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The effect of staurosporine on drug-induced, topoisomerase II-mediated DNA cleavage in human leukemia cells

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Summary. Phorbol-12-myristate 13-acetate (PMA), a stimulator of protein kinase C, dramatically decreased topoisomerase II-reactive drug-induced DNA cleavage in HL-60 human leukemia cells. The effect of staurosporine, an inhibitor of protein kinase C, on drug-induced, topoisomerase II-mediated DNA cleavage was quantified in the same cells. Staurosporine decreased the magnitude of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)- and etoposide-induced DNA cleavage in a dose- and time-dependent fashion. Measurement of several parameters of cell proliferation revealed no clear and uniform correlation between staurosporine's inhibition of these parameters and its effects on drug-induced DNA cleavage. A direct comparison with PMA's effects on drug-induced DNA cleavage showed that whereas PMA's inhibition of etoposide-induced cleavage was much greater than its inhibition of *m*-AMSA-induced cleavage, the magnitude of staurosporine's effect on the cleavage produced by the two topoisomerase II-reactive drugs was similar. Thus, although PMA stimulates protein kinase C and staurosporine inhibits this enzyme, it is unlikely that the actions of either on topoisomerase II-reactive, drug-induced DNA cleavage are mediated directly via protein kinase C. Furthermore, it is likely that the mechanisms by which PMA and staurosporine inhibit topoisomerase II-reactive drug-induced cleavage are different.

Abbreviations: *m*-AMSA, 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide; etoposide, VP-16, 4′-demethylepipodiphyllotoxin 4-(4,6-*O*-ethylidene-β-D-glucopyranoside); STSN, staurosporine; PMA, phorbol-12-myristate 13-acetate; DMSO, dimethylsulfoxide; AD, adherent cells; SN, supernatant cells

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Introduction

The enzyme topoisomerase II plays a crucial role in chromosomal segregation and gene transcription [11]. It is also the target of a number of active antineoplastic drugs, including etoposide, Adriamycin, and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) [7]. Factors regulating the expression of this enzyme and its role in other cellular processes have not been completely described.

Recently, we reported the results of a series of experiments aimed at establishing a cellular system in which the role of topoisomerase II in cellular differentiation could be explored [20, 22, 23]. This was an outgrowth of our observation that phorbol-12-myristate 13-acetate (PMA)-induced monocytoid differentiation [but not dimethylsulfoxide (DMSO)-induced granulocytoid differentiation] of human leukemia cells in culture was accompanied by dramatic decreases in drug-induced topoisomerase II-mediated DNA cleavage. Because the receptor for PMA is protein kinase C [8, 9] and because protein kinase C has been shown to alter topoisomerase II activity in isolated biochemical systems [12, 13], we began to study the action of other drugs affecting protein kinase C on topoisomerase II-mediated events.

Staurosporine is a potent but nonspecific inhibitor of protein kinase C [10, 16, 17]. It probably interferes with the action of the enzyme by binding to it, but the precise mechanism is unknown [2, 3, 10]. This report describes the actions of staurosporine on drug-induced, topoisomerase II-mediated DNA cleavage. Rather than producing the opposite effects of those produced by PMA, staurosporine also decreased topoisomerase II-reactive drug-induced DNA cleavage. However, significant differences between the actions of PMA and those of staurosporine suggest that the mechanism by which each causes the inhibition of topoisomerase II-reactive drug-induced DNA cleavage differs.

Materials and methods

Staurosporine was initially given to us by Dr. H. Nakano of Kyowa Hakko Kogyo, Ltd., Tokyo, Japan. Further amounts of the drug were obtained from Calbiochem Corporation (San Diego, Calif.); a 10^{-3}-M stock was constituted in 100% DMSO. PMA was supplied by LC Services Corporation (Woburn, Mass.); a 10^{-2}-M stock solution was constituted in 100% DMSO. Etoposide and *m*-AMSA were constituted and used as previously described [21].

