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**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1658356> since 2018-01-19T17:29:50Z

*Published version:*

DOI:10.1038/tpj.2017.46

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# Therapeutic targeting of membrane-associated GRP78 in leukemia and lymphoma: preclinical efficacy *in vitro* and formal toxicity study of BMTP-78 in rodents and primates

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**Running title:** Targeting leukemia cells with a pro-apoptotic peptide

**Keywords:** Targeted drug delivery, GRP78, Pro-apoptotic peptide

## Abstract

Translation of drug candidates into clinical settings requires demonstration of preclinical efficacy and formal toxicology analysis for filling an Investigational New Drug (IND) application with the US Food & Drug Administration (FDA). Here, we investigate the membrane-associated glucose response protein 78 (GRP78) as a therapeutic target in leukemia and lymphoma. We evaluated the efficacy of the GRP78-targeted pro-apoptotic drug Bone Metastasis Peptidomimetic 78 (BMTP-78), a member of the  $D(KLAKLAK)_2$ -containing class of agents. BMTP-78 was validated in cells from patients with acute myeloid leukemia (AML) and in a panel of human leukemia and lymphoma cell lines, where it induced dose-dependent cytotoxicity in all samples tested. Based on the *in vitro* efficacy of BMTP-78, we performed formal Good Laboratory Practice (GLP) toxicology studies in both rodents (mice and rats) and nonhuman primates (cynomolgus and rhesus monkeys). These analyses represent required steps towards an IND application of BMTP-78 for theranostic first-in-human clinical trials.

## Introduction

Human cancers are characterized by abnormal activation of stress-response chaperons to inhibit apoptosis and regulate senescence and autophagy.<sup>1</sup> Stress-response chaperons consist in two families: heat-shock proteins and glucose-regulated proteins (GRP). Among the latter, GRP78 has been widely studied as a biomarker of endoplasmic reticulum stress and an activator of the unfolded protein response,<sup>2</sup> these processes have been related to drug resistance in tumor cells.<sup>3,4</sup> Unexpectedly, GRP78 has been found associated to the cell membrane in many cancer types, a feature that enables specific targeting by circulating ligands.<sup>5-8</sup> In addition to our original discovery of cell-surface GRP78, we have recently reported that GRP78 is up-regulated and systemically accessible in aggressive variants of human prostate and breast cancer, and can thereby be exploited for theranostic purposes.<sup>9,10</sup>

High expression of GRP78 and its localization at the cell surface in human B-lineage acute lymphoblastic leukemia (ALL) is associated with disease relapse and chemotherapy resistance.<sup>11</sup> GRP78-knockout mice show reduced multiplication of hematopoietic stem cells, loss of lymphoid and myeloid progenitors, and decreased lymphoid cell populations.<sup>5</sup> Conditional knockout in the hematopoietic system indicates that GRP78 plays a mechanistic role both in leukemogenesis<sup>12</sup> and in hematopoietic stem cell survival.<sup>13</sup> In preclinical studies, GRP78 induces AKT oncogenic signaling and leukemogenesis in PTEN-null mice,<sup>12</sup> and its cell-surface association with Cripto is functional in hematopoietic stem cell quiescence.<sup>14</sup>

In this study, we provide evidence that GRP78 accumulates on the surface of cells from patients with acute myeloid leukemia (AML), as well as on human leukemia and lymphoma cell lines, where it can serve as an accessible molecular target. As a leukemia/lymphoma-targeting agent, we tested Bone Metastasis Targeting Peptidomimetic 78 (BMTP-78), a prototype drug composed by a GRP78-binding peptide, WIFPWIQL,<sup>5,7,15</sup> fused to the pro-apoptotic enantiomer  $D(KLAKLAK)_2$ , which disrupts the mitochondrial membrane causing cell death.<sup>5,7,16</sup> Previous

work from our group revealed that BMTP-78 inhibits tumor growth and prolongs survival in preclinical models of breast and prostate cancer.<sup>5, 7</sup> Our current results indicate that BMTP-78 exerts a dose-dependent cytotoxicity *in vitro* in a panel of human leukemia and lymphoma cell lines, as well as primary cells from patients with AML. The overall positive outcome of BMTP-78 treatment in several preclinical models of human cancers prompted us to conduct exploratory Good Laboratory Practice (GLP) toxicology studies in rodents and nonhuman primates towards formal application of an Investigational New Drug (IND) with the US Food & Drug Administration (FDA).

## Materials and Methods

### **Human leukemia and lymphoma cell lines**

The human cell lines were obtained from the Leukemia Cell and Tissue Bank of the Department of Leukemia at The University of Texas M.D. Anderson Cancer Center (UTMDACC). The panel included: MOLT-4 and CCRF-CEM (T-cell ALL), HL-60 (acute promyelocytic leukemia), OCI-AML3 (AML), THP-1 (monocytic acute leukemia), K562 and KBM7 (chronic myelogenous leukemias, CML), SR-786 (anaplastic large T-cell lymphoma), U937 and TUR (monocytic lymphomas), TF-1 (erythroleukemia), and RPMI-8226 (multiple myeloma). Cells were maintained in standard humidified hypoxia chambers (HeraCell 150, Thermo Electron Corporation) with 5% CO<sub>2</sub> and 5% O<sub>2</sub> at 37°C in RPMI 1640 containing 10% FBS, L-glutamine (0.292 mg/ml), penicillin (100 units/ml), and streptomycin (100 units/ml).

### **Patient-derived leukemia cells and non-malignant controls**

Cells from peripheral blood were obtained from the Human Tissue Repository at the University of New Mexico Comprehensive Cancer Center (UNMCCC). All procedures were reviewed and approved by the Institutional Scientific Review Committee and Institutional Review Board (IRB).

