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Prevention of tumorigenesis of oncogene-transformed rat fibroblasts with DNA site inhibitors of poly(ADP ribose) polymerase

(tumor inhibition/*ras*/1,2-benzopyrone/octadeoxyribonucleotide)

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ABSTRACT **The EJ-*ras* gene was placed under the transcriptional control of the steroid-inducible mouse mammary tumor virus promoter/enhancer and introduced into Rat-1 fibroblasts, yielding the 14C cell line. When these cells were exposed to dexamethasone *in vitro*, EJ-ras mRNA was induced 15- to 20-fold, the cells grew in agar, and, after injection of cells into syngenic Fischer 344 rats, they produced lethal fibrosarcomas. Inhibitors of poly(ADP ribose) polymerase, which prevent the activation of the purified enzyme by a synthetic octadeoxyribonucleotide duplex, inhibited both *in vivo* tumorigenicity and *in vitro* growth in soft agar. The enzyme inhibitor 1,2-benzopyrone, which was studied in detail, and other polymerase inhibitors had no effect on EJ-ras mRNA or p21 protein expression. Poly(ADP ribose) polymerase [NAD$^+$:poly(adenosine diphosphate D-ribose) ADP-D-ribosyltransferase, EC 2.4.2.30] was inhibited by the drug in both untreated and dexamethasone-treated cells both *in vitro* and *in vivo* to the same extent, but biological consequences of enzyme inhibition were manifest only when the cells were in the transformed tumorigenic state.**

We originally reported that transformation of human fibroblasts *in vitro* by apparently nontoxic concentrations of ultimate carcinogens was prevented by molecules that inhibit poly(ADP ribose) polymerase [NAD$^+$:poly(adenosine diphosphate D-ribose) ADP-D-ribosyltransferase, EC 2.4.2.30] (1, 2). Before a cell biological mechanism of action of antitransforming drugs could be formulated, both the mode(s) of action of chemical carcinogens, leading to oncogenesis, and the mechanism of inhibition of the polymerase enzyme by inhibitors, acting as antitransformers, required clarification. To avoid the first problem, we determined the effects of inhibitors of poly(ADP ribose) polymerase on the tumorigenesis of cells containing known oncogenes, since the significance of oncogenes in neoplasia is well documented (3–6). We selected a cell type in which oncogene expression was inducible; therefore, the effects of drugs could be determined before and after overt transformation. This model also avoided the biochemical complications introduced by the toxicity of chemical carcinogens. As we report here, the biochemical action of antitransforming drugs on the polymerase appears to be restricted to the DNA site of the enzyme, and this explains the ineffectivity as antitumorigenic agents of inhibitors acting at the NAD$^+$ site only (2).

Although poly(ADP ribose) polymerase was inhibited *in vivo* by antitumorigenic drugs to the same extent in both nontransformed and neoplastic cells, the cell biological effects (i.e., the arrest of cell proliferation) occurred only in cells expressing oncogenes. Among the drugs that inhibited transformation (2), 1,2-benzopyrone (frequently referred to as coumarin, which is to be distinguished from dicoumarol) was studied in detail because it is readily administered to animals in drinking water (7) and is nontoxic.

