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Potential anticancer activity analysis of piscidin 5-like from *Larimichthys crocea*

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Abstract

Antitumor activity is one characteristic function of some certain antimicrobial peptides (AMPs) found in recent years. In the present study, we attempted to detect potential anticancer activity of a recombinant piscidin 5-like from *Larimichthys crocea* (rLc-P5L) which owned widely antibacterial and strong antiparasitic activity *in vitro*. The light microscope observation indicated rLc-P5L was of antitumor activity to HeLa cells, 293T cells and L929 cells. MTT assay showed the toxic sensitivity of rLc-P5L to three tumor cell strains was 293T>L929>HeLa. Scanning electron microscope (SEM) results showed rLc-P5L behaved like a lytic peptide to cause damage on cells membrane of L929 cells by forming globular clusters, even pores at 60 μmol/L, or degrading membrane to make it completely lose cytoskeleton structure at 80 μmol/L; rLc-P5L treatment also resulted in DNA degradation. Fluorescence observation results indicated rLc-P5L could cause L929 cells at least two obvious changes: one is nucleus, nuclear chromatin condensed in the margin, nuclear volume became smaller and shrank to be out of shape, or lysed to be debris; the other is cytoskeleton, they became disordered and polarized to make cells atrophic shapes, or even lysed to be debris. In summary, rLc-P5L owned potential anticancer activity causing membrane structure damage and genome DNA degradation. Interestingly, treatment with different concentration of rLc-P5L seemingly caused the similar but different changes, whether it indeed gave rise to cancer cells diverse death way, the further studies should be performed, and the detailed mechanisms were still need further explored.

Key words: *Larimichthys crocea*, piscidin 5-like, anticancer activity, membrane destruction, DNA degradation

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1 Introduction

With the development research on antimicrobial peptides (AMPs), scholars found AMPs are one of the most promising candidates to completely or partially replace conventional antibiotics. The distinctive action modes in targeting cells membrane were not some certainly specific receptors, which almost do not cause the development of resistance. Therefore, tremendous efforts have been devoted to this field in the past years (Hoskin and Ramamoorthy, 2008; Schweizer, 2009; Hancock and Sahl, 2006), and more and more AMPs were isolated from bacteria to animals, plants. Biodiverse marine organisms are important reservoirs of abundant AMPs.

The identification of various AMPs as potent and selective antitumor drugs against different kinds of tumor cells has attracted remarkable interests (Lin et al., 2010), and they are of enormous potential to be developed as drug candidates to cure cancer. As we all knew, energy-dependent transporters such as P-glucoprotein on the cancer cell membranes rendered some already existed anticancer drugs to be useless, such as adriamycin or vinca alkaloids, because resistance-acquired P-glucoprotein was able to lower drugs concentration in the cells (Zupkó et al., 2009). In particularly, resistance existence has gradually triggered great concern worldwide about the failure of cancer or inflammation therapies. Previous studies demonstrated that cancer cells or bacteria membranes carry much more net negative charges, such as phosphatidylserine (PS) (Dobrzyńska et al., 2005), O-glycosylated mucins (Dennis, 1991), Lipopolysaccharides (LPS), or lipoteichoic acid (LTA). Most AMPs or called ACPs (anticancer peptides) owning amphipathic structure, preferentially bind and insert themselves into negatively charged cell membranes to disrupt cellular or mitochondrial membranes (Chen et al., 1988; Risso et al., 2002), so it was relatively easy to understand why cationic peptides can target and damage tumor cells or bacteria membranes. Similar to antibacterial mechanisms, there are several pathways describing ACPs action mechanisms, they could target various molecules to induce cell death. For example, CS5931 could activate caspase 9 and 3 of HCT-8 cells to exhibit significant anti-proliferative and pro-apoptotic activities (Cheng et al., 2012). Both members NRC-03, NRC-07 of pleurocidin family could lyse breast cancer cells membrane (Hilchie et al., 2011). Kahalalide F leads to necrosis-like cell death involving in the depletion of ErbB3 (human epidermal growth factor receptor 3) and

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the inhibition of Akt (protein kinase B) signal in the breast cancer cell line SKBR3 that depends on ErbB3 for its survival (Janmaat et al., 2005). Magainin 2, or tachyplesin could activate caspase-9/3 through mitochondrial pathway to induce apoptosis (Chen et al., 2005; Lehmann et al., 2006). A_9K could penetrate into HeLa cells to affect F-actin reconstruction, and thus triggered mitochondrial dysfunction (Xu et al., 2013).

Piscidins, an AMP family, own widely antibacterial, strong antiparasitic and lower hemolytic activity (Pan et al., 2019; Niu et al., 2013). Piscidin 5 is a member of piscidin family, it was obtained occasionally from hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Salger et al., 2011). Piscidin 5-like of *Larimichthys crocea* (termed Lc-P5L) was identified from a comparative transcriptome, it was a head kidney expression gene (Zhou et al., 2014). Previous studies have demonstrated recombinant piscidin 5 like (rLc-P5L) owned widely antibacterial spectrum (Pan et al., 2019), and it was involved in the antiparasitic immune response to *Cryptocaryon irritans* causing membrane rupture and contents leakage (Zheng et al., 2020). Report has found piscidin like of *L. crocea* (termed Lc-pis), another member of piscidin family, is a cancer cell killer, it binds to cancer cells to generate pores to lyse them, and the binding process was mediated by its positive net charge (Zhou et al., 2018). In this work, we tried to explore the potential activity of rLc-P5L against cancer cells *in vitro*.

