Targeted T-Cell Therapy for Human Leukemia: Cytotoxic T Lymphocytes Specific for a Peptide Derived From Proteinase 3 Preferentially Lyse Human Myeloid Leukemia Cells

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Proteinase 3 is present in high concentration in the primary granules of acute and chronic myeloid leukemia blasts, and may represent a potential T-cell target antigen. We screened proteinase 3 against the binding motif of HLA-A2.1. Based on its high predicted binding, a 9-mer peptide, “PR-1,” was synthesized and tested for binding to HLA-A2.1 using the T2 cell line. PR-1 at 100 μg/mL significantly increased expression of HLA-A2.1, with median channel of fluorescence increasing from 22 to 294. Binding half-life was determined to be 1,460 minutes by 125I-labeled β2-microglobulin incorporation. HLA-A2.1+ peripheral blood mononuclear cells from a normal donor were used to generate a T-cell line specific for PR-1. The line demonstrated 85% PR-1-specific lysis at an E:T ratio of 50:1, compared with 20% lysis without PR-1, using T2 cells as targets. It also showed 79% specific lysis to fresh chronic myelogenous leukemia blasts, 54% to fresh acute myelogenous leukemia blasts, and only background lysis (<20%) to HLA-A2.1+ normal allogeneic marrow cells. The amount of lysis of HLA-A2.1+ myeloid cells was proportional to cytoplasmic proteinase 3 expression. Thus, HLA-A2.1-restricted cytotoxic T cells, raised against a peptide contained in proteinase 3, preferentially lysed fresh human leukemic cells.

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MATERIALS AND METHODS

Peptide synthesis. All peptides were synthesized by Biosynthesis (Lewisville, TX) to a minimum of 95% purity as measured by high performance liquid chromatography. The peptide sequences PR-1 and PR-2 were chosen according to their predicted binding to the HLA A2.1 motif based on the known binding affinities of other previously published peptide sequences.24

Patients and donors. After informed consent, cells from patients with leukemia (P1-P5), as well as healthy donors (D1 and D2) were obtained from peripheral blood (PB) or BM. The cells were separated using Ficoll-Hypaque gradient-density (Organon Teknika Co., Durham, NC) and subsequently frozen in RPMI-1640 complete medium (CM, 25 mmol/L HEPES buffer, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin; Gibco BRL, Gaithersburg, MD) supplemented with 20% heat-inactivated fetal bovine serum (FBS; ATLANTA Biologicals, Corvallis, Oregon), or 20% heat-inactivated pooled human AB serum (HS; Pel-Freez, Brown Deer, WI) and 10% dimethyl sulfoxide (DMSO) according to standard protocols. Before use, cells were thawed, washed, and suspended in CM + 10% HS.

Cell lines. T2 cells were kindly provided by Dr Licia Rivoltini (NCI, NIH, Bethesda, MD). T2 cells are a hybrid human cell line which lack most of the MHC class II region including the known TAP (transporter proteins for antigenic peptide) and proteosome genes. They contain the gene HLA-A*0201, but express very low levels of cell-surface HLA-A2.1 and are unable to present endogenous antigens.25 These cells were maintained in culture in CM + 10% FBS.

K562 NK-sensitive leukemia cell line (American Type Culture Collection [ATCC], Rockville, MD), transfected with HLA-A*0201 using previously described methods,24 was used as a target. The HLA-A*0201 gene construct was kindly provided by Dr A. Madrigal (London, UK). To transfet K562 cells with HLA-A*0201, 10 μg of the HLA-A*0201 cDNA gene construct was precipitated and mixed with 5 x 106 subconfluent K562 cells. Electroporation was then performed. Cells were cultured in CM + 10% FBS for 2 days and then transferred to medium containing 1.5 mg/mL (total activity) neomycin (G418; Gibco, Gaithersburg, MD) to select cells expressing the HLA-A*0201 molecule. HLA-A2.1 expression was examined with mouse anti-HLA-A2.1 (supernatant derived from a hybridoma cell line), BB7.2 (ATCC), and stable HLA-A2.1 expression was obtained in greater than 95% of cells after 2 weeks of culture.