Blood samples were obtained from adult AML patients (n=3) and from healthy volunteers (n=3). Mononuclear cells were isolated using Ficoll-Paque Plus (GE Healthcare).

### **Synthetic peptides**

The peptides used in this study were synthesized by Polypeptide Laboratories under Good Manufacturing Practice (GMP) conditions, purified by high-performance liquid chromatography, and lyophilized. Reconstituted material was stable and maintained its potency through preparation and infusion. BMTP-78 is a synthetic peptidomimetic composed of the GRP78-binding motif WIFPWIQL fused to the pro-apoptotic enantiomer <sub>D</sub>(KLAKLAK)<sub>2</sub> through a Gly-Gly linker.

### **Flow cytometry of cell lines**

One hundred thousand cells were blocked in phosphate-buffered saline (PBS) containing 10 µg/ml human IgGs (Sigma) for 30 min on ice. Cells were successively incubated with goat anti-GRP78 antibody (Santa Cruz, Clone C-20 or N-20) in labeling buffer [PBS containing 0.2% bovine serum albumin (BSA), 0.1% sodium azide (NaN<sub>3</sub>), and 5% heat-inactivated donkey serum] on ice for 1 h, washed with washing buffer (PBS containing 0.2% BSA and 0.1% NaN<sub>3</sub>) and incubated with a phycoerythrin (PE)-conjugated anti-goat antibody in labeling buffer for 30 min. After additional rinsing in washing buffer, cells were resuspended in PBS containing 0.2% BSA and analyzed by flow cytometry (BD FACS Canto II).

### **Flow cytometry of primary cells**

Mononuclear cells were isolated from peripheral blood with Ficoll-Plaque PLUS (GE Healthcare) and blocked in human IgGs as described above. The mouse monoclonal anti-GRP78 (clone [10c3], Abcam) and control isotype antibodies were conjugated to PE with the Lightning-Link R-Phycoerythrin kit (Innova Biosciences). Cells were double-stained with anti-CD33 (conjugated to

PercP-Cy5.5) and anti-CD34 (conjugated to allophycocyanin, APC) antibodies (both eBioscience) for 1 h in the dark and on ice. Cells were analyzed with a BD Accuri C6 flow cytometer. Data were processed with FlowJo software package (V.10.0.8, FlowJo LLC).

### **Cell viability and apoptosis assays**

For the viability assays, cells were seeded in 96-well plates ( $2 \times 10^4$  cells/well) in complete medium, and incubated overnight at 37°C with increasing concentrations of either BMTP-78 or an admixture of WIFPWIQL and  $D(KLAKLAK)_2$  at equimolar concentrations (negative control). Viability was determined by measuring the cytoplasmic lactate dehydrogenase (LDH) enzymatic activity with DHL™ Cell Viability & Proliferation Assay Kit (AnaSpec). For the apoptosis assays, OCI-AML cells were seeded in 6-well plates ( $2 \times 10^5$  cells/well), and incubated overnight at 37°C with 20 μM of either BMTP-78 or the control admixture, followed by staining with FITC-conjugated annexin V and propidium iodide (PI, Sigma), and flow cytometry with a BD FACS Canto II.

### **Statistical analysis**

All data are reported as the average mean  $\pm$  standard error of the mean (S.E.M.). Student's *t*-test (unpaired) was used to determine statistical significance. *P*-values are represented as follows: \*  $P < 0.05$  or \*\*  $P < 0.01$ .

### **BMTP-78 toxicology in rodents**

Two studies were conducted with BMTP-78 in CD-1 female mice and Sprague Dawley® male rats at the UTMDACC Michale E. Keeling Center for Comparative Medicine and Research (Bastrop, Texas). The preliminary 5-day range-finding study consisted of 6 groups of 5 CD-1 mice that received a single intravenous (i.v.) or subcutaneous (s.c.) dose of BMTP-78 (30, 100, or 200 mg/kg). A 14-day single-dose range-finding study was performed in Sprague Dawley®

rats. This study was designed as 5-day single dose (s.c.) followed by a 9-day recovery period, and consisted of 4 groups of 5 rats (5, 15, 50 or 150 mg/kg). In both studies, the control group was treated with vehicle alone. All animals were observed prior to dose initiation, and on days 1, 5, 14, 28. Daily observations for signs of toxicity and body weights were recorded. At termination, animals were sacrificed and subjected to complete necropsy with histological evaluation of select tissues. Number of animals, doses and schedule for these studies are shown in Supplementary Table 1.

### **BMTP-78 study in nonhuman primates**

Five GLP toxicology studies were conducted in cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*), at the UTMDACC Michale E. Keeling Center for Comparative Medicine and Research (Bastrop, Texas). Initial average animal weight was 5-9 kg; all animals were initially healthy with no history of chronic illness or injury. BMTP-78 was administered as a single infusion or multiple doses through a peripheral venous catheter. Number of animals, doses and schedules are shown in Table 1. Over the course of the study, the animals were observed twice daily for clinical signs of toxicity, including observations at 1 and 2 h post-infusion on the days of BMTP-78 administration. Body weights were measured weekly, and blood and urine were collected for clinicopathological analysis. At the time of sacrifice, all monkeys were subjected to full necropsy, with tissues collected and processed for histopathological evaluation. Tissues were fixed in 10% neutral-buffered formalin, processed and embedded in paraffin, sectioned at 4-5  $\mu\text{m}$ , and stained with hematoxylin and eosin (H&E). Eyes were fixed in Davidson's solution (30 ml of 95% ethyl alcohol, 20 ml of 10% neutral buffered formalin, 10 ml glacial acetic acid, 30 ml of water), prior to processing and paraffin embedding. All GLP studies (rodents and primates) were registered with the FDA.



