Estrogen-induced Potentiation of DNA Damage and Cytotoxicity in Human Breast Cancer Cells Treated with Topoisomerase II-interactive Antitumor Drugs

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ABSTRACT

Hormone stimulation of responsive neoplasms is a potential strategy for improving the target selectivity of cancer chemotherapy. Using an alkaline DNA-unwinding technique to detect drug-induced DNA strand breakage, we have shown that estrogen stimulation of T-47D human breast cancer cells enhances induction of DNA cleavage by etoposide (VP-16), 4',9-acridinylaminomethanesulfon-*m*-anisidide (m-AMSA), mitoxantrone, and doxorubicin, drugs known to interact with the DNA-modifying enzyme topoisomerase II. No enhancement of DNA cleavage or cytotoxicity was seen in estrogen-treated cells exposed to X-rays or bleomycin. Novobiocin (an inhibitor of topoisomerase II) markedly antagonized the enhancing effect of estrogen on VP-16-induced DNA cleavage, while neutral nucleoid sedimentation detected less than 10% of such strand breaks revealed in estrogen-treated cells by alkaline unwinding. Estrogen did not affect DNA repair of lesions induced by X-rays, VP-16, or ultraviolet radiation. Enhancement of DNA cleavage was accompanied by a corresponding enhancement of cytotoxicity in cells treated with VP-16 or m-AMSA, but only minimal enhancement of cytotoxicity was seen following treatment with mitoxantrone or doxorubicin. Estrogen-treated and control cells treated with VP-16 and m-AMSA sustained similar levels of DNA cleavage for equivalent levels of cytotoxicity. These findings suggest that estrogen potentiates the cytotoxicity of VP-16 and m-AMSA by enhancing topoisomerase II-mediated DNA damage but that such "damage" does not contribute significantly to cytotoxicity induced by mitoxantrone or doxorubicin. Estrogen stimulation of receptor-positive breast cancer may prove to be a clinically relevant strategy for improving the selectivity and cytotoxicity of some, but not all, topoisomerase II-interactive drugs.

INTRODUCTION

Estrogen has been reported to enhance the cytotoxicity of DNA synthesis inhibitors *in vitro* (1, 2), and the idea of "priming" tamoxifen-synchronized cells with estrogen prior to cytotoxic therapy has since proved attractive enough to warrant clinical trials (3–5). The initial promise of this approach has not, however, been fulfilled (6, 7). Estrogen has also been reported to enhance DNA damage induced in estrogen-sensitive cells by m-AMSA[2] (8), an intercalating antitumor drug which appears to act by stabilizing a "cleavable complex" of DNA and topoisomerase II (9). This latter enzyme has been implicated in numerous cellular processes, including gene transcription (10), DNA replication (11), and DNA recombination (12). Proliferating neoplastic cells exhibit markedly elevated topoisomerase II activity (13–16), raising the possibility that drugs interacting with this enzyme could be selectively potentiated by estrogen stimulation of hormone-sensitive target cells. However, a central question underlying such a strategy concerns the extent to which topoisomerase II-mediated DNA cleavage contributes to the cytotoxicity of a given chemotherapeutic agent.

VP-16, a podophyllotoxin derivative, is believed to interact directly with topoisomerase II (17), while doxorubicin (Adriamycin) and mitoxantrone, two DNA-intercalating drugs commonly used in the clinical management of breast cancer, are also known to interact with topoisomerase II *in vitro* (18). Here we compare the DNA-damaging capacity and cytotoxicity of VP-16 and m-AMSA with that of mitoxantrone and doxorubicin, and we examine the way in which estrogen modulates these responses in receptor-positive human breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Hormone Stimulation

Derivation and characteristics of cell lines are presented in Table 1. Cell stocks were maintained as monolayer cultures in RPMI plus 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (subsequently referred to as complete medium) and were incubated at 37°C in 5% CO_2 in air. Prior to hormonal manipulation cells were maintained in RPMI plus 5% dextran-charcoal-stripped (23) fetal calf serum, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, pH 7.3 (BDH Chemicals, Parkstone, Poole, United Kingdom), glutamine, and antibiotics for at least 3 weeks. By using the dextran-charcoal stripping technique twice in succession, more than 99% of tritiated estradiol (Amersham, Aylesbury, Buckinghamshire, United Kingdom) was consistently removed; direct radio-immunoassay (using ¹²⁵I-estradiol and rabbit anti-estradiol bound to a solid phase; Steranti Research, Ltd., St. Albans, United Kingdom) of ether extracts confirmed that the final concentration of estradiol in medium was less than 25 fM. Cells were able to be passaged in charcoal-stripped medium for more than 6 months without discernible change in growth characteristics but were generally used after 3 to 6 weeks of estrogen deprivation. Estrogen receptors were measured by a whole-cell equilibrium binding technique (24), and binding maxima were calculated with the Cambridge IBM 3081 computer using Harwell Library routine VBOIA. 17β-Estradiol (Sigma, Poole, Dorset, United Kingdom) was stored at 0°C as 10^{-5} M stock ethanol and added to medium at a final concentration of 10^{-8} M in 0.1% ethanol 24 h prior to experiments involving hormone stimulation, while control samples received 0.1% ethanol alone.

