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Specificity of Inhibitors of Poly(ADP-Ribose) Synthesis

Effects on Nucleotide Metabolism in Cultured Cells

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SUMMARY

The effects of inhibitors of poly(ADP-ribose) synthesis on cell growth and several parameters of nucleotide metabolism have been determined. At concentrations which produced similar inhibitions of poly(ADP-ribose) synthesis, 3-acetylaminobenzamide (1 mM) had no effect on L1210 cell growth, 3-aminobenzamide (5 mM) was slightly inhibitory and 3-methoxybenzamide (5 mM) was a potent inhibitor of growth. During a 2-h incubation, none of the inhibitors affected ribo- or deoxyribonucleotide concentrations in cells treated with or without *N*-methyl-*N*-nitrosourea; however, *N*-methyl-*N*-nitrosourea treatment reduced dCTP concentrations by 50%. During a 24-hr incubation, 3-aminobenzamide and 3-acetylaminobenzamide did not lower ribonucleotide concentrations in cells grown with either undialyzed or dialyzed serum. In contrast, 3-methoxybenzamide caused a depletion of UTP in cells grown with undialyzed serum and caused a depletion of all purine and pyrimidine ribonucleotides in cells grown with dialyzed serum. 3-Aminobenzamide and 3-acetylaminobenzamide had no effect on the conversion of hypoxanthine to ATP and GTP but did slightly inhibit incorporation of formate into ATP and GTP. 3-Methoxybenzamide inhibited incorporation of both hypoxanthine and formate into purine ribonucleotides. 3-Aminobenzamide, 3-acetylaminobenzamide, and 3-methoxybenzamide all inhibited glycine incorporation into ATP and GTP and reduced both the incorporation of thymidine into DNA and the apparent specific activity of the dTTP pool. We conclude that inhibition of poly(ADP-ribose) synthesis causes little or no growth inhibition and has no effect on purine or pyrimidine nucleotide synthesis *de novo*. The effect of all the inhibitors on glycine and formate metabolism may be related to an inhibition of ADP-ribose synthesis or may be a secondary effect of the inhibitors. The growth inhibition and the reduction in nucleotide concentration caused by 3-methoxybenzamide are apparently secondary effects of this drug and may result from an inhibition of phosphoribosyl pyrophosphate synthesis.

INTRODUCTION

There is evidence for an association between poly(ADP-ribose) synthesis and DNA repair (for review see Ref. 1), some of which is based on the use of inhibitors of poly(ADP-ribose) synthesis such as 3-aminobenzamide and 5-methylnicotinamide. The important assumption of this approach is that the effects of the inhibitors result solely from the inhibition of poly(ADP-ribose) synthesis; however, many of the inhibitors of poly(ADP-ribose) synthesis are nicotinamide or benzamide analogues, and, therefore, they may inhibit other NAD^+-dependent reactions. As well, inhibition of NAD^+ consumption for the synthesis of ADP-ribose may alter nucleotide metabolism. Finally, since the biological roles of ADP-ribose are not completely understood, one cannot predict precisely the metabolic effects of inhibiting ADP-ribose synthesis.

Three recent reports have concluded that inhibitors of poly(ADP-ribose) synthesis inhibit nucleotide synthesis (2–4). In one of these studies the evidence for inhibition of nucleotide synthesis was very indirect in that the incorporation of [^{14}C]methyl groups from [^{14}C]methyl methanesulfonate into DNA purines was used as a measure of purine nucleotide synthesis (2). In another study [^{3}H]methionine and [^{14}C]glucose incorporation into DNA was measured, but the DNA was isolated by direct extraction of the cells with chloroform:isoamyl alcohol (24:1) followed by ethanol precipitation (3). This proce-

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dure as reported would not have removed the RNA and is unlikely to have removed all of the proteins, which would have been labeled by [^{3}H]methionine and [^{3}H] glucose. Finally, none of these studies actually measured the effects of inhibitors of poly(ADP-ribose) synthesis on nucleotide concentrations.

Alterations in nucleotide metabolism have been found to have many effects on DNA metabolism, including the inhibition of DNA replication (for reviews see Refs. 5 and 6) and repair (7, 8) and the induction of mutations and recombinations (for review see Ref. 9). Therefore, the possibility that inhibitors of poly(ADP-ribose) synthesis exert effects on nucleotide metabolism has important implications for studies which depend on the use of inhibitors to determine the biological roles of poly(ADP-ribose). We have, therefore, determined the effects of inhibitors of poly(ADP-ribose) synthesis on cell growth and on several parameters of nucleotide metabolism in cultured cells, treated with or without a DNA-damaging agent.

