LHRH-conjugated BinB pore-forming domain for specific targeting to breast cancer cells

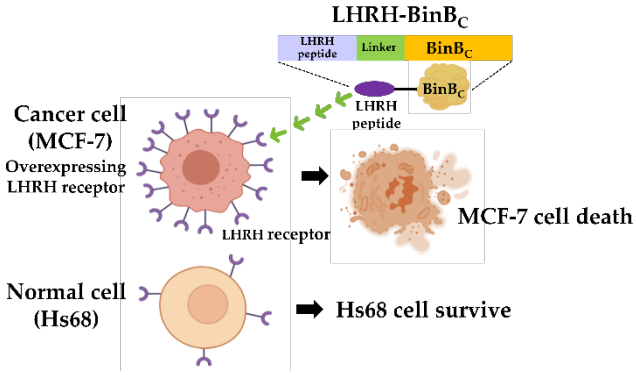
Tipaporn Kumkoon, Monrudee Srisaisup, Chalongrat Noree, Panadda Boonserm *

[1] Institute of Molecular Biosciences, Mahidol University, Salaya, Phuttamonthon, Nakhon Pathom 73170, Thailand; tipaporn.kuk@student.mahidol.edu

* Correspondence: panadda.boo@mahidol.ac.th

Abstract: Breast cancer has a high incidence in females around the world. Treatments of this cancer lack specificity and cause long-term side effects to the patients. As a result, pore-forming toxins from bacteria together with cell targeting peptides (CTPs) have been used as alternative anticancer agents. Here, we aim to increase the target specificity of BinB toxin derived from *Lysinibacillus sphaericus* by fusing a luteinizing hormone-releasing hormone (LHRH) peptide to its pore-forming domain (BinBc) to target MCF-7 (LHRH receptor positive) cells. Hs68 (LHRH receptor negative) cells were used for comparing the degree of LHRH-mediated target specificity. Parasporin-2 (PS2), a pore-forming toxin from *Bacillus thuringiensis* with strong cytotoxicity against human cancer cells, was also used as a positive control. The cytotoxic effects of BinB, PS2, BinBc, and LHRH-BinBc were monitored by MTT assay. We found that BinB at highest concentrations (16 μM) could reduce the viability of MCF-7 and Hs68 cells, whereas PS2 at 0.00125 to 0.08 μM and at 0.5 μM showed strong cytotoxic effects on MCF-7 and Hs68 cells, respectively. However, both BinBc and LHRH-BinBc caused no cytotoxic effects on Hs68 cells, but they could inhibit the proliferation of MCF-7 cells. Moreover, LHRH-BinBc was slightly more active to MCF-7 cells than free BinBc. Our results have suggested that BinBc represents an attractive biotoxin that can be engineered as a potential anti-cancer agent.

Graphical abstract:



Keywords: Binary toxin; C-terminal domain of BinB (BinBc); Parasporin-2 toxin; cell-targeting peptide; LHRH peptide

1. Introduction

Breast Cancer is one of the most lethal diseases, causing thousands of deaths in women around the world. Several cancer treatments have been developed such as chemotherapy, radiation, targeted therapies, and immunotherapy, but those treatments can induce some unfavorable side effects. This has led to the development of new strategies to reduce adverse effects by using bacterial toxins as anticancer agents. *Lysinibacillus*

