# AN INTEGRATIVE APPROACH DISCOVERS A NOVEL ANTI-LEUKEMIC PEPTIDE FROM HUMAN MILK


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## Abstract

Chemotherapy in childhood leukemia is associated with late morbidity in leukemic survivors, while certain patient subsets relatively resistant to standard chemotherapy. Identifying new agents with the sensitivity and selectivity toward leukemic cells with less systemic toxicity is a warrant. Peptide-based therapeutics is gaining attention during the last few years. Here, we used an integrative workflow combining mass spectrometric peptide library construction, *in silico* anticancer peptide screening, and *in vitro* leukemic cell studies to discover a novel anti-leukemic peptide owning 3+charges and alpha-helical structure, namely HMP-S7, from human breast milk. HMP-S7 showed cytotoxic activity against four distinct leukemic cell lines in a dose-dependent manner but had no affected on solid malignancies or representative normal cells. HMP-S7 induced leukemic cell death by

penetrating the plasma membrane into the cytoplasm, causing lactate dehydrogenase leakage, thereby defining membranolytic action. In conclusion, HMP-S7 is the selective anti-leukemic peptide promising for further validation in preclinical and clinical studies.

**Keywords:** Anticancer peptide; Cancer cytotoxicity; Human breast milk; Leukemia; Peptidomics; Virtual screening

## MAIN TEXT

### Introduction

Cancer is a significant cause of death in children and adolescence during the last few years in Asia, Central and South America, Northwest Africa, and the Middle East (*1*). Hematologic malignancies, particularly acute lymphocytic leukemia (ALL), are predominant, accounting for 30% of childhood cancer (*2*). Optimization of chemotherapeutic regimens during the past decades has resulted in more than 90% remission rate in childhood ALL (*3*). However, the burden of late morbidity due to chemotherapeutic treatments, which occur in two-thirds of pediatric cases (*4*), has become important considerations as the number of long-term leukemic survivors increases (*5*). Moreover, specific subsets of pediatric ALL are relatively resistant to standard chemotherapy and have a high risk of relapse (*6*). These challenges pose the need to further improve current treatment while identifying new agents should also be concerned with the sensitivity and selectivity toward leukemic cells with less-to-no toxicity to normal cells.

Peptide-based drugs, or anticancer peptides, can be a new strategy for cancer treatment. Although the exact mechanism and selectivity criteria have yet to be elucidated, anticancer peptides may have oncolytic effects depending on peptide characteristics and target membrane features in selectivity and toxicity (*7*). Cancer cells have high negative transmembrane potential, greater membrane fluidity, more abundant microvilli (increasing outer surface area), the net negative charge from anionic molecules on the surface. In contrast, normal cells are electrically neutral (*8-11*). These negative charges on the cancer cell membrane are attractive to the positive charge peptides to disturb membrane stability, causing the loss of electrolyte and cell death (*8*), while high cholesterol contents of the normal cell membrane can protect cell fluidity and block cationic peptide entry (*12*). Common strategies for anticancer peptide discovery are; i) activity-guided purification from biological/natural products (*13*); ii) examination of antimicrobial peptides for cancer sensitivities (*14, 15*). The former approach tends to deliver positive results, but it is associated with labor-intensive and time-consuming processes. The latter strategy is relatively cost- and time-effective but still limited to antimicrobial peptides known a priori. Investigation of anticancer peptides has progressed slowly in the past decades, indicating gaps for improvement, particularly on the selection of peptide sources, screening methods, and downstream analyses.

Human milk is a promising source of therapeutic peptides. Bioactive milk peptides are released from sourced proteins by enzymatic hydrolysis, fermentation with a proteolytic starter culture, and proteolysis with proteolytic microorganisms (*16, 17*). Although human milk has rarely been investigated for anticancer activities, studies have showed human milk-derived peptides could exhibit various biological effects such as antimicrobial (*18, 19*) and immunomodulatory activities (*20*). Human milk-derived beta-casein fragments have been studied for immunomodulation, antibacterial, antioxidant, opioid agonist, antihypertensive activities, and cell proliferation of human preadipocytes (*21*). These bioactive peptides have differed in amino acid compositions and sequences, and the peptide length can vary from two to twenty amino acid residues (*22*). It is anticipated that human milk also contains

anticancer peptides as that of bovine milk. PGPIPN hexapeptide of bovine β-casein can inhibit invasion and migration of human ovarian cancer cells (*23*). ACFP, an anti-cancer fusion peptide derived from bovine β-casein and lactoferrin, can inhibit viability and promote apoptosis in primary ovarian cancer cells (*24*). Interestingly, breastfeeding for six months or longer was associated with a 19% lower risk of all childhood leukemia than shorter breastfeeding or none (*25, 26*). Identifying novel anti-leukemic peptides from human milk is a warrant for the future development of non-allergic and non-toxic peptide-based drugs to improve childhood leukemia therapy.

This study aimed to discover a novel anti-leukemic peptide with less cytotoxicity toward normal cells from human milk. We applied a robust workflow integrating the strength of liquid chromatography-tandem mass spectrometry to generate a natural-occurring human milk peptide library *in silico* screening based on physicochemical, structural, and predictive anticancer properties by machine learning algorithms to prioritize candidates for functional studies of synthetic peptides to determine anti-leukemic activity and the mode of action. By this strategy, a novel anti-leukemic peptide was identified from human milk as the main outcome.

## Results

To separate human milk peptides and test their cytotoxicity to leukemic cells, ten healthy mothers aged between 25 and 36 years old (32.3±3.27 years old) donated breast milk at once during 6-129 days (63.8±38.97 days) after delivery (details in **Table S1)**. After multiple steps of centrifugation to remove cells, lipids, and extracellular vesicles, and then ultrafiltration (3-kDa cutoff) to remove proteins, the small molecules (<3-kDa), except the peptides, passed through the C18 SPE. Moreover, the C18 bound peptides were eluted by various acetonitrile concentrations and then performed their cytotoxicity. The conceptual framework of this study is illustrated in **Figure 1**.

