bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 # AN INTEGRATIVE APPROACH DISCOVERS A NOVEL ANTI-LEUKEMIC PEPTIDE FROM HUMAN MILK
2
3 **Authors**
4 Wararat Chiangjong,[1] Jirawan Panachan,[2] Thitinee Vanichapol,[2] Nutkridta Pongsakul,[1]
5 Tassanee Lerksuthirat,[3] Pracha Nuntnarumit,[4] Sarayut Supapannachart,[4] Chantragan
6 Srisomsap,[5] Jisnuson Svasti,[5,6] Suradej Hongeng,[2] Somchai Chutipongtanate[1,7,8,*]
7
8 **Affiliations**
9 [1] Pediatric Translational Research Unit, Department of Pediatrics, Faculty of Medicine
10 Ramathibodi Hospital, Mahidol University, Bangkok 10400 Thailand.
11 wararat_01@yahoo.com (W.C.); nutkridta@yahoo.co.th (N.P.)
12 [2] Division of Hematology and Oncology, Department of Pediatrics, Faculty of Medicine
13 Ramathibodi Hospital, Mahidol University, Bangkok 10400 Thailand.
14 jirawan.pan.13@gmail.com (J.P.); thitinee@gmail.com (T.V.);
15 suradej.hon@mahidol.ac.th (S.H.)
16 [3] Research Center, Faculty of Medicine Ramathibodi Hospital, Mahidol University,
17 Bangkok 10400 Thailand. tassanee.lerk@gmail.com (T.L.)
18 [4] Division of Neonatology, Faculty of Medicine Ramathibodi Hospital, Mahidol
19 University, Bangkok 10400 Thailand. pracha.nun@mahidol.ac.th (P.N.);
20 sarayut.sup@mahidol.ac.th (S.S.)
21 [5] Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok 10210 Thailand.
22 chantragan@cri.or.th (C.S.); jisnuson@cri.or.th (J.S.)
23 [6] Applied Biological Sciences Program, Chulabhorn Graduate Institute, Bangkok 10210
24 Thailand
25 [7] Department of Clinical Epidemiology and Biostatistics, Faculty of Medicine
26 Ramathibodi Hospital, Mahidol University, Bangkok 10400 Thailand.
27 [8] Chakri Naruebodindra Medical Institute, Faculty of Medicine Ramathibodi Hospital,
28 Mahidol University, Samut Prakan 10540 Thailand
29 *Corresponding author: schuti.rama@gmail.com, somchai.chu@mahidol.edu
30
31 **Correspondence:**
32 Somchai Chutipongtanate, Ph.D., M.D.
33 Pediatric Translational Research Unit, Department of Pediatrics,
34 Faculty of Medicine Ramathibodi Hospital, Mahidol University
35 270 Rama 6 Rd. Phayathai
36 Ratchathewi Bangkok 10400 Thailand
37 +66-82-3346020
38 schuti.rama@gmail.com, somchai.chu@mahidol.edu
39
40 **Abstract**
41 Chemotherapy in childhood leukemia is associated with late morbidity in leukemic
42 survivors, while certain patient subsets relatively resistant to standard chemotherapy.
43 Identifying new agents with the sensitivity and selectivity toward leukemic cells with less
44 systemic toxicity is a warrant. Peptide-based therapeutics is gaining attention during the last
45 few years. Here, we used an integrative workflow combining mass spectrometric peptide
46 library construction, *in silico* anticancer peptide screening, and *in vitro* leukemic cell studies
47 to discover a novel anti-leukemic peptide owning 3+charges and alpha-helical structure,
48 namely HMP-S7, from human breast milk. HMP-S7 showed cytotoxic activity against four
49 distinct leukemic cell lines in a dose-dependent manner but had no affected on solid
50 malignancies or representative normal cells. HMP-S7 induced leukemic cell death by

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

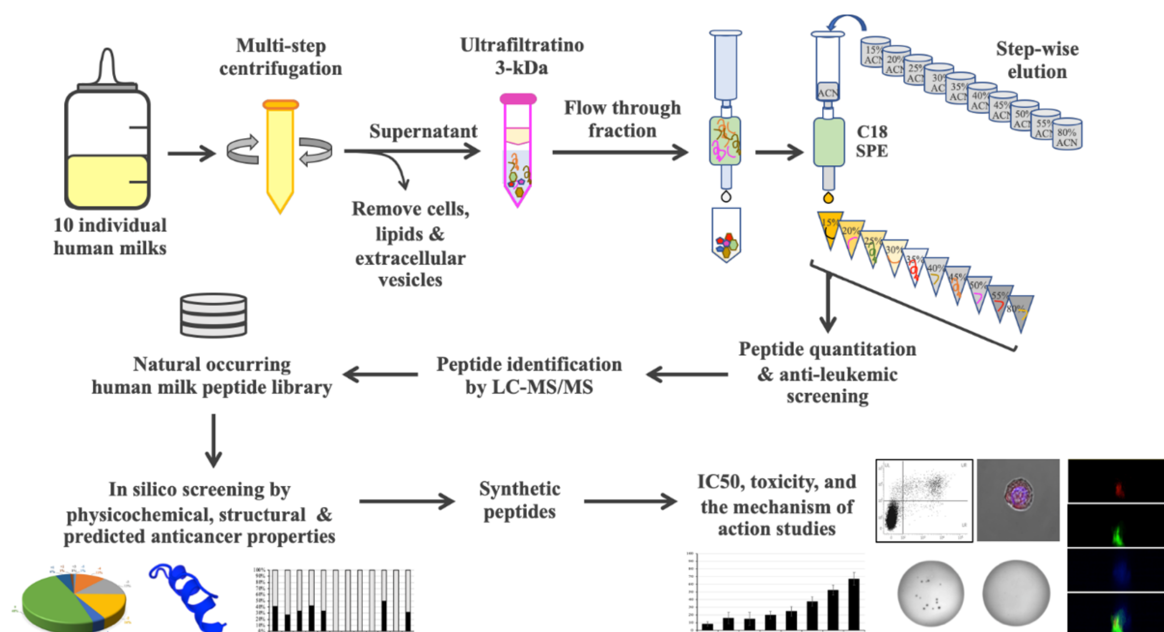
Cancer is a significant cause of death in children and adolescence during the last few years in Asia, Central and South America, Northwest Africa, and the Middle East (*1*). Hematologic malignancies, particularly acute lymphocytic leukemia (ALL), are predominant, accounting for 30% of childhood cancer (*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

