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The AF4-mimetic peptide, PFWT, induces necrotic cell death in MV4-11 leukemia cells

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Abstract

Despite ongoing success in the treatment of childhood acute lymphoblastic leukemia, patients harboring translocations involving the MLL gene at chromosome 11q23 remain resistant to treatment. To improve outcomes, novel therapeutics designed to target the unusual biology of these leukemias need to be developed. Previously, we identified an interaction between the two most common MLL fusion proteins, AF4 and AF9, and designed a synthetic peptide (PFWT) capable of disrupting this interaction. PFWT induced cell death in leukemia cells expressing MLL-AF4 with little effect on the colony forming potential of hematopoietic progenitor cells, suggesting the AF4–AF9 complex is an important pharmacological target for leukemia therapy and PFWT is a promising chemotherapeutic prototype. In these studies, we demonstrate that PFWT induces death by necrosis in MV4-11 cells. Cell death is characterized by rapid loss of plasma membrane integrity with maintenance of nuclear membrane integrity, and is independent of caspase activation, DNA fragmentation, and mitochondrial membrane depolarization. PFWT-mediated necrosis is inhibited by the serine protease inhibitor TLCK, suggesting this death pathway is regulated. Given the resistance of t(4;11) leukemias to conventional chemotherapeutic agents that induce apoptosis, further identification of the molecular events mediating this death process should uncover new avenues for therapeutic intervention.

Keywords

Necrosis; MLL; Targeted therapy; AF4; AF9; Programmed cell death

1. Introduction

With improvements in the treatment of acute lymphoblastic leukemia (ALL) in the last few decades has come the recognition that specific genetic anomalies are tightly linked to therapeutic response and prognosis. Rearrangement of the mixed lineage leukemia (MLL) gene

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Contributions. Christine Palermo designed and performed the majority of the experiments and wrote the manuscript. Cecily Bennett performed the experiments utilizing electron microscopy. Amanda Winters conducted critical control experiments and flow cytometry analysis. Charles Hemenway oversaw the entire project and assisted with preparation of the manuscript.

Conflict of interest statement None.

at 11q23 is one such anomaly. Rearrangements of MLL occur in various subsets of leukemias including 5% of sporadic acute myeloid and acute lymphoblastic leukemias [1]. Furthermore, almost all secondary leukemias associated with exposure to topoisomerase II targeting drugs involve chromosome translocations at 11q23 [1]. MLL rearrangements are encountered in leukemias in all age groups and are remarkably common in infant leukemia. Importantly, MLL gene rearrangement is an adverse prognostic indicator in children and adults with ALL. This problem is particularly relevant to infants with ALL in whom long-term disease-free survival remains less than 50% with conventional chemotherapy [2]. To improve the outcome associated with these cancers, novel therapeutics designed to target the unusual biology of MLL-leukemia cells need to be developed.

One promising chemotherapeutic target we have identified is the protein interaction between two common MLL fusion partners, AF4 and AF9 [3]. Collectively, the MLL rearrangements t(9;11)(p22;q23), leading to the fusion gene MLL-AF9, and t(4;11)(q21;q23) resulting in the MLL-AF4 fusion occur in over 50% of infant and therapy related cases of acute leukemia [4]. Mapping of the AF4-AF9 protein interaction domains revealed these sites are located in regions of AF4 and AF9 that are highly conserved within the protein families to which each belongs [5,6], and these interaction domains are retained in MLL-AF4 and MLL-AF9 fusion proteins. Based on these mapping studies, we synthesized a synthetic peptide (PFWT) capable of disrupting the AF4-AF9 interaction *in vitro* and *in vivo* [7]. This peptide induced cell death in leukemia cell lines expressing the MLL-AF4 fusion protein with little effect on the colony forming potential of hematopoietic progenitor cells [7]. These findings identify the AF4-AF9 protein complex as an important pharmacological target for leukemia therapy and establish PFWT as a promising prototype for the development of new drugs to treat MLL-rearranged leukemias.