Growth Curves and Cytotoxicity

Following trypsin/EDTA detachment, cells were seeded into 6-cm Petri dishes at an initial density of 5×10^4 cells/plate and incubated for 48 h prior to medium changing. Either estrogen or ethanol alone was added to the medium 24 h before experiments. Cell-doubling times were calculated from log linear growth curves. Cytotoxicity was evaluated by growing T-47D cells in complete medium for 5 days following

Table 1 *Characteristics of human breast cancer cell lines*

Cell line	Ref.	Passage numbers	Estrogen receptors (fmol/μg DNA)	T_D/h (control)	T_D/h (estrogen)
T-47D[a]	19	84–95	1.94	120	60
ZR-75-1[a]	20	85–90	1.50	84	54
MCF-7[b]	21	288–295	4.65	31	24
SK-Br-3[a]	22	28–34	0.20	118	122

[a] Supplied by American Type Culture Collection, Rockville MD.
[b] Supplied by Dr. R. Skilton, St George's Hospital, Middlesex London.

experimental treatment, a technique which we[3] and others (25) have previously found to correlate with clonogenic survival of human breast cancer cells; using this technique, the total number of cell doublings differs between estrogen-treated and control cells by less than 5% (since control cells refed with complete medium rapidly reach an identical maximal growth rate to that of cells primed with estrogen). Following 24 h incubation with either estrogen or ethanol, samples were treated and then washed twice in warmed phosphate-buffered saline (in g/liter: NaCl, 8.0; KCl, 0.2; Na$_2$HPO$_4$, 1.15; KH$_2$PO$_4$, 0.2, pH 7.3) supplemented with calcium and magnesium before being refed with complete medium for 5 days of growth. Cell samples were counted in triplicate using a Coulter Counter following detachment with 0.01% EDTA and 0.25% trypsin.

Drug Treatment and X-Irradiation

VP-16 (Bristol-Myers Pharmaceuticals, Syracuse, NY) was stored as a 34 mM stock solution in the dark at room temperature. Mitoxantrone (Lederle, Pearl River, NY) was stored as a 100-μg/ml stock solution and doxorubicin (Adriamycin; Farmitalia, Barnet, Hertfordshire, United Kingdom) as a 500-μg/ml stock solution at $-20°C$. m-AMSA (Parke-Davis, Eastleigh, Hampshire, United Kingdom) was stored as a 15 mM stock solution in dimethyl sulfoxide at $-20°C$ in the dark. Bleomycin sulfate (Lot U9U10AS8; Nippon Kayaku Co., Tokyo, Japan) was a gift of Lundbeck, Ltd. (Luton, United Kindgom) with potency originally assayed as 1.7 mg/mg solid; manufacturer's analysis revealed that 68.7% of the preparation was bleomycin A$_2$ and that copper content was less than 0.008%. Novobiocin (Sigma) was prepared as a 100 mM stock solution in water immediately prior to use and added to samples at a final concentration of 1 mM for 1 h prior to VP-16 treatment. All drugs used in experiments were diluted in water and added to samples at various concentrations for 1 h at 37°C. Following drug treatment, monolayers were washed twice at 4°C and then layered with buffer (10 mM Tris-HCl-100 mM NaCl-10 mM EDTA-1 mg/ml bovine serum albumin, pH 8.0) prior to snap-freezing. Cultures were X-irradiated at a rate of 64.5 cGy/min using a 250-kV 15-mA X-irradiator (Pantak, Windsor, Hertfordshire, United Kingdom) with filtration of 2.32-mm copper half-value thickness.

DNA Damage Assays

Alkaline Unwinding. A modified version (26) of the technique described by Kanter and Schwartz (27) was used. Briefly, after freezing drug-treated monolayers as described above, samples were thawed rapidly and resuspended in the same buffer at 4°C. For each treated sample, quadruplicate measurements were made of total, partial, and background unwinding. Cells were apportioned at a density of 1.25 × 10^5/ml of sample, and Hoechst 33342 was added to a final concentration of 0.25 μM. Fluorescence was measured using a Perkin-Elmer MPF-4 fluorescence spectrophotometer and absolute strand break frequencies calculated from X-ray calibrations (26).

Nucleoid Sedimentation. Based on the method described by Cook and Brazell (28) and modified by Farzaneh et al. (29), this technique quantifies changes in DNA supercoiling due to either strand breaks or intercalation. Following detachment, cells were gently resuspended in ice-cold phosphate-buffered saline using a Pasteur pipet. Fifty μl of suspension containing 2 × 10^5 cells were deposited onto 150 μl of lysis buffer (final concentration, 2 mM EDTA, 0.5% Triton X-100, 100 mM Tris-HCl, and 2 M NaCl, pH 8.0) over 3.8 ml 15–30% sucrose gradients containing 1 mM EDTA, 10 mM Tris-HCl, 2 mM NaCl, and 1 μM Hoechst 33342 at pH 8.0. Cells were lysed for 30 min at room temperature prior to centrifugation for 30 min at 50,000 × g_{max} using an MSE Superspeed ultracentrifuge with a 6 × 4.2-ml swing-out titanium rotor.

Measurement of Unscheduled DNA Synthesis

Cells were grown on autoclaved glass coverslips in 6-cm Petri dishes for 2 days and then treated with either estrogen or ethanol for 24 h.

[3] R. J. Epstein, P. J. Smith, J. V. Watson, N. M. Bleehen, submitted for publication.

