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The Growth Inhibitiory Effect of New Pyrrolo[1,2-α]benzimidazole Derivatives on Human Gastric Cancer Cells

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In the course of screening synthetic compounds to inhibit tumor cell growth, pyrrolo[1,2-α] benzimidazole (PBI), an intermediate of azamitosene, was found to inhibit a proliferation of gastric cancer cell lines. Despite a potential cytotoxic activity against solid tumor cells as opposed to that against rapidly-doubled leukemic cells, there has been no report on the inhibition of gastric cancer cell line by PBI and its' derivatives. The present experiment was designed to determine if PBI derivatives can effectively inhibit the cellular proliferation of gastric cancer cells by using *in vitro* as well as *in vivo* chemosensitivity system (MTT assay, clonogenic assay and human tumor xenografted assay). Of the tested PBI derivatives, PBI (**18**) and PBI (**20**), displayed the effective growth inhibition of cultured gastric cancer cells or even in the xenografted nude mouse model.

Key words : Pyrrolo[1,2-]benzimidazole (PBI), Gastric cancer cell line, Nude mouse

INTRODUCTION

Gastric cancer is the most common type of gastrointestinal tract cancer among men in Korea (Ministry of Health and Social Affairs, 1989). High prevalence and mortality rate from stomach cancer in the Korea have urged to develop newer therapeutic intervention. Specifically, a cytoreductive surgery and chemotherapy have been the mainstay of its treatment (Carrato *et al.*, 1995). Nevertheless, these therapeutic strategies failed to extend significantly survival time in cancer patient. Currently, for the treatment of stage III or higher, chemotherapy was documented to be the most effective (Rougier *et al.*, 1994). In clinical field, some alkylating agents such as mitomycin C, cyclophosphamide have been yet preferred for use to treat various stages of gastric cancer patients in a combined regimen. However, the combined chemotherapy using alkylating agents has been limited in their usefulness owing to their high toxicity (Kelson, 1996). Therefore, the development of safe and effective chemotherapeutic for the treatment of gastric cancer will be warranted. To reduce toxicity of reductive alkylating quinones and optimize their activity, azamitosene was proposed as one of promising candidates (Iyengar *et al.*, 1983; Sami *et al.*, 1984). Thus, alkylating quinone methides, which are formed upon reduction of the quinones, will be highly reactive with DNA (nucleophile) of tumor cells (Kennedy *et al.*, 1980; Keyes *et al.*, 1985; Moore and Czerniak, 1981). A low reduction potential environment within tumor cells would make reductive alkylating quinones, such as azamitosenes to selectively target hypoxic tumor cells. Recently, some preclinical studies using PBI-based antitumor agents (azamitosenes), designed as new DNA cross-linkers, mimicking mitomycin C were reported to show potent cytostatic activity (Ahn and Kim, 1996; Islam and Skibo, 1990; Islam and Skibo, 1991; Skibo and Schulz, 1993). In the course of screening synthetic compounds to inhibit tumor cell growth, some PBI derivatives, as intermediates of azamitosene, were found to inhibit a proliferation of gastric cancer cell lines. Though PBI derivatives may had a cytotoxicity on leukemia, there is no report on the inhibition of gastric cancer cell line by PBI derivatives. Thus, the present study was focused to evaluate if PBI derivatives effectively inhibit the growth of human gastric cancer cells *in vitro* and *in vivo*.

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MATERIALS AND METHODS

Drugs

PBI derivatives were synthesized in our Insititute.

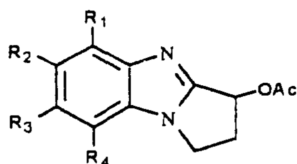

Fig. 1. Structures of PBI derivatives

Compounds	R_1	R_2	R_3	R_4
7	H	CF_3	H	NO_2
9	H	NO_2	H	H
12	H	CH_3	Br	H
13	H	OCH_3	NO_2	H
14	Br	OCH_3	H	H
17	H	CF_3	H	NH_2
18	H	OCH_3	NHOH	H
19	H	OCH_3	NH_2	H
20	H	CF_3	H	

Their structures were depicted as follows (Fig. 1). Their purity was over 99 percent and checked before every experiment. Basic reagents for synthesis of PBI derivatives and other cytotoxic agents for comparative cytotoxicity were obtained from Sigma Co., Ltd (St. Louis, MO, USA) and so on. For the use of *in vitro* and *in vivo* experiment, PBI derivatives were used with dissolution in DMSO and vehicle were made of 0.1% DMSO in saline.

Cancer cell lines

The cancer cell lines for cytotoxicity test were as follows: SNU-16 (gastric cancer, human), KHH-1 (gastric cancer, human), MKN-45 (gastric cancer, human). Each cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and incubated in a humidified 5% CO_2 chamber at 37°C.