EXPERIMENTAL PROCEDURES

Materials. [^{14}C]Formate, 57 mCi/mmol, [8-^{14}C]hypoxanthine, 40–50 mCi/mmol, and [*methyl*-^{3}H]thymidine, 50 Ci/mmol, were purchased from Moravek Biochemicals Inc., Brea, CA. [8-^{3}H]dATP, 9 Ci/mmol, [8-^{3}H]dGTP, 5.6 Ci/mmol, [5-^{3}H]dCTP, 30 Ci/mmol, and [methyl-^{3}H] dTTP, 43 Ci/mmol, were purchased from ICN, Irvine, CA. [^{14}C]Glycine (100 mCi/mmol) was purchased from New England Nuclear.

DNA polymerase I from *Escherichia coli* was purchased from New England Nuclear. Poly[d(IC)], poly[d(AT)], 3-aminobenzamide, and *N*-methyl-*N*-nitrosourea were purchased from Sigma. 3-Methoxybenzamide was purchased from Aldrich. 5-Methylnicotinamide was a gift of Lilly. 3-Acetylaminobenzamide was a gift of Dr. W. R. Kidwell, Laboratory of Pathophysiology, National Cancer Institute, Bethesda, MD.

Cell culture. Chinese hamster ovary-K1 cells were grown in suspension cultures in α-minimal essential medium containing 10% dialyzed fetal calf serum (Grand Island Biological Co., Grand Island, NY), as described (10). The population doubling time was ~12 hr. L1210 cells were grown in static suspension cultures in Fischer's medium containing 10% horse serum (Grand Island Biological Co.). The population doubling time was ~13 hr. Cell density and the population volume distribution were determined using a model Z_F Coulter Counter equipped with a Coulter Channelyzer II.

Damaging with MNU.[2] MNU, freshly dissolved in dimethyl sulfoxide, was added to L1210 cells in culture medium at 37°, usually for 20 min in the absence of inhibitors of ADP-ribose synthesis except in the experiment described in Table 1 in which the exposure was for 1 hr in the presence of the inhibitors. After a 20-min exposure to MNU the cells were centrifuged and resuspended in fresh warm medium containing the inhibitors of ADP-ribose synthesis.

Cell extraction. Cells were centrifuged and extracted on ice for 30 min with 0.4 M perchloric acid containing either [^{3}H]adenosine or [^{14}C] adenine for determination of dilution, as described (10). Extracts were neutralized by extraction with 0.5 M Alamine 336 (tricapryltertiaryamine, Henkel Corp., Kankakee, IL) in Freon-TF (trichlorotrifluoroethane, Dupont Canada, Inc., Maitland, Ontario) (10).

Nucleotide concentrations. Ribonucleotide concentrations were measured using a Spectra-Physics HPLC equipped with an Isco V^4 detector. A Partisil 10 SAX anion exchange column (Whatman, Clifton, NJ) was used, and the nucleotides were eluted isocratically with 0.25 M KH_2PO_4, 0.5 M KCl, pH 4.5, at 1 ml/min which allowed quantitation of ADP and the nucleoside triphosphates. NAD^+ was quantitated separately from the other ribonucleotides using a Resolve C-18 reversed phase column (Waters Scientific Ltd., Mississauga, Ontario) which was eluted using the following gradient system, at a flow of 1 ml/min: solvent A, 100 mM $(NH_4)_3PO_4$, pH 5.1; solvent B, 25% methanol in water; 0–7 min, 100% A; 13 min, 70% A; 16 min, 70% A; 18 min, 100% A. Detection was at 254 nm, and the peaks were integrated automatically by a Spectra-Physics 4100 integrator. The reproducibility of the NAD^+ determinations (standard deviation, %) was 5.1% (independent determinations on five cultures).

Deoxyribonucleoside triphosphate concentrations were measured using a modification of the DNA polymerase procedure (11). Accuracy and reproducibility (standard deviation), respectively, were as follows: 98 and 2.5% for dATP, 96 and 5.8% for dTTP, 93 and 4.3% for dCTP, and 78 and 8.5% for dGTP. The following controls were performed. Background incorporation (i.e., in the absence of the limiting nonradioactive deoxyribonucleotide) was always measured. In addition, standards were added to cell extracts to determine if the assay was affected by the extracts, and checks were made to demonstrate that the assays were independent of the amount of extract used. Finally, time courses were always performed both with standards and with each cell extract to ensure that the maximum incorporation was reached at the same time under all conditions.

Quantitation of 3-ABA, 3-MeOBA, 3-AcABA, and 5-MeNA. Quantitation of the inhibitors of poly(ADP-ribose) synthesis in neutralized perchloric acid extracts of medium or cells was by HPLC using a Waters Resolve C-18 reversed phase column. 3-ABA was eluted with 50 mM NH_4PO_4, pH 5.1, at 1 ml/min. The other three inhibitors were eluted with 40 mM NH_4PO_4, 10% methanol, pH 5.1. Peak detection was at the following wavelengths, in nm: 3-ABA (254), 3-MeOBA and 3-AcABA (230), and 3-MeNA (270). 3-MeNA was detected at its maximum absorbance; the choice of detection wavelengths for the other inhibitors represented a compromise in order to minimize the absorbance of the solvent while maximizing the absorbance of the inhibitor.

