Photoactivation of pheophorbide \( a \) induces a mitochondrial-mediated apoptosis in Jurkat leukaemia cells

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Received 23 October 2002; received in revised form 29 July 2003; accepted 18 May 2004

Available online 8 July 2004

Abstract

The mechanism of cell death by pheophorbide \( a \) (\( \text{Pb}a \)) which has been established to be a potential photosensitizer was examined in experimental photodynamic therapy (PDT) on Jurkat cells, a human lymphoid tumor cell line. In 30–60 min after irradiation, \( \text{Pb}a \) treated cells exhibited apoptotic features including membrane blebbing and DNA fragmentation. \( \text{Pb}a \)/PDT caused a rapid release of cytochrome \( c \) from mitochondria into the cytosol. Sequentially, activation of caspase-3 and the cleavage of poly ADP-ribose polymerase (PARP) were followed. Meanwhile, no evidence of activation of caspase-8 was indicated in the cells. In experiments with caspase inhibitors, it was found that caspase-3 alone was sufficient initiator for the \( \text{Pb}a \)-induced apoptosis of the cells. \( \text{Pb}a \) specific emission spectra were confirmed in the mitochondrial fraction and the light irradiation caused a rapid change in its membrane potential. Thus, mitochondria were entailed as the crucial targets for \( \text{Pb}a \) as well as a responsible component for the cytochrome \( c \) release to initiate apoptotic pathways. Taken together, it was concluded that the mode of Jurkat cell death by \( \text{Pb}a\)/PDT is an apoptosis, which is initiated by mitochondrial cytochrome \( c \) release and caspase-3-pathways.

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Keywords: Silkworm metabolite; Chlorophyll derivatives; Photodynamic therapy; Pheophorbide \( a \); Apoptosis; Cytochrome \( c \)

1. Introduction

Various studies have established photodynamic therapy (PDT) to be a valuable therapeutic method for some cancers and viruses [1–3]. PDT involves the application of a photosensitizing agent and light with a selected wavelength to activate the photosensitizer, which catalyzes the production of the reactive oxygen species to produce cytotoxicity [4–6]. In recent, Photofrin® [7] of various photosensitizers was licensed for a therapeutic tool for some forms of cancer [8–10]. Despite the favorable results of PDT with Photofrin®, a few drawbacks was also found in the clinical application. Hematoporphyrin absorbs very weakly at the therapeutic wavelength of 630 nm, where tissue penetration of light is not optimal, limiting tissue penetrating depths of no more than 5 mm [11,12]. An ideal photosensitizer must absorb at longer wavelengths to treat more deep-seated or larger tumors. In addition, overlapping absorption spectra of hematoporphyrin and melanin in the malignant tissue have made PDT ineffective for cancers as melanoma with pigments [13]. These drawbacks in the use of Photofrin® compelled researchers to search for new second-generation photosensitizers with improved physical, chemical and spectral properties [14]. In this, we discovered that...
chlorophyll metabolites from silkworm excreta have yielded an effective PDT results in vitro and in mice systems [15,16] and that pheophorbide a (Pba) among the metabolite-components is a major active compound. Some studies on tumor-PDT by use of Pba have been conducted [17,18], however, them on Jurkat pound. Some studies on tumor-PDT by use of Pba the metabolite-components is a major active com-
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been reported.

In these experiments, it was revealed that Pba photosensitized Jurkat tumor cells, but not in peripheral blood mononuclear cells (PBMC) used as the normal control, and that apoptosis is the major type of cell death involved in Jurkat cells following Pba/PDT. This occurred via caspase-3 pathways that were initiated by direct mitochondria disruption and immediate release of cytochrome c.