MATERIALS AND METHODS

Plasmid Construction and Transfection. The pMTV-EJras plasmid was made by placing the EJ-*ras* coding domain under the transcriptional control of the mouse mammary tumor virus (MTV; steroid-inducible) promoter/enhancer, and it also contains a neomycin-resistance gene (8). EJ-*ras* is a mutant c-Ha-*ras1* gene isolated from the EJ/T24 bladder carcinoma cell lines (9). The steroid-responsive portion of the MTV long terminal repeat (*Cla* I–*Bam*HI fragment) from plasmid p484 (10) was subcloned between the above restriction sites in the vector pCV108, which carries the neomycin-resistance gene (11), generating pMTV108. A fragment of the pEJ6.6 plasmid (9) [the 4.8-kilobase (kb) fragment from a *Sma* I site located ≈20 base pairs (bp) upstream from the initiation ATG to the *Bgl* II site near the 3′ end of the clone] was converted to *Bgl* II ends with linkers and inserted into the *Bam*HI site of pMTV108, generating pMTV-EJras (see Fig. 1). Rat-1 fibroblasts were transfected with this plasmid by the calcium phosphate technique (12) and selected in the presence of 400 μg/ml of G418 (GIBCO). Drug-resistant colonies were isolated with the aid of a cloning cylinder. Cell line 14C was chosen because it spontaneously formed few and only very small colonies in soft agar, and exposure to 0.1 μM dexamethasone (Dex) greatly increased both the number and size of colonies. EJ-*ras* under the control of the mouse metallothionein (zinc-inducible) promoter/enhancer was constructed by subcloning the same EJ-*ras* fragment as was used for pMTV-EJras into the *Bgl* II and *Bam*HI sites of the vector pMT1 (13), from which the metallothionein coding domain had been removed. Rat-1 fibroblasts were cotransfected with this plasmid, and pCV108 was selected in the presence of G418. Cell line 15A was selected among resultant foci. A temperature-sensitive (ts) Rous sarcoma virus-containing normal rat kidney cell line, tsNY68, was also investigated (14).

Antitransforming Drugs. Those used in soft-agar assays were chosen from a list described earlier (1, 2), and concentrations that had no toxic or growth-inhibitory effects (IC_0) and 50% inhibition (IC_{50}) were determined as reported (2).

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Abbreviations: ts, temperature sensitive; MTV, mouse mammary tumor virus; Dex, dexamethasone; IC_0 concentration with no toxic or growth-inhibitory effects; IC_{50}, concentration giving 50% inhibition.

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These drugs included benzamide, hexamethylenebisacetamide, and 1,2-benzopyrone (Sigma). *In vitro* drug transport studies were performed with highly tritiated (10^5 Bq/μmol) 1,2-benzopyrone in 14C cells. Externally applied 1,2-benzopyrone (100 μM) in cell culture equilibrated with the intracellular drug concentration within 6–8 hr. Based on published results (15, 16), it was estimated that a 200 μM drug concentration in the drinking water for 7 days could maintain a cellular concentration of unmetabolized drug between 50 and 80 μM—that is, in the same order of magnitude as the noncompetitive K_i obtained with purified poly(ADP ribose) polymerase enzyme (see Fig. 3).

Isolation and Preparation of EJ-ras mRNA. A probe complementary to human EJ-ras mRNA was prepared by inserting the 188-bp *Nar* I–*Sma* I fragment of EJ-*ras* [containing part of exon 2 and intron 2, with the *Nar* I-end converted to *Bam*HI with linkers (17)] into the *Hin*cII and *Bam*HI sites of the plasmid pSP64 (Promega Biotec, Madison, WI). The vector was linearized with *Eco*RI prior to its use as a DNA template. [α-^{32}P]UTP (Amersham) and SP6 RNA polymerase (New England Nuclear) were used for the phage SP6-directed synthesis of radiolabeled RNA probe as described (18). The product of the reaction was purified by 6 M urea/PAGE to isolate the full-length probe. Total cellular RNA was purified (19), and quantitation of EJ-ras mRNA was performed by RNase protection of the complementary RNA probe (20).

Cell Culture Conditions. Conditions were the same as described (1, 10), and cell nuclei were isolated as reported (21).

Quantitative Determination of Mono- and Poly(ADP Ribosyl)ation Sites in Isolated Nuclei. This was performed as previously described (22, 23).

Cellular Poly(ADP Ribose) Content of 14C Cells. 14C cells that were exposed to various treatments were assayed directly in monolayer cultures not exceeding 30–50% confluency. Batches of 60–100 × 10^6 cells per experiment were fixed *in situ* with 20% trichloroacetic acid (≈1–2 ml per culture dish of 55 cm^2). Cells were collected with a rubber policeman, and acid extracts (by six successive washings) were prepared by sonication and centrifugal sedimentation of acid-precipitated particles. Acid-soluble nucleotides and poly(ADP ribose) were isolated as reported (24). Complete digestion with phosphodiesterase of tritiated poly(ADP ribose) that was extracted from the cells followed by chromatography identified and quantitated the polymer (24). Cellular NAD^+ (25) and ATP (26) were analyzed by standard micromethods in the acid extracts.