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

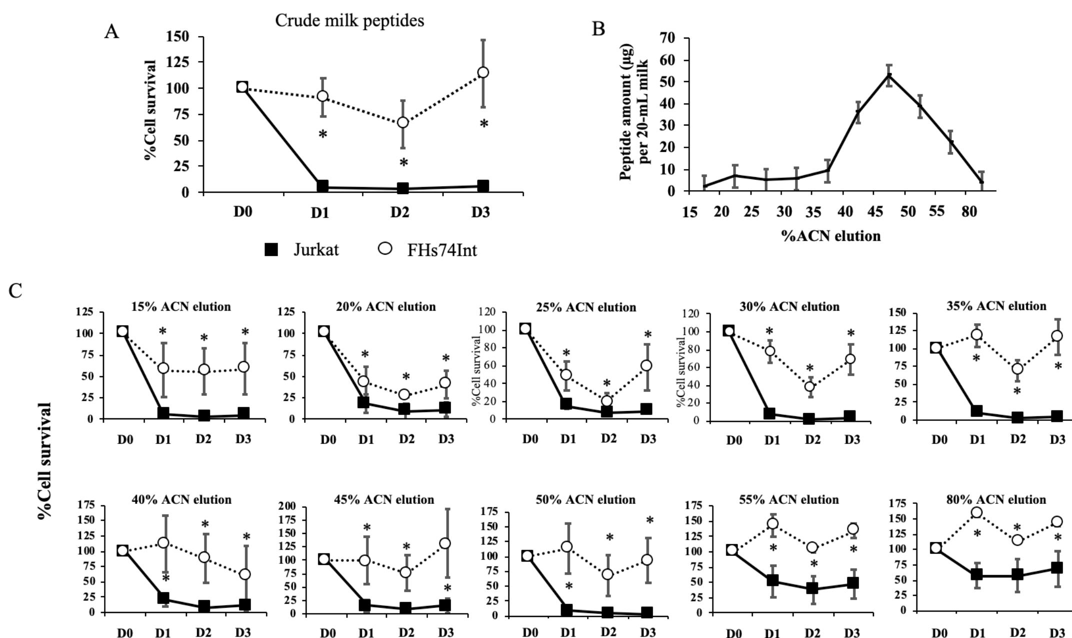
01 anticancer peptides as that of bovine milk. PGPIPN hexapeptide of bovine β-casein can
02 inhibit invasion and migration of human ovarian cancer cells (*23*). ACFP, an anti-
03 cancer fusion peptide derived from bovine β-casein and lactoferrin, can inhibit viability and
04 promote apoptosis in primary ovarian cancer cells (*24*). Interestingly, breastfeeding for six
05 months or longer was associated with a 19% lower risk of all childhood leukemia than
06 shorter breastfeeding or none (*25, 26*). Identifying novel anti-leukemic peptides from human
07 milk is a warrant for the future development of non-allergic and non-toxic peptide-based
08 drugs to improve childhood leukemia therapy.
09 This study aimed to discover a novel anti-leukemic peptide with less cytotoxicity
10 toward normal cells from human milk. We applied a robust workflow integrating the
11 strength of liquid chromatography-tandem mass spectrometry to generate a natural-
12 occurring human milk peptide library *in silico* screening based on physicochemical,
13 structural, and predictive anticancer properties by machine learning algorithms to prioritize
14 candidates for functional studies of synthetic peptides to determine anti-leukemic activity
15 and the mode of action. By this strategy, a novel anti-leukemic peptide was identified from
16 human milk as the main outcome.
17
18 **Results**
19 To separate human milk peptides and test their cytotoxicity to leukemic cells, ten
20 healthy mothers aged between 25 and 36 years old (32.3±3.27 years old) donated breast milk
21 at once during 6-129 days (63.8±38.97 days) after delivery (details in **Table S1)**. After
22 multiple steps of centrifugation to remove cells, lipids, and extracellular vesicles, and then
23 ultrafiltration (3-kDa cutoff) to remove proteins, the small molecules (<3-kDa), except the
24 peptides, passed through the C18 SPE. Moreover, the C18 bound peptides were eluted by
25 various acetonitrile concentrations and then performed their cytotoxicity. The conceptual
26 framework of this study is illustrated in **Figure 1**.
27



28
29
30 **Figure 1**. **A conceptual framework of the integrative strategy for discovering a novel**
31 **human milk-derived anti-leukemic peptide**. This strategy combines the strengths of mass
32 spectrometry for high-throughput peptide identification, *in silico* screening for prioritizing
33 peptide candidates, and experimental validation for anti-leukemic activities. Abbreviations:

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

34 LC-MS/MS, liquid chromatography-tandem mass spectrometry; IC50, half-maximal
35 inhibitory concentration; SPE, solid-phase extraction.
36
37 *Most human milk peptide fractions had cytotoxic effects on leukemic and normal cell lines*
38 After C18 bound peptide elution, the crude milk peptide fraction was tested to
39 observe cytotoxic effects against Jurkat (T lymphoblastic leukemia) and FHs74Int cells (the
40 representative normal intestinal epithelium), respectively (**Figure 2A**). The crude milk
41 peptides showed a potent cytotoxic effect on Jurkat but had less impact on FHs74Int cells.
42 The coarse milk peptide fraction was then fractionated by C18 SPE with the step-wise ACN
43 elution, in which the chromatogram was demonstrated by the peptide amounts (**Figure 2B**).
44 These eluted peptides by 15%-80% ACN were treated to Jurkat leukemic cells and FHs74Int
45 normal cells to observe cell survival as shown in **Figure 2C**. The low peptide amount in
46 15%-30% ACN eluted peptide fractions could decrease Jurkat cell survival more than the
47 highest peptide amount (45% ACN eluted peptide fraction). This indicated that the
48 elimination of Jurkat leukemic cells depended on the specific peptide sequence with
49 hydrophilic property more than peptide amount.
50



51
52 **Figure 2. Human milk peptide fractionation and cytotoxicity profiles against leukemic**
53 **and normal cells.** (**A**) After centrifugation and passing through <3 kDa cut-off column, the
54 milk solution (20 mL) was passed through the C18 column. The milk peptides were bound
55 to the C18 SPE column, eluted with 80% ACN (1 mL), and dried using a SpeedVac
56 concentrator. The crude milk peptides were resuspended in the culture medium and then
57 treated to Jurkat and FHs74Int cells for 1-3 days (n=3 biological replication). The graph
58 shows % cell survival (mean±SEM). Peptides were then fractionated into 10 fractions by
59 C18 SPE column with the step-wise acetonitrile elution. An equal volume (20 ml) of the
60 individual human milk supernatant (<3 kDa) was flown through the C18 SPE column. The
61 human milk peptides in the supernatant were bound to the column and eluted by equal
62 volume (1 mL) of 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, and 80% ACN in
63 subsequently. These peptides were subjected to LC-MS/MS to generate the peptide library.
64 (**B**) The fractionated peptides were resuspended in deionized distilled water and then
65 quantitated using Bradford's assay. The peptide amount in each fraction was calculated as

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

66 mean ± SEM. (**C**) Also, Jurkat and FHs74Int cells were treated with the 10 eluted fractions
67 of human milk peptides of 3 individual volunteer mothers for 1-3 days. The remaining live
68 cells after the 10 peptide fraction treatment were detected by WST-1 solution at 440 nm.
69 The OD was subtracted by culture medium without cells (blank). The percentage of cell
70 survival was calculated by (OD of the sample)/(OD of non-treatment)×100. *; p<0.05
71 comparing to non-treatment condition (n=3 individual milk specimens with 3 technical
72 replicates for each specimen).
73
74 *Peptide identification, library construction, and in silico anti-cancer peptide screening*
75 Eleven fractions (from one crude and ten step-wised ACN eluates) of human milk
76 peptides were identified by LC-MS/MS. A total of 142 naturally occurring human milk
77 peptides with unique sequences were collected into the peptide library, ready for further
78 analyses (**Table S2**). The distribution of all identified peptides by fractions, peptide length,
79 p*I*, and the net charge was shown in **Figure 3A** (upper panel). Overall, most identified natural
80 occurring-milk peptides had less than 20 amino acids in length, contained zero and anionic
81 net charge with inclining acid property. The most frequently detected pattern was the natural
82 peptides with proline-rich sequence (**Figure S1**). This proline-rich peptide is probably crucial
83 to some biological effects of human milk. For example, a ligand containing a proline-rich
84 sequence or a single proline residue is involved in protein-protein interaction (*27*). Proline-
85 rich sequence-contained antimicrobial peptides can kill microorganisms by interacting with
86 70S ribosome and disrupting protein synthesis (*28*).
87 *In silico* screening of the natural-occurring human milk peptide library was performed
88 by a robust workflow. Firstly, we screened the library by physicochemical properties, i.e.,
89 length, *p*I, and net charge (as shown in **Figure 3A**) since most of the known anticancer
90 peptides are small cationic peptides (commonly 5-30 amino acid length with net charge +2 to
91 +9) that bind to the negative charge of phosphatidylserine and sialic acid on the cancer cell
92 membrane (*12, 29, 30*). Secondly, the secondary structure of the peptides were predicted by
93 the PEP-FOLD3 webserver (**Figure 3B**). Peptides can form alpha-helix, beta-sheet, and
94 random coils (*31*). However, most of the known anticancer peptides shared the alpha-helix
95 structure (*32*). Thirdly, the identified peptides were predicted for anticancer properties using
96 machine learning prediction software. Since ACP prediction software could provide different
97 results depending on the algorithms and training datasets, four online-accessible software
98 were then applied in this study, including ACPpred-FL (*33*), antiCP 2.0 (*34*), MLACP (*35*),
99 and mACPpred (*36*) (**Figure 3C**). As a result, eight human milk peptides and one bovine milk
00 peptide (as a positive ACP control) from various prioritization based on net charges,
01 secondary structures, and the predicted anticancer properties were selected for further
02 analysis **(Table S3)**.
03

