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Cytotoxic Effects of Smp24 and Smp43 Scorpion Venom Antimicrobial Peptides on Tumour and Non-tumour Cell Lines

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Abstract

Smp24 and Smp43 are novel cationic AMPs identified from the venom of the Egyptian scorpion *Scorpio maurus palmatus*, having potent activity against both Gram-positive and Gram-negative bacteria as well as fungi. Here we describe cytotoxicity of these peptides towards three non-tumour cell lines ($CD34^+$ (hematopoietic stem progenitor from cord blood), HRECs (human renal epithelial cells) and HACAT (human skin keratinocytes) and two acute leukaemia cell lines (myeloid (KG1a) and lymphoid (CCRF-CEM) leukaemia cell lines) using a combination of biochemical and imaging techniques. Smp24 and Smp43 (4–256 µg/mL) decreased the cell viability (as measured by intracellular ATP) of all cells tested, although keratinocytes were markedly less sensitive. Cell membrane leakage as evidenced by the release of lactate dehydrogenase was evident throughout and was confirmed by scanning electron microscope studies.

Keywords *Scorpio maurus palmatus* · Antimicrobial peptide · Smp24 · Smp43 · Cell death

Introduction

Over the last decades, an increasing number of pathogenic microorganisms have developed resistance to conventional antibiotics posing problems in the management of infection. The rapid increase in drug-resistant infections emphasizes the urgent need to develop novel reagents with new modes of action (see, inter alia Bahar and Ren 2013). The development of agents with a low potential to resistance is technically challenging and is a contributing factor in the declining discovery of novel classes of antibiotics.

Antimicrobial peptides (AMPs) are essential to the innate immune system across all phyla and have the potential as a novel therapeutic class of broad-spectrum antibiotics due to their selectivity for prokaryotic membranes (Bahar and Ren, 2013). AMPs are generally positively charged, amphipathic molecules between 12 and 70 amino acids in length. They target and disrupt prokaryotic membranes due to the initial electrostatic attraction to the negatively charged membrane surface (Huang 2000; Zasloff 2002). A threshold concentration is (often) then required before membrane disruption occurs. A multitude of models and mechanisms have been proposed to account for this subsequent disruption (Teixeira et al. 2012; Heath et al. 2018). One key challenge to developing AMPs as therapeutic agents is to minimize and hopefully eliminate any cytolytic effects on eukaryotes. The lack of overall surface charge on eukaryotic membranes provides hope that this particular goal can be achieved. Although healthy eukaryotic cell membranes are zwitterionic, the transbilayer movement of phosphatidylserine to the outer membrane leaflet in tumour cells (for a review, see Sharma and Kanwar 2018), also provides cancer cells, analogously to prokaryotic cells, with a negatively charged outer membrane. The appearance of phosphatidylserine on the outer membrane leaflet regulates malignant transformation by primarily suppressing anti-tumour immune responses (Utsugi et al. 1991). These observations have led to many suggestions that AMPs might be effective anti-cancer agents (Wang and Wang 2016; Crusca et al. 2018), providing that there is

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a sufficient distinction (effective therapeutic index) between cytolytic effects on tumour and non-tumour cell membranes.

Scorpion venoms are cocktails of diverse biologically active compounds (see, inter alia, Rodriguez de la Vega and Possani, 2005; Rodriguez de la Vega et al. 2010; Cao et al. 2014; Abdel-Rahman et al. 2015; Cid-Uribe et al. 2018; Kazemi and Sabatier, 2019) and provide a rich source of AMPs (for reviews see Harrison et al. 2014; Wang and Wang, 2016; El-Bitar et al. 2019). Abdel-Rahman et al. (2013) identified two novel amphipathic cationic AMPs (Smp43 and Smp24) through cDNA sequencing of the venom gland of the Egyptian scorpion *Scorpio maurus palmatus*. Both peptides showed a potent activity against both Gram-positive and Gram-negative bacteria as well as fungi (Harrison et al. 2016a) and also formed pores in model prokaryotic and eukaryotic phospholipid membranes. The mechanism of membrane disruption caused by Smp24 depended on phospholipid composition; the peptide formed toroidal pores in prokaryotic-like membranes but hexagonal phase non-lamellar phase structures were seen in eukaryotic-like membranes (Harrison et al. 2016b). In comparison, Smp43 disrupted both types of membranes by a common mechanism that involves elements of both the carpet model and the expanding pore mechanism, that we have termed "diffusion-limited disruption" (Heath et al. 2018). Here in more detail, we have set out to study the cytotoxic effects of Smp24 and Smp43 on non-tumour (hematopoietic stem cells, primary renal cells and immortalised keratinocytes) and tumour (myeloid and lymphoid leukaemia) eukaryotic cells lines.