Measurement of cytotoxicity

To evaluate cytotoxicity, modified MTT method was performed essentially as described previously (Ahn and Kim, 1996; Carmichael *et al.*, 1987). Briefly, monocellular suspension was seeded at 10^4 cells per well in 96 well plates with 100 μl of medium per well. To compare cytotoxicity between PBI derivatives and cytotoxic agents (mitomycin C) were added at varying concentrations and cultures were incubated for 72 hours in an incubator maintaining a highly humidified atmosphere, 5% CO_2 and 95% air. Fifty μl of the medium containing MTT (5 mg/ml) were added to each well. After 4 hours of exposure, the medium was partly decanted and the wells were washed with PBS, and then 150 μl of DMSO was added to each well to solubilize the precipitates. The plates were transferred to an ELISA reader to measure the absorbance at 570 nm with a reference wave length, 630 nm. IC_{50} value, 50% inhibition of cell growth, was calculated by regression analysis (plotting the viability versus the concentration of the test compound) using Graphpad Prism 2.0 (GraphPad Software, Inc, San Diego, CA, USA). All experiments were done at least 3 times, with 3 wells for each concentrations of test agents.

Measurement of tumor colony formation

Twenty-four well-clonogenic assay was done by modifying the 96 well based clonogenic assay (Kim *et al.*, 1996; Salmon *et al.*, 1978; Shoemaker *et al.*, 1981). Layers of 0.5 ml, 0.5% noble agar in supplemented RPMI 1640 medium were prepared in a 24 well culture plate. KHH-1 cells to be tested were overlayered on basal agar in 0.5 ml of 0.3% agar containing 20% FCS, double strength-RPMI 1640 medium and various concentrations of the drugs. The final concentration of the cells in each culture was 5×10^3 per well. All experiments were done at least 3 times, with 4 wells for each concentrations of test agents. Colony formation was calculated as follows. C.F.E (colony forming efficiency)=[formed colony number/seeded colony number]×100%.

In vivo evaluation in human tumor xenograft models

Using established KHH-1 gastric cancer model, female nude mice (18~24 g) were obtained from Harlan Sprague Dawley and implanted s.c by 26 gauge with monocellular suspension harvested from *in vitro* growing tumor in culture dishes. When tumors were approximately 5×5 mm (usually about 12 days after implantation), the animals were pair-matched into treatment and control groups (day 1). Each group contained 4 to 5 tumor-bearing mice that were ear-tagged and followed individually throughout the experiment. Treatment of drugs or vehicle was initiated when the primary tumor reached a size of approximately 5×5 mm (day 0). PBI (**18**) and PBI (**20**) were administered by only one i.p. injection on the 2nd and 3rd week of treatment and given i.p with three (2 days interval) injection on the 1st week of treatment. Following tumor implantation, mice were weighed twice weekly and tumor measurements were made using calipers twice weekly beginning on day 1. Tumor measurements were converted to tumor weight(mg) using an established formula:

Weight (mg)=[Width (mm^2)×Length (mm)]/2 and calculated as the mean±S.E. Experiments were terminated when tumors in control animals reached the size of 1~2 g. At termination, all mice were weighed, sacrificed, and their tumors were excised.

Table I. 50% Inhibitory concentrations of PBI derivatives on gastric cancer cell lines measured by MTT assay

Compound	IC_{50} (μg/ml)[a] ± S.D[b]		
	SNU-16	KHH-1	MKN-45
7	24.4±2.3	15.63±4.2	>50.0
9	33.5±5.6	>50.0	11.9±1.8
12	24.9±5.1	13.8±6.2	47.5±2.9
13	20.2±3.3	27.3±0.9	27.48±1.4
14	>50.0	13.9±2.7	>50.0
17	>50.0	>50.0	>50.0
18	0.18±0.03	2.1±1.3	19.3±3.5
19	>50.0	>50.0	>50.0
20	1.14±1.01	10.4±2.8	6.4±1.6
MMC[b]	0.034±0.02	3.51±2.4	0.104±0.08

[a]: IC_{50} value which is defined as the concentration that caused 50% inhibition of cell growth
[b]: Standard deviation
[b]: mitomycin C

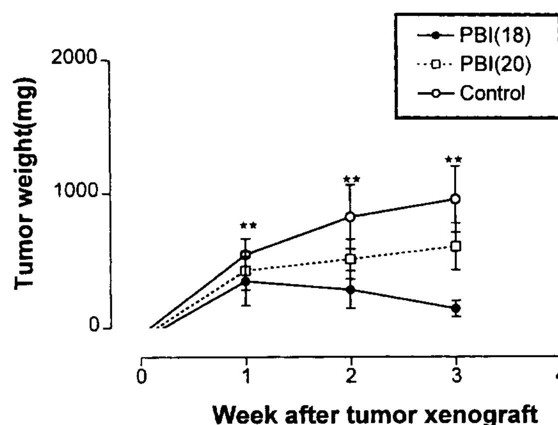

Fig. 2. Growth curve of xenografted KHH-1 tumor treated with PBI (18) ro PBI (20). **P<0.01 compared to corresponding vehicle control group.