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

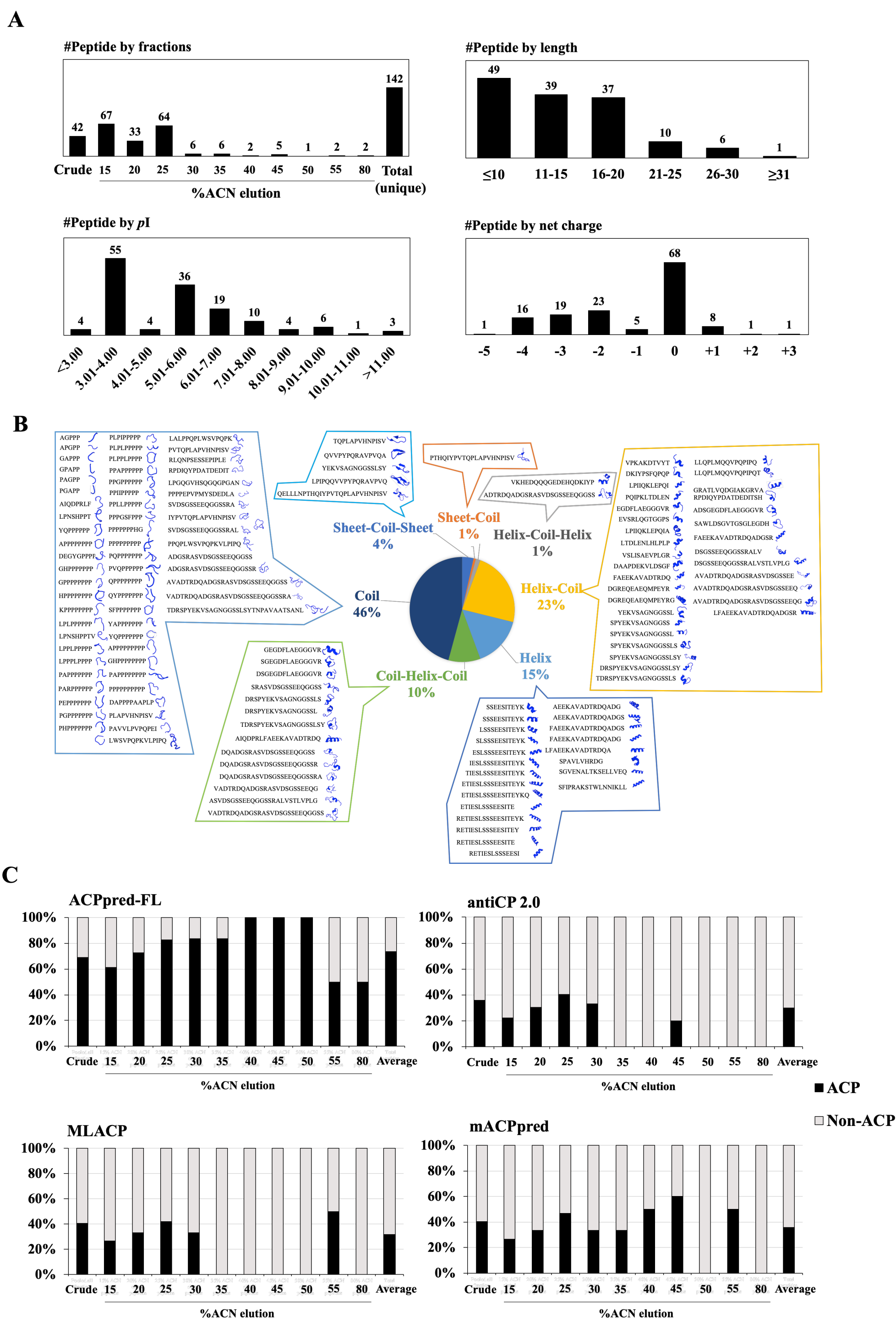
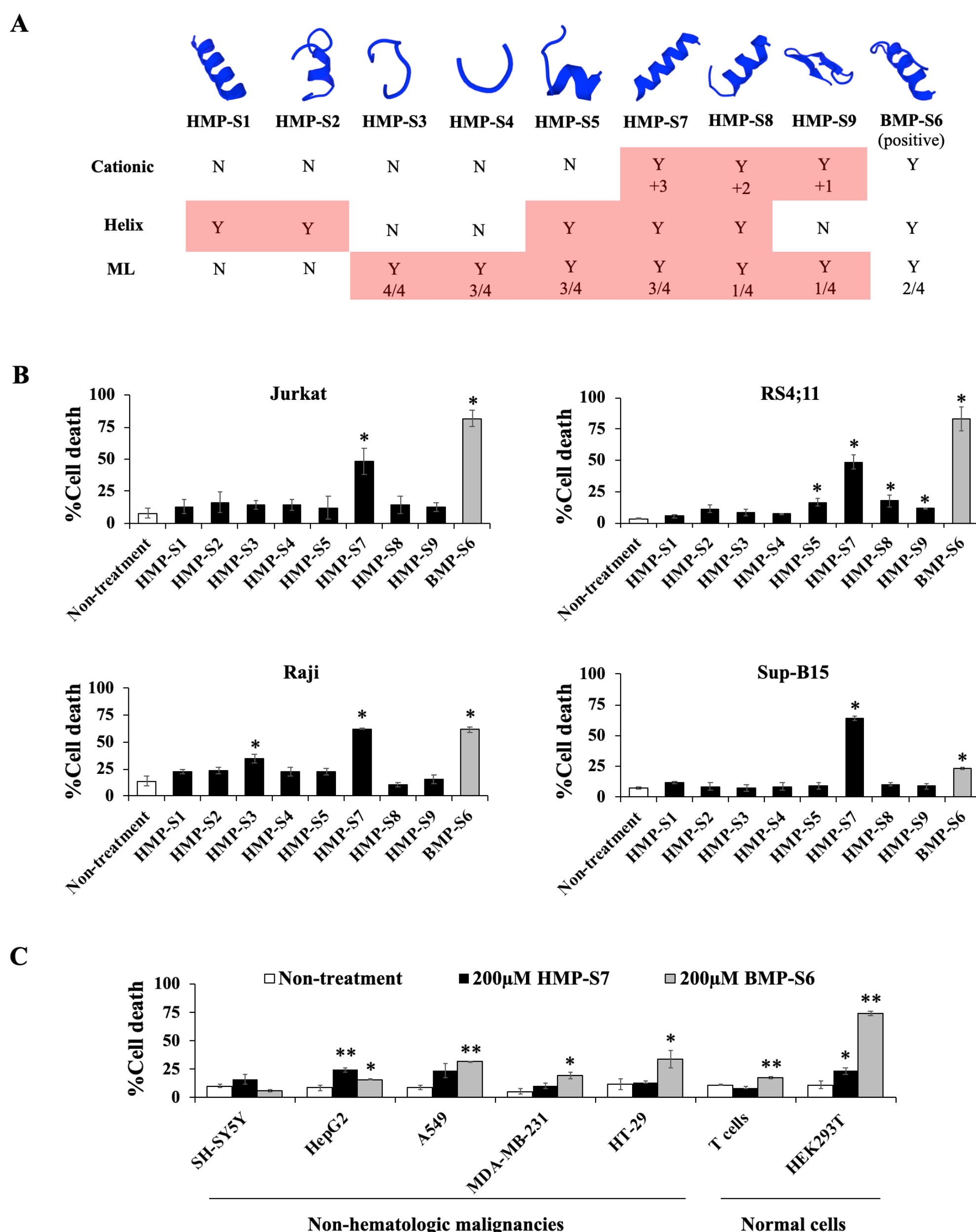