In this study, a series of experiments were performed to characterize PFWT-mediated death in the t(4;11) leukemia cell line, MV4-11. Generally, chemotherapeutics induce cell death by activating apoptotic pathways [8–10]. Interestingly, and contrary to our initial findings [7], neither morphological nor biochemical markers of apoptosis were detected in MV4-11 cells following PFWT exposure. Our observations demonstrate an atypical pattern of cell death independent of caspase activation, DNA fragmentation, and mitochondrial membrane permeabilization that is characterized by the maintenance of nuclear integrity and dramatic disruption of the plasma membrane. PFWT-mediated cell death is prevented by the serine protease inhibitor TLCK, indicating this death process is regulated and thus not accidental or uncontrolled. These data suggest PFWT-mediated necrosis, as defined here by loss of plasma membrane integrity and extrusion of intracellular contents into the extracellular space, is a caspase-independent process in which serine proteases play an essential role. Understanding this necrotic death mechanism may reveal novel therapeutic targets, with important consequences for attempts to treat leukemias that are resistant to apoptotic stimuli.

2. Materials and methods

2.1. Chemicals

PFWT and PFmut peptides were synthesized by Global Peptides (Fort Collins, CO). PFWT corresponds to amino acids 761–773 of human FMR2 (LWVKIDL DLLSRV) [7]. PFmut serves as a negative control and contains two amino acid substitutions (LWEKSDL DLLSRV) responsible for attenuating its ability to bind to AF9 [7]. Both peptides are linked at their amino-termini to the Penetratin protein transduction motif (RQIKIWFQNRRMKWKK) to facilitate intracellular uptake. Peptides were dissolved in tissue culture grade DMSO (25 mg/ml). The broad spectrum caspase inhibitor z-VAD-FMK, was purchased from Promega. Etoposide was purchased from A.G. Scientific. *N*- α -*p*-Tosyl-lysine chloromethyl ketone (TLCK) was

purchased from Sigma–Aldrich Chemical Company. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was obtained from Molecular Probes/Invitrogen.

2.1.1. Cell culture and viability assays—Leukemia cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 110 units/ml penicillin, and 110 µg/ml streptomycin at 37 °C in a humid atmosphere with 5% CO₂. For all assays, cells were treated at a density of 1×10^6 cells/ml. Cell viability was assessed by flow cytometry using ethidium homodimer-1 (ethdr) and calcein AM (Molecular Probes/Invitrogen) according to the manufacturer's protocol with the following changes: cells were re-suspended in PBS containing 1.6nM calcein AM and 0.4 µM ethdr. CountBright™ absolute counting beads (Molecular Probes/Invitrogen) were added to each sample for determination of cell number. Fluorescence was monitored on an LSRII flow cytometer (Becton Dickinson). Doublet discrimination was performed on all samples. Live cells were considered those that were both negative for ethdr and positive for calcein. Experiments were performed in triplicate. Values are expressed as percent of DMSO-treated live cell numbers. Statistical significance was determined using one-tailed *t*-test analysis.

2.1.2. Western blotting—MV4-11 cells were treated as indicated in the figure legends. Cells were collected, washed in PBS, and lysed in Laemmli buffer. Proteins were separated by SDS-PAGE on a 4–12% Bis–Tris gradient gel (Invitrogen), and transferred to a nitrocellulose membrane. Membranes were probed with primary antibodies recognizing PARP (Cell Signaling), cleaved caspase-3 (Asp175, Cell Signaling), or β-actin (Sigma). Proteins were visualized by chemiluminescence (GE Healthcare).

2.2. TUNEL

DNA strand breaks were detected by flow cytometric measurement (LSRII) of 3'-hydroxyl ends using the APO-BrdU™ TUNEL assay kit (Molecular Probes/Invitrogen) according to the manufacturer's protocol with the following exception: cells were stained with a 1:300 dilution of the Alexa Fluor 488 dye-labeled anti-BrdU antibody.

2.2.1. Ultrastructural studies—Electron microscopic studies were performed on MV4-11 cells at a density of 1×10^6 cells/ml treated with PFWT (25 µg/ml), PFmut (25 µg/ml) or an equivalent percentage of DMSO. After treatment, samples were washed in PBS and processed by Professional Histology Services (New Orleans, LA). Samples were fixed in 3% glutaraldehyde and 1.5% OsO₄ and embedded in spur epoxy resin. The samples were sectioned and stained with uranyl acetate and lead citrate prior to visualization on a Philips CM-10 transmission electron microscope. Images were captured using Advanced Microscopy Techniques Corp imaging program and a XR111 (11 Megapixel) Bottom Mount, CCD Camera System.