Quantitation of purines in cell culture medium. Neutralized perchloric acid extracts of Fisher's medium containing 10% horse serum (dialyzed or undialyzed) were analyzed by HPLC using a Waters Resolve C-18 reversed phase column eluted with 50 mM NH_4PO_4, pH 5.5, at 1 ml/min. Peak detection was at 254 nm. Guanine, hypoxanthine, and xanthine were not separated and were, therefore, quantitated together. Adenine eluted as a separate peak.

Purine nucleotide synthesis from [^{14}C]formate, [^{14}C]glycine, and [^{14}C]hypoxanthine. L1210 cells were incubated in Fisher's medium at 37° with [^{14}C]formate (0.94 mM), [^{14}C]glycine (0.1 mM), or [^{14}C]hypoxanthine (36 μM) for 30 min. The cells were then centrifuged and extracted with 0.4 M perchloric acid, as described above. Procedures for the separation and measurement of radioactivity in purine ribonucleotides have been described previously (12).

[^{3}H]Thymidine incorporation into dTTP and DNA and calculation of the apparent rate of DNA synthesis from dTTP. L1210 cells at a density of 3–4 × 10^5 cells/ml were incubated with or without 0.5 mM MNU for 20 min, centrifuged, resuspended in fresh medium, and incubated with or without inhibitors for 2 hr. An aliquot of each culture was used for determination of dTTP pool sizes (see above). [^{3}H]dThd (50 Ci/mmol, 0.2 μM) was added to a second aliquot of each culture and incubated for the final 30 min of the 2-hr incubation followed by extraction with 0.4 M perchloric acid. The neutralized extracts were chromatographed and the radioactivity in dTTP was determined, as described (10). Carrier DNA was added to the perchloric acid-insoluble pellets, and the pellets were washed three times with 0.4 M perchloric acid. The pellets were dissolved in 0.2 N KOH, and aliquots were spotted on polyethyleneimine cellulose thin-layer plates. The plates were washed overnight with 50% methanol in water to remove [^{3}H] dThd. The origins containing the DNA were scraped into scintillation vials and eluted with 1 N NaOH for 3 hr. The NaOH was neutralized with acetic acid, and the radioactivity was determined using Triton X-

[2] The abbreviations used are: MNU, *N*-methyl-*N*-nitrosourea; 3-ABA, 3-aminobenzamide; 3-MeOBA, 3-methoxybenzamide; 3-AcABA, 3-acetylaminobenzamide; 5-MeNA, 5-methylnicotinamide; HPLC, high pressure liquid chromatography; CHO, Chinese hamster ovary.

100 counting fluid (0.4% 2,5-diphenyloxazole, 0.02% 1,4-bis[2-(5-phenyloxazolyl)]benzene, in 1 part Triton X-100 and 2 parts xylene). Corrections were made for the relative counting efficiency of the dTTP and DNA samples.

The apparent specific activity of the dTTP pool was calculated based on the assumption that the dTTP pool is homogeneous and that rapid mixing occurs. In order to calculate an apparent rate of DNA synthesis from dTTP, the rate of incorporation of [^{3}H]dTMP into DNA was divided by the specific activity of the dTTP pool, as described (10).

RESULTS

Inhibition of the MNU-induced depletion of NAD$^+$ by inhibitors of poly(ADP-ribose) synthesis. Since the biological roles of mono- and poly(ADP-ribose) are not known for certain, one cannot predict the perturbations in metabolism which will result from the inhibition of ADP-ribose synthesis. Therefore, in order to detect effects of the inhibitors which were not specific to the inhibition of poly(ADP-ribose) synthesis, it was necessary to use the inhibitors at concentrations which produced similar extents of inhibition of poly(ADP-ribose) synthesis. Metabolic effects of the inhibitors which were not universal could then be ascribed to a secondary effect of the inhibitor.