2 Materials and methods

2.1 *Reagents and cells culture*

FITC-conjugated phalloidin and Hoechst 33342 were obtained from Solarbio® (China) and used following instructions. Note that all water used in this study was processed from a Milli-Q biocel ultrapure water system (Millipore, USA) with a minimum resistivity of 18.2 MΩ.

Human cervical carcinoma cells (HeLa cells), human renal epithelioma cells (293T cells) and mouse fibroblastoma cells (L929 cells) were cultured in Dulbecco's minimum essential medium (DMEM) with 10% fetal bovine serum (FBS). All the cells were maintained in 5% CO_2 at 37°C.

The rLc-P5L was obtained through constructing prokaryotic expression system with pET-28a as vector and *Escherichia coli* BL21 (DE3) as expression host bacteria. After induced by isopropyl β-D-thiogalactoside (IPTG), the denatured rLc-P5L existing in the conclusion bodies was purified through Ni column (GE Healthcare, USA) by gradient imidazole, and then refolded to obtain active part.

2.2 *In vitro cytotoxicity assay*

Cytotoxicity of rLc-P5L against three cell strains was determined by MTT assay. Cells were pre-seeded on sterilized 96-well plates at a density of 2×10^4 cells/well in 100 μL DMEM containing 10% FBS. The plates were then incubated for 24 h at 37°C in an atmosphere containing 5% CO_2 for cells adhering to the wall completely. rLc-P5L (20 μL) was added to a final concentration of 20–100 μmol/L, and the cells were incubated for another 24 h. Wells containing rEGFP-His-tag or without peptides were served as negative or blank controls. All mixtures were removed softly, 100 μL fresh DMEM and 10 μL MTT were added into each well and the plates were incubated for further 4 h at 37°C. Subsequently, the precipitated formazan was dissolved. The absorbance at 490 nm was measured using a microplate autoreader (Infinite M200 Pro). Percentage of cell survival was obtained from the percent ratios of A_{490} readings of treated groups over control cells.

2.3 *Observation of cell destruction by SEM*

L929 cells were pre-seeded on a glass coverslip in a 96-well plate with adjusted concentration in 180 μL DMEM containing 10% FBS, incubated at 37°C for 24 h in an atmosphere containing 5% CO_2. rLc-P5L (20 μL) was added to each well to a final concentration of 60 μmol/L or 80 μmol/L, followed by incubation for 6 h, 12 h, 24 h. Wells containing rEGFP-His-tag or without peptides were served as negative or blank controls. The cells were fixed with 4% glutaraldehyde in phosphate buffered saline (PBS) overnight at 4°C. All coverslips were washed with PBS and dehydrated with a series of graded alcohol. After critical point dried and gold sputtered, the samples were observed under a JSM-6390LV SEM (Japan).

2.4 *Laser scanning confocal microscopy (LSCM) observation of morphological changes*

L929 cells were pre-seeded on a sterilized confocal 96-well plate at adjusted concentration in 180 μL DMEM containing 10% FBS. The plate was then incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. rLc-P5L (20 μL) was added to each well to a final concentration of 60 μmol/L or 80 μmol/L for 6 h, 12 h, 24 h. The cells were fixed in 4% paraformaldehyde overnight at 4°C. Then the cells were washed with PBS, and permeabilized by 0.5% Triton X-100 for 5 min. After washed by PBS, the solution containing FITC-phalloidin and Hoechst 33342 was added and incubated for 3 h in the dark, then washed with PBS, and observed under LSCM.

2.5 *Agarose gel electrophoresis of genome DNA damage*

L929 cells were pre-seeded on sterilized 96-well plate at adjusted concentration in 180 μL DMEM containing 10% FBS. The plate was then incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. rLc-P5L (20 μL) was added to each well to a final concentration of 40 μmol/L, 60 μmol/L or 80 μmol/L for 24 h, or incubated for 6 h, 12 h, 24 h at 60 μmol/L. Then all samples were collected for DNA isolation with TIANamp Marine Animals DNA Kit (TIANGEN, China) according to the instruction, and DNAs were loaded to run in 1.2% agarose gel electrophoresis.

3 Results

3.1 *Anticancer activity of rLc-P5L*

In order to check whether rLc-P5L was poisonous to several cancer cell strains, the simplest way was to observe the changes of cancer cells treated by rLc-P5L. Just as illustration in Fig.1, HeLa cells, 293T cells and L929 cells appeared normal morphology and attachment in the control groups or rLc-P5L-treated groups under 20 μmol/L (data not shown); after being exposed to more than 20 μmol/L rLc-P5L (partial results not shown), cells turned rounded, some even floated in the medium, and the degree presented a concentration dependent tendency, whilst the medium became dirty. Therefore, rLc-P5L was of antitumor activity.

