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INHIBITORS OF POLY(ADP-RIBOSE) POLYMERASE ENHANCE UNSCHEDULED DNA SYNTHESIS IN HUMAN PERIPHERAL LYMPHOCYTES

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SUMMARY: Benzamide and *m*-aminobenzamide, the most potent inhibitors of poly-(adenosine diphosphate ribose) polymerase known, enhanced unscheduled DNA synthesis after ultraviolet irradiation in human lymphocytes. A positive correlation was found between the inhibitory activities and enhancing effects on unscheduled DNA synthesis of inhibitors related to benzamide and nicotinamide. Lymphocytes of a patient with xeroderma pigmentosum did not show enhanced unscheduled DNA synthesis with these inhibitors after ultraviolet irradiation, but like normal lymphocytes, showed enhanced unscheduled DNA synthesis after treatment with N-methyl-N'-nitro-N-nitrosoguanidine.

INTRODUCTION

Poly(ADP-Rib)[1] is formed by ADP-ribosylation catalyzed by poly(ADP-Rib) polymerase in nuclei of eukaryotes (1-4) and it is covalently attached to nuclear proteins such as histone and nonhistone proteins, including poly-(ADP-Rib) polymerase itself (5). The function of poly(ADP-Rib) or poly-(ADP-ribosyl)ation of nuclear protein is unknown, although there are several suggestive reports of its involvement in regulation of cellular mechanisms, including DNA replication, DNA repair, cell differentiation and cell transformation (1-4, 6-11).

We recently found that inhibitors of poly(ADP-Rib) polymerase structurally related to benzamide and nicotinamide induce sister chromatid exchanges (SCEs) and that there is a positive correlation between inhibition of poly(ADP-Rib) polymerase and induction of SCEs (12,13). SCEs might be understood to be a

Abbreviations : Poly(ADP-Rib), poly(adenosine diphosphate ribose) or poly-(ADP-ribose) ; SCEs, sister chromatid exchanges ; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine ; PBS, phosphate buffered saline; Hepes, N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid.

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cellular expression of DNA damage and repair and they are induced by many carcinogens-mutagens (14). We became interested in determining the effects of these inhibitors of poly(ADP-Rib) polymerase on unscheduled DNA synthesis.

Berger and Sikorski (15) and Althaus et al. (16) made the interesting observation that nicotinamide increased unscheduled DNA synthesis of cells after treatment with carcinogenic agents. As nicotinamide is methylated by S-adenosylmethionine to form 1-methylnicotinamide$^+$, it serves as an inhibitor of t-RNA methylase (17). Methylation of DNA or other RNAs, including the 5'-terminal cap structure of mRNAs, may also be inhibited by consumption of S-adenosylmethionine. Nicotinamide is a precursor of NAD^+ and it enhances the NAD^+ content of the cells (18). Although nicotinamide may have several biological effects, as described above, one of its known functions is to inhibit poly(ADP-Rib) polymerase (1-4). Benzamide and *m*-aminobenzamide were found by Shall (19) and Purnell and Whish (20) to be much stronger inhibitors of poly(ADP-Rib) polymerase than nicotinamide. Therefore, we tested the effects of a series of compounds related to benzamide and nicotinamide on the level of unscheduled DNA synthesis in peripheral lymphocytes of a normal adult and a patient with xeroderma pigmentosum.

MATERIALS AND METHODS

Chemicals: Benzamide, *o*-, *m*-, and *p*-aminobenzamide were obtained from Tokyo Kasei Co., Tokyo, Japan. The above compounds each gave a single peak on high performance liquid chromatography. Nicotinamide was from Wako Pure Chemical Industries, Osaka, Japan. 1-Methylnicotinamide (N^1-methylnicotinamide chloride), N'-methylnicotinamide and 3-acetylpyridine were purchased from Sigma Chemical Co., St. Louis, Mo. Nicotinic acid and benzoic acid were obtained from Daiichi Pure Chemicals and Kanto Kagaku Co., Tokyo, Japan, respectively. MNNG was obtained from Aldrich Chemical Co., Milwaukee, Wis.

Lymphocytes: About 20 ml of normal human blood was obtained in a 20 ml syringe with 1.8 ml of 2 % EDTA and 0.2 ml of 10 times concentrated PBS. It was mixed with 1 volume of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and the lymphocytes were obtained by Ficoll-Hypaque gradient centrifugation (21). Peripheral lymphocytes were obtained similarly from a 29-year-old man with xeroderma pigmentosum.

Procedure for assay of unscheduled DNA synthesis: Unscheduled DNA synthesis was assayed by a slight modification of the method of Berger and Sikorski (15). Briefly, the lymphocytes were washed once with PBS and suspended in PBS at 2 x 10^6 cells/ml. For ultraviolet irradiation, the cell suspension was poured into plastic dishes and irradiated with ultraviolet light at 254 nm at 20 J/m^2.

