VOL. 52, NO. 5, MAY 1999 THE JOURNAL OF ANTIBIOTICS pp. 449 — 454

# High-mannose Type Oligosaccharide-dependent Apoptosis in U937 Cells Induced by Pradimicin, a Mannose-binding Antibiotic

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(Received for publication February 8, 1999)

Cell surface oligosaccharides play a role in a variety of biological events such as cell adhesion and signal transduction. We have shown that BMY-28864, a semi-synthetic analog of pradimicin, induced apoptosis of U937 cells which had been incubated with 1-deoxymannojirimycin, an inhibitor of mannosidase I. BMY-28864 was not cytotoxic to the cells which had been cultivated with other glycosidase inhibitors such as castanospermine and swainsonine. We thus propose that BMY-28864 induces apoptosis by acting on a specific mannose-rich oligosaccharide, presumably $(Man)_9(GlcNAc)_2$[†].

Pradimicin, a potent and highly selective antibiotic against fungi and yeasts, was first isolated from *Actinomadura hibisca* in 1988.[1] It belongs to a family of benzo[*a*]naphthacenequinone antibiotics and a member of congeners and derivatives have been reported.[2] Pradimicin BMY-28864 (PRM) has also been reported as a chemically modified derivative for improved solubility and antifungal activity. YAMAMOTO *et al.* found that PRM inhibited the fusion of human immunodeficiency virus to T cells by binding to high-mannose glycans on gp120.[3] Additionally, MORITA *et al.* have shown the inhibition of osteoclast formation from hematopoietic precursors by the treatment with PRM[4] and ITO *et al.* reported the enhancement of the syncytium formation of human parainfluenza virus-infected HeLa cells by PRM.[5] These events are considered to be caused by the interaction of PRM with mannose-rich glycoproteins. Very recently, the interaction of PRM with mannose derivatives was carefully analyzed spectroscopically and some dissociation constants of the complex were determined by LEE *et al.*[6] In the previous paper, we have shown that PRM induced apoptosis in U937 cells which had been incubated in the presence of 1-deoxymannojirimycin (DMJ).[7] DMJ inhibits an α-mannosidase I and promotes the expression of high-mannose type oligosaccharide at the cell surface. We herein report our study on the involvement of cell surface glycans in PRM-induced apoptosis, and show evidences that the binding of PRM to the high-mannose type oligosaccharide is essential to the apoptosis.

