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INHIBITION OF THE DNA CATENATION ACTIVITY OF TYPE II TOPOISOMERASE BY VP16-213 AND VM26

Anil Minocha and Byron H. Long

Bristol-Baylor Laboratory
Department of Pharmacology
Baylor College of Medicine
Houston, Texas 77030

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SUMMARY: Studies suggest that the anticancer drugs VP16-213 and VM26 produce cytotoxicity by inducing protein-associated DNA breakage *in vivo* through interaction with a yet unknown nuclear component. The effects of these drugs and their congeners on topoisomerase activities was investigated. VP16-213, VM26, and congeners active toward inducing DNA breaks also inhibited the catenation activity of eukaryote type II topoisomerase *in vitro* at very low drug concentrations. A structure-activity relationship was obtained for inhibition of catenation that parallels *in vivo* DNA breakage and cytotoxic activities. Type I topoisomerase activity was totally unaffected by these drugs.

DNA breakage activity of VP16-213[1] is well documented (1-6). DNA breaks accumulate rapidly upon exposure of cells to drug and are as rapidly repaired upon removal of drug (1,3-5). Recently, Wozniak and Ross demonstrated that VP16-213 induces DNA-protein crosslinks concommitant with DNA breakage (2). VM26 has similar DNA breakage activity (7) but is approximately 10 times more active than VP16-213 (3-5). The DNA breakage activity of these drugs is likely mediated by an enzyme-like cellular component, since exposure of cells to drug at 4°C prevents DNA breakage (1,2,6). Evidence suggests that the yet unidentified cellular factor is localized in the nucleus (2). The observations that DNA breakage activity requires magnesium ions (8) and is stimulated by ATP (9) are compatible with type II topoisomerase involvement in VP16-213-induced DNA breakage.

[1] Abbreviations: mAMSA, 4'-(9-acridinylamino)-methanesulfon-m-anisidide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; Podo, podophyllotoxin; EP, 4-epipodophyllotoxin; DMEP, 4'-demethyl-4-epipodophyllotoxin; DMEPBG, 4'-demethylepipodophyllotoxin-4-(4,6-O-benzylidene-β-D-glucopyranoside); VM26, 4'-demethylepipodophyllotoxin-4-(4,6-O-thenylidene-β-D-glucopyranoside); and VP16-213, 4'-demethylepipodophyllotoxin-4-(4,6-O-ethylidene-β-D-glucopyranoside).

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The possibility that topoisomerases may be involved in DNA breakage has been suggested as an explanation for the protein-associated DNA breakage produced by adriamycin in vivo (10) and other drugs that intercalate into DNA (11,12). Recently, Marshall et al. reported that novobiocin and coumermycin, inhibitors of type II topoisomerase, inhibit production of double strand DNA breaks by mAMSA in isolated nuclei (13). Although the DNA breakage produced by VP16-213 and VM26 (1-6) is similar to that produced by DNA intercalators (10-12), there is no evidence that either VP16-213 or VM26 intercalate into DNA. We have reported preliminary results demonstrating inhibition of purified type II topoisomerase in vitro by VP16-213 and VM26 (14).

MATERIALS AND METHODS

Phosphocellulose (P-11) and Hydroxylapatite were products of Whatman and Bio-Rad, respectively. PMSF and podophyllotoxin were purchased from Sigma, PM2 DNA was purchased from Boehringer Mannheim, and Polyamin P was obtained from BRL. VP16-213 and VM26 were provided by Bristol Laboratories and other congeners were gifts of Sandoz and NCI.

Topoisomerase Isolation. Novikoff hepatoma cells from the ascites fluid of 8-10 rats were dounce homogenized in reticulocyte swelling buffer containing 1 mM PMSF as described by Miller et al. (15). The nuclei were purified by differential centrifugation and lysed in 1 M NaCl. DNA in the nuclear lysate was removed by aggregation with 0.3% Polyamin P followed by centrifugation. The supernatant was loaded onto a P-11 column (2.6 cm i.d. x 20 cm), which was eluted with a linear gradient of 0.1 to 2.0 M NaCl (16). Topoisomerase activity eluted between 0.5 and 1.5 M, but fractions from 0.5 to 0.8 M were pooled and concentrated by saturation with ammonium sulfate. The dissolved protein was loaded onto an hydroxylapatite column (2.0 cm i.d. x 5 cm) and eluted in steps of 0.25, 0.4, 0.55, and 0.7 M phosphate. Topoisomerase II activity eluted in the 0.55 and 0.7 M fractions whereas type I activity was found only in the 0.7 M fraction as revealed by the absence of relaxation activity in the 0.55 M fraction upon omission of ATP.

