THE POTENTIATION OF THE ANTITUMOR ACTIVITY BUT NOT TOXICITY OF BLEOMYCIN BY 3-AMINOBENZAMIDE

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Our previous studies have demonstrated that 3-aminobenzamide (3AB), an inhibitor of adenosine diphosphate-ribosyl transferase (ADPRT) could enhance the cytotoxicity (*in vitro*) and antitumor activty (*in vivo*) of bleomycin (BLM) A_5 and peplomycin (PEP) against S-180, hepatoma and Ehrlich ascites carcinoma (EAC). In this study, it was shown that the inhibition rates (INR's) of S-180 in two experiments were increased from 42.5 and 46.1% to 66.2 and 75.9% when BLM 2.5 mg/kg/day × 8 was combined with 3AB 385.4 mg/kg/day × 8, while the decrease of body weight could not be enhanced. BLM at a dose of 5 mg/kg/day × 8 gave INR's of 64.8 and 75%, similar to the combined group but decreased the body weight more significantly. However, the addition of 3AB 385.4 mg/kg/day to BLM did not increase the acute toxicity of BLM alone. There was no significant difference of change of the body weight and subacute toxicity between the BLM and BLM + 3AB group. There was no difference of peripheral blood white cell count and the pathomorphological and ultrastructural change, wet weight and hydroxyproline content (to reflect the collagen content) of the lung of the mice between BLM alone and BLM + 3AB group. Therefore, the study provided experimental evidences for the reasonable use of nontoxic ADPRT inhibitors in adjunct to the chemotherapy of BLM in cancers.

The nuclear enzyme adenosine diphosphate-ribosyl transferase (ADPRT) catalyses the formation of polymer $(ADP\text{-}ribose)_n$ from nicotinamide adenine dinucleotide (NAD). $(ADPR)_n$ is related to several biological functions of cells, such as DNA repair, differentiation and others. It has been demonstrated that inhibitors of ADPRT can potentiate the antitumor activity of cytotoxic drugs.[1~3] Our previous experiments demonstrated that inhibitors of ADPRT, *e.g.* 3-aminobenzamide (3AB), 3-methoxybenzamide (3MB) and nicotinamide (NA) can enhance the antitumor activity of bleomycin (BLM) A_5 and peplomycin (PEP) against S-180 and Ehrlich ascites carcinoma (EAC) of mice *in vivo* and *in vitro*.[4~7] We have observed further the effects of 3AB on the toxicity and antitumor activity of BLM in order to provide a reasonable basis for high effective and low toxic combinations of clinical chemotherapy of cancers.

Materials and Methods

Drugs

BLM was a gift of Nippon Kayaku Co., Ltd., Tokyo, Japan.
3AB was the product of Chemi-industry Co., Tokyo, Japan.

Animals

Non-inbred mice weighing 18~22 g were supplied by the animal center of Sun Yat-sen University of Medical Sciences.

Antitumor Experiments

Mice were inoculated with S-180 homogenate under armpit. Each group consisted of 9~10 mice. Drugs were administered intraperitoneally (ip) daily for 8 days, beginning 24 hours after tumor

transplantation. 0.9% NaCl solution was administered to control. All animals were killed by cervical dislocation 9 days after transplantation. Body weight was determined, tumors were carefully isolated and weighed. Antitumor activity was assessed by inhibition rate (INR):

$$INR = (1 - T/C)\%$$

T: Average tumor weight in test group

C: Average tumor weight in control group

Toxicity Experiments

Acute Toxicity Experiment: 100 mice were divided into 10 dose groups. 5 dose groups were administered BLM alone. The highest dose of BLM was 400 mg/kg, the lowest dose was 35 mg/kg. The ratio of the two consecutive doses was 0.55. The other 5 groups of mice were administered by BLM + 3AB 385.4 mg/kg. The doses of BLM were equal to those for BLM given alone. Mice were observed for 1 week, the death of mice was recorded daily. LD_{50} was calculated by simplified probit method.