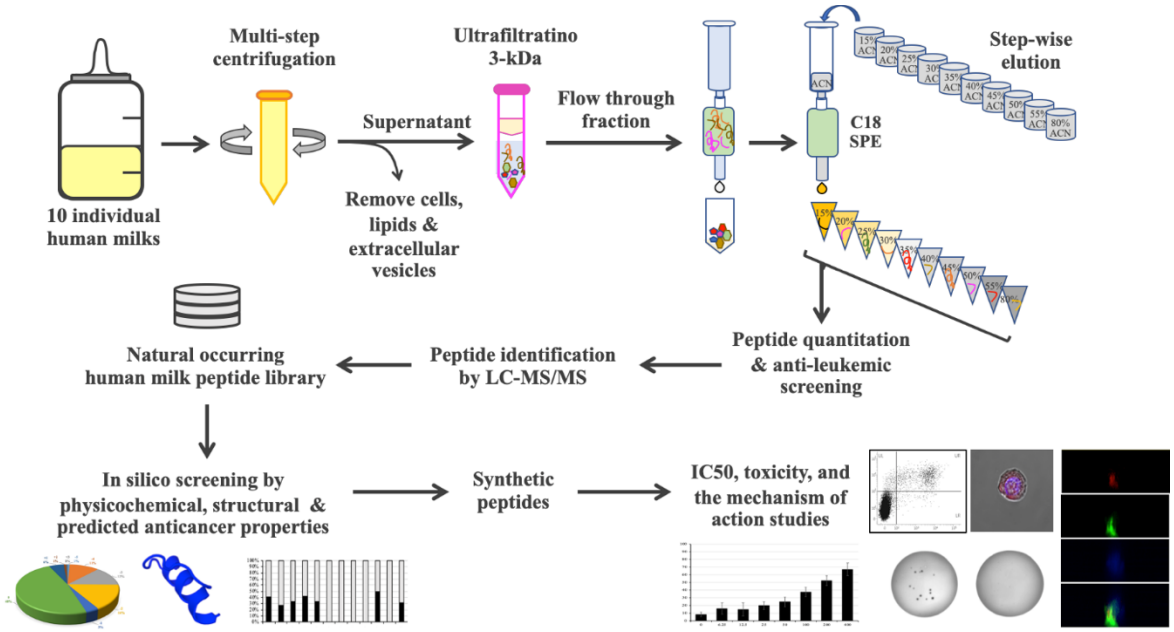


**Figure 1**. **A conceptual framework of the integrative strategy for discovering a novel human milk-derived anti-leukemic peptide**. This strategy combines the strengths of mass spectrometry for high-throughput peptide identification, *in silico* screening for prioritizing peptide candidates, and experimental validation for anti-leukemic activities. Abbreviations:

LC-MS/MS, liquid chromatography-tandem mass spectrometry; IC50, half-maximal inhibitory concentration; SPE, solid-phase extraction.

*Most human milk peptide fractions had cytotoxic effects on leukemic and normal cell lines*

After C18 bound peptide elution, the crude milk peptide fraction was tested to observe cytotoxic effects against Jurkat (T lymphoblastic leukemia) and FHs74Int cells (the representative normal intestinal epithelium), respectively (**Figure 2A**). The crude milk peptides showed a potent cytotoxic effect on Jurkat but had less impact on FHs74Int cells. The coarse milk peptide fraction was then fractionated by C18 SPE with the step-wise ACN elution, in which the chromatogram was demonstrated by the peptide amounts (**Figure 2B**). These eluted peptides by 15%-80% ACN were treated to Jurkat leukemic cells and FHs74Int normal cells to observe cell survival as shown in **Figure 2C**. The low peptide amount in 15%-30% ACN eluted peptide fractions could decrease Jurkat cell survival more than the highest peptide amount (45% ACN eluted peptide fraction). This indicated that the elimination of Jurkat leukemic cells depended on the specific peptide sequence with hydrophilic property more than peptide amount.

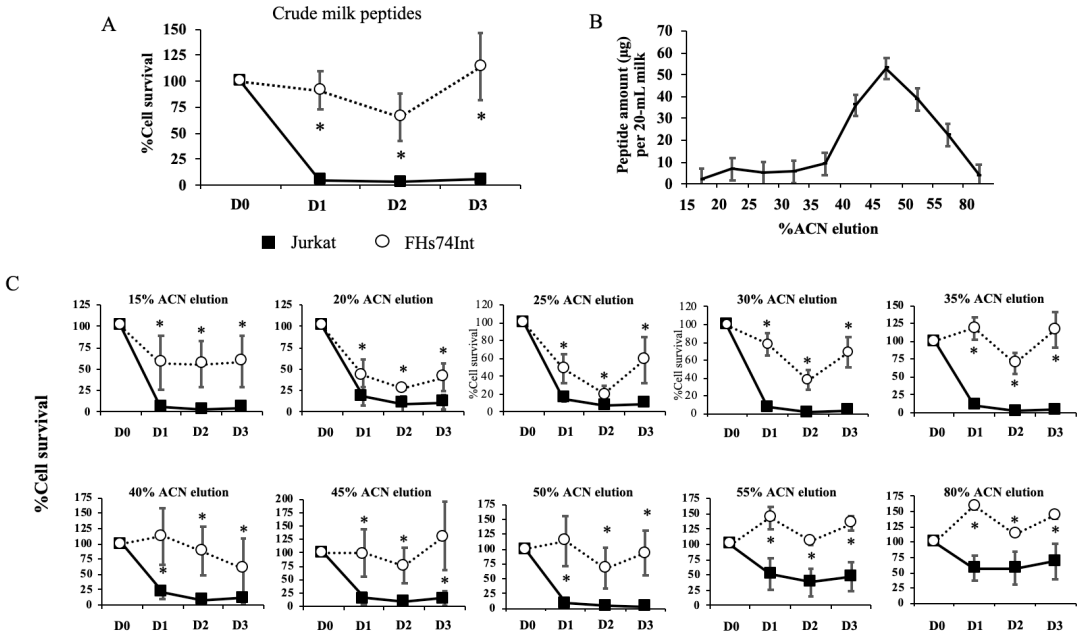


**Figure 2. Human milk peptide fractionation and cytotoxicity profiles against leukemic and normal cells.** (**A**) After centrifugation and passing through <3 kDa cut-off column, the milk solution (20 mL) was passed through the C18 column. The milk peptides were bound to the C18 SPE column, eluted with 80% ACN (1 mL), and dried using a SpeedVac concentrator. The crude milk peptides were resuspended in the culture medium and then treated to Jurkat and FHs74Int cells for 1-3 days (n=3 biological replication). The graph shows % cell survival (mean±SEM). Peptides were then fractionated into 10 fractions by C18 SPE column with the step-wise acetonitrile elution. An equal volume (20 ml) of the individual human milk supernatant (<3 kDa) was flown through the C18 SPE column. The human milk peptides in the supernatant were bound to the column and eluted by equal volume (1 mL) of 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, and 80% ACN in subsequently. These peptides were subjected to LC-MS/MS to generate the peptide library. (**B**) The fractionated peptides were resuspended in deionized distilled water and then quantitated using Bradford's assay. The peptide amount in each fraction was calculated as

mean ± SEM. (**C**) Also, Jurkat and FHs74Int cells were treated with the 10 eluted fractions of human milk peptides of 3 individual volunteer mothers for 1-3 days. The remaining live cells after the 10 peptide fraction treatment were detected by WST-1 solution at 440 nm. The OD was subtracted by culture medium without cells (blank). The percentage of cell survival was calculated by (OD of the sample)/(OD of non-treatment)×100. *; $p<0.05$ comparing to non-treatment condition (n=3 individual milk specimens with 3 technical replicates for each specimen).