2.2.2. Assessing mitochondrial $\Delta\Psi_m$ —Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were determined using the cationic dye JC-1 (Molecular Probes/Invitrogen). MV4-11 cells were treated as indicated in the figure legend and stained for 30 min at 37 °C with JC-1 (1 µM) according to the manufacturer's protocol. Treatment with CCCP, a direct disrupter of electron transport, was used as a positive control. Changes in JC-1 fluorescence were monitored on an LSRII.

2.2.3. Comet analysis—Comet analysis (single cell gel electrophoresis technique) was performed under alkaline conditions [11]. In brief, cells were diluted in low melting agarose, spread onto each well of the Trevigen slides and incubated at 4 °C for 1 h. After adherence to slides, cells were submerged in alkaline lysis buffer and incubated at 4 °C for an additional 1 h. Unwinding was also conducted at 4 °C for 1 h in electrophoresis buffer. Electrophoresis was

conducted at 1 V/cm for 15 min. Following electrophoresis, slides were neutralized and fixed in 70% ethanol for 5 min and stained with SYBR Green I. Comets were photographed at 200× magnification and 75 Comets per sample were analyzed using Komet 6.0 (Kinetic Imaging).

3. Results

3.1. PFWT induces cell death in MV4-11 cells

The MV4-11 cell line harbors the t(4;11)(q21;q23) translocation fusing MLL to AF4 [12,13]. Time course analysis was performed in MV4-11 cells to determine the kinetics of cell death following exposure to PFWT. To monitor cell viability, cells were treated with calcein AM and ethdr and analyzed by flow cytometry as described in Section 2. The PFWT dose utilized in this experiment was based on previously published dose–response viability analyses of MLL-AF4 cell lines [7]. As shown in Fig. 1, cell death, indicated by staining with the membrane-impermeable dye, ethdr, was evident as early as 1 h after exposure to the PFWT peptide. Viability continued to diminish over time, however the primary insult occurred very rapidly with nearly 50% cell loss at 1 h. As anticipated, the negative control peptide, PFmut, had no significant effect on MV4-11 cell viability. For comparison, the response of MV4-11 cells to the well-characterized chemotherapeutic agent, etoposide, was monitored. Etoposide induces double strand DNA breaks and typically triggers apoptosis in sensitive leukemia cells. The kinetics of cell death are considerably delayed in MV4-11 cells exposed to etoposide when compared to PFWT with approximately 50% of cell death occurring after 6 h in etoposide-treated cells. The rapid induction of cell death by PFWT indicates that cellular homeostasis is severely disturbed within minutes of exposure to the peptide. Moreover, the uptake of ethdr within 1 h requires disruption of the plasma membrane on a time scale that is inconsistent with apoptosis.

3.2. PFWT does not trigger molecular changes indicative of apoptosis

To begin to elucidate the molecular cues associated with PFWT-mediated cell death, we evaluated three biochemical hallmarks common to apoptosis; activation of caspases, oligonucleosomal DNA fragmentation, and mitochondrial membrane depolarization.

Caspase-3 is one of the key executioners common among the multiple caspase pathways and has been implicated in mediating the death response associated with many chemotherapeutics through the proteolytic cleavage of numerous biologically important proteins including the nuclear enzyme poly (ADP-ribose) polymerase (PARP) [14]. To establish if PFWT-mediated cell death involves caspase-3 activation, MV4-11 cells were treated with PFWT and caspase-3 cleavage was assessed by Western blotting. As shown in Fig. 2a, the 17 kDa cleaved fragment of caspase-3 was not detected at any time point following treatment with PFWT or PFmut. Caspase-3 was cleaved following treatment with etoposide however, indicating this apoptotic pathway is functional in this leukemia cell line. Similarly, the proteolytic cleavage fragment of PARP, a downstream caspase-3 target, was only detected in etoposide-treated cells. Consistent with these findings, PFWT-mediated cell death was not inhibited following pretreatment with the broad spectrum caspase inhibitor zVAD-FMK (Fig. 2b). The ability of zVAD-FMK to inhibit etoposide-induced cell death confirms the inhibitor was indeed active under our experimental conditions. Taken together, these data suggest caspase activation is not necessary for PFWT-mediated death.

