

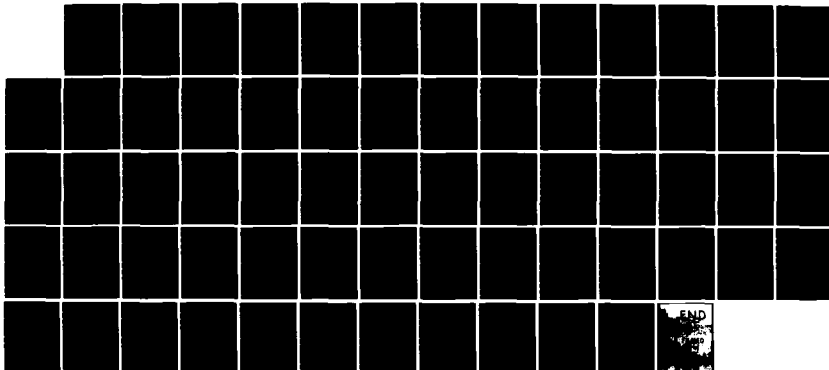
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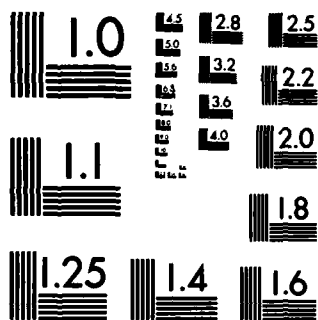
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BENZAMIDE DERIVATIVES AS PROTECTIVE AGENTS AGAINST THE ACTION
OF XENOTOXIC AGENTS ON HUMAN CELLS

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31 May 1984

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The first year of this report was culminated in a manuscript being submitted to the Proceedings of the National Acad. of Sci. The abstract and a copy of the paper were presented to the AFOSR in the final technical report, contract # F49620-80-C-0085. In addition to finding that benzamide can intervene in carcinogen induced neoplastic transformation that is cell cycle dependent, we also developed a procedure for detecting the presence of a malignant pheno- type in sarcoma tumor tissue. Monoclonal antibodies, (McAb) were developed against the transformed phenotype that cross reacted with the chemically		

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transformed cells. We were able also to use this McAb for the detection of sarcomatous invasive lesions in human tissue in the presence of normal stroma. Using these procedures described above combined with the analysis of competitive inhibitory kinetics of poly(ADP-ribose)polymerase activity we have now been able to identify eleven of these inhibitors that interfere in the neoplastic transformation process, (to be published in 1984-85). During this time period we were also able to establish human skin xenografts on nude mice and apply these technologies to human skin in vivo situations. We have also introduced a new technology for examining modification of DNA by the carcinogen, i.e. post labeling of the modified DNA that only requires 2×10^6 cells and ca. 190-200 ng of DNA. ✓

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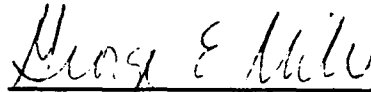
2 x 10⁶ cells per plate

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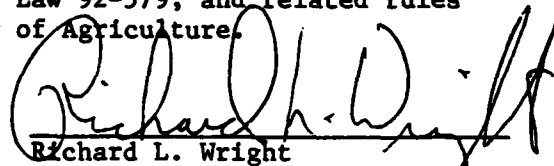
ANIMAL USE STATEMENT

The experiments reported herein were conducted according to the principles described in "Guide for the Care and Use of Laboratory Animals," prepared for the Committee on Care and Use of Laboratory Animals, DHHS Publication No. NIH 78-23, revised 1982.



George E. Milo, Ph.D.
Comprehensive Cancer Center

The Ohio State University has on file with the Office of Protection from Research Risks, NIH, a statement of assurance concerning the care and treatment of laboratory animals. This assurance states that the University complied with NIH "Guide for the Care and Use of Laboratory Animals," applicable portions of Public Law 92-579, and related rules and regulations issued by the Secretary of Agriculture.



Richard L. Wright
Deputy Director for Development
The Ohio State University
Research Foundation



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II. Statement of Work

Our objectives were to investigate how a class of inhibitors interfered in the neoplastic transformation process. We treated the cells here, isolated the nuclei and sent frozen samples to Dr. Kun in San Francisco for enzyme analysis. Then we followed with a representative sample of the treated cells the expression of the transformed phenotype, (see summary).

III. Status of Research

We recognize how protective agents can be used to inhibit a carcinogen induced event when a toxicant is added to human cells. We recognize further that this inhibition is cell cycle related. We have now developed probes that permit us to detect the presence of an abnormal phenotype in the presence of normal cells. Progress towards the objectives is proceeding in a timely fashion.

IV. Chronological List of Written Publications or in Preparation

Kurian, P., Milo, G., Kirsten, E., and Kun, E. Prevention of neoplastic transformation of human fibroblasts by drugs that interact with the poly(ADP-ribose)polymerase system. 1984. Science, in preparation.

Kurian, P., Jeffrey, A., and Milo, G. Preferential binding of benzo(a)pyrene diol epoxide to the linker DNA of human foreskin fibroblasts in S phase in the presence of benzamide, 1984. Proc. of the Nat'l. Acad. Sci. in preparation.

Kumari, H.L., Kurian, P., Beland, F., Howard, P., Kamat, P., Witiak, D.T., and Milo, G.E. Early events of the carcinogenesis process in human foreskin fibroblasts. 1984. 8th International Symposium on Polynuclear Aromatic Hydrocarbons, Battelle Press, Columbus, Ohio, in press.

V. List of Professional Personnel

George E. Milo, Ph.D. in Biochemistry, 1967, Principal Investigator.

Ponnamma Kurian, Ph.D. in Biochemistry 1978, Senior Research Associate. Thesis title: REDUCTION OF OXALIC ACID AND PECTIC ENZYMES BY CRISTULARIELLA PYRIMIDALIS.

VI. Interactions

Kumari, H.L., Kurian, P., Beland, F., Howard, P., Kamat, P., Witiak, D.T., and Milo, G.E. Early events of the carcinogenesis process in human foreskin fibroblasts. 1984. 8th International Symposium on Polynuclear Aromatic Hydrocarbons, Battelle Press, Columbus, Ohio, in press.

VII. New Discoveries

N.A.

VIII. Assessment of Progress Report

Our progress to date is presented here: We report here that cells in G₁/S phase (when maximum number of cells contain calmodulin antigen in the nucleus) are more sensitive to chemical carcinogenic insult than cells in mid S or late S phase of the cell cycle. Benzamide inhibits transformation induced by methylazoxy methanol acetate and 1-nitropyrene only when added to the cells at G₁/S phase. However, treatment with benzamide did not significantly alter the binding of carcinogen to DNA or the specific DNA-carcinogen adducts.

These results and progress on the effect of specific inhibitors on specific carcinogen-DNA adducts formed with nuclear DNA are discussed here: Addition of benzamide (BZ) at the onset of S inhibited expression of the neoplastic phenotype in human foreskin fibroblasts treated in vitro with 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetra-hydrobenzo[a] pyrene, (BPDE), in early S, (PNAS 80: 7219-7223, 1983). Analysis of the specific BPDE-DNA adducts revealed that ca. 65% of the total adducts in BZ and non-BZ carcinogen treated cells was the BPDE-dG adduct. Limited micrococcal nuclease digestion of the early S phase nuclei from cells treated with BPDE indicated that the carcinogen binds equally to linker and core DNA. However, when the cells were predominantly in S, in the presence of BZ, there was ca. three times more binding of BPDE to the linker DNA compared to the core region. The confluent cells in G₁ cell arrest treated only with BPDE also bound the carcinogen preferentially to the linker region. These data indicate that pretreatment of the cells with benzamide at the onset of S established a preferential binding pattern in the linker DNA, similar to that observed in the cells treated with BPDE in G₁ arrest. This phenomenon is probably due to a poly(ADP-ribose) related structural change in the chromatin brought about by the presence of benzamide in the cell in early S when the carcinogenic insult by BPDE was delivered.

The mode of action of the inhibitors, twelve in number, on the cell cycle dependent regulation of carcinogenesis is unknown. However, it can be assumed that the modification of DNA structure in S phase is critical since the preferential reaction appears to be with the replication forks.

EARLY EVENTS OF THE CARCINOGENESIS PROCESS IN HUMAN
FORESKIN FIBROBLASTS

Battelle Symposium
in press

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SUMMARY

We report here that cells in G₁/S phase (when maximum number of cells contain calmodulin antigen in the nucleus) are more sensitive to chemical carcinogenic insult than cells in mid S or late S phase of the cell cycle. Benzamide inhibits transformation induced by methylazoxy methanol acetate and 1-nitropyrene only when added to the cells at G₁/S phase. However, treatment with benzamide did not significantly alter the binding of carcinogen to DNA or the specific DNA-carcinogen adducts.