*Peptide identification, library construction, and in silico anti-cancer peptide screening*

Eleven fractions (from one crude and ten step-wised ACN eluates) of human milk peptides were identified by LC-MS/MS. A total of 142 naturally occurring human milk peptides with unique sequences were collected into the peptide library, ready for further analyses (**Table S2**). The distribution of all identified peptides by fractions, peptide length, p*I*, and the net charge was shown in **Figure 3A** (upper panel). Overall, most identified natural occurring-milk peptides had less than 20 amino acids in length, contained zero and anionic net charge with inclining acid property. The most frequently detected pattern was the natural peptides with proline-rich sequence (**Figure S1**). This proline-rich peptide is probably crucial to some biological effects of human milk. For example, a ligand containing a proline-rich sequence or a single proline residue is involved in protein-protein interaction (*27*). Proline-rich sequence-contained antimicrobial peptides can kill microorganisms by interacting with 70S ribosome and disrupting protein synthesis (*28*).

*In silico* screening of the natural-occurring human milk peptide library was performed by a robust workflow. Firstly, we screened the library by physicochemical properties, i.e., length, *p*I, and net charge (as shown in **Figure 3A**) since most of the known anticancer peptides are small cationic peptides (commonly 5-30 amino acid length with net charge +2 to +9) that bind to the negative charge of phosphatidylserine and sialic acid on the cancer cell membrane (*12, 29, 30*). Secondly, the secondary structure of the peptides were predicted by the PEP-FOLD3 webserver (**Figure 3B**). Peptides can form alpha-helix, beta-sheet, and random coils (*31*). However, most of the known anticancer peptides shared the alpha-helix structure (*32*). Thirdly, the identified peptides were predicted for anticancer properties using machine learning prediction software. Since ACP prediction software could provide different results depending on the algorithms and training datasets, four online-accessible software were then applied in this study, including ACPpred-FL (*33*), antiCP 2.0 (*34*), MLACP (*35*), and mACPpred (*36*) (**Figure 3C**). As a result, eight human milk peptides and one bovine milk peptide (as a positive ACP control) from various prioritization based on net charges, secondary structures, and the predicted anticancer properties were selected for further analysis (**Table S3)**.

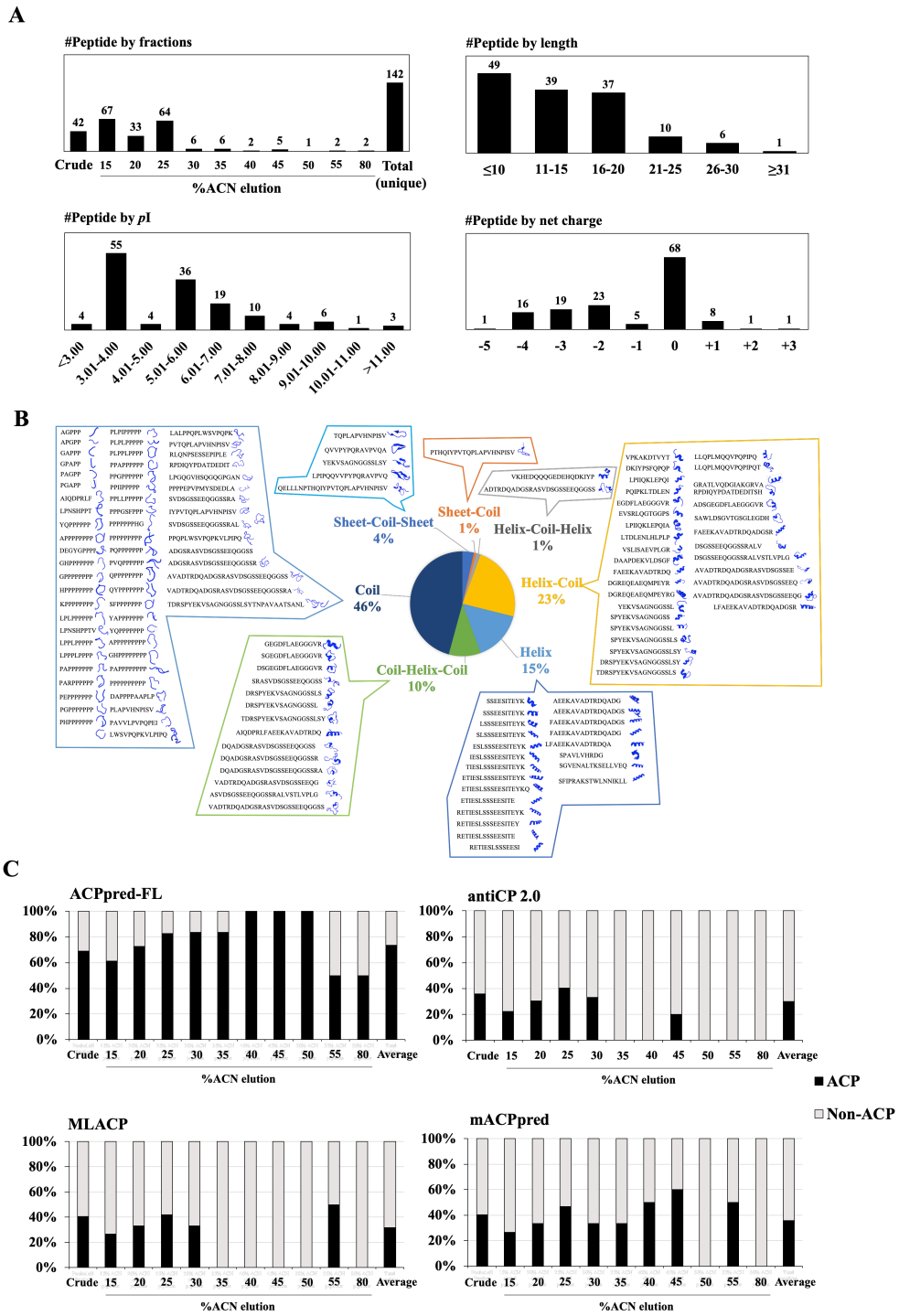


**Figure 3. Predicted physicochemical, structural, and machine learning-based anticancer peptide screening of natural occurring-human milk peptides**. (**A**) Distribution of the unique human milk peptides identified by LC-MS/MS. (**B**) the distribution and the secondary structure of all identified peptides predicted by PEP-FOLD3 software. (**C**) Proportions of the predicted ACP vs. non-ACP peptides by four ACP machine learning programs, including ACPpred-FL, antiCP 2.0, MLACP, and mACPpred. The percentage of ACP (black) and non-ACP (gray) was calculated by (number of ACP or non-ACP prediction/number of total identified peptides in individual fraction) ×100%. Full results of *in silico* ACP screening was provided in **Table S2**.