As another marker of apoptosis, nucleosomal DNA fragmentation was assessed using terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL). Consistent with the above findings, etoposide-treated cells were strongly positive for TUNEL (Fig. 3), whereas treatment with PFWT failed to induce significant TUNEL staining at any time point examined.

The loss of mitochondrial membrane potential ($\Delta\Psi_m$) is often a key event in cell death and has been demonstrated to mediate the death process through many different mechanisms including the release of molecules involved in caspase activation during apoptosis (cytochrome *c*) [14], as well as the release of molecules involved in caspase-independent death pathways (Endo G, Omi/HtrA2, ROS) [15]. To investigate the involvement of mitochondrial depolarization in PFWT-mediated death, $\Delta\Psi_m$ was monitored by flow cytometry using the fluorescent molecule JC-1. JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red upon the formation of red fluorescent J-aggregates in a polarized environment [16]. As shown in Fig. 4, treatment with the mitochondrial membrane disruptor, CCCP, or etoposide caused a dramatic shift in JC-1 fluorescence from red to green in MV4-11 cells, indicative of depolarized mitochondrial membranes in these cells. However, treatment with PFWT did not induce a fluorescence shift, suggesting maintenance of $\Delta\Psi_m$. The number of cells with loss of or reduced $\Delta\Psi_m$ following PFWT treatment did not fluctuate substantially over time. These data suggest that mitochondrial membrane integrity is preserved following PFWT treatment and that the death processes observed are most likely not mediated by mitochondrial signaling molecules.

3.3. PFWT induces distinct morphological changes in MV4-11 cells consistent with necrosis

The lack of caspase activation, TUNEL staining, and mitochondrial membrane depolarization in MV4-11 cells following PFWT treatment strongly suggests the peptide is triggering a death pathway distinct from classically defined apoptosis. To conclusively determine if PFWT-mediated cell death is distinguishable from apoptosis, primary morphological changes of PFWT-treated MV4-11 cells were examined using electron microscopy. As anticipated, PFWT-treated cells exhibited few if any morphological signs of apoptosis such as condensed chromatin, plasma membrane blebbing, or nuclear fragmentation. Instead, necrotic morphology was observed exemplified by plasma membrane disruption with preservation of nuclear architecture (Fig. 5). By 6 h, spilling of cytosolic constituents into the extracellular space was readily apparent (Fig. 5). Both mitochondrial size and integrity were preserved in PFWT-treated MV4-11 cells, again indicating a lack of involvement of this organelle in mediating cell death. Other observed morphological changes include cell clustering and an increase in cytoplasmic granularity. The lack of chromatin condensation, shrunken nuclei, obvious disintegration of the cell body, or the appearance of vacuoles distinguish this death process from either autophagy or apoptosis. In sum, cell morphology is consistent with necrosis following exposure to PFWT.

3.4. PFWT does not induce DNA single or double strand breaks

One recently identified mechanism responsible for necrosis induced by DNA-alkylating chemotherapeutics involves activation of the nuclear enzyme PARP. PARP catalyzes the hydrolysis of NAD⁺ and transfer of the ADP-ribose moiety to nuclear acceptor proteins following DNA damage. In situations of excessive DNA damage, overactivation of PARP leads to necrosis by depleting the cellular NAD⁺ pool [17,18]. To assess the potential involvement of this pathway in PFWT-induced cell death, double- or single-stranded DNA breaks were assessed by the alkaline phosphatase Comet assay [11]. The presence of Comets was easily visible following exposure of MV4-11 cells to etoposide (Fig. 6). However, Comets were not visible following PFWT treatment at any time point examined. These findings implicate a cell death model that does not involve direct DNA damage.