sphaericus (Ls) is a Gram-positive bacterium that produces insecticidal binary toxin (Bin) during the sporulation stage with toxicity against *Culex* and *Anopheles* mosquito larvae [1-3]. Bin toxin is composed of BinA (42 kDa) and BinB (51 kDa) [3]. BinA is essential for toxicity whereas BinB is required for receptor binding, internalization, and translocation of BinA-BinB complex into susceptible larval gut cells [4]. According to the crystal structure of BinB, the N-terminal domain has β-trefoil scaffold with structural similarity to some carbohydrate-binding proteins, suggesting the receptor binding role of this domain. Whereas the C-terminal domain of BinB acts as a pore-forming domain due to its similar structure with those of aerolysin type β-pore forming toxins (β-PFTs), including parasporin-2 (PS2) from *Bacillus thuringiensis* [3]. Previous study has shown that the truncated form of BinB containing only the C-terminal or pore-forming domain (referred to as 'BinBc') showed higher membrane permeability and membrane perturbation, when compared with those of full-length BinB, supporting the important role of this domain for membrane interaction and pore formation [5]. PS2, one of the aerolysin-type pore-forming toxins, causes strong cytotoxicity against several human cancer cells with limited toxicity towards normal cells [6]. According to the structural similarity of Bin toxin and PS2, Bin toxin may also exert cytotoxicity towards human cancer cells, in addition to its possessing mosquitocidal activity. Our previous studies showed that high concentrations (500 μg/ml) of trypsin-activated Bin toxin affected cell viability of human cancer cell lines, including A549, Caco-2, HepG2, HK-1, and KKU-M055. Moreover, Bin toxin altered cell morphology, decreased cell migration and adhesion, and induced cell apoptosis [7]. Although using bacterial toxins could be a promising strategy to reduce undesirable side effects as compared with chemotherapy, its poor specificity to the cancer cells remains a critical concern. To solve this, a new class of targeting moiety called cell-targeting peptides (CTPs) have been employed as peptide-drug conjugates for directing several anticancer agents to the target cancer cells [8,9]. This approach is based on the binding between peptides and receptors that are overexpressed on certain cancer cells. Luteinizing hormone-releasing hormone (LHRH) or gonadotropin-releasing hormone (GnRH), a hormonal decapeptide produced by hypothalamus, has been used as a targeting peptide due to the fact that the LHRH receptors are overexpressed on the cell membrane of various cancer cells, including breast cancer cells, but not expressed or barely expressed on those of the normal cells [10,11].

Here, we aim to increase the specificity of a pore-forming or C-terminal domain of BinB (BinBc) to target the breast cancer cells. BinBc protein was fused with the luteinizing hormone-releasing hormone (LHRH) which acts as a targeting moiety to the LHRH receptor on the breast cancer cells. MCF-7 and Hs68 cell lines which are LHRH receptor positive and negative cells, respectively, were chosen as models to study the specificity and cytotoxicity of engineered LHRH-BinBc toxin, in comparison to other forms of BinB toxin.

2. Materials and Methods

2.1. Construction of BinBc plasmid conjugated with LHRH targeting peptide

To construct an engineered form of BinBc toxin targeting LHRH receptors on the breast cancer cells, a recombinant plasmid (pET28b-LHRH-BinBc) was constructed by fusing LHRH peptide (QHWSYGLRPG) and a linker (GSG) to the C- terminal domain or pore-forming domain of BinB (BinBc) protein using PCR-based site-directed mutagenesis (Non-Quick Change protocol) (Figure 1). Mutagenic forward and reverse primers were phosphorylated at 5′ ends before ligation. LHRH peptide and linker sequences were designed to flank the 5′ end of the forward primer, while the reverse primer was designed to be upstream (reverse-complement) of the forward primer's sequence. After PCR, linear PCR products (pET28b-LHRH-BinBc) were purified using PCR Cleanup Kit (Geneaid column), and treated with *Dpn*I to remove the parental DNA templates prior to ligation to circularize the mutagenized plasmid. After bacterial transformation, the recombinant

plasmids (pET28b-LHRH-BinBc) were isolated from the bacterial transformants, and initially screened by restriction digestion analysis (*Nde*I and *Bam*HI) before validation of the entire sequence again by DNA sequencing (Macrogen, Korea).