3.2 *Effects of rLc-P5L on viability of cancer cells*

MTT assay was applied to analyze the toxicity of rLc-P5L to these three cancer cell strains, the results showed rLc-P5L exhibited significant inhibitory action at more than 20 μmol/L. Just for L929 cells, the cells appeared no significant death, but the other two kinds of cells died significantly at 40 μmol/L ($p<0.05$). When the treated concentration continued to increase to be more than 40 μmol/L, the death rate got high significance ($p<0.01$). Seen from Fig. 2, the toxic sensitivity of rLc-P5L to them was

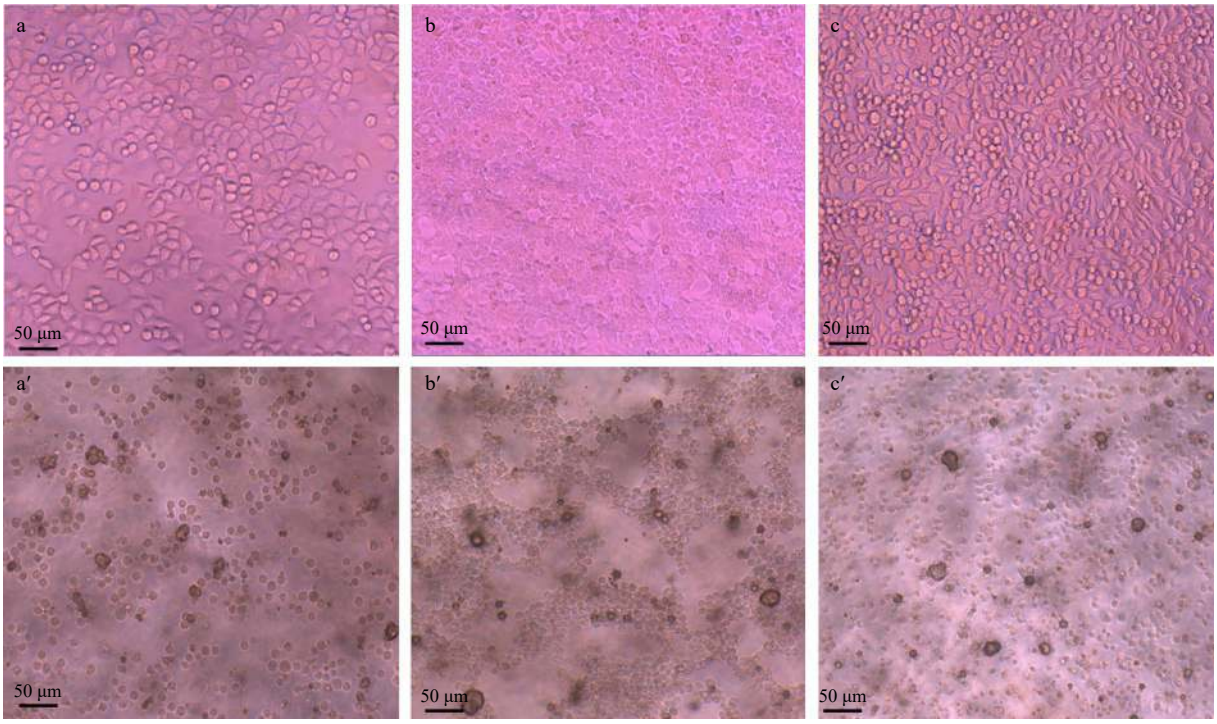

Fig. 1. The anticancer observation of 80 μmol/L rLc-P5L on cancer cells. a and a′. HeLa cells, b and b′. 293T cells, c and c′. L929 cells. a–c indicate control groups, a′–c′ indicate rLc-P5L treated groups at 60 μmol/L.

293T>L929>HeLa, and the anticancer activity appeared a dose-dependent manner.

3.3 *SEM observation effects of rLc-P5L on L929 cells*

3.3.1 *rLc-P5L (60 μmol/L) altered membrane structure of L929 cells*

To examine the damage effect of rLc-P5L on membrane structure, L929 cells were treated for 6 h, 12 h, and 24 h with 60 μmol/L rLc-P5L. Seen from Fig. 3, SEM observed L929 cells showed a normal smooth surface, spread appearance and spindle pattern in the control groups (Figs 3a–c). After treated with 60 μmol/L rLc-P5L, L929 cells appeared crimped boundaries and irregularly morphological structure. Many globular clusters were clearly visible on the cells surface. Such obvious morphological changes hinted cytoskeleton alteration or damage occurrence. With treatment time increasing, pores formed on cell membranes at different positions (Fig. 3e), and the cell membranes became unintegral as a result of falling of some globular clusters. Therefore, rLc-P5L functioned like a lytic peptide in disrupting membranes to form pores or creases on the cell surface.

