Modulation of Nicotinamide Adenine Dinucleotide and Poly(Adenosine Diphosphoribose) Metabolism by the Synthetic "C" Nucleoside Analogs, Tiazofurin and Selenazofurin

A New Strategy for Cancer Chemotherapy

Nathan A. Berger, Sosamma J. Berger, Donna M. Catino, Shirley J. Petzold, and Roland K. Robins

The Hematology-Oncology Division, Departments of Medicine and Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106; The Cancer Research Center, Department of Chemistry, Brigham Young University, Provo, Utah 84601

Abstract

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) and selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide) are synthetic "C" nucleosides whose antineoplastic activity depends on their conversion to tiazofurin-adenine dinucleotide and selenazofurin-adenine dinucleotide which are analogs of NAD. The present study was conducted to determine whether these nucleoside analogs and their dinucleotide derivatives interfere with NAD metabolism and in particular with the NAD-dependent enzyme, poly(ADP-ribose) polymerase. Incubation of L1210 cells with 10 μM tiazofurin or selenazofurin resulted in inhibition of cell growth, reduction of cellular NAD content, and interference with NAD synthesis. Using [^{14}C]nicotinamide to study the uptake of nicotinamide and its conversion to NAD, we showed that the analogs interfere with NAD synthesis, apparently by blocking formation of nicotinamide mononucleotide. The analogs also serve as weak inhibitors of poly(ADP-ribose) polymerase, which is an NAD-utilizing, chromatin-bound enzyme, whose function is required for normal DNA repair processes. Continuous incubation of L1210 cells in tiazofurin or selenazofurin resulted in progressive and synergistic potentiation of the cytotoxic effects of DNA-damaging agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These studies provide a basis for designing chemotherapy combinations in which tiazofurin or selenazofurin are used to modulate NAD and poly(ADP-ribose) metabolism to synergistically potentiate the effects of DNA strand-disrupting agents.

Introduction

Tiazofurin (Taz)[1] (2-β-D-ribofuranosylthiazole-4-carboxamide) and selenazofurin (Sel) (2-β-D-ribofuranosylselenazole-4-carboxamide) are synthetic "C" nucleoside analogs with potent antineoplastic activity in experimental murine and human tumors (1–5). They are highly effective in treating a variety of murine tumors including L1210 leukemia, P388 leukemia, and the highly resistant Lewis lung carcinoma (1–4). They are also effective in vitro against several human tumors including the human colon adenocarcinoma line LoVo/L and the human promyelocytic leukemia cell line HL-60 (6, 7). Sel also induces maturation in HL-60 cells (7). Using human tumor-cloning assays to examine cells from a variety of solid tumors, Taz was found to have significant antitumor effects in ~17% of cases (8) and this agent is now undergoing phase I and II clinical trials (9–11).

The antitumor effects of Taz and Sel are mediated, in part, by their conversion to tiazofurin-adenine dinucleotide (TAD) and selenazofurin-adenine dinucleotide (SAD), which are analogs of NAD (4, 12). TAD and SAD are synthesized by nicotinamide mononucleotide · ATP · adenylyl transferase; they inhibit the NAD-dependent enzyme, inosine 5'-monophosphate (IMP) dehydrogenase, which converts IMP to guanosine 5'-monophosphate (GMP). As a result of this inhibition, cells become depleted of GMP and they accumulate the precursor IMP (4, 12, 13). GMP depletion appears to be part of their cytotoxic mechanism since the effect of these compounds can be overcome by supplying cells with guanosine nucleosides (4). The importance of converting these analogs to their dinucleotide derivatives is demonstrated by the fact that a line of P388 cells found to be deficient in its ability to convert Taz to TAD is resistant to the acute toxic effects of Taz (14). Furthermore, sensitivity of murine tumors to the pharmacologic effects of Taz correlates with their intracellular levels of TAD (15).

Since Taz and Sel are metabolized to NAD analogs by enzymes normally involved in NAD synthesis and since the dinucleotide analogs interfere with NAD-dependent enzymes such as IMP dehydrogenase, it seemed possible that the nucleoside analogs and/or their metabolites might interfere with some other aspects of NAD metabolism and in particular with the NAD-dependent enzyme, poly(ADP-ribose) polymerase. Because of the important requirement for NAD and poly(ADP-ribose) metabolism in the DNA repair process (16–18), we investigated the effects of Taz and Sel on various aspects of this pathway to determine whether these compounds could be used to modulate levels of NAD or poly(ADP-ribose) polymerase activity. Our results indicate that modulation of NAD and poly(ADP-ribose) synthesis by Taz or Sel may be a useful approach to potentiate the activity of other cytotoxic agents whose mechanism of action involves DNA strand disruption.

Received for publication 7 August 1984 and in revised form 25 October 1984.

1. *Abbreviations used in this paper:* 3-AB, 3-aminobenzamide; ADP-ribose, adenosine diphospho-ribose; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DMS, dimethylsulfate; GMP, guanosine 5'-monophosphate; IMP, inosine 5'-monophosphate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NMN, nicotinamide mononucleotide; SAD, selenazofurin-adenine dinucleotide; Sel, selenazofurin; TAD, tiazofurin-adenine dinucleotide; Taz, tiazofurin.

J. Clin. Invest.

0021-9738/85/02/0702/08 $1.00
Volume 75, February 1985, 702–709

Methods

Taz, Sel, TAD, and SAD were synthesized as previously described (1–5) and dissolved in saline for tissue culture experiments or in 50 mM Tris-HCl adjusted to pH 8.0 for enzymatic assays. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 3-aminobenzamide (3-AB) were purchased from Sigma Chemical Co., St. Louis, MO. MNNG was dissolved in dimethyl sulfoxide immediately before use. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was obtained from the Drug Development Branch of the National Cancer Institute and was dissolved in 95% ethanol and diluted into phosphate-buffered saline (PBS) before addition to tissue cultures.

L1210 mouse leukemic cells were maintained in suspension culture at 37°C in α-modified Eagle's medium, with 25 mM Hepes buffered to pH 7.2, and supplemented with 50 μM 2 mercaptoethanol, 7 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Cell numbers were determined microscopically with a hemocytometer and viability was determined by trypan blue exclusion.

Cellular NAD^+ content was measured as previously described (19, 20). Briefly, cell samples were collected from suspension by centrifugation at 3000 *g*, 4°C for 3 min, and the media was removed by aspiration. Cell pellets were rapidly resuspended in 400 μl of 0.35 N $HClO_4$ and incubated in an ice bath for 15 min. Acid extracts were neutralized, and supernatants were collected and stored at −80°C. Samples from multiple points of an experiment were stored frozen and then all assays were performed simultaneously. NAD^+ was assayed using enzymatic cycling with malic and alcohol dehydrogenase as previously described (19, 20). Duplicate samples were taken for each point and then each sample was assayed in duplicate. All values are presented as the means of assays performed in quadruplicate and are representative of complete studies performed at least three times. We calculate that any inhibitor carried over from the cell extract would not exceed a concentration of 0.1 μM in the cycling assay, whereas our preliminary studies showed that this cycling assay was not significantly affected by the presence of Taz, Sel, TAD, or SAD at the highest concentrations tested which were 1 mM for the nucleoside analogs and 100 μM for the dinucleotide analogs.

