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Identification of a *Bacillus thuringiensis* Surface Layer Protein with Cytotoxic Activity against MDA-MB-231 Breast Cancer Cells

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In this work, we isolated a surface layer protein (SLP) from a *Bacillus thuringiensis* (Bt) strain to evaluate it cytotoxic effects against MDA-MB-231 human breast cancer cells. AP11 was selected from a group of Bt strains using SLP oligonucleotides developed from *Bacillus* conserved regions. The AP11 strain was grown in Luria Bertani medium until the late exponential phase; an 86 kDa protein was extracted using 5 M LiCl and identified by liquid chromatography–tandem mass spectrometry. It corresponded to a multispecies SLP highly similar to previously described SLPs in Bt. The MDA-MB-231 breast cancer cells LC_{50} was obtained using 0.25 μg/ml of the isolated SLP. HaCat non-cancerous cells presented 90% survival using the same protein concentration. Our data suggest that SLP cytotoxicity against MDA-MB-231 could be induced by an interaction with the CDH11 cell membrane receptor.

Keywords: *Bacillus thuringiensis*, surface layer protein, breast cancer, cytotoxicity, anticancer, MDA-MB-231

Introduction

Bacillus thuringiensis (Bt) is an important bacterium to agriculture economy due insecticidal activity generated by its Cry proteins, also known as δ-endotoxins [1, 2]. Cry proteins are produced as crystal inclusion bodies at the end of sporulation of Bt and induce strong toxic effects in Lepidoptera, Coleoptera, Diptera, and Hymenoptera orders, by specific interaction with membrane receptors from the midgut epithelium cells [3–8]. Olmos *et al.* in 2011 reported that insecticide Cry proteins from *Bacillus thuringiensis* presented cytotoxic activity on MDA-MB-231 breast cancer cells [9].

Surface layer proteins (SLPs) are present in more than 185 bacterial species. SLPs are found in Archaea, gram-negative and gram-positive bacteria, including species of the genus *Bacillus*. The S-layer is an ordered structure representing a proteinaceous paracrystalline array, which completely covers the surfaces of many pathogenic and probiotic bacteria and could represents more than 15% of the whole cell mass [10–18]. SLPs from *Bacillus* species possess a molecular mass of between 66 and 255 kDa [19–21]. A *Bacillus thuringiensis* SLP involved in toxicity against *Epilachna varivestis* has been isolated and identified [15]. The SLP from *Lactobacillus acidophilus* ATCC 4356 was used as antimicrobial compound inhibiting infections by *Salmonella typhimurium* strain SL1344, which delayed apoptosis by reducing downstream caspase-3 activation in Caco-2 cells [22]. The *Lactobacillus acidophilus* ATCC 4356 SLP inhibits Junin virus entry in dendritic cells, due to an interaction with DC-SIGN, a cell-surface receptor-adhesion factor [23]. *Lactobacillus helveticus* MIMLh5 SlpA exerts anti-inflammatory effects by reducing the activation of NF-kB on the intestinal epithelial Caco-2 cell line. Additionally, the same SlpA stimulates innate immune system by triggering the expression of proinflammatory tumor necrosis factor alpha and COX-2 in human macrophage cell line U937, via recognition through Toll-like receptor 2 [24]. However, no effects have been reported with respect to using SLP as anticancer compounds. Breast cancer is one of the most frequently diagnosed cancer diseases globally and is the leading cause of female cancer deaths worldwide [25,

26]. For this reason, breast cancer is an important target to look for the development of natural and synthetic anticancer compounds.

In this work, an 86 kDa SLP with cytotoxic activity against MDA-MB-231 breast cancer cells was isolated from a Bt strain and characterized. Our data suggest that cytotoxic effects could be induced by an interaction between the isolated SLP and MDA-MB-231 CDH11 cell membrane receptor, which is partially conserved between dendritic cells (DC-SIGN) and *Manduca sexta* (BT-R1) receptors [4, 23, 27].

Materials and Methods

Bacterial Strains and Growth Conditions

Bacillus subtilis W168 strain was used as a negative control because no SLP genes have been identified in this bacterium [12, 28]. Spore-forming gram-positive bacteria were previously isolated from soil samples and identified as Bt strains by production of Cry proteins at the end of sporulation [2]. Bacterial strains were grown at the end of the exponential phase in 500 ml of LB medium at 30°C and 200 rpm, for chromosomal DNA extraction and SLP purification.