3.3.2 *rLc-P5L (80 μmol/L) lyse membrane of L929 cells*

Occasionally, a higher concentration of 80 μmol/L rLc-P5L treatment was carried out to observe the further destructive effects. All control groups presented a normally smooth surface and spindle pattern in Figs 4a–c. Just as showed in Figs 3d–f, 80 μmol/L rLc-P5L treatment resulted in atrophying into sphere of cells, severe degradation of cell membranes. We could observe clearly a lot of entwined skeleton structures which supported the whole cell post treatment at 6 h (Fig. 3d). With time extension, massive skeleton structures continued to become loose, even appeared big holes among the skeleton structures, and inner condition could be observed from the loose structure at 12 h (Fig. 3e). Finally, the skeleton structures almost disappeared, nucleus and

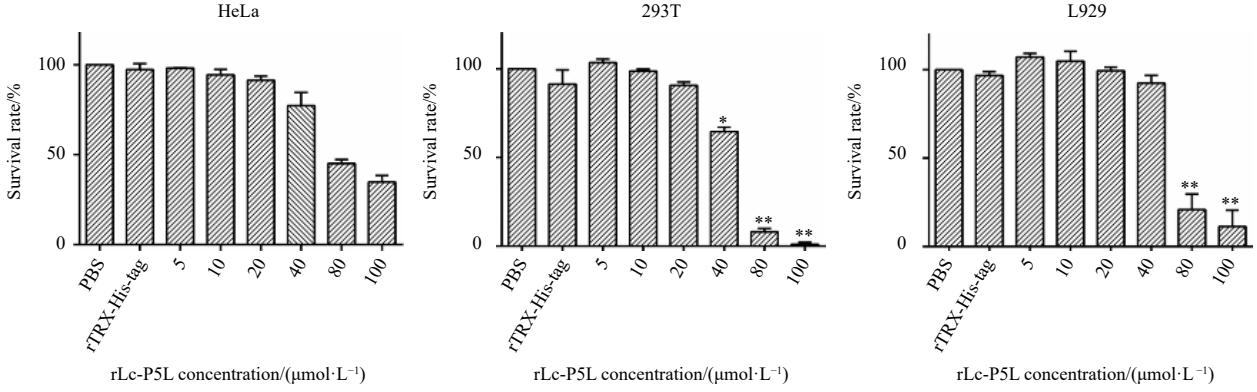

Fig. 2. The toxic effect of rLc-P5L on the cancer cell strains. * indicates significant difference (p<0.05), and **, highly significant difference (p<0.01).

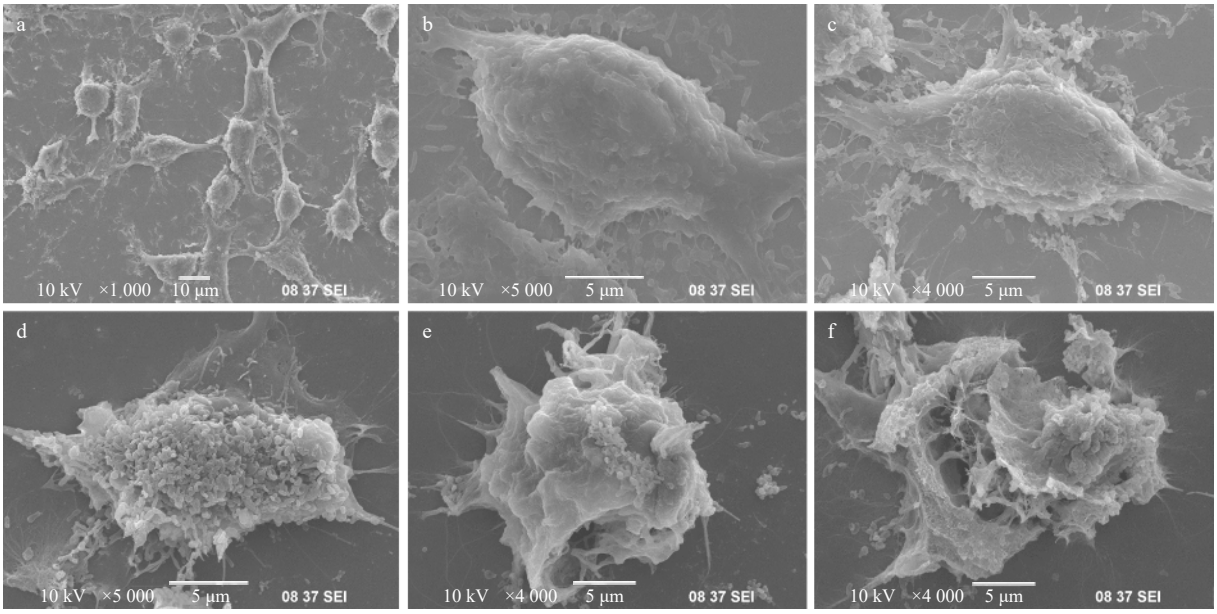

Fig. 3. The SEM observation of L929 cells treated by 60 μmol/L rLc-P5L for different time. a. The blank group; b. L929 cells treated with PBS; c. L929 cells treated with 60 μmol/L rGFP-His-tag; d–f. L929 cells treated with 60 μmol/L rLc-P5L for 6 h (d), 12 h (e) and 24 h (f).