Subacute Toxicity Experiment: Each group consisted of 18~20 mice. The dose schedules were BLM 15 mg/kg and BLM 15 mg/kg + 3AB 385.4 mg/kg ip twice weekly for up to 4 weeks. Control mice were received 0.9% NaCl solution. 3 mice of each group were sacrified at 3 weeks after initiation of treatment. Left lung was isolated and fixed by polyformalin-glutaraldehyde, postfixated with 1% osmic acid and embeded in PDAD, ultrathin sections were cut and stained with uranyl acetate and lead citrate. It was suitable for the studies of electronmicroscopy. Other mice were sacrificed at 6 weeks after initiation of treatment. Peripheral blood white cells were counted. All the right lungs were washed with 0.9% NaCl solution, dried with filter paper and then weighed. The content of hydroxyproline in the right lung, which is almost exclusively derived from collagen, was estimated according to the method of WOESSNER.[8] The left lung was fixed by formalin, embeded in paraffin, sectioned (5 μm), stained with hematoxilin-eosin and VAN GIESON's (V.G.) for the studies under light microscopy. Histopathological studies of other organs were carried out also.

Results

Antitumor Experiments *In Vivo*

The results were shown in Table 1. Two experiments showed that INR's were −6.5 and 0% in 3AB 385.4 mg/kg/day alone group with no significant difference between 3AB group and control. The INR's were enhanced from 46.1 and 42.5% to 75.9 and 66.2%, when BLM 2.5 mg/kg/day was combined with 3AB 385.4 mg (2.5 mmol)/kg/day. There were significant differences between these two groups ($P<0.01$, $P<0.05$). The INR's of BLM 5 mg/kg/day group were 75 and 64.8%. They were significantly different from those of BLM 2.5 mg/kg/day group ($P<0.05$), while there was no significant difference between the INR in BLM 5 mg/kg/day group and BLM 2.5 mg/kg/day + 3AB group ($P>0.05$). These indicated that 3AB at the dose with no antitumor activity by itself could increase the antitumor activity of BLM 2.5 mg/kg to the extent of BLM 5 mg/kg. At the same time, 3AB did not enhance the influence of BLM on the body weight. The percentage of increased body weight in two experiments were 3.5 and −1.8 in BLM 2.5 mg/kg group; 1.2 and −2.9 in BLM 2.5 mg/kg + 3AB group; −11.1 and −8.9 in BLM 5 mg/kg group. There was no difference between BLM 2.5 mg/kg group and BLM 2.5 mg/kg + 3AB group ($P>0.05$), while the body weight in BLM 5 mg/kg group decreased more significant than that of BLM 2.5 mg/kg + 3AB group. Additionally, the INR was enhanced from 64.8 to 72.6% when BLM 5 mg/kg was combined with 3AB 385.4 mg/kg, however, the difference between these two groups was not significant statistically.

Tests for Toxicity

Acute Toxicity Test

The ip LD_{50} of BLM was 106 mg/kg, its 95% confidence limit was 72~155 mg/kg. LD_{50} of BLM

Table 1. Effect of BLM and 3AB on the growth of S-180 in mice.

Groups	Dose (mg/kg) (ip, qd × 8)	*n*	% of increased body weight	*P*	Tumor weight (g) (X±SD)	INR (%)	*P*
1. NS	0.4 ml	10	+11.8		2.14±0.83		
2. 3AB	385.4	10	+12.3	>0.05*	2.28±0.72	−6.5	>0.05*
3. BLM	2.5	10	+3.5	<0.05*	1.23±0.27	+46.1	<0.01*
4. BLM±	2.5	10	+1.2	<0.05*	0.55±0.13	+75.9	<0.01*
3AB	385.4			>0.05**			<0.01**
5. BLM	5	10	−11.1	<0.01*	0.57±0.13	+75.0	<0.01*
				<0.01**			<0.05**
				<0.01***			>0.05***
1. NS	0.4 ml	9	+1.6		1.42±0.38		
2. 3AB	385.4	9	+4.4	>0.05*	1.42±0.46	0	>0.05*
3. BLM	2.5	9	−1.82	>0.05*	0.82±0.24	+42.5	<0.05*
3. BLM+	2.5	9	−2.9	>0.05*	0.48±0.21	+66.2	<0.05*
3AB	385.4			>0.05**			<0.05*
5. BLM	5	9	−8.9	<0.01*	0.50±0.22	+64.8	<0.01*
				>0.05**			<0.05**
				>0.05***			>0.05***
6. BLM+	5	9	−8.7	<0.01*	0.45±0.13	+72.6	<0.01*
3AB	385.4			>0.05****			<0.05**
7. BLM	7.5	9	−21.8	<0.01*	0.15±0.25	+89.4	<0.01*
				<0.05**			<0.05**
				<0.05**			<0.05***
				<0.05****			<0.05****

