Pergamon

Biochemical Pharmacology, Vol. 47, No. 11, pp. 2105–2110, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(93)E0117-P

ANTAGONISTIC EFFECT OF ACLARUBICIN ON CAMPTOTHECIN INDUCED CYTOTOXICITY: ROLE OF TOPOISOMERASE I

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(*Received* 3 *March* 1993; *accepted* 7 *December* 1993)

Abstract—The cellular target of camptothecin and several of its derivatives has been identified as topoisomerase I. Central to the cytotoxic action of camptothecin is the drug's ability to stimulate formation of topoisomerase I mediated DNA cleavages. Here we demonstrate that the intercalating antitumor agent aclarubicin inhibits camptothecin induced DNA single strand breaks in cells as measured by alkaline elution. When purified topoisomerase I was reacted with DNA, aclarubicin inhibited the formation of enzyme mediated DNA breaks induced by camptothecin. High aclarubicin concentrations (10 and 100 μM) caused a slight stimulation of topoisomerase I mediated DNA cleavage at a few distinct DNA sites. The cytotoxicity associated with camptothecin treatment measured in clonogenic assays was antagonized by preincubation with aclarubicin. This inhibitory effect of aclarubicin upon camptothecin action holds implications for the scheduling of aclarubicin in combination therapy with anticancer agents directed against topoisomerase I. Aclarubicin also inhibits the effect of topoisomerase II directed agents [such as etoposide (VP16), amsacrine (mAMSA), etc.] suggesting that aclarubicin acts against the two topoisomerases.

Key words: topoisomerase I; camptothecin; aclarubicin; antagonistic effect

Topoisomerases have been identified as the cellular target of a number of important anticancer agents [1]. Recently, considerable attention has been given to camptothecin and a number of its derivatives of which some have now entered clinical trials [2]. Studies on camptothecin resistant cells have identified topoisomerase I as the cellular target of this drug [3, 4]. Furthermore, a direct interaction between topoisomerase I and camptothecin has been demonstrated by incubation of radioactively labeled drug with the enzyme [5]. Camptothecin affects the interaction between topoisomerase I and DNA thereby leading to an elevated level of topoisomerase I mediated DNA strand breaks [6, 7]. Notable among the effects of camptothecin on mammalian cells are genome fragmentation, inhibition of RNA and DNA synthesis, and S-phase specific cytotoxicity [1]. Topoisomerase II is the cellular target for a number of clinically important anticancer agents (e.g. acridines, epipodophyllotoxins and ellipticines) (for review see Ref. 8). The antitumor activity of several anthracyclines is mediated via an interaction with topoisomerase II [9, 10]. Exposure of cells to anthracyclines such as daunorubicin and Adriamycin® leads to an elevated level of topoisomerase II mediated DNA strand breaks which is believed to be responsible for the induction of cell death [1]. However, profound differences seem to exist among the anthracyclines with respect to their mechanism of action as compounds such as the clinically used anthracycline, aclarubicin, do not show any significant stimulation of topoisomerase II mediated DNA cleavage [11]. These compounds prevent introduction of topoisomerase II mediated DNA strand breaks [12, 13] and effectively antagonize the cytotoxicity of topoisomerase II directed agents such as VP16¶ and mAMSA [12]. In this respect, aclarubicin resembles a novel class of drugs, including the bis(2,6-dioxopiperazine) derivatives [14], and fostriecin [15], which inhibit the introduction of topoisomerase II mediated DNA cleavages. Other agents with strong DNA affinity also inhibit topoisomerase II and antagonize the effect of drugs directed against this enzyme [16]. Antagonism between camptothecin and topoisomerase II directed chemotherapeutic agents has also been described [17]. As much interest is presently focused on drugs targeting topoisomerase I, we have investigated the effect of aclarubicin on camptothecin induced DNA damage. We find that aclarubicin antagonizes the cytotoxicity induced by camptothecin through breaks in DNA. These results are of therapeutic interest with respect to scheduling of aclarubicin in combination with other antitumor agents directed against topoisomerase I.