Oligonucleotide Design and SLP Gene Amplification

SLP gene sequences from several *Bacillus* species (*B. thuringiensis* serovar *vazensis* (HM626285.1), *B. thuringiensis* serovar *poloniensis* (HM626284.1), *B. thuringiensis* serovar *pingluonsis* (HM626296.1), *B. weihenstephanensis* (HM626280.1), *B. cereus* (HM626283.1), and *B. mycoides* (HM626282.1)) were aligned with the ClustalW program for conserved region identification and oligonucleotide design. Chromosomal DNA was purified from Cry producer Bt strains and *B. subtilis* using standard methodology [29]. SLP genes were amplified by PCR using forward 5′TGACAGCAGCAATGGTAG′3 (FwSLP) and reverse 5′GCTGCTTCTGCTTTaGTTA′3 (RvSLP) oligonucleotides. The PCRs were performed in 100 µl containing 50 ng of DNA in TE buffer and 20 ng of FwSLP and RvSLP primers. Amplification was done using 25 cycles; one cycle consisted of 1 min at 95°C, 1 min at 54°C, and 2 min at 72°C. Agarose gels ethidium bromide stained and a UV-light detector were used to evaluate the products. TrackIt 100 bp DNA Ladder from Thermo Fisher Scientific, Invitrogen (USA), was used as the molecular weight marker.

SLP Extraction

Bt-AP11 strain was grown as indicated above and extraction of the SLP was performed with LiCl as described previously, with some modifications [24, 30]. Briefly, AP11 culture was harvested by centrifugation at 2,000 ×*g* for 10 min at 4°C and washed with cold sterile purified water. The pellet was resuspended in 10 ml of 20 mM Tris-HCl (pH 8), homogenized in an ice bath for 5 min with a minipimer, centrifuged at 2,000 ×*g* for 10 min at 4°C, and washed with cold sterile purified water. The bacterial pellet was treated with 0.1 volume of 1 M LiCl solution for 30 min at room temperature and slight agitation, and centrifuged. The SLP was then pellet-extracted with 0.1 volumes of 5 M LiCl solution for 60 min at room temperature and slight agitation, in the presence of 0.001% protease inhibitor (Spectrum, USA). The supernatant was passed through a 0.2 µm-pore-size filter and concentrated using 50 kDa centrifugal amicon filter units (Merck Millipore Ltd, USA). Protein purity was determined by SDS-PAGE and RP-HPLC.

SLP Characterization and Identification

The extracted SLP was suspended in SDS-PAGE sample buffer, boiled for 5 min, and separated in a 10% SDS-PAGE by using Tris-glycine-SDS buffer and a Mini-Protean system (Bio-Rad, USA). The protein was analyzed using silver and Coomassie Brilliant Blue G-250 staining and the precision plus protein dual color standard weight marker from Bio-Rad. The SLP concentration was obtained by Bradford method [31].

RP-HPLC analysis was performed on an Agilent 1100 instrument with a G1365B detector, using a reversed-phase Eclipse XDB-C8 column with 4.6 mm ID × 150 mm and 5 µm particle size (Agilent Technology, USA). The SLP was eluted at room temperature using solvent A (0.1% TFA in Milli-Q-treated water) and solvent B (0.1% TFA in acetonitrile). The elution expressed as the solvent B proportion was as follows: 0–30 min, 0–30%; 30 to 32 min, 100%. The protein was eluted at a flow rate of 0.2 ml/min and monitored at 210 nm.

Mass spectrum acquisition was performed in an ESI positive ion mode. The HPLC-purified SLP was reduced with DTT, treated with iodoacetamide (Sigma-Aldrich, USA) and digested with trypsin (Promega Sequencing Grade Modified Trypsin). Obtained peptides were applied to a LC-MS system with the EASY-nLC II bomb (Thermo-Fisher Co., USA) coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher Co.) with a nano-electrospray system. The HPLC utilized a gradient system of 10–80% of solvent B (water/acetonitrile with 0.1% of formic acid) in 120 min, using a home-made RP-C18 capillary column (ID 0.75 µm × 10 cm) and a flowrate of 300 nl/min. Peptides fragmentation collision-induced dissociation and high-energy collision dissociation methods were used, and only ions with 2+, 3+, and 4+ charges were selected. Spectrums were acquired in a positive detection mode and data acquisition was carried out in a dependent way from the total scanning ions and predetermined charges, with an isolation band of 3.0 (*m/z*), normalized energy collision of 35 arbitrary units, Q activation of 0.250, activation time of 10 msec and maximal injection time of 10 msec by micro-scanning. During automatic data capture, dynamic ion exclusion was used: (i) exclusion list of 400 ions, (ii) pre-exclusion time of 30 sec, and (iii) exclusion time of 300 sec. Results were interpreted using NCBIr and Uniprot from Proteome Discoverer 1.4 software.

