INHIBITORS OF POLY(ADENOSINE DIPHOSPHATE RIBOSE) POLYMERASE POTENTIATE THE ANTITUMOR ACTIVITY OF BLEOMYCIN AGAINST EHRlich ASCITES CARCINOMA

HISAE KAWAMITSU, MASANAO MIWA, YUKO TANAKA, HIROMI SAKAMOTO, MASAOKI TERADA, AKIO HOSHI AND TAKASHI SUGIMURA

Biology Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan

(Received June 15, 1982)

m-Aminobenzamide and benzamide, which are potent inhibitors of poly(adenosine diphosphate ribose) polymerase and have no antitumor activity themselves, potentiated the antitumor activity of bleomycin against Ehrlich ascites carcinoma *in vivo*.

Keywords—bleomycin; inhibitors of poly(ADP-Rib) polymerase; ADP-ribosylation; potentiating effect; Ehrlich ascites carcinoma

INTRODUCTION

Poly(ADP-Rib) is a biopolymer synthesized from NAD⁺ in the nuclei of eukaryotes.¹⁾ Recently poly(ADP-Rib) was suggested to be involved in DNA repair.²⁾ Durkacz *et al.* reported that a potent inhibitor of poly(ADP-Rib) polymerase, *m*-aminobenzamide (3-aminobenzamide), inhibits the rejoining of DNA in mouse L1210 leukemic cells that have been treated with dimethylsulfate and also that it enhances cell killing with dimethylsulfate *in vitro*.^{2e)}

If *m*-aminobenzamide enhances DNA damage by interfering with DNA repair, it should also potentiate the antitumor activity of drugs that damage DNA *in vivo*. However, there are no reports on the potentiating effect of a strong inhibitor of poly(ADP-Rib) polymerase on the antitumor activity of a drug *in vivo*.

Bleomycin is an antitumor drug that is effective against human cancers, especially squamous cell carcinomas.³⁾ It is thought to act by causing double and single strand scissions of DNA.⁴⁾ We examined the effect of *m*-aminobenzamide and its related compounds on the antitumor activity of bleomycin against Ehrlich ascites carcinoma *in vivo*.

MATERIALS AND METHODS

Chemicals — *m*-Aminobenzamide and benzamide were obtained from Tokyo Kasei Co., Tokyo, Japan. Nicotinamide was from Wako Pure Chemical Industry, Osaka, Japan. 1-Methylnicotinamide and *N'*-methylnicotinamide were products of Sigma Chemical Co., St. Louis, Mo. Bleomycin hydrochloride was purchased from Nihon Kayaku Co., Ltd, Tokyo, Japan; it contained 55–70% of bleomycin A₂, less than 35% of bleomycin B group and less than 1% of bleomycin B₄.

Assay of Antitumor Activity — Five million Ehrlich ascites carcinoma cells were transplanted intraperitoneally into female ICR mice, weighing 22 ± 2g. Bleomycin was suspended in physiological saline containing 0.5% carboxymethylcellulose with or without *m*-aminobenzamide or related compounds. They were injected into animals intraperitoneally 24 h after transplantation of Ehrlich ascites carcinoma cells and then once a day for 4 d. Each group consisted of 6 mice and the ascitic fluid was collected 7 d after tumor transplantation.

Antitumor activity was evaluated by measuring the ratio of the average total packed cell

Abbreviation: Poly(ADP-Rib), Poly(adenosine diphosphate ribose).

volume of the treated group to that of the control group and expressed as T/C%⁵⁾

Assay of Poly(ADP-Rib) Polymerase — Reaction mixtures contained in 0.1 ml, 1 mM [¹⁴C]NAD⁺ (2×10^6 cpm/ μ mol NAD⁺), 100 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM dithiothreitol, with or without inhibitor and an appropriate amount of sonicated nuclei of Ehrlich ascites carcinoma cells, obtained from ascitic fluid 1 week after transplantation into ICR female mice. The mixture was incubated for 10 min at 25°C and then acid-insoluble radioactivity of an

aliquot was determined as described previously.⁶⁾

RESULTS

Table I shows that *m*-aminobenzamide itself had no antitumor activity, but that it potentiated the antitumor activity of bleomycin markedly. Its potentiating effect was marked at a dose of 6.5 mmol/kg/d (1000 mg/kg/d) when bleomycin was given at a dose of 0.3 mg/kg/d or 1 mg/kg/d.