U937 (ATCC) is a human cell line derived from the pleural effusion of a patient with diffuse histiocytic lymphoma, and exhibits monocye-like characteristics. It was maintained in CM + 10% FBS.

HLA A2.1 binding assay. Stable HLA-A2.1 expression in T2 cells is observed only when peptides that are capable of binding to and stabilizing the cell surface expression of the HLA-A2.1 molecule are added exogenously.26 T2 cells were washed three times in serum-free CM and suspended at 1 x 10^6 cells/mL. Cells, 1 x 10^6, were incubated for 18 hours in serum-free CM containing 100 μg/mL of each of the peptides and 3 μg/mL of β2-microglobulin (β2m; Sigma, St Louis, MO). The cells were washed with phosphate-buffered saline (PBS) and incubated for 30 minutes on ice with mouse anti-HLA-A2.1 monoclonal antibody (MoAb). The cells were again washed with PBS and incubated for 30 minutes on ice, in the dark, with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (CALTAG Laboratories, San Francisco, CA). The cells were washed three times and then fixed with 4% paraformaldehyde. Cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA).

Peptide binding half-life assay. The peptide binding half-life was determined by measuring the dissociation rate of 125I-labeled β2m from the heterotrimer complex of the HLA-A2.1 heavy chain, peptide, and β2m as previously described.26 Briefly, β2m was labeled for 18 hours at 4°C with 125I and then combined with peptides at 200 μg/mL as well as HLA-A2.1 heavy chain at 20 μg/mL. The labeled heterotrimeric complex was then separated from unincorporated β2m by high-performance liquid chromatography gel filtration, and the half-time of dissociation of β2m was determined by subjecting aliquots of the complex to a second round of gel filtration.26

Generation of peptide-specific T cells from healthy human PB mononuclear cells (PBMC). PBMC from a healthy HLA-A2.1+ donor (D1) were stimulated in vitro with either PR-1 or PR-2 using a protocol adapted from previous studies.24 Briefly, T2 cells were washed three times in serum-free CM and by incubating with peptide at 20 μg/mL for 2 hours in CM. The peptide-loaded T2 cells were then irradiated with 7,500 cGy, washed once, and suspended with freshly isolated PBMC at a 1:1 ratio in CM supplemented with 10% HS. After 7 days in culture, a second stimulation was performed and the following day, 60 IU/mL of recombinant human interleukin-2 (rIL-2) (Biosource International, Camarillo, CA) was added. After 14 days in culture a third stimulation was performed, followed on day 15 by addition of rIL-2. A fourth stimulation was performed on day 21 followed on day 22 by the addition of rIL-2. After a total of 27 days in culture, the peptide-stimulated T cells were obtained and tested for peptide-specific cytotoxicity toward T2 cells, leukemia cell lines, and fresh human leukemia cells.

Cytotoxicity assay. A semi-automated mini-cytotoxicity assay was used to determine specific lysis as previously described.27 Effector cells (D1 cells) were prepared in doubling dilutions from 6 x 104 to 25 x 104 cells/well and were plated in 40 μL, 60-well Terasaki trays (Robbins Scientific, Sunnyvale, CA) with six replicates per dilution. Target cells (T2 cells ≥ peptides, leukemia cell lines, marrow-derived leukemic cells, or marrow-derived cells from a healthy donor) at a concentration of 2 x 105 cells/mL were stained with 10 μg/mL of Calcein-AM (CAM; Molecular Probes Inc, Eugene, OR) for 60 minutes at 37°C. After washing three times in CM + 10% HS, target cells were resuspended at 105 cells/mL (ie, 10 target cells in 10 μL medium were added to each well containing effector cells). Wells with target cells alone and medium alone were used for maximum (max) and minimum (min) fluorescence emission, respectively. After 4 hours incubation at 37°C in 5% CO2, 5 μL Fluorochrome (EB Stain-Quench Reagent; One Lambda, Inc, Canoga Park, CA) was added to each well and the trays were centrifuged for 1 minute at 60g before measurement of fluorescence using an automated Lambda Fluoroscan (One Lambda, Inc). A decrease in the fluorescence emission is proportional to the degree of lysis of target cells, once the released dye is quenched by the hemoglobin contained in the Fluorochrome reagent. The percentage of lysis was calculated as follows:

\[
\text{% Lysis} = \left( \frac{1 - \left( \frac{\text{Mean Experimental Emission} - \text{Mean min}}{\text{Mean max} - \text{Mean min}} \right) \times 100 \right) \times 100\%
\]