Cell Cultures

MDA-MB-231 breast cancer and HaCat non-cancerous cells

were grown in DMEM containing 10% of fetal bovine serum (FBS; Thermo Fisher Scientific, Invitrogen). Cultures were supplemented with 1% of antibiotic-antifungal 100× (Thermo Fisher Scientific, Invitrogen) and incubated at 37°C with 5% of CO_2 in a humidified atmosphere until 90% density was obtained.

SLP Cytotoxicity in MDA-MB-231 Cells

Cell viability was measured by the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. First, 100 µl of MDA-MB-231 and HaCat cultures containing 1×10^4 cells/ml were incubated in triplicates in 96-well plates until 90% density. Cell growth was arrested after 24 h by eliminating FBS from the DMEM. Then, 1.0, 0.5, and 0.25 µg/ml of the purified SLP were added to the cultures, and the plates were incubated two more hours under the conditions mentioned above. A negative control was prepared using cell lines incubated under the same conditions but without SLP addition. Then, 10 µl of DMSO were added in wells to kill cells in positive controls, and plates were incubated under conditions mentioned above. Additionally, 50 µl of MTT at 5 mg/ml was added to all wells, and the plates were incubated for three more hours. The supernatant was eliminated carefully from the wells, formazan crystals produced by the cells were dissolved in 100 µl of isopropanol, and the absorbance was measured at 595 nm.

Cell viability percentage was obtained with respect to the negative control.

% cell viability = (Ab of experimental sample/Ab of negative control) × 100

Statistical Analysis

ANOVA test was used. A p level of <0.05 was considered statistically significant.

Results and Discussion

AP11 Strain Isolation and Identification

Differences among pathogenic, probiotic, and commensal microorganisms could lie in the magnitude of evoked response that can be defined as strong, intermediate, or homeostatic, respectively [32]. In this sense, identification of the bacterial molecules related with that response could represent an approach of key importance to elucidate the molecular mechanisms involved in cell modulation. Knowing natural molecules could induce the development of safer therapies for the treatments of specific diseases, avoiding whole bacterial utilization [33].

The use of Bt as a biopesticide is a viable alternative for insect control, since insecticidal Cry proteins produced by this bacterium are receptor-specific and harmless to humans, animals, and plants [4, 8]. In addition to Cry toxins, Bt produces SLPs also with insect virulence activity [15]. However, the phylogenetic relationship among *Bacillus cereus*, *B. anthracis* and Bt makes difficult to differentiate between them using 16S rDNA sequences [15]. In this sense, AP11 an S-layer producer strain was isolated and identified by the production of crystal inclusion bodies at the end of sporulation [2].

***slp* Gene Identification**

SLP genes have been cloned and their sequences revealed a low similarity, except in the cell wall-targeting SLP homology domain, which involves the anchoring of these proteins to the peptidoglycan-linked polysaccharides [16]. In this sense, we analyzed the presence of *slp* genes in Bt strain AP11 using oligonucleotides designed from *Bacillus* conserved sequences. Interestingly, even when few genes from *Bacillus* were found to be compared, the oligonucleotides obtained were highly conserved. PCRs were performed, and the products were analyzed in an agarose gel stained with ethidium bromide and were also sequenced. In addition to used, *Bacillus subtilis* W168 was used as a negative control because this species does not contain *slp* genes [12, 28]. Obtained results confirm that *Bacillus slp* oligonucleotides amplified a 540 bp expected size product from Bt strain AP11. Moreover, the *B. subtilis* W168 negative control did not show any amplification using *slp* conserved oligonucleotides, confirming the absence of *slp* genes in this bacterium (Fig. 1). Furthermore, it is important to mention that these oligonucleotides are the first ones developed to detect in an easy, precise, and fast way *slp* genes in *Bacillus* species. Finally, a sequence obtained from the PCR product