Materials and Methods

Materials

Cell Titre-Glo® reagent kit was obtained from Promega (Southampton, UK). RPMI media was obtained from Invitrogen (Paisley, Scotland). DMEM media was obtained from Lonza (Cologne, Germany). Epithelial growth media was obtained from Innoprot (Deria, Spain). 96-well white microplates were obtained from Fisher Scientific (Loughborough, UK). All other reagents were the highest grade available and were obtained from Sigma (Gillingham, UK).

Peptides

The sequence of both Smp24 (IWSFLIKAATKLLPSLFGGGKKDS) and Smp43 (GVWDWIKKTAGKIWNSEPVKALKSQALNAAKNFVAEKIGATPS) (Abdel-Rahman et al. 2013) were synthesized (> 90% pure) using solid-phase chemistry and were purchased from Think Peptides® (Oxford, UK).

Cell Lines

Non-tumour haematopoietic progenitor stem cells ($CD34^+$) were obtained from Stem Cell Technologies (Grenoble, France). Primary Human renal epithelial cells (HRECS) were obtained from Innoprot (Spain). Immortalized human skin keratinocytes (HaCaT) was obtained from Thermofisher Scientific (UK). Human lymphoid leukaemia cells (CCRF-CEM, acute lymphoblastic leukaemia, ATCC: CCL-119) and human myeloid leukaemia cells (KG-1a, acute mylogenous leukaemia, ATCC: CCL-243) were obtained from the ATCC (Teddington, UK). MycoAlert™ mycoplasma detection kits were obtained from Lonza (Cologne, Germany).

Culture Conditions

Suspended cells ($CD34^+$, CCRF-CEM and KG-1a) were seeded in T75cm^2 flasks in RMPI 1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 1.5 mM L-glutamine and 100 µg/mL penicillin/streptomycin. HaCaT cells were seeded in T75cm^2 flasks in DMEM medium supplemented with 10% FBS, 1.5 mM L-glutamine and 100 µg/mL penicillin/streptomycin. HRECs were seeded in T75 cm^2 flasks in epithelial cell media containing 2% FBS, 1% epithelial cell growth supplement (EPICGS) and 1% penicillin/streptomycin. Cells were incubated at 37 °C with 5% CO_2 and tested regularly for mycoplasma contamination; all cells were negative throughout the study.

Cell Viability Assay

Cell viability was determined by measuring intracellular ATP levels, using a Cell Titre-Glo® luminescent cell viability assay (Promega). Cells were seeded into white 96-well plates at 2.5×10^4 cells/well and were treated with either Smp24 and Smp43 or water (vehicle control) and 10% Triton X-100 (positive control). Cells were incubated (37 °C) for 24 h, followed by addition of Cell Titer-Glo® reagent (100 µL), according to the manufacturer's instructions. Luminescence (proportional to the amount of ATP present in the cells) was measured using a Victor 1420 luminescence detector (Perkin Elmer, Cambridge, UK). Mean values from three independent experiments were calculated and all treated samples were normalised to controls. The LC_{50} was determined for each peptide in each cell line. LC_{50} was calculated from a linear regression equation of each standard curve for each peptide with each cell line. The percentage of ATP level normalized to vehicle controls.

Lactate Dehydrogenase Assay

Lactic dehydrogenase (LDH) was measured using a Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, UK). Cells were seeded in 96 well plates at 2.5×10^4 cells/well and treated with either Smp 24 and Smp43 or water (negative control) and 10% Triton X-100 (positive control). Cells were incubated (37 °C) for 24 h. A maximum LDH activity control was included by treating cells with 10× lysis buffer (10 μL), included in the kit. Following treatments, cell supernatants (50 μL) were treated with detection reagent (50 μL) according to the manufacturer's instructions. LDH activity was measured at 490 nm and the % LDH released was determined, relative to the maximum activity control. The experiment was performed in triplicate and mean values from three independent experiments were calculated.