## Results

### **Expression of the surface-associated GRP78 in peripheral blood cells of AML patients**

To evaluate GRP78 as a potential target in leukemia, we analyzed its expression on the membrane of patient-derived cells via flow cytometry. The amount of surface-associated GRP78 on peripheral blood cells was markedly increased in all AML samples ( $46.6 \pm 3.9\%$ ) in comparison with normal healthy donors ( $3.6 \pm 1.0\%$ ) (Fig. 1A). To investigate the tumor cell populations expressing GRP78, we used CD33 and CD34 in double-staining with an anti-GRP78 antibody. CD33 is a specific myeloid receptor expressed in leukemic blasts and stem cells in AML,<sup>17, 18</sup> and CD34 is a marker of immature leukemic cells and leukemic stem cells.<sup>19, 20</sup> In patients with AML,  $26.6 \pm 3.3\%$  of the peripheral blood cells positive for CD33 were also positive for GRP78 (Fig. 1B). In healthy donors, the percentage of CD33<sup>+</sup>/GRP78<sup>+</sup> cells was as low as  $1.4 \pm 0.4\%$ . AML patients showed elevated numbers of CD34<sup>+</sup> cells, which were specifically co-stained for GRP78 ( $37.3 \pm 9.4\%$ ; compare with healthy donors,  $1.4 \pm 0.6\%$ ) (Fig. 1C). Given the presence of GRP78 on the surface of leukemia cells from patients, we evaluated its expression in leukemia cell lines, including OCI-AML3 cells derived from the primary blast of an AML patient,<sup>21</sup> and K562 and KBM-7 cells from patients with CML. All cells showed specific staining for surface GRP78 (Fig. 1D). These results show that GRP78 is expressed on the surface of human primary AML cells and leukemia cell lines, confirming previous studies where overexpression of unfolded protein response genes, including *GRP78*, was reported in AML patients.<sup>22</sup>

### **BMTP-78 reduces the viability of human leukemia and lymphoma cells**

We have previously demonstrated that BMTP-78 selectively kills breast and prostate cancer cells, reducing both primary tumor growth and secondary spreading to the lungs and bones in mouse models.<sup>7</sup> Here, we tested whether GRP78 could serve as a target for BMTP-78 in a

panel of human leukemia and lymphoma cell lines. All the cell lines tested were highly sensitive to BMTP-78: markedly reduced cell viability was observed at low molar concentrations, and, in most cases, the maximum cytotoxic effect was reached at  $\leq 10 \mu\text{M}$  (Fig. 2). No detectable effect was observed with the control, which consisted of an equimolar admixture of WIFPWIQL and  $\text{D}(\text{KLAKLAK})_2$ . The effect of BMTP-78 on cell viability was also evaluated in primary cells from patients with AML. Three specimens of peripheral blood with 65-78% blasts were incubated with increasing molar concentrations of BMTP-78 or control peptides (Fig. 3). Notably, higher molar concentrations of BMTP-78 were necessary to decrease cell viability in primary human leukemia cells compared to the above-described cell lines: a complete loss of cell viability was observed at 10-30  $\mu\text{M}$ . The negative control admixture had no detectable effects. Collectively, these results demonstrate the efficacy of BMTP-78 in reducing cell viability of established leukemia and lymphoma cell lines as well as of peripheral blasts from AML patients.

### **BMTP-78 induces apoptosis in human leukemia cells**

We next evaluated morphologic changes in OCI-AML3 cells incubated for 16 h with 20  $\mu\text{M}$  of either BMTP-78 or the control admixture of WIFPWIQL plus  $\text{D}(\text{KLAKLAK})_2$ . On microscopic analysis, cells treated with the control peptides showed a regular morphology, as opposed to cells treated with BMTP-78, which appeared “shrunk” and fragmented into characteristic apoptotic bodies (Fig. 4). The double positivity for annexin V and PI in > 70% of BMTP-78-treated cells confirmed the microscopic assessments. In contrast, cells treated with the control admixture exhibited only background levels of cell death (~5%). We concluded that BMTP-78 induces apoptosis in leukemia cells by specifically targeting cell surface-associated GRP78.

### **Toxicology studies with BMTP-78 in rodents**

An initial dose range-finding study of BMTP-78 was performed in female CD-1 mice (n=5 animals per group) dosed at 30, 100 and 200 mg/kg (i.v. or s.c.). Two control groups were

injected i.v. or s.c. with vehicle only (PBS). Similar signs of toxicity were noted in all treatment groups regardless of the administration route. Affected organs/tissues were: injection sites, lymphoid tissues, lungs, kidneys, and gastrointestinal tract. The lesions were characterized as degenerative, regenerative and/or inflammatory in nature. In most cases, renal and/or immune failure was considered as the cause of death after BMTP-78 administration. Body weights decreased in all mice treated with BMTP-78 (Supplementary Table 1). All mice in control groups appeared normal during the study.