[³H]Thymidine (specific activity, 25 Ci/mmol) was then added to cells for 1 h prior to drainage of plates and washed three times in phosphate-buffered saline. Coverslips were irradiated at 254 nm using a UV fluence rate of 1.089 jm^{-2}/s. Cultures were then refed with medium containing either estrogen or ethanol in addition to [³H]thymidine (specific activity, 6.7 Ci/mmol) at a final concentration of 0.5 μCi/ml for 2 h. Coverslips were then washed twice, fixed using acidified methanol, stained with acetoorcein, dip-coated with nuclear track emulsion (NTB-2; Kodak), stored for 4 days at $-20°C$, and developed. Heavily labeled (S-phase) cells were excluded from the analysis; the number of grains over all other cells in each microscopic field was counted for at least 10 fields at each fluence, and the results are expressed as an average number of grains per non-S-phase cell.

Measurement of Drug Uptake

Cells exposed to estrogen or ethanol for 24 h were treated with doxorubicin for 1 h, washed twice in ice-cold phosphate-buffered saline, layered with freezing buffer, and snap-frozen prior to detachment by rapid thawing. Since doxorubicin is an autofluorescent drug, fluorescence of samples was then able to be determined on a single-cell basis using flow cytometry. To determine whole-cell uptake of mitoxantrone, estrogen-treated and control cells were treated for 1 h with tritiated mitoxantrone (tritium labeling by Amersham, Buckinghamshire, United Kingdom). Cells were detached using trypsin/EDTA and counted with a Coulter Counter, and equivalent cell numbers were then lysed with sodium hydroxide prior to scintillation counting. Autoradiographic analysis of nuclear drug uptake was determined for cells exposed to either tritiated mitoxantrone or tritiated Hoechst 33342 (tritium labeling by New England Nuclear, Boston, MA); the latter drug, a DNA-specific fluorochrome, exhibits similar cell uptake kinetics to other drugs regulated by cytoplasmic efflux pumps (30). Briefly, cells were grown on coverslips with or without estrogen and, following a 1-h exposure to radiolabeled drug at 37°C, washed three times, and allowed to air-dry. Coverslips were then mounted, dip-coated with nuclear track emulsion as described above, and developed after 4 days of exposure at $-20°C$. Slides were poststained with 3% Giemsa in Sorensen's buffer at pH 6.8. The number of autoradiographic grains overlying 100 nuclei (visualized either by Hoechst 33342 DNA fluorescence or by Giemsa staining) was determined and expressed as a mean per nucleus.

RESULTS

Estrogen-induced Enhancement of Drug-induced DNA Damage. Using alkaline unwinding, no damage enhancement was evident in estrogen-treated T-47D cells following X-irradiation (Fig. 1a) or bleomycin exposure (data not shown). Mitoxantrone (Fig. 1b), m-AMSA (Fig. 1c), and VP-16 (Fig. 1d) induced more DNA damage in estrogen-treated cells than in controls, as did doxorubicin, 4'-epidoxorubicin, and aclastomycin (data not shown). Estrogen pretreatment was not associated with increased VP-16-induced damage in receptor-negative SK-Br-3 breast cancer cells (Fig. 2a) or in HT-29 colon carcinoma cells (data not shown). Significant increases in DNA damage were seen in receptor-positive ZR-75-1 (Fig. 2b) and MCF-7 cells (Fig. 2c) following VP-16 exposure of estrogen-treated samples, although this degree of enhancement was less pronounced than that seen in T-47D cells.

Influence of Estrogen on VP-16-induced DNA Damage and Repair. Induction of DNA damage by 5 μM VP-16 reached near-maximum levels in T-47D cells after only 15 min drug exposure (Fig. 3a). Administration of novobiocin, an inhibitor of topoisomerase II (31), reduced VP-16-induced DNA damage in estrogen-treated cells almost to control levels (Fig. 3b). No difference in DNA cleavage could be detected between cells treated with novobiocin alone and untreated controls. When expressed as X-ray equivalents, the amount of DNA damage
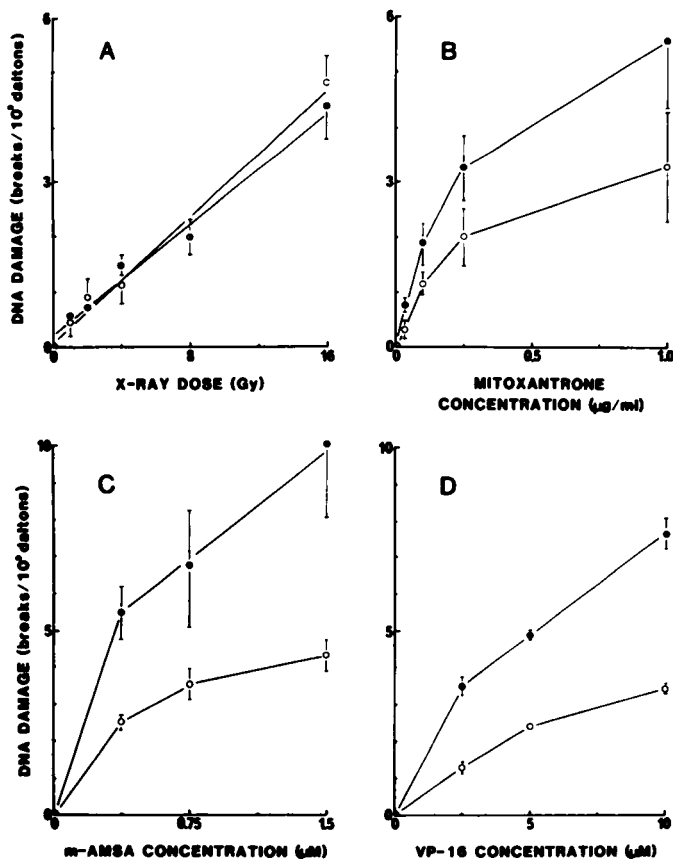
Fig. 1. DNA damage in T-47D cells measured by alkaline unwinding. Drug treatments were for 60 min at 37°C. All measurements are based on quadruplicate determinations. O, control cells; ●, estrogen-treated cells.