For studies to evaluate the effect of Taz or Sel on uptake of nicotinamide and its incorporation into pyridine nucleotides, L1210 cells were incubated with these compounds for 48 h. Initially and after 24- or 48-h incubation in media containing the inhibitors, [carbonyl-^{14}C]nicotinamide (sp act, 56 mCi/mmol; Amersham Corp., Arlington Heights, IL) was added at a final concentration of 1 μCi/ml and cells were incubated for an additional 6 h. Cells were then collected by centrifugation at 2,500 rpm, 5 min at 4°C. The cells were washed with 5 ml ice-cold saline containing 10 mM nicotinamide, collected again by centrifugation, and then treated with 100 μl of 5% TCA. Acid-treated cells were frozen and thawed three times. Supernatant samples were taken to determine the total amount of radioactivity in the extracts and then the components were separated and identified by descending chromatography on Whatman No. 3 paper (Whatman Laboratory Products, Inc., Clifton, NJ) using 2 M NH_4Cl in 0.12 M sodium citrate (pH 5.3):95% ethanol (1:3) as solvent. Chromatograms were run with added standards of NAD, NADP, nicotinamide, nicotinamide mononucleotide (NMN), nicotinic acid, and 1-methyl nicotinamide. Spots were identified by UV absorption; strips were cut into 1-cm sections and radioactivity determined in toluene-based scintillation fluid (20, 21).

Alkaline elution studies were used to evaluate DNA strand breaks (22, 23). L1210 cells were prelabeled by growing in media with 0.02 μCi/ml [2-^{14}C]thymidine (sp act, 57 mCi/mmol) for 24 h. Cells were resuspended in nonradioactive medium and incubated for an additional 24 h to allow all DNA fragments to be chased into high molecular weight material. At intervals following experimental therapy, 5×10^5 cells were removed from the culture, diluted into an excess of cold PBS, and collected onto polyvinyl chloride filters, 25 mm diam, 2 μm pore size. The cells were lysed with 5 ml of 0.1 M glycine, 0.02 M EDTA, and 2% sodium dodecyl sulfate (pH 10) and then washed with 5 ml 0.2 M EDTA (pH 10) and then 5 ml proteinase K (Curtin Matheson Scientific, Cleveland, OH); 0.5 mg/ml in 0.2 M EDTA (pH 10) was added to the filter holder, following which the filters were eluted in the dark with a 2% wt/vol solution of tetrapropyl ammonium hydroxide (pH 12.1), 0.02 M EDTA via a peristaltic pump at a rate of 0.04 ml/min. Fractions were collected directly into scintillation vials at 90-min intervals for 15 h. Samples were mixed with 3.3 vol of Scintiverse (Fisher Scientific Co., Pittsburgh, PA) containing 0.7% glacial acetic acid. Radioactivity remaining on the filters was determined by treating the filters with 0.4 ml 1 N HCl at 70°C for 1 h followed by 2.5 ml 0.4 N NaOH at room temperature for 30 min and then the addition of 10 ml Scintiverse. Radioactivity remaining in the tubing was recovered by five washes with 3 ml 0.4 N NaOH and counted in Scintiverse as above. Data is plotted as the radioactive DNA remaining on the filters as a function of time of elution (22, 23). For irradiation experiments, cells were collected by centrifugation, resuspended in cold PBS at a concentration of 5×10^5/ml, and irradiated in an ice-water bath using a Gamma Cell 5,000 (Atomic Energy Canada Ltd., Ontario, Canada) at an incident dose of 850 rads/min. Following irradiation, cells were applied directly to filters, lysed, and eluted as above.

The effect of inhibitors on poly(ADP-ribose) polymerase was evaluated with enzyme purified from lamb thymus (24). Reaction systems contained 10 μg calf thymus DNA, 10 μg histone H1, 1 mM dithiothreitol, 100 mM Tris-HCl pH 8, either 7 μM [^{32}P]NAD (sp act, 2386 cpm/pmol) or 450 μM [^{14}C]NAD (sp act, 4.6 cpm/pmol), and 30 μl of enzyme solution in a total volume of 100 μl. Reaction mixtures were incubated for 15 min at 37°C, stopped by addition of an excess of 20% trichloracetic acid, and then processed for determination of radioactivity (25).

Results

Fig. 1 *A* shows the effects of Taz and Sel on the growth of L1210 cells in culture. 1 μM Taz had no suppressive effect on the growth rate; in fact, as the cells reached high density, the presence of 1 μM Taz was associated with a slight increase in cell growth. In contrast, 10 μM Taz completely inhibited cell growth. Sel is a more effective inhibitor of cell growth; 1 μM Sel significantly reduced the growth rate and 10 μM Sel completely inhibited it. These effects are undoubtedly mediated, in part, by inhibition of IMP dehydrogenase resulting in depletion of guanine nucleotides and consequent inhibition of DNA, RNA synthesis, and cell growth (12–14). Fig. 1 *B* shows that incubation in Taz and Sel also affects the cellular content of NAD^+. During the 48-h growth period, the control cells showed a reduction in NAD content from 330 to 208 pmol/10^6 cells. We have previously reported this type of decrease in cellular NAD content when cultures of human lymphocytes or mouse L cells grow to high density (18, 23). L1210 cells grown in Taz or Sel showed a more pronounced decrease in cellular NAD content. After 48 h, the final NAD content in L1210 cells incubated in 10 μM Taz or 10 μM Sel was 50 and 30 pmol/10^6 cells, respectively, which constitutes a reduction to 15 or 9% of the initial control level.