* Compared with 1. ** Compared with 3. *** Compared with 4. **** Compared with 5.
n: No. of mice.

with 3AB (385.4 mg/kg) was 107 mg/kg with a 95% confidence limit of 76～165 mg/kg.

Subacute Toxicity Test

(1) Change of Body Weight: In three experiments the body weights of mice in control groups increased from 21.6±1.4, 20.9±1.2 and 23±1.4 to 33.8±3.7, 30.5±2.9 and 28.2±3.5, average increased rate of body weight were 56, 46 and 23% at 6 weeks, respectively. The increases of body weight in treated groups were slower than those of the control. The body weight in BLM alone groups changed from 21.7±1.2, 20.7±1.3 and 22.4±1.6 to 23.3±3.1, 22.0±3.8 and 21.4±1.5, average increase rates of body weight were only 7, 6 and −4%. The body weight in BLM+3AB groups changed from 21.7±1.5, 20.9±1.4 and 22.5±1.4 to 23.0+3.4, 21.7±3.6 and 20.6±3.5 g, average increase rates of body weight were 6, 4 and −8%. There was no significant difference between BLM alone and BLM+3AB group, while there was significant difference between treated and control group. Data of the experiment were shown in Table 2 and Fig. 1.

Fig. 1. Body weight of mice treated with BLM (15 mg/kg) and BLM (15 mg/kg)+3AB (385.4 mg/kg), ip twice weekly.
* Significantly different from control ($P<0.01$).
n: 17 (NS, ○), 16 (BLM+3AB, ▲), 17 (BLM, ●).

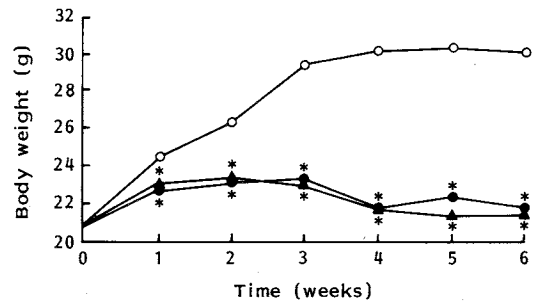

Table 2. The change of body weight (g).

Groups	Dose (mg/kg) (ip, q3d × 8)	Body weight (X±SD)/*n*		
		Begining	1 week	2 weeks
NS	0.4 ml	20.9±1.2/20	24.7±2.2/20	26.6±2.8/20
BLM	15	20.7±1.3/20	22.7±2.1/20*	23.3±3.1/20*
BLM+ 3AB	15 385.4	20.9±1.4/20	23.2±2.2/20*	23.5±3.1/20*
NS	0.4 ml	21.6±1.4/20	25.6±2.4/20	28.5±3.3/20
BLM	15	21.7±1.2/20	23.2±1.6/20*	24.7±2.4/20*
BLM+ 3AB	15 385.4	21.7±1.5/20	23.1±2.3/20*	24.6±2.8/20*
NS	0.4 ml	23.0±1.4/18	23.8±2.4/18	27.1±3.1/18
BLM	15	22.4±1.6/18	21.7±1.7/18*	21.2±1.7/18*
BLM+ 3AB	15 385.4	22.5±1.4/18	21.8±1.7/18*	20.3±2.4/18*