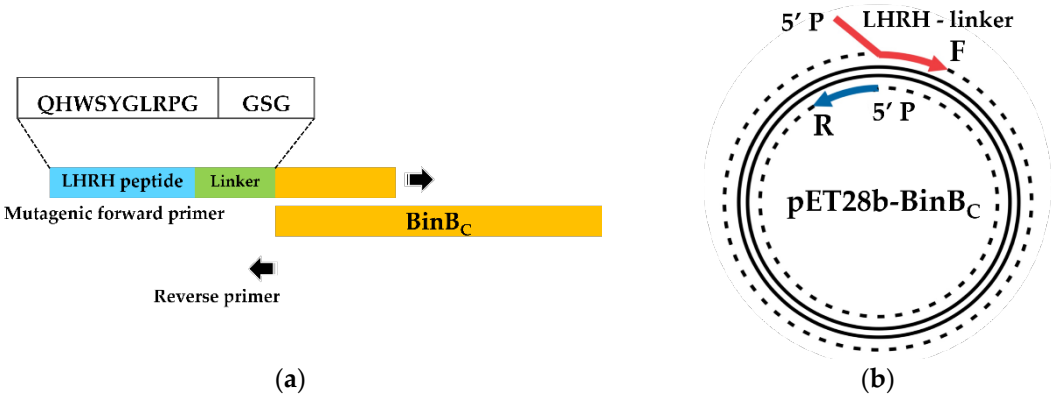


Figure 1. Construction of recombinant plasmid (pET28b-LHRH-BinBc). (**a**) Addition of LHRH peptide and linker to BinBc-encoding gene. Amino acid sequences of LHRH peptide and linker were fused to BinBc coding sequence; (**b**) The pET28b-LHRH-BinBc was constructed via PCR-based site-directed mutagenesis. F and R are 5′ phosphorylated (5′ P) forward and reverse primers, respectively.

2.2. Protein expression and purification of BinB, BinBc, engineered recombinant BinBc (LHRH-BinBc), and PS2

The recombinant plasmid pET28b-LHRH-BinBc was transformed into *E. coli* BL21(DE3) pLysS strain for protein expression. Then, the expression of His-tagged LHRH-BinBc was induced by adding 0.2 mM IPTG to an exponential growth culture and continuously grown at 18 °C for 5 hours. The successful expression of recombinant proteins was detected by 12% SDS-PAGE and western blotting using HRP anti-polyhistidine antibody [HIS-1] (1:5000). After confirmation, cells were harvested using centrifugation at 4 °C, 6000 rpm for 10 minutes, and the cell pellets were kept at -20 °C. The cells expressing LHRH-BinBc were mixed with ice-cold lysis buffer (50 mM Tris-HCl and 200 mM NaCl, pH 8.0) followed by cell lysis using ultra-sonication. The soluble and insoluble fractions were separated by centrifugation at 8000 rpm, 4 °C for 1 hour. After equilibrating HisTrap™ Fast Flow column (prepacked with precharged Ni^{2+}) with lysis buffer, the supernatant was loaded into the column. The non-specific proteins were removed by washing column with a buffer containing low concentration of imidazole (25 and 50 mM). Then, the target proteins were eluted from the resin by using a buffer with increasing concentration of imidazole (100 and 250 mM). All the unbounded and eluted fractions were collected and analyzed with 12% SDS-PAGE. The fractions containing purified recombinant protein (LHRH-BinBc) were pooled together and concentrated by using centrifugal filter units (10 kDa cutoff, Amicon®). The purified concentrated recombinant protein was further purified by using gel filtration chromatography (Superdex 200 HR 10/30 column, GE healthcare Life Science). The eluted protein fractions were collected and analyzed by SDS-PAGE analysis.

The expression and purification of BinB, BinBc and PS2 proteins were prepared following protocols as described previously [5,7]. Briefly, BinB, BinBc, and PS2 were independently cloned and expressed as a His-tagged protein using *E. coli* BL21(DE3) pLysS as a host strain. All of them were purified using Ni–NTA affinity and size-exclusion chromatography. To obtain the activated toxins, purified inactivated PS2 and BinB proteins were subject to activation via proteinase K and trypsin digestion, respectively, to produce an activated form of PS2 (30 kDa) and BinB (45 kDa). The proteinase K and trypsin were subsequently removed by using size-exclusion chromatography which was equilibrated with 50 mM Tris-HCl pH 9 and 1 mM dithiothreitol (DTT).

2.3. In vitro study

2.3.1. Cell culture and cell cytotoxicity analysis

Human breast cancer cell line (MCF-7) was purchased from ATCC (Manassas, VA, USA). Human fibroblast cell line (Hs68) was kindly provided by Dr. Kanokporn Srisucharitpanit. Hs68 and MCF-7 cells were cultured at 37 °C, 5% CO_2, in DMEM (Gibco) and EMEM (ATCC), respectively, both supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). The viabilities of MCF-7 and Hs68 cells were assessed after treated with toxins at different concentrations, having cells treated with PS2 as a positive control, and cells treated with buffer (Tris-HCl pH 9) as a negative control. Briefly, $1x10^4$ cells of MCF-7 and Hs68 cell line were seeded into each well of a 96-well plate and cultured for 48 hours. Different concentrations of toxins (0.5, 1, 2, 4, 8, 16 μM for activated BinB, BinBc, and LHRH-BinBc, and 0.00125, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, and 0.5 μM for PS2) were added to the corresponding well (three replicates) and the plate was incubated for 48 hours. Then, 10 μl of MTT solution (Invitrogen, Carlsbad, California, USA) at a final concentration of 5 mg/ml was added and the plate was incubated for 4 hours. Then, 100 μl of dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) was added to each well to dissolve the formazan crystals in the cells before measuring the absorbance at 595 nm using Beckman Coulter DTX 880 Microplate Reader.