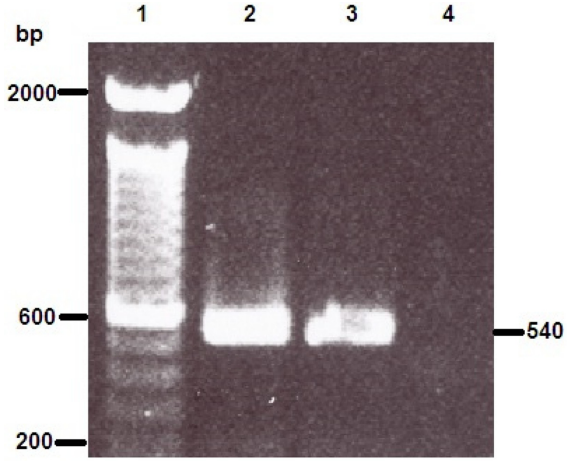

Fig. 1. Agarose gel electrophoresis analysis of *slp* gene amplification.

Lane 1, molecular weight marker; lanes 2 and 3, 540 bp PCR product of Bt AP11 strain; lane 3, PCR product of Bs W168 negative control.

established that a *slp* gene 87% similar to that from *Bacillus thuringiensis* (HM626296.1) was amplified. In addition, this gene codified to the ADQ08580.1 protein [34].

SLP Identification

The SLPs have important roles in growth, survival, and maintenance of cell integrity, enzyme display, and interaction with the host and its immune system. In addition, it was proposed that these proteins are involved in antiviral and antibacterial activities [12, 18].

In pathogenic bacteria like *Bacillus cereus*, the SLP has been reported to promote interactions with human leukocytes, contributing to pathogenicity [35, 36].

On the other hand, in probiotic bacteria like *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus helveticus* MIMLh5, SLP beneficial effects were carried out through the interaction with specific cell-surface receptor-adhesion factors [23, 24]. In Bt, a SLP has been implicated in insect virulence [15]. However, no anticancer effects have been reported for any kind of SLPs. In this sense, Bt strain AP11 was grown at the end of the exponential phase and harvesting to avoid cell debris and to facilitate SLP purification. Bt pellets were processed with LiCl to extract cell wall proteins, and supernatants were treated as described in Material and Methods. LiCl is reported as the cleanest denaturalizing agent, since this solvent allows SLP extraction without bacterial cell disruption [24, 30, 37]. Furthermore, SLP does not contain covalent bridges between its N-terminal domain and other accessory molecules, like lipopolysaccharides, polysaccharides, or peptidoglycan [28, 38, 39]. In this sense, Fig. 2A shows that a single protein of ~86 kDa was extracted with LiCl from the AP11 strain. Additionally, these results were corroborated by HPLC analysis at 210 nm (Fig. 2B). In order to identify this protein, LC-MS/MS was used to sequence it and the obtained peptides were compared through databases. Spectrometry analysis indicated a molecular mass of 86,575.4 Da, which is very similar to the 86 kDa mass obtained from SDS-PAGE gel characterization (Fig. 2A). Furthermore, protein identification presented ~50% coverage with respect to SLP1 (ADU04485.1), a protein previously reported in a Bt strain [40]. In this sense, ADU04485.1 and ADQ08580.1 obtained from the protein and DNA sequences, respectively, presented 98% similarity between them, corroborating the 86 kDa Bt origin (data not shown). However, phylogenetic relationship among SLPs from *Bacillus cereus*, *Bacillus sphaericus*, *B. anthracis*, *B. licheniformis*, and Bt indicate that this protein could belong to a multispecies group with similar origin (WP_001140751.1).

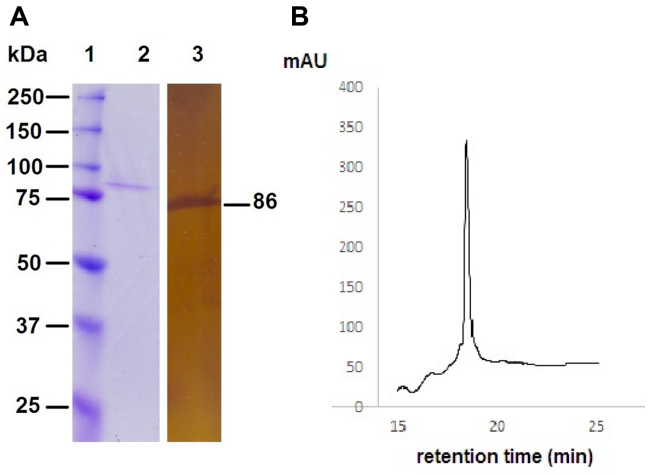

Fig. 2. Characterization of the surface layer protein (SLP) purified from *Bacillus thuringiensis*.
(**A**) SDS-PAGE analysis of the 86 kDa SLP; Lane 1: molecular weight marker; lane 2: 86 kDa protein extracted and stained with Coomassie Blue; lane 3: 86 kDa protein extracted and silver stained. (**B**) Bt AP11 86 kDa SLP detected by HPLC.