Other Procedures. Calf thymus poly(ADP ribose) polymerase was purified to 95% homogeneity by a recently developed method (K.B., E. Kirsten, and E. Kun, unpublished data) and by a published procedure (27). The coenzymic octamer duplex,

5′ AGATCAGT 3′
3′ TCTAGTCA 5′,

was prepared by solid-phase synthesis (28). The composition of the octamer was based on the hormone receptor-binding consensus DNA sequence (29).

Cellular DNA was determined in nuclei dissolved in KOH (24), followed by neutralization and fluorescence assay (30). The exchange of I (in 6,8-diiodo-1,2-benzopyrone) for ^{3}H, to obtain the highly labeled drug, was carried out as reported (31), and the product was purified by chromatography.

RESULTS

Inducible Transformation of 14C Cells and Inhibition of Anchorage-Independent Growth by Polymerase Inhibitors. 14C cells contain the MTV EJ-*ras*, and EJ-*ras* gene expression and its phenotypic consequences are under the control of glucocorticoid hormones. After exposure to 0.1 μM Dex for 2 days, cells became rounded and formed distinct foci, and these changes persisted after removal of Dex. In soft agar, 14C cells formed only a limited number of microcolonies, (0.1 mm in diameter), whereas Dex increased greatly both the number and especially the size of colonies, frequently ≥ 3- to 5-mm diameter. Dex induced a 15- to 20-fold increase in EJ-ras mRNA after 12 hr of exposure (Fig. 1, lanes 2 and 4). Similar changes in morphology and soft-agar colony growth occurred with the 15A (zinc-inducible EJ-*ras*) cell line and the tsNY68 cell line (ts v-*src* gene) (not shown).

The effect of selected drugs on colony formation induced by Dex was determined at IC_0 (nontoxic) concentrations and is summarized in Table 1. The IC_{50} and IC_0 of these selected drugs (cf. ref. 2) acting on 14C cells is shown in Table 1. Notably, these drugs did not prevent the change in morphology induced by Dex. Similar results were obtained with the 15A cell line in soft agar with benzamide and hexamethylenebisacetamide as drugs and with the tsNY68 cell line (14) with benzamide (not shown).

Inhibition of Tumorigenesis. Subcutaneous injection at multiple sites in Fischer 344 rats (male, 150-g body weight) of 10^5 14C cells pretreated or not pretreated with 0.1 μM Dex for 48 hr produced an 80% tumor incidence (Table 2, experiments 1 and 2), and the tumor size in the two groups varied between 3 and 11 g. Glucocorticoids produced *in vivo* were apparently sufficient to activate 14C cells. These tumors were identified as high-grade fibrosarcomas with no contaminating population of reactive cells (not shown), and the tumors eventually killed the animals.

The compounds that were effective in inhibiting soft-agar colony growth were also found to inhibit tumorigenesis *in vivo*. The effects of various treatments were determined 2 weeks after inoculation of 14C cells (Table 2) because it