With a fixed dose of bleomycin of 1 mg/kg/d, the decreases of T/C% indicating the potentiation of bleomycin effect were 55% and 58% at doses

TABLE I. *Effect of m-Aminobenzamide on the Antitumor Activity of Bleomycin on Ehrlich Ascites Carcinoma*

Dose of bleomycin (mg/kg/d)	T/C% ^{a)}		Statistical ^{c)} significance
	<i>m</i> -Aminobenzamide ^{b)}		
	—	+	
0	100± 8 ^{d)} (24)	97± 9 (18)	n.s.
0.1	106± 24 (6)	72± 25 (6)	n.s.
0.3	99± 9 (18)	26± 10 (18)	<i>p</i> < 0.001
1.0	72± 17 (12)	17± 18 (12)	<i>p</i> < 0.05
3.0	16± 14 (12)	2± 2 (12)	n.s.

a) Values are means ± S.E. Values in parentheses are numbers of animals in the group.

b) 6.5 mmol/kg/d (1000 mg/kg/d).

c) n.s., not significant.

d) The total packed cell volume of the control group was $1.1 \text{ ml} \pm 0.1 \text{ ml}$.

TABLE II. *Effect of m-Aminobenzamide and Related Compounds on Poly(ADP-Ribose) Polymerase of Ehrlich Ascites Carcinoma*

Related compounds	Activity of poly(ADP-Rib) polymerase (%) ^{a)}	
	Concentration of compounds	
	0.1 mM	1.0 mM
None	100 ^{b)}	100 ^{b)}
Benzamide	25	3
<i>m</i> -Aminobenzamide	37	6
Nicotinamide	74	28
<i>N'</i> -Methylnicotinamide	103	107

a) Sonicated nuclear preparation of Ehrlich ascites carcinoma.

b) The enzyme activity without inhibitors was 2.0 nmol (4100 cpm) ADP-Rib incorporated into acid-insoluble material / 10 min / 2.4×10^6 sonicated nuclei.

of 6.5 mmol/kg/d (1000 mg/kg/d) and 4.9 mmol/kg/d (750 mg/kg/d) of *m*-aminobenzamide, respectively (Tables I and III). However the decrease of T/C% was only 11% at a dose of 3.3 mmol/kg/d (500 mg/kg/d) of *m*-aminobenzamide.

m-Aminobenzamide and its related compounds were examined to determine whether there is any correlation between the inhibition of poly(ADP-Rib) polymerase *in vitro* and the potentiation of the antitumor activity of bleomycin on Ehrlich ascites carcinoma *in vivo*. As shown in Table II, the inhibitory activity of these compounds was consistent with the published data from experiments using rat liver nuclei.⁶⁾

Table III shows the effect of related compounds on the antitumor activity of bleomycin. Benzamide, another potent inhibitor of

poly(ADP-Rib) polymerase (Table II), also caused 55% decrease of T/C% when given at a dose of 4.9 mmol/kg/d (600 mg/kg/d) (Table III). Benzamide seemed to be more effective than *m*-aminobenzamide, because benzamide caused 44% decrease of T/C% at a dose of 3.3 mmol/kg/d (400 mg/kg/d), whereas at the same molar dose *m*-aminobenzamide caused only 11% decrease, as described above. Nicotinamide, which is a moderately strong inhibitor (Table II) had no potentiating effect at a dose 6.5 mmol/kg/d (800 mg/kg/d) (Table III). 1-Methylnicotinamide, which does not inhibit the enzyme, was not effective at 3.3 mmol/kg/d (550 mg/kg/d) and was toxic to mice at 4.9 mmol/kg/d (820 mg/kg/d). Therefore, we could not evaluate the effect of combined treatment with bleomycin and 1-methylnicotinamide. *N'*-Methylnicotinamide which also does not inhibit the enzyme (Table II),