While the control L1210 cells showed a decrease in NAD^+ content per cell, the accompanying increase in cell numbers actually resulted in a net increase of NAD^+ in the culture system. This is shown in Fig. 1 *C* where net values for NAD^+ in the culture are determined as a product of the cell count multiplied by the cellular content of NAD^+. Thus during the 48-h culture period, the control, untreated L1210 cells produced a net increase in NAD^+ from 70 to 262 pmol/ml. In the presence of 1 μM Taz, the NAD content of the culture also

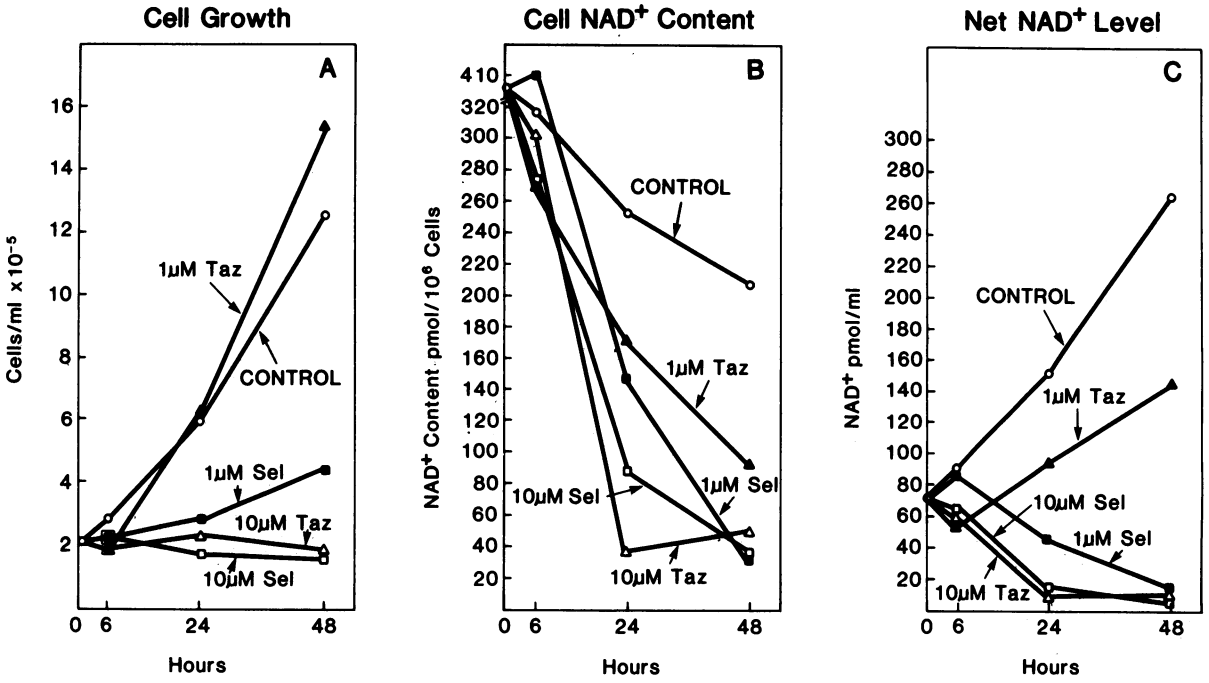

Figure 1. Effect of Taz and Sel on L1210 cell growth and NAD^+ levels. L1210 cells were grown in control media (○) or in media supplemented with 1 μM Taz (▲), 10 μM Taz (△), 1 μM Sel (■), or 10 μM Sel (□). *A* shows cell counts. *B* shows NAD^+ content on a per cell basis determined by enzymatic cycling techniques. *C* shows the net NAD^+ content per milliliter cell suspension. It is determined by the product of (cells/milliliter) × (NAD^+/cells).

increased but only to half the level that it did in control cells. This ability to continue NAD synthesis in the presence of 1 μM Taz correlated with the ability to maintain cell growth. The presence of 10 μM Taz resulted in a decrease in both the NAD content per cell and the net NAD content of the culture. Similarly, NAD content per cell and net NAD content of the culture also decreased when the cells were incubated with 1 or 10 μM Sel. The amount of NAD^+ in the culture is dependent on both the synthesis and turnover of this compound. L1210 cells synthesize NAD by the nicotinamide salvage pathway in which nicotinamide is converted by the enzyme nicotinamide phosphoribosyl transferase to NMN, which is then condensed with ATP by the enzyme NMN·ATP·adenylyl transferase to form NAD (26, 27). In the presence of restricted nicotinamide, cells avidly reuse their nicotinamide to maintain a constant, net NAD content in culture (20). In the present experiments, the overall decrease of net NAD content suggests that Taz or Sel may interfere with NAD synthesis.

Studies of NAD synthesis. Fig. 2 shows the effect of incubating cells in Taz or Sel on the components of the nicotinamide salvage pathway. In these experiments, cells were continuously incubated in the presence of 1 or 10 μM Taz or Sel. At the beginning of the experiment or after 24 or 48-h preincubation with these agents, [^{14}C]nicotinamide was added and its uptake and conversion to [^{14}C]NAD studied during a subsequent 6-h incubation. In each panel, the initial time point represents the value in control untreated cells. The first panel shows that in the presence of 1 or 10 μM Sel there was an initial increase in the incorporation of [^{14}C]nicotinamide into NAD. However, after 24 or 48 h of incubation in 10 μM Sel, there was a progressive suppression in the amount of [^{14}C]nicotinamide converted to NAD. After 48 h in 1 μM Sel, NAD synthesis from nicotinamide was also suppressed relative to control. 10 μM Taz was similar to Sel in that it stimulated the rate of NAD formation from nicotinamide during the first 24 h. However, incubation in 10 μM Taz for 48 h resulted in a decrease in NAD synthesis from nicotinamide. 1 μM Taz had a negligible effect during the first 24 h; however, after 48-h preincubation, it produced a decrease in utilization of nicotinamide for NAD synthesis.

Fig. 2 *B* shows that the initial increase in NAD synthesis in the Sel-treated cells was associated with an increase in uptake of nicotinamide. After 24-h incubation with 1 μM Sel, there was a progressive increase in accumulation of intracellular nicotinamide with a slight decrease after 48 h. Similarly, incubation in 10 μM Taz resulted in increased accumulation of [^{14}C]nicotinamide, whereas 1 μM Taz was associated with an initial increase and then a decrease in nicotinamide accumulation. At 10 μM Sel, nicotinamide accumulation increased during the first 6 h and then remained slightly elevated relative to control.

The significance of this data is shown most clearly in Fig. 2 *C* which presents the ratio of the intracellular [^{14}C]nicotinamide relative to the amount incorporated into [^{14}C]NAD. A decrease in the ability to synthesize NAD from exogenous nicotinamide is associated with an increase in accumulation of intracellular nicotinamide relative to NAD. This increase in ratio of nicotinamide to NAD indicates that during continuous incubation in Taz or Sel, cells retain the ability to transport nicotinamide but they develop a block in the pathway for converting nicotinamide to NAD, resulting in a decrease in newly synthesized NAD and an accumulation of intracellular nicotinamide. This is most clearly shown in the case of 10 μM Sel, which causes the greatest depression in NAD synthesis, the highest levels of intracellular nicotinamide