Two lines of HL-60 human leukemia cells were used in this study. The first (Tables 1–3) was a gift from Dr. R. Hall, Guthrie Research Institute (Sayre, Pa.) [1, 6]. The second (Figs. 1, 2) was a line we previously described that was obtained from ATCC (see [20]). The effects of staurosporine were somewhat greater in the first cell line, but this line became spontaneously unresponsive to the actions of staurosporine to produce cellular adherence prior to our performing the experiments shown in Fig. 1 and 2, thus necessitating the use of another HL-60 line. The cells were propagated and radiolabeled for alkaline elution as previously described [20, 22, 23].

DNA cleavage was quantified using the alkaline elution method of Kohn et al. [5], with murine leukemia L1210 cells serving as internal standards as previously described [19]. Measurement of DNA synthesis was carried out as previously described [20]. The percentage of cells in S phase was quantified using flow cytometric analysis of DNA content as previously described [18].

Results

Staurosporine (30 nM for 24 h) decreased the DNA cleavage produced by a 1-h treatment with either *m*-AMSA or etoposide (Table 1). Prolongation of the staurosporine exposure to 48 h magnified this decrease and produced some adherence of the cells to the plastic surface of the tissue-culture flasks (Table 1). The cleavage decrease was greater in the adherent cells than in the supernatant cells.

We have previously shown that phorbol ester treatment can also lower topoisomerase II-reactive drug-induced DNA cleavage in HL-60 cells [20, 22, 23]. The decrease following PMA treatment was always greater for etoposide-induced cleavage than for *m*-AMSA-induced cleavage [20, 22]. Following incubation with staurosporine, however, the difference between the effects produced by these two drugs was not as dramatic (Table 1). This suggests that staurosporine's effects on topoisomerase II-reactive drug-induced DNA cleavage might be mediated by mechanisms different from those that produce PMA's effects.

The data shown in Tables 2 and 3 and in Figs. 1 and 2 were generated in an attempt to explain the effects of staurosporine on drug-induced DNA cleavage and to compare these effects with those of PMA. The data from each of the experiments for which results are displayed in Table 2 were obtained from aliquots of one group of cells on the same day. No pooled data were involved. The previously reported drug-specific difference in the effect of 10 nM PMA on DNA cleavage was seen. The lower concentration of PMA had a weaker effect on DNA cleavage as well as on the other parameters quantified [20]. The DNA of cells that were exposed to staurosporine also displayed a decreased susceptibility to drug-induced cleavage, but the magnitude of that decrease was similar for *m*-AMSA- and etoposide-induced cleavage. Neither the amount of DNA synthesis inhibition, the amount of adherence, the minimal inhibition of cell growth, nor the alteration of the cell-cycle stage distribution predicted the magnitude of DNA cleavage decline in any uniform fashion (Table 2; Figs. 1, 2).

A more extensive examination using three concentrations of staurosporine (Table 3) *did* show a dose-response effect for staurosporine on *m*-AMSA- and etoposide-induced DNA cleavage; however, again we detected no

Table 1. The effect of 30 nM staurosporine on the DNA cleavage produced by 1 h incubation with 0.5 μM *m*-AMSA or 5 μM etoposide in HL-60 human leukemia cells

Duration of staurosporine exposure (h)	*m*-AMSA	Etoposide
24	59.7±8.4 ($n=4$)	49.6±15.2 ($n=5$)
48 (Adherent cells)	14.3±2.5 ($n=5$)	7.3± 3 ($n=6$)
48 (Supernatant cells)	27.9±9.6 ($n=5$)	23.7± 9.8 ($n=6$)

Data represent mean values ± 1 SD of the ratios of the drug-induced DNA cleavage frequency (expressed in rad-equivalents) [20, 22, 23] produced in cells that were exposed to 30 nM staurosporine divided by the cleavage frequency produced in cells that were not exposed to staurosporine (expressed as a percentage). At the 48-h time point, adherent and supernatant cells were separated prior to the 1-h drug treatment. *n*, Number of independent determinations