2.3.2. Statistical analysis

After completing three independent replicates, the data are presented as mean ± standard deviation (SD). Statistical analysis (paired t-test) was performed by using GraphPad Prism Version 9.1.2 (226). Statistically significant was considered when $p = 0.05$ or lower.

3. Results

3.1. Expression and purification of BinB, BinBc, LHRH-BinBc and PS2 proteins

The LHRH-BinBc and BinBc proteins were expressed and analyzed using 12% SDS-PAGE and western blotting (Figure 2). The BinBc protein was highly expressed as a soluble form with a molecular mass of about 28 kDa (Figure 2a), but small amount of this protein was also detected as an insoluble form (Figure 2b). LHRH-BinBc protein was mainly expressed as a soluble protein with a molecular mass of about 30 kDa, as expected (Figures 2a and 2b). To determine the cytotoxic activity of PS2 and BinB proteins, in comparison with BinBc and LHRH-BinBc, inactive forms of PS2 and BinB proteins were activated by proteinase K and trypsin, respectively, giving protein fragments of 30 kDa for PS2 and 45 kDa for BinB (data not shown).

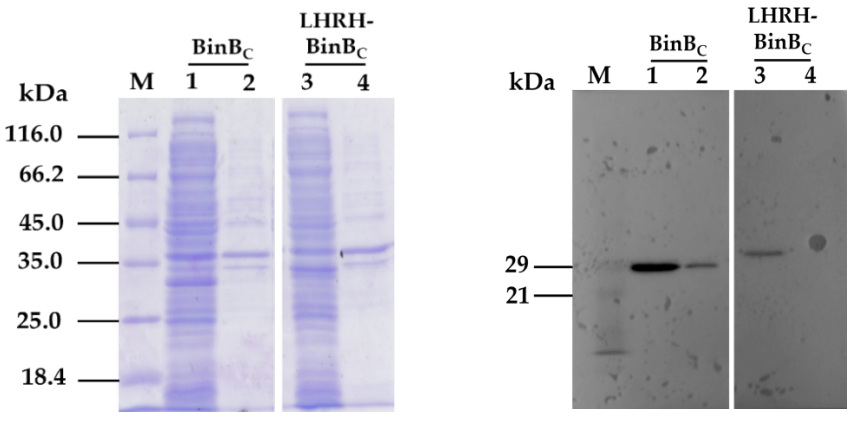


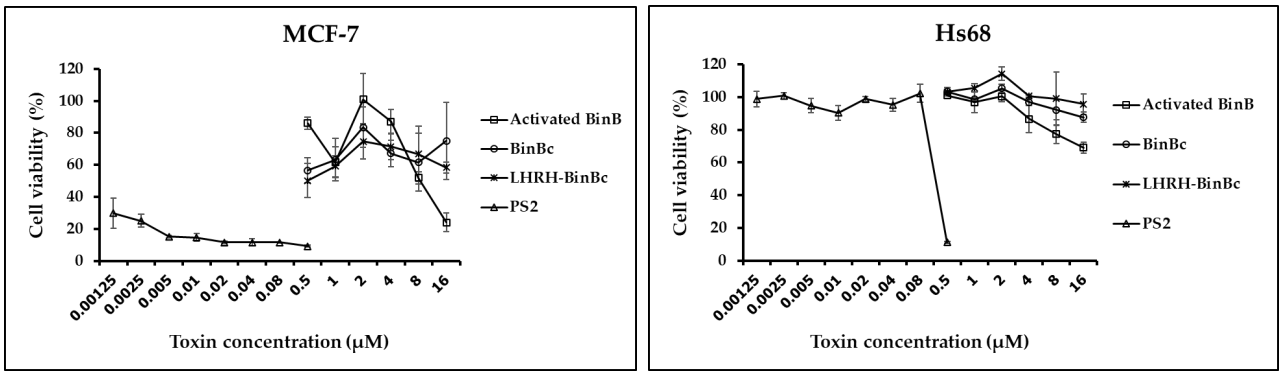
Figure 2. 12% SDS-PAGE and western blot analysis of protein expression in *E. coli* BL21(DE3) pLysS strain. (**a**) 12% SDS-PAGE analysis of BinBc and LHRH-BinBc protein expression; Lane M: unstained protein MW marker; Lanes 1a and 3a:

soluble fractions of BinBc and LHRH-BinBc, respectively; Lanes 2a and 4a: insoluble fractions of BinBc and LHRH-BinBc, respectively; (**b**) Western blot analysis of BinBc and LHRH-BinBc protein expression using HRP anti-polyhistidine antibody; Lane M: pre-stained protein MW marker; Lanes 1b and 3b: soluble fractions of BinBc and LHRH-BinBc, respectively; Lanes 2b and 4b: insoluble fractions of BinBc and LHRH-BinBc, respectively.

3.2. Cell cytotoxicity analysis

To investigate the cytotoxic effects of engineered LHRH-BinBc, compared to BinBc and other toxins (BinB and PS2) on MCF-7 and Hs68 cells, cells were treated with activated BinB, BinBc, LHRH-BinBc toxins at concentrations ranging from 0.5-16 μM, and 0.00125-0.5 μM for PS2 toxin. The results of MTT assay showed that trypsin-activated BinB, especially at 16 μM, reduced the viability of MCF-7 and Hs68 cells (Figures 3a and 3b). Both BinBc and LHRH-BinBc showed no adverse effects on Hs68 cells but they could inhibit cell proliferation of MCF-7 cells by about 50% even at the lowest concentration used in this experiment (0.5 μM). In addition, LHRH-BinBc was slightly more active against MCF-7 cells, compared to BinBc (Figure 3a). As a positive control, PS2 at 0.00125 to 0.5 μM showed strong cytotoxicity on MCF-7 cells in a dose-dependent manner (Figure 3a) whereas at 0.5 μM, PS2 appeared to be toxic against Hs68 cells (Figure 3b).

Under the microscope, the morphologies of MCF-7 and Hs68 cells were observed. We found that neither BinBc nor LHRH-BinBc caused any cell morphological changes to the MCF-7 cells, whereas cell shrinkage, a characteristic of apoptotic cell death [12], was observed after treating MCF-7 cells with all concentrations of PS2 (0.00125-0.5 μM) and at 16 μM of trypsin-activated BinB (Figure S1). No morphological alteration of HS68 cells was observed after treated with all concentrations of activated BinB, BinBc and LHRH-BinBc (0.5-16 μM) and at 0.00125 to 0.08 μM of PS2 proteins (Figure S2). However, PS2 at 0.5 μM could induce cell morphological change of Hs68 cells (Figure S2), in consistent with our data from the cytotoxicity assay of HS68 cells when treated with this same concentration (Figure 3b).



(**a**) (**b**)

Figure 3. Cell viability of MCF-7 (**a**) and Hs68 (**b**) cell lines in response to trypsin-activated BinB, BinBc, LHRH-BinBc, and proteinase K-activated PS2 treatments at different concentrations. All data are presented as mean ± SDs from the triplicate analyses.

4. Discussion

Many bacterial toxins have been developed as new drugs to treat cancers, for example, *Clostridium perfringens* enterotoxin (CPE) employed to target the breast cancer cells by inducing cytolysis through binding to its receptors, the tight junction proteins CLDN 3 and 4 [13]. Truncated diphtheria toxin (DT) produced by a pathogenic strain of *Corynebacterium diphtheria* appeared to be highly active against various human cancer cells [14]. Previous studies showed that Bin toxin, produced by *Lysinibacillus sphaericus* (Ls), has the potential to be developed as an anticancer agent by induction of cell apoptosis in human cancer cells [7]. To improve the specificity of toxins, cell-targeting peptides (CTPs) have

been used to deliver toxins to specific cancer cells [8,9]. It has been found that more than 50% of human breast cancers express LHRH receptor (LHRH-R) [15,16]. Thus, in this study we fused LHRH peptide with the pore-forming domain of BinB or BinBc with the intention to target MCF-7 cells (LHRH-R positive), compared with HS68 cells (LHRH-R negative). Due to the small size and target specificity potential of LHRH peptide, the BinBc coupled with LHRH (LHRH-BinBc) is, therefore, expected to have better target specificity, membrane penetration and intracellular trafficking as compared with the full-length BinB.