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¶ Abbreviations: DTT, dithiothreitol; mAMSA, amsacrine, 4′-(9-acridinylamino)metanesulfon-*m*-anisidide; PBS, phosphate-buffered saline (150 mM NaCl, 50 mM phosphate, pH 7.2); TCA, trichloroacetic acid; Tris–HCl, tris(hydroxymethyl)aminomethane; VP16, etoposide, demethylepipodophyllotoxin ethylidene-β-D-glucoside.

MATERIALS AND METHODS

Quantitation of DNA single strand breaks by alkaline elution. DNA damage in cells was assessed by alkaline elution methods on L1210 cells as described previously [18], except that control cells were exposed to 100 μM H_2O_2 for 60 min, corresponding to irradiation with 300 rad as described [19].

Clonogenic assay. Drug induced cytotoxicity was assessed by colony formation in soft agar with a feeder layer containing sheep red blood cells as described previously [20]. The cell lines used in these experiments were the human "wild type" small cell carcinoma of the lung designated OC-NYH [21] and the daunorubicin resistant subline of NCl-H69 designated H69/DAU4 [22]. Single-cell suspensions (2×10^4 cells/mL) in RPMI 1640 supplemented with 10% fetal calf serum were exposed to drugs and then washed twice in PBS at 20°. To obtain 2000–3000 colonies in control dishes 2×10^4 cells were plated. In each experiment the drug combinations were tested on the same batch of cells to reduce the interexperimental variation. Following 3 weeks of incubation the colonies were counted.

Effect of aclarubicin on DNA synthesis. Single cell suspensions of 1.0×10^6 viable cells/mL in a total volume of 2 mL (2.0×10^6 cells) were incubated in medium with aclarubicin or the DNA synthesis inhibitor aphidicolin (the Sigma Chemical Co., St Louis, MO, U.S.A.) for 1 or 24 hr (aphidicolin was employed as a control). The cells were pulse labeled by adding 2 μCi/mL [^{3}H]thymidine (Amersham International, Amersham, U.K.) for the last 10 min of the drug exposure. Subsequently, 2 mL of 10% TCA was added to the cell suspension, the precipitates were spun down, resuspended in 5% TCA and the pellet was solubilized with 0.8 mL 0.5 M KOH at 70° for 1 hr and analysed in a Packard scintillation spectrometer.

Effect of aclarubicin on [^{3}H]camptothecin accumulation. Cell suspensions of 2.5×10^6 viable cells in 2 mL (5×10^6 cells) were incubated for 30 min with DNase I (Sigma) (described in Ref. 23) and then exposed to [^{3}H]camptothecin (Moravek Biochemicals, Brea, CA, U.S.A.) with or without aclarubicin for 60 min in phosphate buffer (57 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 51 mM Na_2HPO_4, 9.0 mM NaH_2PO_4, pH 7.45) to which 5% (v/v) fetal calf serum and 10 mM glucose were added. The cells were then spun down (150 g) for 5 min and washed twice with 10 mL of PBS (4°). The cell pellets were solubilized in 0.8 mL of 0.5 M KOH at 70° for 1 hr and analysed in a Packard scintillation spectrometer.

Topoisomerase I mediated DNA cleavage. Topoisomerase I was purified from Daudi cells according to a previously published procedure [24]. As DNA substrate was used pUC19 DNA liniarized with Hind III. The DNA was 3'-end-labeled at both ends by using the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs Inc., Beverly, MA, U.S.A.) and [α-^{32}P]dATP (Amersham). The labeled DNA fragment was incubated with purified topoisomerase I (100 U) at 30° in a buffer containing 10 mM Tris–HCl (pH 8.0), 3 mM $CaCl_2$, 1 mM DTT, 5% DMSO, 5% glycerol and drug as indicated. The reactions were terminated by addition of SDS to a final concentration of 1%, treated with proteinase K (500 μg/mL) and precipitated with ethanol. Finally, the samples were redissolved in 10 mM Tris–HCl (pH 8.0). After addition of 1 vol. of loading buffer (50% formamide, 0.05% bromophenol blue, 0.03% xylene cyanole, 5 mM EDTA), the samples were analysed by electrophoresis in 6% denaturing polyacrylamide gels as described previously [25]. Fuji RX film was used for autoradiography.