2. Materials and methods

2.1. Pheophorbide a (Pba) from silkworm excreta

Stocks of silkworm excreta stored in nature were used to extract chlorophyll derivatives (CpD). The stocks were emulsified in pure acetone and kept over night in dark. The liquid fractions were collected separately from the sediments by centrifugation at 16,000g for 15 min in 4 °C and concentrated by use of concentrator. Acetone free materials from the sediments were prepared in evaporator and named CpD. The CpD was extracted with chloroform, and the chloroform layer was then concentrated to a small volume. Thereafter it was concentrated to dryness, and the residue was dissolved in a small volume of solvent system of chloroform:methanol (9:1). The solution was applied to a silicagel column and eluted with solvent system of chloroform:methanol (9:1). The active fraction was collected, concentrated to dryness, and further purified by preparative TLC with solvent system of chloroform:methanol (9.5:0.5). The purified substance was identified as pheophorbide a (Pba) (with more than 97% purity) with LC-MS analysis. Aliquots of Pba were prepared in N,N-dimethyl formamide (DMF) as stock solutions. The final concentration was adjusted to be 5 mg/ml of DMF as stock solutions and stored in dark at −20 °C until use.

2.2. Cell culture

A human T cell leukemia cell line, Jurkat (ATCC, Rockford, MD, USA) was selected for the target tumor cell for PDT in vitro. Since the origin of Jurkat cells was lymphoid tissue, primary human peripheral blood mononuclear cells (PBMC) from healthy person were prepared by use of Ficoll (Amersham–Pharmacia Bio-
tech, Uppsala, Sweden) for normal control. PBMCs were obtained instantly from a donor and applied to the test without the treatment of stimulators. As basal media for the maintenance of cells, RPMI-1640 (Life Technologies, Gaithersburg, MD, USA) had been used. The growth media for the experiments were prepared by supplementing the basal media with 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin and 100 g/ml of streptomycin. The cells in the growth media had been incubated at 37 °C under 5% CO2 in a humidified incubator.

2.3. In vitro PDT by use of Pba

Jurkat cells (1 × 10^6 in a 35 mm flask) were incubated with 1.5 ml of RPMI containing Pba (0.5 μg/ml) for 1 h at 37 °C. Following incubation, the cells were irradiated with the light for 10 min. For the light source, a 200 W halogen lamp (MVI Micro Video Instruments Inc., MA, USA) attenuated by a 630 nm cut off filter was used. The total power out put for the irradiation was adjusted to 1.2 J/cm² at 20 mW/cm² by use of a Laser power meter (Metrologic Instruments, Inc., Blackwood, NJ, USA).

Following the completion of Pba/PDT, the cells were further incubated at 37 °C for 1 h in a humidified incubator. The changes in morphology of photo-damaged cells were photographed in 1 h after Pba/PDT in phase-contrasted microscopy (400×) (Carl Zeiss, Oberkochen, Germany).

The types of cell death were characterized by FACS (Becton–Dickinson, Sunnyvale, CA, USA) analysis of the cells stained with propidium iodide (PI) and Annexin V (Biosource, Camarillo, CA, USA). Briefly, 1 h after PDT, cell pellet was suspended in 500 μl Annexin V–HEPES solution (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2) and was kept on ice for 30 min in the dark. The cells were then washed once in ice-cold HEPES buffer. PI was added just before FACS analysis.

2.4. Target cell specificity of Pba/PDT

To confirm tumor specificity of Pba/PDT, responses of Jurkat cells, a leukemia cell line and fresh PBMC from healthy donor was studied. The responses were evaluated by the differences in number of survived cells after Pba/PDT (according to varied PDT-post incubation times and Pba concentrations) by use of the trypan blue dye exclusion test. Briefly, both the tumor and normal cells (1 × 10^6) in 35 mm flasks were treated with the same protocols of Pba/PDT. Following the treatment, the cells were further incubated for 2, 4, 6 and 8 h at 37 °C in a humidified incubator, and then their viability was evaluated. In addition, the cells (1 × 10^6 in a 35 mm flask) were incubated with 1.5 ml of RPMI added with Pba varied from 0.01 to 2 μg/ml for 1 h at 37 °C.
These cells were then exposed to light, and their viability was evaluated.

2.5. DNA fragmentation

After PDT treatments, the cellular DNA of the treated cells was isolated and applied onto 2% agarose gel to identify the formation of DNA ladder using standard procedure. Briefly, 0, 15, 30, 60, 120 and 240 min after PDT, cells suspended in 100 μl of PBS and cytolysis were induced by adding an equal volume of 2x lysis buffer (200 mM HEPES, pH 7.5, 2% Triton X-100, 400 mM NaCl, and 20 mM EDTA). Following a 45 min digestion with 1 μg of RNase A at 37 °C, samples were extracted by phenol/chloroform, precipitated by ethanol, and analyzed on a 2% agarose gel.