Groups	Body weight (X±SD)/*n*			
	3 weeks	4 weeks	5 weeks	6 weeks
NS	29.8±3.1/17	30.6±1.9/17	30.7±2.9/17	30.5±2.9/17
BLM	23.5±3.7/17*	21.9±3.3/17*	22.6±4.5/17*	22.0±3.8/17*
BLM+ 3AB	23.2±3.2/16*	21.9±3.0/16*	21.7±4.0/16*	21.7±3.6/16*
NS	29.6±4.1/20	30.9±2.1/12	30.4±2.7/12	33.8±3.7/12
BLM	24.4+2.5/20*	24.5±3.0/13*	24.3±2.9/13*	23.3±3.1/13*
BLM+ 3AB	24.6±2.7/20*	23.5±4.0/16*	23.7±3.6/13*	23.0±3.4/11*
NS	26.7±2.9/17	28.2±3.4/17*	28.1±2.3/16	28.2±3.5/16
BLM	21.1±1.4/18*	21.8±1.6/18*	20.7±1.9/17*	21.4±1.5/17*
BLM+ 3AB	20.1±2.6/18*	20.7±3.1/17*	20.4±3.1/16*	20.6±3.5/16*

* Significantly different from control ($P<0.01$).
No significant difference between BLM and BLM+3AB ($P>0.05$).
n: No. of mice.

Table 3. Number of white blood cells/mm^3 ($\bar{x}$±SD) at week-6.

Treatment (ip, q3d × 8)	Dose (mg/kg)	Expt 1	*n*	Expt 2	*n*	Expt 3	*n*
NS	0.4 ml	14,316±3,496	12	14,032±3,950	17	7,365±3,257	16
BLM	15	14,969±3,314	13	14,064±4,194	17	9,476±2,188	17
BLM+ 3AB	15 385.4	16,554±2,599	11	15,168±5,197	16	8,240±2,790	16

No significant difference between every two groups ($P>0.05$).
n: No. of mice.

(2) Counts of White Blood Cells: Three experiments showed that the numbers of peripheral white blood cells were 14,969±3,314, 14,064±4,194 and 9,476±2,188/mm^3 in BLM alone group, 16,554±2,599, 15,168±5,197 and 8,240±2,790 in BLM+3AB group, 14,316±3,496, 14,032±3,950 and 7,365±

Fig. 2. BLM group.

Bleb of capillary increased, some blebs were confluent to form large cavity (a closed arrow), elastic fibrils increased (an open arrow).

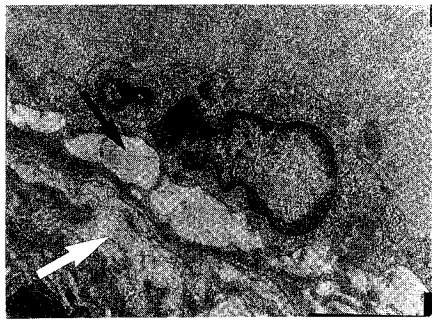

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Fig. 3. BLM+3AB group.

Elastic fibrils increased (an open arrow).

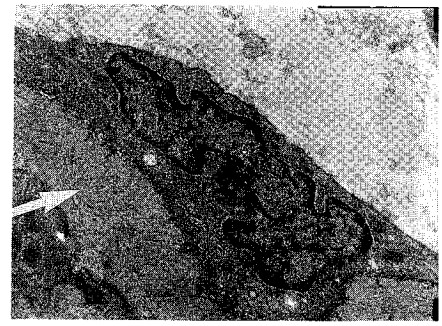

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Fig. 4. BLM+3AB group.

Bleb of capillary increased (a closed arrow), collagen increased (an open arrow).

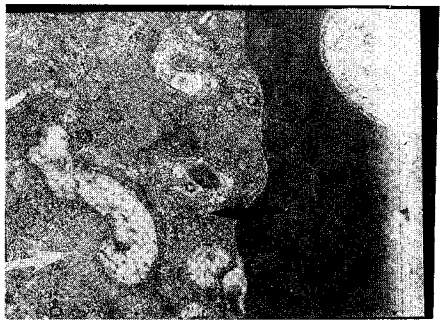

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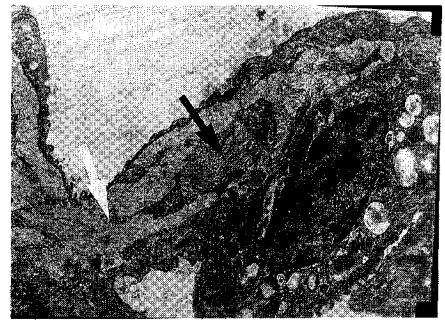
Fig. 5. BLM group.