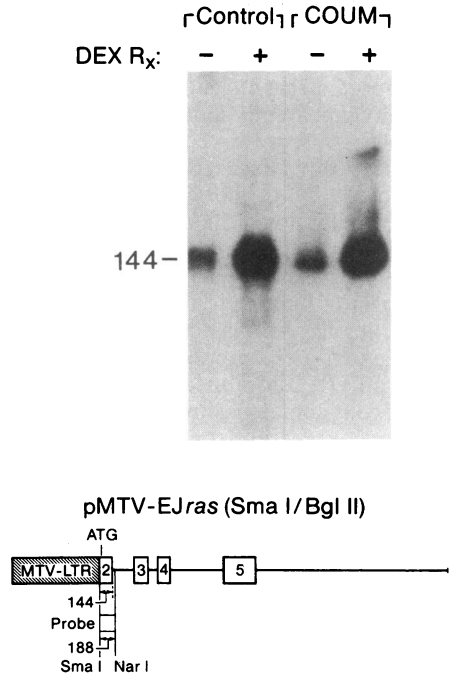

FIG. 1. (*Upper*) Hybridization of SP64 EJ-ras complementary DNA probe to the 144-bp segment of the mRNA encoded by exon 2 of EJ-*ras*. Dex R_x indicates the absence (−) or presence (+) of the drug; COUM refers to pretreatment of cells with 0.1 mM 1,2-benzopyrone (coumarin) for 72 hr. (*Lower*) Schematic representation of the transfecting plasmid DNA. The *Sma* I–*Nar* I fragment was treated with RNase T and A, yielding the 144-bp sequence (shown on the gel). Numbers 2, 3, 4, and 5 are exons of the EJ-*ras* oncogene. LTR, long terminal repeat.

Table 1. Inhibitory effect of drugs on the Dex-induced growth of 14C cells in soft agar

Exp.	Condition	No. of colonies*	*n*	IC_{50}, mM	IC_0, mM
1	Control				
	− Dex	42 ± 48	10	—	—
	+ Dex	308 ± 69	10		
2	Benzamide (1 mM)				
	− Dex	48 ± 48	5	4.2	1
	+ Dex	143 ± 53	5		
3	Hexamethylenebisacetamide (1 mM)				
	− Dex	48 ± 48	5	7.2	1
	+ Dex	117 ± 48	5		
4	1,2-benzopyrone (0.1 mM)				
	− Dex	56 ± 53	2	0.5	0.1
	+ Dex	74 ± 18	2		

The assay for anchorage-independent growth in 0.35% agar and the methods for the determination of drug toxicity are described in *Materials and Methods*.
*Average of three plates inoculated with 1000 cells each (±SD).

allowed maximal tumor growth without the appearance of central tumor necrosis. Tumor growth was defined as zero, if the injected mass did not increase above 1.0 g in 2 weeks.

Treatment of 14C cells in culture with 100 μM 1,2-benzopyrone for 120 hr (Table 2, experiment 3) or *in vivo* administration of the drug in drinking water (experiment 4) significantly reduced tumor size and tumor incidence, and a combination of drug pretreatment and *in vivo* drug feeding (experiment 5) resulted in maximal reduction of tumor incidence and size. The effectivity of these drugs was not altered by Dex-induced transformation of cells prior to animal inoculation (experiment 6). Fig. 2 demonstrates the distribution of tumor weights in untreated rats and the effect of drug treatment. The drug treatment shifted the distribution of tumor weights towards smaller and fewer tumors.

Inhibitors Do Not Affect EJ-*ras* Expression. EJ-ras mRNA and p21 protein levels were determined in order to ascertain whether these drugs prevented the soft-agar growth and tumorigenesis of 14C cells by blocking EJ-*ras* gene expression. A large increase in the level of EJ-ras mRNA after Dex (lane 2) was not diminished by 1,2-benzopyrone treatment (lane 4). Other drugs that counteract Dex-induced tumorigenesis in rats also failed to diminish the glucocorticoid-induced augmentation of EJ-ras mRNA (32). Immunoprecipitation of p21 in 14C cells exposed to Dex failed to show an effect of drugs on the augmented p21 protein contents (not shown).

Inhibition of Poly(ADP-Ribose) Polymerase by 1,2-Benzopyrone *in Vitro* and *in Vivo*. By varying the concentration of coenzymic octadeoxynucleotide (Fig. 3) and keeping NAD^+ concentration constant, the purified enzyme was inhibited by 1,2-benzopyrone by an apparent noncompetitive mechanism. *In vitro* enzymatic activity of isolated nuclei was also inhibited by 100 μM 1,2-benzopyrone by 80–90% (not shown).