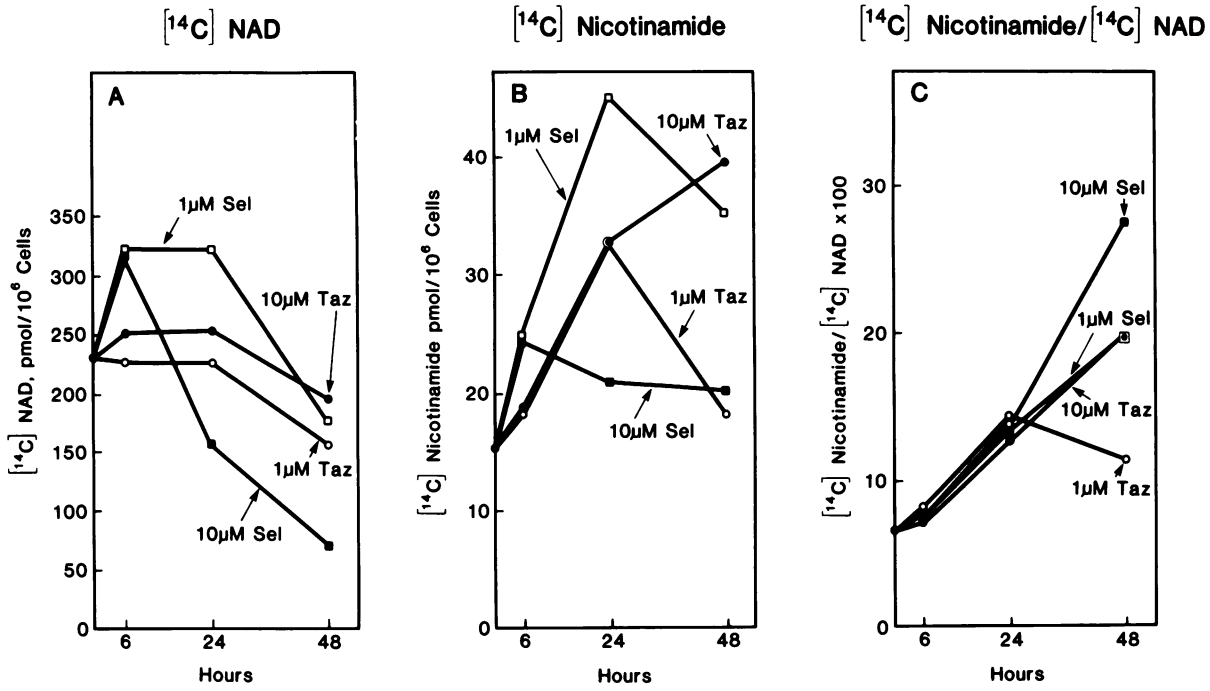

Figure 2. Effect of Taz and Sel on L1210 cell uptake of [^{14}C]nicotinamide and its conversion to [^{14}C]NAD. L1210 cells in the logarithmic phase of growth were diluted to 2×10^5 cells/ml and incubated in complete media or in media supplemented with Sel or Taz. At the beginning of the study and at 24 and 48 h, nicotinamide uptake was evaluated by a 6-h pulse with [^{14}C]nicotinamide. Extracts of cells from each point were analyzed by paper chromatography to determine distribution of radioactivity into nicotinamide metabolites. Uptakes in control cells were performed at each time point and were essentially the same as the point indicated in each figure at 0 time. Treated cells were incubated in media supplemented with 1 μM Sel (□), 10 μM Sel (■), 1 μM Taz (○), or 10 μM Taz (●). *A* shows incorporation of [^{14}C]nicotinamide into NAD. *B* shows intracellular [^{14}C]nicotinamide and *C* shows the ratio of intracellular [^{14}C]nicotinamide to [^{14}C]NAD.

accumulation, and therefore, the highest ratio of nicotinamide to NAD. Thus, prolonged exposure to Taz or Sel progressively interferes with the cell's ability to utilize nicotinamide for the synthesis of NAD contributing to the decreased cellular NAD pools and increased accumulation of the precursor nicotinamide.

To further identify the location of the Taz- and Sel-induced block in NAD synthesis, we examined the intermediates on the pathway between nicotinamide and NAD. Table I shows the distribution of these intermediates in control cells and in cells pretreated with 10 μM Taz or 10 μM Sel. The decreased incorporation into NAD is associated with a relatively constant level of labeling of NADP as a percent of NAD, indicating that there is a decrease in synthesis of all pyridine nucleotides and not merely a shift from NAD to NADP pools. Under these conditions of decreased pyridine nucleotide synthesis, there is a relative increase in labeling of nicotinamide pools with no significant increase in NMN. Thus the block appears to be at the level of nicotinamide phosphoribosyl transferase, the enzyme responsible for converting nicotinamide to NMN. It is interesting to note that the cells do not shift much of the intracellular nicotinamide into the alternate pathways of synthesizing nicotinic acid or 1-methyl nicotinamide (20, 21, 28).

Effects of inhibitors on poly(ADP-ribose) polymerase. Since Taz and Sel are metabolized into the NAD analogs, TAD and SAD, we examined each of these compounds for their effect on poly(ADP-ribose) polymerase. Fig. 3 compares the effects of these agents on the activity of purified poly(ADP-ribose) polymerase in the presence of two different substrate concentrations, above and below the enzymes Michaelis constant (K_m) for NAD which is ~150 μM (24). In the presence of 450 μM NAD, Taz and Sel were weak inhibitors producing only 10 and 20% inhibition at inhibitor concentrations of 5 mM. 5 mM TAD was significantly more effective, producing 70% inhibition. SAD produced a similar inhibition curve at lower concentrations but was not tested at 5 mM due to problems

Table I. Effects of Taz and Sel on Intracellular Metabolites of [^{14}C]Nicotinamide in L1210 Cells

Metabolite	Control	10 μM Taz	10 μM Sel
	pmol/10^6 cells	*pmol/10^6 cells*	*pmol/10^6 cells*
Nicotinamide	15.4	39.2	20.4
NMN	1.9	1.9	0.5
NAD	231	196	72.6
NADP	11.3	10.6	3.5
Nicotinic Acid	1.6	3.2	1.1
1-Methylnicotinamide	1.1	2.9	0.9
Total	262.3	244.8	99.0

L1210 cells were grown for 48 h in complete media (control) or in the presence of 10 μM Taz or 10 μM Sel. After 48 h, [^{14}C]nicotinamide was added and the cells were incubated for an additional 6 h. Cells were then collected, washed, and acid extracts were prepared and analyzed by paper chromatography as described in Methods. Values in this table represent distribution of metabolites derived from [^{14}C]nicotinamide during the 6-h incubation period.

