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Cytotoxic properties of a new synthetic demethylpodophyllotoxin derivative, BN 58705, against human tumor cell lines*

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Abstract. The in vitro cytotoxic properties of a newly synthesized demethylpodophyllotoxin derivative, 4-*o*-butanoyl-4′-demethylpodophyllotoxin (BN 58705), were determined by using several human tumor cell lines of different histological origin and of different sensitivity to conventional chemotherapeutic drugs (Adriamycin and *cis*-diammine-dichloride platinum). BN 58705 is shown to be cytotoxic against various human tumor cell lines as assessed by the MTT assay. Furthermore, BN 58705 is shown to be cytotoxic against several drug-resistant tumor cell lines. BN 58705 is cytotoxic at concentrations 100- to 1000-fold lower than those of Adriamycin or *cis*-diammine-dichloride platinum required to achieve similar cytotoxicity. BN 58705 did not mediate DNA fragmentation of target cells, whereas the epipodophyllotoxin-like etoposide induced DNA cleavage by stabilizing the DNA-enzyme intermediate. Like vinca alkaloids, BN 58705 induced a block in the mitotic phase of the cell cycle. By comparison, BN 58705 exerted a stronger cytotoxic activity in vitro than did either etoposide, an epipodophyllotoxin, or vincristine, a vinca alkaloid. When BN 58705 was applied in vivo in mice, it resulted in low toxicity (50% lethal dose, 150 mg/kg). These results demonstrate than BN 58705 is cytotoxic to drug-resistant human tumor cell lines and is manyfold more potent than conventional drugs. The cytotoxic potency and low toxicity of BN 58705 are important criteria to establish its potential chemotherapeutic efficacy in vivo.

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Abbreviations: cpm, counts per minute; BN 58705, 4-*o*-butanoyl-4′-demethylpodophyllotoxin; MTT,3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide; OD, optical density; TRIS, TRIS (hydroxymethyl) aminomethane; EDTA, ethylenediaminetetraacetic acid; FITC, flouresceinisothiocyanate; PI, propidium iodide

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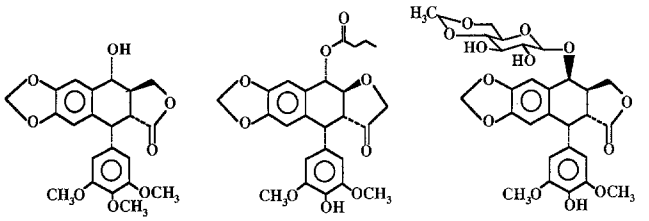
Introduction

Plant alkaloids are naturally occurring nitrogenous bases. The cytotoxic alkaloid derivatives of the pink periwinkle, vinca alkaloids, such as vincristine and vinblastine, arrest cell division during mitosis through their ability to bind to the mitotic spindle [7]. The active principle podophyllotoxin, derived from the roots and rhizomes of the may-apple or mandrake plant, has also been found to be antimitotic agent that binds to tubulin at a site distinct from that occupied by the vinca alkaloids [1]. Thus, several podophyllotoxin derivatives have been synthesized and tested to determine whether a relationship exists between their chemical structure and their antineoplastic activity [9–12]. The epipodophyllotoxins, glycosidic derivatives of podophyllotoxin such as etoposide and teniposide, have been reported to exert stronger antitumor activity than the other derivatives tested thus far. For instance, etoposide is currently used in the treatment of some malignancies, including lymphomas and small-cell lung cancer [2, 18, 20], although it is not very effective against several other tumors. Teniposide remains a research investigative drug, although its activity against childhood acute lymphocytic leukemia is well established [21]. Accordingly, there is considerable effort to synthesize new derivatives that encompass a wide spectrum of antineoplastic activity and thus overcome the limitations of currently used epipodophyllotoxins.

The present study examined the cytotoxic activity of a new demethylpodophyllotoxin derivative, BN 58705. The antitumor cytotoxic activity of BN 58705 in vitro was investigated against a variety of human tumor cell lines of different sensitivity to conventional chemotherapeutic drugs. Furthermore, several properties of the BN 58705-mediated cytotoxic activity were compared with those mediated by etoposide and vincristine.

Materials and methods

Reagents. BN 58705, 4-*o*-butanoyl-4′-demethylpodophyllotoxin, was synthesized at Seripharm, France, and was provided by the Institut Henri



PODOPHYLLOTOXIN BN 58705 ETOPSIDE (VP-16)

Fig. 1. Chemical structures of podophyllotoxin, BN 58705, and etoposide. BN 58705 is 4-*o*-butanoyl-4′-demethylpodophyllotoxin (470.05 Da) and belongs to the family of demethylpodophyllotoxins

Beaufour, Le Plessis Robinson, France. The structures of BN 58705 and etoposide as well as their parent compound, podophyllotoxin, are illustrated in Fig. 1. After its reconstitution with absolute ethanol, BN 58705 was stored at 200 μ*M* at 4° C. Prior to its use, BN 58705 was dissolved in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1% L-glutamine (Gibco), 1% pyruvate (Gibco), 1% nonessential amino acids (Gibco), and 1% Fungibact solution (Irvine Scientific), which contains 10,000 U penicillin/G/ml, 10 mg streptomycin sulfate/ml, and 25 μg Fungizone/ml. Etoposide (Sigma), vincristine (Sigma), and podophyllotoxin (Sigma) were reconstituted with phosphate-buffered saline at 200 μ*M* and 2 m*M*, respectively. Prior to their use, etoposide and vincristine were diluted in the medium described above to achieve the desired final concentrations. The MTT dye, 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide, was purchased from Sigma and was stored dry at 4° C. For use, it was reconstituted at 5 mg/ml in phosphate-buffered saline. Isopropyl alcohol (Sigma) was acidified with 0.05 *N* hydrochloride. Trig(Hydroxymethyl)aminomethane (TRIS), Triton X-100, Trizma-hydrochloride, sodium chloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma. Nonidet P40, fluorescein isothiocyanate (FITC), and propidium iodide (PI) were purchased from Sigma and stock solutions of 1 mg/ml were prepared.