Figure 3. Predicted physicochemical, structural, and machine learning-based anticancer peptide screening of natural occurring-human milk peptides. (**A**) Distribution of the unique human milk peptides identified by LC-MS/MS. (**B**) the distribution and the secondary structure of all identified peptides 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**.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

16
17
18 *HMP-S7 killed leukemic cells but not normal cells or solid tumor cell lines*
19 **Figure 4A** illustrated eight selected human milk-derived peptides and a known bovine
20 milk-derived anticancer peptide synthesized for cancer cytotoxicity screening using trypan
21 blue staining. These results showed that HMP-S7, the highest cationic charged peptide (+3
22 net charge) with α-helical structure, had higher inhibitory activity than the other human milk-
23 derived peptides against four leukemic cell lines, including Jurkat, Raji, RS4;11, and Sup-
24 B15 cells **(Figure 4B)**. To elucidate the selectivity of HMP-S7, the effect of HMP-S7 was
25 further observed on normal cells, i.e., T cells and HEK293T embryonic kidney cells, and solid
26 tumor cell lines, i.e., SH-SY5Y neuroblastoma, HepG2 hepatoblastoma, A549 lung
27 adenocarcinoma, MDA-MB-231 breast cancer, and HT-29 colorectal adenocarcinoma by
28 trypan blue staining (**Figure 4C**). Compared to BMP-S6, the known anti-cancer peptide,
29 HMP-S7 had less cytotoxicity toward the normal cells and most cancer cells. This result
30 suggested that HMP-S7 was more selective to leukemic cells than the BMP-S6. Although
31 HMP-S7 had no cytotoxicity on normal T cells, it showed a mild cytotoxic effect on
32 HEK293T cells. Based on these activity profiles, HMP-S7 was chosen to further examine the
33 IC50 against four leukemic cell lines (**Figure 5A**). The results demonstrated anti-leukemic
34 activity of HMP-S7 was dose-dependent, with the IC50 ranged from 89.2 to 186.3 μM
35 depending on leukemic cells to be tested. Furthermore, the inhibitory activity of the HMP-S7
36 treated Jurkat leukemic cell lines was confirmed by the colony-forming assay using soft agar,
37 as shown in **Figure 5B**. Because HMP-S7 could induce leukemic cell death; thereafter, its
38 cell death mechanism should be further investigated.
39

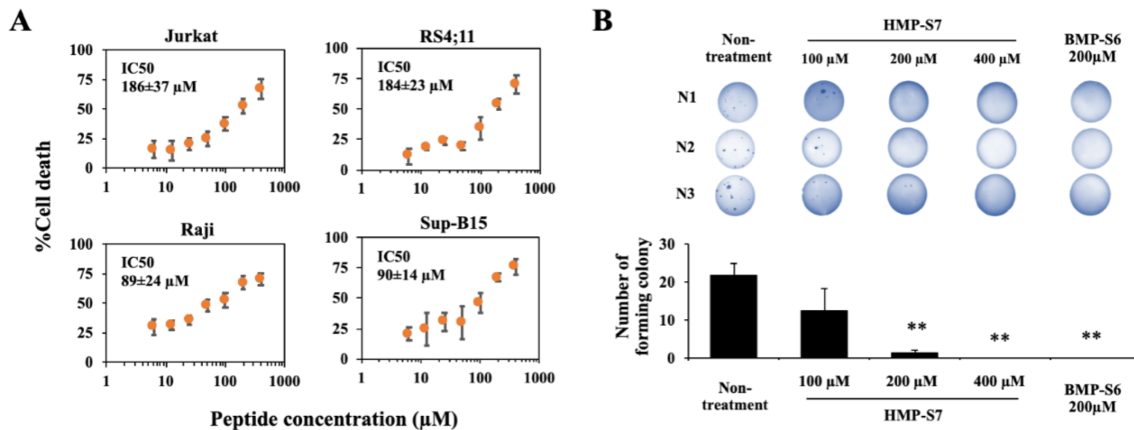
bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



40
41 **Figure 4. Effect of the 8 synthetic human milk peptides on leukemic and non-
42 leukemic cell lines.** (**A**) After the combination of physicochemical properties with cationic
43 peptides, helical structure, and machine learning prediction, the 8 synthetic human milk
44 peptides (HMPs) and the positive control (BMP-S6) (the details of the selected peptides
45 were summarized in **Table S3**) were examined for anti-leukemic activity. (**B**) Four
46 leukemic cell lines, including Jurkat, Raji, RS4;11 and Sup-B15, were treated with 8
47 synthetic HMPs and BMP-S6 at 200 µM to observe %cell death after 24 h treatment using
48 trypan blue exclusion assay under light microscope. The % cell death was calculated by
49 (number of death cells/total cell number) ×100. The percentage of cell death of all 4
50 leukemic cell lines after HMP-S7 treatment can significantly increase. (**C**) Besides
51 leukemic cell treatment, HMP-S7 was treated to non-hematological malignancy cell lines,
52 including neuroblastoma (SH-SY5Y), hepatoblastoma (HepG2), lung cancer (A549),
53 triple-negative breast cancer (MDA-MB-231), colon cancer (HT-29), and normal cells
54 including T cells and HEK293T embryonic kidney cells. Statistical significance was
55 calculated by %cell death of three biological replicates. *p<0.05, ** p<0.01 compared to
56 non-treatment condition.

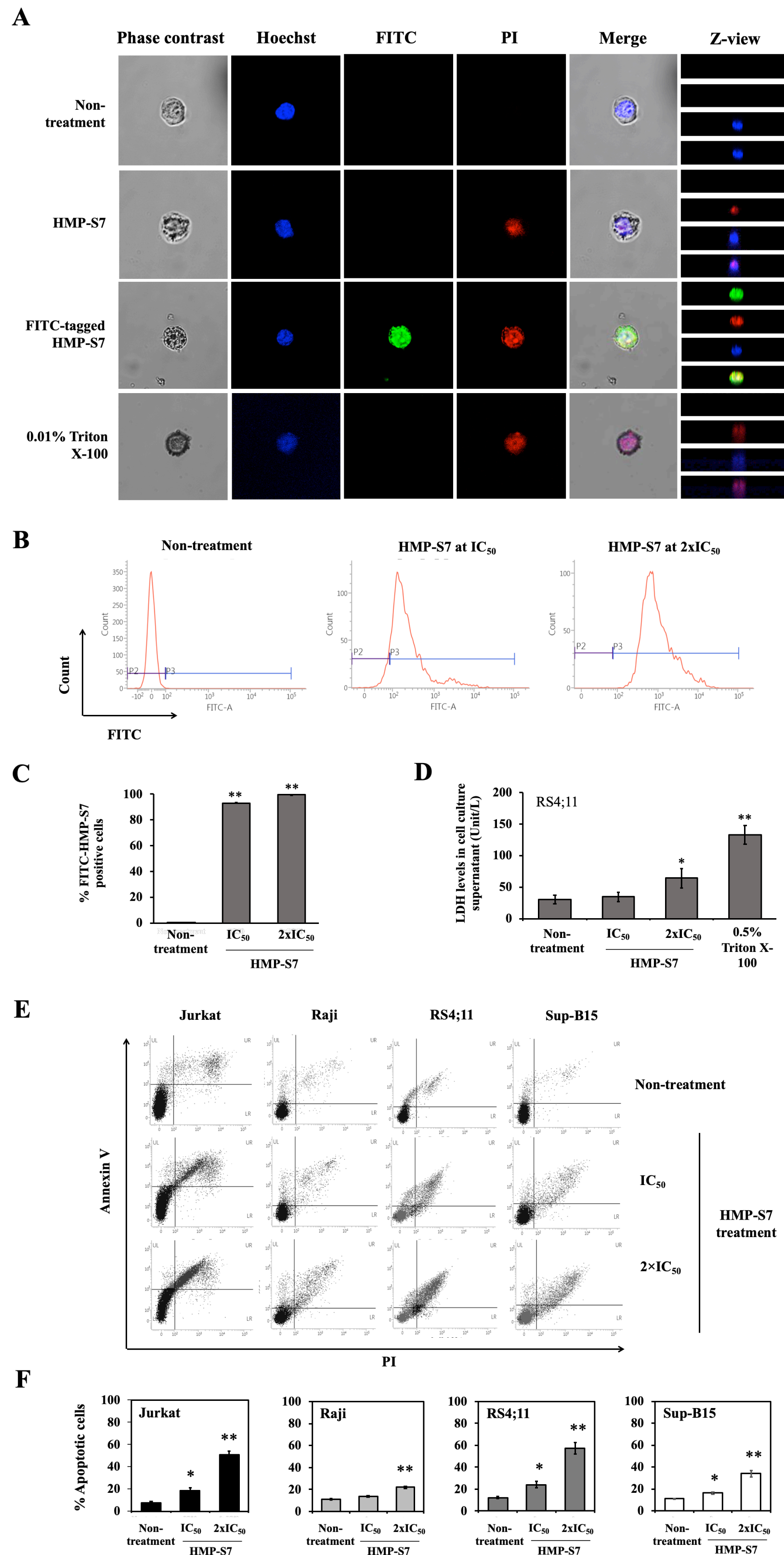
bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