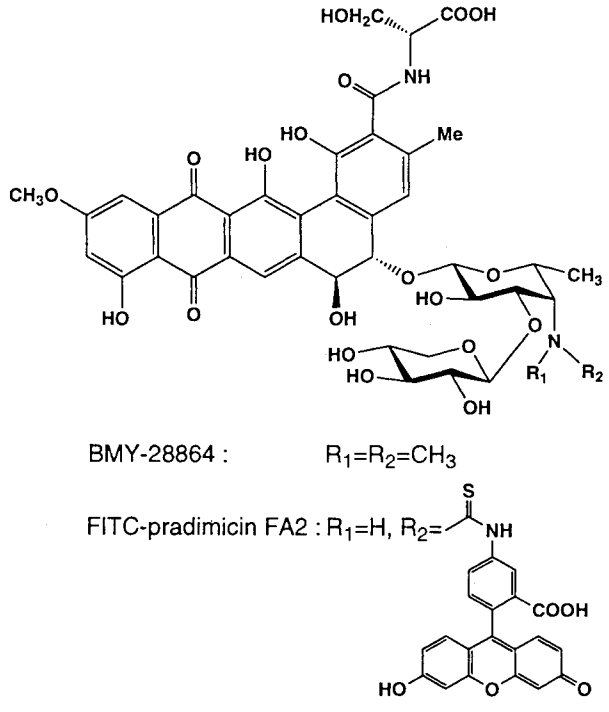
## Materials and Methods

### Materials

1-Deoxymannojirimycin (DMJ), castanospermine (CAS), and swainsonine (SW) were purchased from Sigma Chemical Co. (St.Louis, MO, USA) and diluted with sterilized water to provide 10 mg/ml stock solution. BMY-28864 was prepared from pradimicin FA1 according to the reported procedure[8,9] and diluted with PBS (+) to give 1 mg/ml stock solution. FITC-concanavalin A (FITC-Con A) was from Seikagaku Corp. (Tokyo, Japan) and dissolved in PBS (+) to provide 1 mg/ml stock solution. FITC-pradimicin (FITC-PRM) was prepared by the reaction of pradimicin FA2[8] and FITC isomer-I (Dojindo Lab., Kumamoto, Japan) and dissolved in DMSO to give 10 mg/ml stock

† Abbreviations: Man, mannose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; NeuAc, *N*-acetylneuraminic acid; Gal, galactose.

Fig. 1. Structures of BMY-28864 and FITC-pradimicin FA2.




solution. MTS and PMS were from Promega Corp. (Madison, WI, USA) and diluted with Dulbecco's phosphate buffer (pH 7.35) to provide 2 mg/ml and 0.92 mg/ml stock solutions respectively. All stock solutions were kept at −20°C.

### Cell Culture

U937 human myeloid leukemia cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, JRH Bioscience, Tokyo, Japan) and 100 μg/ml kanamycin sulfate at 37°C in a 5% $CO_2$ incubator.

### Glycosidase Inhibitor Treatment

Cells ($1 \times 10^5$/ml) were cultured with DMJ (50 ~ 1000 μg/ml), CAS (20 ~ 500 μg/ml) or SW (10 ~ 100 μg/ml) under the same conditions described above. After incubation for up to 72 hours, cells were washed with PBS (−) and used for further experiments.

### Pradimicin Treatment

Cells ($1 \times 10^5$/ml) were cultured with various concentrations of BMY-28864 (0 ~ 100 μg/ml) under the same conditions described above. After incubation for up to 72 hours, cells were washed with RPMI 1640 and the cell viability was determined by MTS method.

### Binding of FITC-Con A and FITC-PRM

After the glycosidase inhibitor treatment, U937 cells ($5 \times 10^5$) were incubated with FITC-Con A (50 μg/ml) for 24 hours in PBS (+) (1 ml) or FITC-PRM (50 μg/ml) for a given length of time in RPMI 1640 (2 ml) at 37°C in dark. After washing with PBS (+) three times, the cells were suspended in PBS (+) (3 ml) and the fluorescence emission at 522 nm was measured with an excitation wavelength of 494 nm.

### Cell Viability Assay

Cell viability was determined by MTS method according to the supplier's protocol (Promega Corp.). Cells were plated in a 96-well plate ($2 \times 10^5$ cells/well) and cultured with MTS/PMS (20 : 1, 20 μl) for up to 4 hours. Reaction was stopped by the addition of 10% SDS (25 μl) and the optical density was determined using an ELISA reader at 490 nm. The percent viability was calculated.