Table I. Effects of inhibitors of poly(ADP-Rib) polymerase on unscheduled DNA synthesis after ultraviolet irradiation in human peripheral lymphocytes

Compound	[^{3}H]dThd incorporation (cpm/10^6cells/4 hours)		Enhancement (fold)
	-UV*	+UV	+UV
Control	166	707	1.0
Benzamide	209	3,507	5.0
m-Aminobenzamide	164	4,484	6.3
Nicotinamide	212	4,465	6.3
o-Aminobenzamide	254	1,678	2.4
p-Aminobenzamide	178	2,479	3.5
3-Acetylpyridine	208	1,181	1.7
1-Methylnicotinamide$^+$	135	890	1.3
N'-Methylnicotinamide	156	831	1.2
Nicotinic acid	216	771	1.1
Benzoic acid	118	857	1.2

* Ultraviolet irradiation.

Control lymphocyte suspensions were treated similarly but without ultraviolet irradiation. After irradiation, the cells were collected from the dish and suspended at 4 x 10^6 cells/ml in McCoy 5A medium containing 20 % fetal calf serum, 50 mM Hepes buffer (pH 7.4) and 20 mM hydroxyurea. A volume of 250 µl of lymphocyte suspension containing 10^6 cells was incubated with 50 µl of each inhibitor solution of 20 mM concentration for 30 min at 37°C. Then, 200 µl of [methyl-^{3}H]-dThd (45 Ci/mmole, Radiochemical Centre, Amersham, England) was added at a final concentration of 200 µCi/ml and the mixture was incubated for 4 hours at 37°C in a CO_2-incubator. For treatment with MNNG, the cell suspension in McCoy 5A medium containing 20 % fetal serum and 50 mM Hepes buffer (pH 7.4) was treated with MNNG at 20 µg/ml for 30 min at 37°C. Then hydroxyurea and *m*-aminobenzamide or nicotinamide were added and incubation was continued for 30 min. Then the cells were incubated with [^{3}H]dThd as described above. After incubation, an equal volume of 2 mM cold dThd in PBS was added to stop the labeling and the cells were washed three times with PBS and three times with 5 % trichloroacetic acid using a sonicator to suspend the precipitate. Acid-insoluble material was dissolved in 0.5 ml of Protosol (New England Nuclear, Boston, Mass.). Then 5 ml of Aquasol-2 (New England Nuclear) was added and the acid-insoluble radioactivity was determined in a Packard liquid scintillation spectrometer 1 day later when chemiluminescence had subsided. All assays were performed in duplicate, and the acid-insoluble radioactivities at zero time on incubation of [^{3}H]dThd with the cells in the absence of inhibitors was subtracted from observed values.

RESULTS

Enhancement of unscheduled DNA synthesis in normal lymphocytes by inhibitors

Table I shows data on [^{3}H]dThd incorporation in the presence and absence of the inhibitors of poly(ADP-Rib) polymerase. The incorporations of [^{3}H]dThd

in the presence of the various inhibitors without ultraviolet irradiation of the lymphocytes were almost the same and may represent some residual DNA repair occurring in normal physiological conditions or some trace amount of replicative DNA synthesis that was not suppressed by 10 mM hydroxyurea. In a separate experiment, in which the lymphocytes were incubated with and without hydroxyurea in the incubation medium, incorporations of 240 and 1,300 cpm/10^6 cells/4 hours, respectively, were observed in the absence of ultraviolet irradiation. As shown in Table I, ultraviolet irradiation caused 4.3-fold increase of [^{3}H]dThd incorporation in the absence of inhibitors. In the presence of 2 mM concentration of benzamide, *m*-aminobenzamide and nicotinamide, the incorporations after ultraviolet irradiation were 5.0, 6.3 and 6.3 times, respectively, that of the control without inhibitor. *o*-Aminobenzamide, *p*-aminobenzamide and 3-acetylpyridine caused similarly 2.4-, 3.5- and 1.7-fold increase, respectively. 1-Methylnicotinamide$^+$, N'-methylnicotinamide, nicotinic acid and benzoic acid did not significantly enhance the incorporation.

The inhibitions by these inhibitors of poly(ADP-Rib) polymerase in a preparation of disrupted nuclei from rat liver have been reported (13); the negative logarithms of the concentrations of the compounds that produced 50 % inhibition of the poly(ADP-Rib) polymerase reaction were expressed as $pM_{50}I$ values. When the logarithms of the enhancements shown in Table I were plotted against the $pM_{50}I$ values of the inhibitors (13), a positive correlation was obtained, as shown in Fig. 1.

