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ORIGINAL ARTICLE

The antibacterial peptide ABP-CM4: the current state of its production and applications

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Abstract The increasing resistance of bacteria and fungi to currently available antibiotics is a major concern worldwide, leading to enormous efforts to develop new antibiotics with new modes of actions. Antibacterial peptide CM4 (ABP-CM4) is a small cationic peptide with broad-spectrum activities against bacteria, fungi, and tumor cells, which may possibly be used as a promising candidate for a new antibiotic. For pharmaceutical applications, a large quantity of antimicrobial peptides needs to be produced economically. In this communication, the progress in the structural characteristics, heterologous production, and biological evaluation of ABP-CM4 are reviewed.

Keywords Antibacterial peptide · ABP-CM4 · Heterologous expression · Antimicrobial · Anticancer

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Introduction

The increasing resistance of bacteria and fungi to currently available antibiotics is a major concern worldwide, leading to enormous efforts to develop new antibiotics with new modes of actions (Makovitzki et al. 2006). One potential source of novel antibiotics is the antimicrobial peptides (AMPs) which are relatively small molecules that are less than 100 amino acids in length and have a broad spectrum of antimicrobial activity. They serve as an ancient defense mechanism against pathogenic microorganisms that easily come in contact with the host through the environment (Sugiarto and Yu 2004). AMPs are now considered a fundamental component of the innate immune system (Radek and Gallo 2007). In contrast to the adaptive immune response, the innate immune response is immediate, nonspecific, and diverse (Gallo and Nizet 2003; Ganz 2003; Nizet and Gallo 2003).

In the past decades, a large number of AMPs have been isolated and purified from various species of organisms, such as defensins, cathelicidins, histatins, and cecropins (Koczulla and Bals 2003). Defensins are a broadly dispersed group of cationic peptides containing cysteine-rich conserved motifs originally identified in human and rabbit neutrophils (Radek and Gallo 2007). In contrast to the defensins, Cathelicidins were identified solely in mammalian species (Dorschner et al. 2001; Gallo et al. 1997). PR-39 became the first AMP found in mammalian skin,

specifically porcine wound fluid (Gallo et al. 1994). Histatins are a group of AMPs, found in the saliva of man and some higher primates, which possess antifungal properties (Helmerhorst et al. 1997). Cecropins are positively charged peptides that were originally isolated from the blood lymph of the giant silk moth (Hultmark et al.1980). Cecropins have the ability to form specific amphipathic alpha-helices which allow them to target nonpolar lipid cell membranes. Upon membrane targeting, they form ion-permeable channels subsequently resulting in cell depolarization, irreversible cytolysis, and finally death (Boman 2003). ABP-CM4, an antibacterial peptide isolated from the hemolymph of the silkworm *Bombyx mori*, belongs to the cecropins family (Tu et al. 1989). ABP-CM4 kills bacteria, tumors, and fungi by permeabilizing the cell membranes without being toxic to mammalian cells (Chen et al. 2010; Zhang et al. 1997; Xu and Zhang 2001). It would be important for control of resistant pathogen bacterial and fungal infections, but would allow conducting additional studies on their molecular interactions and antimicrobial mechanisms, as well on their eventual use in public health care. Therefore, the purpose of this communication is to revise the state of the art on the study of ABP-CM4 concerning its structural characteristics, expression systems, and biological activities of the recombinant products.

Structural characteristics of ABP-CM4

AMPs are small, positively charged, amphipathic molecules (which possess both hydrophobic and hydrophilic regions) of variable amino acid composition and length (6–100 amino acids). Based on their secondary structure, AMPs are grouped into four major classes: β-sheet, α-helical, loop, and extended peptides (Giuliani et al. 2007). The molecular mass of ABP-CM4 was 3876.64 confirmed by MALDI-TOF MS. ABP-CM4 is a cationic peptide containing $Arg^{1,16}$, $Lys^{3,6,7,10,21,}$ Glu^{9}, and Asp^{17}, which results in a net charge of +5 at neutral pH (Chen et al. 2010).