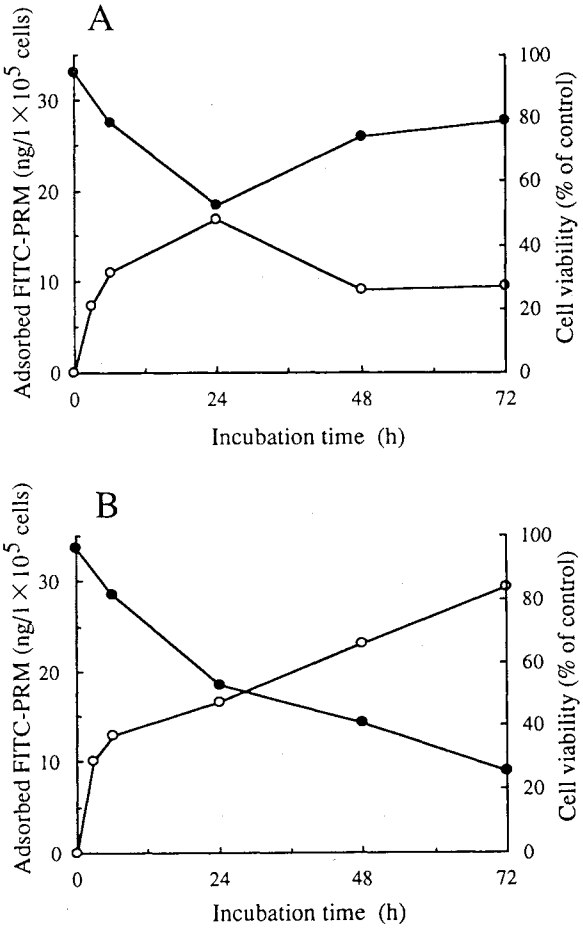
## Results

### Preparation of DMJ-treated U937 Cells

It has been reported that coincubation of mammalian cells with 1-deoxymannojirimycin (DMJ), a specific inhibitor of mannosidase I, induces the accumulation of $(Man)_9(GlcNAc)_2$, resulting in the expression of high-mannose type oligosaccharides at the cell surface.[10,11] The growth of U937 cells was initially examined in the presence of various concentrations of DMJ. Coincubation with 100 ~ 500 μg/ml DMJ did not affect the doubling time (18 hours). At 1000 μg/ml of DMJ the growth was slightly inhibited and the doubling time was prolonged from 18 to 20 hours. Morphological change was not observed even at 1000 μg/ml DMJ. Above results confirmed that U937 cells were not affected by less than 500 μg/ml DMJ regarding to the growth rate and the morphology.

The induction of high-mannose type oligosaccharide was evaluated by measuring the binding of FITC-concanavalin A (FITC-Con A) to the cells.[7] The binding of FITC-Con A increased intensively up to 50 times over control after 3 hours of incubation and slightly after

Fig. 2. Time course of binding of FITC-PRM to DMJ-treated cells (○) and the effect of PRM on apoptosis (●).



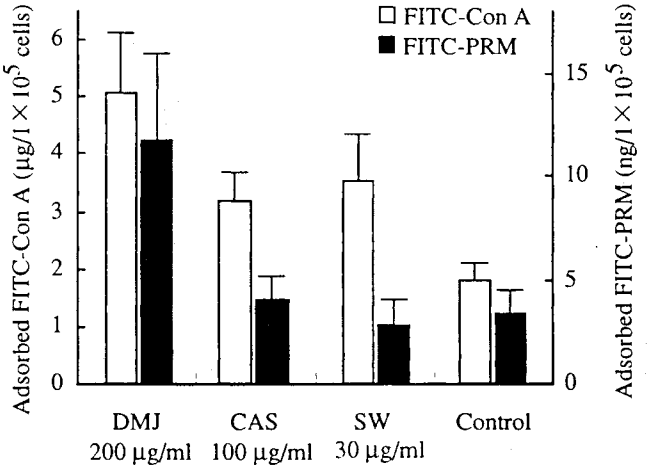

A: in the absence of DMJ
B: in the continuous presence of DMJ

18 hours.

## Effect of PRM on DMJ-treated Cells

PRM-induced cell death was investigated time-dependently in parallel with the measurement of binding of pradimicin. Pradimicin BMY-28864 (PRM) and FITC-labeled pradimicin FA2 (FITC-PRM) were used for the cytotoxic assay and the binding study respectively. After 48 hours of incubation with DMJ, cells were treated with 50 μg/ml PRM in the absence of DMJ (Fig. 2A). The percentage of the living cells decreased by 53% after 24 hours, and increased by 79% after 72 hours. The binding of FITC-PRM peaked around 24 hours after incubation and then decreased. This recovery of the

Fig. 3. Binding of FITC-Con A and FITC-PRM to glycosidase inhibitor-treated cells.