57



58
59 **Figure 5. The inhibitory concentration of HMP-S7 treated leukemic cells.** (**A**) Four
60 leukemic cell lines i.e., Jurkat, Raji, RS4;11, and Sup-B15 cells, were treated with HMP-
61 S7 in various concentrations (0-400 µM) for 24 h and their %cell death (mean ± SD) was
62 counted by using trypan blue exclusion assay. (**B**) The number of the forming colony of
63 HMP-S7 (100 µM, 200 µM, and 400 µM) treated Jurkat cells was confirmed the anti-
64 leukemic activity of HMP-S7. The positive control peptide was BMP-S6 at 200 µM
65 concentration. After 24 h treatment, the cell suspension was equally volume taken into soft
66 agar to form colonies for 20 days. The colonies in the soft agar were stained with crystal
67 violet and counted. **, $p<0.01$ compared to non-treatment condition (n=3 biological
68 replicates).
69
70 *Mechanisms of HMP-S7-induced leukemic cell death*
71 To elucidate whether HMP-S7 can act as the membranolytic peptide against
72 leukemic cells, Jurkat cells were treated with HMP-S7 (untagged or FITC-tagged) at the IC50
73 for 24 h. By confocal microscopy, PI staining of cytoplasmic RNA was observed in the
74 untagged HMP-7 and the positive control Triton X-100 (**Figure 6A**). The FITC-tagged HMP-
75 S7 was located in the cytoplasm as PI stain of cytoplasmic RNA. Flow cytometry showed
76 that the HMP-S7 could be more cumulative into cytoplasm when increasing the
77 concentrations from IC50 to 2×IC50 (**Figure 6B** and **6C**). By lactate dehydrogenase (LDH)
78 release assay, intracellular LDH leakage into the cell culture supernatant was evidence at 24
79 h in HMP-S7-treated RS4;11 cells (at IC50 and 2×IC50) and Triton X-100 treated cells
80 (**Figure 6D**). Finally, HMP-S7-induced leukemic cell death was confirmed by flow cytometry
81 using Annexin V/PI co-staining. The number of apoptotic cells was significantly increased
82 compared to non-treatment condition, while a dose-response relationship was observed in
83 four leukemic cell lines with IC50 and 2×IC50 of HMP-S7 treatment (**Figure 6E** and **6F**).
84 Taken together, our findings suggested that HMP-S7 attacked leukemic cells to form
85 micropores on cellular membranes and penetrated into the cytoplasm to perturb leukemic
86 cells until the death.

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



87
88
89 **Figure 6. HMP-S7 action on internalization and leukemic cell death.** Jurkat cells were
90 treated with HMP-S7 conjugated with or without FITC at IC50 and 0.01% Triton X-100
91 (positive control) and stained with PI to observe membrane permeability **(A)**. The FITC
92 tagged HMP-S7 (at IC50 and 2×IC50) were internalized into the cytoplasm of Jurkat cells.
93 (**B**) Flow cytometry of FITC tagged HMP-S7-treated Jurkat cells. (**C**) The bar graph showed

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

44 integrative workflow; nonetheless, only HMP-S7 had the cytotoxic effect against leukemic
45 cells. From this observation, we learned that this integrative approach is more accurate than
46 using a single preferred anticancer property to prioritize peptide candidates. When one
47 peptide candidate met all three criteria, the difference in one positive charge (and perhaps
48 two votes from machine learning models) did matter for the anti-leukemic prediction (**Figure**
49 **3**). In future studies, this integrative approach of ACP screening can be applied to larger data
50 sets, either the mass spectrometric-based or the *in silico* generated peptide libraries, to speed
51 up the discovery of ACP against multiple cancer types.

52 Looking forward, HMP-S7 should be further validated in preclinical models. Further
53 peptide modifications using HMP-S7 as a prototype, for example, amino acid substitution
54 (*40*), homing motif tagging (*41*), and PEGylation (*42*), may improve its anticancer efficacy,
55 tumor targeting, and biocompatibility and stability. Furthermore, the human milk peptide
56 library can be expanded by further profiling relevant biospecimens or by *in silico* peptide
57 generation from the proteins of interest. Aforementioned *in vitro* study support, machine
58 learning for ACP prediction might be further improved by a combination of ensemble
59 models, physicochemical property, and secondary peptide structure.

60 This study had limitations. It was expected that milk peptides should present a more
61 significant number of unique identities than those identified in this study. Losing of peptides
62 during mass spectrometric detection because of their neutral net charge may be one reason.
63 Another possibility is that the milk peptides may be degraded during the fractionation
64 process. These limitations could be overcome by the combined methods using fractionations
65 and high-resolution mass spectrometry detection. The issues related to the small peptide
66 library because of experimental bottlenecks could be addressed by *in silico* generated
67 peptide database as aforementioned, given that the custom-made peptides are commercially
68 available and ready for anticancer activity screening. Lastly, it should be emphasized that
69 *ex vivo* experiments of HMP-S7 by using the patient's derived leukemic cells should be
70 further explored in the future.
71

72 ## Conclusions

73 This study discovered HMP-S7 (SFIPRAKSTWLNNIKLL) as the novel anti-
74 leukemic peptide derived from human breast milk. This anti-leukemic peptide is a cationic
75 and alpha-helical structure peptide that selectively kills leukemic cells but not normal cells.
76 Future research on peptide modification, together with the efficacy studies in preclinical
77 animal models or patient's derived leukemic cells *ex vivo*, is warranted to develop HMP-S7
78 as peptide-based cancer therapeutics in the future.
79

80 ## Materials and Methods

81 **Human milk collection.** Human milk (60 ml) from 10 healthy volunteer mothers of full-
82 term infant, whose blood test has no infection of HIV, Hepatitis B or C viruses, and Syphilis,
83 were collected by a breast pump and stored in a freezer (-20°C) as per the regulation of
84 Ramathibodi Human Milk Bank (RHMB) before transferring to the laboratory (*43*). All
85 subjects gave their informed consent for inclusion before they participated in the study. The
86 study was conducted in accordance with the Declaration of Helsinki, and the protocol was
87 approved by the Human Research Ethics Committee, Faculty of Medicine Ramathibodi
88 Hospital, Mahidol University (Protocol ID-11-60-13).
89

90 **Peptide isolation and fractionation.** Three pooled milk specimens were produced from 3,
91 3, and 4 individual specimens (**Table S1**) before further processing. Twenty milliliters of
92 pooled milk specimens were centrifuged at 4°C, 1,500 g for 10 min, and then 5,000 g for 30
93 min twice to remove cells and lipids. The collected supernatant was centrifuged at 4°C,