2.6. Preparation of cytosolic protein extracts

Biochemical changes by PDT including fragmentation of nuclear DNA, cytosolic appearance of cytochrome c, activation of caspases, and PARP cleavage were studied. Whole cell extracts were obtained from PDT treated cells washed twice in PBS by sonicating them in ice chamber. Cytochrome c released from mitochondria was confirmed by detecting the enzyme in cytosolic protein that was extracted following the method reported by Yang et al. [19]. In brief, cell pellets obtained from washed cells (twice with PBS) were suspended in 700 μl ice-cold buffer at pH 7.5 containing 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1m M EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF. Cells in the buffer solution were disrupted using a homogenizer. Lysate was centrifuged at 10,000 g for 10 min and the supernatant was further centrifuged at 100,000 g for 1 h in an ultracentrifuge (Beckman Palo Alto, CA, USA) using a Type 100 TL-100 rotor.

2.7. Immunoblot analysis

The cell lysate (approximately 40 μg of protein) was separated in 10% polyacrylamide SDS-gels and transferred to the nitrocellulose membrane [20]. This was then imersed in blocking buffer (5% skim milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h at room temperature and incubated with primary antibodies (BD Pharmingen, San Diego, CA, USA), cytochrome c (1:500), caspase-3 (1:500), caspase-8 (1:1000) and PARP (1:2000) in blocking buffer overnight at 4 °C. After the incubation, the membrane was probed with horseradish peroxidase-labeled anti-rabbit IgG antibody (1:5000) in PBS (containing of 0.05% Tween 20 and 5% skim milk powder) for 30 min at room temperature. The proteins in the membrane were detected by enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK) and bands were visualized by autoradiography using X-ray film (Fujifilm Co.).

2.8. Caspase inhibition experiments

Inhibition of caspase activity was examined by use of caspase inhibitors to clarify the significance of caspase in the cell death. The general caspase inhibitor, ZVAD-fmk (Calbiochem, Cambridge, MA, USA) and caspase-3 inhibitor, Z-DEVD-fmk (Calbiochem, Cambridge, MA, USA) were added to the cells to give final concentration of 50 or 100 μM for the final 60 min before photoactivation. Jurkat cells (1 × 10⁶) were treated with Pba/PDT following the original protocols described.

To assess cell viability in 2 h after the incubation, the cells were harvested from 35 mm tissue culture dish and 100 μl of a MTT solution (1 mg/ml) was added to the cell pellets. The solution (100 μl) was incubated in 96-well microtitre plates. After 4 h, the cells were centrifuged and the supernatant was discarded. One hundred μl of DMSO was added to the pellets, and then, the reaction was stopped by the addition of 100 μl of isopropanol. The absorbance was measured with an automted spectrophotometric microtitre plate reader (Spectra Max 340/Molecular Devices, Sunnyvale, CA, USA) using a 570 nm filter.

2.9. Spectrometry for Pba accumulated on mitochondrial membrane and changes in mitochondrial membrane potential (ΔΨₘ)

The mitochondrial fraction was prepared to detect the Pba specific spectrophotometric patterns. Briefly, Jurkat cells were treated with 10 μg/ml Pba without light irradiation. After 1 h, cells were homogenized in 9 ml of homogenizing buffer (0.25 M sucrose in 1 mM HEPES buffer, pH 7.5) and centrifuged at 4500g for 10 min. The supernatant was re-centrifuged at 16,000g for 25 min at 4 °C and precipitates were suspended in 20 ml of suspension buffer (0.25 M sucrose in 10 mM HEPES buffer with 1 mM EDTA, pH 7.5). These fractions were then subjected to the photometry analysis using a luminescence spectrophotometer (LS-5, Perkin-Elmer, Torrance, CA, USA). In photometry analysis, the light of 677 nm was found to be the light of maximum wavelength emitted by Pba (in DMF) irradiated with the light of 420 nm. ΔΨₘ was analyzed by the method described by Cossarizza et al. [21,22], which allows the direct measurement of ΔΨₘ in isolated mitochondria from cells treated with Pba/PDT. Pba/PDT-treated cells were loaded with 10 μg/ml JC-1 (Molecular Probes, Leiden, Netherlands) for 15 min at 37 °C in PBS buffer. After loading, cells were washed twice with fresh PBS. Flow cytometry was performed using a FACScan flow cytometer (Beeton-Dickinson, Franklin Lakes, NJ, USA) equipped with a single 488 nm argon laser. A minimum
of 10,000 cells per sample was acquired in list mode and analyzed using LYSYS II software as reported [23].