Effects of inhibitors on unscheduled DNA synthesis in peripheral lymphocytes of a normal subject and a patient with xeroderma pigmentosum

The effects of the inhibitors of poly(ADP-Rib) polymerase on unscheduled DNA synthesis in peripheral lymphocytes of a patient with xeroderma pigmentosum were examined. The data in Table II are consistent with the fact that patients with xeroderma pigmentosum are known to show much reduced unscheduled DNA syn-

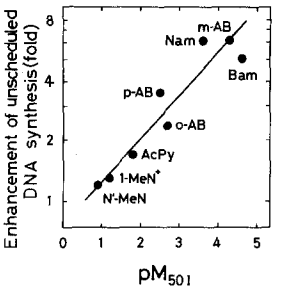

Fig. 1. Correlation between inhibition of poly(ADP-Rib) polymerase and enhancement of unscheduled DNA synthesis after ultraviolet irradiation. pM_{50I} = -log[molar concentration of the compound causing 50 % inhibition of poly(ADP-Rib) polymerase activity]. The abbreviations for chemicals and their pM_{50I} values are as follows(13). Benzamide (Bam), 4.6 ; *m*-aminobenzamide (m-AB), 4.3; nicotinamide (Nam), 3.6; *o*-aminobenzamide (o-AB), 2.7; *p*-aminobenzamide (p-AB), 2.5; 3-acetylpyridine (AcPy), 1.8; 1-methylnicotinamide (1-MeN^+), 1.2; and N'-methylnicotinamide (N'-MeN), 0.9. The pM_{50I} values for nicotinic acid and benzoic acid were less than 0.9 and could not be determined under the usual assay conditions (13) and are not plotted here. The ordinate shows enhancement of unscheduled DNA synthesis after ultraviolet irradiation on a log scale.

thesis after ultraviolet irradiation. It should be emphasized that the peripheral lymphocytes of the patient did not show any response to the potent inhibitors *m*-aminobenzamide and nicotinamide, in unscheduled DNA synthesis after ultraviolet irradiation. Contrary to the case of ultraviolet irradiation however, the lymphocytes of the patient, like those of the normal subject, showed marked responses to the inhibitors enhancing unscheduled DNA synthesis after MNNG treatment.

DISCUSSION

We demonstrated in this work that the potent inhibitors of poly(ADP-Rib) polymerase, benzamide and *m*-aminobenzamide, greatly enhanced unscheduled DNA synthesis after ultraviolet irradiation and MNNG treatment (Tables I and II). Moreover the enhancing activities of these compounds correlated well with their inhibitions of poly(ADP-Rib) polymerase (Fig. 1). These facts strongly indicate that enhancement of unscheduled DNA synthesis after ultraviolet irradiation is due to inhibition of poly(ADP-Rib) polymerase. Therefore, the findings of Berger and Sikorski (15) and Althaus et al. (16) that nicotinamide

Table II. Effects of inhibitors of poly(ADP-Rib) polymerase on unscheduled DNA synthesis after ultraviolet irradiation and MNNG treatment in peripheral lymphocytes of a normal subject and a patient with xeroderma pigmentosum

Lympho-cytes	Addition	[^{3}H]dThd incorporation (cpm/10^6cells/4 hours)		Enhancement (fold)
		-UV*	+UV	+UV
Normal	None	154	463	1.0
	m-Aminobenzamide	241	3,054	6.6
	Nicotinamide	162	2,999	6.5
XP**	None	177	303	1.0
	m-Aminobenzamide	187	406	1.3
	Nicotinamide	226	317	1.0
		-MNNG	+MNNG	+MNNG
Normal	None	223	983	1.0
	m-Aminobenzamide	210	2,533	2.6
	Nicotinamide	209	1,379	1.4
XP	None	269	839	1.0
	m-Aminobenzamide	359	1,913	2.3
	Nicotinamide	378	1,354	1.6

* Ultraviolet irradiation.
** Xeroderma pigmentosum.

enhanced unscheduled DNA synthesis after treatment with several carcinogenic agents might be understood as due to inhibition of poly(ADP-Rib) polymerase.

Yoshihara et al. reported that Ca^{++}, Mg^{++}-dependent endonuclease was inactivated by formation of poly(ADP-Rib)(22). Yamada et al. also reported a nuclease that is inhibited by poly(ADP-Rib)(23). As there was no enhancement of DNA synthesis without ultraviolet irradiation or MNNG treatment in the presence of the potent inhibitors, there may be endonucleases that are inhibited by poly(ADP-Rib) formation, like the nucleases described above (22,23), but that are specific for the damages by ultraviolet irradiation or MNNG treatment. Then these endonucleases may be stimulated by inhibition of poly(ADP-Rib) synthesis to make several times more cuts for repair synthesis to start.

The lack of response of the peripheral lymphocytes of the patient with xeroderma pigmentosum to the inhibitors after ultraviolet irradiation can be explained by supposing that these cells do not have such an endonuclease to excise pyrimidine dimers (24), but do have an endonuclease to excise the DNA damage caused by MNNG. Although pyrimidine dimer-specific endonucleases have been purified from prokaryotes (25), no dimer-specific endonuclease has been purified from mammalian cells. In this connection it is interesting that Waldstein et al. reported a labile endonuclease activity from bovine thymus that does recognize pyrimidine dimers (26), but it is unknown whether this activity is inhibited by poly(ADP-Rib) formation. Durkacz et al. showed that DNA rejoining was inhibited by *m*-aminobenzamide (7). Therefore it is also conceivable that when poly(ADP-Rib) formation is inhibited by inhibitors, unscheduled DNA synthesis does not stop at the correct point but continues, resulting in a longer chain of DNA displacing the parental chain.

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