INTRODUCTION

Proliferating cell populations of mammalian cells are more responsive to chemical transforming agents than non-dividing cells (1). Moreover human cells when treated in early S of the cell cycle are the most responsive to a carcinogenic insult (2). Cordeiro-Stone *et al.* (3) have reported that alkylation of rapidly replicating DNA is heightened 2.6 to 5.0 times per nucleotide residue in responsive cells in mid-S of C₃H 10 T 1/2 clone 8 cells than in non-proliferating cells. In human cells, events leading up to early S must be programmed so the conditions for optimum responsiveness to the carcinogenic insult may be present. It is possible that these cells that are actively transcribing chromatin in the late G₁ transition stage 2-4 hours prior to the onset of S may have an altered regulatory effect on selective gene function (4). Changes in the conformational structure of DNA via modification of the acidic nuclear proteins could alter the gene function of these cells. One such mechanism for modification of non-histone nuclear proteins is by poly(ADP) ribosylation of the protein. The enzyme poly(ADP-ribose) polymerase appears to be under the control of Ca²⁺. Alterations in intranuclear calcium have been

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known to influence transformation events by viruses (5), physical carcinogens (6), and chemical agents (7). It has been known also that calcium influences cellular differentiation in human keratinocytes (8). These pleiotypic effects of calcium appear to be appropriately orchestrated via an intracellular protein, calmodulin. The uniqueness of this protein as a functional cellular protein is its allosteric properties (9). It has been suggested that calcium blockers and other calcium antagonists such as felodipine and prenylamine may alter the allosteric configuration of the calmodulin so the subsequent calcium modified events in early S may proceed. We intend to follow these events and determine how an antagonist of calcium alters calmodulin and how benzamide, an inhibitor of the expression of carcinogenesis, are programmed in concert and in tandem in a proper sequence to alter the carcinogen induced initiation of events that result in the cell responding to the carcinogenic insult as a toxic insult.

MATERIALS AND METHODS

Cell Culture

Neonatal foreskin (NFS) cell cultures were established as described previously(10). Fibroblasts were separated from the mixed cell culture by selective detachment from the substratum by trypsin treatment. The fibroblast cultures were passaged routinely using 0.1% trypsin and were maintained on Eagle's Minimum Essential Medium (MEM) prepared with Hank's Balanced Salt Solution, 25.0 mM Hepes buffer (Gibco, Grand Island, NY) at pH 7.2, supplemented with 1.0 mM sodium pyruvate, 1X non-essential amino acids (Microbiological Associates, Rockville, MD), 2.0 mM glutamine (Micro. Assoc.), 0.2% sodium bicarbonate, 5.0 ug/ml gentamicin, and 10% fetal bovine serum (FBS, Reheis, Kankakee, ILL). This growth medium was designated CM. The cultures were incubated in an atmosphere of 4% CO₂-enriched air at 37°C.

Cytotoxicity Assays

To determine the transforming dose to be used in the experiments it was necessary to examine the cytotoxic effects of the carcinogens on the cells at risk. Cells were seeded at 40/cm² in MEM supplemented with 1X essential amino acids (Micro. Assoc.), 1X vitamins (Micro. Assoc.) and 20% FBS; this medium was designated CMV. After the attachment, the cells were treated for 24 hours with

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varying doses of a carcinogen (MAMA was obtained from Sigma Chemical Corp. St. Louis, MO, and 1-nitropyrene was supplied by Dr. Fred Beland, NCTR, Jefferson, ARK) in CM supplemented with 10% FBS. The treatment medium was removed and the cells were washed twice to remove the residual extracellular carcinogen. The cultures were refed with CMV and incubated for 7 to 10 days. Colonies were fixed with 3% buffered formalin and stained with hematoxylin; those with ≥ 100 cells were scored to obtain the relative colony forming efficiency (RCF, the number of treated colonies relative to the number of untreated colonies). The dose (ED₅₀) which yielded a 50% reduction from control values in the RCF, was then calculated. A non-cytotoxic carcinogenic dose was used to transform the cells. The cells were treated with a transforming dose of the carcinogen(s) in the following manner.

Transformation Protocol

Neonatal foreskin fibroblasts at a population doubling (PDL) of < 5 were passaged at 8000 cells/cm² into MEM minus arginine and glutamine (Biolabs, Northbrook, ILL), supplemented with 1.0 mM sodium pyruvate, 5.0 ug/ml gentamicin, 0.2% sodium bicarbonate, and 10% dialyzed FBS. This nutrient deficient blocking medium was designated DM. After 24 hours incubation in a 4% CO₂ environment, the cells were released from the nonproliferating state by changing to CM containing 10% FBS and 0.5 U/ml insulin (IN). In these experiments two hours after the onset of S (12 hours after release from the nutrient deficient block) ca. 20% of the cells were in S and at this time the carcinogens were added to the cell cultures.

At the end of the treatment period the experimental carcinogenic agent was removed in either S₁ (early S), S₂ (mid S), or S₃ (late S), and the cells were passaged at a 1:2 split ratio into CM containing 2X vitamins, 8X nonessential amino acids, and 20% FBS, hereafter referred to as 8X medium. When these and subsequent cultures reached confluent density (80-90%), they were passaged at a 1:10 split ratio into 8X medium. Benzamide (BZ), an inhibitor of carcinogenesis, was added at the onset of S and removed when the carcinogen was removed (11) from the cultures.

Characterization of the Transformed Cells, Transitional Stage Growth in Soft Agar

Carcinogen-treated and control fibroblast populations were passaged in 8X medium at 1:10 split ratios until they reached PDL 20

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to 24. They were then seeded at 50,000 cells/25 cm² area in 2 ml 0.33% agar prepared in Dulbecco's LoCal medium (Biolabs) supplemented with 1 mM sodium pyruvate, 1X non-essential amino acids, 1X essential amino acids, 2.0 mM glutamine, 1X vitamins, 0.2% sodium bicarbonate, 5.0 ug/ml gentamicin, and 20% FBS. These cells were layered over 5 ml of a 2% agar base prepared in RPMI 1629 medium (GIBCO) supplemented with 1X sodium pyruvate, 1X nonessential amino acids, 1X essential amino acids, 2.0 mM glutamine, 1X vitamins, 0.2% sodium bicarbonate, 5 ug/ml gentamicin, and 20% FBS. The cultures were kept in a high humidity incubator in a 4% CO₂ atmosphere; 0.5 ml of 0.33% agar (prepared in supplemented modified Dulbecco's LoCal medium) was added every 3 to 5 days. The cultures were examined for colony (bolus) growth weekly, and they were considered negative when no growth was seen in 4 weeks. Cultures were scored as positive when colonies of ≥ 50 cells were observed and the number of colonies per plate were counted. Monolayer cultures were then reestablished by removing the colony from the agar with a tuberculin syringe (20 gauge needle) and seeding it into 8X medium. After attachment and sufficient growth, the cells were removed by trypsinization and reseeded to distribute evenly the cells from the monolayer colonies that had developed.

Radiolabeling of Nuclear DNA

In order to check cell synchronization, about 1.25×10^5 cells were seeded into P₆₀mm diameter plates directly into synchrony medium. Twenty-four hours later, cells were released from synchrony by refeeding the plates with growth medium containing 10% FBS and 0.5 U/ml insulin. At 60 minute intervals after release, triplicate plates were incubated with (³H-CH₃)-thymidine (S.A. 60 Ci/mMole), 1 uCi/ml for 20 minutes at 37°C. At the end of this time cells were washed twice at 4°C with 5.0 ml medium containing cold thymidine (0.6 mM) and precipitated with cold 10% (w/v) TCA. The precipitate was recovered on Whatman GE/C filter paper, washed further with ice cold 5% TCA and ethanol and dried. The radioactivity was determined in a Beckman LS9000 scintillation counter using 10 ml of Instagel scintillation cocktail.

Binding of ¹⁴C-Methyl Azoxy (¹⁴C)-Methanol Acetate to Cellular DNA

Ten hours after release from the G₁ block, monolayer cultures (8000 cells/cm²) were treated for 6 hours with either (¹⁴C)-MAMA

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(2.5 $\mu\text{Ci/ml}$) at 0.03 mM or (^{14}C)-MAMA, 0.03 mM and benzamide, 1.0 mM. At the end of treatment, after washing the cultures twice, the cells were detached with 0.1% trypsin and collected by centrifugation at 600 x g for 5 minutes. The cells were lysed with 1% Sodium Dodecyl Sulfate, (SDS) in Standard Saline Citrate, (SSC) buffer. Following dilution of SDS to 0.5%, the lysate was incubated with heat treated ribonuclease (100 $\mu\text{g/ml}$) for 1 hour at 37°C. The DNA was precipitated with cold 5% TCA, and collected on GF/C filter paper, 2.4 cm circles. After washing with cold 5% TCA and ethanol (-19°C), the filter paper circles were air dried and counted in LS9000 liquid scintillation counter using 10 ml instagel. An aliquot of cell suspension was treated with 20 mM NaOH for 60 minutes at 21°C and DNA of this digest was determined by fluorimetry.

Analysis of (^{14}C -Me) DNA and Nitrosopyrene-DNA Adducts

Ten hours after release from block, monolayers were treated for 3 hours with either (^{14}C)-MAMA at 0.022 mM (S.A. 55 mCi/mmol) or (^{14}C)-MAMA 0.022 mM and benzamide, 1.0 mM. After treatment, cell cultures were washed with CM minus FBS, scraped from the culture dish and collected by centrifugation at 600 x g for 5 minutes. The cells were suspended in SSC buffer (10⁷ cells/ml) and lysed with 1.0 % SDS. The lysate was extracted with equal volume of phenol reagent, (phenol 500 g, m-cresol 55 ml, 8-hydroxyquinoline 0.5 g, water 70 ml) three times. The DNA was precipitated with two volumes of cold ethanol (-19°C) in the presence of 0.2 M sodium acetate (pH 5.0) at -20°C overnight. The DNA was further purified by successive treatment with RNase A (50 $\mu\text{g/ml}$) for 30 min at 37°C followed by pronase (100 $\mu\text{g/ml}$) for 60 min at 37°C. The DNA was reextracted as described earlier. The DNA was resuspended in deionized water; an aliquot was removed for determination of radioactivity and DNA content. The rest was used for adduct analysis.