Poly(ADP-ribose) synthesis is difficult to measure in intact cells both because of the lack of a specific precursor and because of rapid turnover of poly(ADP-ribose) in cells treated with DNA-damaging agents (13, 14). The rapid turnover of poly(ADP-ribose) argues against the validity of using poly(ADP-ribose) accumulation as a measure of its synthesis. Since previous studies have provided evidence that the NAD$^+$ depletion caused by alkylating agents is the result of poly(ADP-ribose) synthesis and turnover (13–17), we have used inhibition of MNU-induced depletion of NAD$^+$ as a measure of the inhibition of poly(ADP-ribose) synthesis. 3-ABA, 3-MeOBA, and 5-MeNA have been reported to inhibit poly(ADP-ribose) synthesis with approximately equal efficiency (18), and we, therefore, tested all at 5 mM, a concentration commonly used in studies on DNA repair. 3-AcABA, synthesized by Purnell and Whish (19), is a more potent inhibitor of poly(ADP-ribose) synthetase with a K_i ~0.1-fold that of 3-ABA and was, therefore, tested at 1.0 mM.[3]

As shown in Table 1, a 1.5-hr incubation of L1210 cells with the inhibitors alone caused a slight elevation of NAD$^+$. A 1-hr treatment with 1.0 mM MNU reduced the NAD$^+$ concentration to 32% of control, consistent with previous studies (for review see Ref. 1). The inhibitors prevented the depletion of NAD$^+$ with approximately equal efficiency.

Since the inhibitors were present during the incubation with MNU, one mechanism by which the inhibitors might have prevented the MNU-induced depletion of NAD$^+$ would have been to react with the MNU, thus reducing damage to DNA and the subsequent synthesis of poly(ADP-ribose). Therefore, 3-ABA, 3-MeOBA, and 5-MeNA at 5 mM and 3-AcABA at 1 mM were each incubated with 1 mM MNU in culture medium for 1 hr at 37°. Neither a loss of the parent peaks nor the appearance of new peaks was detected by HPLC analysis (see "Experimental Procedures").

[3] W. R. Kidwell, personal communication.

TABLE 1

The effect of inhibitors of poly(ADP-ribose) synthesis on MNU-induced NAD$^+$ depletion in L1210 cells

Cells were incubated 30 min with or without inhibitors of poly(ADP-ribose) synthesis and then incubated 60 min with or without 1.0 mM MNU. The cells were then centrifuged and extracted on ice with 0.4 M perchloric acid.

MNU treatment	Drug	NAD$^+$	
		Per cent of untreated control[a]	Per cent of inhibitor-treated control
–	5 mM 3-ABA	128	100
–	1 mM 3-AcABA	135	100
–	5 mM 3-MeOBA	126	100
–	5 mM 5-MeNa	104	100
+		32	
+	5 mM 3-ABA	95	74
+	1 mM 3-AcABA	109	81
+	5 mM 3-MeOBA	106	84
+	5 mM 5-MeNA	79	76

[a] The untreated control value for NAD$^+$ in pmol/10^6 cells was 355.

Cell growth. The first parameter used to test the specificity of the inhibitors was cell growth rate. L1210 cell growth rate was unaffected over a 24-hr period by 1 mM 3-AcABA but was inhibited by 5 mM 3-ABA and 5 mM 3-MeOBA to 80 and 66% of control, respectively. Furthermore, after 24 hr the growth rate of the 3-MeOBA-treated cells declined to less than 10% of control, whereas cells treated with 3-ABA or 3-AcABA maintained constant growth rates to at least 48 hr. In CHO cells 5 mM 3-ABA and 1 mM 5-MeNA had no effect on growth rate but 5 mM 5-MeNA reduced growth rate to 74% of control. Since, at the concentrations used, these inhibitors were approximately equally effective at inhibiting the MNU-induced depletion of NAD$^+$, these results suggest that inhibition of poly(ADP-ribose) synthesis causes little or no inhibition of cell growth and that the inhibition of growth observed with 3-ABA, 3-MeOBA, and 5-MeNA is probably the result of the inhibition of other metabolic processes.

Metabolism of the inhibitors. The possibility was considered that the lack of effect of 3-AcABA and the small inhibitory effect of 3-ABA on cell growth may have resulted from metabolic inactivation of the drugs and conversely that the potent growth inhibitory effects of 3-MeOBA may have resulted from metabolic activation. In order to determine their metabolic fate, 5 mM 3-ABA, 1 mM 3-AcABA, and 5 mM 3-MeOBA were incubated with L1210 cells in culture medium at 37°. Samples were removed at 0 and 24 hr and extracted on ice with 0.4 M perchloric acid. The extracts were neutralized and analyzed by HPLC (see "Experimental Procedures") to determine the amount of drug remaining and to look for metabolites. The extraction procedure did not induce breakdown of the drugs; however, 3-ABA did decompose in neutralized perchloric acid extracts when stored for several days at −20°. The decomposition product appeared as a new peak on the HPLC. The percentage of each drug remaining after a 24-hr incubation with L1210

cells was: 3-ABA (91%), 3-AcABA (90%), and 5-MeOBA (102%). No new HPLC peaks appeared, although it is possible that a metabolite might have had the same retention time as the parent drug. Therefore, a second test was performed in which L1210 cells were incubated with or without 5 mM 3-ABA for 0.5 or 24 hr followed by a 1-hr incubation with 1.0 mM MNU. 3-ABA was equally effective under both conditions in inhibiting the depletion of NAD^+.