The secondary structure and three-dimensional structure prediction of ABP-CM4 showed the N-terminus to be a typical amphipathic α-helix (Jones 1999; Ward et al. 2004; Zdobnov and Apweiler 2001), which was an important structural parameter for anticancer activity (Yang et al. 2004). Charged residues are grouped on one face of the helix and hydrophobic residues are grouped on the opposite face (Fig. 1a). Then the secondary structure of ABP-CM4 was estimated by CD spectroscopy in a solution containing different concentrations of 2,2,2-trifluoroethanol (TFE) from 0 to 50% (v/v). It was found from the CD spectra that ABP-CM4 displayed a negative band at 200 nm in water, suggesting largely random coil conformation. However, in the presence of 20% TFE in water, the shape of the curves was increasingly characteristic of α-helical secondary structure. This peptide exhibited a positive band at 195 nm and two negative bands at 208 and 222 nm in 30 and 50% TFE in water, suggesting a well-defined α-helical conformation (Fig. 1b). Then the CD spectra of ABP-CM4 in POPG vesicles (at peptide:lipid ratios of 1:25 and 1:50) were estimated (Supplementary Fig. 1S). These spectra suggest that the peptide does not have the same structure in POPG or in TFE. These spectral differences were (1) the dichroic intensities in 200 nm are very different (positive in POPG but negative in TFE); (2) the negative band in 222 nm is displaced to a larger wavelength in POPG than in TFE; and (3) the α-helical signature is weaker in POPG than in TFE. The CD study confirms the molecular feature of ABP-CM4 as a linear α-helix in membrane-mimicking solvents, which is consistent with other α-helix ABPs (Bechinger et al. 1997; Wu et al. 2009). Owing to its special sequence and dimensional structure, ABP-CM4 is insensitive to denaturants, detergents, organic solvents, extreme temperatures, and pH. The results of the thermal stability test showed that temperatures as high as 95°C for 30 min had no influence on the activity of ABP-CM4; however, temperatures above 100°C (30 min) decreased the antimicrobial activity of ABP-CM4 by 15.5%. Varying the pH from 2.0 to 10.0 had no effect on the activity of ABP-CM4; however, pH values above 12.0 decreased the antimicrobial activity of ABP-CM4 by 12.5%. And 25.5% loss of potency was observed at pH 12.0 above 100°C (30 min) (Fig. 2 and Supplementary Fig. 2S). These features strengthen its potential as a microbicide, as well as providing a simple heat precipitation process to separate it from other host proteins when it is expressed in heterologous bacteria or cells.

Heterologous expression of ABP-CM4

ABP-CM4 is a promising candidate for a new antibiotic. For pharmaceutical applications, a large quantity of AMPs needs to be produced economically. A few years ago, the ways of obtaining ABP-CM4 were (1) isolation from the silkworm, which requires large amounts of material from the source with the problem of having very low yields of the interest peptide; and (2) chemical synthesis of peptides, which generate high costs. Therefore, genetic engineering became a great strategy to produce large amounts of interesting ABP-CM4 with low cost, to produce possible variants using site-directed mutagenesis, and to elucidate the antimicrobial mechanisms and to improve the final yields.