Successively, we performed a 5-day single dose followed by a 9-day recovery period in Sprague Dawley® male rats (n=5 animals per group) administered s.c. with BMTP-78 at 5, 15, 50 and 150 mg/kg. In each group, 2 animals were sacrificed at day 5, and 3 at day 14. The incidence and severity of BMTP-78-related lesions occurred in a dose-dependent manner, and were more severe in the two high-dose groups (50 and 150 mg/kg), where all animals were dead or moribund at day 3. The 15 mg/kg dose appeared to be the midpoint of BMTP-78-related toxicity: 3 out of 5 animals were found dead by day 3. All animals in the 5 mg/kg treatment group survived to study termination with minimal lesions. Body weights in the high-dose groups (50 and 150 mg/kg) were lower compared to the low-dose (5 mg/kg) and vehicle groups. Most affected were the kidneys, but alterations were also seen in the spleen, lymph nodes, and at injection sites. Kidney lesions were generally degenerative/necrotic in nature in the high-dose groups and regenerative in the low-dose groups. Renal failure was considered a cause of spontaneous death in these rats. Given the promising toxicology profile of BMTP-78 at low doses observed in rodents, we used these results to enable a dose-range selection for subsequent studies in nonhuman primates.

### **Toxicology studies with BMTP-78 in nonhuman primates**

Within the class of  $D(KLAKLAK)_2$ -containing compounds, we have previously evaluated GLP toxicities of agents against obesity (adipotide<sup>23, 24</sup>) and cancer (BMTP-11<sup>25, 26</sup>). We reported that

these BMTP-78-like compounds induce reversible renal toxicity and no adverse side effects in monkeys.<sup>24, 26</sup> Inspired by these findings and by the above-described efficacy *in vitro* and in animal models, we evaluated BMTP-78 toxicology profiles in nonhuman primates. Five studies were designed, including single and multiple dose regimens, along with dose-range determination and repeated infusion for the assessment of BMTP-78 safety (Table 1).

Preliminarily, a dose-escalating study (study #1) was performed in cynomolgus monkeys (n=3) serially dosed with BMTP-78 at 0.3, 3, 100 and 200 mg/kg via s.c. administration every 3 days for 2 weeks. The study was terminated after the administration of 100 mg/kg dose due BMTP-78-related toxicity at the injection site and in the kidneys of all monkeys. In the subsequent study (study #2), cynomolgus monkeys (n=3) were scheduled to receive weekly i.v. doses (n=4) of BMTP-78 at 3 mg/kg. Two animals had complications and were euthanized after receiving a single dose of BMTP-78; one received all doses, but presented with necrotizing and inflammatory lesions at the i.v. injection site. Kidney lesions (cortical tubular necrosis) were also observed in one of the monkeys after a single dose. A substantially lower dose (0.75 mg/kg) of BMTP-78 was administered to cynomolgus monkeys (n=2) (study #3) and no adverse effects were detected.

A second dose-ranging study (1-9 mg/kg) in rhesus monkeys (study #4) included 4 animals that were scheduled to receive 4 weekly i.v. doses of BMTP-78. The animal that received the highest dose (9 mg/kg) completed only 2 infusions and was euthanized shortly after the second week of treatment; the necropsy revealed a cardiac lesion not seen in the previous GLP toxicology studies. The lesion was present in the myocardium of the intraventricular septum and was degenerative and fibrotic. Although the animals that received the low (1 mg/kg), mid-low (3 mg/kg) and mid-high (6 mg/kg) doses completed the 4 weeks of treatment, kidney toxicity was observed in the animals treated with 3 and 6 mg/kg of BMTP-78, including tubular necrosis/degeneration and/or regeneration. Few necrotic foci were observed in the mid-low dose (3 mg/kg), minimal necrotic changes in the mid-high dose (6 mg/kg) and mild necrotic changes

in the high dose (9 mg/kg) animal. Similar to the monkeys in study #2, lesions were also observed at the injection site of the monkeys in the mid-low and mid-high doses. A description of protocols and the summary of results are presented in Table 1.

Finally, we tested BMTP-78 safety in a large cohort of female rhesus monkeys (n=20), with 4 i.v. infusions over a 22-day period, followed by a 29-day recovery period (study #5, Supplementary Table 2). The 20 animals were randomized in one control group (45% saline) and low (3 mg/kg) to high (30 mg/kg) dose groups. Each monkey was scheduled to receive BMTP-78 as a 2-h infusion once weekly. Two animals in the high dose group (30 mg/kg) presented with fatal cardiac arrhythmias shortly after the initial injection. A third animal received a single dose of 15 mg/kg and also developed heart arrhythmia with irreversible cardiac arrest. Two additional animals receiving the mid-high dose (9 mg/kg) of BMTP-78 developed fatal arrhythmias, one after the first infusion and one after the third. The other animals in the group developed tachycardia during all 4 infusions. At the low dose (3 mg/kg), all 5 animals displayed sinus tachycardia after the fourth infusion. In contrast with higher doses, this sinus tachycardia did not convert to life-threatening ventricular arrhythmias. Due to the severe heart toxicity and the risk of fatal cardiac arrhythmias, the GLP toxicology studies were terminated early in an abrogated fashion, as required by the FDA.

Altogether these studies led to the conclusion that, despite the promising anti-tumor effect of BMTP-78 *in vitro* and in preclinical cancer models, the current formulation is not suitable to be pursued for cancer treatment because of unexpected cardiac toxicity resulting in unanticipated morbidity and mortality.