Nucleotide pools in L1210 cells. The lack of inhibition of cell growth by 3-AcABA and the small growth inhibition caused by 3-ABA did not rule out the possibility that these drugs altered nucleotide metabolism. For example, 100 μM dThd has been found to increase dTTP and dGTP pools substantially in CHO cells but was not growth inhibitory (10). Alterations in nucleotide metabolism which are insufficient to inhibit cell growth might still either inhibit DNA repair or induce artifacts in the quantitation of DNA repair. Therefore, we determined the effect of the inhibitors on ribo- and deoxyribonucleotide pool sizes in L1210 cells. Both undamaged and MNU-damaged cells were used to determine if the inhibitors had different effects depending on whether or not DNA repair and poly(ADP-ribosylation) had been stimulated. Cells were incubated 20 min with or without 0.5 mM MNU, centrifuged, and resuspended in fresh medium, and then incubated 2 hr with or without the inhibitors. A 2-hr incubation period was chosen both in order to minimize any changes in cell cycle distribution which might be induced by 3-MeOBA or MNU and because many studies on DNA repair have used relatively short incubation periods when measuring changes in DNA strand breaks or DNA repair synthesis.

As shown in Table 2, in the absence of MNU treatment, the inhibitors had little effect on either ribo- or deoxyribonucleotide pools. Treatment of cells with 0.5 mM MNU for 20 min followed by a 2-hr incubation caused the dCTP pool to decline to ~50% of control, but the other pools remained at approximately control levels. Incubation of MNU-treated cells with the inhibitors had no additional effect on the pools.

Cleaver (4) has suggested that purines in culture medium compensate for inhibition of purine synthesis *de novo* and thus account for the lack of cytotoxicity of 3-ABA in cultured cells. Although the Fisher's medium used in the experiments described here contained no purines, the presence of 10% undialyzed horse serum produced a final purine concentration of ~1.0 μM, consisting of guanine, hypoxanthine, and xanthine, as determined by HPLC (see "Experimental Procedures"); no other purines were detected.

We tested the proposal of Cleaver by determining the effects of the inhibitors on ribonucleotide and NAD^+ pool sizes in L1210 cells grown for 24 hr in medium containing either undialyzed or dialyzed horse serum. No purines were detected in dialyzed serum by HPLC (see "Experimental Procedures"). As shown in Table 3, 5 mM 3-ABA and 1 mM 3-AcABA did not lower ribonucleotide pools in cells grown with undialyzed or dialyzed serum; however, 5 mM 3-MeOBA caused a significant depletion of UTP in cells grown with undialyzed serum and caused a depletion of all purine and pyrimidine ribonucleotide pools in cells grown with dialyzed serum.

Although the growth rate of the cells with dialyzed serum was only 53% of the rate with undialyzed serum, the inhibitors had no additional growth inhibitory effects in undialyzed serum.

Incorporation of formate, hypoxanthine, and glycine into purine nucleotides. Although the nucleotide pool size data argue that 3-ABA and 3-AcABA do not inhibit purine nucleotide synthesis, these inhibitors might still either cause a transient inhibition of nucleotide synthesis of insufficient duration to reduce nucleotide concentrations or alter radioactive precursor metabolism without inhibiting nucleotide synthesis. Therefore, the effect of the inhibitors on formate and hypoxanthine incorporation into nucleotides in L1210 cells treated with or without MNU was also determined. As shown in Table 4, 3-ABA and 3-AcABA had no effect on the conversion of hypoxanthine to ATP and GTP but did slightly inhibit incorporation of formate into ATP and GTP, suggesting either an inhibition of purine synthesis *de novo* or an effect on folate metabolism. 3-MeOBA inhibited incorporation of both hypoxanthine and formate into purine ribonucleotides, with more effect on formate incorporation. MNU treatment had little effect on nucleotide synthesis, and MNU in combination with the inhibitors produced the same effects as the inhibitors alone.

TABLE 2

The effect of inhibitors of poly(ADP-ribose) synthesis on ribo- and deoxyribonucleotide pool sizes in L1210 cells with or without prior MNU treatment

Cells were treated with or without 0.5 mM MNU for 20 min, centrifuged, resuspended in fresh medium, and incubated with or without inhibitors of poly(ADP-ribose) synthesis for 2 hr. Values are average of two experiments.