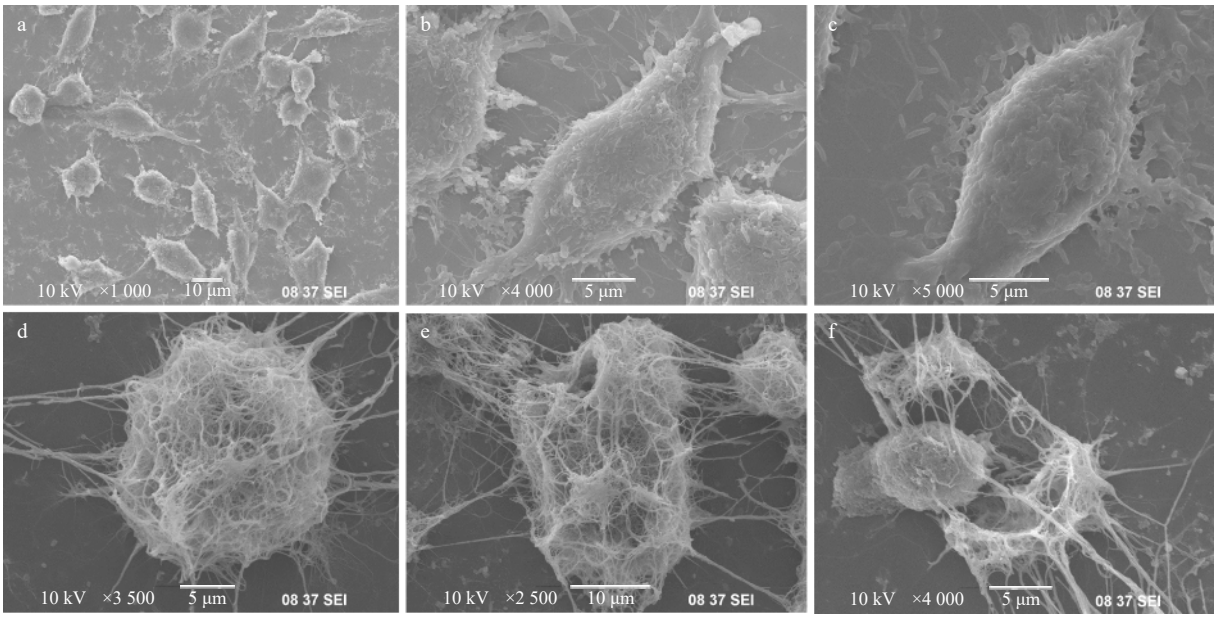

Fig. 4. The SEM observation of L929 cells treated by 80 μmol/L rLc-P5L for different time. a. The blank group; b. L929 cells treated with PBS; c. L929 cells treated with 80 μmol/L rGFP-His-tag; d–f. L929 cells treated with 80 μmol/L rLc-P5L for 6 h (d), 12 h (e) and 24 h (f).

other contents fell out from the large gaps, and the whole cell structure disintegrated severely at 24 h (Fig. 3f).

3.4 *rLc-P5L treatment caused L929 DNA degradation*

After treated with rLc-P5L, the agarose gel electrophoresis of DNAs was carried out and showed in Fig. 5. The blank or negative control groups presented tidy, bright bands without any degradation or fragmentation. Different concentration of rLc-P5L at 40 μmol/L, 60 μmol/L, 80 μmol/L treatment caused DNA bands weakening to be hardly observed with trailing phenomenon, which meant rLc-P5L could lead to L929 cells genome DNA degradation (Fig. 5a).

In addition, different treatment time under 60 μmol/L rLc-P5L resulted in similar phenomenon (Fig. 5b). Therefore, rLc-P5L treatment could cause L929 cells genome DNA degradation in dose- and time-dependent manners.

3.5 *Fluorescence observation damage effect of rLc-P5L on nucleus and cytoskeleton of L929 cells*

3.5.1 *rLc-P5L (60 μmol/L) treatment effects on nucleus and cytoskeleton of L929 cells*

Fluorescence staining was employed to mark cell nucleus and cytoskeleton. Compared to the control cells with smooth nuclear edge and clear boundary. In the 60 μmol/L treated groups (Fig. 6), nuclear chromatin got congealed, some cells presented highly