## Discussion

The expression of stress-related proteins has been reported in many solid tumors as a response to unfavorable microenvironment conditions such as hypoxia and glucose deprivation.<sup>1, 27</sup> In the bone marrow, low oxygen levels are observed in the endosteal area where hematopoietic stem cells are maintained in a quiescent niche while maintaining a pluripotent state.<sup>28</sup> This phenomenon results from the activation of hypoxia inducible factor 1 (HIF-1) and other targets including VEGFA, CXCR4 and the Cripto/GRP78 complex.<sup>14, 29, 30</sup> The levels of surface-associated GRP78 have been used to distinguish two populations of hematopoietic stem cells: one, characterized as GRP78<sup>+</sup>/CD34<sup>-</sup>, seems to be a myeloid-biased lineage with slow engraft reconstitution ability, while the other (GRP78<sup>-</sup>/CD34<sup>-</sup>) tends to generate a compromised lymphoid lineage.<sup>31</sup> In hematopoietic malignancies, disease progression has been linked to vast expansion of hypoxic areas within the bone marrow and overexpression of the GRP78-binding partner Cripto<sup>32</sup> to form a supramolecular complex on the cell surface. Consistent with these findings, elevated expression of surface-associated GRP78 has been described in several tumor types,<sup>5-10, 33</sup> most of which are characterized by a hypoxic microenvironment. The mechanism of GRP78 relocation from the ER to the plasma membrane seems to be dependent on hypoxia and nutrient deprivation;<sup>34</sup> therefore, its association with co-receptors that trigger cell proliferation and prevent apoptosis is considered a hallmark of cancer cells.

Here we report the expression of surface-associated GRP78 in cells from patients with AML, as well as in different human leukemia cell lines. Our results explore whether GRP78 would be an accessible molecular target for drug delivery, by extending previous results in which expression of surface-associated GRP78 was shown in B-cell ALL<sup>11</sup> and CLL.<sup>35</sup> To address this hypothesis, we tested the GRP78-targeted peptidomimetic BMTP-78 *in vitro* in a panel of human leukemia and lymphoma cell lines and primary cells from patients with AML. BMTP-78 reduced cell viability in all conditions tested and at a lower concentration (< 10  $\mu$ M) than BMTP-11 (60-80

$\mu\text{M}^{36}$ ), an IL11-R $\alpha$ -targeting drug that has been recently evaluated in a first-in-man clinical trial.<sup>26</sup> BMTP-78 treatment resulted in apoptosis, confirming that this compound, like BMTP-11<sup>25, 26</sup> and adipotide,<sup>23, 24</sup> induces cell death by a ligand-specific pathway.

Despite the promising anti-tumor effects of BMTP-78 *in vitro* and in preclinical tumor models,<sup>5, 7</sup> and a favorable toxicity profile in rodents at low doses, its current formulation has shown unexpected side effects in primates, thereby deeming this drug candidate unsuitable for clinical testing. Of note, BMTP-78-related toxicity was not observed in therapeutic studies with tumor-bearing mice, probably due the dose used (15 mg/kg).<sup>6, 8</sup> Toxic effects in mice were observed at 30 mg/kg and above, although in rats 15 mg/kg was already considered a midpoint for the drug test. Interestingly, no cardiac abnormalities were observed in any toxicology studies performed in rodents. The  $\text{D}(\text{KLAKLAK})_2$  pro-apoptotic moiety has been extensively used by us<sup>5, 7, 16, 37</sup> and others<sup>38-41</sup> to develop ligand-directed therapeutics, and two of our most advanced  $\text{D}(\text{KLAKLAK})_2$ -based formulations have been successfully granted safe-to-proceed and translated into FDA clinical trials.<sup>24, 26</sup> These observations allow excluding a causative involvement of  $\text{D}(\text{KLAKLAK})_2$  in the observed adverse events. We speculate that the biochemical features of WIFPWIQL, e.g. its high content in hydrophobic residues (Ile, Phe, Pro, Leu, Trp) might contribute to its insolubility and tissue-specific toxicity. Of note, WIFPWIQL was originally identified by an *in silico* analysis,<sup>15</sup> as opposed to other targeting peptides that our group has selected via *in vivo* screenings and that have been exploited in formulations compatible with preclinical and/or clinical studies. Further investigation is needed to assess a potential toxicity profile of WIFPWIQL. Meanwhile, we have recently identified a new GRP78-targeting motif from an *in vivo* phage display selection in a patient-derived xenograft model of prostate cancer.<sup>10, 42, 43</sup> This peptide, SNTRVAP, might therefore resolve the toxicity issues of the current BMTP-78 formulation.

In the present work, we describe and confirm the presence of membrane-associated GRP78 in human leukemia cells and investigate the targeting of GRP78 with BMTP-78. We conclude

that the first generation of BMTP-78 should no longer be pursued in clinical testing because of its unacceptable toxicity profile in nonhuman primates. However, several aspects merit further comment. First, BMTP-78 will likely still be useful as a molecular imaging probe, because the molar doses used would be several orders of magnitude lower. As such, a study of WIFPWIQL conjugated to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for PET imaging is currently under planning with the lead labeled compound already synthesized in GMP grade (unpublished observations). Moreover, as mentioned, a next-generation GRP78 ligand has been selected and validated for therapeutic applications.<sup>10, 42</sup> Collectively, this GLP-toxicology study serves as a cautionary note in which species-specific toxicity must be taken into account during preclinical drug development. The fact that the toxicity profile of BMTP-78 in rodents was essentially indistinguishable from adipotide<sup>24</sup> and BMTP-11,<sup>26</sup> but the nonhuman primate study showed unexpected cardiac toxicity in the form of lethal heart arrhythmias, is quite remarkable and should give pause to drug development efforts. Nevertheless, despite the toxicity of BMTP-78 described herein, the accessibility of GRP78 and its potential as a target remain a path to be pursued for the treatment of hematopoietic malignancies.



## Acknowledgments

This work was supported by the Specialized Program in Research Excellence (SPORE) in Leukemia from The University of Texas M. D. Anderson Cancer Center (R.P. and W.A), the Gillson-Longenbaugh Foundation (R.P. and W.A), and The Kimberly Patterson Fellowship for Leukemia Research (D.E.J).