3.5. PFWT-mediated death is prevented by serine protease inhibitors

Despite the view that necrosis is an uncontrolled process, accumulating evidence suggests this may not always be true. A number of studies demonstrate that a cell can initiate its own suicide by necrosis in a manner that involves distinct signaling pathways [17,19–21]. To differentiate

between spontaneous and programmed necrosis it is crucial to demonstrate that PFWT-mediated cell death can be prevented by interfering with intracellular signaling. To do this, MV4-11 cells were pretreated with a variety of inhibitors previously demonstrated to interfere with signaling pathways implicated in caspase-independent cell death. Pretreatment with 3-methyladenine (inhibitor of autophagy [22]), PJ34 (PARP inhibitor [23]), SB203580 (MAP kinase inhibitor [24,25]), or the antioxidants *N*-acetylcysteine and PDTC failed to inhibit PFWT-mediated cell death as assessed by intracellular esterase activity (data not shown). However, pretreatment with the serine protease inhibitor TLCK [26,27] inhibited PFWT-induced cell death almost completely (Fig. 7a). This is an important finding because it demonstrates that PFWT-induced cell death is not an incidental occurrence involving passive breakdown of cellular integrity, but it is a controlled and modifiable process involving serine proteases. The inability of PFWT to induce erythrocyte lysis *in vitro* [28,29] further illustrates that this peptide does not directly damage the cell membrane (data not shown).

3.6. PFWT induces similar morphologic changes in the MLL-leukemia cell line, Molm13

To further establish the significance of the PFWT death mechanism, it is important to demonstrate the peptide evokes the same death pathway in other sensitive cell types. To do this, the t(9;11)(p22;q23) Molm13 leukemia cell line [30] was assessed by electron microscopy. This cell line expresses the MLL-AF9 fusion protein and has an EC₅₀ of ~30 µg/ml PFWT when measured by calcein AM/ethdr fluorescence (Fig. 7b). Similar to the MV4-11 cell line, treatment of Molm13 cells with PFWT resulted in loss of plasma membrane integrity and extrusion of intracellular contents into the extracellular space (compare Fig. 5–Fig. 7c). Additionally, PFWT-mediated cell death was inhibited with the serine protease inhibitor, TLCK (Fig. 7b). We conclude that PFWT induces necrotic cell death in both MLL-rearranged leukemia cell lines, MV4-11 and Molm13.

4. Discussion

In this report, we describe a form of cell death in which the cell actively initiates necrosis in response to the synthetic peptide, PFWT. This death occurs very rapidly and is effectively inhibited by the serine protease inhibitor TLCK. Although the involvement of serine proteases has been documented in many different experimental models of cell death, in most cases neither the specific serine protease involved nor the precise mechanism of action has been clarified [26,27,31–35]. Likewise, we have not yet identified the serine protease(s) that may be involved here. Among the few serine proteases that have been linked to cell death are granzyme A [36], and the mitochondrial protein Omi/HtrA2 [37–39]. The involvement of granzyme A in this death process is unlikely due to lack of expression in MV4-11 cells (data not shown). Similarly, Omi/HtrA2 does not appear to be involved in this death pathway as its release from the mitochondria into the cytosol was not detected following Western blotting of mitochondrial enriched fractions of PFWT-treated MV4-11 cells (data not shown). Currently studies are underway to try and identify the serine proteases or potential protein targets involved in mediating this rapid cell death process.

Previously, our lab published data concluding PFWT-mediated cell death occurs through an apoptotic mechanism [7]. However, the extensive analysis of the PFWT-mediated death pathway shown here clearly demonstrates that this is not the case. We believe our previous data were misinterpreted due to changes in the autofluorescent characteristics of the cells that result following PFWT treatment. These fluorescent properties often caused our entire PFWT-treated population to increase in fluorescence in our flow cytometry analyses and when interpreted in the absence of extensive controls led us to conclude positive cell staining. Extensive exploration of this phenomenon and inclusion of etoposide controls led us to realize this effect was nonspecific. This misinterpretation was emphasized further upon re-

examination of the original flow cytometry endpoint (caspase activation) using additional techniques (Western blotting, and chemical inhibition).

Understanding this necrotic cell death mechanism may imply novel therapeutic targets with important consequences for attempts to treat diseases that are currently resistant to apoptotic stimuli. Previous studies emphasizing that t(4;11) leukemia cell lines are resistant to apoptosis [40,41] suggest these cancers may benefit from treatments capable of activating other death pathways. In fact, preliminary data from our lab demonstrates synergy in MLL-leukemia cells between PFWT and other known pro-apoptotic agents including 17AAG, etoposide, and cytarabine (Bennett, CA et al., in preparation). Thus identifying agents that trigger regulated necrotic cell death is not only important for the understanding of programmed cell death itself, but has potential implications in the treatment of leukemia either on their own or to complement current apoptosis-based therapies. Given the guarded prognosis of individuals with MLL-rearranged leukemias, new approaches to the treatment of these diseases are welcome.