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

44 separated with a gradient of 5-45% acetonitrile/0.1% formic acid for 30 min at a flow rate
45 of 300 nl/min. The isolated peptides were identified by the amaZon speed ETD ion trap
46 mass spectrometer (Bruker Daltoniks, Billerica, MA, USA). Peptide sequences and
47 identification were interpreted using in-house MASCOT software version 2.4.0 with the
48 SwissProt database, against *Homo sapiens*, no fixed and no variable modifications, no
49 enzymatic digestion with no missed cleavage allowed, monoisotopic, ±1.2 Da for peptide
50 tolerance, ±0.6 Da for fragment ion tolerance, 2+ and 3+ charge state for ESI-TRAP
51 instrument. Identified peptides were provided ion scores higher than 20 (significance
52 threshold at $p < 0.05$).
53
54 ***In silico* anticancer peptide screening.** Physicochemical properties of all identified human
55 milk peptides were predicted by using the PepDraw tool
56 (http://www.tulane.edu/~biochem/WW/PepDraw/), including peptide length, mass, net
57 charge, isoelectric point (p*I*), and hydrophobicity. Peptide structures were predicted using
58 the PEP-FOLD3 De novo peptide structure prediction tool (*45-47*). Anticancer properties
59 were predicted by four web-based machine learning programs, including i) ACPred-FL
60 (Tianjin University, School of Computer Science and Technology, China), a sequence base
61 predictor for identifying anti-cancer peptides from proteins, by using classification mode at
62 confidence 0.5 as default setting (http://server.malab.cn/ACPred-FL/#) (*33*); ii) AntiCP 2.0,
63 an ensemble tree classifier on amino acid composition
64 (https://webs.iiitd.edu.in/raghava/anticp2/index.html) (*34*); iii) MLACP, a random forest-
65 based prediction of anticancer peptides (www.thegleelab.org/MLACP.html) (*35*); and iv)
66 mACPpred, a Support Vector Machine-Based Meta-Predictor (http://www.thegleelab.org/
67 mACPpred) (*36*).
68
69 **Cytotoxicity assay.** For cell treatment, the fractionated milk peptides (10 μg/reaction) or
70 the synthetic peptides (varied concentrations as indicated) were resuspended in 100 μL of
71 culture media with appropriated supplements. For floating cells, the peptide solution was
72 mixed with cell suspension (10,000 cells/10 μL/well) in a well of 96-well plate flat bottom.
73 For adherent cells, 10,000 cells were seeded into a 96-well plate (flat bottom) and cultured
74 until they reached 80% confluence before adding 100 μL of peptide solution into the well
75 containing 80% FHs 74 Int cell confluence (3 technical replication). The peptide-treated
76 cells were incubated for 24, 48, or 72 h in 5% CO_2 incubator at 37°C with humidity as
77 indicated. Cell viability was measured by trypan blue exclusion or WST-1 assays (Roche
78 Diagnostics GmbH, Mannheim, Germany).
79 The two synthetic peptides at 200 μM final concentration, i.e., HMP-S7 and BMP-
80 S6 were selected to treat with normal (T cells and HEK293T (human embryonic kidney
81 cells)) and cancer cell lines (SH-SY5Y (neuroblastoma), MDA-MB-231 (breast
82 adenocarcinoma), HT29 (colorectal adenocarcinoma), A549 (lung carcinoma), HepG2
83 (hepatocellular carcinoma).
84 Human peripheral blood mononuclear cells (PBMCs) were prepared from 10 mL
85 EDTA peripheral blood collection as aforementioned for T cell culture. The protocol was
86 approved by the Human Research Ethics Committee, Faculty of Medicine Ramathibodi
87 Hospital, Mahidol University, based on the Declaration of Helsinki (Protocol ID-06-61-09).
88 The 1×10^4 floating cells (in 10 μL), i.e., T cells, were added to 96-well plate, flat bottom
89 containing 100 μL of their culturing medium with or without 200 μM HMP-S7 and 200 μM
90 BMP-S6. While, 1×10^4 adherent cells were seed in 96-well plate, flat bottom and then
91 cultured 1-2 overnight at 37°C under a humidified atmosphere with 5% CO_2 incubator until
92 90-100% cell confluent. The cultured medium was removed, and then added the medium
93 containing 200 μM HMP-S7 and 200 μM BMP-S6. All treated and untreated cells were

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

94 incubated for 24 h before trypsinization (for adherent cells) and split to cell death
95 measurement using trypan blue exclusion assay (3 technical replications).
96
97 **Half maximum inhibitory concentration (IC_{50}) by trypan blue assay.** Four distinct
98 leukemic cell lines, i.e., Jurkat, Raji, RS4;11, and Sup-B15 cell lines were cultured in the
99 medium containing HMP-S7 in vary concentrations (0, 6.25, 12.5, 25, 50, 100, 200, and 400
00 µM) in 96-well plate, flat bottom for 24 h (1×10^4 cells/100 µL/well). The %cell death was
01 estimated by trypan blue exclusion assay using (cell death number/total cell number) × 100.
02 IC_{50} calculation used linear (y=ax+c) or parabolic ($y=ax^2+bx+c$) equation for y=50 value
03 and x value= IC_{50} concentration. IC_{50} concentration of HMP-S7 to the 4 leukemic cell lines
04 was reported in mean±SD.
05
06 **Soft agar assay for colony formation.** Base agar (1.5 mL of 0.5% agar containing 1×RPMI
07 supplement with 10% FBS and 1× penicillin/streptomycin) were plated on each well of a 6-
08 well plate and set aside for 5 min to allow the agar to solidify. Jurkat cells were treated with
09 100 µM, 200 µM, 400 µM HMP-S7, and 200 µM BMP-S6 in a 96-well plate (3 technical
10 replications, 100 µL/10,000 cells/well) for 24 h. After 24 h, the treated/untreated cells in the
11 96-well plate were thoroughly mixed and equally taken (20 µL) to mix in 1 mL of the top
12 agar solution (0.3% agarose containing 1×RPMI supplement with 10% FBS and 1×
13 penicillin/streptomycin). The cell suspension was plated on top of the base agar (3 biological
14 replication) and then allowed agarose to form solid. The medium (0.5 mL) was added on
15 top of the agar to prevent agar dry. The plate was incubated at 37°C in a humidified
16 incubator for 20 days with feed the medium twice a week. The agar was stained with 1 mL
17 of 0.005% crystal violet in 20% ethanol for 1 h, destained with 20% ethanol overnight, and
18 then counted the colonies under stereomicroscope SZ61 (Olympus Corporation, Tokyo,
19 Japan).
20
21 **Apoptosis assay.** Four leukemic cell lines (1×10^5 cells/500 µL/well/cell type) were cultured
22 in 24 well-plate in the medium containing HMP-S7 with IC_{50} and $2\times IC_{50}$ concentrations and
23 non-treatment condition for 24 h (biological triplication). The untreated cells were divided
24 into 3 tubes that are unstained, cells stained with FITC tagged annexin V (no propidium
25 iodide (PI)), and cells stained with PI (no FITC tagged annexin V) to set up compensation
26 and quadrants. The untreated and treated cells were collected and wash cells with cold PBS
27 twice. Thereafter, the cells were resuspended with 100 µL of 1× binding buffer and then
28 added 5 µL of FITC tagged annexin V and 5 µL of PI. The cell suspensions were gently
29 mixed and incubated at room temperature for 15 min in dark. Then, 400 µL of 1× binding
30 buffer was added to each tube, and then the cells were analyzed by using flow cytometry.
31
32 **Lactate dehydrogenase (LDH) release assay.** RS4;11 leukemic cell lines (2×10^5 cells/1
33 mL/well/cell type) were cultured in 24 well-plate in the complete medium containing HMP-
34 S7 at IC_{50} and $2\times IC_{50}$ concentrations, without HMP-S7 condition (non-treatment), and 0.5%
35 Triton X-100 for 24 h incubation (biological triplication). The cell suspension was collected
36 and centrifuged at 200 g for 5 min at room temperature. The 1 mL of supernatant was
37 collected and measured LDH isoenzyme by measuring lactate to pyruvate and NADH,
38 which increased absorbance at 340 nm using Abbott Architect C16000 clinical chemistry
39 analyzer (Holliston, MA, USA) at Clinical Chemistry Unit of Department of Pathology at
40 Ramathibodi Hospital, Thailand. LDH level (U/L) of the culture media was subtracted from
41 the background of the fresh medium and reported as mean±SD.
42