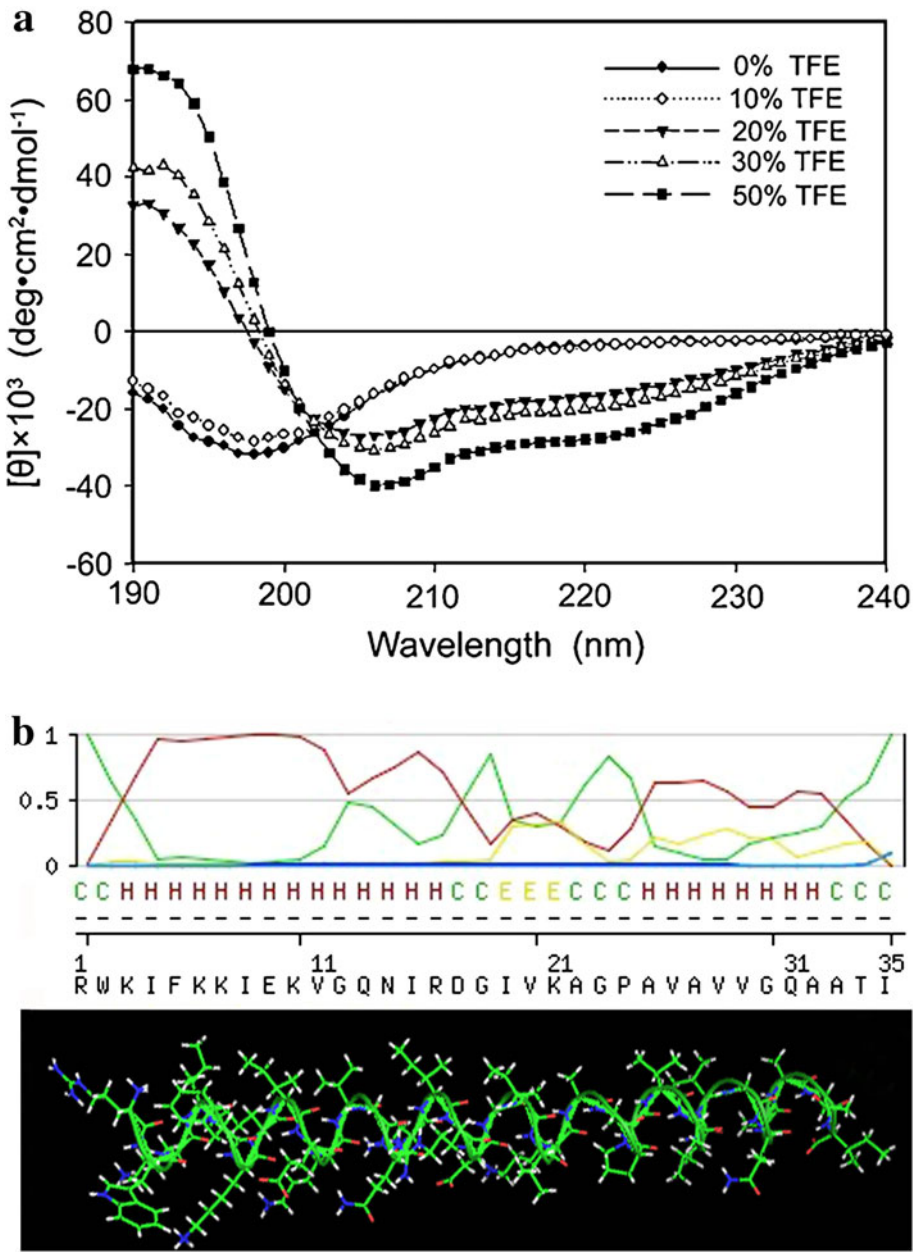

Fig. 1 The secondary structure analysis and three-dimensional structure prediction of ABP-CM4. **a** Secondary structure analysis of ABP-CM4 by CD spectroscopy. Far-UV CD spectra were conducted at room temperature in TFE/water mixtures at various concentrations of TFE in the range of 0–50% (v/v). Adopted from Chen et al. (2010). **b** The secondary and three-dimensional structure prediction of ABP-CM4. *H* helix, *E* extended-beta, *E. coil*

Expression of ABP-CM4 in *Escherichia coli*

The *Escherichia coli* expression system is still the most commonly used because of its high level of expression, the relative simplicity of the DNA manipulations, and the short time required to produce a product. However, alleviating the toxicity and proteolytic degradation of an expressed antibacterial peptide in the *E. coli* system and purifying the peptide easily are common problems. Many fusion partners working as a carrier have been used to express and purify ABP-CM4. The function of the carrier protein is to protect the small cationic peptides against proteolytic degradation (Piers et al. 1993). In addition, the fusion system provides several advantages: (1) they protect the hybrid when proteases are exported to the external environment, (2) the carrier protein might show affinity for a specific ligand, which enables easy purification not only might prevent AMP toxicity against the host cell, and (3) the carrier protein can finally be released by specific proteolytic action, leaving no extra amino acids on the C- or N-terminal regions.

Earlier attempts to produce recombinant ABP-CM4 in our laboratory using the *Escherichia coli* expression system with the PET-28a vector and synthesized as a fusion protein have been only partially successful for an extra fusion partner left at the N-terminus (Li et al. 1999). Subsequently, we synthesized ABP-CM4 using the plasmid of pET-32a, with T7 promoter and thioredoxin (TrxA) as a