induced by 10 μM VP-16 as measured by neutral nucleoid sedimentation was markedly lower than that measured by alkaline unwinding, and this damage was unaffected by estrogen pretreatment (Fig. 3c).

Estrogen did not significantly affect the rate of repair of either X-ray (8 Gy; Fig. 4a)- or VP-16-induced (10 μM; Fig. 4b)

DNA damage: neither the fast initial phase nor the slower secondary phase of repair were modified. Although very little X-ray-induced DNA damage was measurable following 60 min repair, a substantial proportion of VP-16-induced DNA damage remained following an equivalent duration of repair. While this may simply indicate the presence of residual drug despite washing and refeeding [as described by Ross et al. (32) for repair of ellipticine-induced lesions], these findings are also consistent with VP-16 preferentially damaging a subset of cells, leading to impaired repair capacity for a corresponding subset of lesions. In view of this latter consideration VP-16 was administered to control and estrogen-treated cells in the same absolute dose, since exposure designed to give comparable initial break frequencies would not induce comparable levels of damage in the majority of the cells. Unscheduled DNA synthesis induced by UV radiation was not measurably affected by estrogen (Fig. 4c). This suggests that estrogen stimulation is not associated with any overall change in DNA repair capacity for DNA lesions requiring extensive repair synthesis events (e.g., bulky adduct removal following UV irradiation) or for lesions dependent upon efficient strand break ligation (e.g., following X-irradiation).

Whole-Cell and Nuclear Drug Uptake following Estrogen Stimulation. Flow cytometry of estrogen-treated and control cells exposed for 1 h to doxorubicin revealed no difference in cellular fluorescence levels (Fig. 5a). Whole-cell uptake of tritiated mitoxantrone similarly revealed no difference in estrogen-treated samples (Fig. 5b). Nuclear uptake of tritiated Hoechst 33342 and tritiated mitoxantrone in intact cells also failed to reveal any difference between estrogen-treated and control samples (Fig. 5c). Chromatin accessibility, as measured by strand breakage induced by DNase II nicking of freeze-thaw-permeabilized cells (33), was not altered by estrogen pretreatment; moreover, the failure of bleomycin to induce higher levels of DNA scission in estrogen-treated cells further suggests that changes in chromatin accessibility are unlikely to underlie the observed major enhancement of drug-induced DNA cleavage (Ref. 34; data not shown).

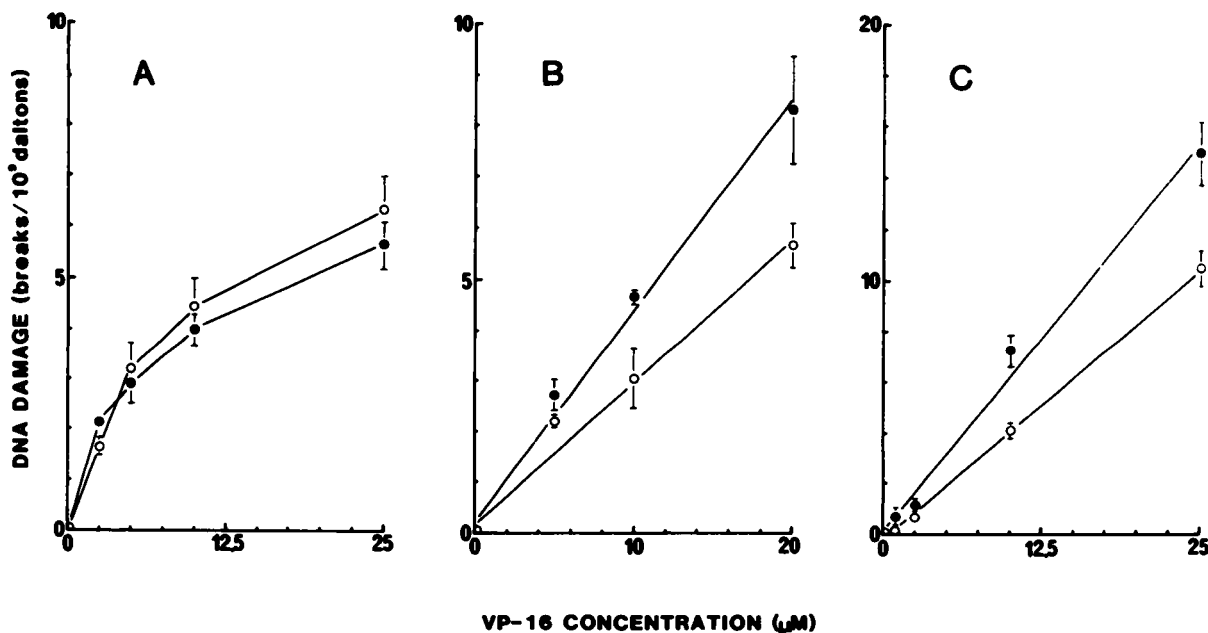Correlation of DNA Damage Enhancement with Cytotoxicity.

