 minutes at 45°C. Then, an aliquot was applied to 1.2% agarose plate and separated by electrophoresis at 40 Volt and 4°C for 15 hours. The electrophoretic patterns were photographed after the agar plate was stained with ethidium bromide. The amounts of inhibitors in the reaction mixtures were indicated at the top of each column.

The compound, izumenolide provided us from Dr. R. B. SYKES of the Squibb Institute for Medical Research, showed a similar inhibitory activity against topoisomerase I at concentrations of over 2.6 µg/ml (Fig. 4B). However, below 1.3 µg/ml, no inhibition was observed, although this compound inhibited DNase I activity at less than one tenth of the concentration of that of dotriacolide.

Topoisomerase I (ω protein) from E. coli was completely inhibited at 20 µg dotriacolide/ml and partially inhibited at 10 µg/ml but no inhibition was observed at 5 µg/ml. On the other hand, the topoisomerase I from calf thymus was completely inhibited at 5 µg dotriacolide/ml and partially inhibited at 2 µg/ml.

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