The purified DNA obtained by phenol extraction was hydrolyzed to purine bases and pyrimidine oligo-nucleotides by heating at 70°C for 30 minutes in 0.1 M HCl and chromatographed with appropriate standards on cation exchange high pressure liquid chromatography (HPLC). Chromatographic separation by HPLC was performed using an LDC-HPLC system with a Partisil-10 SCX M9 column (9.0 x 250 mm) in series with Partisil-10 SCX column (4.6 x 250 mm). Separation of purines and apurinic acid was achieved by using the following solvent program: Solvent A, 20 mM ammonium formate in 6% methanol, pH 4.0. Solvent B, 200 mM ammonium formate in 8% methanol, pH 4.0. A concave gradient of 100% solvent A to 100% solvent B over 25 min with a flow rate of 4.0 ml/min was used.

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Elution of bases was monitored by absorbance at 254 nm. One ml fractions were collected and radioactivity measured by counting in Instagel or NEN 963 cocktail. The C₈1-(deoxyguanosine-8-yl)-amino pyrene adduct analysis was conducted under experimental conditions as reported elsewhere (13).

Calmodulin Localization

Cells seeded at ca. 5000 cells per cm² were fixed in 3% phosphate buffered formalin at pH 7.2 for 30 minutes. The slides were then postfixated in methanol at -20°C for 10 minutes, rinsed with PBS and incubated 1 hr at 37°C with the primary antibody towards calmodulin. This antibody was prepared against rat testes calmodulin protein in sheep. After rinsing the fixed cultures extensively with PBS, the secondary reagent, FITC conjugated rabbit anti-sheep IgG, was incubated with the cells for 1 hr at 37°C. Again the slides were washed with PBS and examined under a Zeiss epifluorescent microscope with 485 nm excitation filter and 520-560 nm barrier filter. Quantitation of calmodulin in the cell was done by radioimmunoassay (12).

RESULTS

Treatment of fibroblasts at a low cell density of 10³ cells per 25 cm² (40 cells per cm²) yielded data that permitted us to select cytotoxic doses of the compounds that would adequately transform the cells at a high cell density from 5-10 x 10³ cells per cm². For these compounds evaluated here the toxicity values were the same in either randomly proliferating or synchronized cultures (8 x 10³ cells/cm²) subsequently subpassaged at 40 cells per cm² following the completion of treatment with the carcinogen. The time of exposure of the drug for cytotoxicity experiments was 24 hrs while exposure to the drug in transformation experiments was for 1 hr, at 2 hr after the onset of scheduled DNA synthesis, (S₁); 4 hrs into S for 1 hr, (S₂); and late S, 7 hrs into S for 1 hr, (S₃), Fig. 1. This procedure permitted us to establish non-cytotoxic carcinogenic doses where concentration effects could be studied in a range of drug that was non-cytotoxic.

In two experiments when cells (8 x 10³ cells per cm²) were treated in either S₁, S₂, S₃ or G₁ release the number of transformants was highest when cells were treated in S₁ compared to S₂ or S₃ and lowest when treated in S₃ or G₁ release. The total number of transformants detected following carcinogen treatment in S₁ ranged

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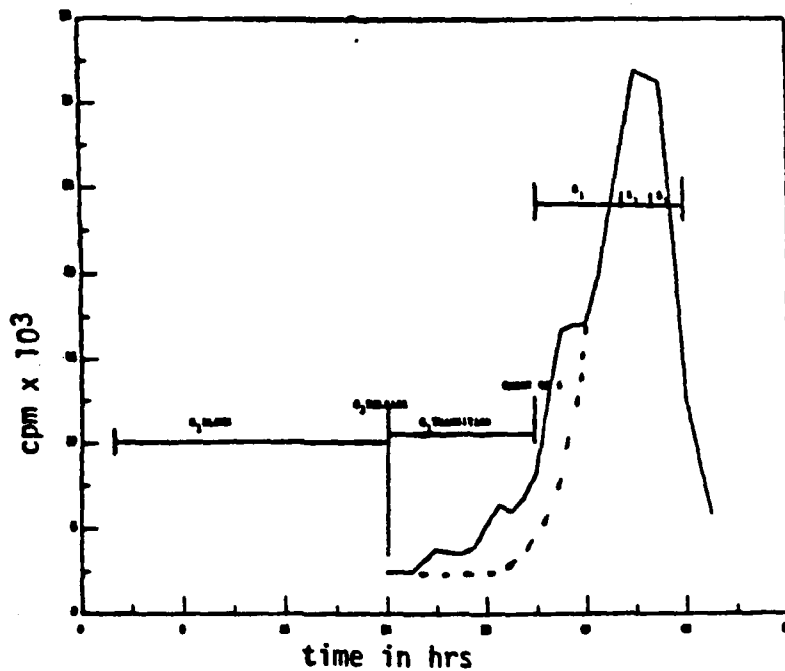


Figure 1. The profile of incorporation of (³H-CH₃)-thymidine into total DNA following release from a nutritional deficient block was followed over a 32 hr period from G₁ release through to the end of S. Samples were taken every hr over the 32 hr period following release from the G₁ block. The dotted line represents the typical profile of incorporation of the radiolabel thymidine into the nucleus when radiolabeled nuclei are counted by autoradiography. The solid line represents the profile of incorporation of (³H-CH₃)-thymidine when the radiolabel as detected by scintillation counting.

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from 382 ± 17.6 to 574 ± 31.4 colonies formed in soft agar per 10^5 cells seeded, (Table 1). The level of transformants observed in populations treated during G_1 release or S_3 ranged from 0 to 5 ± 2.3 colonies (Table 1). Treatment of cells at G_1/S border inhibited the transformation induced by MAMA, whereas treatment in late S or early G_1 had no significant inhibitory effect (unpublished data). The total modification of DNA following treatment with ^{14}C -MAMA of cells in S_1 was not altered in the presence of (BZ), an inhibitor of expression of transformation, (Table 2). Specific carcinogen DNA adduct modification using either MAMA or 1-nitropyrene as the carcinogen revealed that specificity of modification of the cellular DNA was not significantly altered in the presence of BZ, (Table 3). However, we recognize that these analyses were approaching the limits of our detection procedures and that more sensitive procedures may be necessary to detect alterations. In other work not reported here, we have found that modification of DNA by other carcinogens in G_1 release does not vary that much from the extent of modification of DNA in S phase of the cell cycle (11).

Moreover, the absolute number of cells committed to enter S was optimal 6 hrs after the release from the G_1 block (14). At this time there appears to be an optimum number of cells that contain calmodulin antigen in the nuclei of the cells. No further increase in the number of calmodulin positive nuclei in cells was observed prior to the onset of S or into early S, (S_1). When these cells were treated with prenylamine there was an increase in intensity of fluorescence to 4+ without a concomitant increase in cells committed to traversing from G_1 release into S. Furthermore, the total level of calmodulin per 10^6 cells remained constant at 150 ng/ 10^6 cells. There was no further increase in intensity of fluorescence as the cells entered S, (S_1).

To check if endogenous methylation was enhanced by MAMA it was administered to the cells at the onset of S in the presence and absence of benzamide. The extent of endogenous methylation was not altered by the presence of the carcinogen or inhibitor compared to the untreated or carcinogen-alone treated cells over the time of carcinogen treatment in S_1 . We conclude that the minor degree of modification of DNA by the aforementioned alkylation reagents did not influence in situ methylation of DNA.

DISCUSSION

The normal foreskin fibroblast cell population using the procedures we have defined is blocked in G_1 by a nutritional deficient

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TABLE 1

NUMBER OF COLONIES FORMED IN SOFT AGAR (0.33%) PER 10⁵ SEEDED CELLS

	S ₁	S ₂	S ₃	G ₁ Release
Exp. 1	574±31.4	130±8.0	5± 2.3	2 ± 0.7
Exp. 2	382±17.6	75±3.2	1± 0.8	---0---

This table presents data of 2 experiments where MAMA was used to treat cells in either S₁, S₂, S₃ or G₁ Release for 1 hr. The data here was collected from 8 wells, (n=8) of cells seeded at a cell density of 50,000 treated cells at PDL 20 into 2 ml of 0.33% soft agar supplemented with growth medium (See materials and methods). These data are presented as $\bar{M}$ (mean) values ± sigma (standard deviation).

TABLE 2

TOTAL INCORPORATION OF ¹⁴C-MAMA INTO DNA

Exp.#	Treatment	dpm/mg of DNA
1	MAMA	22,917
	MAMA + BZ	21,238
2	MAMA	11,428
	MAMA + BZ	11,440

See Materials and Methods for details.