Scanning Electron Microscopy (SEM)

Suspended cells were seeded in 6 well plates at 0.5×10^6 cells/well while adherent cells were cultured on a cover slip in 6 well plates at 0.5×10^6 cells/well. The cells were then treated for 24 h with different concentrations of Smp24 or Smp43 and water as vehicle control. The suspended cells were harvested and centrifuged at 5000 rpm at 4 °C (5 min) and the supernatant was removed and cell pellets were collected. The cell pellets and cover slips (carrying adherent cells) were washed twice in 200 μL cold 0.1 M phosphate buffer solution (PBS). Then these cells were fixed by adding 100 μL of 3% glutaraldehyde in 0.1 M PBS 24 h at 4 °C. The cells were washed twice with cold 0.1 M PBS each for 15 min at RT but without spinning. Secondary fixation was carried out in 1% aqueous osmium tetraoxide for one hour at RT. Cells were then washed twice with 0.1 M PBS at 4 °C followed by ascending series of ethanol for dehydration (75%, 95%, and then twice in 100% each step 15 min). After discarding ethanol, equal amount (1:1) of 100% ethanol and 100% hexamethyldisilazane were added for 30 min followed by 100% hexamethyldisilazane for another 30 min at RT to ensure complete removal of water from samples. After completion of drying, the samples were mounted on 12.5 mm diameter stumps and attached with Carbon-Sticky Tabs and then coated in an Edwards S150B sputter gold coater with approximately 25 nm of gold. Finally, the cells were then examined and photographed by a scanning electronic microscope (Philips XL20, Germany).

Statistical Analysis

Data were analysed (unpaired student-*t* test) using Prism 6 software (Graph Pad). Means and standard errors were calculated and results were considered statistically significant at $P \leq 0.05$ and highly significant at $P \leq 0.001$.

Results

Smp24 and Smp43 (at concentrations between 4 and 256 μg/mL) both significantly reduced cell viability after 24 h in all cells tested ($CD34^+$, HREC, HaCaT, KG1-a and CCRF-CEM), as evidenced by concentration-dependent reductions in intracellular ATP levels (Fig. 1). Both myeloid (KG1-a) and lymphoid (CCRF-CEM) leukemic tumour cell lines (Fig. 1a and b respectively) were more sensitive to Smp peptides than non-tumour cell lines (Fig. 1c, d and e). The mean LC_{50} value of the two tumour cell lines (16 and 14.5 μg/mL for Smp24 and Smp43, respectively) was significantly different ($p \leq 0.05$) from the mean LC_{50} value of the three non-tumour cells (36.7 and 70 μg/mL for Smp24 and Smp43, respectively). There was a considerable variation in the sensitivity to non-tumour cells ($CD34^+$ stem cells, primary renal cells (HREC) and the transformed keratinocyte cell line, HaCaT) and no apparent correlation between the cytotoxic profiles of Smp24 and Smp43 (Table 1).

The cytotoxic effects of Smp24 and Smp43 were also evidenced by the concentration-dependent release of lactate dehydrogenase (LDH) from all cells tested (Fig. 2). In parallel to measurements of intracellular ATP content (Fig. 1), leakage of LDH from the two leukaemia cell lines was more sensitive to Smp43 than either $CD34^+$ stem cells or primary renal cells. In comparison, the cytotoxic effects of Smp24 were indistinguishable. Interestingly, HaCaT plasma cell membranes were much more resistant to the cytotoxic effects of both Smp24 and Smp43 (Fig. 2e) and this was also reflected in the ATP assay (Fig. 1e). Scanning electron micrographs of cells treated with either Smp24 or Smp43 showed evidence of loss of cell filipodia and membrane microvilli, as well as pore formation and the appearance of cell membrane blebs (Fig. 3).