Cells. A total of 13 human tumor cell lines (see Table 1) were maintained in the medium described above and were employed in the present studies. The SKOV-3, Raji, HT29, and U937 lines were obtained from the American Type Culture Collection (Rockville). The other lines were derived from different sources: A2780, AD10, and C30, from Dr. R. F. Ozols, Philadelphia; M14 and C2, from Dr. S. Golub of UCLA; U251 and 226-P59, from Dr. E. Grimm, Houston; and 222 and 222TR, from Dr. J. Zighelboim of UCLA. These tumor lines have different histological origins and different sensitivities to chemotherapeutic drugs. The AD10 line is a multidrug-resistance phenotype-expressing subline of A2780 and expresses gp170 [13]. The C30 line is a cisplatin-resistant subline of A2780 but does not express the gp170 multidrug-resistance phenotype. The 222TR line is a tumor necrosis factor-resistant subline of 222 [17]. All lines except U937 and Raji were maintained as adherent cultures. U937 and Raji were cultured in suspension. For use, the adherent lines in subconfluent states were trypsinized, washed, and resuspended in medium. The nonadherent cells were washed and resuspended in the medium for use.

MTT cytotoxicity assay. Cytotoxicity was assessed by the MTT assay [6]. Briefly, target tumor cells were resuspended in the medium at 1 million cells/ml after verification of the number and viability of the cells by the trypan blue (Sigma) dye exclusion test. In all, 100 μl cell suspension was distributed into each well of a 96-well flat-bottom microtiter plate (Costar). The plate was incubated in a humidified incubator with 5% CO_2 added at 37° C overnight to allow cells to adhere. The next day, another 100 μl of either the reagent solutions at desired concentrations or the medium alone (positive control) were distributed into each well; 200 μl of the medium alone without cells or reagents were also distributed for the negative or zeroing control. The microtiter plates were incubated for 72 h as described above unless otherwise mentioned. Then, 20 μl of the MTT dye solution was added to each well, including the positive and negative control wells, at 4 h before the end of incubation. The MTT dye, a yellow tetrazolium salt, is reduced by a live cell by mitochondrial dehydrogenases forming a purple formazan [6, 24]. The unreactive MTT dye and the reagent solution in wells were carefully aspirated, and 100 μl of the acidified isopropyl alcohol was added to each well to solubilize the reactive dye. All assays were set up in triplicate. The optical density (OD) values for each well were read at 540 nm using an automatic multiwell spectrophotometer (Titertek Multiskan MCC/340, Flow Laboratories). The negative control wells (medium alone) were used for zeroing of the OD. The percentage of cytotoxicity was calculated using the background-corrected OD as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{OD of experimental well}}{\text{OD of positive control well}}, \times 100.$$

The mean value for triplicate determinations was used for analysis.

Table 1. BN 58705-mediated cytotoxicity against various human tumor cell lines

Tumor line	Histology	% Cytotoxicity					
		BN 58705		Adriamycin		*cis*-diammine-dichloride platinum	
		0.01 μ*M*	0.1 μ*M*	0.1 μ*M*	1 μ*M*	0.1 μ*M*	1 μ*M*
222	Ovarian cancer	41.4±3.5	92.3±4.3	57.6±3.5	83.7±5.5	13.5±2.1	61.4±3.7
222TR	Ovarian cancer	42.8±2.9	93.6±4.5	51.5±4.1	74.7±5.8	8.3±1.5	55.0±3.4
SKOV-3	Ovarian cancer	35.5±3.9	85.3±2.8	14.5±4.1	30.4±3.3	9.5±1.8	18.2±2.5
A2780	Ovarian cancer	45.3±2.5	95.7±3.5	33.2±2.9	65.0±4.6	30.7±3.4	75.6±5.8
AD10	Ovarian cancer	38.6±3.1	76.3±2.6	0.3±0.2	10.7±1.3	0.7±0.3	15.0±2.2
C30	Ovarian cancer	30.7±2.7	80.1±3.7	11.5±1.7	31.5±2.6	0	0.8±0.3
M14	Melanoma	33.8±3.0	75.7±3.1	ND	ND	ND	ND
U251	Brain tumor	17.4±1.5	73.3±2.9	ND	ND	ND	ND
C2	Colon cancer	40.7±3.3	85.7±4.1	25.7±3.5	66.3±5.1	3.5±1.3	35.6±3.0
226-P59	Lung cancer	25.7±3.0	68.6±2.6	ND	ND	ND	ND
Raji	B-cell lymphoma	37.7±2.6	88.2±3.5	25.3±2.7	73.3±4.2	8.7±2.1	35.0±3.6
U937	Promonomyelocytic lymphoma	48.7±3.3	93.7±4.0	38.6±1.5	85.3±3.8	3.5±1.1	53.9±4.5

The cytotoxicity by BN 58705, Adriamycin, and cisplatin was tested at different concentrations as measured in a 72-h MTT assay and is expressed as mean values ± SD. ND, Not determined