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medium (11). The populations in block contained less than 10^{-3} cells, (Radiolabeling index for 1 hr using ($^3\text{H-CH}_3$) thymidine is less than 0.1%) actively proceeding through the cell cycle. These cells when released from this block proceeded into scheduled DNA synthesis ca. 10 hr later. For convenience of discussion, this transitional period from the release of the G_1 block to the onset of S we label as the G_1 transitional stage, (G_{1t}). The S phase of scheduled DNA synthesis we have divided into three stages, S_1 period (2 hrs after S for 1-2 hrs), S_2 period (4 hrs after the onset of S for 1-2 hrs), S_3 period (7 hrs after the onset of S for 1-2 hrs). Following the release from the block in G_1 , the cells were examined for the presence of calmodulin by the use of a polyclonal antibody against calmodulin. Between the time of G_1 release and 6 hrs into G_1 transition the pattern of fluorescence was primarily associated with the cytoplasm. The nuclei did not pick up the FITC-secondary antibody. The calmodulin concentration at this time period was a 150 ng/ 10^6 cells (12). Cells 6 hrs after release from the G_1 block were treated with prenylamine (calmodulin antagonist), an effector of a physical conformational change in the allosteric protein, calmodulin. Treatment with prenylamine did not cause an increase the amount of calmodulin per 10^6 cells, however, there was an increase in fluorescence to 4+ in the cytoplasm. Moreover, there was no increase in the number of nuclei fluorescing for the presence of calmodulin at this time.

As the cells enter early S (S_1), the number of cells committed to go through S in the presence and absence of prenylamine was correspondingly the same. The number of cells incorporating ($^3\text{H-CH}_3$)-thymidine at this time was ca. 20% for the two different populations whereas the number of calmodulin positive nuclei was ca. 56%. The incorporation of ($^3\text{H-CH}_3$)-thymidine, (30 min pulses) in the presence of the modulator exhibit the maximum distortion of the S_1 part of the curve when compared to a typical curve generated by counting nuclei containing grains over the nuclei. As reported elsewhere, (11) the optimum response to treatment with an alkylating carcinogen revealed that treatment in S_1 was the period most responsive to the carcinogen. The modification of the nuclear DNA of cells in early S was found to be quantitatively and qualitatively similar to the modification of DNA of cells in G_1 either early or late G (G_1 release) a few hours prior to the entry of the cells into S_1 . The specific carcinogen-DNA adduct, O⁶methyl guanine (O⁶-MeG) formed in cells treated with a carcinogenic non-toxic dose was similar to the O⁶-MeG adduct formed in cells that would not form transformants, (benzamide treated-MAMA treated cells).

General methylation of the nuclear DNA of the cells using ($^{14}\text{C-methyl}$) -methionine was not altered when the cells were treated with the carcinogen in the presence and absence of

TABLE 3

EFFECT OF BENZAMIDE ON SPECIFIC ADDUCT FORMATION AND TRANSFORMATION

Treatment	Specific Adducts per 10 ⁶ Bases	Incidence of Colony Formation per 10 ⁵ Cells Seeded in Soft Agar
MAMA	1.9 (O ⁶ MeG)	350-600
MAMA + BZ	1.1 (O ⁶ MeG)	---0---
1-Nitropyrene	1.4 (1-aminopyrene- 8-yl C ₈ G)	16-34
1-Nitropyrene + BZ	1.5 (1-aminopyrene- 8-yl C ₈ G)	---0---

The concentrations of these compounds were used at non-toxic carcinogenic doses, (MAMA-see Materials and Methods) and 1-nitropyrene (see Howard *et al.* Carcinogenesis 4:353-355, 1983, table 1).

benzamide, (11). It is interesting to note that treatment of cells with benzamide at the onset of S followed by treatment 2 hrs later in S₁ with the carcinogen did not alter the formation of specific-DNA adducts but did result in a reduction of transformants to zero. Moreover, there was no reduction in the numbers of cells in S₁ that incorporated (¹⁴C-CH₃)-thymidine (unpublished results, Milo, 1984). We suspect that the (Ca²⁺) influence on poly-ADP ribosylation of the protein protecting the DNA is modulated by calmodulin. Benzamide alters the interaction of poly(ADP) ribosylation process thereby altering the physiological function of chromatin at the structural level. These cellular events appear to be regulated by molecular events set in motion during G₁ release presumably by calmodulin-calcium manipulation of poly-(ADP) ribosylation process.

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KEY WORDS

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**Preferential Binding of Benzo[a]pyrene Diol Epoxide
to the Linker DNA of Human Foreskin Fibroblasts
in S Phase in the Presence of Benzamide**

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Classification

Cell Biology

Key Terms

**Cell Synchronization/ Specific Adducts/
S phase/ Nucleosome**

Abbreviations footnote

**BPDE-[(+)-7 α ,8 β -dihydroxy-9 β -10 β -epoxy-
7,8,9,10-tetrahydro benzo[a]pyrene],
BZ [Benzamide], HNF [Human Neonatal
Foreskin]**

ABSTRACT

Addition of benzamide (BZ) at the onset of S inhibited expression of the neoplastic phenotype in human foreskin fibroblasts treated in vitro with 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetra-hydrobenzo[a]pyrene,(BPDE), in early S, (PNAS 80: 7219-7223, 1983). Analysis of the specific BPDE-DNA adducts revealed that ca. 65% of the total adducts in BZ and non-BZ carcinogen treated cells was the BPDE-dG adduct. Limited micrococcal nuclease digestion of the early S phase nuclei from cells treated with BPDE indicated that the carcinogen binds equally to linker and core DNA. However, when the cells were predominantly in S, in the presence of BZ, there was ca. three times more binding of BPDE to the linker DNA compared to the core region. The confluent cells in G₁ cell arrest treated only with BPDE also bound the carcinogen preferentially to the linker region. These data indicate that pretreatment of the cells with benzamide at the onset of S established a preferential binding pattern in the linker DNA, similar to that observed in the cells treated with BPDE in G₁ arrest. This phenomenon is probably due to a poly(ADP-ribose) related structural change in the chromatin brought about by the presence of benzamide in the cell in early S when the carcinogenic insult by BPDE was delivered.

INTRODUCTION

The covalent interaction of a carcinogen with the target cell DNA is considered as one of the major events in neoplastic transformation (1-3). Proliferating cells in vivo and in vitro are found to be more sensitive to transformation by chemical carcinogens than non-dividing cells, (4,5) and this sensitivity is increased when the cells are treated in early S phase (6,7) presumably due to the higher binding of the carcinogen to the newly synthesized DNA (8). A good correlation between carcinogenicity and DNA binding has been observed for a series of chemicals (2).

Benzo[a]pyrene, a potent environmental carcinogen, requires metabolic activation by the microsomal enzymes to the ultimate carcinogen metabolites, (9,10). The bay region epoxide, ($\pm$) 7 α ,8 β -dihydroxy-9 β ,10 β -epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, (BPDE), has been identified as the ultimate metabolite that binds to the DNA. One of the major adducts formed results by the trans addition at the 10-position of BPDE-I to the exocyclic amino group of guanine (11). A correlation between the biological activity of BPDE-I and the presence of BPDE-guanine adducts in the modified DNA has been identified (12). Furthermore, recent studies on the BPDE-DNA adducts in the chromatin have revealed a non-random distribution, the linker DNA being modified to a greater extent than the nucleosome core DNA (13,14). It seems likely that BPDE is capable of forming site specific adducts under favorable conditions and this might have a profound effect on gene expression.

Previous studies from this laboratory have shown that the in vitro treatment of human foreskin fibroblasts, in early S phase, with BPDE induced a neoplastic transformation in the low passage responsive cell populations, but not in the high passage refractory cell populations. Moreover, we observed no significant difference in the level of DNA modification, or in the specific carcinogen-DNA adduct profiles of both of these treated cell types (15). Recently benzamide, a specific inhibitor of poly ADP-ribose polymerase, (16), has been shown to inhibit the transformation of the responsive treated cells initiated by BPDE (17). Preliminary results, upon examination of specific carcinogen-DNA adduct formation indicated that benzamide did not alter the DNA modification. These data presented here expand on the preliminary report, (18) and attempt to explain how benzamide may play a role on the BPDE-DNA adduct formation as well as distribution.

MATERIALS AND METHODS

Chemicals

[G-³H]-BPDE (479 Ci/mole) was supplied by the Midwest Research Institute (Kansas City, Missouri). Methyl [¹⁴C]-thymidine (56 Ci/mole) and methyl [³H]-thymidine (S.A. 80 Ci/mole) were purchased from New England Nuclear (Boston, Massachusetts). Micrococcal nuclease, pancreatic RNase, Proteinase K and Sarkosyl NL were purchased from Sigma Chemicals (St. Louis, Missouri).

Cell Culture and Treatment

Human neonatal foreskin fibroblast (HNF) cells were cultured and serially passaged by previously established procedures, (19). These cells were maintained in 75 cm² flasks in complete growth medium (CM), consisting of Eagle's minimum essential medium (MEM): 25 mM Hepes buffer (pH 7.2), supplemented with a 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 50 µg/ml gentamicin, 0.2% sodium bicarbonate and 10% fetal bovine serum (FBS). Cells at PDL-5 were blocked in the G₁ phase using a nutrient deficient medium, released from the block and treated in early S phase with [G-³H]-BPDE, (15). The final concentration of [G-³H]-BPDE used in these experiments was 0.34 µg/ml of the treatment medium. After 3 hrs treatment, the cells either were harvested by mild trypsinization and pelleted by centrifugation at 1000 x g, or the experimental medium was removed and the cultures refed with CM and harvested 8 hrs later.

In experiments where the cells were treated with [G-³H]-BPDE in the presence of BZ, 1mM BZ was added to the release medium at the onset of S phase of the cell cycle, 2 hrs prior to the carcinogen treatment and was maintained at this concentration all through carcinogen treatment. The cytotoxic effects of BZ and BPDE on the cells were determined in separate experiments by a colony formation toxicity assay, (20). The effect of 1 mM BZ on the S phase of the cell cycle during the treatment period, was measured by the incorporation of thymidine into the nuclear DNA by autoradiography (20), in the presence and absence of BZ.