*HMP-S7 killed leukemic cells but not normal cells or solid tumor cell lines*

**Figure 4A** illustrated eight selected human milk-derived peptides and a known bovine milk-derived anticancer peptide synthesized for cancer cytotoxicity screening using trypan blue staining. These results showed that HMP-S7, the highest cationic charged peptide (+3 net charge) with α-helical structure, had higher inhibitory activity than the other human milk-derived peptides against four leukemic cell lines, including Jurkat, Raji, RS4;11, and Sup-B15 cells **(Figure 4B)**. To elucidate the selectivity of HMP-S7, the effect of HMP-S7 was further observed on normal cells, i.e., T cells and HEK293T embryonic kidney cells, and solid tumor cell lines, i.e., SH-SY5Y neuroblastoma, HepG2 hepatoblastoma, A549 lung adenocarcinoma, MDA-MB-231 breast cancer, and HT-29 colorectal adenocarcinoma by trypan blue staining (**Figure 4C**). Compared to BMP-S6, the known anti-cancer peptide, HMP-S7 had less cytotoxicity toward the normal cells and most cancer cells. This result suggested that HMP-S7 was more selective to leukemic cells than the BMP-S6. Although HMP-S7 had no cytotoxicity on normal T cells, it showed a mild cytotoxic effect on HEK293T cells. Based on these activity profiles, HMP-S7 was chosen to further examine the IC50 against four leukemic cell lines (**Figure 5A**). The results demonstrated anti-leukemic activity of HMP-S7 was dose-dependent, with the IC50 ranged from 89.2 to 186.3 μM depending on leukemic cells to be tested. Furthermore, the inhibitory activity of the HMP-S7 treated Jurkat leukemic cell lines was confirmed by the colony-forming assay using soft agar, as shown in **Figure 5B**. Because HMP-S7 could induce leukemic cell death; thereafter, its cell death mechanism should be further investigated.

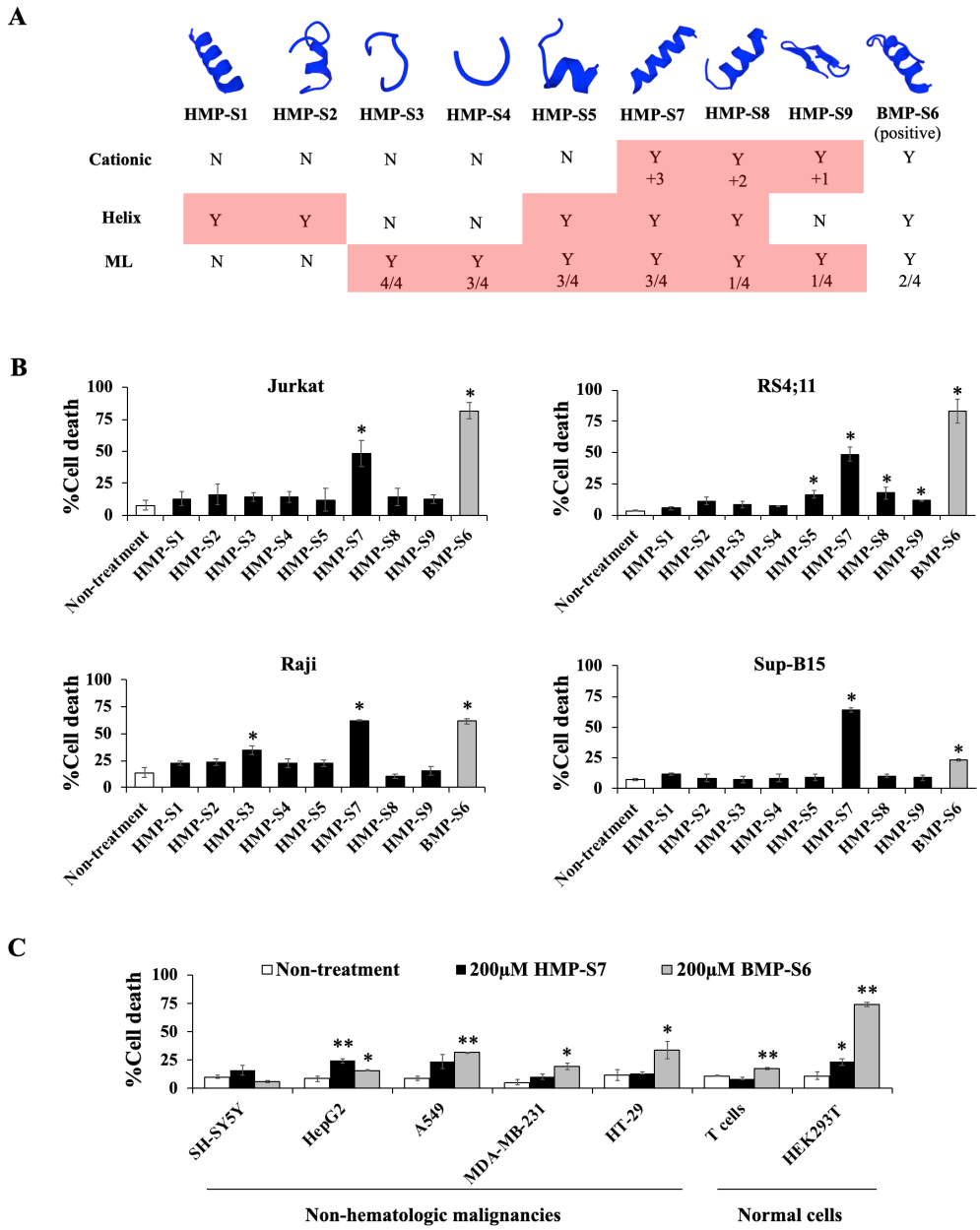


**Figure 4. Effect of the 8 synthetic human milk peptides on leukemic and non-leukemic cell lines.** (**A**) After the combination of physicochemical properties with cationic peptides, helical structure, and machine learning prediction, the 8 synthetic human milk peptides (HMPs) and the positive control (BMP-S6) (the details of the selected peptides were summarized in **Table S3**) were examined for anti-leukemic activity. (**B**) Four leukemic cell lines, including Jurkat, Raji, RS4;11 and Sup-B15, were treated with 8 synthetic HMPs and BMP-S6 at 200 μM to observe %cell death after 24 h treatment using trypan blue exclusion assay under light microscope. The % cell death was calculated by (number of death cells/total cell number) ×100. The percentage of cell death of all 4 leukemic cell lines after HMP-S7 treatment can significantly increase. (**C**) Besides leukemic cell treatment, HMP-S7 was treated to non-hematological malignancy cell lines, including neuroblastoma (SH-SY5Y), hepatoblastoma (HepG2), lung cancer (A549), triple-negative breast cancer (MDA-MB-231), colon cancer (HT-29), and normal cells including T cells and HEK293T embryonic kidney cells. Statistical significance was calculated by %cell death of three biological replicates. *$p$<0.05, ** $p$<0.01 compared to non-treatment condition.