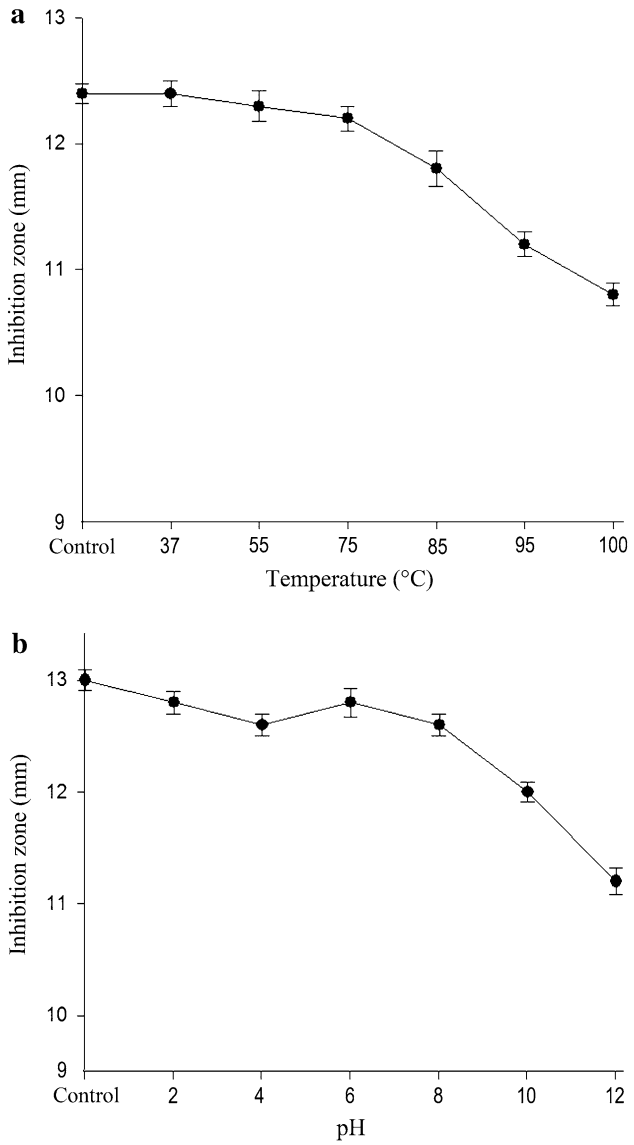

Fig. 2 Effects of temperature (**a**) and pH (**b**) on ABP-CM4. **a** The peptide sample kept at 4°C was used as a control. **b** The sample kept in the original culture (pH 6.5) was used as a control. *E. coli* $K_{12}D_{31}$ was used as the indicator strain

fusion partner because of its reported compatibility with expression of foreign proteins in soluble form (LaVallie et al. 2000). However, this expression system yielded only 1.2–1.4 mg target protein per liter of culture and an extra proline or glycine left at the N-terminus (Li et al. 2007; Zhou and Zhang 2009). Substitution of the TrxA fusion partner with intein-mediated expression system resulted in intact ABP-CM4, but this expression system yielded only 2.1 mg target protein per 6 g wet weight (Chen et al. 2008a, b). In addition, the purification process based on HPLC could not meet the requirement of mechanism determination and other large-scale operations.

To increase the yield of ABP-CM4, inclusion body (IB) expression in the cytoplasm of *E. coli* was attempted with N^{pro} mutant (EDDIE) fusion technology (Cheng et al. 2010). Following refolding by a conventional process and purification by Ni^{2+}-chelating chromatography column and cation exchange chromatography column, about 6 mg/L of ABP-CM4 was produced in shaking flask culture. Although it offers an alternative way of producing large quantities of antibacterial peptides, the cleavage rate of His-EDDIE-CM4 fusion protein reached only 50%, which was caused by the first amino acid of ABP-CM4. How to enhance the cleavage rate of His-EDDIECM4 fusion protein need be further studied. The heterogeneity of ABP-CM4 from IB exposes the weakness of this type of expression. Another new technology of biosynthesis of ABP-CM4 using the elastin-like peptide (ELP) tags was also tried, but the low yield and cleavage rate were disappointing (Shen et al. 2010). Finally, we turned our attention to the chaperon-fused expression system. Fortunately, this system with hexahistidine and small ubiquitin-related modifier (SUMO) double-tagged ABP-CM4 leads to a soluble fusion protein in the cytoplasm of *E. coli* at a level of >18% of the total soluble protein. With this construct, intact and native ABP-CM4 can be rapidly purified in a soluble and biologically active form by two rounds of affinity chromatography with one round of SUMO protease digestion in between according to Gao et al. (2010). In a 5-L bioreactor using conventional fed-batch culture (FBC) in a manner similar to that described previously (Ma et al. 2006), about 24 mg/L native ABP-CM4 was afforded (Li et al. 2009). On this basis, we described a procedure of producing a larger quantity of recombinant ABP-CM4 by multimerization of ABP-CM4 gene with the fusion partner TrxA or SUMO, about 26–48 mg/L of ABP-CM4 was produced in shaking flask culture (Zhou et al. 2009; Li et al. 2011). These were the highest yield of ABP-CM4 reported to date. Overexpression of soluble SUMO–CM4 in the cytoplasm of *E. coli* to obtain intact, homogeneous, and activated ABP-CM4 by simple downstream processes makes this system promising for practical applications. By comparison of the expression of ABP-CM4 with other cecropin peptides (Campo et al. 2008; Chen et al. 2009; Hong et al. 2008; Jan et al. 2010; Zhang et al. 2010), the SUMO fusion technology potentially could be employed as a new way for the production of recombinant cecropin peptides (Supplementary Table 1S).