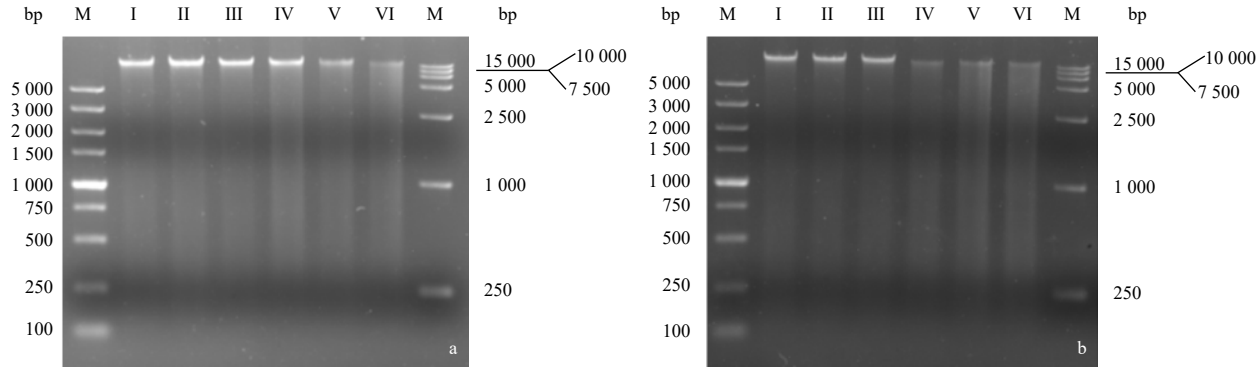

Fig. 5. The agarose gel electrophoresis of L929 cells genome DNA treated by rLc-P5L. a. Genome DNA of L929 cells treated at different concentration of rLc-P5L for 24 h; b. genome DNA of L929 cells treated at 60 μmol/L rLc-P5L for different time. M represents the standard DNA marker; Ⅰ, the blank group; Ⅱ, genome DNA of L929 cells treated by PBS; Ⅲ, genome DNA of L929 cells treated by rGFP-His-tag; Ⅳ in a, 40 μmol/L rLc-P5L; Ⅴ in a, 60 μmol/L rLc-P5L; Ⅵ in a, 80 μmol/L rLc-P5L; Ⅳ in b, treated 6 h; Ⅴ in b, treated 12 h; Ⅵ in b, treated 24 h.

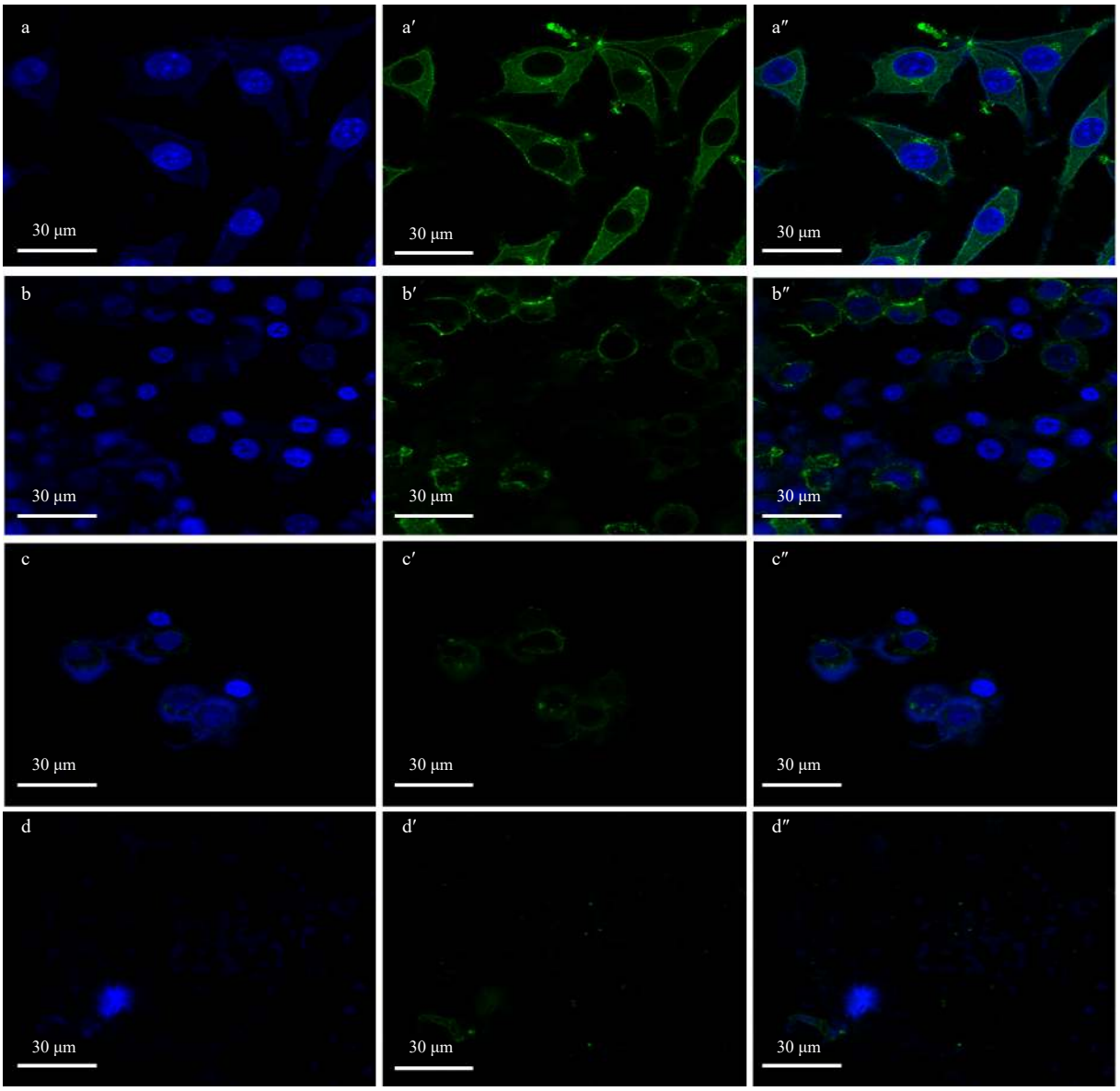

Fig. 6. Fluorescence observation damage effects of 60 μmol/L rLc-P5L on L929 cells. a. The blank group, L929 cells treated with PBS, and 60 μmol/L rEGFP-His-tag; b–d. L929 cells treated with 60 μmol/L rLc-P5L for 6 h (b), 12 h (c) and 24 h (d). Left panel showed that dyeing in Hoechst 33342; central panel in FITC-Phalloidin; right panel in merge.