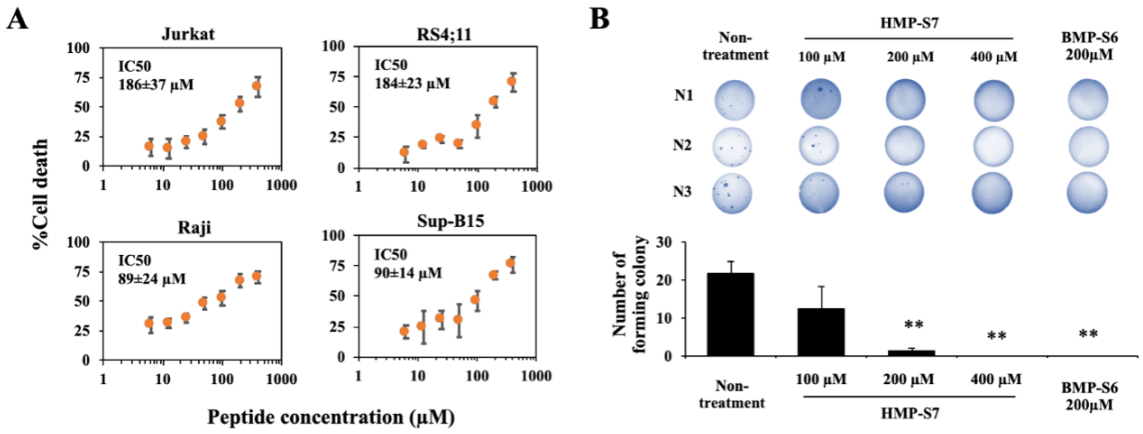


**Figure 5. The inhibitory concentration of HMP-S7 treated leukemic cells.** (**A**) Four leukemic cell lines i.e., Jurkat, Raji, RS4;11, and Sup-B15 cells, were treated with HMP-S7 in various concentrations (0-400 μM) for 24 h and their %cell death (mean ± SD) was counted by using trypan blue exclusion assay. (**B**) The number of the forming colony of HMP-S7 (100 μM, 200 μM, and 400 μM) treated Jurkat cells was confirmed the anti-leukemic activity of HMP-S7. The positive control peptide was BMP-S6 at 200 μM concentration. After 24 h treatment, the cell suspension was equally volume taken into soft agar to form colonies for 20 days. The colonies in the soft agar were stained with crystal violet and counted. **, $p<0.01$ compared to non-treatment condition (n=3 biological replicates).

*Mechanisms of HMP-S7-induced leukemic cell death*

To elucidate whether HMP-S7 can act as the membranolytic peptide against leukemic cells, Jurkat cells were treated with HMP-S7 (untagged or FITC-tagged) at the IC50 for 24 h. By confocal microscopy, PI staining of cytoplasmic RNA was observed in the untagged HMP-7 and the positive control Triton X-100 (**Figure 6A**). The FITC-tagged HMP-S7 was located in the cytoplasm as PI stain of cytoplasmic RNA. Flow cytometry showed that the HMP-S7 could be more cumulative into cytoplasm when increasing the concentrations from IC50 to 2×IC50 (**Figure 6B** and **6C**). By lactate dehydrogenase (LDH) release assay, intracellular LDH leakage into the cell culture supernatant was evidence at 24 h in HMP-S7-treated RS4;11 cells (at IC50 and 2×IC50) and Triton X-100 treated cells (**Figure 6D**). Finally, HMP-S7-induced leukemic cell death was confirmed by flow cytometry using Annexin V/PI co-staining. The number of apoptotic cells was significantly increased compared to non-treatment condition, while a dose-response relationship was observed in four leukemic cell lines with IC50 and 2×IC50 of HMP-S7 treatment (**Figure 6E** and **6F**). Taken together, our findings suggested that HMP-S7 attacked leukemic cells to form micropores on cellular membranes and penetrated into the cytoplasm to perturb leukemic cells until the death.