## Conflict of Interest

RP and WA are founders and equity stock-holders of Alvos Therapeutics (Arrowhead Pharmaceuticals Research Corporation, Pasadena, CA). Arrowhead Pharmaceuticals has licensed rights to patents and technologies described in this article. RP and WA are inventors on patent applications and intellectual property related to this work and will be entitled to standard royalties if licensing and/or commercialization occur. The University of New Mexico Health Sciences Center manages and monitors these arrangements, along with the University of Texas M.D. Anderson Cancer Center. The other authors declare that they have no competing interests.

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## Figure Legends

**Figure 1.** Detection of cell surface-associated GRP78 in leukemia. (A) The expression of GRP78 was studied in mononuclear cells isolated from peripheral blood of patients with AML compared with healthy donors. Ficoll-purified cells were stained with PE-conjugated anti-GRP78 or control isotype antibody. Cells were double-stained with PE-conjugated anti-GRP78 plus (B) PercP-Cy5.5-conjugated anti-human CD33 or (C) APC-conjugated anti-human CD34 antibody. (D) Leukemia cell lines (OCI-AML3, K562 and KBM-7) were stained with PE-conjugated anti-GRP78 or control isotype antibody and analyzed by flow cytometry. Non-stained cells were used as a negative control. The percentage of double-stained cells is represented. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

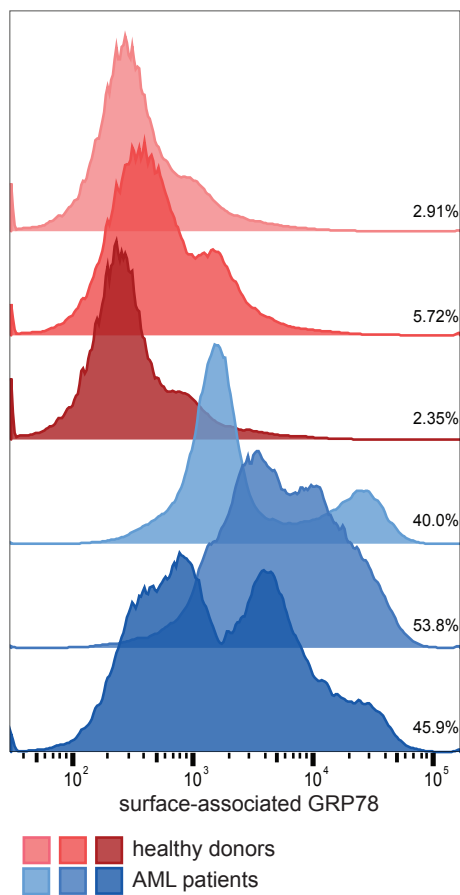
**Figure 2.** Effect of BMTP-78 on cell viability. A panel of human leukemia and lymphoma cell lines was treated with increasing concentrations of BMTP-78 or control peptides. Cell viability was measured by LDH activity. Results represent the average of three independent experiments.

**Figure 3.** Drug activity of BMTP-78 in primary human leukemia cells. Peripheral blood cells purified from three AML patients were incubated with increasing concentrations of BMTP-78 or control peptides. Cell viability was measured via LDH enzymatic activity.

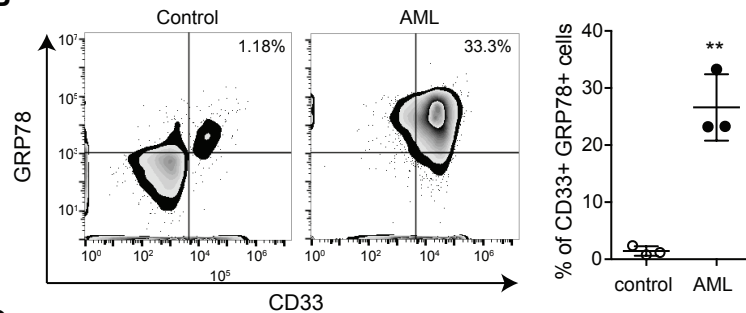
**Figure 4.** Effect of BMTP-78 on cell death by apoptosis. OCI-AML3 cells were treated for 16 h with BMTP-78 or control peptides (20  $\mu\text{M}$ ), followed by staining with Annexin V/PI to detect the percentage of apoptotic cells by flow cytometry. Untreated OCI-AML3 cells served to determine background levels of cell death under the same experimental conditions.