In the present study, both BinBc and LHRH-BinBc at 0.5 μM showed no cytotoxic effects on Hs68 cells, but they did inhibit the proliferation of MCF-7 cells by about 50%. Moreover, LHRH-BinBc showed slightly more cytotoxicity towards MCF-7 cells than free BinBc, probably due to the presence of LHRH-R overexpressed on MCF-7 breast cancer cells. However, the viabilities of MCF-7 cells after treated with LHRH-BinBc and free BinBc were not significantly different over a wide range of concentrations used (p = 0.2485), suggesting that BinBc itself is cancer cell specific and the cytotoxicity could still be maintained after fusing with LHRH peptide. Alternatively, the fusion of LHRH peptide with BinBc may interfere the interaction of LHRH peptide with its receptor, hence reducing the cytotoxicity to the target cancer cells. Therefore, the specific binding of BinBc and LHRH-BinBc to MCF-7 cells, in comparison with other LHRH-R negative cells, remains to be investigated further. Previously, we demonstrated the inhibition of cell proliferation of various human cancer cells (A549, Caco-2, HepG2, HK-1, and KKU-M055) upon treatments with trypsin-activated Bin toxin. In this study, we also compared the cytotoxic effects of cells upon treatments with trypsin-activated BinB and proteinase K-activated PS2 to those of LHRH-BinBc and BinBc. Activated BinB at 0.5 μM was less active to MCF-7 cells compared to both BinBc and LHRH-BinBc whereas at 16 μM, activated BinB showed strong cytotoxicity against MCF-7 cells (Figure 3a) together with cell shrinkage induction (Figure S1). Similarly, activated BinB at 16 μM also inhibited the proliferation of HS68 cells, suggesting the undesirable effects of BinB to normal cells when used at this concentration. Moreover, proteinase K-activated PS2, which is highly active against several human cancer cells, showed dose-dependent cytotoxicity towards MCF-7 cells. The same trend was reported in the previous study that PS2Aa1 showed strong cytotoxic effects on HepG2, MCF-7, KLE, Hec-1A, MDA-MB231 and PC-3 cells, whereas no cytotoxic effect was observed on normal cell lines (IOSE-144, HIEEC, HIESC and MCF-10A) [6]. However, in the present study, PS2 when challenged up to 0.5 μM, significantly decreased viability of Hs68 cells, indicating the non-target specificity of PS2 at this concentration. It has been indicated that the cytocidal effect of PS2 is not mediated through apoptosis, but rather its pore-forming activity by forming an oligomeric complex in lipid raft on the plasma membrane via GPI-anchored protein recognition [17]. It is possible that PS2 at high levels may spontaneously insert and form pores in a receptor-independent manner, leading to the off-target cytotoxicity.

The selective cytotoxicity of LHRH-BinBc and BinBc towards breast cancer cells (MCF-7) together with the absence of cytotoxicity to normal cells (Hs68) suggested that these forms of BinB are promising to be developed as anticancer agents. Further studies are still required to improve the specificity and efficacy of Bin toxin for its potential application as an anticancer agent.

5. Conclusions

In the present study, the pore-forming domain of BinB or BinBc was successfully fused with LHRH peptide to specifically target the breast cancer cells expressing LHRH receptors. Our results demonstrated that both BinBc and LHRH-BinBc showed cytotoxic effects on the breast cancer cells (MCF-7) but not on the normal cells (Hs68). In comparison with BinBc, LHRH-BinBc showed slightly more active to MCF-7 cells, suggesting the potential development of BinB pore-forming domain as targeted anticancer agent in the future.

Supplementary Materials: Figure S1: Morphological changes of MCF-7 cell lines after treated with activated BinB, BinBc, LHRH-BinBc, and PS2 for 48 h, Figure S2: Morphological changes of Hs68 cell lines after treated with activated BinB, BinBc, LHRH-BinBc, and PS2 for 48 h.