Collagen deposition (a closed arrow), elastic fibrils increased (an open arrow).



1 μm

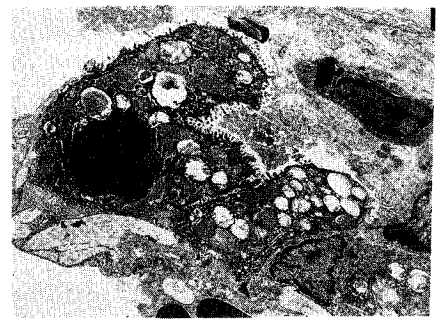
Fig. 6. BLM+3AB.

Collagen deposition (a closed arrow), elastic fibrils increased (an open arrow).

500 nm

Fig. 7. BLM group.

Type II cell of lung proliferated.



1 μm

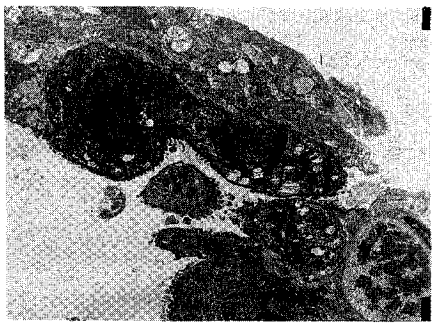
3,257/mm^3 in control group. There was no significant difference between the BLM and BLM+3AB group ($P>0.05$). It indicated that 3AB did not increase the myelosuppression of BLM.

(3) Pulmonary Toxicity Test: The dose schedule used was accorded to the dose and duration of BLM resulting in pulmonary toxicity reported in the literatures.[9]

Histopathological Change of Lung under Electronmicroscopy

The early damage in lung can be observed with electronmicroscopy. As compared with control, two treated groups (BLM alone and BLM+3AB) showed increased blebs of capillary, some blebs were confluent to form large cavities, type II cell of lung proliferated, collagen and elastic fibrils in the interstitium of lung increased (Figs. 2~8). The histopathological changes in BLM alone and

Fig. 8. BLM+3AB group.
Type II cell of lung proliferated.



2 μm

Fig. 9. BLM group.
Change of focal interstitial pneumonitis.

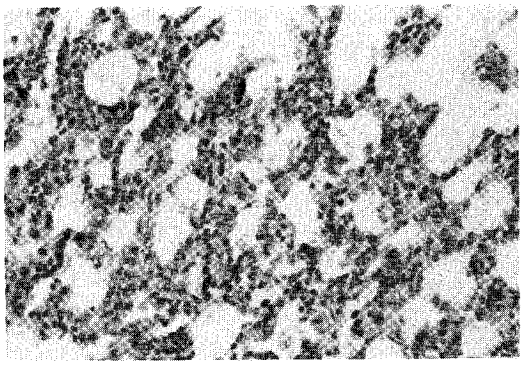


Fig. 10. BLM+3AB group.
Change of focal interstitial pneumonitis.

Fig. 11. BLM group.
Red collagen deposition could be observed with V. G. stain.

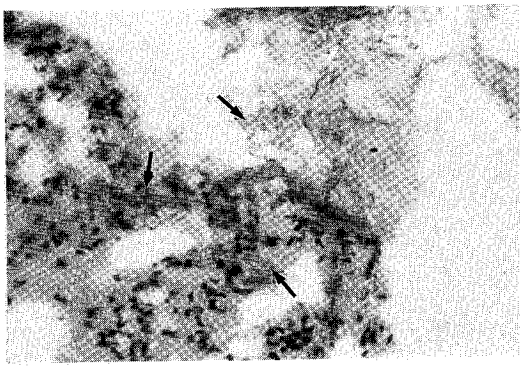
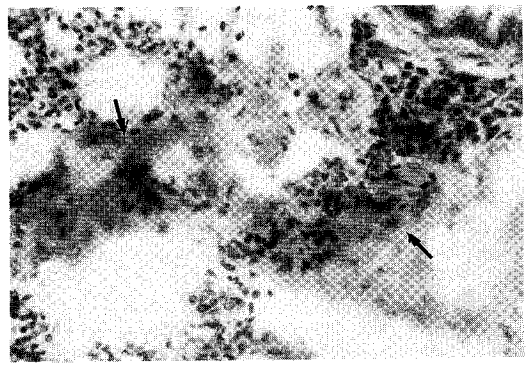


Fig. 12. BLM+3AB group.
Red collagen deposition could be observed with V. G. stain.