Discussion

Scorpion venoms have provided a rich source of AMPs and the possibilities of developing their therapeutic potential is enhanced when it can be demonstrated that such peptides have limited cytotoxic effects on mammalian cells. For example, the considerably lower cytotoxic effects of Smp24 against stem cells (e.g. $CD34^+$) and primary cells (e.g. HRECs) (LC_{50} 20–37 μg/mL) described here is in contrast with the significantly higher antimicrobial activity of Smp24 against strains of *Staphylococcus* and *Bacillus* (LC_{50} 4–8 μg/mL) (Harrison et al. 2016a).

The present investigation of the cytotoxic effects of Smp24 and Smp43 revealed a concentration-dependent

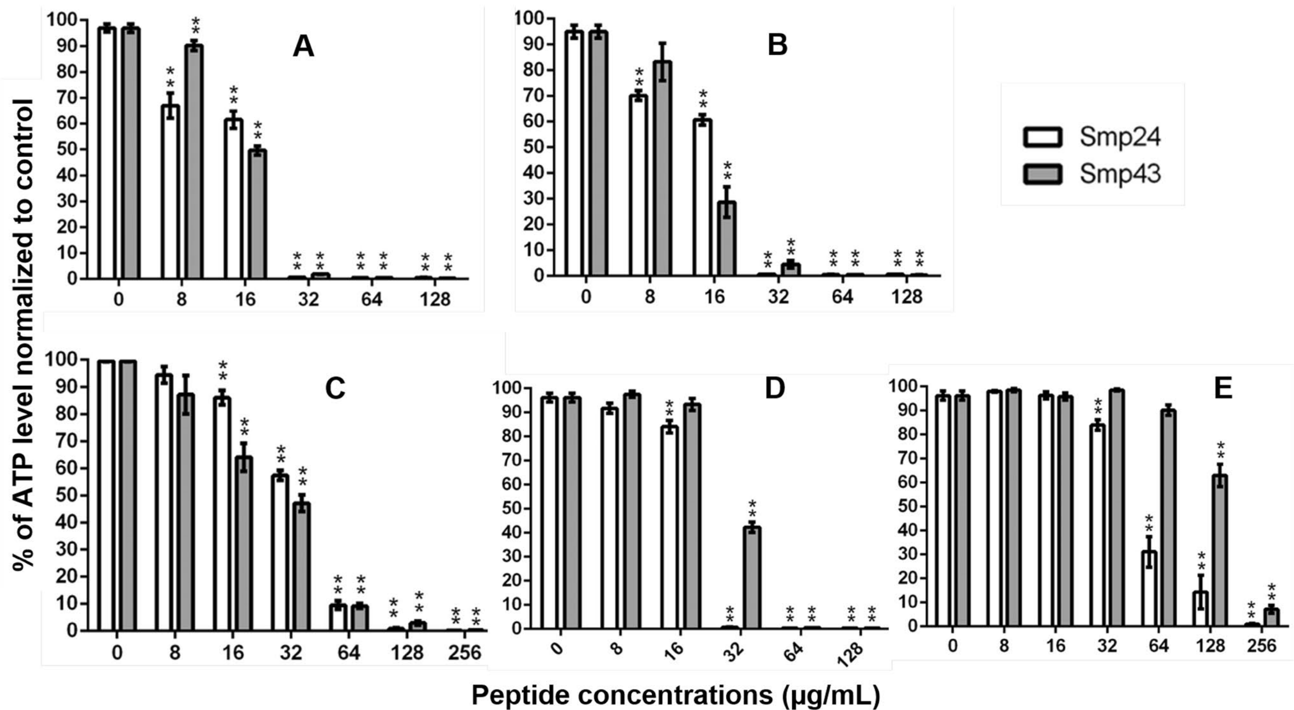

Fig. 1 Effects of Smp24 and Smp43 on cellular ATP levels of tumour and non-tumour cell lines. Tumour (KG-1a and CCRF-CEM; Panels **a** and **b**, respectively) and non-tumour (HREC, $CD34^+$ and HaCaT; Panels **c**, **d** and **e**, respectively) cell lines were treated with either Smp24 or Smp43 and cellular ATP levels measured with a Cell Titre-Glo assay. Treated cells showed a significant (**P ≤ 0.001) decrease from untreated controls. Data presented as mean ± SE, using an unpaired student t-test

Table 1 LC_{50} values (50%ATP levels, normalized to controls) of cell lines treated with Smp peptides (μg/mL)