2.10. Statistics

The statistical evaluation was done by using the Mann–Whitney U test with a GraphPad Prism software (Ver. 3.0). Results are presented as means ± the standard errors of the means (SEMs).

3. Results

3.1. Pbα/PDT induces rapid death of Jurkat cells

In phase-contrasted microscopy, morphologic changes of Jurkat cells treated with Pbα/PDT were significant in 30 min. Membrane blebbing and cell shrinkage was prominent in more than a half of the cells photosensitized with Pbα (Fig. 1).

In order to assess the importance of the apoptotic process in Pbα-mediated cytotoxicity, Jurkat cells were analysed by Annexin V/PI staining method which is commonly used to differentiate necrotic and apoptotic cell populations. The apoptotic cell death was scored 1 h after the light irradiation (Fig. 2). As shown in Fig. 2, cells treated with PDT were found to be double-stained with Annexin V and PI via early apoptotic process. Morphologic changes of the PDT treated cells shown in Fig. 1 further supported the result above. Whereas, in the experiment to compare the difference of cell death with Jurkat cells and PBMC by Pbα/PDT, more than 90% of control PBMC had survived (Fig. 3). This result indicates that the normal cells are not cytotoxic by Pbα/PDT. A Pbα dose of 0.5 µg/ml adequate for the apoptosis studies had been used throughout the experiments. Tumor cell killing was clearly influenced by the time assayed after PDT as well as the concentration of the photosensitizer applied to the cells. Light dependency of Pbα mediated PDT cytotoxicity was demonstrated by observing survival of the Pbα treated cells when it was protected from light. More than 95% of the cells survived in the cases. In order to determine the nature of cell death, photosensitized cells were assessed for DNA fragmentation by agarose gel electrophoresis. In a time-course study, DNA laddering was detectable by 60 min with a 1.2 J/cm² light dose (Fig. 4).

Fig. 1. Apoptosis response of Jurkat cells by Pbα/PDT. Jurkat cells were photographed 1 h after Pbα/PDT using a phase-contrasted microscope (400×). The control (a) and the cells treated with only DMF solvent (b), light only (c) and Pbα only (0.5 µg/ml) (d) exhibited normal cell morphology. Only in cells treated with Pbα (0.5 µg/ml)/PDT (e), apoptotic changes including membrane blebbing were prominent.

Fig. 2. Death of Jurkat cells by Pbα/PDT in Annexin V staining. Jurkat cells were incubated with 0.5 µg/ml Pbα for 1 h and then irradiated with a 1.2 J/cm² light dose. Cells were harvested 1 h after photosensitization and stained with Annexin V/PI for FACS analysis. Lower left panel represents living cells, lower right panel represents apoptotic cells.
3.2. Pba/PDT induces apoptosis via caspase-3 activation

Confirming laddering of fragmented DNA from the Jurkat cells treated with Pba/PDT, a processing of caspase-3 was examined. Activation of caspase-3 was confirmed 30 min after the treatment (Fig. 5). Cleavage of poly ADP-ribose polymerase (PARP), a target of activated caspase-3, was consequently observed (Fig. 5). To confirm that caspases were involved in Pba/PDT-induced cell death of Jurkat cells, 1 h prior to PDT, cells were treated with increasing concentration of the general caspase inhibitor, ZVAD-fmk (Calbiochem, USA) and caspase-3 inhibitor, Z-DEVD-fmk (Calbiochem, USA). In cultures treated with ZVAD-fmk, cell viability was significantly decreased, and then, further enhancement of the viability by ZVAD-fmk was not measured by increased concentration of the ZVAD-fmk up to 100 µM. Whereas, in cells treated with Z-DEVD-fmk that inhibits caspase 3, cell viability was the nearly same that as PDT untreated control (Fig. 6). Thus, caspase-3 was implied as a key enzyme in apoptosis of PDT treated Jurkat cells. On the other hand, caspase-8 was not involved in this pathway (Fig. 5). Activation of caspase-8 is known to trigger cytochrome c release in receptor-mediated (Fas or TGF receptor) pathways. Our results led us to the assumption that Pba/PDT triggers direct release of cytochrome c from mitochondria which activates caspase-3 in Jurkat cell. In Western blot analysis, Pba photosensitization gave rise to the appearance of cytochrome c in the cytosol. Cytosolic cytochrome c was demonstrated 15 min after the Pba/PDT. In 30 min following the cytochrome c release, processing of caspase-3 was confirmed in Western blot (Fig. 5).