Cells used to isolate nuclei for limited micrococcal nuclease digestion were pre-labeled with [¹⁴CH₃]-thymidine as described. PDL-3 cells were serially seeded at 10,000 cells per cm² into 75 cm² flasks and 16-18 hrs later 0.2 nCi of [¹⁴CH₃]-thymidine/ml was added for 48 hrs. At this time the experimental radioactive medium was replaced with fresh CM and the cells were allowed to grow for 2-3 more days to a confluent state. These prelabeled confluent cells were subpassaged at 10,000 cells per cm² into a nutrient deficient blocking medium for 24 hrs, released from the block and treated with the carcinogen or benzamide and carcinogen (described earlier). When confluent cell cultures in G₁ arrest were required, the [¹⁴CH₃]-thymidine prelabeled cells were allowed to grow in the fresh CM for 5-6 days. They were then treated with 0.34 µg [G-³H]-BPDE per ml of CM minus FBS, for 3 hrs and then harvested.

Isolation of Nuclei

All isolation procedures were carried out between 0-4°C. The pelleted HNF fibroblast cells were suspended in 0.25 M sucrose, 10 mM Tris-maleate, at pH 7.5 containing 1 mM dithiothreitol, 3 mM Ca^{+2} -acetate, 2 mM Mg^{+2} -acetate, pH 7.5, (buffer A), at 10^7 cells/ml and homogenized in a stainless steel mortar with a teflon pestle with a 0.009" clearance connected to a Tri-R homogenizer. When 80% or more of the cells were broken, as evidenced by examining the cells under phase microscopy at 430X following staining by toluidine blue, the cells were prepared for nuclei isolation in the following manner: Six and one half ml of 1.8 M sucrose in buffer A was added to 1 ml of homogenate, mixed by two up and down strokes, layered over 5 ml of 1.8 M sucrose in buffer A and centrifuged at 40,000 x g in vacuo at 4°C, for 1 hr. The nuclei pellet was resuspended in 1.0 M sucrose in buffer A by gentle homogenization and centrifuged at 10,000 x g for 10 min. The pellet at the bottom was resuspended in the desired solution and quick frozen at -70°C.

Analysis of DNA Adducts

Nuclei prepared from cells harvested immediately after the carcinogen treatment (3 hrs) as well as those refed with the CM for 8 hrs after treatment were suspended in 10 mM Tris-HCl-1mM Na_2EDTA buffer (pH 7.5) containing 0.1% sodium dodecyl sulfate (SDS), ($6-8 \times 10^6$ nuclei/ml buffer) and quick frozen at -70°C until used for adduct analysis. Nuclear DNA was purified and analyzed by the procedure described by Tejwani et al. (15).

Micrococcal Nuclease Digestion and Assay

The digestion and assay of the digested products were carried out by a modification of the combined procedures of Jack and Brookes (13) and Kaneko and Cerruti (21). Briefly, the nuclei were suspended in 0.25 M sucrose, 1 mM Tris-HCl, 0.5 mM CaCl₂, pH 7.4, (digestion buffer) at ca. 5×10^6 nuclei/100 μ l, treated with micrococcal nuclease (2.5 μ g/ml) at 37°C and 30 μ l aliquots removed at various times between 0-40 min. The reaction was stopped by the addition of an equal volume of 10 mM Tris-HCl -10 mM EDTA, pH 7.4, followed by Sarkosyl NL to a final concentration of 0.5%. The nuclear lysate was then incubated with 20 μ g/ml of RNase (previously heat treated in the dark for 10 min at 100°C) at 37°C for 30 min followed by treatment with 100 μ g/ml of proteinase K for 60 min at 37°C. The incubation medium was mixed with an equal volume of redistilled phenol made up as a phenol reagent (phenol: chloroform: isoamyl alcohol-24:24:1 v/v), by vortexing for 2 min. After mixing the phases, the homogeneous suspension was centrifuged at 5,000 rpm for 5 min in a Beckman microfuge, Model 11. The top aqueous layer was removed and the phenol layer extracted twice with half the volume of 10 mM Tris HCl-10 mM EDTA at pH 7.4. The combined aqueous buffer phases were extracted once again (v/v), with the phenol reagent as above and the final aqueous phase was extracted twice with water saturated ether to remove traces of phenol. Excess ether was removed by placing the tubes in a 60°C water bath for 5 min. Twenty-five μ l of 2 mg/ml of calf thymus DNA was added as a carrier and the DNA was precipitated with 2 volumes of cold ethanol at -20°C overnight. The precipitate was

recovered by centrifugation at 10,000 rpm (in microfuge) for 7 min. The precipitate was washed once carefully with cold 80% ethanol and the pellet dissolved in 50 μ l of 1 M HCl, by heating at 70°C for 1 hr. Following neutralization with 50 μ l of 1 M KOH, (CO₂ free), the solution was quantitatively transferred to a counting vial using 150 μ l of 50 mM Tris HCl, pH 7.5. Ten ml of scinti verse II (Fisher Scientific, Fairlawn, New Jersey) were used for assaying radioactivity in a Beckman 9000 Scintillation counter. The phenol phase and the combined ethanol supernatant also were assayed for the presence of radioactivity. The tritium counts were corrected for spillover of the ¹⁴C radiation. The ratio of ³H/¹⁴C dpm in the precipitate was used as a measure of the concentration of BPDE-DNA adducts.

Determination of Adduct Concentration in Linker DNA

The concentration of the adducts on the linker DNA was calculated using the equation:

$$200x = 146y + 54z \quad (13)$$

where, x= concentration of the adducts on undigested DNA

and y= concentration of the adducts on core DNA (after 27% digestion)

and z= concentration of the adducts on the linker DNA

200= base pairs in nucleosome

146= base pairs in core DNA

To compute these concentrations of adducts in [¹⁴CH₃]-thymidine prelabeled [G-³H]-BPDE treated cells, the [³H]/[¹⁴C] ratio of ethanol precipitable nuclear material at definite time, (t₅ to 40 min), during

limited micrococcal nuclease digestion was divided by the $[^3\text{H}]/[^{14}\text{C}]$ ratio measured at $t=0$ min digestion.

RESULTS

Earlier studies in this laboratory have revealed that 1 mM benzamide inhibits the carcinogenic response of BPDE treated HNF cells (17). The relative cloning efficiency (20), a measure of cytotoxicity, was determined over a range of concentrations of BZ from 0.01-4 mM. No inhibition of the relative cloning efficiency compared to the untreated control was observed with 1 mM benzamide, the concentration used in our experiments. At 4 mM, approximately 15% inhibition was observed. Although 0.34 μ g/ml concentration of BPDE used in the binding studies was toxic to the cells at low cell density employed in the cloning assay, at the high cell density conditions used in our experiment the cells grew well after a short lag phase and transformants were observed. However, the low specific activity and the low percentage of base modification of DNA by [G- 3 H]-BPDE necessitated the use of this concentration.

In order to investigate the effect of BZ on the early S phase of these cells, the time period at which they are very susceptible to the carcinogen, [3 H-CH $_3$]-thymidine incorporation in the presence and absence of BZ was measured by autoradiography, (Fig. 1). BZ was added 10 hrs after release from the G $_1$ block by the nutrient deficient medium, at the onset of S. Radiolabeled nuclei containing 50 grains or more for a total of 1000 nuclei were counted for each point on the graph. The percentages of radiolabeled nuclei were calculated, up to 21 hrs after release from synchrony. No difference was observed in the number of nuclei going through S phase in the presence or absence of benzamide.

DNA was isolated from the nuclei of the cells treated with [G-³H]-BPDE, or [G-³H]-BPDE and 1 mM BZ. The levels of specific adducts were 4.5 ± 7.6 and 3.7 ± 2.8 modified bases per 10^6 bases, respectively. The slight variations could not be correlated with the extent of inhibition of the transformations (17). The HPLC profiles of the modified deoxyribonucleosides, shown in Figs. 2a and 2b, were qualitatively and quantitatively similar. Eight hrs after concluding the BPDE treatment, when 50% of the radiolabeled carcinogen was removed from the cells, nuclei were isolated and the DNA was analyzed as described previously. The specific BPDE-DNA modified adducts were 1.2 and 0.75 modified bases of 7 β -BPDE-dG per 10^6 bases in the presence and absence of BZ, respectively. Moreover, the ratios of specific modified bases were also qualitatively and quantitatively similar in the BZ and non-BZ treated cells, Fig. 3a and 3b. These data suggested that preferential removal of the specific carcinogen-DNA adducts was not a contributing factor to the induction of a carcinogenic event.