Peptides	Non-tumour cell lines			Tumour cell lines	
	$CD34^+$	HREC	HaCaT	KG1-a	CCRF-CEM
Smp24	20 ± 0.3	37 ± 1.2	53 ± 1.5	16 ± 1.3	16 ± 0.9
Smp43	32 ± 0.6	28 ± 0.3	150 ± 2.9	16 ± 0.7	13 ± 1.5

Tumour or non-tumour cell lines were treated with either Smp24 or Smp43 for 24 h at 37 °C. LC_{50} values presented as mean ± SE

reduction in cellular ATP levels in both tumour and non-tumour cell lines, resulting in the loss of cell viability. Although Smp24 and Smp43 appeared to inhibit the viability of all cells, both lymphoid and myeloid leukaemia cell lines were more sensitive (LC_{50} values 13–16 μg/mL) in comparison to the non -tumour cell lines (stem cells, primary renal cells and keratinocytes), with LC_{50} values ranging from 20 to 53 μg/mL). The loss of membrane integrity, suggested by these ATP studies, was confirmed by complementary studies demonstrating leakage of LDH from all the cells tested. Previous studies have shown that scolopendrasin-VII, an AMP identified by de novo RNA sequencing of the centipede *Scolopendra subspinipes mutilans* (Lee et al 2015), ABP-CM4, a cecropin-like AMP from the haemolymph of the Chinese silkworm, *Bombyx mori* (Chen et al. 2010) and polybia-MP from the venom of the Brazilian social wasp, *Polybia paulista* (Wang et al 2009) all reduced the viability of different leukaemia cell lines in concentration-dependent manner.

Moreover, the SEM studies were conducted to gain more details about morphological disruption induced by Smp24 and Smp43. The results showed that both peptides induced various concentration-dependant morphological alternations on the cell membrane of all cells tested. These alternations were represented by losing cell membrane microvilli and filopodia, pore formation, development of membrane blebs and disintegrated cell membranes. These data confirmed the results obtained from the ATP and LDH assays. Previously, similar results were reported by different AMPs. For instance, Lu et al. (2016) observed that leukemia cell lines treated with the antimicrobial peptide of PFR displayed irregular shape, corrugated surface, cell swelling, bubbles protruding from their membranes and membrane disruption. Moreover, temporin-1CEa AMP incubated with breast cancer cells showed shriveled, invaginated and disrupted cell membranes (Wang et al. 2013). Similarly, hepcidin1–5 AMP induced pore formation and cell swelling in HepG2, HeLa, and HT1080 cell lines (Chang et al. 2011). Pore formation and membrane disruption were also reported in human