Taken together, these results indicate that Jurkat cell death by Pba/PDT may be occurred by the apoptosis
initiated from mitochondria, via not receptor-mediated pathways.

3.3. Pba is detected on mitochondrial membrane and Pba/PDT induces loss of mitochondrial membrane potential

Accumulation of Pba on mitochondrial membrane was evidenced in the Pba treated Jurkat cells. The cells emitted light of 677 nm, when excited by a light of 420 nm. The emitted light was peaked at 677 nm and the native Pba in DMF solvent recorded the same peak (Fig. 7(a)). The emitted light of 677 nm was revealed to be specific to Pba since the spectrum was detected only in Pba treated cells. The intensities of the light of 677 nm were positively correlated with the amount of Pba in the samples tested. Pba-specific light emitted by the mitochondria fractions of Pba treated cells following light activation indicates the mitochondrion is the primary target for the Pba. The loss of mitochondrial membrane potential was also occurred in the Pba/PDT treated Jurkat cells (Fig. 7(b)). As shown in Fig. 7(b), Distinct populations of cells with different extents of mitochondrial depolarization are detectable following apoptosis-inducing treatment with Pba/PDT. This result reveals that the loss of mitochondrial cytochrome c from Jurkat cells stimulated to undergo apoptosis is associated with a loss of the mitochondrial membrane potential.

4. Discussion

We have isolated pheophorbide a (Pba) from silkworm excreta stocks stored in nature for months and demonstrated its photosensitizing efficacy against Jurkat leukemia. In the experiment to compare the difference of cell death with Jurkat cells and PBMC by Pba/PDT, more than 90% of control PBMC had survived (Fig. 3). This result indicates that the normal cells are not cytotoxic by Pba/PDT, and that PDT using a photosensitizing chemical such as Pba can be applied safely to selective cancer treatment. Cell death is achieved by two fundamentally different mechanisms; apoptosis and necrosis. Apoptosis for a number of different cell types sensitized with various photosensitizers has been shown [24,25]. PDT induced-apoptosis has been claimed to both cell type specific and sensitizer dependent [26]. Our results provide evidence that the cytotoxic response of light-irradiated Jurkat cells pre-treated with Pba is an apoptosis. One hour after light irradiation of the cells treated with Pba, membrane blebbing, shrinkage, and DNA fragmentation were demonstrated. In Annexin V
staining for apoptosis assays, the cells were scored positive. These observations reproducibly demonstrate apoptosis of Jurkat cells treated with Pbα/PDT.