ASSAY FOR TOPOISOMERASE I ACTIVITY. The relaxation of form I PM2 supercoiled DNA in the absence of ATP was employed to assess topoisomerase I activity, as described by Liu and Miller (17). The conversion of form I to form I^{o} DNA was followed by electrophoresis in 1% agarose gel.

ASSAY FOR TOPOISOMERASE II ACTIVITY. The catenation activity of the type II enzyme was used as an assessment of enzyme activity. The assay buffer comprised of 50 mM Tris HCl, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 30 µg/ml of BSA (pH 7.0) (17). The incubation mixtures consisted of 20 µl of assay buffer containing 2 mM ATP, 0.5 µg histone I, and 0.5 µg of PM2 DNA. Incubation was for 1 hr at 37°C, after which the reaction was stopped by the addition of 1/5 vol of 5% SDS, 25% glycerol, and 0.25% bromophenol blue. For inhibition studies, appropriate dilutions of drugs were added 15 min prior to addition of enzyme.

RESULTS

Congeners of Podophyllotoxin. The structures of the different congeners of podophyllotoxin and their structure-activity relationship for DNA breakage

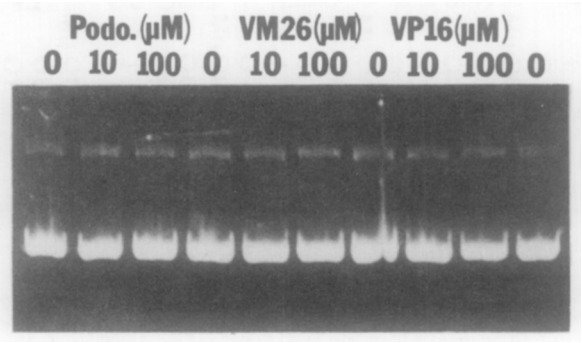

Fig. 1. VP16-213, VM26, and podophyllotoxin have no effect on PM2 DNA *in vitro*. PM2 DNA was incubated with concentrations of the drugs displayed above the lanes for 30 min at 37° in assay buffer, followed by addition of SDS-glycerol-bromophenol blue, electrophoresis in 1% agarose gel, and visualization by UV light of the DNA stained with 1 μg/ml ethidium bromide.

and cytotoxicity have been described (3,4). Congeners selected for this study include Podo and EP, which are highly active as antimicrotubule agents but have no DNA breakage activity; DMEP, which has both antimicrotubule and DNA breakage activities; and VP16-213, VM26, and DMEPBG, which have little antimicrotubule activity but are highly active toward DNA breakage (3,4). Podo, VP16-213 and VM26 had no apparent effect on DNA *in vitro*, an indication that the drugs alone did not break, bind to, or intercalate into DNA (Fig. 1). Under the conditions employed, DNA breakage activity would be reflected by a conversion of form I DNA to forms II and III and binding or intercalation would be indicated by an altered migration of the DNA (18).

Inhibition of DNA Catenation Activity. Purified topoisomerase II catalyzed an ATP-dependent catenation of PM2 DNA *in vitro* that was dependent upon enzyme concentration. Little topoisomerase I activity in this preparation was detected even at the highest enzyme concentrations, as determined by incubation of enzyme with DNA in the absence of ATP and histones (unpublished results).