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Fig. 1. PFWT treatment of MV4-11 cells induces cell death. MV4-11 cells were treated with either 20 μ M etoposide, 25 μ g/ml PFWT, or 25 μ g/ml PFmut for the time indicated. Live cells were detected by flow cytometry as described in Section 2. Values are expressed as a percent of untreated live cell number. Data from three different experiments were compiled for this figure.

Fig. 2. PFWT-mediated cell death does not involve caspase activation. (a) MV4-11 cells were left untreated (U) or treated with either 20 μ M etoposide (E), 25 μ g/ml PFWT (P), or 25 μ g/ml PFmut (M) for the time indicated. Proteins were separated by SDS-PAGE and blotted for cleaved PARP or cleaved caspase-3. Actin was used as a loading control. The Western blots are representative of three experiments. (b) MV4-11 cells were pre-treated with the indicated concentration of zVAD-FMK for 1.5 h followed by an additional 6 h treatment with DMSO, 25 μ g/ml PFWT, 25 μ g/ml PFmut, or 20 μ M etoposide. Live cells were detected by flow cytometry as described in Section 2. Asterisks indicate a statistically significant increase in live cell numbers over etoposide treatment alone ($p < 0.05$). Etoposide + 20 μ M zVAD-FMK treatment gave a p -value of 0.052.

Fig. 3.

PFWT-induced cell death does not involve DNA fragmentation as detected by TUNEL staining. MV4-11 cells were treated with either 20 μ M etoposide, 25 μ g/ml PFWT, or 25 μ g/ml PFmut for the time indicated. DNA fragments were detected using TUNEL staining. The numbers in the top right corner of each plot represent the percent of TUNEL positive cells.

Fig. 4.

PFWT treatment does not induce mitochondrial depolarization. MV4-11 cells were treated with 50 μ M CCCP (30 min), 20 μ M etoposide (6 h), 25 μ g/ml PFWT (time indicated), or 25 μ g/ml PFWmut (time indicated) and stained with the mitochondria selective JC-1 dye. The numbers on each dot plot represent the percentage of gated events with decreased red/green fluorescence intensity ratios attributable to cells with reduced $\Delta\Psi_m$.

Fig. 5. PFWT induces ultrastructural changes indicative of necrosis. MV4-11 cells were treated with either DMSO, 25 μ g/ml PFWT (WT), or 25 μ g/ml PFmut for 6 h and observed by electron microscopy. Arrowhead denotes loss of membrane integrity. Membrane disruption was observed in ~ 20% of PFWT-treated cells. (a) Magnification: 2950x. Bars are 2 μ m. (b) Magnification: 2200x.

Fig. 6.

PFWT does not induce double or single strand DNA breaks in MV4-11 cells. Single and double strand DNA breaks were detected using the alkaline Comet assay following treatment of MV4-11 cells with either 25 $\mu\text{g/ml}$ PFWT (WT), or 25 $\mu\text{g/ml}$ PFWmut (Mut) for the times indicated. Comets were photographed at 200x magnification and 75 Comets per sample were analyzed using Komet 6.0 (Kinetic Imaging). The plot represents the average tail length of the Comets (micrometer) for each treatment condition. Cells were exposed to DMSO or 20 μM etoposide for 6 h as controls.

Fig. 7.

The serine protease inhibitor TLCK prevents PFWT-mediated cell death in leukemia cells. (a) MV4-11 or (b) Molm13 cells were pre-treated with the indicated concentration of TLCK for 1 h followed by an additional 1 h treatment with either DMSO, 25 μ g/ml PFWT, or 25 μ g/ml PFmut. Live cells were detected by flow cytometry as described in Section 2. Asterisks indicate a statistically significant increase in live cell numbers over PFWT treatment alone ($p < 0.05$). (c) Molm13 cells were treated with DMSO, 25 μ g/ml PFWT, or 25 μ g/ml PFmut for 6 h and observed by electron microscopy. Magnification: 2200x. Inset magnification: 11,500x. Bars are 2 μ m.