condensed chromatin with strongly blue fluorescence, some chromatin condensed in the margin to form crescent-shaped hat structure, and some even emerged blue debris.

In addition, the green fluorescence marking F-actin indicated the cytoskeleton, they scattered and distributed throughout the cells to support its complete cellular morphology in the control groups. After treated with rLc-P5L, F-actin became disordered and polarized to make cells shrink to be clumps, which is a typical characteristic of cytoskeleton changes; the whole cells seemed thin and small, cytoskeleton structure was destroyed badly with shorter and more disordered microtubule bundles, and there were a lot of relatively small green debris in the microscopic field after 24 h treatment. Therefore, cells with different morphologies could be observed in the same microscopic field, which suggested rLc-P5L owned double active against cancer cells, and the damage appeared a time-dependent damage manner.

3.5.2 *rLc-P5L (60 μmol/L) treatment effects on nucleus and cytoskeleton of L929 cells*

Similar to above described, smooth nuclear edge and clear

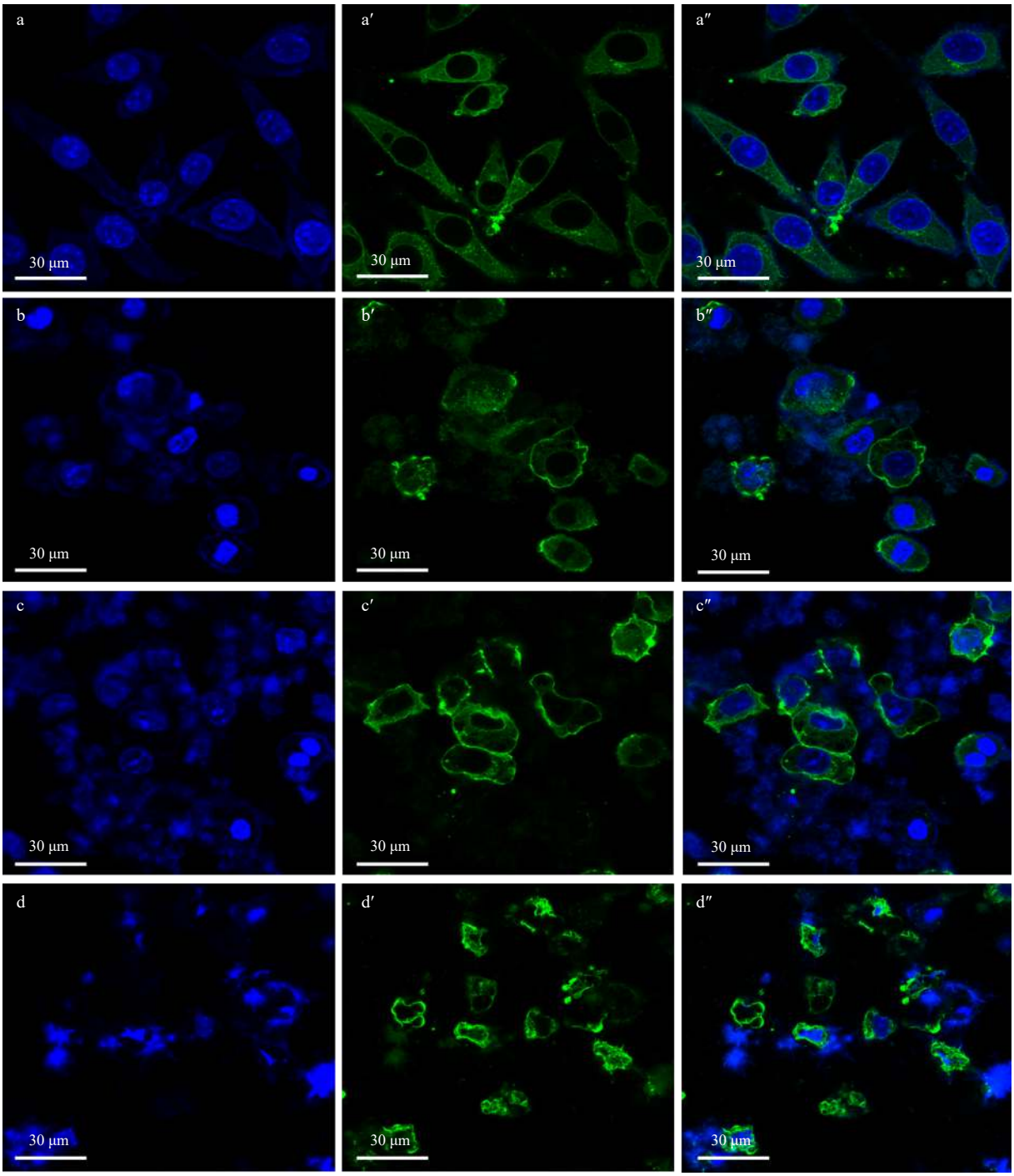

Fig. 7. Fluorescence observation damage effects of 80 μmol/L rLc-P5L on L929 cells. a. The blank group, L929 cells treated with PBS, and 80 μmol/L rEGFP-His-tag; b–d. L929 cells treated with 80 μmol/L rLc-P5L for 6 h (b), 12 h (c) and 24 h (d). Left panel showed that dyeing in Hoechst 33342; central panel in FITC-Phalloidin; right panel in merge.

boundary, tidy cytoskeleton and well-rounded cells were presented in the control groups. However, most nuclear chromatins condensed, nuclear atrophied and volume became smaller with irregular morphology after 80 μmol/L rLc-P5L treatment (Fig. 7) for 6 h; almost all nuclear chromatin appeared flocculent structure with no any nuclear morphology after treated for 12 h, and continuously, they were lysed to be debris after treated for 24 h.

Meanwhile, 80 μmol/L rLc-P5L treatment resulted in cytoskeleton appeared irregular outsets, and some of them have disappeared at different positions; after treated for 24 h, cytoskeleton presented rather atrophic shape, and some of them have been lysed to be debris.

4 Discussion

As to anticancer activity, studies have indicated some ACPs played multiple resistant roles: ACPs could mediate humoral immunity to prevent tumor cells invasion; ACPs could destroy cell, mitochondria or nuclear membranes to kill tumor cells through direct contact with tumor cells (Chen et al., 2005; Papo et al., 2006; Papo and Shai, 2005; Al-Hajj and Clarke, 2004); in addition, ACPs could also prevent tumor cells metastasis (Papo et al., 2006). In brief, ACPs play antitumor activity through cytolytic or other mechanisms, and the selectivity depends on the structure and components of membrane which ACPs will target to 26.