Since no significant difference in specific DNA modification could be detected, the distribution of the adducts on the linker versus core DNA regions was examined by the limited micrococcal nuclease digestion technique. The time course of the excision of the adducts on the nuclear DNA was followed by limited digestion of the nuclei prelabeled with [¹⁴CH₃]-thymidine and treated with [G-³H]-BPDE or BZ and [G-³H]-BPDE, and purification of the undigested DNA. Confluent cells, (G₁), prelabeled with [¹⁴CH₃]-thymidine and treated with [G-³H]-BPDE for 3 hrs, were also analyzed similarly for comparison, as it has been shown by

earlier workers that BPDE preferentially binds to the linker region in quiescent cells in a confluent dense state (13). The extent of residual radiolabel remaining in the EtOH precipitate is measured as a function of time from 0 to 40 min. The values obtained, following the limited micrococcal nuclease digestion were computed as percentage of the precipitable counts before digestion. The [^{14}C] counts remaining in the ethanolic precipitate following the limited digestion are a measure of the residual undigested DNA and are an indirect measurement of the rate of digestion of the DNA in the linker and core regions. A measurement of residual [^3H] counts in the ethanol precipitate following limited micrococcal nuclease digestion represents the residual modified DNA. This is an indirect measurement of the rate of total DNA-adduct removal. The data obtained in one experiment is shown in Figure 4. Similar patterns were obtained in two other experiments also. There was no wide variation in the rates of digestion of the nuclei [^{14}C removal) from the three samples (Fig.4a). Nuclei from the cells treated with [$\text{G-}^3\text{H}$]-BPDE in early S phase were better substrates for limited micrococcal nuclease digestion than the nuclei from cells exposed to 1 mM BZ-carcinogen treatment. This is evident by the gradual removal of the [^{14}C] label over the 40 min period. Nuclei prepared from confluent cells exposed to [$\text{G-}^3\text{H}$]-BPDE were more resistant to the limited micrococcal nuclease digestion than the other two types of treated nuclei. In contrast to the [$^{14}\text{CH}_3$]-thymidine removal, there were significant differences in the rate of early adduct excision ([^3H] removal) from the DNA of the three samples (Figure 4b). This rate of adduct removal was lower (Fig. 4b) from

the DNA of [G-³H]-BPDE treated S-phase nuclei than from the DNA of the cells pretreated with BZ at the onset of S before the addition of BPDE 2 hrs later. Nuclei from BPDE treated confluent cells, (G₁), exhibited an intermediate rate of DNA-adduct excision during the early period of digestion. The differences in the rate of removal of the radiolabel [³H] were negligible after 10-20 min digestion, when approximately 25% of the modified DNA (the linker DNA) was digested. Chromatography of the digests by slab gel electrophoresis on 6% poly acrylamide gel did not show any detectable, distinct difference in the band patterns of the DNA of the three differently treated nuclear preparations. We observed a prominent band of ca. 146 base pairs in all three nuclear preparations following a 10-20 min digestion. The appearance of this band was indicative of the presence of nucleosome core DNA. Though [³H] and [¹⁴C]-radiolabels present in the phenol and ethanol layer were measured, they did not give any valuable data mainly because ca. 95% of the [³H] label in the nuclei was bound to the proteins and hence appeared to be associated with the phenol phase. Also, a definite fraction of the [¹⁴C] (approx. 20%) was found associated with the phenol layer, at zero minute digestion. This could probably be due to the metabolism of the nucleotides and the incorporation of the [¹⁴C] label in the various nuclear components during the period the cells are allowed to grow to confluency. Furthermore, the digestion products after 5-40 min digestion were distributed between the phenol layer and the ethanol supernatant when extracted after digestion.

A plot of the relative adduct concentration, ($[\text{³H}]/[\text{¹⁴C}]$ ratio in the ethanol precipitable material at various times of digestion divided by

the [^3H]/[^{14}C] ratio in the ethanol precipitable material before digestion) versus the percentage of DNA digested, is shown in Figure 5. There is a rapid decrease in the adduct concentration in the confluent cell nuclei as well as in the BZ and BPDE treated S-phase nuclei. The decrease was greater in the BZ -BPDE treated nuclei when the slope of the first 10-20 min of removal of adducts is quickest when plotted as a function of the percent digested DNA. After the linker DNA was digested (27% digestion), the adduct concentrations became constant, indicating that BPDE binds preferentially to the linker DNA. Nuclei isolated from carcinogen only treated S-phase cells exhibited only a slight decrease in adduct concentration during digestion. The rapid removal of the adducts from the BZ and BPDE treated nuclei in the beginning of the digestion indicates a higher binding to the regions in the linker DNA easily accessible to micrococcal nuclease, probably the replicating forks in the linker DNA.

At 27% digestion, when all the linker DNA was solubilized, the relative adduct concentration in the core DNA of the confluent cells exposed to the BPDE was 70% of the undigested DNA, (Fig. 5). This is equivalent to 2.6 times more binding to the linker DNA compared to the nucleosome core DNA (see Materials and Methods for calculation), and is in close agreement with the data of Jack and Brookes (13) for primary mouse embryo cells. S phase cells, exposed to the carcinogen and BZ also, exhibited a similar binding, the relative adduct concentration in the core DNA being 68% of the total DNA and hence 2.7 fold more binding on the linker DNA. However, in the absence of BZ, this binding was considerably

decreased, only 1.4 times more binding to the linker region of the nucleosomes. Since only 80% of the cells went through the S phase (Fig. 1) the preferential binding observed in this case may be due to that fraction of the cells which did not undergo replication.

DISCUSSION

Several chemical carcinogens including BPDE, can induce neoplastic transformation of human diploid fibroblasts (6,15,17). Benzamide, a competitive inhibitor of chromatin associated poly ADP-ribose polymerase, has been shown to inhibit transformation by a variety of chemicals such as BPDE, as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methylazoxymethanol acetate (MAMA) (17,22). The specificity of this inhibition appears to involve the covalent molecular binding of the inhibitor to the poly(ADP-ribose)polymerase-poly(ADP-ribose)-DNA complex (unpublished data).

Earlier studies (17) have shown that 1.0 mM, of benzamide does not have any effect on MAMA-induced strand breaks, or the extent of methylation of O⁶-guanine, though it inhibits poly(ADP-ribose) synthesis and also the expression of neoplastic transformation by MAMA. A similar finding has been reported by Borek et al. (22) for the analogue, 3-aminobenzamide. A 1.0 mM concentration of 3-aminobenzamide significantly inhibited transformation by MNNG as well as poly(ADP-ribose) synthesis, whereas it had no significant effect on repair replication, DNA synthesis by salvage pathways and strand breakage frequencies in X-ray damaged cells. However, contrary to our results, they found that strand break frequencies in carcinogen damaged cells increased with increasing concentration of 3-aminobenzamide. Moreover, in a more recent study, we have shown that benzamide had no effect on UV induced thymidine dimer formation or the removal of these dimers

(unpublished data). However, it did inhibit transformation. The above data suggest that factors other than DNA damage and repair may be involved in the inhibition of transformation by carcinogen in the presence of BZ.

The present study was designed for detecting any possible qualitative or quantitative difference in the formation of the carcinogen-DNA adduct in the presence of BZ. The cells were treated at early S phase of the cell cycle with the carcinogen, or BZ and carcinogen. Determination of the percentage of the radiolabeled nuclei by autoradiography, after [^3H -CH $_3$]-thymidine treatment revealed that BZ did not alter the extent of incorporation of [^3H -CH $_3$]-thymidine in the nuclei involved in DNA replication during the time period of the experimental treatment. Analysis of the specific BPDE-DNA adducts by HPLC, from the carcinogen modified DNA isolated at the conclusion of the experimental treatment with either the carcinogen or carcinogen and BZ treatment and also at 8 hrs after the treatment did not reveal any qualitative or quantitative difference in the presence of specific carcinogen adducts formed at a transforming dose of the carcinogen.

Recent reports on the distribution of the carcinogen modifications in the DNA of the chromatin using the limited micrococcal nuclease technique have indicated preferential binding of carcinogens to the linker DNA of chromatin (13,21). This localized binding of the carcinogen in specific regions of DNA may play an important role in the regulation of gene expression. Jack and Brookes (13), have shown that BPDE binds three times more to the linker DNA compared to the core DNA when

confluent mouse embryo cells were treated. Our studies with treated confluent HNF cells also revealed similar results. There was 2.6 times more binding to the linker versus core DNA. Moreover, no transformant was observed (unpublished data). HNF fibroblasts, when treated in S phase with BPDE in the presence of BZ, behave similarly to the cells in G₁ arrest, with 2.7 times more binding to the linker DNA than to the core DNA. But preferential binding of the BPDE to the linker DNA was very low (1.4X) in the absence of BZ. It has been shown (24,25), that nucleosomes are redistributed randomly along the nuclear DNA during replication. The mechanism proposed to account for this redistribution during DNA synthesis is that of proteins sliding along the DNA, and dissociation and reassociation of the nuclear proteins. If so, we can easily envision how preferential binding of BPDE to the linker DNA of the chromatin observed in the confluent cells may be absent when the cells at S phase are treated with the carcinogen. The re-establishment of the preferential binding of the carcinogen to the S-phase chromatin in the presence of BZ, a potent ADP-ribose polymerase inhibitor and an anticarcinogen, suggests that poly ADP ribosylation of the nuclear protein may be involved in an as yet unknown manner, in retaining the integrity of the linker and core regions of the DNA during replication in early S. This, in turn, might mask the modification of certain critical sites by the carcinogen, thereby inhibiting the neoplastic transformation.

Figure 1: Effect of 1 mM benzamide on the incorporation of [^3H -CH $_3$]-thymidine into the nuclei. Cells seeded at 10,000/cm 2 into T-25 flasks, using amino acid deficient medium to block them at G $_1$ were released from the block after 24 hrs. Benzamide (O—O, 1 mM) was added to these flasks at 8 hrs after release from G $_1$ block. (●—●, untreated control). Flasks were removed at every 4 hrs after release from the G $_1$ block until the onset of S and then every hr for a 12 hr period. These cultures were refed with (^3H -CH $_3$)-thymidine containing medium for 30 min (1 $\mu\text{Ci/ml}$, S.A. 80 Ci/mmole). After the treatment the cells were fixed, washed and dried. The labeled nuclei were detected by autoradiography. Each point represents the percent of radiolabeled nuclei obtained by counting the labeled nuclei in a total of 1000 nuclei counted.