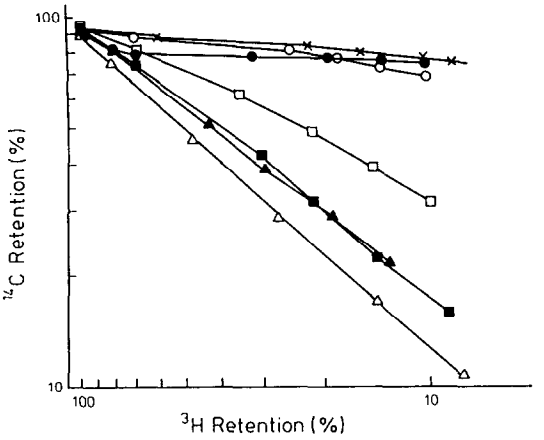

Fig. 1. Cellular DNA degradation associated with camptothecin treatment is prevented by aclarubicin. DNA single strand breaks were measured by alkaline elution technique as described in Materials and Methods. The effect of treating the cells with 1 (▲) or 2 μM (△) camptothecin is illustrated. Incubation with 1 (■) or 5 μM (□) aclarubicin for 10 min before the addition of 2 μM camptothecin antagonized the DNA degradation. Control cells (x) and cells treated with either 1 (●) or 5 μM (○) aclarubicin is included.

RESULTS

Aclarubicin inhibits camptothecin induced DNA single strand breaks

To study the influence of aclarubicin on DNA damage in cells induced by camptothecin, an alkaline elution filter technique was used employing L1210 cells. This cell line is routinely used in these assays because of its high viability [18]. Cells were treated with camptothecin for 1 hr at 37° and examined for production of DNA single strand breaks (Fig. 1), which demonstrates that camptothecin causes extensive DNA single strand breaks at a concentration of 2 μM, whereas a slightly lower level was observed at 1 μM. However, treatment of the cells with 5 μM aclarubicin for 10 min prior to the addition of 2 μM camptothecin abolished camptothecin induced DNA single strand breaks. This effect of aclarubicin was most pronounced at 5 μM but a significant protection could also be observed at 1 μM. Incubation with 1 or 5 μM aclarubicin in the absence of camptothecin did not result in the formation of DNA breaks to any

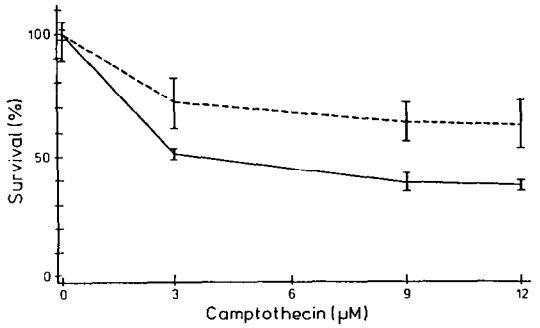

Fig. 2. Aclarubicin antagonizes the cytotoxicity of camptothecin. Dose–response curve obtained by incubating OC-NYH cells with increasing concentrations of camptothecin for 60 min (——). The effect of treatment with 0.5 μM aclarubicin for 20 min before camptothecin addition is illustrated (— — —). The aclarubicin cytotoxicity was normalized to 100% in order to visualize the antagonism in OC-NYH cells (0.5 μM aclarubicin displayed a cytotoxicity of 58% in this cell line). Points = means, bars = SEM from triplicate cultures.

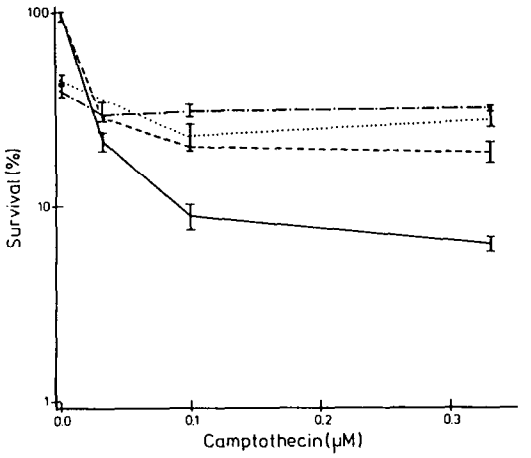

Fig. 3. Aclarubicin prevents the cytotoxicity of camptothecin in the cell line H69/DAU4. Dose–response curves were obtained with cells incubated for 24 hr. Effects of camptothecin alone (——) and in combination with aclarubicin at concentrations of 0.1 (— — —), 0.25 (· · · · ·) or 0.4 μM (· - · - · -) are shown. Points = means, bars = SEM from triplicate cultures.

significant extent (Fig. 1). This was also observed when the concentration of aclarubicin was increased to 10 μM (data not shown).