Figure 1

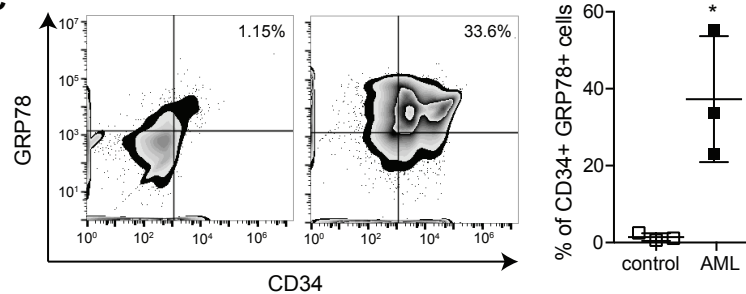
**A**



**B**



**C**



**D**

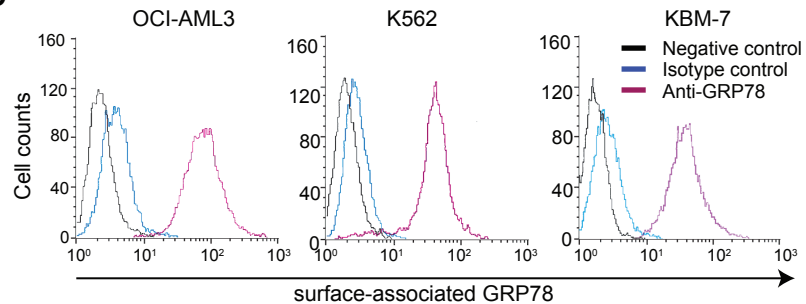




Figure 2

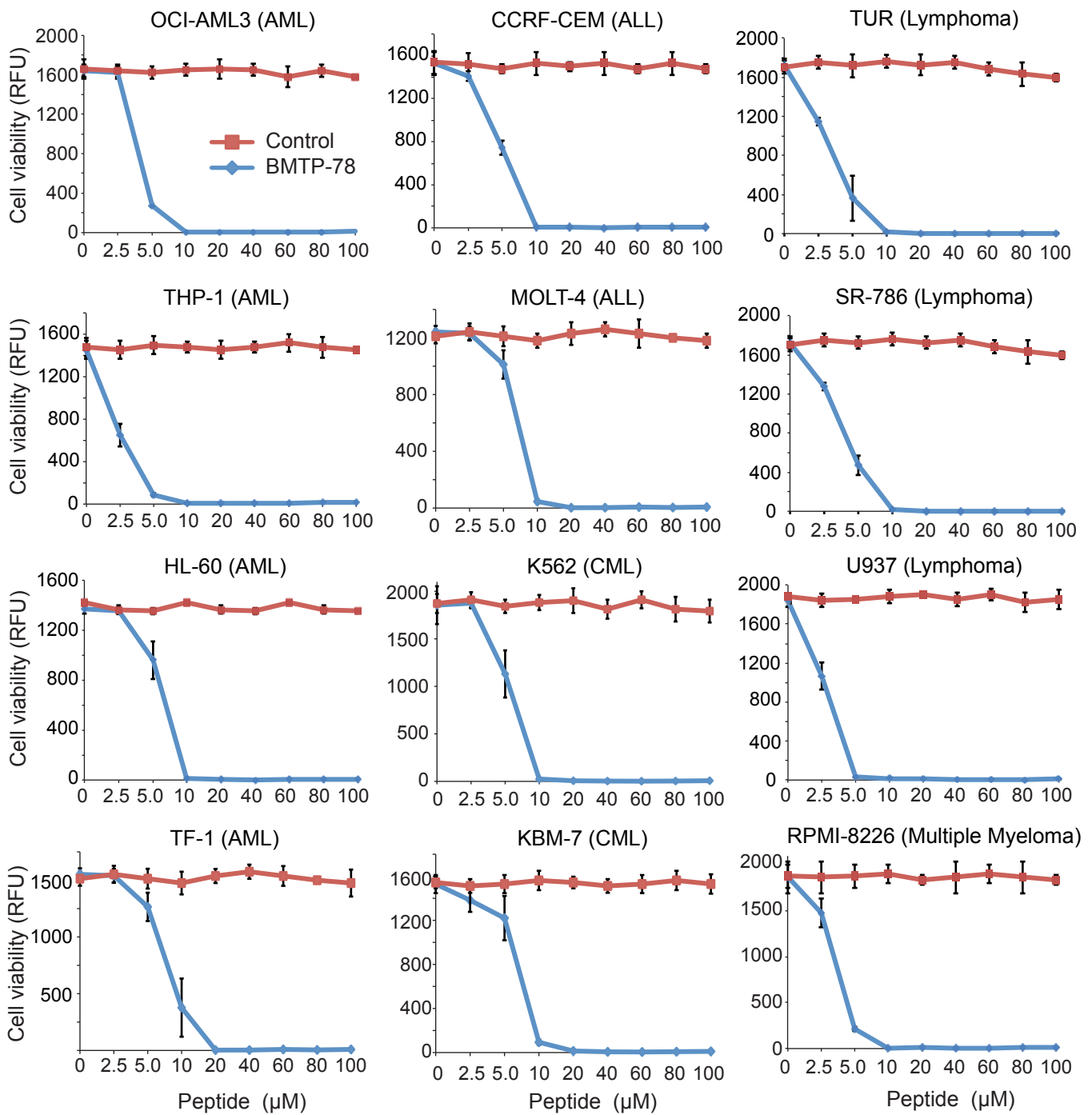


Figure 3

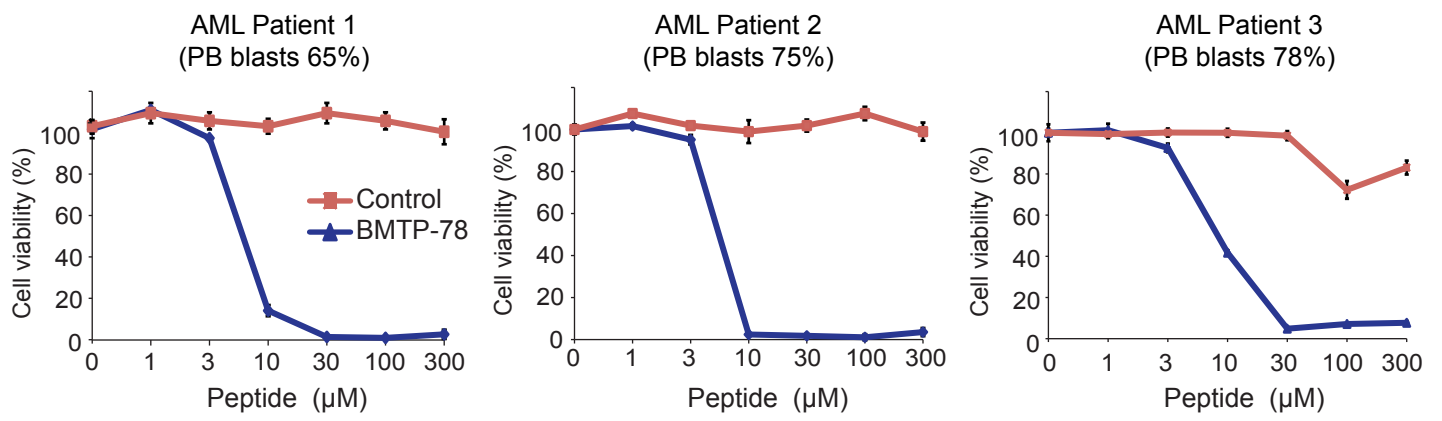
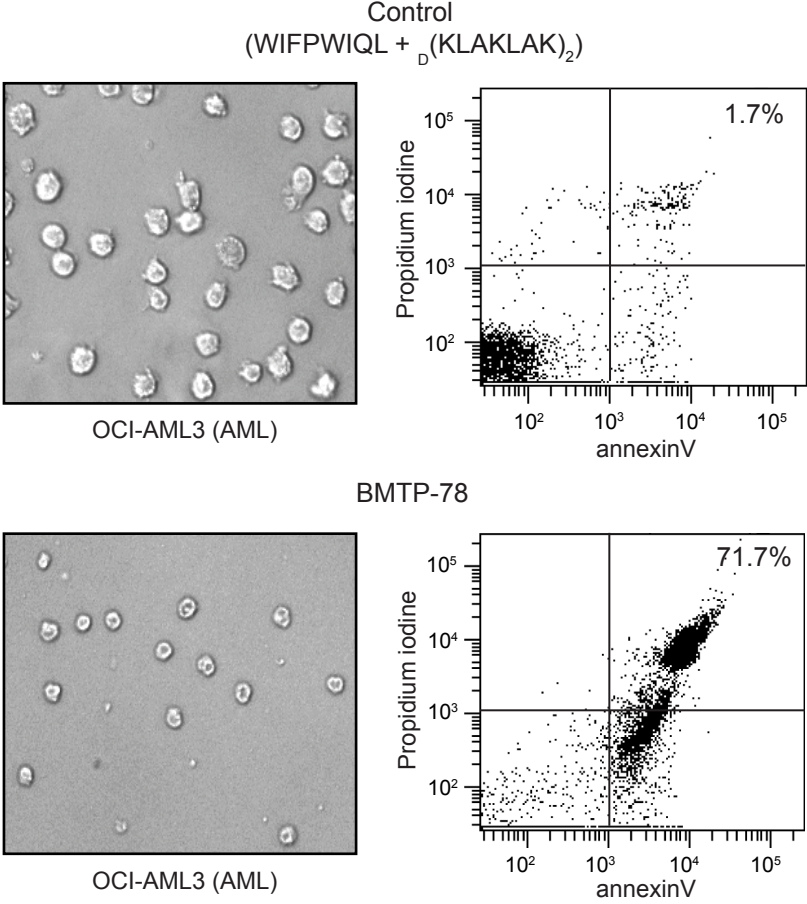


Figure 4



**Table 1.** Toxicology studies of BMTP-78 in nonhuman primates

| Study | Protocol   | Animals                    | Sex | Number of animals | Doses schedule  | Dose (# animals)   | Adverse effects and necropsy results (# affected animals)   |
|-------|--|----------------------------|-----|-------------------|---|--|---|
| 1     | Multiple dose range study s.c. injections of BMTP-78 | <i>Macaca fascicularis</i> | M   | 3                 | 1 dose every 3 days for 2 weeks s.c.                      | 0.3, 3, 30, 100, 200 mg/kg (3)   | Kidney toxicity (after 100 mg/kg dose)<br>Lesions at s.c. injection site  |
| 2     | Multiple dose i.v. infusion                          | <i>Macaca fascicularis</i> | M   | 3                 | 4 weekly i.v.   | 3 mg/kg (3)  | Necrotizing and inflammatory lesions at i.v. injection site (3)<br>Kidney lesions (1)   |