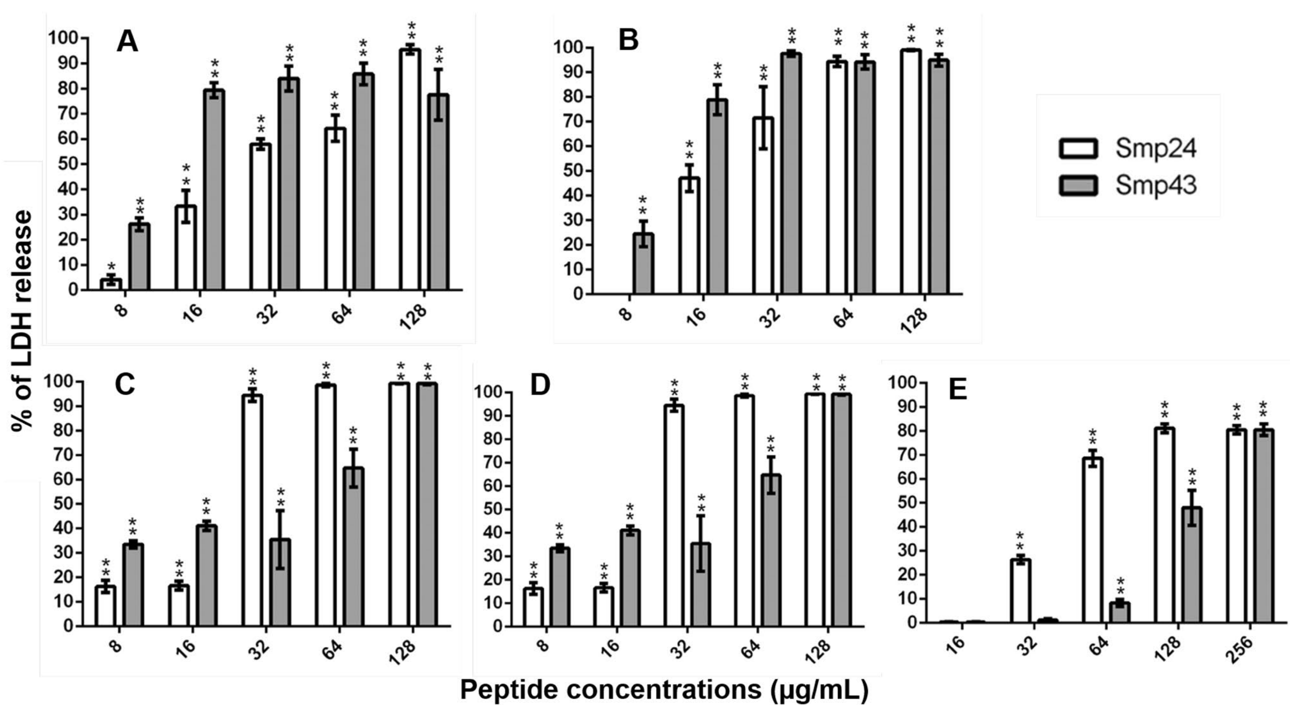

Fig. 2 Effects of Smp24 and Smp43 on cell membrane integrity of tumour and non-tumour cell lines, as evidenced by LDH release. Tumour (KG-1a and CCRF-CEM; Panels A and B, respectively) and non-tumour (HREC, $CD34^+$ and HaCaT; Panels C, D and E, respectively) cell lines were treated with either Smp24 or Smp43 and the release of LDH was measured. Treated cells showed significant (**P ≤ 0.001) effects with respect to untreated controls, in a concentration dependent manner. Data presented as mean ± SE, using an unpaired student t-test

bladder cancer cells treated with magainin II and cecropin B AMPs (Lehmann et al. 2006; Suttmann et al. 2008).

It has previously been suggested that AMPs might be effective anti-cancer agents (Wang and Wang 2016; Crusca et al. 2018), due to surface membrane charge differences. The outer plasma membrane leaflet in normal cells exhibits an overall neutral charge as its main components are the zwitterionic phospholipids, phosphatidylcholine and sphingomyelin (Dolis et al. 1997; Hoskin and Ramamoorthy, 2008; Riedl et al. 2014). In contrast, membrane of cancer cells is negatively charged, due to the exposure of the negatively charged phospholipid phosphatidylserine on their outer leaflet (Ran and Thorpe. 2002; Márquez et al. 2004; Schroder-Borm et al. 2005; Dobrzyn´ska et al. 2013). The electrostatic interaction between the positively charged AMPs and the negatively charged components in a cell membrane was believed to play a major role in target selectivity (Lu et al. 2016). Therefore, the unique membrane composition in tumour cells may make them more susceptible to targeting by cationic AMPs such as the scorpion venom peptides studied here.

It is clear that a range of mammalian cell types need to be assayed when testing Smp peptides for toxicity. In a previous study (Harrison et al 2016a), we have shown that Smp43 has very low haemolytic activity (1.2% ± 0.5% lysis at 512 μg/mL) in comparison with the release of ATP from all the cell lines (tumour and non-tumour) tested here (ca. 100% at the same concentration). In comparison, Smp24 caused significant erythrocyte disruption (89.6% ± 5.6%) at 512 μg/mL and the dose-dependent effect of ATP release from the non-tumour cell lines studied here (Fig. 1) paralleled both the haemolytic activity and the ATP release from HepG2 cells studied previously (Harrison et al 2016a). Although Smp24 and Smp43 have highest potency against gram positive bacteria (MIC values 4–32 μg/mL), both myeloid (KG1-a) and lymphoid (CCRF-CEM) leukemic tumour cell lines were more sensitive to Smp peptides than the non-tumour cell lines; however the sensitivity to the tumour cell lines was more akin to that of gram-negative bacteria (Harrison et al 2016a).

