and vocalization was excessive. This pattern of behavior, characteristic of a fear response, was in striking contrast to that observed in saline-injected rats, who slept for almost the entire 6-hour observation period. Specificity of the behavioral response to hpGRF is indicated by the finding that it differs widely, both in time course and pattern, from that described after administration of other hypothalamic releasing and inhibiting peptides, including somatostatin, into the CNS (*4*).

While the doses of hpGRF that I used were large, it is difficult to estimate what percentage of the peptide actually reaches particular neuronal sites when administered through the cerebrospinal fluid. Furthermore, the doses were in the range of those used in most previous studies of actions of hypothalamic peptides in the CNS (*4*). In view of the lack of effect of hpGRF on plasma prolactin—a sensitive monitor of stress in the rat (*18*)—it is unlikely that the hpGRF-induced responses were due to nonspecific stress. It is also unlikely that any of the effects reported here were mediated systemically by leakage from the brain, since similar doses of hpGRF administered peripherally fail to affect either plasma glucose or behavior and have opposite effects on plasma GH (*19*). Our findings support the hypothesis that hpGRF exerts direct actions in the brain that are independent of its effects at the level of the pituitary gland.

Involvement of the CNS in glucoregulation has been recognized since the classic observation of Bernard (*20*). In particular, the ventromedial hypothalamus has been implicated as a critical CNS locus for carbohydrate metabolism by a variety of techniques; stimulation of this region elevates plasma glucose whereas lesions facilitate insulin secretion (*21*). It is also widely believed that the ventromedial hypothalamus is a major center for integration of emotional behavior. Electrical stimulation of the ventromedial nucleus produces an affective defense response in cats, and rats display fear-related behaviors consisting of vocalization, rearing, and escape attempts (*22*), behaviors remarkably similar to those we observed in response to central injection of hpGRF. Immunohistochemical studies have revealed the presence of hpGRF-immunoreactive neurons in arcuate and ventromedial nuclei of the primate and rat hypothalamus (*23*), neural loci consistent with physiological findings on hypothalamic regulation of GH secretion (*24*). In addition, hpGRF-immunoreactive fibers were found projecting to several regions of the hypothalamus outside the characteristic termination sites on median eminence portal capillaries (*25*). It is possible that GRF is the neural substrate subserving all these functions and plays an important neurotransmitter or neuromodulatory role in the basal hypothalamus to coordinate the neuroendocrine, visceral, and behavioral responses of the organism.

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Adriamycin-Induced DNA Damage Mediated by Mammalian DNA Topoisomerase II

Abstract. *Adriamycin (doxorubicin), a potent antitumor drug in clinical use, interacts with nucleic acids and cell membranes, but the molecular basis for its antitumor activity is unknown. Similar to a number of intercalative antitumor drugs and nonintercalative epipodophyllotoxins (VP-16 and VM-26), adriamycin has been shown to induce single- and double-strand breaks in DNA. These strand breaks are unusual because a covalently bound protein appears to be associated with each broken phosphodiester bond. In studies in vitro, mammalian DNA topoisomerase II mediates DNA damage by adriamycin and other related antitumor drugs.*

Because of the clinical importance of adriamycin in the treatment of many common tumors, extensive studies have been performed to determine the possible antitumor mechanism or mechanisms of adriamycin and other related antitumor anthracyclines (*1, 2*). Adriamycin binds tightly to DNA and interferes with many DNA-related functions such as DNA replication and RNA synthesis (*1, 2*). It has been shown that, when linked to agarose beads, adriamycin can exert its cytotoxic effect without entering cells (*3*). Adriamycin can also be reduced to a semiquinone radical that damages macromolecules such as DNA and cell mem-

branes (*4*). Whether this damage is related to toxic side effects (for example, cardiac toxicity) or to the antitumor effect has not been established. Many intercalative antitumor drugs, including adriamycin, induce protein-linked DNA breaks in cultured mammalian cells by a mechanism that is probably independent of radical formation (*5*). The nonintercalative antitumor drugs VP-16 and VM-26 also induce protein-linked DNA breaks (*6, 7*). In studies on *m*-AMSA [4′-(9-acridinylamino) methane-sulfon-*m*-anisidide], ellipticines, and epipodophyllotoxins, there is evidence that the putative protein target may be mammalian DNA topoisomerase II (*7–9*).