TABLE III. Effect of *m*-Aminobenzamide and Related Compounds on the Antitumor Activity of Bleomycin on Ehrlich Ascites Carcinoma

Dose of bleomycin (mg/kg/d)	T/C % ^{a)}		Statistical ^{c)} significance
	Related compounds ^{b)}		
	—	+	
Benzamide			
0	100±4 (24)	96± 7 (12)	n.s.
1	69±8 (18)	14± 7 (12)	<i>p</i> < 0.001
<i>m</i> -Aminobenzamide			
0	100±4 (24)	120± 5 (24)	n.s.
1	69±8 (18)	11± 8 (12)	<i>p</i> < 0.001
Nicotinamide			
0	100±4 (24)	85± 15 (6)	n.s.
1	69±8 (18)	62± 7 (6)	n.s.
<i>N'</i> -Methylnicotinamide			
0	100±4 (24)	96± 7 (12)	n.s.
1	69±8 (18)	72± 9 (12)	n.s.

a) Values are mean ± S.E. Values in parentheses are numbers of animals in the group.

b) 4.9 mmol/kg/d for benzamide, *m*-aminobenzamide and *N'*-methylnicotinamide. 6.5 mmol/kg/d for nicotinamide.

c) n.s., not significant.

did not show any potentiating effect even at 4.9 mmol/kg/d (680 mg/kg/d) (Table III).

DISCUSSION

The most potent inhibitors of poly(ADP-Rib) polymerase, benzamide and *m*-aminobenzamide, which did not have any antitumor activity by themselves (Tables I and III), potentiated the antitumor activity of bleomycin against Ehrlich ascites carcinoma *in vivo* (Tables I and III). *N*'-Methylnicotinamide, which does not inhibit poly(ADP-Rib) polymerase, did not have any potentiating effect (Tables II and III). Thus there is an apparent correlation between inhibition of poly(ADP-Rib) polymerase and the potentiating effect. Although the mechanism of the potentiating effects of these compounds is not known, it probably involves inhibition of poly(ADP-Rib) formation *in vivo*.

Durkacz *et al.* reported that *m*-aminobenzamide probably inhibits the rejoining step in DNA repair and thus enhances cell killing *in vitro* by a DNA damaging drug like dimethylsulfate.^{2,e)} Nduka *et al.* also reported enhancement of the cell killing effect of *N*-methyl-*N*-nitrosourea by other inhibitors of poly(ADP-Rib) polymerase, namely 5-methylnicotinamide, caffeine, theophylline, theobromine and thymidine.⁷⁾ The failure of nicotinamide, which is a moderately strong inhibitor of poly(ADP-Rib) polymerase, to potentiate the effect of bleomycin *in vivo* might be due to the fact that nicotinamide increases NAD⁺,⁸⁾ since NAD⁺ is a substrate of poly(ADP-Rib) polymerase and increase in its level may counteract the inhibitory effect of nicotinamide on poly(ADP-Rib) polymerase *in vivo*.

Bleomycin is a DNA-damaging antitumor drug that has some specificity for certain cancers.³⁾ A drug that has no antitumor activity itself but potentiates the antitumor activity of bleomycin should be of value in cancer chemotherapy. This *in vivo* study suggests that a potent inhibitor of poly(ADP-Rib) polymerase, such as benzamide or *m*-aminobenzamide may be useful for this purpose *in vivo*.

Acknowledgement We wish to thank Dr. K. Kureitani and Dr. M. Iigo of this Institute for valuable suggestions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, from the Ministry of Health and Welfare, and from the Society for Promotion of Cancer Research of Japan.