The cytotoxicity of camptothecin is antagonized by aclarubicin

A clonogenic assay was conducted to evaluate the cytotoxicity of camptothecin in the presence and absence of aclarubicin. Previously an antagonistic effect of aclarubicin on the cytotoxicity induced by topoisomerase II targeting drugs in OC-NYH cells was observed [12]. Thus, OC-NYH cells were incubated with drugs for 1 hr and the drugs were then removed by washing in drug free PBS. The cells were then seeded in agar to assess their colony forming ability. The clonogenic assay demonstrated that treatment of cells with increasing concentrations of camptothecin resulted in 50% cell death at a concentration of 3 μM (Fig. 2). At higher camptothecin concentrations this effect leveled off. Such saturation is in accordance with the distinct S phase specificity of camptothecin induced cell death [1, 26]. Figure 2 shows that aclarubicin protects the OC-NYH cells from the cytotoxicity associated with camptothecin treatment. However, aclarubicin displays by itself a high degree of cytotoxicity in the OC-NYH cells (58% cell death in 0.5 μM aclarubicin) and in order to visualize the antagonism, the toxicity due to aclarubicin itself was subtracted in Fig. 2. In order to increase the cytotoxicity of camptothecin and thereby improve the possibility of demonstrating the effect of aclarubicin it was necessary to prolong the exposure. However, this also increases the cytotoxicity of aclarubicin itself and we therefore took advantage of another cell line where the exposure to aclarubicin was better tolerated. Accordingly, the studies were performed in H69/DAU cells as it has previously been shown that this multidrug resistant cell line is 4-fold resistant to aclarubicin, as compared to its parent cell line, but retains its sensitivity towards camptothecin, [22, 23]. The reduced aclarubicin toxicity in this cell line made it possible to increase the drug exposure time to 24 hr thereby increasing the toxicity of camptothecin (Fig. 3). A clonogenic assay conducted as described for the OC-NYH cells with increasing concentrations of camptothecin for 24 hr resulted in 90% cell death at a concentration of approximately 0.1 μM (Fig. 3). There is a significant antagonistic effect of aclarubicin on the camptothecin induced cytotoxicity. The antagonistic effect of aclarubicin is more pronounced with increasing amounts of aclarubicin. However, toxicity of aclarubicin is not a prerequisite as the non-toxic dose of 0.1 μM aclarubicin also exerts an inhibitory effect on camptothecin induced cell death. The antagonistic effect was also observed at camptothecin concentrations from 0.03 to 3 μM, and aclarubicin concentrations between 0.1 and 0.5 μM (data not shown). The effect of aclarubicin on camptothecin cytotoxicity was also analysed by an isobologram method using the median effect plot principle [27]. The analysis utilized data obtained with four different mixtures of camptothecin and aclarubicin (two 1:10 and two 3:1 mixtures). Computations were performed with the Multiple-drug effect analysis software (Biosoft), which demonstrated an antagonistic effect in all four experiments.

The influence of camptothecin on topoisomerase I mediated DNA cleavage is prevented by aclarubicin

The influence of aclarubicin on the formation of topoisomerase I mediated DNA cleavages observed in the presence of camptothecin was investigated by reacting purified human enzyme with DNA in the presence and absence of these agents. Topoisomerase I induced DNA cleavages were trapped by the addition of 1% SDS. The cleavages introduced in