In the presence of adenosine triphosphate (ATP) or deoxyadenosine triphosphate (dATP), mammalian DNA topoisomerase II can catalyze a number of topological isomerization reactions (for example, knotting and unknotting, catenation and decatenation, and relaxation of superhelical twists) presumably via a transient intermediate involving protein-linked DNA double-strand breaks (*10–13*). We have identified a topoisomerase II–DNA complex, termed the "cleavable complex," which may be related to this putative transient intermediate (*8, 9, 14*). Treatment of the cleavable complex with protein denaturants results in both single- and double-strand breaks in DNA. Like prokaryotic DNA topoisomerase II, eukaryotic DNA topoisomerase II also cleaves DNA to produce a four-base stagger and becomes covalently linked to the protruding 5′-phosphoryl end through a tyrosyl phosphate linkage (*11, 12, 14–17*).

To test whether protein-linked DNA breaks induced by adriamycin and other antitumor drugs also involve mammalian DNA topoisomerase II, we conducted studies in vitro using purified calf thymus DNA topoisomerase II. Linear pBR322 DNA labeled at one end with ^{32}P was fragmented to smaller pieces in the presence of calf thymus topoisomerase II and antitumor drugs such as anthracyclines [adriamycin, daunomycin, and 5-iminodaunorubicin (IDR)], related synthetic drugs (bisantrene and mitoxantrone), and the antitumor antibiotic actinomycin D (Fig. 1). The presence of ATP (1 m*M*) stimulated DNA cleavage two to three times more than did control (data not shown). For comparison, the antitumor drugs *m*-AMSA and the 2-methyl-9-hydroxy derivative of ellipticine, which have been shown to promote topoisomerase II–mediated DNA cleavage, were included (*8, 9*). Ethidium bromide, which is an intercalator but not an antitumor drug, was included as a negative control. As shown in Fig. 1B, ethidium bromide did not stimulate topoisomerase-mediated DNA cleavage.

Two interesting features of topoisomerase II–mediated cleavage were noted. (i) At higher concentrations of intercalators, DNA cleavage was actually inhibited (Fig. 1). Adriamycin (10 ng/ml; Fig. 1A, lane g) was one of the most potent drugs in stimulating topoisomerase II–mediated DNA cleavage at low concentrations. At doses of more than 0.25 μg of adriamycin per milliliter (Fig. 1A, lane i), topoisomerase II–mediated DNA cleavage was abolished. (ii) Although antitumor anthracyclines (adriamycin, daunomycin, and IDR) stimulated cleavage at similar sites, antitumor drugs of different chemical classes showed strikingly different cleavage patterns (compare, for example, *m*-AMSA, 2-methyl-9-hydroxyellipticinium acetate, and adriamycin in Fig. 1).