Mitochondria of the cells were found to be primary target for the Pbα in initiating the apoptotic process of the cells. This speculation that Pbα/PDT triggers the mitochondrial pathway of apoptosis has been supported by confirming Pbα specific emission spectra in the mitochondrial fraction by use of spectrophotometry. Uptake of photosensitizer to mitochondria membrane and lysosome had been also reported in other studies [26,27]. Release of mitochondrial cytochrome c into the cytoplasm was observed by confirming cytochrome c in the cytosolic fraction of the cells. Release of cytochrome c from PDT treated cells has been reported in others [20,28]. Involvement of caspase in the process of the cell death implied by the cytochrome c in cytosol was examined by use of caspase inhibitors. In cultures treated with ZVAD-fmk, cell viability was significantly decreased, and then, further enhancement of the viability by ZVAD-fmk was not measured by increased concentration of the ZVAD-fmk up to 100 μM. Whereas, in cells treated with Z-DEVD-fmk that inhibits caspase-3, which is known to be probably the most important effector molecules in inducing the DNA fragmentation, cell viability was the nearly same that as PDT untreated control (Fig. 6). Thus, caspase-3 was implied as a key enzyme in apoptosis of PDT treated Jurkat cells. Reports on the involvement of activated caspase-8 as an early upstream effector caspase in PDT-induced cell death are controversial. It has been well documented that caspase-8 is activated in Fas-, TRAIL- and TNF-induced apoptosis and the activated caspase-8 is involved as an early upstream effector caspase in the receptor mediated apoptosis [29–31]. In contrast, it was also shown that caspase-8 activation is not an initiating event in PDT induced apoptosis in HeLa cells sensitized with benzoporphyrin derivative monoacid ring A (BPD-MA) as a photosensitizer [32,33]. Nevertheless, our results demonstrated that Pbα/PDT induced Jurkat cell death is not by receptor-mediated pathway (Figs. 5 and 6). For the activation of caspase-3 in Jurkat cells treated with Pbα/PDT, direct cytochrome c release from mitochondria was assumed and rapid release of cytochrome c into cytosol 15 min after PDT was confirmed by Western blotting (Fig. 5). The processing of cytochrome c released was observed and cleavage of PARP in 1 h after PDT was confirmed (Fig. 5). The rapid appearance of cytochrome c in the cytosol following Pbα/PDT might be attributed to direct action of Pbα localized to mitochondria [32-34]. In addition, localization of Pbα onto mitochondrial membrane was ensured by observing Pbα specific-emission spectra in mitochondrial fractions by using spectrophotometry. These results indicate that rapid and direct mitochondrial uptake of Pbα affects release of cytochrome c in the mitochondria. PARP cleavage followed by immediate activation of caspase-3 was also evidenced. These sequential events are responsible for the apoptosis of Jurkat cells. Thus, Pbα/PDT mediated apoptosis in Jurkat cells is not mediated by a receptor mediated pathway via caspase-8.

In conclusion, we demonstrated that Pbα, a silkworm metabolite, is a putative photosensitizer for PDT. In case of Jurkat cells, Pbα was accumulated on mitochondrial membrane and triggered cytochrome c release that activates caspase-3 and cleaves PARP to drive the cells into the process of apoptosis (Fig. 5).

Dai et al. [35] determined 10-hydroxypeophytin to be a unique metabolic product of silkworm, however, we was able to isolate Pbα from silkworm metabolites regarding agricultural waste, demonstrate its photosensitizing activity against tumor cells, and reveal a mitochondrial apoptotic mechanism in Jurkat tumor cell death by Pbα/PDT. These suggest a possibility being developed as the photodynamic antitumor agent in aspects of its biological activity and costs. In addition, we demonstrated that Pbα showed the photosensitizing effect approximately 50 times as much as Photofrin® [7] against Jurkat and HL 60 tumor cell lines (data not shown).

We propose the following sequence of events in response to PDT with Pbα in Jurkat tumor cell line. Rapid release of cytochrome c from mitochondria into the cytosol was induced from the disruption of mitochondrial membrane, and then, caspase-3 was activated by cytochrome c. Activated caspase-3 induced PARP cleavage, and then, PARP translocated into nucleus catalyzed DNA fragmentation. This sequential process demonstrated an apoptosis in Jurkat cell.

5. Abbreviations

PDT photodynamic therapy
Pbα pheophorbide a from silkworm excreta
PARP poly ADP-ribose polymerase
HPD hematoporphyrin derivative
CpD chlorophyll derivative
DMF N,N-dimethyl formamide
PBMC peripheral blood mononuclear cell
PI propidium iodide
JC-1 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyan-4,4-dimethyldiimidazolylcarboxyanine iodide
TCLD50 50% lethal dose for tissue culture
Z-DEVD-fmk Z-Asp-Glu-Val-Asp-fluoromethylketone
ZVAD-fmk Z-Val-Ala-Asp(OMe)-fluoromethylketone

Acknowledgements

This work was supported by the research grants from the Ministry of Commerce, Industry and Energy, and the Ministry of Agriculture and Forestry of Korea.
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