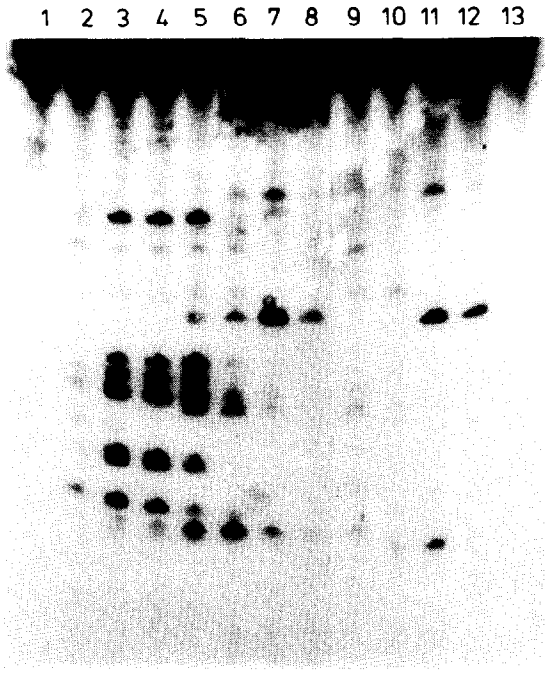

Fig. 4. Aclarubicin prevents the formation of camptothecin induced topoisomerase I mediated breaks in DNA. Purified human topoisomerase I was incubated with radioactively labeled DNA in the absence or presence of drugs. The reactions were terminated by the addition of SDS, and the samples were treated as described in Materials and Methods. Lane 1 shows the DNA substrate without topoisomerase I addition. In lane 2 DNA breaks are generated by topoisomerase I in the absence of drug, and in lane 3 in the presence of 2 μM camptothecin. The effect of pretreatment with aclarubicin at different concentrations before the addition of 2 μM camptothecin is demonstrated [(concentrations are μM) lane 4: 0.1, lane 5: 1, lane 6: 10, lane 7: 100, lane 8: 500]. The effect of aclarubicin on topoisomerase I mediated DNA cleavage in the absence of camptothecin is shown in lanes 9–13 [(concentrations are μM) lane 9: 0.1, lane 10: 1, lane 11: 10, lane 12: 100, lane 13: 500].

the absence of drug are shown in Fig. 4, lane 2. Inclusion of camptothecin in the reaction mixture results in a pronounced stimulation of topoisomerase I mediated DNA cleavage and maximal DNA cleavage was observed with 2 μM camptothecin (Fig. 4, lane 3). Similar experiments in which the reaction mixtures were pretreated with increasing concentrations of aclarubicin before addition of 2 μM camptothecin were performed. This demonstrated that 10 μM aclarubicin prevented the formation of camptothecin induced DNA breaks (Fig. 4, lane 6). When the concentration of aclarubicin was lowered the camptothecin induced topoisomerase I mediated DNA breaks reappeared (Fig. 4, lanes 4 and 5). However, at high aclarubicin concentrations, topoisomerase I mediated DNA cleavage was stimulated at few sites. Incubation of topoisomerase I with aclarubicin in the absence of camptothecin demonstrated that these cleavages were induced by aclarubicin (Fig. 4, lanes 9–13). The stimulatory effect of aclarubicin on topoisomerase I mediated DNA breaks was only observed at high drug concentrations (10 and 100 μM) (Fig. 4, lanes 11 and 12). Further increase in the aclarubicin concentration resulted in inhibition of the enzyme mediated breaks in DNA (Fig. 4, lane 13).