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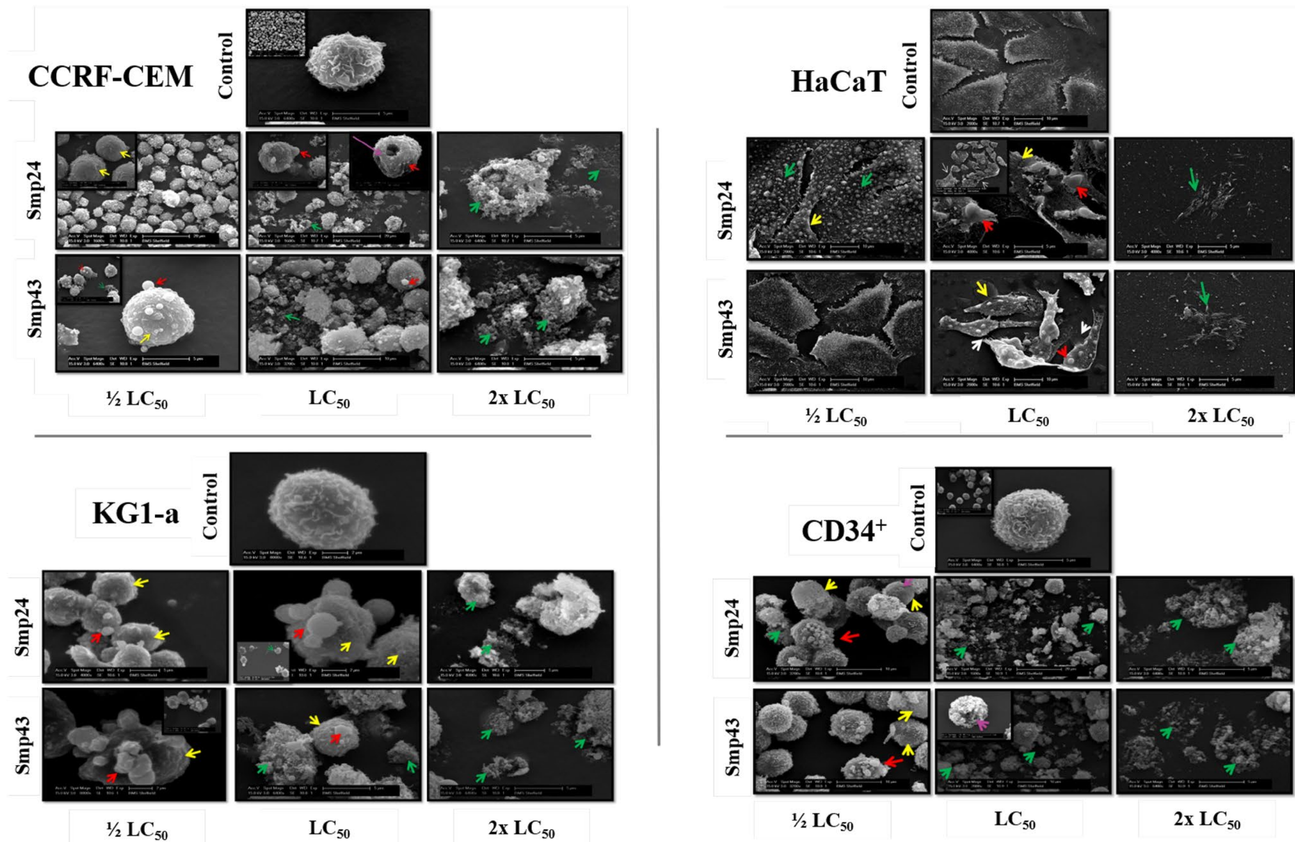

Fig. 3 Scanning electron micrograph of CD34$^+$, HaCaT, CCRF-CEM and KG1-a cells showing the effects of Smp24 and Smp43 treatment on their cell membrane. Treated cells revealed increases in microvilli loss (yellow arrow), appearance of cell membrane blebs (red arrow), losing cell membrane filopodia (white arrow), cell membrane pore formation (purple arrow) and disintegrated cell membranes and destroyed cells (green arrow) (Color figure online)

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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