Fig. 2. DNA damage following 60 min VP-16 exposure in (a) SK-Br-3, (b) ZR-75-1, and (c) MCF-7 cells. All measurements are based on quadruplicate determinations. O, control cells; ●, estrogen-treated cells.
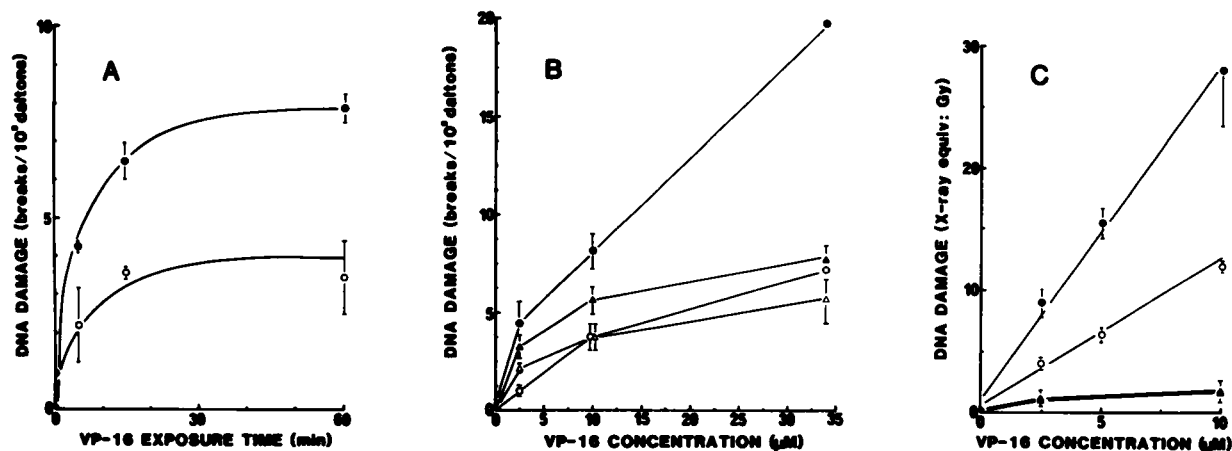
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Fig. 3. *a*, time course of 5 μM VP-16-induced DNA damage induction in T-47D cells. O, control cells; ●, estrogen-treated cells. *b*, effect of novobiocin treatment on estrogen-induced DNA damage enhancement following VP-16 exposure of T-47D cells. Cells were stimulated with estrogen (or ethanol alone) for 24 h prior to addition of 1 mM novobiocin for 1 h, followed in turn by addition of VP-16 for a further 1 h. O, control cells; Δ, control cells plus novobiocin; ●, estrogen-treated cells; ▲, estrogen-treated cells plus novobiocin. *c*, comparison of VP-16-induced DNA damage measured in X-ray equivalents by alkaline unwinding and neutral nucleoid sedimentation. O, control cells measured by alkaline unwinding; ●, estrogen-treated cells measured by alkaline unwinding; Δ, control cells measured by nucleoid sedimentation; ▲, estrogen-treated cells measured by nucleoid sedimentation. Nucleoid results based on triplicate determinations, all others based on quadruplicates.
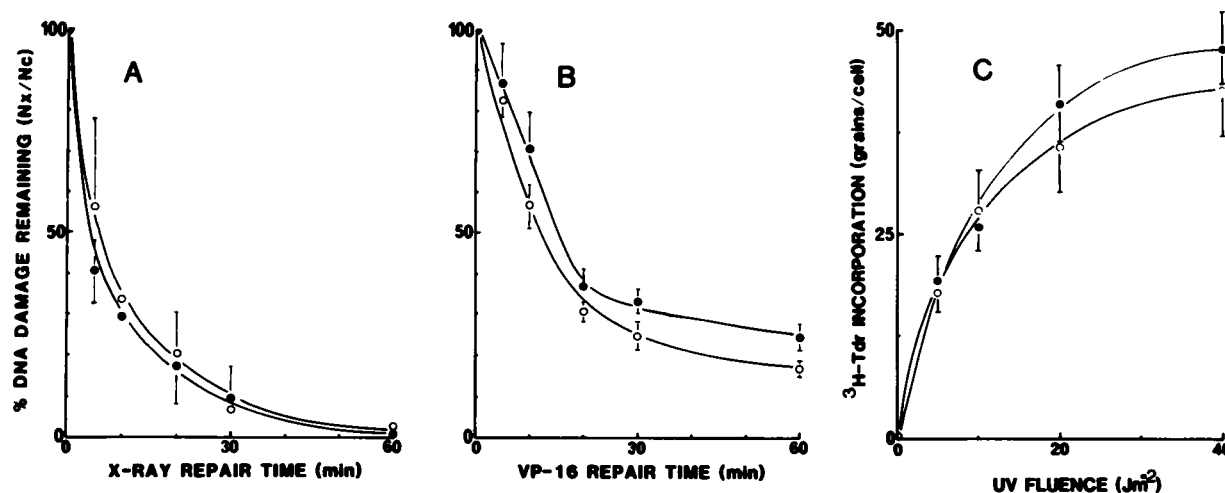
Fig. 4. DNA repair time course of T-47D cells following (*a*) 8 Gy X-irradiation, and (*b*) 10 μM VP-16 treatment. Either estrogen or ethanol alone were present for 24 h prior to treatment, during treatment, and for the 60-min repair period following. All measurements based on quadruplicate determinations. *c*, unscheduled DNA synthesis following UV irradiation in estrogen-treated and control cells. Standard errors are based on ten readings at each fluence. O, control cells; ●, estrogen-treated cells.