BLM+3AB group were similar.

Histpathological Change of Lung under Light Microscopy

As compared with control, the pathological change of lung of mice in BLM alone and BLM+3AB group occurred similarly at 6 weeks after the initiation of treatment.

a) Changes of Focal Interstitial Pneumonitis: Edema, infiltration of lymphocytes and macrophages in interstitium were found in both BLM alone and BLM+3AB group (Figs. 9 and 10). Collagen deposition in interstitium could be observed with V.G. stain (Figs. 11 and 12).

b) Number of Pulmonary Cells under Light Microscopy: Total number of pulmonary cells in three visual fields on each slide of lung was estimated. The number of cells were increased markedly in BLM and BLM+3AB group as compared to control, while there was no significant difference between BLM alone and BLM+3AB group (Table 4). These indicated that 3AB did not potentiate the infiltration of cells induced by BLM.

c) Wet Weight of Right Lung and Relative Wet Weight: In three experiments, the wet weight of right lung which reflected the severity of edema, infiltration of cell and fibrosis in lung were increased in BLM alone and BLM+3AB group. The relative wet weight, which means the ratio of the wet weight of right lung (mg) to body weight (g), were increased markedly in BLM alone and BLM+3AB group. There

Table 4. Number of pulmonary cells/3 visual fields.

Groups	Dose (mg/kg) (ip, q3d×8)	No. of cells (X±SD)					
		Expt 1	*n*	Expt 2	*n*	Expt 3	*n*
NS	0.4 ml	305±65	12	269±50	17	282±55	16
BLM	15	549±86**	13	556±78**	17	439±78**	17
BLM+3AB	15+385.4	512±62**	11	533±64**	16	444±92**	16

** Significantly different from control ($P<0.01$).
No significant difference between BLM and BLM+3AB ($P>0.05$).
n: No. of mice.

Table 5. Effect of BLM and BLM+3AB on body weight and right lung wet weight of mice.

Expt	Groups	Dose (mg) (ip, q3d×8)	*n*	Body weight (X±SD) (g)	Right lung wet weight (X±SD) (mg)	Relative lung weight (X±SD)
1	NS	0.4 ml	12	30.90±2.12	101.82±10.10	3.00±0.16
	BLM	15	13	24.50±3.00**	132.07±27.54**	5.72±1.22**
	BLM+3AB	15+385.4	11	23.54±3.87**	125.96±16.24**	5.56±0.09**
2	NS	0.4 ml	17	30.52±2.90	93.72±9.71	3.06±0.20
	BLM	15	17	22.01±3.79**	104.94±10.72**	4.54±1.89**
	BLM+3AB	15+385.4	16	21.71±3.57**	106.46±9.76**	4.97±0.60**
3	NS	0.4 ml	16	28.58±3.09	86.67±12.75	3.03±0.30
	BLM	15	17	21.59±2.17**	108.96±24.71*	5.06±1.17**
	BLM+3AB	15+385.4	16	20.09±3.20**	101.04±17.26*	5.03±0.28**

* Significantly different from control ($P<0.05$).
** Significantly different from control ($P<0.01$).
No significant difference between BLM and BLM+3AB ($P>0.05$).
n: No. of mice.

Table 6. Hydroxyproline content of whole right lung.