In eukaryotic host

Pichia pastoris is an alternative host that, like *E. coli*, can be grown cheaply and rapidly, possesses certain post-translational modification pathways, is able to secrete more efficiently, and permits the production of r-proteins without intense process development. Introduction of the coding sequence for ABP-CM4 into *P. pastoris* demonstrates the potential for expression of ABP-CM4 in a eukaryotic

system. After inducing about 72 h with 0.5% methanol at 20°C, supplied with 2% casamino acids to avoid proteolysis, approximately 40 mg ABP-CM4 was secreted into 1 L of medium. Recombinant ABP-CM4 was purified through size-exclusion chromatography and 15 mg pure active ABP-CM4 was obtained from 1 L culture (Zhang et al. 2006). During the production of ABP-CM4, we found that the antibacterial activity of secreted ABP-CM4 obtained by liquid fermentation, 250 rpm, 20°C in YPD media for 144 h, was dropping along the culture time, from 17 mm inhibition zone at 72 h to 9 mm zone at 144 h. The diminished antibacterial activity was correlated with the low stability of ABP-CM4 at 20°C and proteolytic activity inside the fermentation media more than with the inhibition of *P. pastoris* growth due to ABP-CM4 expression. Furthermore, the yield in *P. pastoris* was 15 mg/L, which is low compared with cytoplasmic expression in *E. coli*. Another eukaryotic expression of ABP-CM4 with green fluorescent protein (GFP) fusion protein using the expression vectors pcDNA3 was also tried, but the low yield and an extra GFP fusion partner left at the N-terminus were disappointing (Chen et al. 2008a, b).

In conclusion, ABP-CM4, a promising microbicide candidate with a special primary structure that is sensitive

Table 1 Compilation of the key features of recombinant ABP-CM4 from various expression systems

Expression type	Purification process and yield	Key features	References
Silkworm Natural product	CM-Sepharose chromatography, Phenyl-Sepharose. Yield not determined	Natural product. Low yield	(Tu et al. 1989)
E. coli Cytoplasmic IB Expression	Inclusion body refolding, Ni-IDA AC, Autoprotease by self-cleavage, IEC. Yield 15 mg/L, (optimal conditions)	N^{pro}. Inclusion body. High yield. Obtained intact and homogeneous native CM4 from the His-EDDIE–CM4 fusion.	(Cheng et al. 2010)
Cytoplasmic soluble Expression	Ni-NTA AC, Formic acid or Hydroxylamine hydrochloride cleavage, RP-HPLC. Yield 1.2–1.4 mg/L	Trx. Soluble expression in the cytoplasm of *E. coli*. Low yield. An extra residue left at the N-terminus of the CM4 from the His–TRX–CM4 fusion	(Li et al. 2007; Zhou and Zhang 2009)
Cytoplasmic soluble expression	Chitin AC, DTT cleavage, RP-HPLC. Yield 2.1 mg/L, (unoptimal conditions)	CBD. Soluble expression in the cytoplasm of *E. coli*. Low yield. Obtained intact and homogeneous native CM4 from the Intein–CBD–CM4 fusion	(Chen et al. 2008a, b)
Cytoplasmic soluble expression	Ni-NTA AC, Hydroxylamine hydrochloride cleavage, RP-HPLC. Yield 12 mg/L, (optimal conditions)	Trx. Soluble expression in the cytoplasm of *E. coli*. High yield. An extra glycine residue left at the N-terminus of the CM4 from the His–TRX–nCM4 fusion	(Zhou et al. 2009)
Cytoplasmic soluble expression	ITC, Self-cleavage. Yield 0.6 mg/L, (unoptimal conditions)	Elp, Soluble expression in the cytoplasm of *E. coli*. Low yield. Obtained intact and homogeneous native CM4 from the ELP–CM4 fusion	(Shen et al. 2010)
Cytoplasmic soluble expression	Ni-NTA AC, SUMO protease cleavage. Yield 24 mg/L, (optimal conditions)	Sumo. Soluble expression in the cytoplasm of *E. coli*. Simplified purification process and high yield. Obtained intact and homogeneous native CM4 from the His–SUMO–CM4 fusion	(Li et al. 2009)
Cytoplasmic soluble expression	Ni-NTA AC, Hydroxylamine hydrochloride cleavage. Yield 48 mg/L, (optimal conditions)	Sumo. Soluble expression in the cytoplasm of *E. coli*. Simplified purification process and high yield. An extra glycine residue left at the N-terminus of the CM4 from His–SUMO–2CM4 fusion	(Li et al. 2011)
Yeast and fungi Pichia pastoris Secreted expression	Size-exclusion chromatography. Yield 15 mg/L, (optimal conditions)	Simplified purification process and high yield. Obtained intact and homogeneous native CM4 as extracellular protein. But proteolytic activity inside the fermentation media	(Zhang et al. 2006)
Human myeloid leukemia K562 cells	NA	Low yield and an extra GFP fusion partner left at the N-terminus	(Chen et al. 2008a, b)

to proteolytic activity, has been expressed in *E. coli*, yeast, and human myeloid leukemia K562 cells (Table 1). At this moment though, the soluble expression in the cytoplasm of *E. coli* might be the only feasible option.