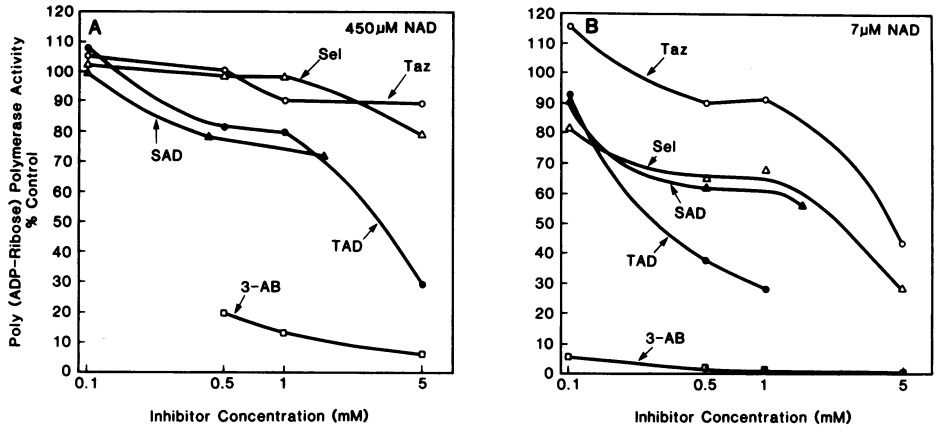

Figure 3. Effects of Taz, Sel, and their dinucleotide analogs on activity of purified poly(ADP-ribose) polymerase. 100% activity is the amount of radioactive ADP-ribose incorporated into polymer in 15 min at 37°C using purified lamb thymus poly(ADP-ribose) polymerase as described in Methods. Reactions in *A* were conducted in the presence of 450 μM NAD; reactions in *B* were conducted in 7 μM NAD. Inhibitors are Taz (○), Sel (△), TAD (●), SAD (▲), and 3-AB (□).

with solubility and limited material. By way of comparison, 3-AB, which is one of the most effective inhibitors of poly(ADP-ribose) polymerase, produced 94% inhibition at 5 mM. Since these agents are presumably competitive with NAD and since they also appear to cause reduced cellular NAD levels, we evaluated their effects at concentrations of NAD which were below K_m. Fig. 3 *B* shows that at 7 μM NAD, 5 mM Taz or Sel produced 54 and 60% inhibition of enzyme activity. Even at 0.5 mM, Sel produced 35% inhibition. Inhibition by the dinucleotide analogs SAD and TAD were also greater at the lower substrate concentration. In contrast to 3-AB, Taz, Sel, and their dinucleotide derivatives were weak to moderate inhibitors of poly(ADP-ribose) polymerase.

Poly(ADP-ribose) polymerase synthesis is required for normal DNA repair processes (16–18). Cells with restricted activity of poly(ADP-ribose) polymerase due to low NAD levels or the presence of enzyme inhibitors are deficient in their ability to repair DNA strand breaks (16–18). To determine whether the metabolic effects of Taz and/or Sel were associated with the development of DNA strand breaks, we incubated cells with these analogs and analyzed DNA integrity by alkaline elution after 6, 24, and 48 h of incubation. Fig. 4 shows that continuous incubation of L1210 cells with Taz and Sel for 24 and 48 h resulted in dose-dependent increases in DNA strand breaks. No significant DNA strand breaks occurred when cells were incubated with these compounds for only 6 h. By comparing the curves produced in the presence of the inhibitors to those produced by graded doses of γ-irradiation (Fig. 4 *C*), it is apparent that incubation in 10 μM Taz or Sel produced strand break curves that fell between those produced by 75 and 150 rad. The breaks which occur in the presence of Taz or Sel may be spontaneous breaks which fail to be repaired because of the presence of these agents. While the radiation-induced breaks are usually rapidly repaired (29), those produced by incubation of cells with Taz or Sel appear to be more persistent and therefore potentially more toxic.

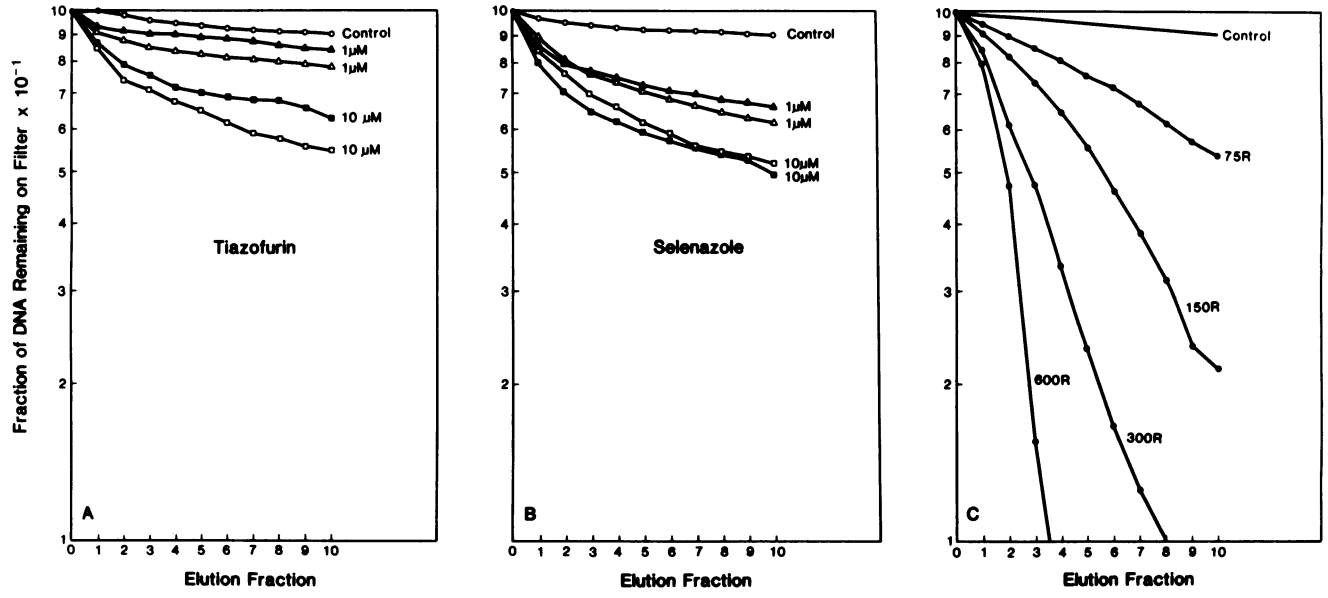

Figure 4. Alkaline elution studies were performed with cells containing [^{14}C]thymidine-labeled DNA as described in Methods. The curves for control cells (○) were the same at the beginning and end of the experiment. In *A*, cells were incubated with 1 μM Taz for 24 h (△) or 48 h (▲) or with 10 μM Taz for 24 h (□) or 48 h (■). In *B*, cells were incubated with 1 μM Sel for 24 h (△) or 48 h (▲) or with 10 μM Sel for 24 h (□) or 48 h (■). In *C*, cells were treated with 75 R–600 R as indicated in the figure.