SLP Cytotoxic Activity against MDA-MB-231

Breast cancer is the leading cause of cancer-related deaths in women globally. In this sense, the search for bioactive compounds against breast cancer is an ongoing project worldwide [26, 41, 42]. Recently, some Cry and PS proteins produced by Bt strains have shown cytotoxic effects against human cancer cell lines [9, 43–46]. However, no reports have been published about SLP effects against human cancer cells. Nevertheless, results available demonstrate DC-SIGN$_{CTL}$ and Toll-like receptors directly interact with SLPs from *Lactobacillus acidophilus* and *L. helveticus,* inhibiting pathogen virulence and stimulating the immune system, respectively [23, 24]. Moreover, Cry1A proteins specifically interact with midgut cell receptor (Bt-R1) of insects without affecting humans, plants, or animals [4, 27]. In this sense, our SLP was evaluated in triplicates in MDA-MB-231 breast cancer and HaCat non-cancer cell lines (Fig. 3). MDA-MB-231 is a triple-negative mesenchymal cell with CDH11 cadherin as the principal membrane receptor. On the other hand, HaCat is a keratinocyte epithelial cell line mainly expressing the CDH1 receptor. CDH1 is known as a tumor suppressor cadherin, whereas CDH11 increases the cancer cell ability to migrate [47, 48]. Obtained results shown that the 86 kDa SLP isolated from strain AP11 presented MDA-MB-231 cytotoxicity at all concentrations assessed, and a LC_{50} value of 0.25 µg/ml (Fig. 4A). On the other hand, HaCat non-cancerous cells assayed with the same SLP

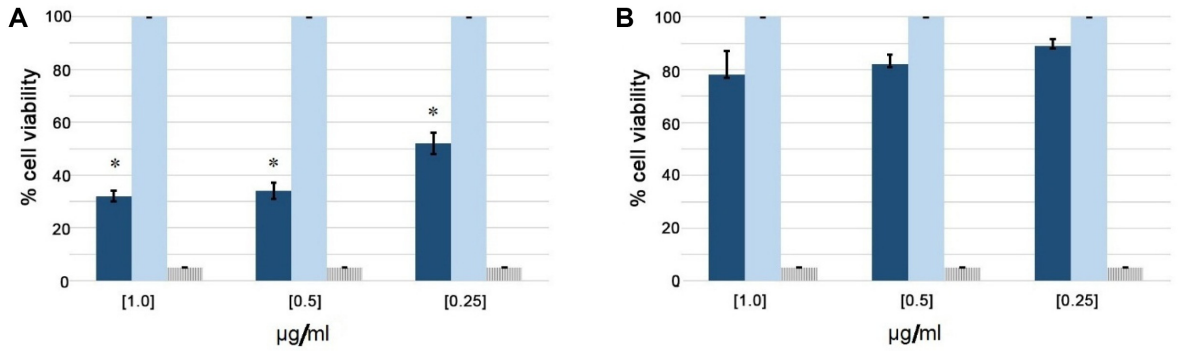

Fig. 3. Cytotoxic effects of the 86 kDa SLP on MDA-MB-231 and HaCat cells.
(**A**) Cell viability of MDA-MB-231 using 1.0, 0.5, and 0.25 µg/ml of the SLP (dark blue bar); Negative control: MDA-MB-231 cells without SLP (light blue bar); Positive control: MDA-MB-231 cells with 10 µl of DMSO (striped gray bar). (**B**) Cell viability of HaCat using 1.0, 0.5, and 0.25 µg/ml of the SLP (dark blue bar); Negative control: HaCat cells without SLP (light blue bar); Positive control: HaCat cells with 10 µl of DMSO (striped gray bar). *$p < 0.05$.