Table 2 The minimal inhibitory concentration of recombinant CM4 to selected microorganisms

Microorganisms	Minimum inhibitory concentration (μM)	
	Recombinant CM4	Native CM4
E. coli $K_{12}D_{31}$	12	12
Salmonella spp.	14–16	15
P. aeruginosa	20	20
A. niger	4–8	5
T. viride	4–8	4–8
G. saubinetii	2–4	2–4
P. chrysogenum	16–32	20

Antimicrobial and anticancer activity assays of ABP-CM4

The most intriguing feature of ABP-CM4 is its destructive effect on bacteria, fungi, and tumors without damaging normal cells.

Antibacterial activity assays

The antibacterial activity of the ABP-CM4 was evaluated by determining its minimal inhibitory concentration (MIC) against selected bacteria (Lee et al. 2002). The results indicated that recombinant ABP-CM4 and native ABP-CM4 have almost the same antimicrobial activity against *E. coli* K12D31, *Salmonella* spp. and *P. aeruginosa* (Table 2). The antibiotic mechanism of amphipathic antibacterial peptides with α-helical structures is not clearly understood. Cell structure disrupted by pore formation (Ludtke et al. 1996; Shai and Oren 2001) or ion channel

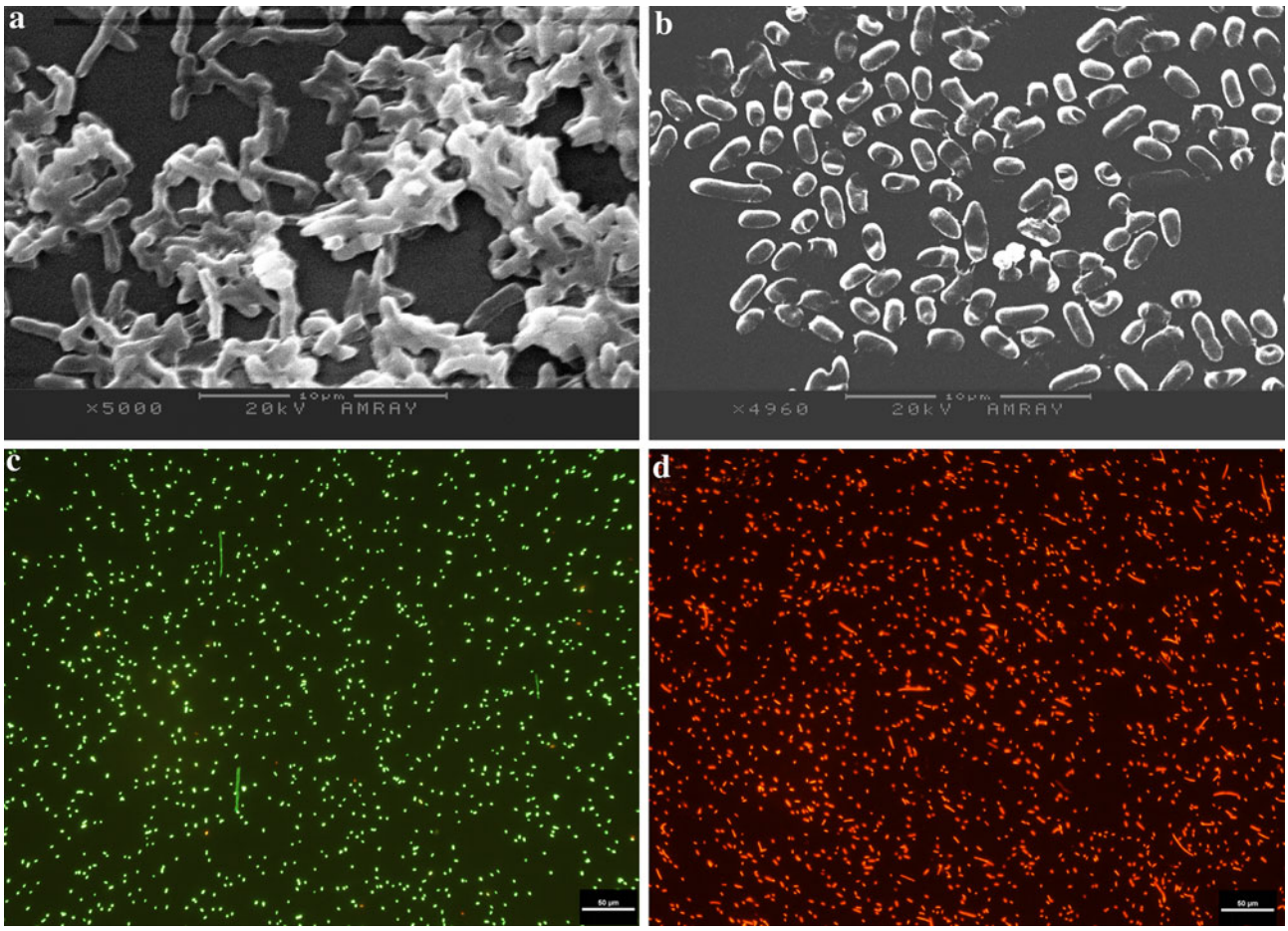

Fig. 3 Scanning electron micrographs (SEM, X5, 000) of ABP-CM4's effect on bacteria surface morphology and live/dead assay. **a** SEM of *E. coli* $K_{12}D_{31}$ incubated with PBS. **b** SEM of *E. coli* $K_{12}D_{31}$ incubated with 10 μM ABP-CM4. **c** Live/dead staining assay of normal *E. coli* $K_{12}D_{31}$. **d** Live/dead staining assay of *E. coli* $K_{12}D_{31}$ incubated with 10 μM ABP-CM4