Poly(ADP-ribose) synthesis is required for the repair of DNA damage induced by agents such as MNNG or BCNU (16–18). Since Taz and Sel can interfere with poly(ADP-ribose) synthesis by lowering levels of the substrate NAD and by direct inhibition of the enzyme, it seemed possible that these agents would potentiate the cytotoxic effects of MNNG and BCNU. To evaluate this possibility, L1210 cells were continuously treated with Taz and Sel for 48 h. At the beginning of the experiment and again after 24 and 48 h, separate groups of cells were treated with 5 μg/ml MNNG or 100 μM BCNU. For each group, cell viability was determined by trypan blue analysis 6 h after treatment with the various combinations of agents. Fig. 5 shows that as cells are incubated in Taz or Sel they become progressively more susceptible to the cytocidal effects of MNNG and BCNU. Fig. 5 *A* shows that the control cells were 98% viable at the beginning and they retained their high level of viability throughout the experiment. At the beginning of the experiment, treatment of the control cells with 5 μg/ml MNNG or 100 μM BCNU reduced their viability to 81 and 88%, respectively. When cells were maintained in control growth medium for 24 or 48 h and then treated with MNNG or BCNU, the decrease in viability was approximately the same as it was at the beginning of the experiment and the viability in all cases remained >80%.

Preincubation of cells with Taz or Sel for 24or 48 h resulted in a marked increase in their susceptibility to MNNG or BCNU. Fig. 5 *B* shows that after 24 h in 10 μM Taz, viability was 92%, and after 48 h, it was reduced to 64%. Addition of MNNG after 24-h preincubation with 10 μM Taz resulted in a decrease in viability to 55%. Similarly, there were marked decreases in viability when MNNG or BCNU were added to cells which had been preincubated with 10 μM Taz for 48 h. The decrease in viability that occurred when BCNU or MNNG were added to cells that were preincubated in Taz was much greater than that which occurred when the same agents were added to control cells. This suggests a synergistic interaction between preincubation with Taz and treatment with BCNU or MNNG.

Fig. 5 *C* shows that incubation in 10 μM Sel had the greatest, single agent effect in reducing cell viability, with 70% viability after 24 h and 38% after 48 h. The cytocidal effects of MNNG or BCNU were potentiated by pretreatment with Sel in a manner similar to the potentiation by Taz. Thus, while MNNG or BCNU did not significantly affect viability of control cells, their cytotoxic effects were significantly potentiated by 48-h preincubation with 10 μM Taz or Sel. At 1 μM, Taz and Sel were not as effective as they were at 10 μM in potentiating the effects of BCNU and MNNG.

Discussion

Taz and Sel are promising new antitumor agents (6–9). Understanding their mechanism of action and metabolic effects should provide an important basis for developing strategies for their optimal use in single agent and combination chemotherapy protocols. The acute effects of these agents appear to be mediated by their conversion to analogs of NAD which subsequently interfere with IMP dehydrogenase, resulting in the depletion of guanine nucleotides and the consequent inhibition of DNA, RNA synthesis, and cell growth (4, 12–14). Our studies indicate that on a chronic basis, treatment with Taz or Sel also interferes with several aspects of pyridine nucleotide and poly(ADP-ribose) metabolism. Thus Taz, Sel, and their metabolites interfere with NAD synthesis and also interfere with activity of poly(ADP-ribose) polymerase. Their effects on NAD synthesis appear to be at the level of nicotinamide phosphoribosyl transferase. This enzyme is known to be inhibited by NAD (30), and thus, it is possible that the dinucleotide derivatives, TAD and SAD, function as NAD analogs in this system and inhibit conversion of nicotinamide to NMN. Our preliminary studies, using a new cycling assay to measure nicotinamide phosphoribosyl transferase, indicate that the enzyme is inhibited by TAD. Taz, Sel, and their metabolites also presumably function as competitive inhibitors of poly(ADP-ribose) polymerase since they are more effective inhibitors at low concentrations of NAD. Our studies suggest

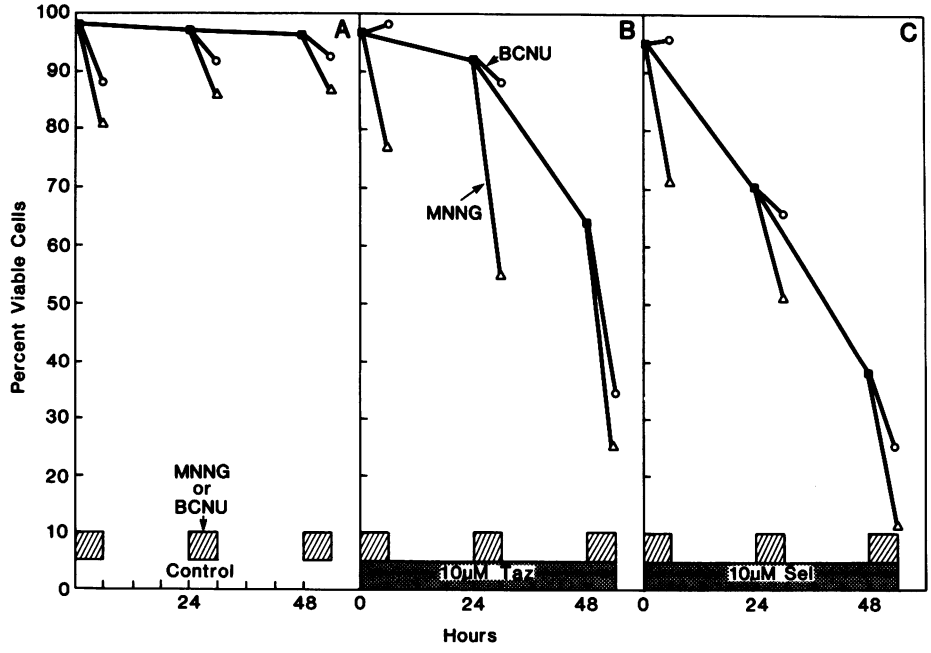

Figure 5. Effect of preincubation in Taz or Sel on MNNG or BCNU toxicity. In each panel, the solid squares (■) and continuous line shows the effect on viability of incubating cells with no addition (*A*), with 10 μM Taz (*B*), or with 10 μM Sel (*C*). The duration of incubation with each of these agents is indicated by the stippled box at the bottom of each panel. At 0, 24, or 48 h, 100 μM BCNU (○) or 34 μM MNNG (△) was added to separate samples removed from the main culture and the effect on viability determined after 6 h. The duration of each exposure to BCNU or MNNG is indicated by the cross-hatched boxes at the bottom of the figures.

that modulation of NAD and poly(ADP-ribose) synthesis with Taz or Sel may be useful in potentiating the antitumor effects of chemotherapeutic agents that function by disrupting DNA.

NAD and poly(ADP-ribose) metabolism are required for normal DNA repair processes (16–18). The polymerase is present in cells in reserve quantities (17, 18, 31). The enzyme is activated by DNA strand breaks to cleave its substrate NAD between the nicotinamide and ADP ribose moieties. This same enzyme is capable of forming a covalent linkage between ADP-ribose and protein acceptor molecules (24, 32). Subsequent ADP-ribose residues are then added by *O*-glycosidic linkages to form homopolymers which have been found to occur in excess of 100 residues. Poly(ADP-ribosylation) in response to DNA damage has been shown to alter chromatin structure and to affect the activity of several enzymes including the polymerase itself, DNA ligase, topoisomerase, and a Ca^{++}, Mg^{++}-dependent endonuclease (32–35). Thus, poly(ADP-ribosylation) may affect the DNA repair process by altering chromatin structure and also by affecting the activities of the enzymes involved in this process.