DISCUSSION

Topoisomerase I has previously been identified as the primary cellular target of camptothecin [3, 6, 7]. The involvement of topoisomerase I in camptothecin cytotoxicity has also been demonstrated in a yeast model system where it was shown that cells lacking topoisomerase I were insensitive to camptothecin while introduction of human topoisomerase I restored camptothecin sensitivity [4]. Drug stabilized topoisomerase I–DNA complexes are central to the cytotoxic action of camptothecin in cells [6, 28]. Similarly, a number of clinically important anticancer agents targeting topoisomerase II such as VP16, daunorubicin and Adriamycin have been demonstrated to enhance the level of topoisomerase II–DNA complexes [9, 29, 30]. Recently, much interest has focused on drugs including aclarubicin [12, 31], the bis(2,6-dioxopiperazine) derivatives [14] and fostriecin [15] which inhibit the enzyme without increasing the level of topoisomerase II–DNA cleavable complexes. These agents do not only inhibit topoisomerase II but antagonize also the stimulation of enzyme mediated DNA breaks normally found associated with agents such as VP16 and mAMSA. In this paper we investigated the effect of the clinically used anticancer agent, aclarubicin on the action of the topoisomerase I targeting drug camptothecin and have demonstrated that aclarubicin is an efficient modulator of the cytotoxicity displayed by camptothecin. No inhibitory effect was exerted by aclarubicin on camptothecin accumulation in H69/DAU cells (data not shown), and the antagonism thus seems to occur intracellularly. Aclarubicin protected the cells from the extensive DNA degradation associated with camptothecin treatment, and aclarubicin suppressed the introduction of camptothecin induced DNA breaks generated by purified human topoisomerase I. This inhibition of camptothecin induced DNA breaks mediated by aclarubicin may thus represent the basis for the inhibition of camptothecin cytotoxicity. However, aclarubicin has also been found to be an efficient inhibitor of RNA and DNA synthesis. This suggests an alternative mechanism of cellular protection from camptothecin cytotoxicity, as it has previously been demonstrated that inhibition of either RNA or DNA synthesis protects cells from the cytotoxicity induced by drugs directed against topoisomerase I and II [26, 32, 33]. However, studies on the effect of aclarubicin on DNA synthesis showed that aclarubicin at concentrations of 0.1 and 1.0 μM only reduced the DNA synthesis 10 and 25%, respectively, in H69/DAU cells. Thus, the results demonstrate that the aclarubicin concentrations which antagonize the cytotoxicity of camptothecin have virtually no effect on DNA synthesis. Irrespective of the mechanism of action

the finding reported here is that the clinically employed anticancer drug aclarubicin inhibits the effect of camptothecin. This observation holds important implications for using these two drugs in combination.

Aclarubicin at high concentrations caused an enhancement of topoisomerase I mediated DNA cleavage at a few sites when purified topoisomerase I was employed. However, no DNA single strand breaks were generated by aclarubicin at concentrations up to 10 μM as determined by alkaline elution assays. A stimulation of topoisomerase I and II activity by distamycin has been observed and it has been suggested to be caused by local alterations in the structure of DNA by this compound which binds to the minor groove of the double helix [34]. Such changes in DNA structure induced by aclarubicin might also explain the enhancement of topoisomerase I mediated DNA breaks observed at a few sites. Aclarubicin has been reported to preferentially inhibit ribosomal gene transcription mediated by RNA polymerase I [35]. Topoisomerase I is enriched in the nucleolus where it seems to be tightly associated with RNA polymerase I [36] and with actively transcribing genes [37–39]. Thus, the profound influence of aclarubicin on topoisomerase I activity presented here could explain the inhibition of rRNA synthesis displayed by aclarubicin, as has also been suggested for other drugs affecting topoisomerase I [40].

The inhibition of camptothecin action by aclarubicin resembles the effect of the minor groove binding compounds distamycin, Hoechst 33258, and 4′,6-diamidino-2-phenylindole which also affect topoisomerase I and II and their interactions with drugs [41–44]. DNA binding studies of several anthracyclines suggest that aclarubicin binds to the minor groove via the three sugar rings of this drug [45, 46]. Furthermore like the minor groove binders aclarubicin inhibits the cytotoxicity of both topoisomerase I targeting drugs (this study) and topoisomerase II targeting drugs [12]. This effect of these DNA binding drugs on camptothecin action is contrasted by the bis(2,6-dioxopiperazine) derivatives [14], which specifically inhibit topoisomerase II and have no effect on topoisomerase I. We are currently investigating whether these differences between various topoisomerase I and II inhibitors have any impact on the sensitivity patterns in cell lines with different forms of drug resistance. There is great clinical interest in topoisomerase I directed drugs as they offer a new target in cancer chemotherapy. However, the present study demonstrates that simultaneous administration of aclarubicin and agents targeting topoisomerase I to patients may be unfavorable.

Acknowledgements—We wish to thank Dr Ole Westergaard for helpful discussions and Kirsten Andersen, Eva Høj and Annette Nielsen for excellent technical assistance. This work was supported by contract BI-6-0171-DK EURATOM, CEC, Brussels, the Danish Cancer Society, the Aarhus University Bioregulation Centre, the Carlsberg Foundation, the Danish Natural Science Research Council (11-5724/12-6011), the Aarhus University Research Foundation, the Thaysen Foundation, the Danish Insurance Association and the Lundbeck Foundation.

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