The NAD^+ and ATP content of cells during various drug treatments varied only marginally from those of untreated 14C cells (Table 3), indicating no cellular toxicity. The poly(ADP ribose) content (with an average chain length of 50–70 ADP ribose units) of untreated 14C cells was about 8% of that of NAD^+ on a DNA basis. Estimation of intracellular NAD^+ concentration, considering cell volume, yielded 160 μM; thus, poly(ADP ribose) concentration was close to 5 μM (in terms of ADP ribose). Mono(ADP ribose) was consistently 10% of the polymer (not shown). Incubation of 14C cells with 0.1 μM Dex reduced cellular poly(ADP ribose) concentration by 12-fold, whereas exposure of cells to 100 μM 1,2-benzopyrone for 5 days caused a reduction of only 50%. The combination of treatment with both the drug and Dex, which reduces tumorigenicity, diminished polymer content somewhat below that found as the result of drug treatment alone (Table 3, experiment 4), but polymer content was 4 times higher than after hormone treatment alone (experiment 2). The apparent antagonism between 1,2-benzopyrone and Dex (compare experiments 2 and 4 in Table 3) is probably related to macromolecular consequences of drug action at the level of DNA–protein binding. Dex *in vitro* had no direct inhibitory effect on the purified calf thymus enzyme or on isolated nuclei (not shown). Enzyme inhibition by 1,2-benzopyrone produced in the cell was reversible (Table 4, experiment 3), with repeated washing of nuclei during their isolation restoring enzymatic activity (compare experiments 3 and 1 in Table 4). In contrast, the apparent decrease of enzymatic activity of nuclei isolated from Dex-treated cells

Table 2. Effect of 1,2-benzopyrone (1,2-BZP) treatment on tumorigenesis by 14C cells

	Treatment					
Exp.	Dex (*in vitro*)*	1,2-BZP (*in vitro*)†	1,2-BZP (water supply)‡	No. of injections§	Growing tumors,¶ no.	Average tumor weight, g
1	−	−	−	49 (24)	40 (81%)	3.0 (± 2.4)
2	+	−	−	10 (5)	8 (80%)	4.2 (± 3.9)
3	−	+	−	24 (12)	9 (38%)	0.8 (± 1.1)
4	−	−	+	46 (23)	21 (45%)	1.1 (± 1.7)
5	−	+	+	25 (12)	6 (24%)	0.7 (± 1.3)
6	+	+	+	10 (5)	3 (30%)	0.9 (± 1.6)

*14C cells were treated in culture with 0.1 μM Dex for 48 hr.
†14C cells were treated in culture with 100 μM 1,2-benzopyrone for 120 hr.
‡Rats were given 200 μM 1,2-benzopyrone in the drinking water for 7 days prior to injection of 10^5 14C cells.
§The number in parentheses is the number of rats used.
¶The percentage in parentheses is the tumor incidence.

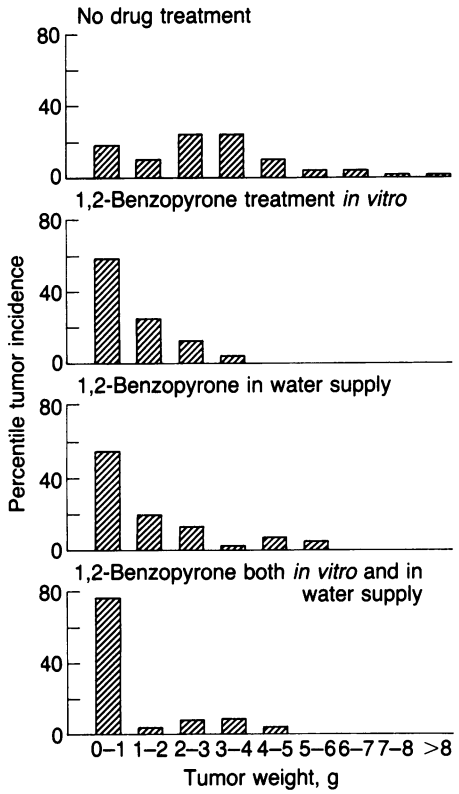

FIG. 2. The effect of 1,2-benzopyrone on tumor-size distribution in Fischer 344 rats (data given in Table 2). The number of injections per condition is shown in Table 2. Percentile tumor incidence (ordinate) means the percentage distribution of tumor size as a function of treatment. Tumor incidence (i.e., the percentage of tumor development from injection of 14C cells) is shown in Table 2.

was maintained at 46–47% (Table 4, experiments 2 and 4, compared with experiments 1 and 3). As seen from Table 4, DNase I abolished Dex-induced differences in enzymatic rates; therefore, they were due to differences in the structural regulation of the enzyme, not to differences in enzyme content (33).