Treatment of cells with inhibitors of poly(ADP-ribose) polymerase can be used to potentiate the cytotoxicity of DNA-damaging agents. For example, 3-AB potentiates the toxic effects of treating cells with the alkylating agent dimethyl sulfate (DMS). Similar treatment with 3-amino benzoic acid (a noninhibitory analog) has no effect on the toxicity of DMS (16, 36). At the molecular level, the inhibition of poly(ADP-ribose) synthesis has been shown to prevent the resealing of DNA strand breaks (16, 37).

Poly(ADP-ribose) polymerase has recently been shown to be a potential target for cancer chemotherapy, especially in protocols where enzyme inhibitors can be combined with agents whose primary mechanism of action is mediated by their ability to produce DNA strand disruptions. For example, the poly(ADP-ribose) polymerase inhibitor, 6-aminonicotinamide, has been shown to potentiate the antitumor effects of BCNU against L1210 leukemia in vivo and in vitro (38). Another poly(ADP-ribose) polymerase inhibitor, 3-AB, has been shown to potentiate the antitumor effect of bleomycin in mice bearing the Ehrlich ascites carcinoma (39). As compared with 3-AB, Taz, Sel, and their intracellular metabolites were found to be weak to moderate inhibitors of poly(ADP-ribose) polymerase. However, their ability to interfere with NAD synthesis produces a reduction in cellular NAD pools, which enhances their ability to act as competitive inhibitors of poly(ADP-ribose) polymerase. This inhibition was associated with the development and persistence of DNA strand breaks and also sensitized the cells to the cytotoxic effects of BCNU or MNNG. These experiments show that continuous exposure to Taz or Sel results in increasing sensitization to MNNG or BCNU. This sensitization is probably further enhanced by the effects of these compounds on guanine nucleotide pools due to inhibition of IMP dehydrogenase (12–15). It is also probable that these agents affect other metabolic processes by interfering with pyridine nucleotide-dependent dehydrogenases. In addition, we have previously shown that the depletion of pyridine nucleotide levels leads to an inability of cells to maintain ATP pools and energy-dependent functions (40). Thus, continuous exposure to Taz or Sel may be a useful approach to modulate multiple biochemical pathways to make cells more sensitive to cytotoxic chemotherapeutic agents. This type of exposure should be possible in clinical situations since the feasibility of giving Taz or Sel by continuous infusion has already been demonstrated in clinical trials (9).

The studies outlined above provide a basis for developing synergistic chemotherapy combinations using Taz and/or Sel plus DNA strand-disrupting agents. The possibility needs to be examined that even more effective regimens can be developed by using DNA strand-disrupting agents in combination with these nucleoside analogs to modulate NAD synthesis along with nicotinamide analogs that inhibit poly(ADP-ribose) synthesis to provide sequential blockades of this pathway.

Acknowledgments

These studies were supported by National Institutes of Health grants CA35983 and GM32647 at Case Western Reserve University and by National Institutes of Health grant CA34384 at Brigham Young University. Some of the studies were performed in facilities supported by the Sohio Foundation.

References

1. Srivastava, P. C., M. V. Pickering, L. B. Allen, D. G. Streeter, M. T. Campbell, J. T. Witkowski, R. W. Sidwell, and R. K. Robins. 1977. Synthesis and anti-viral activity of certain thiazole C-nucleosides. *J. Med. Chem.* 20:256–262.

2. Robins, R. K., P. C. Srivastava, V. L. Narayanan, J. Plowman, and K. D. Paull. 1982. 2-β-D-Ribofuranosylthiazole-4-carboxamide, a novel potential antitumor agent for lung tumors and metastases. *J. Med. Chem.* 25:107–108.

3. Srivastava, P. C., and R. K. Robins. 1983. Synthesis and antitumor activity of 2-β-D-ribofuranosylselenazole-4-carboxamide and related derivatives. *J. Med. Chem.* 26:445–448.

4. Streeter, D. G., and R. K. Robins. 1983. Comparative *in vitro* studies of Tiazofurin and a Selenazole analog. *Biochem. Biophys. Res. Commun.* 115:544–550.

5. Gebeyehu, G., V. E. Marquez, J. A. Kelley, D. A. Cooney, H. N. Jayaram, and D. G. Johns. 1983. Synthesis of thiazole-4-carboxamide adenine dinucleotide. A powerful inhibitor of IMP dehydrogenase. *J. Med. Chem.* 25:922–925.

6. Avery, T. L., and R. K. Robins. 1984. Experimental antitumor activity of tiazofurin. *Proc. Am. Assoc. Cancer Res.* 25:345.

7. Lucas, D. L., R. K. Robins, R. D. Knight, and D. G. Wright. 1984. Induced maturation of the human promyelocytic leukemia cell line, HL-60, by 2-β-D-ribofuranosyl-selenazole-4-carboxamide. *Biochem. Biophys. Res. Commun.* 115:971–980.

8. Melink, T. J., D. D. Von Hoff, G. M. Clark, and C. A. Coltman. 1984. Antitumor activity of tiazofurin in a human tumor cloning system. *Proc. Am. Assoc. Cancer Res.* 25:375.

9. Trump, D. L., K. D. Tutsch, J. M. Koeller, G. Ramirez, R. R. Love, and D. C. Tormey. 1984. Phase I trial and pharmacokinetics of tiazofuran (NSC 286193) administered as a 5-day continuous infusion. *Proc. Am. Assoc. Cancer Res.* 25:344.

10. Roberts, J. D., C. A. Ackerly, R. A. Newman, I. H. Krakoff, and J. A. Stewart. 1984. Phase I study of tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide; NSC 286193). *Proc. Am. Assoc. Cancer Res.* 25:166.

11. Raghavan, D., J. Bishop, J. Levi, R. Fox, D. Hedley, R. Woods, K. Cox, A. Coates, A. Tattersal, and B. Hillcoat. 1984. Phase I and pharmacokinetic study of tiazofurin. *Proc. Am. Assoc. Cancer Res.* 25: 167.

12. Cooney, D. A., H. N. Jayaram, G. Gebeyehu, C. R. Betts, J. A. Kelley, V. E. Marquez, and D. G. Johns. 1982. The conversion of 2-β-D-ribofuranosylthiazole-4-carboxamide to an analogue of NAD with potent IMP dehydrogenase-inhibitory properties. *Biochem. Pharmacol.* 31:2133–2136.

13. Kuttan, R., R. K. Robins, and P. P. Saunders. 1982. Inhibition of inosinate dehydrogenase by metabolites of 2-β-D-ribofuranosylthiazole-4-carboxamide. *Biochem. Biophys. Res. Commun.* 107:862–868.