Table 2. The effect of phorbol ester or staurosporine treatment on drug-induced, topoisomerase II-mediated DNA cleavage and its relationship to cell adherence, DNA synthesis, cell growth, and cell-cycle-phase distribution

	PMA, 10 nM for 24 h (AD)	PMA, 1 nM for 24 h (AD)	STSN, 30 nM for 48 h	
			(AD)	(SN)
Experiment 1:				
m-AMSA cleavage[a]	38.3	49.6	16.8	21.2
Etoposide cleavage	9.2	39.7	8.1	15.6
% Adherence	89	10	24	
DNA synthesis (% control)[b]	8 ±1	66 ±1	5 ±1	6 ±1
Fraction of control growth	0.74	1.25	0.64	
Experiment 2:				
m-AMSA cleavage	25.7	65.8	10.7	43.2
Etoposide cleavage	8.3	41.7	11.7	42
% Adherence	38	6	41	
DNA synthesis (% control)[b]	3 ±1	96 ±18	40 ±7	52 ±11
% Cells in S phase[c]	9	25	14	21

[a] As in Table 1. These data came from experiments in which all of the parameters were quantified on aliquots of cells from a single PMA or staurosporine treatment. PMA, Phorbol-12-myristate 13-acetate; STSN, staurosporine; AD, adherent cells; SN, supernatant cells

[b] Means of 3 independent determinations

[c] Control values were 25%–33% of cells in S phase

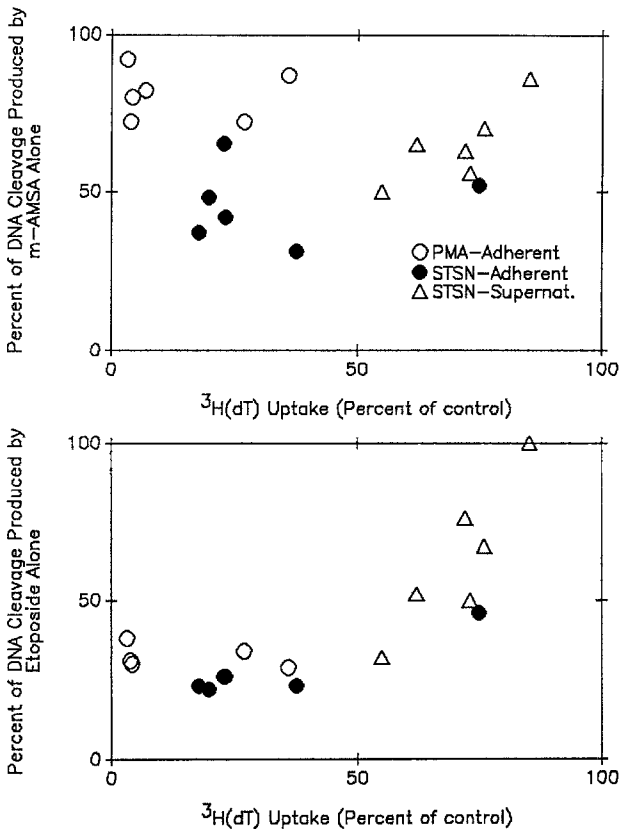

Fig. 1. The absence of a uniform relationship between the inhibition of DNA synthesis and the inhibition of *m*-AMSA- (*top*) or etoposide-induced DNA cleavage (*bottom*) in human leukemia HL-60 cells following their exposure to PMA or staurosporine. Cells were exposed to various concentrations of PMA (1, 2, or 10 nM; *open circles*) for 24 h or to staurosporine (10, 20, or 30 nM; *closed circles*, adherent cells; *open triangles*, supernatant cells) for 48 h followed by a 1-h treatment with either 0.5 μM *m*-AMSA or 5 μM etoposide. DNA cleavage was quantified by alkaline elution with proteinase and was expressed as the percentage of the DNA cleavage produced by *m*-AMSA or etoposide in cells that were not exposed to PMA or staurosporine. Additionally, tritiated thymidine ([^{3}H]-dT) uptake was quantified in an aliquot of the PMA- or staurosporine-treated cells (see [20]. Measurements were made on either adherent or supernatant cell populations