REFERENCES

- 1) a) T.Sugimura: Poly(adenosine diphosphate ribose), *Prog. Nucleic Acid Res. Molec. Biol.*, **13**, 127–151 (1973); b) H.Hilz and P.R.Stone: Poly(ADP-ribose) and ADP-ribosylation of proteins, *Rev. Physiol. Biochem. Pharmacol.*, **76**, 1–59 (1976); c) O.Hayaishi and K.Ueda: Poly(ADP-ribose) and ADP-ribosylation of proteins, *Annu. Rev. Biochem.*, **46**, 95–116 (1977); d) M.R.Purnell, P.R.Stone and W.J.D.Whish: ADP-ribosylation of nuclear proteins, *Biochem. Soc. Trans.*, **8**, 215–227 (1980).
- 2) a) M.E.Smulson, P.Schein, D.W.Mullins, Jr. and S.Sudhakar: A putative role for nicotinamide adenine dinucleotide-promoted nuclear protein modification in the antitumor activity of *N*-methyl-*N*-nitrosourea, *Cancer Res.*, **37**, 3006–3012 (1977); b) H.Juarez-Salinas, J.L.Sims and M.K.Jacobson: Poly(ADP-ribose) levels in carcinogen-treated cells, *Nature* (London) **282**, 740–741 (1979); c) R.C.Benjamin and D.M.Gill: ADP-ribosylation in mammalian cell ghosts, *J. Biol. Chem.*, **255**, 10493–10501 (1980); d) N.A.Berger, G.W.Sikorski, S.J.Petzold and K.Kurohara: Defective poly(adenosine diphosphoribose) synthesis in xeroderma pigmentosum, *Biochemistry*, **19**, 289–293 (1980); e) B.W.Durkacz, O.Omidiji, D.A.Gray and S.Shall: (ADP-ribose)_n participates in DNA excision repair, *Nature* (London), **283**, 593–596 (1980).
- 3) T.Ichikawa: "Fundamental and clinical studies of bleomycin," ed. by S.K.Carter, T.Ichikawa, G.Mathé and H.Umezawa, Japan Scientific Societies Press, Japan, 1976, pp. 99–113.
- 4) T.Terashima, M.Yasukawa and H.Umezawa: Breaks and rejoining of DNA in cultured mammalian cells treated with bleomycin, *Gann*, **61**, 513–516 (1970).
- 5) E.N.Sassenrath: Total packed cell volume as a measure of growth retardation in mouse ascites tumors, *Ann. N. Y. Acad. Sci.*, **76**, 601–609 (1958).
- 6) a) S.Fujimura, S.Hasegawa, Y.Shimizu and T.Sugimura: Polymerization of the adenosine 5'-diphosphate-ribose moiety of nicotinamide-adenine dinucleotide by nuclear enzyme. I. Enzymatic reactions, *Biochim. Biophys. Acta*, **145**, 247–259 (1967); b) A.Oikawa, H.Tohda, M.Kanai, M.Miwa and

- T.Sugimura: Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges, *Biochem. Biophys. Res. Commun.*, **97**, 1311–1316 (1980).
- 7) N.Nduka, C.J.Skidmore and S.Shall: The enhancement of cytotoxicity of *N*-methyl-*N*-nitrosourea and γ -radiation by inhibitors of poly(ADP-ribose) polymerase, *Eur. J. Biochem.*, **105**, 525–530 (1980).
- 8) a) N.O.Kaplan, A.Goldin, S.R.Humphreys, M.M.Ciotti and F.E.Stolzenbach; Pyridine nucleotide synthesis in the mouse, *J. Biol. Chem.*, **219**, 287–298 (1956); b) M.Terada, H.Fujiki, P.A.Marks and T.Sugimura: Induction of erythroid differentiation of murine erythroleukemia cells by nicotinamide and related compounds, *Proc. Natl. Acad. Sci. USA*, **76**, 6411–6414 (1979).