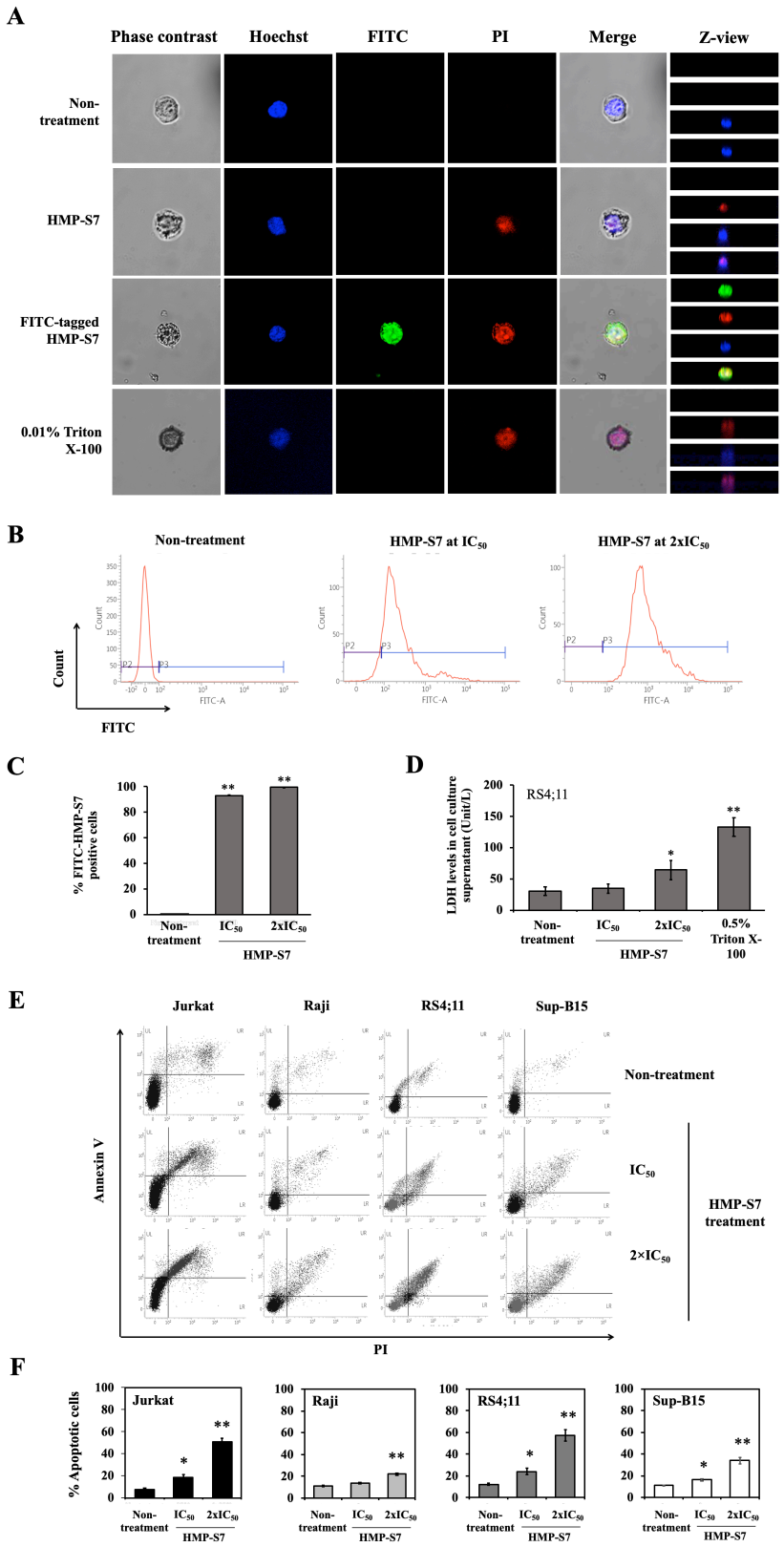


**Figure 6. HMP-S7 action on internalization and leukemic cell death.** Jurkat cells were treated with HMP-S7 conjugated with or without FITC at IC50 and 0.01% Triton X-100 (positive control) and stained with PI to observe membrane permeability **(A)**. The FITC tagged HMP-S7 (at IC50 and 2×IC50) were internalized into the cytoplasm of Jurkat cells. (**B**) Flow cytometry of FITC tagged HMP-S7-treated Jurkat cells. (**C**) The bar graph showed

flow cytometric results as %FITC-positive Jurkat cells. (**D**) LDH release assay. LDH level was detected as the evidence of cellular membrane disruption in the culture supernatant of HMP-S7-treated RS4;11 cells at IC50 and 2×IC50 for 24 h. Triton X-100 treated cells were the positive control. (**E**) Flow cytometric cell death assay using Annexin-V/PI co-staining. Four leukemic cells, i.e., Jurkat, Raji, RS4;11, and Sup-B15 cell lines were treated with HMP-S7 at IC50 and 2×IC50 for 24 h. (**F**) Bar graphs showed %apoptotic cell death composed of early apoptosis (upper left quadrant), late apoptosis (upper right quadrant), and necrosis (lower right quadrant). *$p<0.05$ and **$p<0.01$ compared to non-treatment condition. All experiments were performed in three biological replicates. *$p<0.05$, **$p<0.01$ compared to non-treatment condition.

## Discussion

This study aimed to explore the novel ACP from naturally occurred-human milk peptides. Several reasons supported human milk as a good source for this exploration. First, there is evidence that human breast milk can significantly reduce leukemia in children with more 6-month breastfeeding (*25, 26*). Second, HAMLET (human alpha-lactalbumin made lethal to tumor cells), a protein-lipid complex that induces apoptosis-like death in tumor cells, was discovered from a human milk protein (*37*). Alpha1H (the alpha1 domain of α-lactalbumin in complex with oleic acid), which was further developed from HAMLET, has entered the First-in-Human trial in patients with bladder cancer (clinicaltrials.gov identifier NCT03560479) (*38*). Third, ACP has never been explored from human milk. Fourth, recent advances in mass spectrometric-based proteomics allow high-throughput peptide identification from human milk, and *in silico* ACP prediction algorithms based on the peptide amino acid sequences have been progressed during the last several years.

Therefore, this study developed an integrative workflow for ACP discovery by combining the strengths of mass spectrometry, *in silico* screening, and experimental validation. This strategy replaces labor-intensive and time-consuming processes of the activity-guided purification by applying mass spectrometric peptide identification of the fractionated human milk samples, and the results were compiled into the peptide library of natural occurring-human milk peptides. Peptide amino acid sequences were subsequently fed into *in silico* screening to shorten the time-to-discovery and lessen the cost burden of the large-scale experiment. Finally, the prioritized peptide candidates could be validated for anti-leukemic activities by *in vitro* cellular studies.

To prioritize the peptides for experimental screening and validation, we hypothesized that the consensus results from the physicochemical property (positive charge), secondary peptide structure (alpha-helix), and ACP predictive results from different machine learning models would provide the best chance of ACP identification from the given peptide library. This hypothesis was grounded on previous evidence that; i) the net positively charged peptides are attractive to the net negatively charged cancer cell membranes (*39*); ii) most known oncolytic peptides share alpha-helical structure (*32*); iii) different machine learning models were trained and tested upon various data sets of known ACPs (*33-36*), thereby these models could have varied predictive performance against the new unknown peptide data set. More votes would provide more confidence in the predictive results. Since it was unknown at the initial stage of this study if this integrative approach would be successful, we, therefore, explored each of the preferred ACP properties. For this reason, eight selected peptide candidates were identified and screened for anti-leukemic effect (**Figure 3** and **Table S3**). BMP-S6, the positive control of the experimental validation, has met all three criteria and showed the anti-leukemic effect with a trade-off of more toxicity toward normal cells. Six out of eight selected peptide candidates were not met all three criteria and showed no activity *in vitro*. HMP-S7 and HMP-S8 were the top 2 candidates that met all three criteria of the

integrative workflow; nonetheless, only HMP-S7 had the cytotoxic effect against leukemic cells. From this observation, we learned that this integrative approach is more accurate than using a single preferred anticancer property to prioritize peptide candidates. When one peptide candidate met all three criteria, the difference in one positive charge (and perhaps two votes from machine learning models) did matter for the anti-leukemic prediction (**Figure 3**). In future studies, this integrative approach of ACP screening can be applied to larger data sets, either the mass spectrometric-based or the *in silico* generated peptide libraries, to speed up the discovery of ACP against multiple cancer types.

Looking forward, HMP-S7 should be further validated in preclinical models. Further peptide modifications using HMP-S7 as a prototype, for example, amino acid substitution (*40*), homing motif tagging (*41*), and PEGylation (*42*), may improve its anticancer efficacy, tumor targeting, and biocompatibility and stability. Furthermore, the human milk peptide library can be expanded by further profiling relevant biospecimens or by *in silico* peptide generation from the proteins of interest. Aforementioned *in vitro* study support, machine learning for ACP prediction might be further improved by a combination of ensemble models, physicochemical property, and secondary peptide structure.

This study had limitations. It was expected that milk peptides should present a more significant number of unique identities than those identified in this study. Losing of peptides during mass spectrometric detection because of their neutral net charge may be one reason. Another possibility is that the milk peptides may be degraded during the fractionation process. These limitations could be overcome by the combined methods using fractionations and high-resolution mass spectrometry detection. The issues related to the small peptide library because of experimental bottlenecks could be addressed by *in silico* generated peptide database as aforementioned, given that the custom-made peptides are commercially available and ready for anticancer activity screening. Lastly, it should be emphasized that *ex vivo* experiments of HMP-S7 by using the patient's derived leukemic cells should be further explored in the future.

## Conclusions

This study discovered HMP-S7 (SFIPRAKSTWLNNIKLL) as the novel anti-leukemic peptide derived from human breast milk. This anti-leukemic peptide is a cationic and alpha-helical structure peptide that selectively kills leukemic cells but not normal cells. Future research on peptide modification, together with the efficacy studies in preclinical animal models or patient's derived leukemic cells *ex vivo*, is warranted to develop HMP-S7 as peptide-based cancer therapeutics in the future.