Author Contributions: Conceptualization, T.K., C.N. and P.B.; methodology, T.K., M.S. and P.B.; validation, T.K. and P.B.; formal analysis, T.K. and P.B.; investigation, T.K. and P.B.; resources, P.B.; data curation, P.B.; writing—original draft preparation, T.K.; writing—review and editing, P.B.; visualization, T.K. and P.B.; supervision, P.B.; project administration, P.B.; funding acquisition, P.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary Materials

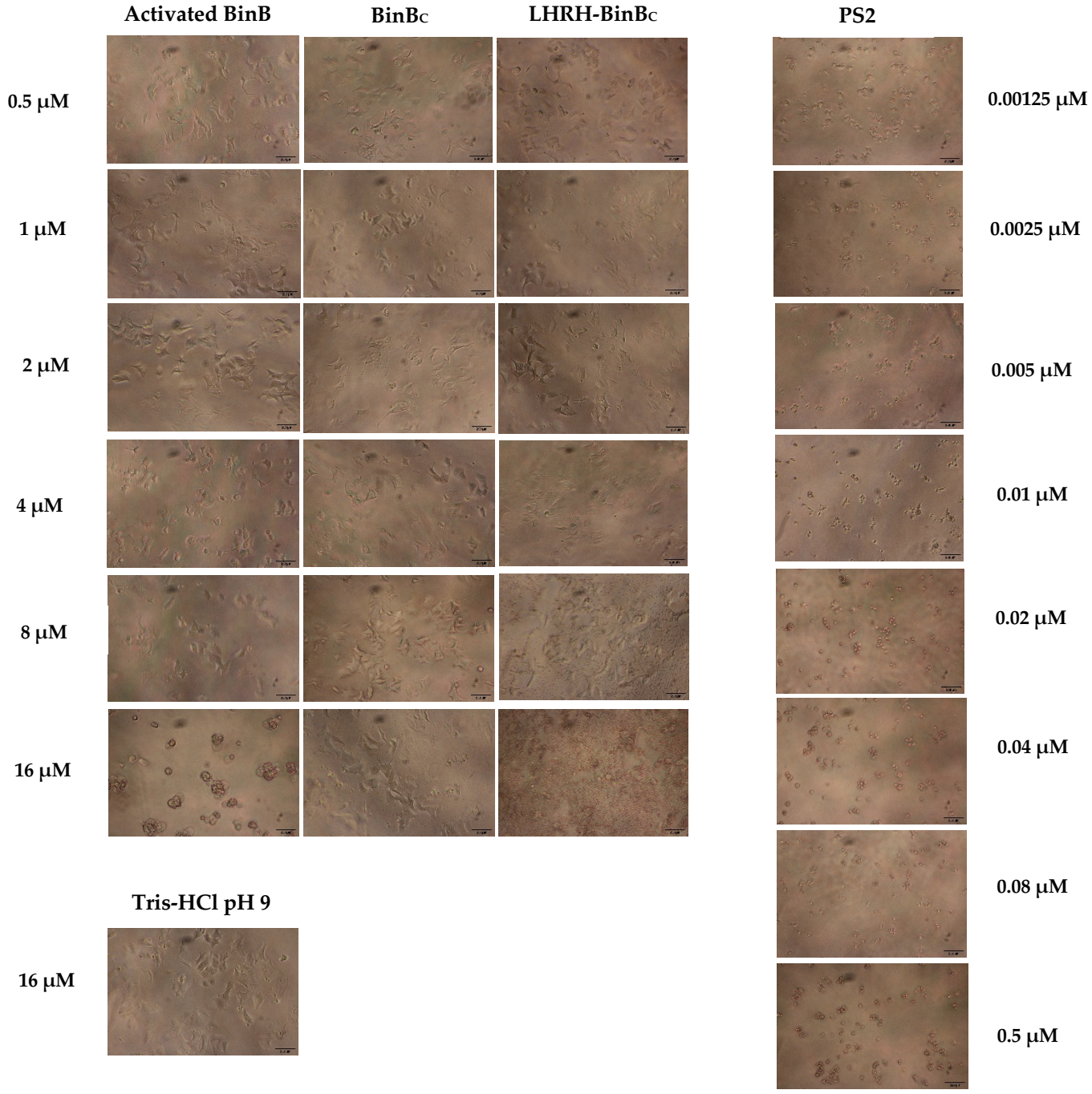

Figure S1. Morphological changes of MCF-7 cell lines after treated with activated BinB, BinBc, LHRH-BinBc, and PS2 for 48 h. Cells were treated with 0.5 to 16 μM of activated BinB, BinBc, and LHRH-BinBc, and 0.00125 to 0.5 μM of PS2 (positive control). Buffer (Tris-HCl pH 9) was used as a negative control. Cell morphological changes were observed under an inverted microscope with 10X objective lens.