Antibodies and flow cytometry. The surface phenotype of the cells was determined by flow cytometry. One million cells were incubated with labeled MoAbs on ice, in the dark, for 30 minutes. After washing three times in PBS, cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The FITC-conjugated MoAbs used were: Goat anti-mouse IgG antibody and mouse anti-HLA class I (CALTAG Laboratories), mouse IgG1 isotype-matched control (Becton Dickinson, San Jose, CA), and CD80 and CD95 (Immunotech S.A., Marseille Cedex, France). Mouse monoclonal anti-HLA-A2.1 antibody...
BB7.2 was derived from culture supernatant of a hybridoma cell line (ATCC) and was not labeled. Cytoplasmic proteinase 3 staining was determined by the following procedure: Cells were permeabilized and fixed with Ortho PermeaFix (Ortho Diagnostics, Raritan, NJ) according to the manufacturer's directions. The cells were stained with 5 μL of proteinase 3 antibody (Accurate Chemical and Scientific Corp, Westbury, NY) and incubated for 40 minutes at room temperature. The cells were washed once with PBA (PBS with 0.18% NaN₃ and 0.1% bovine serum albumin [BSA]). The cells then were labeled with FITC-labeled goat anti-mouse IgG and analyzed by flow cytometry. Ten thousand events were acquired with the Becton Dickinson FACScan for each cell marker, and data were analyzed using Lysis II (Becton Dickinson, San Jose, CA) software.

RESULTS

Peptide synthesis. Peptide sequences contained within proteinase 3 were analyzed for motifs with the potential to bind to the most common HLA-A molecule, HLA-A2.1, present in 49% of the population. Sequences that contained the known anchor motifs at amino acid positions 2 and 9 as well as preferred amino acids at other positions, were sought. A total of 38 peptides were predicted to potentially bind to HLA-A2.1. The first two peptides identified, PR-1 and PR-2, were synthesized (Table 1).

Peptides from influenza B nuclear protein (aa 85-94) and from HTLV-I tax (aa 11-19) were synthesized based on their known binding to HLA-A2.1 to serve as positive controls.

MHC binding and binding half-life assays. Peptides PR-1 and PR-2, together with control influenza peptide, were each pulsed onto T2 cells for 18 hours and the subsequent HLA-A2.1 expression was measured by flow cytometry where increased MHC class I expression corresponds to the degree of binding of the pulsed peptide. T2 cells pulsed with either PR-1 or PR-2 showed strong peptide binding with fluorescence intensities greater than that of the influenza control peptide (Table 1). Low background MHC class I expression was demonstrated by the low fluorescent intensity of the cells without added peptide.

The dissociation half-life of the HLA-A2.1-β2m-peptide complex was measured by monitoring the dissociation of 125I-labeled β2m from the HLA-A2.1 complexes formed in the presence of the peptide to be tested. The two peptides, PR-1 and PR-2, and complexes containing these peptides, bound to HLA-A2.1 and dissociated with half-lives that are typical for antigenic peptides.

Induction of primary cytotoxic T lymphocytes (CTL) responses to peptides. PBMC from a normal healthy donor heterozygous for HLA-A2.1 (D1 cells, Table 2) were stimulated with peptide-pulsed T2 cells. The T2 cell line has been used by others as an antigen-presenting cell for the generation of peptide-specific CTL. The T2 cells were incubated with 10 μg/mL of either PR-1 or PR-2 at 37°C for 2 hours, irradiated with 7,500 cGy, and combined with DI PBMC to induce PR-1-specific and PR-2-specific CTL, respectively. After 4 weeks in culture with weekly restimulation, CTL were tested for specific lysis against Calcein AM-labeled T2 cells loaded or not loaded with peptide.