We also tested the structural integrity of DNA in 14C cells with a highly sensitive fluorometric method (34) and found no detectable single- or double-strand breaks after hormone treatment (results not shown; experiments performed by A. Sooki-Toth).

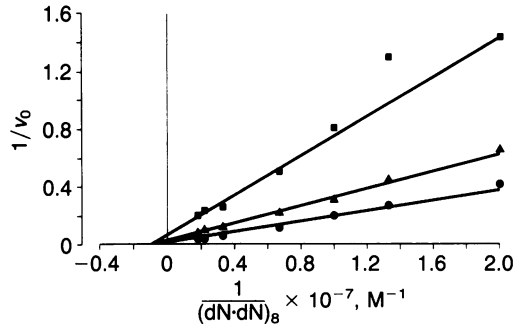

FIG. 3. Kinetics of inhibition at 24°C of purified poly(ADP ribose) polymerase (10 pmol per assay) by 1,2-benzopyrone at 200 μM (■), 50 μM (▲), and 0 (●). The concentration of NAD^+ was constant (100 nM), while the concentration of the double-stranded octamer was varied (abscissa). Reaction time was 2 min (v_0 = velocity in 2 min). The details of the assay are described in ref. 23. K_m (octamer) = 1×10^{-6} M; $K_i = 4.7 \times 10^{-5}$ M.

Table 3. Analyses of intact 14C cells

Exp.	Cell treatment	Metabolite content of intact and treated 14C cells, pmol/μg of cellular DNA		
		NAD	Poly(ADP ribose)	ATP
1	No treatment	46.1 ± 1	3.8 ± 0.2	360 ± 46
2	Dex*	58.3 ± 3	0.3 ± 0.4	360 ± 58
3	1,2-BZP†	46.4 ± 2	1.8 ± 0.7	420 ± 84
4	Dex*/1,2-BZP†	60.0 ± 6	1.2 ± 0.2	415 ± 64

Results represent an average of duplicate analyses (±SD) made on the following batches of cells: 70×10^6 and 50×10^6 (experiment 1); 86×10^6 and 150×10^6 (experiment 2); 120×10^6 and 70×10^6 (experiment 3); and 180×10^6 and 100×10^6 (experiment 4). Dex (0.1 μM) and 1,2-benzopyrone (1,2-BZP; 0.1 mM) were added from stock solutions in ethanol (0.1 mM Dex and 0.1 M 1,2-benzopyrone), controls receiving only ethanol in appropriate dilutions. Seeding cell density was 1.5×10^4 cells per 75 cm^2, and drug treatments commenced after 15 hr to allow cells to attach. The medium was not changed during the entire period of 120 hr. Cells were pretreated for 72 hr with 1,2-benzopyrone (experiments 3 and 4), and then Dex was added for 48 hr.

*Treatment for 48 hr.

†Treatment for 120 days.