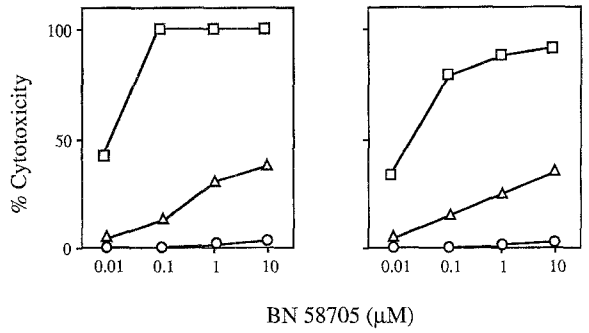


Fig. 2. Kinetics of BN 58705-mediated cytotoxicity. BN 57705-mediated cytotoxicity was measured using the MTT assays for 4 (○), 24 (△), and 72 h (□) of incubation. Each point represents the mean value for triplicate determinations. *Left panel*, the A2780 cell line; *right panel*, the AD10 cell line, a multidrug-resistant subline of A2780 expressing the gp 170 phenotype

The MTT assay relies on the ability of viable cells to reduce tetrazolium to formazans, and the OD value measured is a function of cell number per well, although it may be subject to other conditions [22]. For example, the OD values significantly correlated with the number of tumor cells in the tumor systems examined, namely:

$\log[OD] = 0.524 \times \log[\text{cell number}] - 2.712$ ($r = 0.993$) for 222 cells,
$\log[OD] = 0.537 \times \log[\text{cell number}] - 2.735$ ($r = 0.993$) for 222TR cells, and
$\log[OD] = 0.596 \times \log[\text{cell number}] - 2.964$ ($r = 0.994$) for SKOV-3 cells.

Therefore, the values for percentage of inhibition obtained in the MTT assay represent both the extent of cytolysis achieved following a short-term incubation in which cells remain undivided and the cytostatic activity following a long-term incubation in which most cells proliferate.

DNA fragmentation. DNA fragmentation in target cells was determined as described elsewhere [5, 23, 25], with some modifications [16]. Tumor cells were labeled with 740 kBq [^{125}I]-deoxyuridine (NEN, Dupont) for 24 h at 37° C. Labeled cells were washed three times, resuspended in BN 58705 or etoposide solutions at designated concentrations (or in the medium alone for controls) in a polypropylene tube (Falcon, Becton Dickinson), and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37° C for designated times. At the end of the incubation period, the cells were pelleted by centrifugation (400 *g*, 10 min) and the supernatants were removed (supernatant 1). The cells were lysed in 1 ml hypotonic lysing buffer (10 m*M* TRIS, 1 m*M* EDTA, pH 7.5) containing 0.2% Triton X-100. The lysates were centrifuged (13,000 *g*, 15 min) and the resulting supernatants (supernatant 2) and pellets as well as supernatants 1 were measured for radioactivity using a gamma-counter (Gamma 4000, Beckman). The assay was set up in triplicate. The background radioactivity was subtracted from each sample. The percentage of fragmented DNA and the percentage of specific fragmented DNA for both spontaneous (or control) and experimental conditions were calculated as follows:

$$\%\ \text{Fragmented DNA} = \frac{\text{supernatant 1 cpm} + \text{supernatant 2 cpm}}{\text{supernatant 1 cpm} + \text{supernatant 2 cpm} + \text{pellet cpm}} \times 100.$$

The percentage of specific fragmented DNA was calculated as follows:

$$\%\ \text{Specific fragmented DNA} = \frac{\text{experimental \% fragmented} - \text{spontaneous \% fragmented}}{100 - \text{spontaneous \% fragmented}} \times 100,$$

where *experimental % fragmented* represents the percentage of fragmented DNA in the reagent solutions and *spontaneous % fragmented* represents the percentage of fragmented DNA in the medium alone.

Fragmentation of cell-free DNA was determined using a similar method, with some modifications. Labeled cells were lysed with a hypotonic lysing buffer as described above and were then incubated with the testing reagents (or with the medium for spontaneous DNA fragmentation). Following incubation, the samples were centrifuged (13,000 *g*, 15 min) and both the supernatants and the pellets were collected separately and measured for radioactivity. The background radioactivity was subtracted from each sample. The assay was set up in triplicate. The percentage of DNA fragmentation and the percentage of specific fragmented DNA were calculated as follows:

$$\%\ \text{Fragmented DNA} = \frac{\text{supernatant cpm}}{\text{supernatant cpm} + \text{pellet cpm}} \times 100 \text{ and}$$

$$\%\ \text{Specific fragmented DNA} = \frac{\text{experimental \% fragmented} - \text{spontaneous \% fragmented}}{100 - \text{spontaneous \% fragmented}} \times 100,$$

where *experimental % fragmented* represents the percentage of fragmented DNA in the reagent solutions and *spontaneous % fragmented* represents the percentage of fragmented DNA in the medium alone.

Cell-cycle analysis. The interference by BN 58705 with tumor cell kinetics was studied using a dual-parameter flow-cytometric analysis for nuclear protein versus DNA content [19]. HT29 human colonic cancer cells in their exponential phase of growth were treated with 6–24 μg BN 58705 or vinblastine/ml at 37° C for 18 h. Prior to staining for the flow-cytometric analysis, treated and untreated HT29 cells were trypsinized, washed twice with phosphate-buffered saline, and then resuspended at 1 million cells/ml in nuclear isolation buffer (0.5% Nonidet P40, 0.05 *M* Trizmahydrochloride pH 7.4, 0.05 *M* sodium chloride, 1.0 m*M* EDTA). Staining was carried out by adding to each sample sodium chloride-bicarbonate buffer (pH 8.1), FITC, and PI. Nuclear suspensions were filtered through nylon meshes before being analyzed with a Fac-