MNU treatment	Drug	Per cent of control[a]							
		UTP	CTP	ATP	GTP	dTTP	dCTP	dATP	dGTP
−	5 mM 3-ABA	94	101	104	110	104	127	105	100
−	1 mM 3-AcABA	100	101	108	114	104	109	109	101
−	5 mM 3-MeOBA	73	102	112	122	81	84	108	94
+		86	88	95	96	92	48	112	112
+	5 mM 3-ABA	85	94	99	107	96	55	120	124
+	1 mM 3-AcABA	80	94	96	99	74	43	99	101
+	5 mM 3-MeOBA	72	105	106	121	78	42	109	103

[a] Control values in pmol/10^6 cells were: UTP, 1180; CTP, 330; ATP, 2940; GTP, 630; dTTP, 17.8; dCTP, 16.6; dATP, 6.7; dGTP, 3.8.

TABLE 3

The effect of inhibitors of poly(ADP-ribose) synthesis on ribonucleotide pool sizes in L1210 cells grown 24 hr in medium containing horse serum or dialyzed horse serum

Cells were incubated with or without inhibitors of poly(ADP-ribose) synthesis for 24 hr in Fisher's medium containing either 10% horse serum or 10% dialyzed horse serum.

Serum	Drug	Per cent of control[a]				
		UTP	CTP	ATP	GTP	NAD
Undialyzed	5 mM 3-ABA	106	95	106	122	150
Undialyzed	1 mM 3-AcABA	103	92	105	118	126
Undialyzed	5 mM 3-MeOBA	56	84	106	117	118
Dialyzed	5 mM 3-ABA	99	138	125	121	158
Dialyzed	1 mM 3-AcABA	88	106	98	97	124
Dialyzed	5 mM 3-MeOBA	30	75	75	63	102

[a] Control values in pmol/10^6 cells in undialyzed and dialyzed serum, respectively, were: UTP, 1015, 680; CTP, 385, 160; ATP, 3028, 2190; GTP, 708, 694; NAD, 420, 340.

TABLE 4

The effect of inhibitors of poly(ADP-ribose) synthesis on purine nucleotide synthesis from [^{14}C]formate and [^{14}C]hypoxanthine in L1210 cells with or without prior MNU treatment

Cells were treated with or without 0.5 mM MNU for 20 min, centrifuged, resuspended in fresh medium, and incubated with or without inhibitors of poly(ADP-ribose) synthesis for 2 hr. [^{14}C]Formate or [^{14}C]hypoxanthine were added 0.5 hr before the end of the 2-hr incubation.

MNU treatment	Drug	Radioactivity incorporated (% of control)			
		[^{14}C] Hypoxanthine		[^{14}C] Formate	
		ATP	GTP	ATP	GTP
−	5 mM 3-ABA	99	123	62	82
−	1 mM 3-AcABA	98	116	86	88
−	5 mM 3-MeOBA	67	85	48	56
+		91	103	98	83
+	5 mM 3-ABA	100	112	68	65
+	1 mM 3-AcABA	104	100	84	70
+	5 mM 3-MeOBA	80	83	46	46

TABLE 5

The effect of inhibitors of poly(ADP-ribose) synthesis on purine nucleotide synthesis from [^{14}C]glycine in L1210 cells

Cells were incubated with or without drugs for 2 hr. [^{14}C]Glycine was added 0.5 hr before the end of the 2-hr incubation.

Drug	[^{14}C]Glycine radioactivity incorporated (% of control)	
	ATP	GTP
5 mM 3-ABA	54	45
1 mM 3-AcABA	78	60
5 mM 3-MeOBA	47	44

In order to further define the effects of the inhibitors on precursor metabolism, [^{14}C]glycine, another substrate for purine synthesis *de novo*, was used. As shown in Table 5, all three drugs inhibited [^{14}C]glycine incorporation into ATP and GTP. The possibility of a transient effect of the inhibitor was tested by incubating L1210 cells for 2 and 24 hr with 5 mM 3-ABA and then measuring [^{14}C]glycine incorporation into purine nucleotides during the final 0.5 hr of the incubation. The results, expressed as per cent of control were: 2-hr incubation, ATP:52, GTP:43; 24-hr incubation, ATP:52, GTP:40.