DISCUSSION

A striking biological property of drugs that (*i*) inhibit the activation of poly(ADP ribose) polymerase by synthetic analogs of coenzymic DNA (Fig. 3) *in vitro* and (*ii*) depress enzymatic activity *in vivo* is their relatively indiscriminate antitumorigenicity with respect to the nature of cellular oncogenes. Results similar to those described here were recently obtained also with MS-7 cells (10) containing the glucocorticoid-inducible v-*src* oncogene (unpublished data). The drugs were effective in oncogene-containing cells with various enhancer/promoter sequences (metallothionein or MTV), and in 14C cells the level of EJ-*ras* expression was unaffected by drugs that inhibited tumorigenesis (Fig. 1). The sum of these results indicates that not neoplastic transformation but a late stage of the tumorigenic phenotype may be altered by DNA-oriented poly(ADP ribose) polymerase inhibitors. The reasonable agreement of the K_i of polymerase inhibitors with their cellular concentrations that prevent tumorigenesis, coinciding with polymerase inhibition *in vivo*, provides the most direct argument that implicates poly(ADP ribose) polymerase in the regulation of cellular proliferation of tumor cells. Poly(ADP ribose) polymerase may participate in an as-yet-unknown manner in a cell proliferation-regulating signal pathway (e.g., ref. 35), probably at the DNA level—a mechanism that appears to be confined to tumor cells. This tumor cell specificity is consistent with the observation that inhibition of poly(ADP ribose) polymerase in nonneoplastic cells seems relatively inconsequential except for the induction of certain enzymes (36, 37).

Poly(ADP ribosyl)ation of certain nuclear proteins may be essential for the expression of the tumorigenic phenotype, and inhibition at these sites by 1,2-benzopyrone could explain the depression of tumorigenesis. Numerous nonhistone proteins can serve as physiologic poly(ADP ribose) acceptors (23), and certain protooncogene products may be acceptors. The enzymatic target of drugs appears to be the specific DNA–enzyme complex (Fig. 3). This site is distinct from the NAD^+ binding site of the enzyme and may regulate the DNA-binding activity of the polymerase protein to form ternary complexes with histone, DNA, and the polymerase (S. S. Sastri and E. Kun, unpublished data). Benzamide, which was originally believed only to be a competitive analog of nicotinamide (38), equally binds to the DNA-related site and to the NAD^+ site of the enzyme (unpublished data); therefore its antitransforming effect (Table 1) can be ratio-

Table 4. Protein mono- and poly(ADP ribosyl)ation *in vitro* in nuclei of 14C cells isolated following treatment of cells with Dex and 1,2-benzopyrone (1,2-BZP), alone or in combination

Exp.	Cell treatment	Mono(ADP ribose)		Poly(ADP ribose)	
		− DNase I	+ DNase I	−DNase I	+ DNase I
1	None	24.6 ± 2	78.8 ± 8	21.5 ± 2	85 ± 8
2	Dex*	12.2 ± 3	73.5 ± 7	11.6 ± 1	76 ± 8
3	1,2-BZP†	22.5 ± 2	79.5 ± 6	21.0 ± 1.5	80 ± 4
4	Dex*/1,2-BZP†	9.5 ± 3	73.5 ± 7	8.9 ± 1.0	74 ± 4

Results are expressed as fmol/μg of DNA for mono(ADP ribose) and pmol/μg of DNA for poly(ADP ribose) in terms of ADP ribose units attached to proteins during 4 min at 25°C. Experimental conditions were the same as described in the legend of Table 3.
*Treatment for 48 hr.
†Treatment for 120 hr.

nalized on the same basis as that of 1,2-benzopyrone. Hexamethylenebisacetamide has no inhibitory action at the NAD^+ binding site (2).

It has been suggested (7, 39) that the previously observed anticarcinogenic effect of 1,2-benzopyrone in animals could be explained by induction of carcinogen-detoxifying enzymes (40)—specifically of quinone reductase (41)—a supposition made plausible because chemical carcinogenesis was studied. There is little doubt that metabolism of environmental carcinogens can modify their toxicity and mutagenicity. However, 14C cells, besides being unresponsive to the induction of quinone reductase by 1,2-benzopyrone (not shown), require no external toxic carcinogenic factors for tumorigenicity. The hormonal induction of tumorigenesis and its antagonism by 1,2-benzopyrone in 14C cells both *in vivo* and *in vitro* points to a basic cellular mechanism that appears to be remote from drug metabolism. Another hypothesis, explaining the antitumor activity of 1,2-benzopyrone by the activation of the tumoricidal action of macrophages, has been disproved experimentally (42).

The clinical efficacy of 1,2-benzopyrone in human patients with metastatic renal cancer has been reported (43). The cell biological and biochemical results described here represent an experimental approach for the development of new drugs with antitumorigenic effect.

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