Table 2. Failure of BN 58705 to mediate DNA fragmentation of tumor cells

Drug	Concentration	% Specific DNA fragmentation			
		DNA in cells		Cell-free DNA	
		4 h	7 h	4 h	7 h
BN 58705	0.1 μ*M*	0.3 ± 0.2	0.5 ± 0.3	0.2 ± 0.1	0.4 ± 0.3
	10 μ*M*	1.0 ± 0.3	2.1 ± 0.5	0.6 ± 0.4	1.0 ± 0.5
Etoposide	0.1 μ*M*	8.8 ± 1.2	32.0 ± 3.3	1.3 ± 0.7	1.1 ± 0.6
	10 μ*M*	20.8 ± 2.7	62.7 ± 5.3	0.9 ± 0.6	2.1 ± 0.7

DNA breakdown of the 222 line was measured using [^{125}I]-deoxyuridine as described in Materials and methods, and the degree of DNA breakdown was expressed in percentage of specific DNA fragmentation (mean values ± SD). DNA breakdown of cell-free DNA was determined as described in Materials and methods

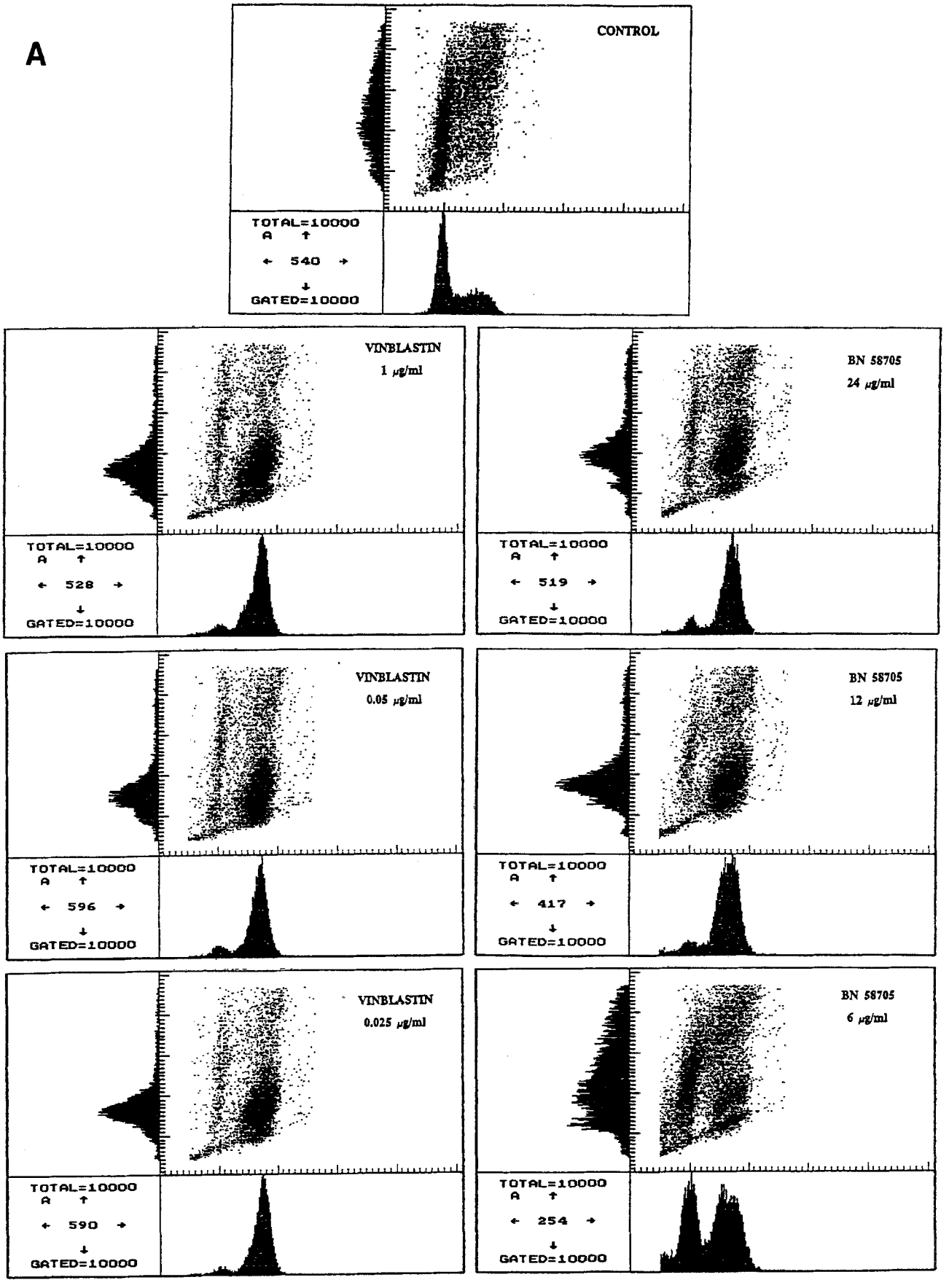

A
CONTROL
TOTAL=10000
A
540
GATED=10000
VINBLASTIN
1 μg/ml
TOTAL=10000
A
528
GATED=10000
BN 58705
24 μg/ml
TOTAL=10000
A
519
GATED=10000
VINBLASTIN
0.05 μg/ml
TOTAL=10000
A
596
GATED=10000
BN 58705
12 μg/ml
TOTAL=10000
A
417
GATED=10000
VINBLASTIN
0.025 μg/ml
TOTAL=10000
A
590
GATED=10000
BN 58705
6 μg/ml
TOTAL=10000
A
254
GATED=10000