[^{3}H]Thymidine incorporation into dTTP and DNA. It has been reported that inhibitors of ADP-ribose synthesis either stimulate [^{3}H]dThd incorporation during DNA excision repair (18, 20, 21) or have no effect (12, 22), depending on the nature and dose of the DNA-damaging agent. We have, therefore, determined the effect of these inhibitors on the incorporation of [^{3}H]dThd into dTTP and DNA. As well, the apparent specific activity of the dTTP pool was calculated, and this value together with the incorporation of [^{3}H]dThd into DNA were used to calculate the apparent rate of DNA synthesis, as described previously (10). These calculations are based on the assumptions that the dTTP pool is homogeneous and that rapid mixing occurs. As shown in Table 6, 3-ABA, 3-AcABA, and 3-MeOBA all reduced both the incorporation of [^{3}H]dThd into DNA and the specific activity of the dTTP pool. When the [^{3}H]dThd incorporation into DNA was corrected for the reduced specific activity of dTTP, 3-ABA and 3-AcABA had little effect on the apparent rate of DNA synthesis, consistent with the small effect of 3-ABA and the lack of effect of 3-AcABA on cell growth. In the case of 3-MeOBA, an apparent rate of DNA synthesis of 39% of control occurred under conditions which, in a separate experiment, produced a 24-hr growth rate of 66% of control. All three drugs reduced the total amount of [^{3}H]dThd which was phosphorylated (i.e., radioactivity in dTTP + DNA), suggesting an inhibition of either membrane transport or phosphorylation.

Treatment with 0.5 mM MNU reduced both the rate of DNA synthesis and the specific activity of the dTTP pool. Addition of the inhibitors to the MNU-treated cells caused a further reduction in both [^{3}H]dThd incorporation into DNA and the rate of DNA synthesis. In MNU-treated cells, 3-AcABA produced an increase in both the incoporation of [^{3}H]dThd into dTTP and the specific activity of dTTP, whereas 3-AcABA alone had no effect on incorporation of [^{3}H]dThd into dTTP and caused a slight reduction in the specific activity of dTTP.

DISCUSSION

3-ABA, 3-MeOBA, and 3-MeNA at 5 mM and 3-AcABA at 1 mM inhibited poly(ADP-ribose) synthesis, as measured by prevention of MNU-induced NAD^+ depletion, with similar efficiency, consistent with previous results obtained with permeable cells (18) and with purified poly(ADP-ribose) synthesis (19).[3] No reaction between the inhibitors and MNU was detected, which argues that prevention of NAD^+ depletion resulted from inhibition of poly(ADP-ribose) synthesis rather than by reduction of MNU concentration.

The lack of effect of 1 mM 3-AcABA on L1210 and CHO cell growth suggests that partial inhibition of poly(ADP-ribose) synthesis is not growth inhibitory and, therefore, that the growth inhibition observed with 5 mM 3-ABA, 5-MeNA, and 3-MeOBA resulted from the inhi-

TABLE 6

The effect of inhibitors of poly(ADP-ribose) synthesis on [^{3}H]thymidine incorporation into dTTP and DNA in L1210 cells with or without prior MNU treatment

Cells were treated with or without 0.5 mM MNU for 20 min, centrifuged, resuspended in fresh medium, and incubated with or without inhibitors of poly(ADP-ribose) synthesis for 2 hr. [^{3}H]dThD was added 0.5 hr before the end of the 2-hr incubation.

MNU treatment	Drug	Percent of control[a]			
		Radioactivity incorporated		Apparent specific activity of dTTP	Apparent rate of DNA synthesis
		dTTP	DNA		
–	5 mM 3-ABA	86	63	60	105
–	1 mM 3-AcABA	99	78	75	104
–	5 mM 3-MeOBA	69	30	76	39
+		128	68	86	79
+	5 mM 3-ABA	114	48	83	58
+	1 mM 3-AcABA	133	54	143	38
+	5 mM 3-MeOBA	100	20	83	24

[a] The control value for the rate of DNA synthesis in pmol of dTMP/min/10^6 cells was 7.2.

bition of other metabolic processes. In support of this conclusion Nakanishi *et al.* (23) found that benzamide, another inhibitor of poly (ADP-ribose) synthesis stimulated the growth of cultured chick bud limb cells. We found that 3-MeOBA differed from the other drugs in that it was a more potent inhibitor of cell growth and also had a delayed effect; L1210 cell growth rate was 66% of control over the first 24 hr of incubation, after which growth was almost completely inhibited. This abrupt effect on cell growth occurred after a single doubling of the cell population, which would be consistent with a block in the cell cycle during early G1 phase. Kidwell *et al.* (24) have reported an abrupt delayed effect of 5 mM 5-MeNA on HeLa cells, but the delay was only 3 hr and resulted from a block in early G2 phase (24). If poly(ADP-ribose) is involved only in DNA repair, then in the absence of a DNA-damaging agent, one would not expect inhibitors of poly(ADP-ribose) to inhibit cell growth. The results of Boorstein and Pardee (25) are consistent with this possibility; they found that while 4 mM 3-ABA or 0.5 mM methyl methanesulfonate had little effect on the cell cycle distribution of normal human fibroblasts over a 72-hr period, addition of 3-ABA to methyl methanesulfonate-treated cells caused a large accumulation of cells in the G2 compartment (25). Inhibitors of poly(ADP-ribose) synthesis also inhibit mono(ADP-ribose) transferase[3] and, therefore, the growth inhibitory effects of some of the inhibitors may result from the inhibition of cytoplasmic mono(ADP-ribose) synthesis.