Estrogen pretreatment did not affect the subsequent growth of T-47D cells treated with either X-rays (Fig. 6*a*) or bleomycin (Fig. 6*b*). A major enhancement of VP-16-induced growth delay was seen in estrogen-treated cells (Fig. 6*c*), and similar enhancement occurred in cells exposed to m-AMSA (Fig. 6*d*). Mitoxantrone (Fig. 6*e*) and doxorubicin (Fig. 6*f*) treatments were associated with only marginal enhancement of growth delay in estrogen-treated cells, and this occurred at drug concentrations associated with very little measurable DNA damage. From these data the ID_{50} (assessed after 5 days of growth in complete medium posttreatment) was determined for estrogen-treated and control cells. These results are presented in Table 2 together with the corresponding amount of initial DNA damage quantified by alkaline DNA unwinding. Between 7 and 8 breaks per 10^9 daltons M. Wt. DNA were induced by VP-16 in both estrogen-treated and control cells at ID_{50}. Estrogen-treated cells exposed to m-AMSA sustained levels of DNA damage per ID_{50} comparable to those seen in cells treated with VP-16, while control cells incurred approximately one-third less. X-rays and bleomycin were 6 times more potent than VP-16 or m-AMSA

in producing growth delay per unit break, while mitoxantrone and doxorubicin induced 30 times greater toxicity than did VP-16 or m-AMSA for each unit of DNA damage detected by alkaline unwinding (Table 2).

DISCUSSION

DNA damage assays based on high pH denaturation measure both "true" DNA strand breaks and protein (presumably topoisomerase II)-associated breaks (35). The extent to which these latter "breaks" (detected only in alkali- or proteinase-based assays) reflect significant DNA damage is therefore problematic, since strand interruptions seem likely to be concealed *in vivo*. This uncertainty is particularly relevant to an understanding of anthracycline-induced cytotoxicity, since these drugs intercalate and distort the DNA double helix in addition to their putative effect on topoisomerase II; indeed, the cytotoxicity of these drugs has been directly related to their ability to condense nucleic acids (36). The podophyllotoxin derivatives VP-16 and VM-26, on the other hand, appear to exert their

Fig. 5. Drug uptake in estrogen-treated and control T-47D cells. *a*, doxorubicin fluorescence measured by flow cytometry. *b*, whole-cell uptake of tritiated mitoxantrone (³*H-Mx*). O, control cells; ●, estrogen-treated cells. Standard errors are based on triplicate measurements. *c*, nuclear uptake of tritiated Hoechst 33342 (³*H-HO-342*) and mitoxantrone as determined by autoradiography. □, control samples; ■, estrogen-treated samples. Standard errors (*bars*) are based on means of 10 readings.
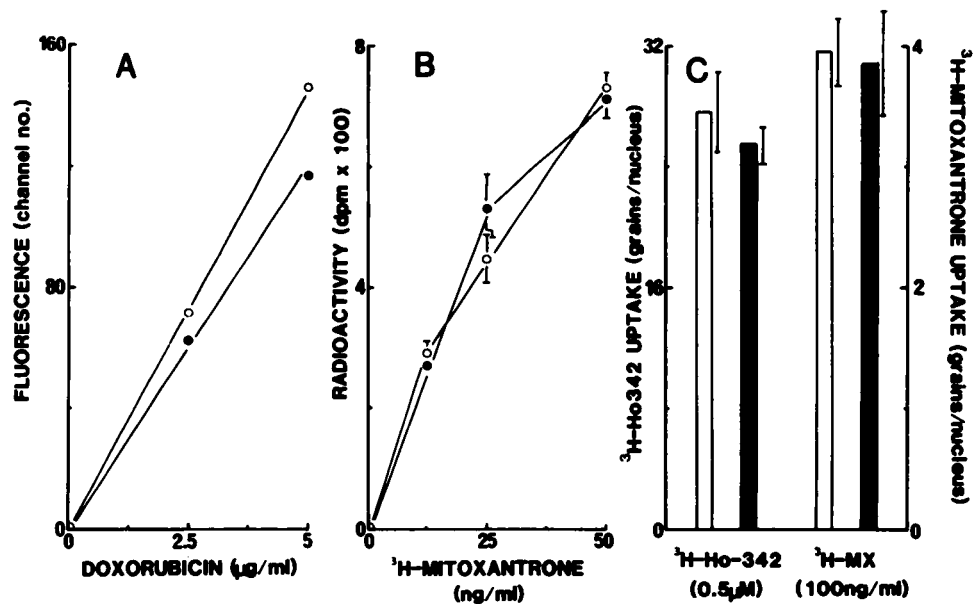
toxicity via a predominant interaction with topoisomerase II (17, 37), although some evidence exists for free radical damage as a potential contributing mechanism (38). This latter observation helps explain the small amount of VP-16-induced damage seen on neutral nucleoid sedimentation, a technique which does not detect protein-associated breaks in the absence of proteinase (39); at least 90% of VP-16-induced "breaks" seen in estrogen-treated cells by alkaline unwinding are therefore revealed only under alkaline conditions. The conclusion, that these latter breaks reflect topoisomerase II-mediated DNA damage, is further supported by the response to novobiocin administration. Although novobiocin cannot be regarded as a specific inhibitor of topoisomerase II in mammalian cells (40), our conclusion is also consistent with reports of VP-16-induced damage measured by alkaline elution correlating with cellular topoisomerase II activity (16).