Fig. 3A

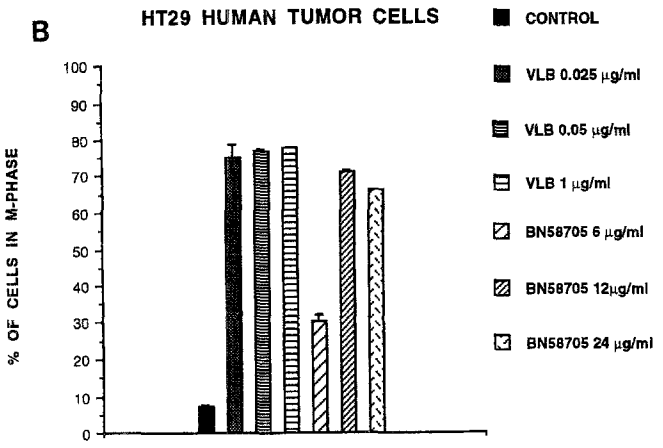


Fig. 3 A, B. Flow-cytometric analysis of the effect of BN 58705 on the cell cycle. The cytograms represent the simultaneous flow-cytometric analysis of nuclear protein (ordinate) versus DNA content (abscissa). **A** The cytograms are derived from the flow-cytometric measurement of nuclear protein (ordinate; FITC fluorescence) versus DNA content (abscissa; PI fluorescence). In each cytogram the monoparametric distributions relative to each fluorochrome are reported. The *upper panel* refers to untreated HT29 cells. The *left panels* show the bivariate distributions obtained with decreasing concentrations of a known antimiotic agent, vinblastine. The *right panels* correspond to BN 58705. **B** Quantitative analysis of the data obtained from the flow-cytometric procedure. The percentage of mitotic cells (ordinate) is reported for each drug concentration tested. *VBL*, Vinblastine

star + flow cytometer (Becton-Dickinson). The latter was equipped with a 2-W argon-ion Laser operating at 250 mW and emitting at a wavelength of 488 nm. The FITC (green) fluorescence, directly proportional to the nuclear protein content, was collected with a 530 ± 30 nm dichroic filter; the PI (red) fluorescence, directly proportional to the nuclear DNA content, was collected with a 630 ± 30 nm dichroic filter.

Toxicity of BN 58705 in mice. Various amounts of BN 58705 were reconstituted in 50 µl 100% ethanol and applied intraperitoneally into groups of adult (8–10 weeks) SJL/J mice (Jackson Laboratories, Maine) weighing 20–25 g each. Controls were given 50 µl 100% ethanol alone. The animals were observed daily for 3 days after the injection of five different amounts of BN 58705 (50, 100, 150, 200, and 250 mg/kg). Each group consisted of 5–10 mice, and mortality was recorded.

Results

Cytotoxicity of BN 58705 in various human tumor cell lines

Initial experiments were designed to determine the cytotoxic effect of different concentrations of BN 58705 against several human tumor cell lines. BN 58705 was found to be cytotoxic against all 12 tumor cell lines tested (Table 1). However, depending on the tumor cell line tested, there were some differences in their sensitivity to different concentrations of BN 58705. Clearly, for all the lines, the concentrations of BN 58705 needed to kill the cells were lower than those of either Adriamycin or cisplatin. Furthermore, the Adriamycin- and/or cisplatin-resistant lines were sensitive to BN 58705. For instance, the AD10 line, which is resistant to Adriamycin and expresses the gp170 multidrug-resistance (MDR) phenotype, was sensitive to concentrations of 0.01 µ*M* BN 58705, whereas 10 µ*M* Adriamycin (1,000-fold concentration) was needed to achieve the same cytotoxicity. The same pattern was seen for the cisplatin-resistant line C30. These studies demonstrate that BN 58705 is cytotoxic against a variety of human tumor cell lines of different histological origin, regardless of whether the tumor cells were sensitive or resistant to conventional cytotoxic chemotherapeutic drugs. Furthermore, the concentration of BN 58705 needed to achieve significant cytotoxic activity was 10–1,000 times lower than those of Adriamycin or cisplatin.

Time kinetics of BN 58705-mediated cytotoxicity

Experiments were designed to determine the time kinetics of BN 58705-mediated cytotoxicity at different concentrations (10 n*M* to 10 µ*M*) against the Adriamycin-sensitive A2780 ovarian cell line and the Adriamycin-resistant AD10 cell line. As shown in Fig. 2, cytotoxicity was not observed following 4 h incubation, but significant cytotoxicity was detected following 24 h incubation and reached a higher level after 72 h incubation. The cytotoxicity of BN 58705 at ≥ 100 n*M* was >99% when the incubation period was extended to 120 h. The cell-doubling time for A2780 and AD10 was 16.4 and 28.8 h, respectively. The absence of cytotoxicity by BN 58705 at 4 h suggests that BN 58705 does not damage the target cell membrane directly.

Failure of BN 58705 to mediate DNA fragmentation of tumor cells

Compounds related to BN 58705, such as the epipodophyllotoxins, have been reported to induce topoisomerase II-mediated DNA cleavage of target cells. The following experiment was designed to determine whether BN 58705 can also mediate DNA fragmentation of target tumor cells. Unlike etoposide, BN 58705 did not mediate significant DNA fragmentation of a sensitive 222 tumor cell line (Table 2). Similar findings were obtained with other cell lines treated with these two agents. Furthermore, there was no sign of blebbing or nuclear condensations that accompany apoptotic cell death following treatment of tumor cells by BN 58705. In tests against free DNA, no nuclease activity was detected as has been shown for diphtheria toxin [4]. These results demonstrate that lysis of tumor cells by BN 58705 is not the result of apoptosis or programmed cell death.