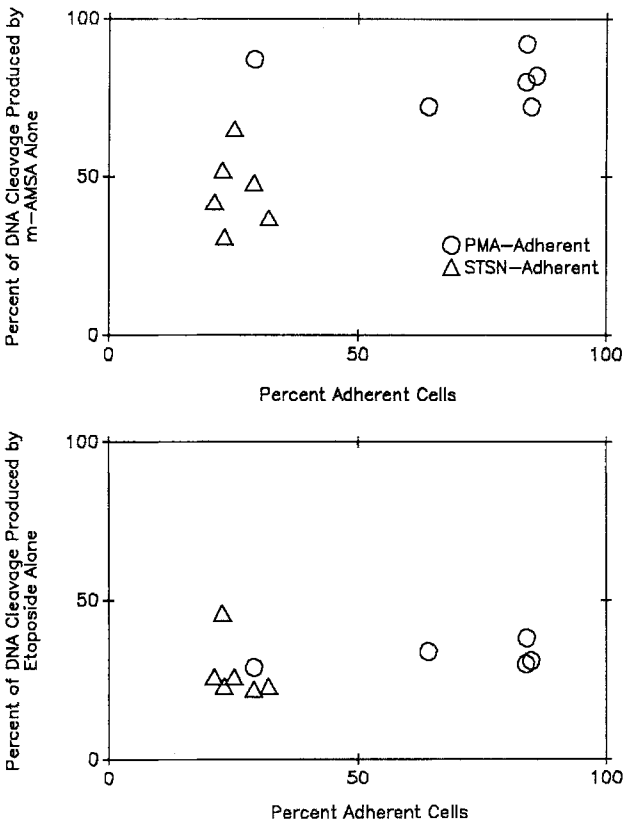

Fig. 2. The absence of a uniform relationship between the amount of adherence produced by PMA or staurosporine and the inhibition of *m*-AMSA- (*top*) or etoposide-induced DNA cleavage (*bottom*). These data came from the same experiments that produced the results shown in Fig. 1. The amount of cleavage inhibition is expressed as a percentage of the cleavage produced by *m*-AMSA or etoposide in cells that were not exposed to PMA or staurosporine

simple uniform relationship between the various measurements of growth inhibition and the degree to which staurosporine decreased topoisomerase II-reactive drug-induced DNA cleavage.

Discussion

We have demonstrated that the ability of phorbol ester treatment to decrease HL-60 topoisomerase II-mediated DNA cleavage in a drug-specific manner is associated with a modest decrease in topoisomerase II activity, an alteration of the structure of immunoreactive topoisomerase II, and a decrease in the steady-state levels of topoisomerase II mRNA [22]. These effects on a line of HL-60 cells that exhibited a proclivity toward monocytoid differentiation were stronger than those on a line that lacked such a proclivity [22].

Initial results obtained in the HL-60 system [20] caused us to hypothesize that the phorbol-induced decline in topoisomerase II-mediated cleavage occurred after the phorbol-induced signal to differentiate. Thus, a mechanistic connection between an early protein kinase C-mediated phosphorylation event (e.g., of topoisomerase II) and the decline of drug-induced cleavage was considered to be unlikely. However, we recently used bryostatin 1, a protein kinase C stimulator that does not induce monocytoid differentiation of HL-60 and that can interfere with the differentiating action of PMA, to uncouple the effects of PMA on DNA cleavage and on differentiation [23]. Bryostatin 1 could prevent adherence when it was added as late as at 6 h after PMA treatment, whereas the PMA-induced decline in etoposide-induced cleavage escaped bryostatin 1 reversal much earlier. These findings suggested that the PMA *signal* that produced the decline in DNA cleavage actually occurred prior to the PMA *signal* to adhere and, presumably, to differentiate. However, since bryostatin 1 itself exerted a minimal effect on drug-induced, topoisomerase II-mediated DNA cleavage, stimulation of protein kinase C alone did not seem likely to be the direct cause of the decline in DNA cleavage.