## Materials and Methods

**Human milk collection.** Human milk (60 ml) from 10 healthy volunteer mothers of full-term infant, whose blood test has no infection of HIV, Hepatitis B or C viruses, and Syphilis, were collected by a breast pump and stored in a freezer (-20°C) as per the regulation of Ramathibodi Human Milk Bank (RHMB) before transferring to the laboratory (*43*). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Human Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital, Mahidol University (Protocol ID-11-60-13).

**Peptide isolation and fractionation.** Three pooled milk specimens were produced from 3, 3, and 4 individual specimens (**Table S1**) before further processing. Twenty milliliters of pooled milk specimens were centrifuged at 4°C, 1,500 g for 10 min, and then 5,000 g for 30 min twice to remove cells and lipids. The collected supernatant was centrifuged at 4°C,

12,000 g for 1 h, 32,000 g for 1 h, and finally, 200,000 g for 1 h to remove extracellular vesicles, including microvesicles and exosomes. Thereafter, peptides (<3 kDa) were separated from high molecular size proteins by a 3 kDa cutoff ultrafiltration column (Amicon®, Merck Millipore Ltd., Cork, Ireland). The crude peptides (<3 kDa) in the flow-through fraction were injected into the C18 solid-phase extraction (SPE) column (Waters, Waters Corporation, Massachusetts, USA). The bound peptides were then step-wise eluted by 1 mL each of 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, and 80% acetonitrile (ACN), respectively. The eluate peptides were dried by SpeedVac concentrator (Labconco, Labconco Corporation, Missouri, USA). The dried peptides were resuspended in deionized (d*I*) water for peptide estimation using Bradford's assay (Bio-rad) (*44*).

**Synthetic peptides.** Nine synthetic peptides (>90% purity) (**Table S3**) were custom ordered from GL Biochem (GL Biochem (Shanghai) Ltd., Shanghai, China) and resuspended in the appropriate culture medium to the final concentration of 200 μM before used.

**Cell culture.** Cell lines were obtained from the American Type Culture Collection (ATCC). Jurkat (ATCC® TIB-152™), RS4;11 (ATCC® CRL-1873™) and Raji (ATCC® CCL-86™) cells were maintained in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1× penicillin/streptomycin (Gibco) in 5% $CO_2$ at 37°C, while Sup-B15 (ATCC® CRL-1929™) cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.05 mM 2-mercaptoethanol (Sigma) and 20% FBS (Gibco) and 1× penicillin/streptomycin (Gibco). SH-SY5Y (ATCC® CRL-2266™), MDA-MB-231 (ATCC® HTB-26™), A549 (ATCC® CCL-185™) cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Gibco) supplemented with 10% FBS and 1× penicillin/streptomycin in 5% $CO_2$ at 37°C. HT-29 cells (ATCC® HTB-38™) and HepG2 (ATCC® HB-8065™) were grown in DMEM-F12 (Gibco) supplemented with 10% FBS and 1× penicillin/streptomycin in 5% $CO_2$ at 37°C.

For representative normal cells, FHs74Int (ATCC® CCL-241™) fetal intestinal epithelial cells were cultured in Hybri-Care Medium (ATCC® 46-X™) supplemented with 30 ng/ml epidermal growth factor (EGF), 10% FBS (Gibco) and 1× penicillin/streptomycin (Gibco) in 5% CO2 at 37°C. HEK293T human embryonic kidney cells (ATCC® CRL-3216™) were maintained in DMEM-high glucose (Gibco) supplemented with 10% FBS and 1× penicillin/streptomycin in 5% $CO_2$ at 37°C. Human peripheral blood mononuclear cells (PBMCs) were prepared from 10 mL EDTA blood for T cell cultivation. The whole blood was diluted in PBS at a ratio of 1:1, and then carefully layer the diluted blood into Ficoll-Paque solution (Robbins Scientific Cooperation, Norway) with a ratio of 2:1 (diluted blood:Ficoll-Paque solution). The tube containing layer solution was centrifuged at 400 g, 20°C for 35 min with no break. One million PBMCs were cultured in the OKT3 and CD28 coated plate. Briefly, 1 μg each of OKT3 and CD28 was added into 1 ml PBS in a 24-well plate and incubated at room temperature for 2 h. The coated well was washed with PBS once before adding PBMCs. The PBMCs in coated well were cultured in RPMI-1640 supplemented with 100U/mL IL-2, 10% FBS and 1× penicillin/streptomycin in the presence of OKT3 and CD28 for 3 days at 37°C under a humidified atmosphere with 5% $CO_2$ before used.

**Human milk peptide identification by mass spectrometry.** Dried human milk peptides (2 μg) were resuspended in 0.1% formic acid and centrifuged at 14,000 rpm for 30 min. The supernatant was collected and injected into the C18 column (75 μm i.d. × 100 mm) by using Easy-nLC (Thermo Fisher Scientific, Inc.) to desalt and concentrate. The peptides were

separated with a gradient of 5-45% acetonitrile/0.1% formic acid for 30 min at a flow rate of 300 nl/min. The isolated peptides were identified by the amaZon speed ETD ion trap mass spectrometer (Bruker Daltoniks, Billerica, MA, USA). Peptide sequences and identification were interpreted using in-house MASCOT software version 2.4.0 with the SwissProt database, against *Homo sapiens*, no fixed and no variable modifications, no enzymatic digestion with no missed cleavage allowed, monoisotopic, ±1.2 Da for peptide tolerance, ±0.6 Da for fragment ion tolerance, 2+ and 3+ charge state for ESI-TRAP instrument. Identified peptides were provided ion scores higher than 20 (significance threshold at $p < 0.05$).

***In silico* anticancer peptide screening.** Physicochemical properties of all identified human milk peptides were predicted by using the PepDraw tool (http://www.tulane.edu/~biochem/WW/PepDraw/), including peptide length, mass, net charge, isoelectric point (p*I*), and hydrophobicity. Peptide structures were predicted using the PEP-FOLD3 De novo peptide structure prediction tool (*45-47*). Anticancer properties were predicted by four web-based machine learning programs, including i) ACPred-FL (Tianjin University, School of Computer Science and Technology, China), a sequence base predictor for identifying anti-cancer peptides from proteins, by using classification mode at confidence 0.5 as default setting (http://server.malab.cn/ACPred-FL/#) (*33*); ii) AntiCP 2.0, an ensemble tree classifier on amino acid composition (https://webs.iiitd.edu.in/raghava/anticp2/index.html) (*34*); iii) MLACP, a random forest-based prediction of anticancer peptides (www.thegleelab.org/MLACP.html) (*35*); and iv) mACPpred, a Support Vector Machine-Based Meta-Predictor (http://www.thegleelab.org/mACPpred) (*36*).