BN 58705-induced cell-cycle block in the mitotic phase

The interference of BN 58705 with tumor cell-growth kinetics was studied using a dual-parameter flow-cytometric analysis of nuclear protein versus DNA content,

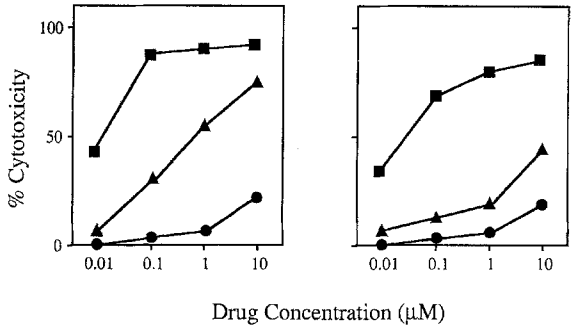


Fig. 4. Cytotoxic activity of BN 58705, etoposide, and vincristine. The cytotoxicity of BN 58705 (■), etoposide (▲), and vincristine (●) was measured using a 72-h MTT assay. Each point represents the mean value for triplicate determinations. *Left panel,* the A2780 cell line; *right panel,* the AD10 cell line, a multidrug-resistant subline of A2780 expressing gp 170

and BN 58705 was compared with vinblastine, a known mitotic inhibitor. HT29 human tumor cells in their exponential phase of growth were treated at 37° C for 18 h with different concentrations of BN 58705 and vinblastine as described in Materials and methods. The results of a representative experiment are shown in Fig. 3 and show both the flow-cytometric analyses (Fig. 3A) and the percentage of cells in the M phase (Fig. 3B) for each sample analyzed. The data clearly show that BN 58705 blocks the mitotic phase of the cell cycle as compared with a known inhibitor, vinblastine. For a fixed incubation time, 12 μg BN 58705/ml gave the maximal blocking effect.

Comparison of BN 58705-mediated cytotoxicity with that mediated by etoposide and vincristine

BN 58705, a demethylpodophyllotoxin derivative, and etoposide, an epipodophyllotoxin, are derivatives of podophyllotoxin and are related structurally, although the cytotoxic mechanisms of these two compounds are different (Table 2). Vincristine, however, shares some properties with BN 58705, as both are mitotic inhibitors. Thus, the cytotoxic activity of BN 58705 was compared with those mediated by etoposide and vincristine. BN 58705 exerted a more potent cytotoxic activity than did etoposide or vincristine against the drug-sensitive A2780 line and the Adriamycin-resistant AD10 cell line (Fig. 4). Against these cell lines, vincristine was not effective, even at high concentrations of the drug. Etoposide was minimally effective at high concentrations against the AD10 cells, which express the gp 170 MDR phenotype. These results together with those shown in Table 1 indicate that BN 58705 is a more potent cytotoxic agent against various human tumor cell lines, including tumor cells refractory to conventional chemotherapeutic drugs such as Adriamycin, cisplatin,

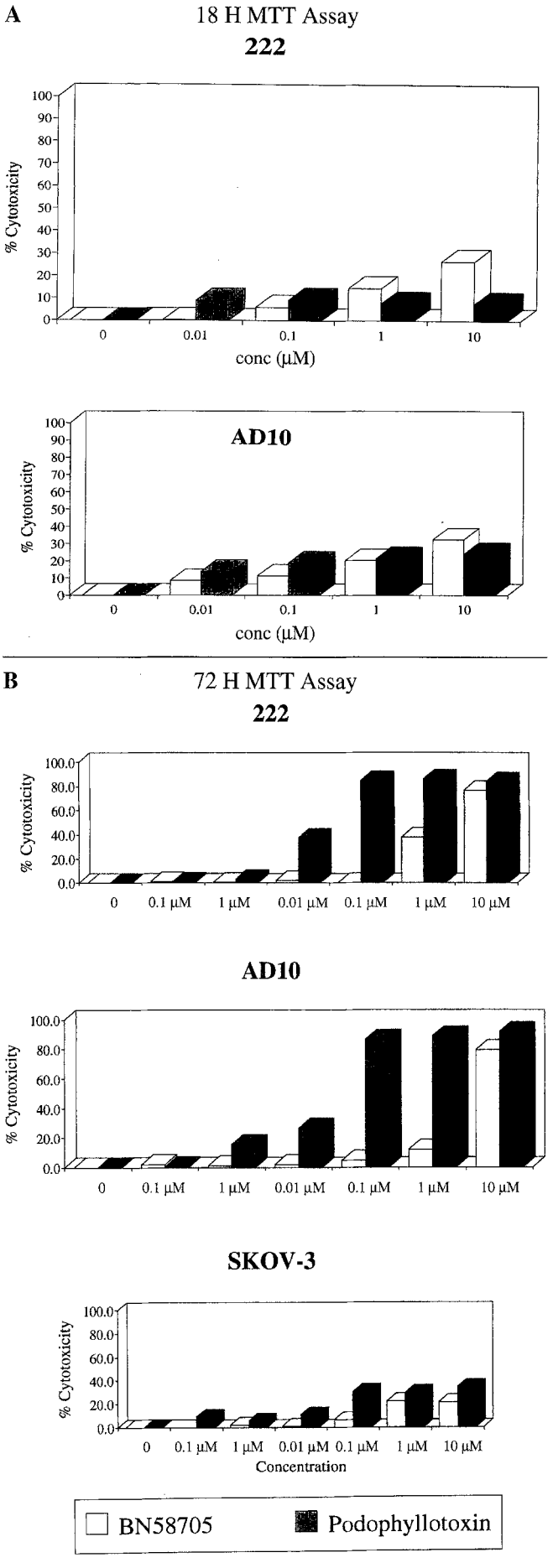


Fig. 5 A, B. Cytotoxic activity of BN 58705 and podophyllotoxin. The cytotoxic activity was determined by the MTT assay in 24-h (**A**) or 72-h (**B**) cultures

Table 3. In vivo toxicity of BN 58705 in mice[a]

Amount of BN 58705 (mg/kg)	Morbidity (number of deaths total)
0	0/ 5
50	0/ 7
100	1/ 8
150	5/10
200	6/ 8
250	6/ 7

[a] SJ/J mice 8–10 weeks old were injected i. p. with different concentrations of BN 58705 and were observed daily for 3 days for survival

vincristine, and etoposide. Furthermore, unlike other chemotherapeutic drugs, significant cytotoxicity was achieved by BN 58705 at low concentrations.