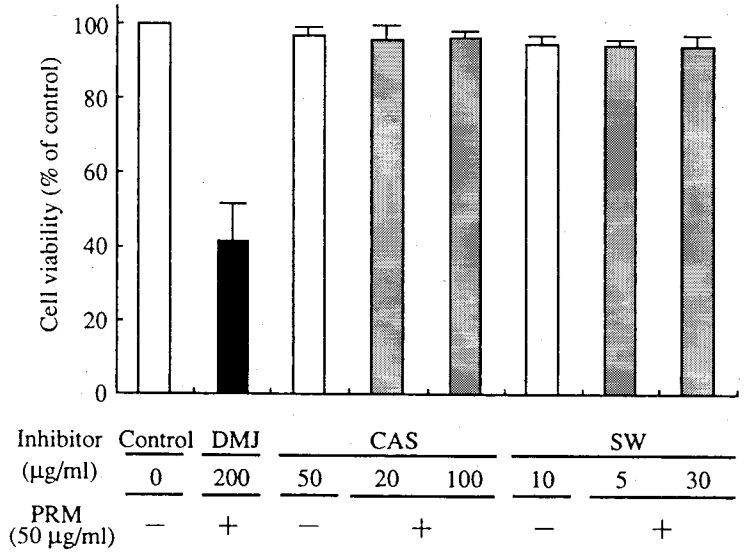



cell growth and the decrease of the binding amount of FITC-PRM could be explained by the regeneration of normal (complex type) oligosaccharides at the cell surface in the absence of DMJ. To prove this hypothesis we incubated the cells with PRM in the continuous presence of DMJ (Fig. 2B). The viability was 26% after 72 hours and the binding took place rapidly until 6 hours and continued slowly up to 72 hours.

## Effect of Glycosidase Inhibitors on PRM-induced Apoptosis

Castanospermine (CAS) and swainsonine (SW) inhibit glycosidases responsible for *N*-linked oligosaccharide processing. We studied the effect of PRM on the cells which had been treated with CAS and SW in addition to DMJ. The alteration in types of the cell surface oligosaccharides in U937 cells were analyzed by comparing the binding of FITC-Con A and FITC-PRM (Fig. 3). The optimal concentration for CAS and SW was determined by measuring the cell growth in the range of 20～500 μg/ml and 10～100 μg/ml respectively with referring to the reported values. FITC-Con A bound to CAS-treated cells 80% over control whereas the binding amount of FITC-PRM was not increased. The binding of FITC-Con A to SW-treated cells, which were considered to possess hybrid type oligosaccharides, was increased 100% over control, but that of FITC-PRM was not. Conversely, FITC-PRM and FITC-Con A bound 250% and 140% over control respectively to

Fig. 4. Effect of PRM on glycosidase inhibitor-treated cells.




DMJ-treated cells. The above result obviously indicates that PRM recognizes and binds to the oligosaccharides of which expression was induced by DMJ treatment. The effect of PRM on the glycosidase inhibitor-treated cells was examined next. After the incubation with an appropriate concentration of each glycosidase inhibitor for 48 hours, cells were treated with 50 μg/ml PRM for 24 hours (Fig. 4). The viability of DMJ-treated cells was 42% of control, whereas CAS- and SW-treated cells were completely insusceptible to PRM.