| 3     | Single dose i.v. infusion                            | <i>Macaca fascicularis</i> | M   | 2                 | Single infusion   | 0.75 mg/kg (2)   | None  |
| 4     | Dose range study of weekly infusions of BMTP-78      | <i>Macaca mulatta</i>      | M   | 4                 | 4 weekly i.v.   | Low: 1 mg/kg (1)<br>Mid-low: 3 mg/kg (1)<br>Mid-high: 6 mg/kg (1)<br>High: 9 mg/kg (1)                           | Dose-dependent kidney toxicity (3 at $\geq 3$ mg/kg doses)<br>Lesions at i.v. injection site (3 at $\geq 3$ mg/kg doses)<br>Heart toxicity (1 at 9 mg/kg doses) |
| 5     | 22-day repeated infusion of BMTP-78                  | <i>Macaca mulatta</i>      | F   | 20                | 4 infusions every 5 days i.v. followed by 29-day recovery | Control: vehicle (4)<br>Low: 3 mg/kg (5)<br>Mid-low: 9 mg/kg (8)<br>Mid-high: 15 mg/kg (1)<br>High: 30 mg/kg (2) | Heart toxicity (15 at $\geq 3$ mg/kg doses), lethal at mid-high doses (5 at $\geq 9$ mg/kg doses)   |