In the present study, alkaline DNA unwinding was used in preference to elution in order to obviate interpretational difficulties arising from differential isotope incorporation in estrogen-stimulated and control cells. The estrogen-induced enhancement of DNA damage measured here by alkaline unwinding in T-47D cells is more pronounced than that reported for MCF-7 cells examined by alkaline elution (8); in the latter study, however, cells were deprived of estrogen for only 24 h prior to hormonal manipulation, a suboptimal regimen presumably deployed to minimize confounding differences in isotope incorporation. Control data from that study confirmed no difference in m-AMSA uptake in estrogen-stimulated cells, and a similar finding reported by Sullivan *et al.* (16) for VP-16 uptake in proliferating *versus* quiescent cells is consistent with our own results for whole-cell drug uptake in estrogen-stimulated and control cells (Fig. 5, *a* and *b*). The finding that nuclear drug uptake [as determined by autoradiography (Fig. 5*c*)] is also unaffected by estrogen treatment extends these observations and suggests further that differences in drug uptake are unlikely to underlie the major enhancement of DNA damage seen in estrogen-treated cells exposed to topoisomerase II-interactive drugs. Chromatin accessibility to m-AMSA has also been found by others to be less important than drug-enzyme interaction in modulating the DNA-damaging effects of that drug (15), and this again is consistent with our own control observations for VP-16. Hence, given that no increase in drug uptake or chro-

matin accessibility can be demonstrated in estrogen-treated cells, the rapid achievement of two different steady-state levels of DNA strand breaks detected in VP-16-exposed cells by alkaline unwinding (Fig. 3*a*) seems likely to reflect equilibration of enzymatically mediated DNA damage in two cell populations with differing enzyme availability, which in this context suggests damage mediated by the availability of topoisomerase II.

The enhancement of DNA damage seen in estrogen-treated T-47D cells exposed to the topoisomerase II-interactive drugs VP-16 and m-AMSA (Fig. 1, *c* and *d*) is accompanied by enhancement of cytotoxicity (Fig. 6, *c* and *d*), contrasting with the lack of damage enhancement and cytotoxicity seen in estrogen-treated cells exposed to the putative free-radical-damaging action of X-rays and bleomycin (Figs. 1*a* and 6, *a* and *b*). Yet despite significant increases in drug-induced DNA damage associated with estrogen stimulation, neither mitoxantrone nor doxorubicin exposure is accompanied by major enhancements of cytotoxicity in estrogen-treated cells (Fig. 6, *e* and *f*). This suggests that the additive cytotoxicity due to estrogen-enhanced DNA damage is minimal in cells treated with these agents. Indeed, cytostatic doses of these drugs induce relatively low (less than one strand break per 10⁹ daltons DNA) damage on assay, explaining the large differences in abscissa scales between Figs. 1*b* and 6*e*. We therefore submit that topoisomerase II-mediated DNA damage does not play a significant role in modulating cytotoxicity induced in T-47D cells by these intercalating agents, although conceivably such "damage" could contribute to cytotoxicity if the impact of competing lesions was altered. Conversely, if the lesion inducing the initial assayed damage is responsible for toxicity, then the nature of such lesions must differ substantially from those induced by VP-16 in order to explain the massive difference in resultant cytotoxicity. Similar conclusions have been suggested by the work of Ross *et al.* (32), who have documented the markedly longer duration of doxorubicin-induced DNA lesions when compared to those induced by drugs associated with lower cytotoxicity per assayed protein-associated break.