In the present study, we found that rLc-P5L was cytotoxic against several tumor cell strains *in vitro*. The morphologic effects of rLc-P5L on L929 cells were similar to A549 cells treated by Mere15 extracted from *Meretrix meretrix* (Wang, 2011). SEM or fluorescence observed rLc-P5L acted through a lytic mechanism involving in pores formation on L929 cells, and high concentration aggravated the damage extent. These results were in accordance with other findings, such as the members NRC-03 or NRC-07 of pleurocidin from *Paralichthys dentatus*, they killed breast cancer cells by lysing cell membranes (Hilchie et al., 2011); *Musca domestica* ACPs could lead to perforated damage of Eca109, K562 or T24 cell strains (Zhao et al., 2007). rLc-P5L incubation caused cytoskeleton disorder of L929 cells. Ceropin B, B1, B3 could also destroy cell microtubules to affect integrity of cytoskeleton and normal function (Chen et al., 2003). Zhao et al. (2000) observed that recombinant *Bombyx mori* AMP could result in microtubule system of HeLa cells shrinking to be irregular net structure. Now researchers believed that ACPs destroy cancer cells membrane structure through electrostatic or hydrophobic interaction. Because cancer cells carry higher negative charges which are more benefit for forming strongly electrostatic interaction making membranes more unstable (Schweizer, 2009; Shai, 1999; Hilchie et al., 2011). Secondly, a TUNEL assay observed DNA degradation could be caused by rLc-P5L. The recently reported salivary histatin 5 showed nuclease activity and binds/cleaves DNA (Melino et al., 2006).

In addition, we detected that rLc-P5L killed cancer cells by destroying the membranes and causing DNA degradation. Interestingly, it looked like that there were imperceptible changes, which hinted different deaths ways occurred under different concentration of rLc-P5L treatment? There were typical features of apoptosis, such as chromatin condensed in the margin, or vesicular bulge on the membrane (Doonan and Cotter, 2008; Wyllie, 1980; Zhu et al., 2006), there were also some certain characteristics of necrosis, such as DNA degradation (Zhang, 2009). However, the actually details about how it kills cancer cells are still not understood very well, the further studies needed to be carried out to determine its anticancer mechanisms. For example, Mere 15 of M. *meretrix* resulted in upregulation of the level of pro-apoptotic factor Bax, downregulation of the content of antiapoptotic factor Bcl-2, decrease of mitochondrial membrane potential and increase of Cytochrome *c* content, and it could also activate mitochondrial pathway (Wang, 2011).

In summary, we herein first discussed Lc-P5L is a kind of fish AMP owning anticancer activity to several cancer cell strains. Our studies clearly demonstrated that Lc-P5L was active against L929 cells through disrupting cell membrane, destroying cytoskeleton and degrading DNA. We believed that the crude Lc-P5L would be a candidate which might be modified to be a new peptide possessing stronger antitcancer activity at a relatively low concentration.

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