Comparison between BN 58705 and podophyllotoxin

The cytotoxic activity of BN 58705 and podophyllotoxin was tested against cell lines that were either sensitive or resistant to Adriamycin (see Table 1). The MTT assay was run for 24 and 72 h. The results are shown in Fig. 5. As indicated in Fig. 5A, BN 58705 is more cytotoxic than podophyllotoxin in the 24-h MTT assay. However, at 72 h, podophyllotoxin was more sensitive than BN 58705 at drug concentrations of <1 μM.

Toxicity of BN 58705 in mice

Several groups of normal mice were inoculated with different amounts of BN 58705 and the mice were observed for 3 days for survival. The results are shown in Table 3. The 50% lethal dose (LD_{50}) was estimated to be approximately 150 mg/kg. The intraperitoneal administration of 50 µl 100% ethanol solvent (control) into mice did not result in any death. The LD_{20} values for other drugs have been reported to be 30 mg/kg for etoposide, 8 mg/kg for cisplatin and 8 mg/kg for Adriamycin [14], whereas the LD_{20} for BN 58705 is >100 mg/kg. Thus, the low in vivo toxicity of BN 58705 together with the strong in vitro cytotoxicity of the compound suggest that BN 58705 may be potent in the treatment of tumor-bearing animals.

Discussion

The present study demonstrates that a newly synthesized demethylpodophyllotoxin derivative, BN 58705, is endowed with potent cytotoxic activity against a broad spectrum of sensitive and resistant human tumor cell lines, including ovarian carcinoma, lung cancer, and lymphomas. The cytotoxic activity of BN 58705 was time-dependent and reached optimal activity following >72 h incubation. BN 58705 was found to share similar time kinetics in the killing of target tumor cells with other cytotoxic chemotherapeutic drugs such as etoposide and vincristine. Several properties unique to BN 58705 were established. BN 58705 was found to be cytotoxic to both drug-sensitive and drug-resistant tumor cell lines. BN 58705-mediated cytotoxicity was achieved at concentrations 10–1,000 times lower than those required of the other drugs tested. As compared with a related compound such as the epipodophyllotoxin etoposide, BN 58705 was shown to be a more potent cytotoxic agent and was capable of killing etoposide-resistant tumor cells. The in vivo toxicity of BN 58705 in mice was low. These findings establish that BN 58705 is a new cytotoxic agent that has the potential to be effective against drug-resistant tumor cells and is endowed with poor murine toxicity in vivo.

Epipodophyllotoxins such as etoposide were originally believed to exert their antineoplastic activity as microtubule inhibitors, as has been found for the parent compound podophyllotoxin [9]. Although tubulin binding is common to all the alkaloids, it is not essential for their antineoplastic activity [1]. Epipodophyllotoxins bind to tubulin with a low affinity, and their antineoplastic activity has been shown to be unrelated to their antimitotic activity [10]. It has been confirmed that epipodophyllotoxins induce topoisomerase II-mediated DNA cleavage by stabilizing the DNA-enzyme complex, resulting in DNA fragmentation [10, 15, 22, 26]. The absence of DNA fragmentation by BN 58705 suggests that the cytotoxicity of the compound is independent of this process. Thus, the mechanism of BN 58705-mediated cytotoxicity appears to be different from that mediated by epipodophyllotoxins.

BN 58705 arrests the cells at a specific phase of the cell cycle, as do the vinca alkaloids [1, 7] and the parent compound podophyllotoxin [8]. The colchicine and vinca alkaloids, such as vincristine, do not significantly affect each other's binding to tubulin, and both compounds can be bound simultaneously [3]. Most antimitotic agents, including the podophyllotoxins, bind at the colchicine site. This observation is in keeping with the description of at least four distinct binding sites on the tubulin molecule [1]. Although the accurate binding site of BN 58705 to tubulin has not been studied, BN 58705 appears to inhibit microtubule assembly, thereby blocking the formation of the mitotic spindle and causing an accumulation of cells in mitosis. The absence of DNA breakdown by BN 58705 may result from the nonepimerization of BN 58705 at the C-ring structure as in etoposide, which is epimerized and mediates DNA breakdown in target cells. BN 58705 may share a similar propertiy of cell-cycle arrest with both the podophyllotoxin and the demethylpodophyllotoxin. These three compounds have the same structure at a C-ring portion of the molecule. Since BN 58705 is an ester, it may be subject to de-esterification by esterases within the cells. These esterases convert it into an analog of demethylpodophyllotoxin, which in turn results in blockage of the cell cycle.

It has been demonstrated that some podophyllotoxin derivatives have little antitumor activity in vivo in spite of their stronger in vitro cytotoxicity as compared with etoposide, an epipodophyllotoxin [1]. However, the LD_{50} value for BN 58705 in mice was approximately 150 mg/kg. This compound has a relatively low in vivo toxicity as compared with other drugs and may have a

wider range between its maximal tolerable dose and its minimal effective dose when used in vivo. Its low toxicity together with its strong in vitro antineoplastic activity suggests that BN 58705 may be considered a potent and attractive antitumor drug.

BN 58705 exhibited more potent cytotoxic activity against several tumor lines tested than did etoposide or vincristine. In addition, our findings indicate that BN 58705 is cytotoxic to Adriamycin-, cisplatin-, and other drug-resistant tumor cells that either express or do not express the phenotype of gp170 MDR. Thus, BN 58705 may be considered a useful drug against tumor cells refractory to conventional drugs.