Figure 2: HPLC profile of [G-³H]-BPDE DNA adducts formed in the presence and absence of 1 mM benzamide. PDL 5 cells were blocked at G₁, released and treated 2 hrs after the onset of S phase with [G-³H]-BPDE for 3 hrs as described in the Materials and Methods section. DNA was isolated and enzymatically digested. The modified deoxyribonucleosides were separated by Sephadex LH-20 chromatography and subsequently co-chromatographed on HPLC with authentic standards.

- A. DNA from non-benzamide treated cells.
- B. 1.0 mM benzamide was added to the incubation medium 4 hrs prior to BPDE treatment.

Figure 3: HPLC profile of specific [G-³H]-BPDE DNA adducts remaining in the cells at 8 hrs after conclusion of 3 hr treatment with [G-³H]-BPDE in the presence and absence of benzamide. PDL-5 cells were incubated with BPDE or benzamide and BPDE as described in Figure 2. At the end of the 3 hr treatment period, the cells were refed with CM for 8 hrs. DNA was then isolated and analyzed by HPLC as described in Figure 3.

- A. DNA from BPDE treated cells.
- B. 1.0 mM benzamide was added to the incubation medium 4 hrs prior to BPDE treatment.

Figure 4: Kinetics of micrococcal nuclease (MNase) digestion of the nuclei from HNF cells after treatment with [G-³H]-BPDE under three different conditions. PDL-5 cells were incubated with [G-³H]-BPDE, 2 hrs after the onset of S for 3 hrs with or without benzamide, the nuclei were isolated and digested with micrococcal nuclease as described under Materials and Methods. Nuclei isolated from confluent cells treated with [G-³H]-BPDE at PDL 5 were also used for MNase digestion. Each experimental point is an average value of triplicates.

- A. percentage of the total precipitable DNA ([¹⁴C]-label)
- B. percentage of the total BP-DNA adducts ([³H]-label), appearing in the ethanol precipitate at various times of digestion.

- : Nuclei from S-phase cells treated with [G-³H]-BPDE.
- : Nuclei from S-phase cells treated with benzamide and [G-³H]-BPDE.
- △—△: Nuclei from confluent cells (G₁ arrest) treated with [G-³H]-BPDE.

Figure 5: Relative adduct concentration in the residual DNA, ($[^3\text{H}]/[^{14}\text{C}]$ ratio of ethanol precipitable material at various times of digestion, divided by the $[^3\text{H}]/[^{14}\text{C}]$ ratio of ethanol precipitable material before digestion) during MNase digestion, as a function of the percentage of DNA digested in nuclei from BPDE treated cells. Values from three independent experiments are used.

- : Nuclei from S-phase cells treated with $[\text{G}-^3\text{H}]$ -BPDE.
- : Nuclei from S-phase cells treated with benzamide and $[\text{G}-^3\text{H}]$ -BPDE-I.
- △—△: Nuclei from confluent cells (G_1 arrest) treated with $[\text{G}-^3\text{H}]$ -BPDE.

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Prevention of Neoplastic Transformation of Human Fibroblasts by
Drugs that Interact with the Poly(ADP-ribose) Polymerase System.

Exposure of intact synchronized human fibroblasts in the early S phase to a non toxic concentration of benzamide, a reagent that inhibits poly(ADP-ribose) polymerase of isolated nuclei or of permeabilized cells (1), prevented neoplastic transformation by DNA methylating, benzoylating, propionylating and sulfonylating ultimate carcinogens (2). Enzymatic assays for poly(ADP-ribose) polymerase in permeabilized cells, that had been exposed to ultimate carcinogens in the presence of benzamide, showed that coincidental with the prevention of transformation, enzyme activity was augmented rather than inhibited (2). For this reason we concluded that the biological effect of benzamide was probably related to a more complex action of the antitransforming drug on the poly(ADP-ribose) polymerase system and enzyme kinetics would be expected to provide some information regarding the nature of this interaction. If this assumption is correct then: a.) antitransforming drugs should be predictable on the basis of their mode of binding to the nuclear poly(ADP-ribose) polymerase system as determined by kinetics; b.) drugs which are in various in vivo systems anti-promoters or act as cancer preventing substances should interact in vitro with the poly(ADP-ribose) polymerase system and should also prevent neoplastic transformation in the same test where benzamide is effective (2). A further refinement of a.) follows from recent results which show that benzamide binds to a probably selective sequence of DNA (Bauer, P.I. and Kun, E. in preparation), indicating that antitransforming drugs should also exhibit a DNA binding propensity. This prediction is feasible on the basis of the known obligatory coenzymic function of an enzyme associated DNA in the poly-

(ADP-ribose) polymerase system (cf. 3). The third prediction c.) is that the concentration of non toxic antitransforming drugs (defined as RCF_1 cf. 2) need not to correlate with the enzyme inhibitory indices (I_{50}) obtained in artificial enzymatic systems determined by initial velocity kinetics (4).

As demonstrated in Table I these predictions were tested with 11 drugs representing examples. Drugs that had no in vitro effect on poly(ADP-ribose) polymerase proved to be ineffective as antitransformers, but not all types of enzyme inhibitors are necessarily antitransformers in all cell types. It is of importance that purine or nicotinamide type (including 1-methyl nicotinamide) molecules, that are known ^{competitive} inhibitors of poly(ADP-ribose) polymerase (cf.3), at non toxic concentration do not serve as antitransformers in human fibroblasts (not shown) in contrast to the reported antitransforming effect of 0.25 to 1.5 mM caffeine in BALB-3T3-714 cells, where the carcinogen was 4-nitroquinoline-N-oxide (5). It is apparent that models of human neoplasia are stringently cell-type specific, and on the basis of animal cell cultures alone, especially with immortalized cell lines (e.g. 3T3), experimental results cannot be generalized. Even the choice of the transforming agent is critical, e.g. ~~ethyl-methane~~ ^{methyl-methane} sulfonate or methyl-N-nitrosourea are ineffective in human fibroblasts while effective in 3T3 lines and methylazoxymethanol acetate, while a highly potent transformer in human fibroblasts is ineffective in many cell lines of animal origin**.

Benzamide and two substituted analogs show competitive binding with slightly varying I_{50} values (Table I, 1-3). Benzamides are competitive inhibitors at doses that prevent transformation and benzamide

itself is toxic at about 5 mM extracellular concentration while substituted benzamides are non toxic. On the other hand, the drug concentrations that completely prevent transformation (Table II, No. 1-3) are nearly the same for all benzamides. These results argue against the biological significance of the nicotinamide binding site oriented competitive inhibition and suggest the

participation of a second type of binding site which also recognizes the aromatic moiety of benzamides. The rate of association of polymerase inhibitors with biologically effective--presumably DNA related--sites may be much slower than the binding to the competitive acid-amide recognizing sites (compare legends of Tables I and II). For this reason initial velocity kinetics with antitransformers that exhibit competition with NAD, may primarily detect apparent competitive binding of molecule~~s~~

containing groups that bind to the acid amide recognizing sites, but have additional molecular structures that also bind to DNA sites ^(but at a slower rate.) This

may be the case with Coumarin (No.9, Table I) and Isoquinoline (No.11, Table I) which are powerful antitransforming agents, effective at a much lower concentration than their competitive I_{50} . All other highly effective antitransforming drugs lack the reactivity with the competitive nicotinamide recognizing site, and show mixed or non-competitive kinetics. It is of interest that the antibiotics Nalidixic Acid (No.6, Table I) and Novobiocin (No.7, Table I) inhibit eukaryotic DNA-topoisomerases (6,7) at the same concentrations as they inhibit poly(ADP-ribose) polymerase in vitro, (I_{50} , Table I) therefore DNA related binding sites for these drugs are apparent. Prevention of transformation by the antibiotics occurs at about 3 to 4 orders of magnitude lower concentrations than I_{50} , further suggesting that enzyme inhibition may not be germane to the

regulation of phenotypic expression. The apparent kinetic effect of Quercetin (No. 10, Table I) on the poly(ADP-ribose) polymerase system is of special interest as it was reported that Quercetin inhibits the action of the tumor promoter 12-O-tetradecanoly-phorbol 13-acetate in human fibroblasts (8). Levimasole (No. 8, Table I) is known to support cancer chemotherapy in certain cases (9,10) and its kinetic effect on poly(ADP-ribose) polymerase system suggests the participation of poly ADP-ribosylations in the mode of action of this drug. Butylated hydroxyanisole (No.4, Table I) is an agent known to inhibit carcinogenesis in animal models (11). This substance and its methyl ether (No.5, Table I) which is not an antioxidant, prove to be antitransformers of nearly equal potency in the human fibroblast model, as shown in Table I, and also interact with the poly(ADP-ribose) polymerase system, exhibiting mixed type of kinetics. Isoquinoline (No. 11, Table I) as a potent ^(antitransformer) may represent a parent molecule for the synthesis of a new family of antitransforming drugs.