**Cytotoxicity assay.** For cell treatment, the fractionated milk peptides (10 µg/reaction) or the synthetic peptides (varied concentrations as indicated) were resuspended in 100 µL of culture media with appropriated supplements. For floating cells, the peptide solution was mixed with cell suspension (10,000 cells/10 µL/well) in a well of 96-well plate flat bottom. For adherent cells, 10,000 cells were seeded into a 96-well plate (flat bottom) and cultured until they reached 80% confluence before adding 100 µL of peptide solution into the well containing 80% FHs 74 Int cell confluence (3 technical replication). The peptide-treated cells were incubated for 24, 48, or 72 h in 5% $CO_2$ incubator at 37°C with humidity as indicated. Cell viability was measured by trypan blue exclusion or WST-1 assays (Roche Diagnostics GmbH, Mannheim, Germany).

The two synthetic peptides at 200 µM final concentration, i.e., HMP-S7 and BMP-S6 were selected to treat with normal (T cells and HEK293T (human embryonic kidney cells)) and cancer cell lines (SH-SY5Y (neuroblastoma), MDA-MB-231 (breast adenocarcinoma), HT29 (colorectal adenocarcinoma), A549 (lung carcinoma), HepG2 (hepatocellular carcinoma).

Human peripheral blood mononuclear cells (PBMCs) were prepared from 10 mL EDTA peripheral blood collection as aforementioned for T cell culture. The protocol was approved by the Human Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital, Mahidol University, based on the Declaration of Helsinki (Protocol ID-06-61-09). The $1\times10^4$ floating cells (in 10 µL), i.e., T cells, were added to 96-well plate, flat bottom containing 100 µL of their culturing medium with or without 200 µM HMP-S7 and 200 µM BMP-S6. While, $1\times10^4$ adherent cells were seed in 96-well plate, flat bottom and then cultured 1-2 overnight at 37°C under a humidified atmosphere with 5% $CO_2$ incubator until 90-100% cell confluent. The cultured medium was removed, and then added the medium containing 200 µM HMP-S7 and 200 µM BMP-S6. All treated and untreated cells were

incubated for 24 h before trypsinization (for adherent cells) and split to cell death measurement using trypan blue exclusion assay (3 technical replications).

**Half maximum inhibitory concentration ($IC_{50}$) by trypan blue assay.** Four distinct leukemic cell lines, i.e., Jurkat, Raji, RS4;11, and Sup-B15 cell lines were cultured in the medium containing HMP-S7 in vary concentrations (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μM) in 96-well plate, flat bottom for 24 h ($1\times10^4$ cells/100 μL/well). The %cell death was estimated by trypan blue exclusion assay using (cell death number/total cell number) × 100. $IC_{50}$ calculation used linear ($y=ax+c$) or parabolic ($y=ax^2+bx+c$) equation for y=50 value and x value= $IC_{50}$ concentration. $IC_{50}$ concentration of HMP-S7 to the 4 leukemic cell lines was reported in mean±SD.

**Soft agar assay for colony formation.** Base agar (1.5 mL of 0.5% agar containing 1×RPMI supplement with 10% FBS and 1× penicillin/streptomycin) were plated on each well of a 6-well plate and set aside for 5 min to allow the agar to solidify. Jurkat cells were treated with 100 μM, 200 μM, 400 μM HMP-S7, and 200 μM BMP-S6 in a 96-well plate (3 technical replications, 100 μL/10,000 cells/well) for 24 h. After 24 h, the treated/untreated cells in the 96-well plate were thoroughly mixed and equally taken (20 μL) to mix in 1 mL of the top agar solution (0.3% agarose containing 1×RPMI supplement with 10% FBS and 1× penicillin/streptomycin). The cell suspension was plated on top of the base agar (3 biological replication) and then allowed agarose to form solid. The medium (0.5 mL) was added on top of the agar to prevent agar dry. The plate was incubated at 37°C in a humidified incubator for 20 days with feed the medium twice a week. The agar was stained with 1 mL of 0.005% crystal violet in 20% ethanol for 1 h, destained with 20% ethanol overnight, and then counted the colonies under stereomicroscope SZ61 (Olympus Corporation, Tokyo, Japan).

**Apoptosis assay.** Four leukemic cell lines ($1\times10^5$ cells/500 μL/well/cell type) were cultured in 24 well-plate in the medium containing HMP-S7 with $IC_{50}$ and $2\times IC_{50}$ concentrations and non-treatment condition for 24 h (biological triplication). The untreated cells were divided into 3 tubes that are unstained, cells stained with FITC tagged annexin V (no propidium iodide (PI)), and cells stained with PI (no FITC tagged annexin V) to set up compensation and quadrants. The untreated and treated cells were collected and wash cells with cold PBS twice. Thereafter, the cells were resuspended with 100 μL of 1× binding buffer and then added 5 μL of FITC tagged annexin V and 5 μL of PI. The cell suspensions were gently mixed and incubated at room temperature for 15 min in dark. Then, 400 μL of 1× binding buffer was added to each tube, and then the cells were analyzed by using flow cytometry.

**Lactate dehydrogenase (LDH) release assay.** RS4;11 leukemic cell lines ($2\times10^5$ cells/1 mL/well/cell type) were cultured in 24 well-plate in the complete medium containing HMP-S7 at $IC_{50}$ and $2\times IC_{50}$ concentrations, without HMP-S7 condition (non-treatment), and 0.5% Triton X-100 for 24 h incubation (biological triplication). The cell suspension was collected and centrifuged at 200 g for 5 min at room temperature. The 1 mL of supernatant was collected and measured LDH isoenzyme by measuring lactate to pyruvate and NADH, which increased absorbance at 340 nm using Abbott Architect C16000 clinical chemistry analyzer (Holliston, MA, USA) at Clinical Chemistry Unit of Department of Pathology at Ramathibodi Hospital, Thailand. LDH level (U/L) of the culture media was subtracted from the background of the fresh medium and reported as mean±SD.

**FITC tagged HMP-S7 internalized into leukemic cells.** The $2\times10^4$ Jurkat cells/100 µl/well in the 96-well plate was treated with HMP-S7 tagged with/without FITC at $IC_{50}$ for 24 h. Thereafter, the treated and untreated Jurkat cells were washed with 1×PBS once before staining with propidium iodide (PI) cell stain kit (Invitrogen) and Hoechst 33342 (Cell Signaling Technology, Inc, Massachusetts, USA), for 30 min at room temperature in dark. After incubation, the cells were centrifuged at 200 g for 5 min and removed the excess dye solution before washing with 1×PBS twice. The cell pellet was mixed with 20% glycerol/PBS and mounted on a glass slide for confocal microscopy (Nikon Instruments, Inc, NY, USA).

**Statistical Analysis**. The number and percentage of death or live cells, OD and $IC_{50}$, were calculated and statistical testing using Student's *t-test* as statistical significance at $p < 0.05$.


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