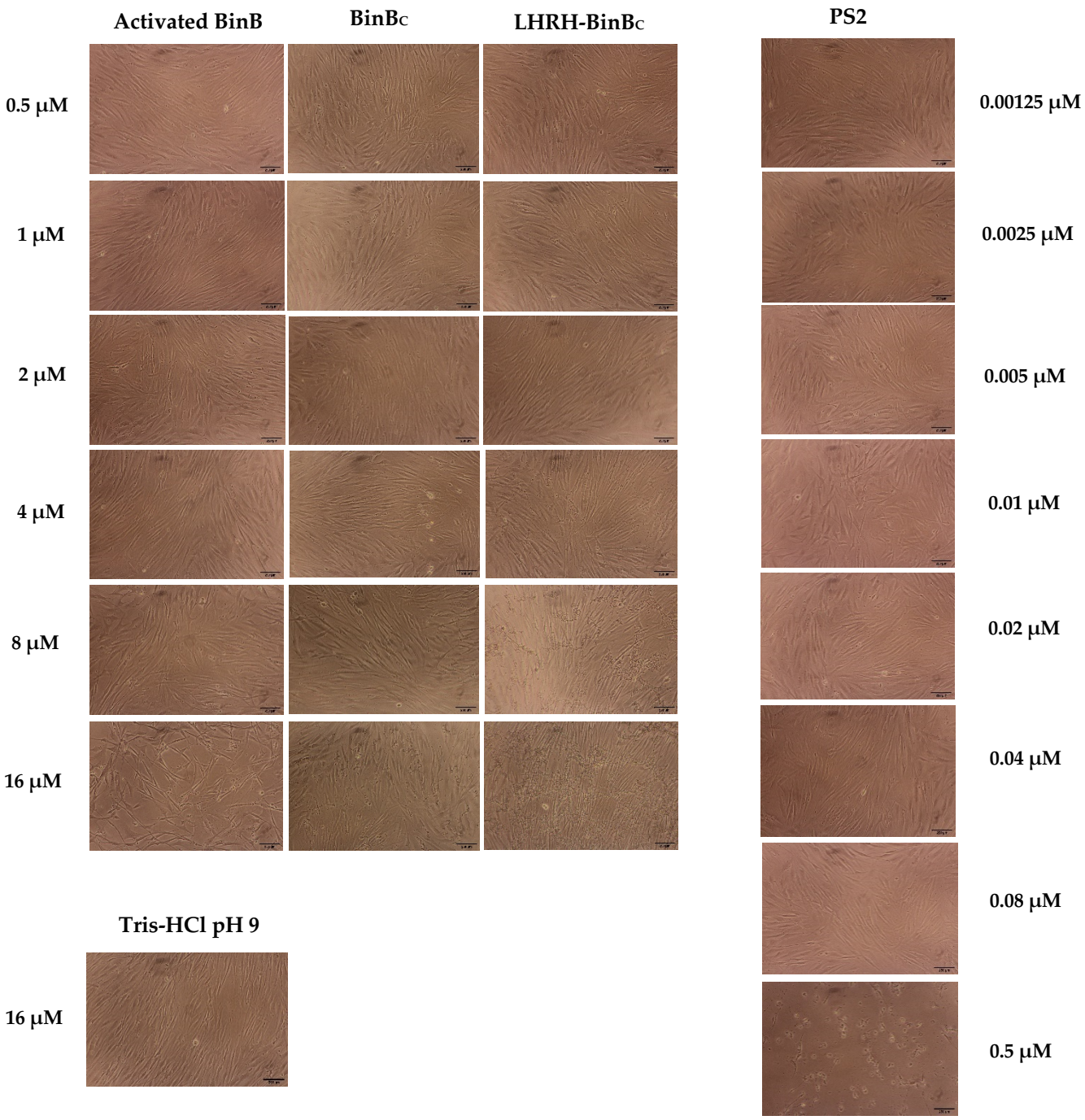

Figure S2. Morphological changes of Hs68 cell lines after treated with activated BinB, BinBc, LHRH-BinBc, and PS2 for 48 h. Cells were treated with 0.5 to 16 μM of activated BinB, BinBc, and LHRH-BinBc, and 0.00125, to 0.5 μM of PS2 (positive control). Buffer (Tris-HCl pH 9) was used as a negative control. Cell morphological changes were observed under an inverted microscope with 10X objective lens.