14. Jayaram, H. N., D. A. Cooney, R. I. Glazer, R. L. Dion, and D. G. Johns. 1982. Mechanism of resistance to the oncolytic C-nucleoside 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC-286193). *Biochem. Pharmacol.* 31:2257–2560.

15. Ahluwalia, G. S., H. N. Jayaram, J. P. Plowman, D. A. Cooney, and D. G. Johns. 1984. Studies on the mechanism of action of 2-β-D-ribofuranosylthiazole-4-carboxamide-V. Factors governing the response of murine tumors to tiazofurin. *Biochem. Pharmacol.* 33: 1195–1203.

16. Durkacz, B. W., O. Omidiji, D. A. Gray, and S. Shall. 1980. (ADP-ribose)n participates in DNA excision repair. *Nature (Lond.).* 283:593–596.

17. Berger, N. A., G. W. Sikorski, S. J. Petzold, and K. K. Kurohara. 1979. Association of poly(adenosine diphosphoribose) synthesis with DNA damage and repair in normal human lymphocytes. *J. Clin. Invest.* 63:1164–1171.

18. Berger, N. A., and G. W. Sikorski. 1981. Poly(adenosine diphosphoribose) synthesis in ultraviolet-irradiated xeroderma pigmentosum cells reconstituted with micrococcus luteus UV endonuclease. *Biochemistry.* 20:3610–3614.

19. Kato, T., S. J. Berger, J. A. Carter, and O. H. Lowry. 1973. An enzymatic cycling method for nicotinamide-adenine dinucleotide with malic and alcohol dehydrogenases. *Anal. Biochem.* 53:86–97.

20. Berger, N. A., S. J. Berger, G. W. Sikorski, and D. M. Catino. 1982. Amplification of pyridine nucleotide pools in mitogen stimulated human lymphocytes. *Exp. Cell. Res.* 137:79–88.

21. Barton, R. A., A. Schulman, E. L. Jacobson, and M. K. Jacobson. 1977. Chromatographic separation of pyridine and adenine nucleotides on thin layers of poly(ethyleneimine) cellulose. *J. Chromatogr.* 130:145–150.

22. Kohn, K. W., L. C. Erickson, R. A. G. Ewig, and C. A. Friedman. 1976. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry.* 15:4629–4637.

23. Kohn, K. W., R. A. G. Ewig, L. C. Erickson, and L. A. Zwelling. 1981. Measurement of strand breaks and cross-links by alkaline elution. *In* DNA Repair, A Laboratory Manual of Research Procedures, Vol. 1. E. C. Friedberg, and P. C. Hanawalt, editors. Marcel Dekker Inc., New York. 379–401.

24. Petzold, S. J., B. A. Booth, G. A. Leimbach, and N. A. Berger. 1981. Purification and properties of poly(ADP-ribose) polymerase from lamb thymus. *Biochemistry.* 20:7075–7081.

25. Berger, N. A., S. J. Petzold, and S. J. Berger. 1979. Association of poly(ADP-rib) synthesis with cessation of DNA synthesis and DNA fragmentation. *Biochim. Biophys. Acta.* 564:90–104.

26. Preiss, J., and P. Handler. 1958. Biosynthesis of diphosphopyridine nucleotide. I. Identification of intermediates. *J. Biol. Chem.* 233: 488–492.

27. Preiss, J., and P. Handler. 1958. Biosynthesis of diphosphopyridine nucleotides II. Enzymatic aspects. *J. Biol. Chem.* 233:493–500.

28. Jacobson, E. L., R. A. Lange, and M. K. Jacobson. 1979. Pyridine nucleotide synthesis in 3T3 cells. *J. Cell. Physiol.* 99:417–426.

29. Zwelling, L. A., D. Kerrigan, and Y. Pommier. 1982. Inhibitors of poly(adenosine diphosphoribose) synthesis slow the resealing rate of x-ray induced DNA strand breaks. *Biochem. Biophys. Res. Commun.* 104:897–902.

30. Elliott, G. C., and M. C. Rechsteiner. 1982. Evidence for a physiologically active nicotinamide phosphoribosyltransferase in cultured human fibroblasts. *Biochem. Biophys. Res. Commun.* 104:996–1002.

31. Berger, N. A., J. W. Adams, G. W. Sikorski, S. J. Petzold, and W. T. Shearer. 1978. Synthesis of DNA and poly(adenosine diphosphate ribose) in normal and chronic lymphocytic leukemia lymphocytes. *J. Clin. Invest.* 62:111–118.

32. Hayaishi, O., and K. Ueda. 1977. Poly(ADP-ribose) and ADP ribosylation of proteins. *Ann. Rev. Biochem.* 46:95–116.

33. Ohashi, Y., K. Ueda, M. Kawaichi, and O. Hayaishi. 1983. Activation of DNA ligase by poly(ADP-ribose) in chromatin. *Proc. Natl. Acad. Sci. USA.* 80:3604–3607.

34. Jongstra-Bilen, J., M. E. Ittel, C. Niedergang, H. P. Vosberg, and P. Mandel. 1983. DNA Topoisomerase I from calf thymus is inhibited in vitro by poly(ADP-ribosylation). *Eur. J. Biochem.* 136: 391–396.

35. Yoshihara, K., Y. Tanigawa, and S. S. Koide. 1974. Inhibition of rat liver Ca^{2+}, Mg^{2+} dependent endonuclease activity by nicotinamide adenine dinucleotide and poly(adenosine diphosphate ribose) synthetase. *Biochem. Biophys. Res. Commun.* 59:658–665.

36. Purnell, M. R., and W. J. D. Whish. 1980. Novel inhibitors of poly(ADP-ribose) synthetase. *Biochem. J.* 185:775–777.

37. Lehmann, A. R., and B. D. Broughton. 1984. Poly(ADP-ribosylation) reduces the steady-state level of breaks in DNA following treatment of human cells with alkylating agents. *Carcinogenesis.* 5: 117–119.

38. Berger, N. A., D. M. Catino, and T. J. Vietti. 1982. Synergistic antileukemic effect of 6-aminonicotinamide and 1,3-bis(2-chloroethyl)-1-nitrosourea on L1210 cells in vitro and in vivo. *Cancer Res.* 42: 4382–4386.

39. Sakamoto, H., H. Kawamitsu, M. Miwa, M. Terada, and T. Sugimura. 1983. Enhancement of antitumor activity of bleomycin by benzamide in vitro and in vivo. *J. Antibiot. (Tokyo).* 36:296–300.

40. Sims, J. L., S. J. Berger, and N. A. Berger. 1983. Poly(ADP-ribose) Polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5′-triphosphate pools in DNA-damaged cells: mechanism of stimulation of unscheduled DNA synthesis. *Biochemistry.* 22:5188–5194.