Figure 1 shows the peptide-specific lysis of the CTL lines against T2 cells loaded with either 1.0 μg/mL of PR-1 or PR-2, or T2 cells without added peptide, at varying effector to target (E:T) ratios. The CTL line generated against the

| Table 1. Peptide Sequences and Binding Data |
| Peptide Name | Amino Acid Sequence | Median Channel of Fluorescence | T 1/2 (min) |
| PR-1 (aa 169-177) | VLQELNVTV | 294 | 1,460 |
| PR-2 (aa 227-236) | RLFPDFTRV | 273 | 140 |
| HTLV-1 tax (aa 11-19) | LLI GFYVPVY | — | 8,000 |

| Table 2. Patient and Donor Cell HLA Types |
| Patient/Donor | Cell Description | HLA-A | HLA-B | HLA-DR |
| D1 | Normal PBMC | 1, 2 | 8, 27 | 3, 16 |
| D2 | Normal marrow | 2, 26 | 13, 58 | 7, 7 |
| P1 | U937 cell line | 3, 3 | 18, 51 | 14, 16 |
| P2 | CML-CP | 2, 28 | 35, 51 | 9, 14 |
| P3 | CML-AP | 1, 2 | 63, 63 | 8, 13 |
| P4 | CML-BC | 2, 26 | 13, 58 | 7, 7 |
| P5 | AML | 2, 3 | 35, 52 | 4, 13 |
| P6 | MDS (RAEB) | 2, 24 | 27, 62 | 1, 13 |

Abbreviations: CML-CP, chronic myelogenous leukemia, chronic phase; CML-AP, chronic myelogenous leukemia, accelerated phase; CML-BC, chronic myelogenous leukemia, blast crisis; AML, acute myelogenous leukemia; MDS (RAEB), myelodysplastic syndrome, refractory anemia with excess of blasts.
PR-1 peptide demonstrated high specific lysis against PR-1-loaded target cells, whereas the CTL line generated against PR-2 did not show any significant cytotoxicity. The data shown are the combined results from three separate experiments using three separately generated CTL lines and displayed as the mean percent specific lysis ± the standard deviation. Cytotoxicity toward T2 cells loaded with HTLV-1 tax (aa 11-19), an irrelevant peptide with high binding affinity to HLA-A2.1, was also measured (data not shown) and resulted in less than 20% specific lysis at E:T ratios of 50:1 by CTL specific for either PR-1 or PR-2.

Table 3 shows the phenotype of the resulting CTL lines after 4 weeks in culture. The data are the results of surface phenotyping by flow cytometry of two separate PR-1-specific CTL lines, and are displayed as the mean of % positive cells ± the standard deviation. Of the total nucleated cell population, 50% ± 4% stained with antibody to CD4 and 21% ± 4% stained with antibody to CD8. No lymphocytes displaying the CD16+ CD56+ natural killer (NK) cell-surface phenotype were detected.

CTL responses to PR-1-loaded T2 cells depend on exogenous peptide concentration. We sought to determine whether there was a dose effect on the observed cytotoxicity of CTL lines against peptide-loaded target cells by incubating T2 cells for 2 hours with varying concentrations of peptide from 0.1 µg/mL to 50 µg/mL and then testing for specific lysis. Figure 2 shows the effect of peptide concentration on specific lysis for the PR-1-specific CTL line, plotted as the difference in the percent specific lysis of T2 cells loaded with peptide minus the percent specific lysis of T2 cells without peptide, at varying E:T ratios. A negative result indicates that the lysis observed when no peptide was present is greater than the observed lysis when the peptide was present at that particular concentration. A clear dose-effect response is demonstrated for PR-1, with the optimal concentration at which to test for lysis being 1 or 0.1 µg/mL of exogenous PR-1 peptide. A similar experiment was performed using PR-2; however, no dose-effect of decreased specific lysis at varying peptide concentrations was demonstrated (data not shown).

CTL responses are HLA-A2.1-restricted. To further show that the CTL response toward PR-1 is specific for target cells expressing the HLA-A2.1 molecule, we prepared T2 cells loaded or not loaded with 1.0 µg/mL PR-1 and again used the CTL line generated against PR-1 to test for specific lysis. Mouse MoAb against HLA-A2.1 was used to block HLA-A2.1-restricted recognition by the CTL line. T2 cells without peptide, but with antibody present, were used to control for any potential nonspecific antibody-mediated cytotoxicity.