Several pieces of evidence indicated

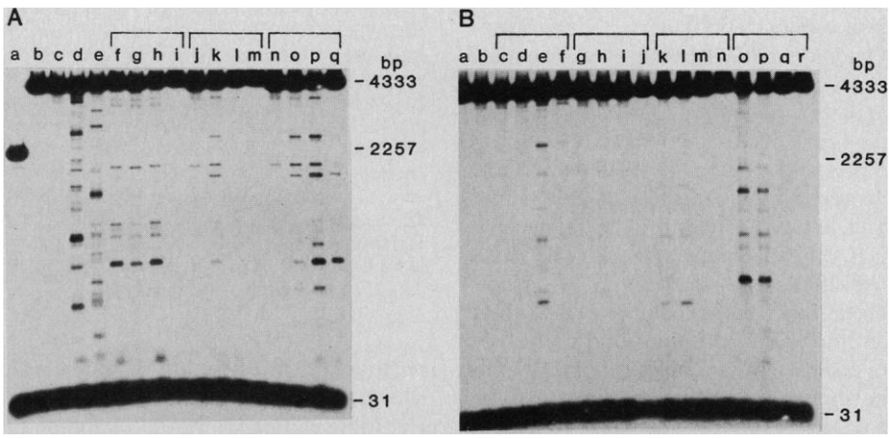

Fig. 1. DNA breakage by antitumor drugs mediated by DNA topoisomerase II. Assays for topoisomerase-mediated DNA cleavage were performed as described (*7–9*). Briefly, pBR322 DNA was linearized with Eco RI and then end-labeled at the 3′ termini with the large fragment of *E. coli* DNA polymerase I in the presence of [α-^{32}P]dATP and three other unlabeled deoxyribonucleoside triphosphates. End-labeled pBR322 DNA was then digested again with Hind III to remove a 31-bp (base pair) small fragment. The uniquely end-labeled pBR322 DNA was used in the cleavage assay. Topoisomerase II cleavage reactions were done in reaction mixtures (20 μl each) containing tris (40 m*M*, *p*H 7.5), KCl (100 m*M*), $MgCl_2$ (10 m*M*), dithiothreitol (0.1 m*M*), EDTA (0.5 m*M*), bovine serum albumin (30 μg/ml), calf thymus DNA topoisomerase II (20 ng), end-labeled (^{32}P) pBR322 DNA (50 ng), and drugs as indicated. Reactions were incubated at 37°C for 30 minutes and then treated with 10 percent SDS (1 μl) and proteinase K (1.5 mg/ml, 1 μl) at 50°C for 30 minutes. DNA samples were analyzed on a 1 percent agarose gel in TBE buffer [89 m*M* tris borate (*p*H 8.3) and 2.0 m*M* EDTA] (*9*). (A) (Lane a) marker DNA; (lane b) DNA control (no enzyme); (lane c) no drug; (lane d) *m*-AMSA (0.25 μg/ml); (lane e) 2-methyl-9-hydroxyellipticinium acetate (0.25 μg/ml); (lanes f to i) adriamycin (0.05, 0.01, 0.25, and 1.25 μg/ml, respectively); (lanes j to m) daunomycin (0.05, 0.25, 1.25, and 6.25 μg/ml, respectively); (lanes n to q) IDR (0.25, 1.25, 6.25, and 31.25 μg/ml, respectively). (B) (Lane a) DNA control (no enzyme); (lane b) no drug; (lanes c to f) actinomycin D (0.04, 0.4, 4.0, and 40 μg/ml, respectively); (lanes g to j) ethidium bromide (0.04, 0.4, 4.0, and 40 μg/ml, respectively; (lanes k to n) bisantrene (0.05, 0.25, 1.25, and 6.25 μg/ml respectively); (lanes o to r) mitoxantrene (0.05, 0.25, 1.25 and 6.25 μg/ml, respectively).

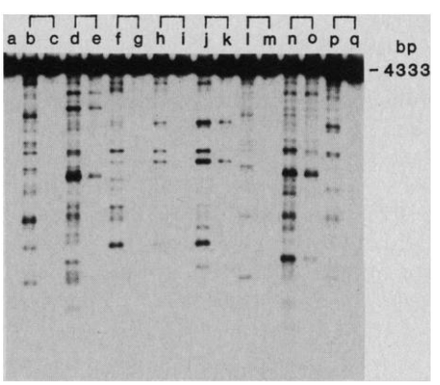

Fig. 2. Salt reversal of drug-induced DNA cleavage. Assays for drug-induced DNA cleavage were conducted as described in the legend to Fig. 1. After the first incubation (37°C, 30 minutes), half of each sample was treated with SDS–proteinase K (the NaCl concentration was adjusted to 0.5*M* during SDS-protease treatment) and the other half was treated with NaCl (0.5*M*, final concentration) for another 15 minutes before SDS–proteinase K treatment. DNA samples were analyzed as described in the legend to Fig. 1. (Lane a) DNA control (no enzyme, no drug); (lane b) *m*-AMSA (0.25 μg/ml); (lane d) 2-methyl-9-hydroxyellipticinium acetate (0.25 μg/ml); (lane f) adriamycin (0.05 μg/ml); (lane h) daunomycin (0.25 μg/ml); (lane j) IDR (6.25 μg/ml); (lane l) bisantrene (0.25 μg/ml); (lane n) mitoxantrone (0.25 μg/ml); (lane p) actinomycin D (4.0 μg/ml). Lanes c, e, g, i, k, m, o, and q were the same as lanes b, d, f, h, j, l, n, and p, respectively, except that NaCl (0.5*M*, final concentration) was added before SDS–proteinase K treatment.

that DNA double-strand breaks induced in vitro by topoisomerase II and anthracycline antitumor drugs are protein-linked. (i) In the absence of DNA topoisomerase II, anthracyclines alone did not produce any DNA double-strand breaks (data not shown). (ii) When proteinase K treatment was omitted, extraction of the reaction products with phenol led to the complete loss of the DNA fragments seen in the gel, suggesting the presence of tightly bound protein on these DNA fragments (data not shown). (iii) Addition of high salt (0.5*M* NaCl, final concentration) to a reaction mixture that had already been incubated reduced DNA cleavage significantly (Fig. 2). This apparent reversal of DNA cleavage by high salt seems incompatible with any postulated mechanism that involves irreversible DNA damage. On the basis of the known properties of mammalian DNA topoisomerase II, we favor the hypothesis that, like *m*-AMSA, ellipticines, and epipodophyllotoxins, these anthracycline antitumor drugs affect the breakage-reunion reaction of DNA topoisomerase II by stabilizing the cleavable complex formed between topoisomerase II and DNA (*8, 14*). Addition of a protein denaturant such as sodium dodecyl sulfate (SDS) traps the cleavable complex and thus reveals the strand breaks that are protein-linked (*8, 14*). Dissociation of the drug from the topoisomerase or the topoisomerase-DNA complex (for example, high-salt treatment or dilution of the reaction mixture) distorts the equilibrium, thereby favoring the formation of a noncleavable complex. An apparent reversal of DNA cleavage can thus be explained.