The present study supports the idea that an effect on protein kinase C per se is unlikely to be a direct modulator of the action of topoisomerase II-reactive drugs in cells. Staurosporine, an inhibitor of protein kinase C [10, 16] and other kinases [16, 17], did not increase drug-induced DNA

Table 3. The effect of various concentrations of staurosporine on drug-induced, topoisomerase II-mediated DNA cleavage and its relationship to cell adherence, DNA synthesis, cell growth, and cell-cycle-phase distribution

Staurosporine exposure (concentration × time)	DNA cleavage[a]		Adherence (%)	DNA synthesis[a] (% control)	Fraction of control growth	% in S phase[a]
	m-AMSA	Etoposide				
30 nM × 24 h	62.3 ±8 ($n = 3$)	47 ±16.2 ($n = 4$)	0	62±5	0.83	30
20 nM × 24 h	61.8 ±7.7 ($n = 3$)	55.1 ±9.7 ($n = 3$)	0	75±8	1.02	30
10 nM × 24 h	72.4 ±9.6 ($n = 4$)	65.7 ±11.7 ($n = 5$)	0	81±3	1.12	35
30 nM × 48 h (AD)	14.4 ($n = 2$)	6.4 ±2.8 ($n = 3$)	38 ±18 ($n = 6$)	17±3	0.78	16
20 nM × 48 h (AD)	7 ($n = 2$)	4.7 ($n = 2$)	32 ($n = 2$)	17±4	0.89	15
10 nM × 48 h (AD)	21.6 ±7.6 ($n = 3$)	11 ±2.2 ($n = 4$)	17 ±12 ($n = 4$)	39±4	1.07	24
30 nM × 48 h (SN)	21.8 ($n = 2$)	22.9 ±3 ($n = 3$)		64±3		23
20 nM × 48 h (SN)	34.2 ($n = 2$)	37.7 ($n = 2$)		92±3		17
10 nM × 48 h (SN)	43.2 ±13 ($n = 3$)	54.4 ±15.1 ($n = 4$)		98±8		34

These data include measurements of DNA cleavage from several staurosporine dose-response experiments along with other measurements from separate experiments. Adherence was calculated from all experiments done on this cell line

[a] As in Tables 1 and 2. If fewer than 3 determinations were involved, the mean is shown without the SD

cleavage. In fact, the opposite was true. However, unlike the effects of phorbol esters, which decreased etoposide-induced DNA cleavage to a greater extent than *m*-AMSA-induced cleavage, the magnitude of staurosporine's effects on the actions of the two topoisomerase II-reactive drugs was similar. This suggests that although both PMA and staurosporine act on protein kinase C and decrease drug-induced, topoisomerase II-mediated DNA cleavage, they probably produce cleavage reduction via different, albeit unexplained, molecular mechanisms. Simple relationships between the effects of staurosporine on DNA cleavage and its effects on some common growth parameters did not reveal a clear and uniform correlation and thus did not aid in identifying potential mechanisms. However, it is possible that growth inhibition and cell-cycle-stage perturbations played some role in the actions of staurosporine on drug-induced DNA cleavage mediated by a proliferation-associated enzyme such as topoisomerase II.

The effects of staurosporine are not limited to those on protein kinase C [10]. Staurosporine has been shown to enhance the uptake of vincristine in P-glycoprotein-containing multidrug-resistant cells [14]. Additionally, staurosporine can enhance the antiproliferative activity of cisplatin and nitrogen mustard [4]. It is thus conceivable that at some future date, staurosporine or its analogs may be considered for combination with more conventional chemotherapeutic agents, particularly in light of the former's recently described inhibition of tumor invasion [15]. As judged from the present data, however, topoisomerase II-reactive drugs may not be among the best choices for combination with staurosporine.

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