Groups	Dose (mg/kg) (ip, q3d×8)	Hydroxyproline (μg) (X±SD)			
		Expt 1	*n*	Expt 2	*n*
NS	0.4 ml	232±25	12	240±37	16
BLM	15	301±71**	13	310±41**	17
BLM+ 3AB	15 385.4	285±41**	11	317±39**	16

** Significantly different from control ($P<0.01$).
No significant difference between BLM and BLM+3AB ($P>0.05$).
n: No. of mice.

was significant difference between treatment and control, no significant difference between BLM alone and BLM+3AB group (Table 5).

d) Content of Hydroxyproline: In two experiments the content of hydroxyproline in whole right lung, which reflected the severity of increase of collagen fibrils in lung, were higher in two treatment groups than in control group. There was no significant difference between BLM alone and BLM+3AB group. These indicated that pulmonary fibrosis induced by BLM did not be potentiated by the addition of 3AB. However, the contents of hydroxyproline per g of lung were 2.29±0.26 and 2.28±0.75 in control, 2.34±0.59 and 2.95±0.59 in BLM alone, 2.08±0.72 and 3.21±0.56 in BLM+3AB group. There was no significant difference between all these three groups, which suggested that the increase of hydroxyproline balance the increase of wet weight of right lung (Table 6).

e) No significant pathological changes of other organs including heart, liver, kidney were found in mice of all three groups.

Discussion

Our experiments have demonstrated that 3AB under subtoxic dose with no antitumor activity by itself could enhance the antitumor activity of BLM to the extent that obtained by double the dose of BLM alone. At the same time, 3AB did not potentiate the toxicity of BLM on the change of body weight. Our previous studies have demonstrated that 3AB, NA, 3MB enhanced the effect of BLM A_5 and PEP against S-180, hepatoma and EAC, 3AB enhanced the tumor inhibition rate of PEP 1.5 mg/kg, the INR equalled to that induced by PEP 3 mg/kg alone.[4~7]

KAWAMITSU demonstrated that 3AB 6.5 mmol/kg enhanced the effect of BLM against EAC.[1] KATO demonstrated that 3AB enhanced the cytotoxicity of BLM on L1210 cells *in vitro* and the percent increase of lifespan on mice inoculated with EAC.[10] Our results were similar to these reports. It has been demonstrated that inhibitors of ADPRT may inhibit the ligation of DNA fragments in the process of damaged DNA repair.[11~13] Our previous works[14] showed that 3AB potentiated the inhibition of BLM on the replication. 3AB stimulated the incorporation of [^{3}H]TdR in DNA repair process of S-180 cells treated by BLM A_5, which may be the consequence after inhibition of DNA ligation. The mechanism of action may be relative to nick translation, thus 3AB blocks the repair ligation of damaged DNA by inhibiting the formation of $(ADP\text{-}R)_n$, this induced the increase of nicks of DNA, which stimulated the synthesis of new fragments, so the incorporation of [^{3}H]TdR increased, but the repairment of damaged DNA was not succeeded. It was well known that BLM mainly makes DNA strand breakage. Thus, it is very possible that 3AB enhance the antitumor activity of BLM by inhibiting the ligation of damaged DNA induced by BLM. Our experiments showed that 3AB in the dose capable to enhance the antitumor activity of BLM did not potentiate the acute toxicity of BLM. The LD_{50} was not changed with the addition of 3AB. SCHEIN demonstrated that 500 mg/kg NA protected the lethality of mice caused by streptozotocin.[15] Our previous works showed also that 3AB 1 mmol/kg did not potentiate the lethality of BLM.[6] These

results indicated that the inhibitors of ADPRT did not potentiate the acute toxicity of some cytotoxic drugs.

We observed especially the effect of 3AB on the subacute and pulmonary toxicity of BLM in our experiments. 3AB did not potentiate the influence of BLM on the body weight and white blood cell count. The histopathological changes and various biological changes of lung, which was induced by a pulmonary toxic dose of BLM, were not enhanced by the addition of 3AB. As we know, the pulmonary toxicity is the main lethal cause of BLM. KATO *et al.*[10] wrote in his paper that "Since bleomycin has serious lung toxicity, the question of whether 3AB increases this lung toxicity should be investigated." Our experiments have answered this question and showed that 3AB did not potentiate the pulmonary toxicity of BLM. Since the toxicity of BLM is dose-dependent, it can be decreased by dose lowering, however, the same extent of antitumor effect of BLM can be maintained when it was combined with ADPRT inhibitors. Therefore, this study provided experimental evidence for the highly effective and low toxic combination of chemotherapy, when BLM was combined with some of the inhibitors of ADPRT.

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