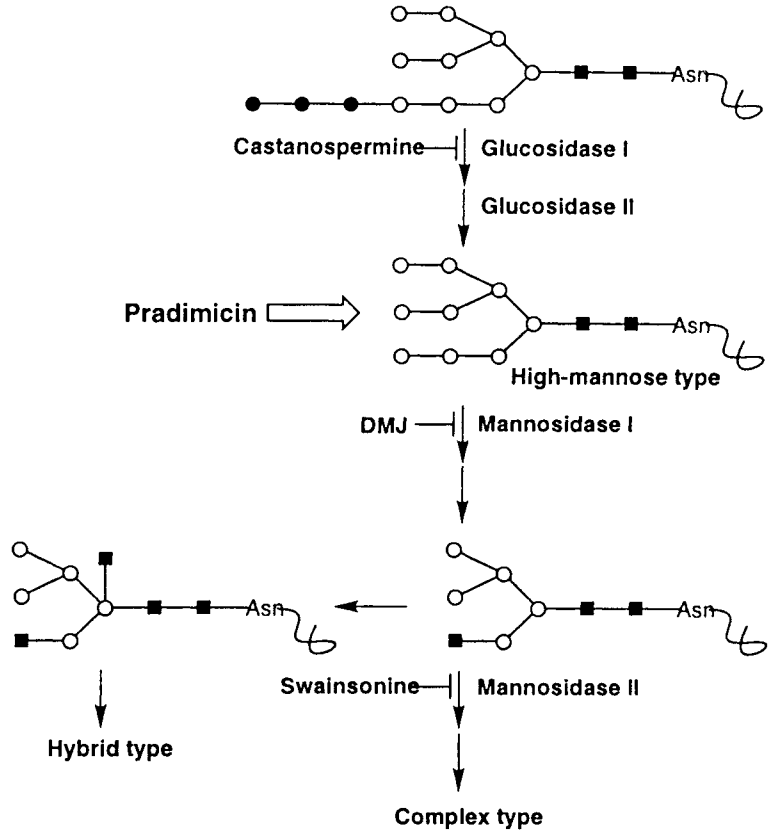
## Discussion

We have shown that PRM induced apoptosis of DMJ-treated U937 cells dose-dependently and the cytotoxic effect of PRM was enhanced depending on the DMJ concentration at the preincubation.[7] The similar effect of PRM was observed in HL-60 and PC12 cells, suggesting the presence of a general mode of action. Since the role of DMJ in this system was to inhibit mannosidase I, it seemed obvious that the cell surface glycan was critical for apoptosis induction. Additionally, the involvement of mannose-containing oligosaccharides was strongly suggested by the property of PRM which recognizes D-mannosides in the presence of $Ca^{2+}$, and PRM was thought to bind to terminal mannose residues at the cell surface since the binding was dependent on the DMJ treatment. In fact, Fig. 2 clearly shows that the apoptosis was induced in response to the PRM binding. Under a fluorescence microscope, FITC-PRM bound to the cell surface uniformly and any localization was not observed (data not shown).

Furthermore, we studied the effect of PRM on other types of oligosaccharide containing terminal mannoses (Fig. 5). In the biosynthesis of *N*-linked oligosaccharide, $(Glc)_3(Man)_9(GlcNAc)_2$ is first transferred from dolichol pyrophosphate to asparagine residues of proteins, and three glucoses are removed by glucosidases I and II to afford a high-mannose type oligosaccharide, $(Man)_9(GlcNAc)_2$. Castanospermine (CAS) inhibits both glucosidases I and II and it is reported that CAS promotes the formation of $(Glc)_3(Man)_9(GlcNAc)_2$ more than 70% over control in virus[12] and mammalian cells.[13] The high-mannose type glycan is transformed into complex type one through the processing by mannosidases I and II. In the presence of swainsonine (SW), a potent inhibitor of mannosidase II, the conversion of high-mannose type to complex type is blocked and the formation of hybrid type such as (NeuAc)(Gal)(GlcNAc)$(Man)_5(GlcNAc)_2$ is preferred.[14~16] CAS- and SW-treated U937 cells were not sensitive to PRM and the level of FITC-PRM binding to those cells was same as that to the control. This result indicates the recognition of a mannose-containing glycan by PRM was specific.

Fig. 5. Biosynthesis of *N*-linked oligosaccharide and plausible target of pradimicin.

■ *N*-Acetylglucosamine, ● glucose, ○ mannose.




The glycosidase inhibitors employed in this study are effective on the processing of *N*-linked oligosaccharides, but not on the proteins. Though it is still under investigation whether PRM functions extracellularly or intracellularly, it is supposed that PRM binds to a high-mannose type oligosaccharide on a cell surface protein, and the protein transmits the apoptotic signal to the cell. In summary, we demonstrated evidences suggesting that PRM induces apoptosis in U937 cells through the interaction with high-mannose type oligosaccharide, presumably $(Man)_9(GlcNAc)_2$, at the cell surface.

## Acknowledgments

This work was partly supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan and the Uehara Memorial Foundation.

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