RESULTS AND DISCUSSION

During the search for synthetic inhibitor of gastric cancer cell growth, a part of pyrrolobenzimidazole (PBI) derivatives we had synthesized, showed a growth inhibition in SNU-1 gastric cancer cell line. Therefore, we wondered if any type of PBI derivatives could effectively abrogate gastric cancer cell growth. On the result of MTT test, IC_{50} of PBI derivatives except (**18, 20**) is over 20 μg/ml on SNU-16 cell line. Among PBI compounds, PBI (**18,20**) displayed excellent cytotoxicities on KHH-1 and MKN-45 cell lines (Table I). In particular, PBI (**18, 20**) were considered to have such a comparable cytotoxicity as tested mitomycin C against KHH-1 cell line. All PBI derivatives revealed different cytotoxicity profiles depending on tested cell line. These concentration-effect curve patterns by PBI derivatives on tested cell line were similar. In colony formation assay using KHH-1 cells, 50% colony forming abilities of PBI (**18,20**) were less than 12.5 μg/ml and at least higher than that of mitomycin C (Table II). Among PBI derivatives, PBI (**18**) compound gave the highest inhibition on colony formation of KHH-1 cells. PBI (**20**) compound also strongly reduced the numbers in KHH-1 colonies (Table II). These data suggest that PBI (**18,20**) may be active *in vivo*. Prior to nude mouse xenograft assay, we had already observed a significant prolongation of life span in PBI (**18,20**)-treated mice than that of saline-treated mice, adopting *in vivo* P388 syngeneic tumor model (unpublished data). To verify an inhibition of human gastric cancer cell growth *in vivo* by PBI (**18,20**), the KHH-1 xenograft model we recently had established was chosen. This model is anticipated to effectively evaluate an efficacy of newer chemotherapeutics against domestic type of gastric cancer because KHH-1 cell line was established from Korean gastric cancer patient. PBI (**18**) or PBI (**20**) administered at 2 mg/kg on a weekly one or two schedule produced the suppression in a tumor weight gain compared to untreated mice group (Fig. 2). In particular, PBI (**18**) decreased profoundly the xenografted-tumor weight (146±32.8 mg) 2nd week post injection five times as high as that (824±237.7 mg) of control mice group. PBI (**20**) also revealed a weaker inhibition on xenografted-tumor growth (477±186.5 mg) on 2nd week's evaluation. However, 4 mg/kg or higher of PBI (**18,20**) was thought to be somewhat toxic on treated mice because from 3rd week post injection, the mice grew cachetic. Because PBI (**18,20**) were obtained in small amount, *in vivo* efficacy test using different doses was partly performed. Therefore, to modulate the dose and schedule of PBI (**18,20**) treatment will remain to be further tested. Despite pre-

Table II. Percent colony formation of KHH-1 cancer cell lines after treatment with PBI derivatives

Compound	% C.F.E[a] in drug concentration±S.D[b]				
	50	12.5	3.125	0.78	0.19 (μg/ml)
18	0	12±4.1	55.5±12.4	79.1±7.5	97.5±3.7
20	0	17.2±8.6	65.3±6.3	88.1±11.1	96.7±5.3
MMC[c]	0	18.3±6.7	68.8±15.5	87.3±9.2	97.2±8.4

[a]: % of Colony Forming Efficiency compared to that of control culture (not any drug added).
[b]: Standard deviation
[c]: mitomycin C

ferential activity *in vitro* and *in vivo* of PBI (**18,20**), how PBI (**18,20**) could inhibit the cellular growth was unsettled. In the previous report, we demonstrated that azamitosene compound could potently inhibit ovarian cancer cells with a change of their cell cycle (Cha *et al.*, 1997). But, whether PBI (**18,20**) can exert the cytotoxicity through the similar or same mechanism like azamitosenes remains to be persued. Collectively, some PBI derivatives have a growth inhibition on human gastric cancer cells *in vitro* as well as *in vivo.* From these results, it seems likely that some structures such as PP2 (**18,20**) are able to evoke an enhancement in cytotoxicity. But, an elucidation of a structure-activity relationship as well as a cytotoxic mechanism by PBI(**18,20**) will lead to develop new lead compound with the potential anti-gastric cancer activity.

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