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

43 **FITC tagged HMP-S7 internalized into leukemic cells.** The 2×10^4 Jurkat cells/100
44 µl/well in the 96-well plate was treated with HMP-S7 tagged with/without FITC at IC_{50} for
45 24 h. Thereafter, the treated and untreated Jurkat cells were washed with 1×PBS once before
46 staining with propidium iodide (PI) cell stain kit (Invitrogen) and Hoechst 33342 (Cell
47 Signaling Technology, Inc, Massachusetts, USA), for 30 min at room temperature in dark.
48 After incubation, the cells were centrifuged at 200 g for 5 min and removed the excess dye
49 solution before washing with 1×PBS twice. The cell pellet was mixed with 20%
50 glycerol/PBS and mounted on a glass slide for confocal microscopy (Nikon Instruments,
51 Inc, NY, USA).
52
53 **Statistical Analysis**. The number and percentage of death or live cells, OD and IC_{50}, were
54 calculated and statistical testing using Student's *t-test* as statistical significance at $p < 0.05$.
55
56 **Acknowledgments**
57 **General**: We would like to thank Mrs. Sirimon Kongthaworn, for assisting milk collection
58 at RHMB, and staff at the Laboratory of Biochemistry, Chulabhorn Research Institute, and
59 at Pediatric Department and Research Center at Faculty of Medicine Ramathibodi Hospital
60 for supporting research instrument facility.
61
62 **Funding:** This study was supported by BRAND'S Health Research Award 2017 (Cerebos
63 Award 2017 to W.C.), Children Cancer Fund under the Patronage of HRH Princess
64 Soamsawali and Ramathibodi Foundation (to S.H.), and the New Discovery and Frontier
65 Research Grant of Mahidol University (NDFR19/2563 to SC). SC was financially supported
66 by the Faculty Staff Development Program of Faculty of Medicine Ramathibodi Hospital,
67 Mahidol University, for his research activities.
68
69 **Author contributions:** Conceptualization, S.C.; methodology, W.C., S.C.; formal
70 analysis, W.C., J.P., T.V., N.P., T.L., C.S., S.C.; resources, P.N., S.S., J.S., S.H., S.C.;
71 writing – original draft preparation, W.C., writing – review and editing, J.P., T.V., N.P.,
72 T.L., P.N., S.S., C.S., J.S., S.H., S.C.; visualization, W.C., S.C.; supervision, J.S., S.H. All
73 authors have read and agreed to the published version of the manuscript.
74
75 **Competing interests:** All authors declared no conflicts of interest.
76
77 **Data and materials availability:** The dataset used or analyzed during this study is included
78 in this published article and its supplementary information files.
79
80 **References**
81 1. L. A. Torre, R. L. Siegel, E. M. Ward, A. Jemal, Global Cancer Incidence and Mortality Rates and
82 Trends--An Update. *Cancer Epidemiol Biomarkers Prev* **25**, 16-27 (2016).
83 2. R. L. Siegel, K. D. Miller, A. Jemal, Cancer statistics, 2016. *CA Cancer J Clin* **66**, 7-30 (2016).
84 3. H. M. Kantarjian, M. J. Keating, E. J. Freireich, Toward the potential cure of leukemias in the next
85 decade. *Cancer* **124**, 4301-4313 (2018).
86 4. L. L. Robison, S. Bhatia, Late-effects among survivors of leukaemia and lymphoma during childhood
87 and adolescence. *Br J Haematol* **122**, 345-359 (2003).
88 5. D. A. Mulrooney, G. Hyun, K. K. Ness, N. Bhakta, C. H. Pui, M. J. Ehrhardt, K. R. Krull, D. B. Crom,
89 W. Chemaitilly, D. K. Srivastava, M. V. Relling, S. Jeha, D. M. Green, Y. Yasui, L. L. Robison, M. M.
90 Hudson, The changing burden of long-term health outcomes in survivors of childhood acute
91 lymphoblastic leukaemia: a retrospective analysis of the St Jude Lifetime Cohort Study. *Lancet
92 Haematol* **6**, e306-e316 (2019).
93 6. D. Bhojwani, S. C. Howard, C. H. Pui, High-risk childhood acute lymphoblastic leukemia. *Clin
94 Lymphoma Myeloma* **9 Suppl 3**, S222-230 (2009).

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

95 7. F. Harris, S. R. Dennison, J. Singh, D. A. Phoenix, On the selectivity and efficacy of defense peptides
96 with respect to cancer cells. *Med Res Rev* **33**, 190-234 (2013).
97 8. N. Papo, Y. Shai, Host defense peptides as new weapons in cancer treatment. *Cell Mol Life Sci* **62**, 784-
98 790 (2005).
99 9. T. Utsugi, A. J. Schroit, J. Connor, C. D. Bucana, I. J. Fidler, Elevated expression of phosphatidylserine
00 in the outer membrane leaflet of human tumor cells and recognition by activated human blood
01 monocytes. *Cancer Res* **51**, 3062-3066 (1991).
02 10. K. Kozlowska, J. Nowak, B. Kwiatkowski, M. Cichorek, ESR study of plasmatic membrane of the
03 transplantable melanoma cells in relation to their biological properties. *Exp Toxicol Pathol* **51**, 89-92
04 (1999).
05 11. M. Sok, M. Sentjurc, M. Schara, Membrane fluidity characteristics of human lung cancer. *Cancer Lett*
06 **139**, 215-220 (1999).
07 12. F. Schweizer, Cationic amphiphilic peptides with cancer-selective toxicity. *Eur J Pharmacol* **625**, 190-
08 194 (2009).
09 13. I. Murota, S. Taguchi, N. Sato, E. Y. Park, Y. Nakamura, K. Sato, Identification of antihyperuricemic
10 peptides in the proteolytic digest of shark cartilage water extract using in vivo activity-guided
11 fractionation. *J Agric Food Chem* **62**, 2392-2397 (2014).
12 14. X. Chen, L. Zhang, C. Ma, Y. Zhang, X. Xi, L. Wang, M. Zhou, J. F. Burrows, T. Chen, A novel
13 antimicrobial peptide, Ranatuerin-2PLx, showing therapeutic potential in inhibiting proliferation of
14 cancer cells. *Biosci Rep* **38**, (2018).
15 15. R. Ma, S. W. Wong, L. Ge, C. Shaw, S. W. I. Siu, H. F. Kwok, In Vitro and MD Simulation Study to
16 Explore Physicochemical Parameters for Antibacterial Peptide to Become Potent Anticancer Peptide.
17 *Mol Ther Oncolytics* **16**, 7-19 (2020).
18 16. A. Pihlanto, H. Korhonen, Bioactive peptides and proteins. *Adv Food Nutr Res* **47**, 175-276 (2003).
19 17. D. P. Mohanty, S. Mohapatra, S. Misra, P. S. Sahu, Milk derived bioactive peptides and their impact on
20 human health - A review. *Saudi J Biol Sci* **23**, 577-583 (2016).
21 18. Y. Fu, C. Ji, X. Chen, X. Cui, X. Wang, J. Feng, Y. Li, R. Qin, X. Guo, Investigation into the
22 antimicrobial action and mechanism of a novel endogenous peptide beta-casein 197 from human milk.
23 *AMB Express* **7**, 119 (2017).
24 19. D. C. Dallas, A. Guerrero, N. Khaldi, P. A. Castillo, W. F. Martin, J. T. Smilowitz, C. L. Bevins, D.
25 Barile, J. B. German, C. B. Lebrilla, Extensive in vivo human milk peptidomics reveals specific
26 proteolysis yielding protective antimicrobial peptides. *J Proteome Res* **12**, 2295-2304 (2013).
27 20. I. Politis, R. Chronopoulou, Milk peptides and immune response in the neonate. *Adv Exp Med Biol* **606**,
28 253-269 (2008).
29 21. X. Cui, Y. Li, L. Yang, L. You, X. Wang, C. Shi, C. Ji, X. Guo, Peptidome analysis of human milk from
30 women delivering macrosomic fetuses reveals multiple means of protection for infants. *Oncotarget* **7**,
31 63514-63525 (2016).
32 22. H. Meisel, R. J. FitzGerald, Biofunctional peptides from milk proteins: mineral binding and
33 cytomodulatory effects. *Curr Pharm Des* **9**, 1289-1295 (2003).
34 23. M. Zhao, C. Wei, X. Yang, J. Zhou, J. Wang, F. Gu, T. Lei, Y. Qin, The milk-derived hexapeptide
35 PGPIPN inhibits the invasion and migration of human ovarian cancer cells by regulating the expression
36 of MTA1 and NM23H1 genes. *Int J Oncol* **48**, 1721-1729 (2016).
37 24. J. Zhou, M. Zhao, Y. Tang, J. Wang, C. Wei, F. Gu, T. Lei, Z. Chen, Y. Qin, The milk-derived fusion
38 peptide, ACFP, suppresses the growth of primary human ovarian cancer cells by regulating apoptotic
39 gene expression and signaling pathways. *BMC Cancer* **16**, 246 (2016).
40 25. V. B. Smulevich, L. G. Solionova, S. V. Belyakova, Parental occupation and other factors and cancer
41 risk in children: I. Study methodology and non-occupational factors. *Int J Cancer* **83**, 712-717 (1999).
42 26. E. L. Amitay, L. Keinan-Boker, Breastfeeding and Childhood Leukemia Incidence: A Meta-analysis
43 and Systematic Review. *JAMA Pediatr* **169**, e151025 (2015).
44 27. B. K. Kay, M. P. Williamson, M. Sudol, The importance of being proline: the interaction of proline-rich
45 motifs in signaling proteins with their cognate domains. *FASEB J* **14**, 231-241 (2000).
46 28. L. Holfeld, D. Knappe, R. Hoffmann, Proline-rich antimicrobial peptides show a long-lasting post-
47 antibiotic effect on Enterobacteriaceae and Pseudomonas aeruginosa. *J Antimicrob Chemother* **73**, 933-
48 941 (2018).