In summary, BN 58705 is endowed with potent antineoplastic activity against various human tumor lines of various histological origin through an antimitotic mechanism like that of the parent compound podophyllotoxin. However, BN 58705 exhibits properties different from those of a related compound such as etoposide. Current studies are examining the in vivo therapeutic efficacy of BN 58705 in nude mice bearing human tumor xenografts. These studies will help to establish the potential therapeutic use of BN 58705 in human cancer therapy.

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References

1. Bender RA, Hamel E, Hande KR (1990) Plant alkaloids. In: Chabner BA, Collins JM (eds) Cancer chemotherapy. J. B. Lippincott, New York, pp 253–275
2. Bosl G, Yagoda A, Golbey RB, Whitmore W Jr, Herr H, Sogani P, Morse M, Vogelzang N, MacDonald G (1985) Role of etoposide-based chemotherapy in the treatment of patients with refractory or relapsing germ cell tumors. Am J Med 78: 423–428
3. Bryan J (1972) Definition of three classes of binding sites in isolated microtubule crystals. Biochemistry 11: 2611–2616
4. Chang MP, Baldwin RL, Bruce C, Wisnieski BJ (1989) Second cytotoxic pathway of diphtheria toxin suggested by nuclease activity. Science 246: 1165–1168
5. Duke RC, Chervenak R, Cohen JJ (1983) Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. Proc Natl Acad Sci USA 80: 6361–6365
6. Green LM, Redde JL, Ware CF (1984) Rapid coloremetric assay: application to the quantitation of cytotoxic and growth inhibitory lymphokines. J Immunol Methods 70: 257–268
7. Johnson IS, Armstrong JG, Groman M, Burnett JP Jr (1963) The vinca alkaloids: a new class of oncolytic agents. Cancer Res 23: 1390–1427
8. Krishan A, Paika K, Frei E (1975) Cytofluorometric studies on the action of podophyllotoxins and epipodophyllotoxins (VM 26, VP 16213) on the cell cycle transverse of human lymphoblasts. J Cell Biol 66: 521–530
9. Loike JD (1982) VP-16-213 and podophyllotoxin: a study on the relationship seen in chemical structure and biological activity. Cancer Chemother Pharmacol 7: 103–111
10. Loike JD, Horwitz SB (1976) Effect of podophyllotoxin and VP-16 on microtubule assembly in vitro and nucleoside transport in HeLa cells. Biochemistry 15: 5435–5442
11. Loike JD, Horwitz SB (1976) Effects of VP-16-213 on the intracellular degradation of DNA in HeLa cells. Biochemistry 15: 5443–5448
12. Long BH, Musial ST, Brattain MG (1984) Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP-16-213 and VM-26: a quantitative structure activity relationship. Biochemistry 23: 1183–1188
13. Louie KG, Hamilton TC, Winker MA, Behrens BC, Tsuruo T, Klecker RW Jr, Mckoy WM, Grotzinger KR, Myers CE, Young RC, Ozols RF (1986) Adriamycin accumulation and metabolism in Adriamycin-sensitive and -resistant human ovarian cancer cell lines. Biochem Pharmacol 35: 467–472
14. Mattern J, Bak M, Hahn EW, Volur M (1988) Human tumor xenografts as model for drug testing. Cancer Metastasis Rev 7: 263–284
15. Minocha A, Long B (1984) Inhibition of the DNA catenation activity of type II topoisomerase by VP-16-213 and VM-26. Biochem Biophys Res Commun 122: 165–170
16. Morimoto H, Safrit J, Bonavida B (1991) Synergistic effect of tumor necrosis factor- and diphtheria toxin-mediated cytotoxicity in sensitive and resistant human ovarian tumor cell lines. J Immunol 147: 2609–2616
17. Nio Y, Zighelboim J, Berek J, Bonavida B (1990) Sensitivity of ovarian tumor cells to effector cells generated by various biological response modifiers. Nat Immun Cell Growth Regul 9: 283–296
18. O'Dwyer PJ, Leyland-Jones B, Alonso MT, Marsoni S, Wittes RE (1985) Etoposide (VP-16-213): current status on an active anticancer drug. N Engl J Med 312: 692–697
19. Pollack A, Moulis H, Block NL, Irvin GL III (1984) Quantitation of cell kinetic responses using simultaneous flow cytometric measurements of DNA and nuclear protein. Cytometry 5: 473–481
20. Radice PA, Bunn PA, Ihde DC (1979) Therapeutic trials with VP-16-213 and VM-26: active agents in small cell lung cancer, non-Hodgkin's lymphomas and other malignancies. Cancer Treat Rep 63: 1231–1239
21. Rivera G, Dahl GV, Bowman WP, Avery TL, Wood A, Aur RJ (1980) VM26 and cytosine arabinoside combination chemotherapy for initial induction failures in childhood lymphocytic leukemia. Cancer 46: 1727–1730
22. Ross W, Rowe T, Glisson B, Yalowich J, Liu L (1984) The role of topoisomerase II in mediating epipodophyllotoxin-induced cleavage. Cancer Res 44: 5857–5860
23. Schmid DS, Tite JP, Ruddle NH (1986) DNA fragmentation: manifestation of target cell destruction mediated by cytotoxic T cells, lymphotoxin-secreting T cell clones and cell-free lymphotoxin-containing supernatant. Proc Natl Acad Sci USA 83: 1881–1885
24. Vistica DT, Skehan P, Scudiero DA, Monks A, Pittman A, Boyd MR (1991) Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. Cancer Res 51: 2515–2520
25. Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555–556
26. Yang L, Rowe TC, Lui LF (1985) Identification of DNA topoisomerase II as an intracellular target of anti tumor epipodophyllotoxins in simian virus 40-infected monkey cells. Cancer Res 45: 5872–5876