Figure 3 shows that with the addition of antibody to HLA-A2.1, specific lysis was blocked. Further, there was only background lysis of T2 cells in the presence of antibody alone. Data shown are the combined results from three separate experiments using three separately generated CTL lines. This demonstrates that the observed cytotoxicity was HLA-A2.1-restricted.

PR-1-specific CTL responses toward HLA-A2.1 human...
myeloid leukemia cells. We next sought to determine if the PR-1-specific CTL line was capable of lysing alloge neic human myeloid leukemia cells from HLA-A2.1+ individuals. Table 2 lists the HLA type and leukemia type of target cells used. As controls, two cell lines expressing low levels of proteinase 3 were used: HLA-A2.1 transfect ed K562 cells and U937 cell line which lack HLA-A2.1 and would therefore be incapable of presenting peptides in an HLA-A2.1-restricted manner.

Cryopreserved BM cells from patients P1-P4, and marrow cells from a healthy normal volunteer (D2, a BM donor for an ABMT performed on patient P3) were thawed and used as targets for the PR-1-specific CTL line.

Figure 4 shows the combined results of three separate experiments from three PR-1-specific CTL lines. In Fig 4A, the specific lysis by PR-1-specific CTL, at various E:T ratios of either U937 cells, HLA-A2.1-transfected K562 cells, or T2 cells with or without exogenously added PR-1 peptide at 1.0 μg/mL is shown. The specific lysis of U937 and HLA-A2.1+ K562 cells by PR-1-specific CTL was lower than the background lysis observed against T2 cells without added peptide.

Figure 4B shows the cytotoxicity of the CTL line against HLA-A2.1+ human myeloid leukemia cells. Marrow cells from three patients with CML (P1, P2, and P3) as well as one patient with AML M4 (P4) were readily lysed, with 53% specific lysis against P1 (a patient with CML in chronic phase) at an E:T ratio of only 6:1. Marrow cells taken from a normal healthy donor (D2) show only background lysis (<20% lysis), similar to that of the control T2 cells without added peptide.

**DISCUSSION**

There is evidence that tissue-restricted proteins, overexpressed proteins, or differentiation antigens can serve as targets for T-cell responses to certain tumors. Tumor-infiltrating lymphocytes (TIL) inducing regressions in malignant melanoma recognize several tissue-restricted normal proteins (Mart-1, gp-100, and tyrosinase). Patients who develop autoimmune manifestations of vitiligo during TIL cell therapy for melanoma often have favorable antitumor responses indicating a melanocyte tissue-restriction rather than a tumor-specific restriction of the T-cell response. TIL cells from patients with other solid tumors also show specificity for
self proteins (eg, HER-2/neu in breast cancer and wild-type p53 in colon cancer).

Numerous examples of proteins with tissue-restricted expression also occur in both myeloid and lymphoid ontogeny. Peptides derived from some proteins (CD45 and CD19) have already been investigated for their ability to stimulate T-cell responses specific for tumors bearing the parent protein. We sought to generate T-cell responses toward myeloid leukemias using a myeloid-specific protein overexpressed in myeloid malignancies as the target. We chose to study the primary granule protein proteinase 3, because the same protein is the target of cytoplasmic antineutrophil cytoplasmic antibodies (cANCA) in Wegener’s granulomatosis. Furthermore, T-cell proliferative responses toward azurophilic granule extracts as well as purified proteinase 3 have been shown in patients with Wegener’s granulomatosis. Thus, if peptides derived from proteinase 3 are contained in the T-cell receptor (TCR) repertoire, it might be easier to generate T-cell responses to peptides contained in this protein in vitro.

In this study we selected from a series of proteinase 3–derived peptides those which were predicted to have high affinity for the anchor residues of the HLA-A2.1 antigen-binding region. Two of these peptides were synthesized and tested in a binding assay. Because of its high binding affinity and long half-life, PR-1 (VLQELNVTV) was chosen to generate a peptide-specific CTL line from a healthy individual. The PR-1 peptide was previously studied by other investigators for its ability to stimulate peptide-specific T-cell responses in patients with Wegener’s granulomatosis. However, they failed to elicit T-cell proliferative responses using peptide concentrations of 20 μg/mL with autologous antigen-presenting cells. In our experiments, peptide-specific cytotoxicity was detectable at peptide concentrations between 0.1 μg/mL to 10.0 μg/mL, but higher PR-1 peptide concentrations did not induce cytotoxicity (Fig 2). Other investigators have found similar dose effects with high peptide concentration leading to decreased cytotoxicity, although the mechanism for this is not understood.