Although Berger *et al.* (26) suggested that the lack of effect of some inhibitors of poly(ADP-ribose) synthesis on cell viability might be the result of metabolic inactivation, our data argue against this possibility since there was no evidence for metabolism of the inhibitors, and 5 mM 3-ABA was equally potent after 0.5- and 24-hr incubation in preventing the MNU-induced depletion of NAD^+.

Since DNA-damaging agents have been reported to alter deoxyribonucleotide pools and metabolism (27, 28), we determined the effects of inhibitors of poly(ADP-ribose) synthesis on nucleotide pools in cells treated with or without MNU. During a 2-hr incubation, the inhibitors had little effect on ribo- or deoxyribonucleotide triphosphate pools in damaged or undamaged cells. In agreement, Kidwell and Burdette (29) found that 5-MeNA had no effect on ATP pools in HeLa cells and Nakanishi *et al.* (23) found that benzamide had no effect on ATP pools in cultured chick limb bud cells. Our finding that MNU treatment caused a specific depletion of dCTP, which was unaffected by the inhibitors, differs somewhat from that of Newman and Miller (27) who found that dimethyl sulfate treatment of CHO cells depleted dCTP but elevated dTTP.

Cleaver reported that 3-ABA inhibited the incorporation of radioactive glucose, methionine, and methyl methanesulfonate into adenine and guanine in DNA and concluded that 3-ABA inhibited the *de novo* synthesis of DNA purines (2–4). They also found that a CHO cell line resistance to thioguanine was more sensitive to the cytotoxic effects of 3-ABA than the parental line (4). They suggested that purines in the culture medium compensated for the inhibition of purine synthesis *de novo* by 3-ABA and that the sensitivity of the thioguanine-resistant cells to 3-ABA resulted from the absence of hypoxanthine-guanine phosphoribosyltransferase. We found that 3-ABA of 3-AcABA did not deplete nucleotide pools in L1210 cells grown for 24 hr in medium containing either undialyzed or dialyzed horse serum. Furthermore, the use of dialyzed serum did not potentiate the growth inhibitory effects of any of the inhibitors. This suggests that 3-ABA and 3-AcABA do not cause a significant inhibition of purine synthesis *de novo*. In contrast, 3-MeOBA specifically depleted UTP pools in cells grown with undialyzed serum and depleted all four ribonucleoside triphosphate pools with the largest effect on UTP, in cells grown in dialyzed serum. This overall reduction in nucleotide concentrations is consistent with an inhibition in phosphoribosyl pyrophosphate synthesis; however, since it was observed only with 3-MeOBA it appears not to be related to an inhibition of poly(ADP-ribose) synthesis.

5 mM 3-MeOBA was the only condition which inhibited the conversion of hypoxanthine to ATP and

GTP. Again, this is consistent with an inhibition of phosphoribosyl pyrophosphate synthesis. All three inhibitors reduced the incorporation of radioactive formate and glycine into ATP and GTP in cells treated with or without MNU. Two possible effects of the inhibitors consistent with these results are: an inhibition of purine synthesis *de novo* or an alteration in folate metabolism. Since it is well established that inhibitors of purine synthesis *de novo* reduce purine nucleotide concentrations (30, 31), the lack of effect of 3-ABA and 3-AcABA on nucleotide concentrations is inconsistent with an inhibition of purine synthesis *de novo*; however, an effect of the inhibitors on folate metabolism would be consistent with all our results. For example, a stimulation of 5,10-methylene tetrahydrofolate and glycine synthesis from tetrahydrofolate and serine would dilute radioactive glycine and formate as well as other precursors which interact with the one-carbon pool, such as [*methyl*-^{14}C] methionine. This explanation is also consistent with previous reports that 5 mM 3-ABA inhibited the incorporation of radioactive methionine and methyl methanesulfonate into purines in DNA (2–4). Further studies will be necessary to determine if this explanation is consistent with the reported inhibition of [^{14}C]glucose incorporation into purines in DNA (3, 4), since this precursor can potentially label purine nucleotides via several routes, including glycine and the one-carbon pool.

Our findings that the inhibitors had effects on thymidine metabolism and that these effects differed depending on whether or not the cells had been damaged with MNU suggest that the use of [^{3}H]dThd incorporation as a means of quantitating DNA replication or repair synthesis may give erroneous results in the presence of these inhibitors. All three inhibitors reduced the total amount of [^{3}H]dThd which was phosphorylated, suggesting an inhibition of thymidine transport or phosphorylation. Further work will be required to determine whether these effects resulted from a direct effect of the inhibitors on thymidine metabolism or from the inhibition of mono- or poly(ADP-ribose) synthesis.

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