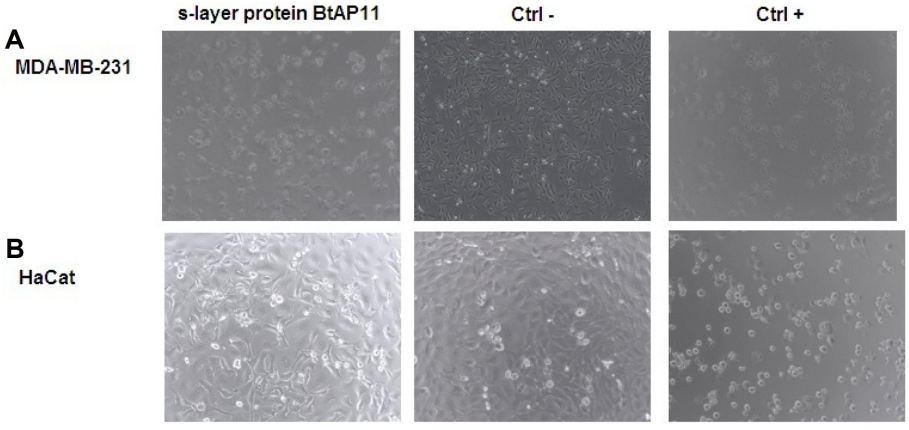

Fig. 4. Cytotoxic effects of the 86 kDa SLP in (**A**) MDA-MB-231 and (**B**) HaCat cells, using 0.25 µg/ml.
Negative control: cells without SLP; Positive control: cells with DMSO.

concentrations only presented 10% of mortality (Fig. 4B). These results demonstrate specific cytotoxic effects on breast cancer cells using the 86 kDa protein produced by Bt. However, its mechanisms of action are not well understood yet. In this sense, we hypothesize that similar to SLP and Cry proteins from *Lactobacillus* and *Bacillus* strains [4, 9, 23–25], the 86 kDa protein could be involved in an interaction with a specific membrane receptor present in MDA-MB-231, but missing in HaCat cells. In this sense, sequence comparison between $BtR1_{Msexta}$, $BtR1_{Hvirescens}$, CDH11, $DC\text{-}SIGN_{CTL}$, and CDH1 receptors shows a great similarity among them, with the exception of CDH1 (Fig. 5A). The region analyzed represents one of the most important sites for Cry1Ab-$BtR1_{Ms}$/Cry1Ac-$BtR1_{Hv}$ toxins-receptors interaction, and is localized adjacent to the membrane-proximal extracellular domain in cadherin's and cadherin-like receptors [27, 49]. Thus, missing of conserved valine amino acids in CDH1 could explain why the 86 kDa SLP does not interact with HaCat with the same intensity than with MDA-MB-231 cells (Fig. 5B). Therefore, interaction of the 86 kDa protein with the CDH11 receptor could activate a signaling transduction pathway, inducing a stronger death response in MDA-MB-231 breast cancer cells. Additionally, CDH11 and $DC\text{-}SIGN_{CTL}$ receptors are highly conserved in this region (Fig. 5B). Hence, this also could be the site where *Lactobacillus acidophilus* SLPs interact with dendritic cells to inhibit Junin virus entry [23]. However, this hypothesis remains to be demonstrated with future assays.

In conclusion, we have demonstrated that the 86 kDa protein produced by Bt AP11 strain is an SLP with specific cytotoxic effects in MDA-MB-231, placing this protein as a promising molecule to develop anticancer compounds. Based on sequence comparison between several cell receptors, we are suggesting that CDH11 could be the target in MDA-

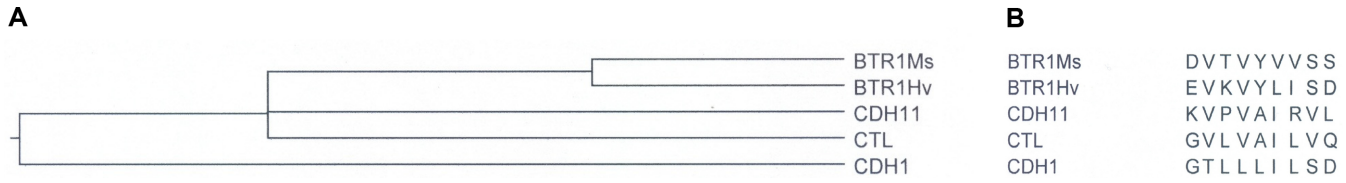

Fig. 5. Receptors sequence comparison.
(**A**) Phylogenetic analysis; (**B**) sequence alignment.

MB-231; for this reason, the next step will be to corroborate this possibility. This information will be useful to understand the mechanism of action of the SLPs and their putative role in modulating signaling transduction pathways in cancer and other kind of cells. However, it is important to remember that the magnitude of the signal generated will depend on the ligand (SLP) concentration and the receptor sequence conservation.

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