In three separate stimulation experiments, peptide specificity was determined by testing for cytotoxicity against the peptide loaded and nonloaded T2 cell line. We further confirmed by blocking studies that the mechanism of cytotoxicity was via MHC class I and therefore most likely involved CD8+ T cells. The involvement of NK cell–mediated lysis in the CTL line was excluded by the absence of cytotoxicity to K562 cells and the absence of detectable cells of NK phenotype in the effector population. HLA-A2.1 specificity was also shown by the absence of cytotoxicity toward proteinase 3-expressing HLA-mismatched targets.

The CTL line was found to be cytotoxic to HLA-A2.1+ allogeneic myeloid leukemia cells but not to normal marrow cells expressing proteinase 3. These experiments provide the first evidence that CTL specific for peptide sequences found in a normal myeloid granule protein exhibit preferential cytotoxicity to myeloid leukemia cells which overexpress proteinase 3. We found that CML cells were more susceptible targets than AML or myelodysplastic syndrome (MDS) cells. Cytotoxicity corresponded closely to the degree of proteinase 3 expression, as measured by a cytoplasmic fluorescence assay. In contrast to the proteinase 3–expressing leukemia cells, normal marrow cells with relatively low levels of proteinase 3 fluorescence were not killed by the CTL line. Factors other than proteinase 3 expression may have determined the lytic susceptibility of the leukemic targets. However, the cells all expressed high levels of MHC class I and the B7.1 costimulatory molecule, which are cell-surface molecules important for directing T-cell killing.

Thus, it is possible that CTL exhibiting in vitro cytotoxicity to proteinase 3–expressing cells may have antileukemic
activity in vivo. Such CTL lines could be used in adoptive immunotherapy strategies to eradicate residual disease, for example, after marrow transplantation. However, anti–PR-3 CTL interactions with myeloid leukemia cells have yet to be fully characterized. The therapeutic efficacy of such T-cell lines might be reduced for example if leukemic progenitors not overexpressing proteinase 3 escape immune destruction. Alternatively, leukemia cells could escape T-cell killing by downregulating proteinase 3 expression during clonal evolution. Some of these issues could be resolved by leukemia colony-inhibition assays’ and studies of adoptively transferred PR-1–reactive CTL in a human AML/severe combined immunodeficient mouse model. It would also be important to determine whether CTL with specificity for proteinase 3 inhibit normal marrow cell proliferation. A low level of expression of proteinase 3 in normal CD34+ cells would suggest that proteinase 3–reacting cytotoxic T cells may spare early progenitors and might only cause at worst a reversible cytopenia. It is also possible that the proteinase 3 gene is polymorphic. In the context of marrow transplantation, T-cell donors not expressing a common polymorphism of proteinase 3 could be used to raise CTL recognizing only the alternative proteinase 3 allele on the recipient myeloid cells, and not the proteinase 3 allele on the donor-derived hematopoietic cells.

CTL lines recognizing overexpressed myeloid-restricted proteins such as proteinase 3 could eventually be used in clinical trials; initially, for example, in the treatment of leukemic relapse after marrow transplantation. Leukemia cells from most patients with CML at all stages of disease evolution over-express proteinase 3, as do approximately 50% of patients with AML. To avoid the risk of damage to normal donor hematopoiesis, T cells transferred to the recipient could be transfected with the thymidine kinase (Tk) gene, allowing their elimination by administration of ganciclovir along with the retransfusion of further donor CD34+ cells, should marrow aplasia develop. Alternatively, such CTL could be used to purge autologous BM ex vivo, or to prevent leukemic relapse in patients transplanted for high-risk myeloid leukemias by using such peptide-specific CTL as part of the T-cell component of the graft.

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Targeted T-cell therapy for human leukemia: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells

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