When congeners of podophyllotoxin were added to the incubation mixture before addition of enzyme, an inhibition of topoisomerase II activity was observed only for those drugs capable of causing DNA breakage *in vivo* (Fig. 2). In the absence of drug, there was an almost complete conversion of form I DNA

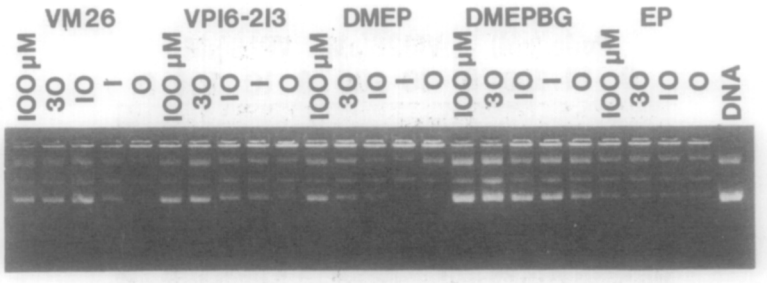

Fig. 2. Effect of different concentrations of congeners of podophyllotoxin on the consumption of form I DNA in the DNA catenation reaction catalized by topoisomerase II. Drug was added to the incubation mixture followed by addition of enzyme. Incubation conditions are described in Materials and Methods and conditions for visualization of the DNA are described in Fig. 1.

to the catenated product (Fig. 2). EP had no effect toward inhibiting this conversion, even at 100 μM, whereas, 1 μM VM, VP, and DMEPBG produced observable inhibition of Form I DNA depletion and 10 to 30 μM DMEP was required to produce the same effect (Fig. 2). Inhibition of enzyme activity was reflected by an inhibition of form I DNA consumption and concommitant inhibition of DNA catenation. The relaxation activity of topoisomerase I was not affected by even the most active drugs, in that no inhibition of DNA relaxation to form I^o DNA was observed either by conducting the reaction in the presence or absence of ATP and histones (unpublished results). These observations expand on a recent report by Ross et al. that adriamycin has no effect on type I topoisomerase (19).

Abilities of the different congeners to inhibit catenation is compared with cytotoxic and DNA breakage activities (3) of these compounds (Fig. 3).

ORDER OF DECREASING ACTIVITY

CYTOTOXICITY

VM26,DMEPBG>EP>VP16-213>DMEP

DNA BREAKAGE

VM26,DMEPBG>VP16-213>DMEP>EP

INHIBITION OF CATENATION

VM26,DMEPBG>VP16-213>DMEP>EP

Fig. 3. Comparison of activities of the different congeners of podophyllotoxin including VP16-213 and VM26 on cytotoxicity, DNA breakage, and inhibition of catenation. Drugs with greatest activity are on the left.

Of the five drugs, only EP failed to display correspondence between cytotoxicity and DNA breakage activity because of its high antimicrotubule activity. However, the ranking of the five drugs is identical for DNA breakage and catenation inhibition activities, suggesting that the inhibition of type II topoisomerase may be related to the DNA breakage and cytotoxicity caused by these drugs.

DISCUSSION

Evidence has been published suggesting that VP16-213 and VM26 produce cytotoxicity by inducing DNA breakage *in vivo* (1-6), which is predominantly double strand DNA breakage (4,5). However, the mechanism by which these breaks are introduced remains undefined. The drugs alone have no effect on DNA *in vitro* (Fig. 2) but probably act through a mechanism involving an enzyme, since DNA breakage is inhibited by incubation of cells with drug at 4^{o} (1,2,7). It is likely that this enzyme is located in the nucleus, since VP16-213 can induce DNA breakage in isolated nuclei (2). These observations, plus the demonstrations that VP16-213-induced DNA breakage activity in isolated nuclei is dependent upon magnesium ions (8) and stimulated by ATP (9) and DNA-protein crosslink formation is associated with DNA break formation (2), are compatible with type II topoisomerase involvement in VP16-213-induced DNA breakage. The observation that VP16-213 causes concommitant formation of DNA-protein crosslinks and DNA breakage in eukaryote cells (2) is similar to reports that oxolinic and nalidixic acids produce DNA-protein crosslinks along with double strand DNA breaks in *E. coli* by inhibiting ligase activity of gyrase *in vivo* (20,21). A covalent association of eukaryote type II topoisomerase with the 5' termini of DNA has recently been reported (22). In addition, type II topoisomerase has recently been shown to cause the introduction of breaks in pBR322 DNA in the presence of mAMSA *in vitro* (23). The results presented here provide the first direct demonstration that eukaryote type II topoisomerase is inhibited by the anticancer drugs VP16-213 and VM26, and thus, may be the primary target of these drugs.

ACKNOWLEDGMENTS

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