The biological effectivity of agents that bind to the poly(ADP-ribose) polymerase system and are listed in Table I is illustrated in Table II. All drugs that have been selected on the basis of the above proposed predictions (a.-c.) proved to be highly effective antitransformers. Coumarin (No. 7, Table II) and Quercetin (No.8, Table II) are weak transforming agents when applied alone. This agrees with their reported relatively feeble carcinogenicity (12,13) in some animal systems. However, if combined with a potent carcinogen, as shown in Table II, transformation is almost completely inhibited, reminiscent of the apparent antagonism between 4-nitroquinoline-N-Oxide and 3,4 benz^ppyrene as skin tumor initiators (14). In agreement with the powerful antitransforming effect of Quercetin (No. 10, Table I) in human fibroblasts (No. 8,

Table II) it was recently shown that this bioflavonoid, in spite of its mutagenicity, suppresses tumor promotion in mouse epidermal membranes (15) and contrary to previous views (13) ~~is not~~ ^{has been reported not to be} a carcinogen in the rat urinary bladder (16). The pragmatic usefulness of our experimental approach is illustrated by the fact that in several models (8, 12-16) experimental results agree with predictions based on enzymological and cell biological tests made with human fibroblasts (2).

The mode of action of poly ADP-ribosylation on the cell cycle dependent regulation of carcinogenesis (2, 17, 18, 19) is unknown. However, it may be assumed that the modification of DNA structure in S phase is critical since carcinogens preferentially react with replication forks (20) and a chromatin structure related role of poly ADP-ribosylation is probable in the S phase (2).

The frequently observed large discrepancy between I_{50} and RCF_1 values argues against a direct ~~participation~~ ^{role} of the inhibition of poly(ADP-ribose) polymerase in the antitransforming cellular effect, at least at the nicotinamide binding site. The possibility of participation of cell wall associated receptors in the mediation of drug effects to chromatin, involving ultimately poly ADP-ribosylation, cannot be ruled out particularly for substances that poorly penetrate cells. This subject is being further studied.

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Table I

Comparison of drug concentrations affecting cell biological responses
in intact cells (RCF_1 , RCF_{50}) with inhibition indices (I_{50}) and apparent
kinetic mechanisms of poly(ADP-ribose) polymerase inhibition in vitro
in permeabilized cells. (All data are given in mol/l).

No.	Drug	(intact cells)		(permeabilized cells)	NAD concentration related inhibition type [†]
		RCF_1	RCF_{50}	I_{50}	
1.	Benzamide	1.0×10^{-3}	4.6×10^{-3}	2.0×10^{-5}	competitive
2.*	BA-analog 1	1.1×10^{-3}	non toxic Δ	1.0×10^{-4}	competitive
3.*	BA-analog 2	1.1×10^{-3}	non toxic Δ	1.0×10^{-4}	competitive
4.	BHA	1.0×10^{-4}	3.5×10^{-4}	5.0×10^{-3}	mixed
5.*	me-BHA	1.0×10^{-4}	5.0×10^{-4}	1.0×10^{-2}	mixed
6.	Nalidixic Acid	8.0×10^{-7}	4.2×10^{-4}	2.0×10^{-3}	non-competitive
7.	Novobiocin	8.0×10^{-7}	6.4×10^{-5}	1.0×10^{-3}	non-competitive
8.**	Levamisole	2.0×10^{-7}	2.1×10^{-4}	1.0×10^{-3}	non-competitive
9.	Coumarin	6.9×10^{-5}	non toxic Δ	1.0×10^{-3}	competitive
10.	Quercetin	3.3×10^{-6}	2.8×10^{-5}	2.5×10^{-4}	mixed
11.	Isoquinoline	4.0×10^{-7}	3.0×10^{-5}	4.0×10^{-3}	competitive

2* 2-acetonyloxy-5-chlorobenzamide (Both 2 and 3 were gifts received from Dow-
Merrell Research Centre, Cincinnati, OH)
3* 2(phenylacetonyloxy)-5-chlorobenzamide

Δ at saturated levels

5* received as a gift from Dr. Paul Talalay, Johns Hopkins Univ., School of Medicine

8* a gift of Janssen Pharmaceuticals, Piscataway, N.J.

[†] this correlation is equivalent with nicotinamide binding sites, as referred to
in the text.

The S.D. of averages of at least 3 tests reported in Table I does not exceed $\pm 20\%$
of the average.

Table II

Inhibition of carcinogen induced neoplastic transformation by drugs shown in Table I

No.	Transforming Carcinogen	No. of Experimental Series	Experimental Conditions	Number of Transformed Colonies per 5×10^4 cells
1.	MNNG(0.7 μ M)	2	a.) carcinogen b.) a.)+ BA c.) a.)+ BA-1 d.) a.)+ BA-2 e.) BA f.) BA-1 g.) BA-2	279.0 $\pm$ 16.0 0 0 0 0 0 0
2.	MNNG(0.7 μ M)	2	a.) carcinogen b.) a.)+ BHA c.) BHA	278.0 $\pm$ 16.0 1.4 $\pm$ 0.6 1.3 $\pm$ 0.3
3.	MAMA(7.0 μ M)	3	a.) carcinogen b.) a.)+ Me-BHA c.) Me-BHA	200.0 $\pm$ 15.0 1.0 $\pm$ 0.4 0
4.	MNNG(0.7 μ M)	2	a.) carcinogen b.) a.)+ NAL c.) NAL	232.0 $\pm$ 16.0 2.0 $\pm$ 1.5 0
5.	MNNG(0.7 μ M)	2	a.) carcinogen b.) a.)+ NOV c.) NOV	228.0 $\pm$ 11.0 8.0 $\pm$ 2.3 0
6.	MNNG(0.7 μ M)	5	a.) carcinogen b.) a.)+ Lev c.) Lev	214.0 $\pm$ 6.0 6.0 $\pm$ 1.8 0
7.	MNNG(0.7 μ M)	5	a.) carcinogen b.) a.)+ COU c.) COU	279.0 $\pm$ 16.0 1.0 $\pm$ 0.7 13.0 $\pm$ 4.0*
8.	MAMA(7.0 μ M)	2	a.) carcinogen b.) a.)+ QU c.) QU	262.0 $\pm$ 18.0 2.0 $\pm$ 0.8 27.0 $\pm$ 3.3*
9.	MNNG(0.7 μ M)	2	a.) carcinogen b.) a.)+ ISO c.) ISO	210.0 $\pm$ 8.0 0 0

* relatively low levels of transformation by the enzyme inhibitors alone.

Legend to Table I

RCF_1 = the drug concentration that does not alter relative cloning frequency (2) and inhibits neoplastic transformation by 80-95%. RCF_{50} = the drug concentration that inhibits relative cloning frequency by 50% (2). Inhibitory indices (I_{50}) were determined with permeabilized human fibroblasts (2) in the presence of 500 μ M NAD and varying concentrations of drugs under assay conditions described previously (2). Types of inhibitions with respect to NAD were determined from initial velocity plots obtained at 25° within 2 minutes in a test system composed of 50 mM Tris-Cl, pH = 8.5, 10 mM EDTA, 20 mM $CaCl_2$, 0.1 mM phenyl methyl sulfonyl fluoride, NAD (varied from 20 to 500 μ M) labeled with ^{14}C in the adenine moiety. The specific radioactivity of NAD in these tests varied from 5×10^5 to 2×10^4 dpm/n mol. Incubation^{volume} was 25 μ l containing about 10 μ g nuclear protein as a source of poly(ADP-ribose) polymerase, that was added last to initiate the reaction. The inhibition mechanisms were identical with either permeabilized human fibroblasts (2) or isolated rat liver nuclei as sources of poly(ADP-ribose) polymerase. Inhibitory mechanisms were identified by standard kinetic models (4).

Legend to Table II

The cell biological effects of selected molecules were determined in synchronized human fibroblasts exactly as reported earlier (2). Briefly, G_1 block was produced in freshly isolated and subcultured human fibroblasts (5×10^3 cell/cm²) by nutritional deprivation (cf. 2) then S induced by refeeding + insulin, and transforming agents and benzamide were added in early S phase. The window of effectivity of both agents was the same as described (cf. 2). Passages for 20 PDL were continued and treated and control cultures seeded (3 to 20×10^6 cells) into a semi-solid medium (0.33% agar, cf. 2) to score for anchorage independent colony growth (1 colony a minimum of 50 cells). This highly sensitive assay exhibits a quantitative relationship to the concentration of benzamide or other antitransformers, as will be reported elsewhere. In order to reduce the number of culture plates per experiment to a reasonable size (ca. 60-80 plates per test) the antitransforming concentration of various drugs (RCF_1) that prevent transformation by 85-100% is given only, providing an approximate basis of comparison of drug effectivity. However, the large differences observed are not significantly affected by this procedure. Two DNA-methylating ultimate carcinogens: methylazoxy-methanol acetate (MAMA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were employed interchangeably because their transforming potency at a non-toxic (RCF_1) concentration was indistinguishable in the presently developed human cell line.

The antitransforming effect of substances listed under "Experimental Conditions" was determined at RCF_1 concentrations (Table I) exposing cells

Table II legend cont.

for 10 hours in S phase to either the carcinogen or the drug alone or in combination exactly as reported (2). BA = benzamide; BA-1 = 2-acetonyloxy-5-chlorobenzamide; BA-2 = 2(phenylacetonyloxy)-5-chlorobenzamide; BHA = butylated hydroxy anisole; Me-BHA = its methyl ether; NAL = nalidixic acid; NOV = novobiocin; LEV = levimasol; COU = coumarin; QU = quercetin; ISO = isoquinoline; 1 colony is a cell aggregate of a minimum of 50 cells.

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