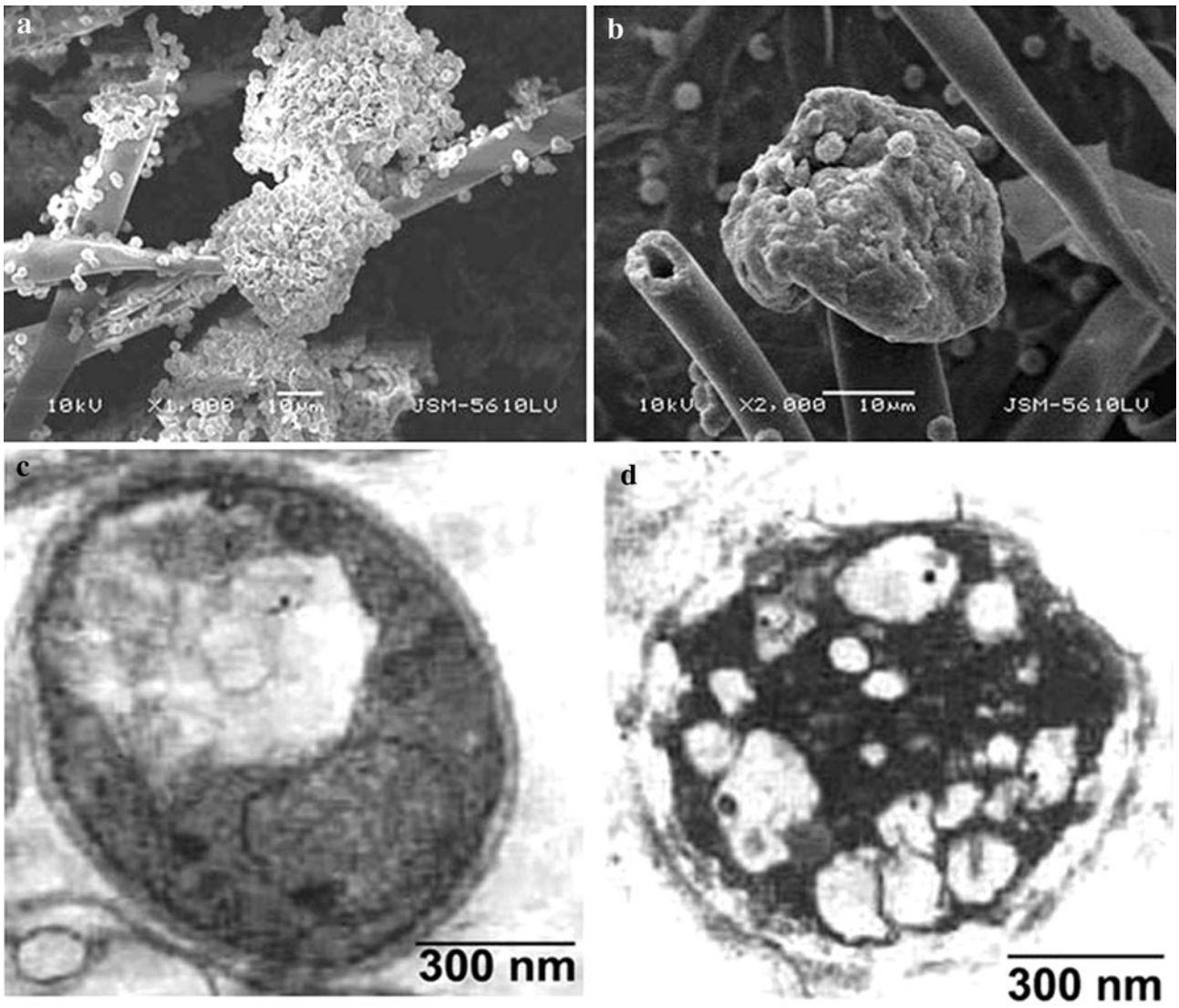

Fig. 4 Scanning electron micrographs (SEM, X3, 000) and Transmission electron micrographs (TEM, X12, 000) of ABP-CM4's effect on cell surface morphology and cellular organelles. *A. niger* cells were incubated with PBS (**a**) or 8 μM ABP-CM4 at 30°C for 16 h (**b**), normal ultrastructure of *A. niger* (**c**) or *A. niger* treated with ABP-CM4 for 8 h (**d**). Adopted from Zhang et al. (2008)

generation (Huang 2000) maybe is the most likely mechanism. In our study, ABP-CM4 can kill the bacteria by forming transmembrane pores on the biofilm (Fig. 3).