To investigate the effect of adriamycin on the catalytic activity of mammalian DNA topoisomerase II, we monitored the strand-passing activity of mammalian DNA topoisomerase II. Like ellipticine, adriamycin strongly inhibited the unknotting activity of calf thymus DNA topoisomerase II in the P4 unknotting assay (Fig. 3) (*18*). However, ethidium bromide, which did not induce cleavage (see Fig. 1B), also strongly inhibited the strand-passing activity of calf thymus DNA topoisomerase II (Fig. 3). As suggested previously, the strand-passing activity of DNA topoisomerase II is sensitive to intercalators (*8, 9*). In contrast, studies on the nonintercalative epipodophyllotoxins VP-16 and VM-26 have shown that drug stabilization of the cleavable complex leads to inhibition of the catalytic activity (*7*). It thus seems possible that the inhibition of activity by intercalative antitumor drugs may result from both drug stabilization of the cleavable complex and drug intercalation into DNA. Intercalation may block the binding of topoisomerase II to DNA and thus inhibit the strand-passing activity. This interpretation is also consistent with the observation that at higher concentrations of intercalative antitumor drugs DNA cleavage was actually inhibited (Fig. 1). Our result is consistent with the earlier reports that the antitumor activity is closely related to drug stabilization of the cleavable complex rather than to drug inhibition of the enzymatic activity of mammalian DNA topoisomerase II (*7–9*).

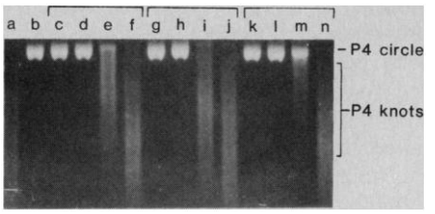

Fig. 3. Inhibition of DNA strand-passing activity by DNA intercalators. The strand-passing activity of calf thymus DNA topoisomerase II was monitored by the P4 unknotting assay (*18*). Reactions (20 μl each) were conducted under the same conditions as those described for the cleavage assay (see Fig. 1) except that P4 knotted DNA (20 μg/ml) and calf thymus DNA topoisomerase II (30 ng/ml) were used. After incubation at 37°C for 30 minutes, reactions were stopped with 5 μl of 20 percent Ficoll, 5 percent Sarkosyl, and 50 m*M* EDTA and then analyzed on a 0.6 percent agarose gel in TBE buffer (*7*). (Lane a) P4 knotted DNA control (no enzyme, no drug); (lane b) no drug; (lanes c to f) ellipticine (0.04, 0.2, 1, and 5 μg/ml, respectively); (lanes g to j) adriamycin (0.04, 0.2, 1, and 5 μg/ml, respectively); (lanes k to n) ethidium bromide (0.4, 0.2, 1, and 5 μg/ml, respectively).

The effect of adriamycin and other related antitumor drugs on topoisomerase II–DNA complexes resembles the effect of nalidixic (or oxolinic) acid on *Escherichia coli* DNA gyrase (*11, 12*). Nalidixic acid stabilizes a gyrase-DNA complex which, upon protein-denaturant treatment, results in DNA double-strand breaks and the covalent linking of the *gyrA* subunit to each 5′-phosphoryl end of the broken DNA strand (*11, 12*). We have suggested that drug stabilization of the cleavable complex may lead to cell killing by a mechanism analogous to the strong bactericidal effect of nalidixic (or oxolinic) acid on bacterial cells (*8*). Nalidixic acid selectively kills growing bacterial cells presumably by a mechanism related to its specific stabilization of the gyrase-DNA complex (*11, 12, 19*). Many potent antitumor drugs affect the breakage-reunion reaction of mammalian DNA topoisomerase II by stabilizing the cleavable complex. Whether this unusual DNA damage is related to drug-induced cytotoxicity, sister chromatid exchange, or chromosomal aberration is still not clear. Our studies suggest a possible role of topoisomerase II in the action of these antitumor drugs.

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