Since it is recognized that intercalators may interact with topoisomerase II in more than one way (41), such conclusions are not altogether surprising. Other workers have observed that cytotoxicity does not correlate with total DNA strand breakage induced by these drugs and that anthracycline derivatives are
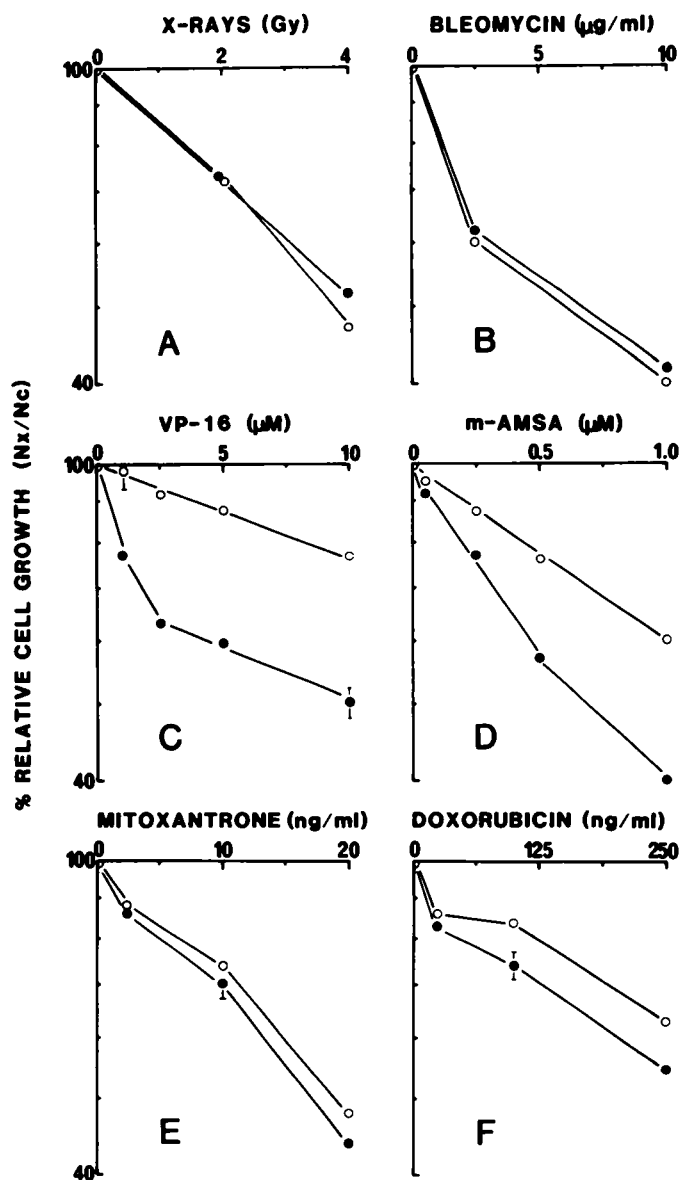
Fig. 6. Growth delay induced in T-47D cells by various treatments. All measurements are based on triplicate determinations. O, control cells; ●, estrogen-treated cells.

Table 2 *Quantitative comparison of equitoxic levels of DNA-damaging agents and resultant DNA damage measured by alkaline unwinding in estrogen-treated and control cells*

Treatment	ID$_{50}$[a]			DNA damage[b] at ID$_{50}$ (breaks/10^9 daltons)	
	Control		Estrogen-treated[c]	Control	Estrogen-treated
X-rays	3.9	(Gy)	4.1	1.2	1.3
Bleomycin	6.0	(µg/ml)	6.5	1.0	1.2
m-AMSA	1.4	(µM)	0.7	4.2	6.7
VP-16	21.5	(µM)	10.1	7.5	7.9
Doxorubicin	370	(ng/ml)	320	0.1	0.2
Mitoxantrone	18	(ng/ml)	17	0.19	0.32

[a] Treatment exposure required to induce 50% growth delay measured after 5 days of growth in complete medium following treatment.

[b] Initial level of DNA strand breakage measured by alkaline unwinding after 60 min of drug treatment (or immediately following irradiation).

[c] For 24 h prior to drug exposure or irradiation.

more cytotoxic per strand break than m-AMSA (42, 43), but no coherent explanation for these findings has yet emerged. Interestingly, Robson *et al.* (44) have recently described a variant cell line characterized by sensitivity to both intercalating and nonintercalating topoisomerase II-interactive drugs; in this system, however, increased background levels of protein-associated DNA strand breaks in these cells did not correlate with measurable toxicity, raising the possibility that the phenotype reflects an abnormal drug-enzyme interaction rather than an increased number of damaged sites *per se*.

The finding that estrogen-treated and control cells sustain similar levels of DNA damage for equivalent levels of VP-16-induced cytotoxicity (Table 2) strongly suggests that these assayed lesions are directly responsible for the cytotoxicity of this agent and that estrogen enhances the cytotoxicity of similar drugs (such as m-AMSA; Table 2) by inducing a parallel increase in the number of such lesions. However, "breaks" induced by such agents appear to exert only one-sixth the cytotoxicity of breaks induced by ionizing radiation or the radiomimetic drug bleomycin; since repair of these latter lesions seems at least as efficient as that of VP-16-induced DNA cleavage, this suggests that the observed difference in cytotoxicity reflects corresponding differences in cellular response to the respective DNA lesions. On the other hand, the apparent 30-fold greater cytotoxicity of strand breaks induced by mitoxantrone and doxorubicin seems likely to reflect an alternative major mechanism of toxicity for these drugs. For although persistence of the protein-associated DNA damage alone could account for the markedly increased cytotoxicity (*i.e.*, per unit of initially assayed DNA damage) of these drugs relative to VP-16 and m-AMSA, it does not explain the failure of estrogen to potentiate their cytotoxicity in parallel with the observed increase in assayed DNA cleavage. This suggests that the toxicity of these agents may arise predominantly not from cleavable complex formation *per se* but from other, perhaps unrelated, consequences of DNA intercalation.

In summary, DNA damage induced by either VP-16 or m-AMSA is strongly enhanced by estrogen stimulation, and this is expressed as a highly significant potentiation of cellular toxicity; however, although anthracycline-induced DNA damage also appears to be enhanced by estrogen stimulation, potentiation of cytotoxicity is only marginal. We suggest that drug-induced growth inhibition in estrogen-responsive human breast cancer cells provides a system for quantifying the relative contribution of topoisomerase II-mediated DNA damage to drug-induced cytotoxicity. Moreover, the findings presented here may help explain the disappointing results of clinical trials based on estrogen stimulation of tumors treated with anthracycline-based regimens and suggest rational new approaches for the design of breast cancer chemotherapy protocols.

ACKNOWLEDGMENTS

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