Antifungal activity assays

The ABP-CM4 showed a twofold and fourfold greater antifungal activity against *A. niger* than did cecropin A (Delucca et al. 1997) and cecropin B (Delucca et al. 1998), and was also more effective against *A. niger* than AMPs from mammals, such as defensins (DeLucca and Walsh 1999). In addition to *A. niger*, ABP-CM4 is also effective against *T. viride*, *G. saubinetii*, *P. chrysogenum* (Table 2).

Lee et al. (2002) reported that HP (2-20) may exert its antifungal activity by disrupting the structure of the cell membrane via pore formation or directly by interacting with the lipid bilayer in a salt-dependent manner. In our study, the cell wall regeneration capacity of protoplasts treated with ABP-CM4 was much lower than that of the control and suggested that the peptide act on the fungal plasma membrane. These results demonstrated that the prime target of ABP-CM4 action was the plasma membrane, not the cell wall. ABP-CM4 antifungal activity is characterized by the disruption of cell membranes and the cellular cytoskeleton, internal structural changes within the cell including decreased mitochondrial integrity, and

Table 3 Anticancer activity of CM4

Cells	Inhibitory concentration 50% (μM)
Cancer cells	
K562	15.8
U937	17.5
THP-1	14.2
SHG-44	10.2
HeLa	20.1
HepG2	18.2
Normal cells	
PBMCs	>80
HEK-293	>80

binding to fungal DNA (Zhang et al. 2008). The sum of these interactions can lead to cell death (Fig. 4).

Anticancer activity assays

Recently, a growing number of studies have shown that some of the cationic ABPs, which are toxic to bacteria but not to normalmammalian cells, exhibit a broad spectrum of cytotoxicity against cancer cells (Hoskin and Ramamoorthy 2008). Until now, several amphiphilic helical peptides, such as BMAP-27, BMAP-28, cecropin B, maganins, LL-37, and aurein 1.2, exhibited anticancer activity. Our studies have shown ABP-CM4 has a selective anticancer activity in several leukemia cell lines but does not affect normal cells (Table 3). Difference of binding activity between leukemia cells and normal cells results from membrane differences implicated in contributing to the selective cytotoxicity (Chen et al. 2010).

So far, at least three anticancer mechanisms have been proposed, including (1) cell membrane lytic effect; (2) activation of intrinsic pathways of apoptosis via mitochondrial membrane disruption; (3) certain peptides are potent inhibitors of blood vessel development, which is associated with tumor progression (Zaiou 2007). In our study, ABP-CM4 can disturb the tumor cellular membrane leading to leakage (Chen et al.2010; Wang et al. 1998; Zhang et al. 1997). By comparison of the anticancer activities of ABP-CM4 with other known antitumor peptides (Chen et al. 2005; Eliassen et al. 2006; Lehmann et al. 2006; Müller et al. 2002; Papo et al. 2006; Sharma 1992; Shin et al. 1999), most antitumor peptides are membrane-targeted leading to cell lysis (supplementary Table 2S), which shows potential for synergy with current cancer treatments. In addition to leukemia cells, ABP-CM4 is also effective against the growth of SHG-44, HeLa, and HepG2 cells (Cheng et al. 2010; Li et al. 2010). Our results indicated that ABP-CM4 has the potential for development as a novel anticancer agent.

Conclusions and perspectives

ABP-CM4 is a promising microbicide candidate that was discovered 22 years ago. It has displayed an impressive potential as a microbicide and could even be a novel anticancer agent. Using the present technology, it may be possible to produce ABP-CM4 on a large scale and at low cost. The soluble cytoplasm expression of native ABP-CM4, plus the established know-how of the bioprocesses of *E. coli* produced biologics in the industrial sector, might provide a ready solution for the manufacture of this microbicide candidate. Other approaches provide a backup for future manufacturing of the drug.

Recent studies have demonstrated that ABP-CM4 has the potential to inhibit cellular cytokine and NO released by binding directly to LPS or by blocking the binding of LPS to LPS-binding protein (Lin et al. 2008). This makes it an attractive drug candidate for treatment of endotoxin shock and sepsis caused by bacterial infection. Further studies are needed to elucidate the exact mechanism involved in its cytotoxicity and its selectivity tested in different assay and pharmacological models.

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