bioRxiv preprint doi: https://doi.org/10.1101/2021.03.07.434306; this version posted March 8, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

49 29. D. Gaspar, A. S. Veiga, M. A. Castanho, From antimicrobial to anticancer peptides. A review. *Front*
50 *Microbiol* **4**, 294 (2013).
51 30. M. R. Felicio, O. N. Silva, S. Goncalves, N. C. Santos, O. L. Franco, Peptides with Dual Antimicrobial
52 and Anticancer Activities. *Front Chem* **5**, 5 (2017).
53 31. M. Mahlapuu, J. Hakansson, L. Ringstad, C. Bjorn, Antimicrobial Peptides: An Emerging Category of
54 Therapeutic Agents. *Front Cell Infect Microbiol* **6**, 194 (2016).
55 32. S. R. Dennison, M. Whittaker, F. Harris, D. A. Phoenix, Anticancer alpha-helical peptides and
56 structure/function relationships underpinning their interactions with tumour cell membranes. *Curr*
57 *Protein Pept Sci* **7**, 487-499 (2006).
58 33. L. Wei, C. Zhou, H. Chen, J. Song, R. Su, ACPred-FL: a sequence-based predictor using effective
59 feature representation to improve the prediction of anti-cancer peptides. *Bioinformatics* **34**, 4007-4016
60 (2018).
61 34. P. Agrawal, D. Bhagat, M. Mahalwal, N. Sharma, G. P. S. Raghava, AntiCP 2.0: an updated model for
62 predicting anticancer peptides. *Brief Bioinform*, (2020).
63 35. B. Manavalan, S. Basith, T. H. Shin, S. Choi, M. O. Kim, G. Lee, MLACP: machine-learning-based
64 prediction of anticancer peptides. *Oncotarget* **8**, 77121-77136 (2017).
65 36. V. Boopathi, S. Subramaniyam, A. Malik, G. Lee, B. Manavalan, D. C. Yang, mACPpred: A Support
66 Vector Machine-Based Meta-Predictor for Identification of Anticancer Peptides. *Int J Mol Sci* **20**,
67 (2019).
68 37. C. Svanborg, H. Agerstam, A. Aronson, R. Bjerkvig, C. Duringer, W. Fischer, L. Gustafsson, O.
69 Hallgren, I. Leijonhuvud, S. Linse, A. K. Mossberg, H. Nilsson, J. Pettersson, M. Svensson, HAMLET
70 kills tumor cells by an apoptosis-like mechanism--cellular, molecular, and therapeutic aspects. *Adv*
71 *Cancer Res* **88**, 1-29 (2003).
72 38. T. T. Hien, I. Ambite, D. Butler, M. L. Y. Wan, T. H. Tran, U. Hoglund, M. Babjuk, C. Svanborg,
73 Bladder cancer therapy without toxicity-A dose-escalation study of alpha1-oleate. *Int J Cancer* **147**,
74 2479-2492 (2020).
75 39. Q. Liu, H. Zhao, Y. Jiang, M. Wu, Y. Tian, D. Wang, Y. Lao, N. Xu, Z. Li, Development of a lytic
76 peptide derived from BH3-only proteins. *Cell Death Discov* **2**, 16008 (2016).
77 40. C. Hu, X. Chen, W. Zhao, Y. Chen, Y. Huang, Design and Modification of Anticancer Peptides. *Drug*
78 *Des* **5**, 138 (2016).
79 41. Z. J. Li, C. H. Cho, Peptides as targeting probes against tumor vasculature for diagnosis and drug
80 delivery. *J Transl Med* **10 Suppl 1**, S1 (2012).
81 42. V. Gupta, S. Bhavanasi, M. Quadir, K. Singh, G. Ghosh, K. Vasamreddy, A. Ghosh, T. J. Siahaan, S.
82 Banerjee, S. K. Banerjee, Protein PEGylation for cancer therapy: bench to bedside. *J Cell Commun*
83 *Signal* **13**, 319-330 (2019).
84 43. P. Kanaprach, N. Pongsakul, N. Apiwattanakul, C. Muanprasat, S. Supapannachart, P. Nuntnarumit, S.
85 Chutipongtanate, Evaluation of Fetal Intestinal Cell Growth and Antimicrobial Biofunctionalities of
86 Donor Human Milk After Preparative Processes. *Breastfeed Med* **13**, 215-220 (2018).
87 44. S. Chutipongtanate, K. Watcharatanyatip, T. Homvises, K. Jaturongkakul, V. Thongboonkerd,
88 Systematic comparisons of various spectrophotometric and colorimetric methods to measure
89 concentrations of protein, peptide and amino acid: detectable limits, linear dynamic ranges,
90 interferences, practicality and unit costs. *Talanta* **98**, 123-129 (2012).
91 45. A. Lamiable, P. Thevenet, J. Rey, M. Vavrusa, P. Derreumaux, P. Tuffery, PEP-FOLD3: faster de novo
92 structure prediction for linear peptides in solution and in complex. *Nucleic Acids Res* **44**, W449-454
93 (2016).
94 46. Y. Shen, J. Maupetit, P. Derreumaux, P. Tuffery, Improved PEP-FOLD Approach for Peptide and
95 Miniprotein Structure Prediction. *J Chem Theory Comput* **10**, 4745-4758 (2014).
96 47. P. Thevenet, Y. Shen, J. Maupetit, F. Guyon, P. Derreumaux, P. Tuffery, PEP-FOLD: an updated de
97 novo structure prediction server for both linear and disulfide bonded cyclic peptides. *Nucleic Acids Res*
98 **40**, W288-293 (2012).
99 48. J. S. Mader, A. Richardson, J. Salsman, D. Top, R. de Antueno, R. Duncan, D. W. Hoskin, Bovine
00 lactoferricin causes apoptosis in Jurkat T-leukemia cells by sequential permeabilization of the cell
01 membrane and targeting of mitochondria. *Exp Cell Res* **313**, 2634-2650 (2007).

02