Article

Cathelicidin-DM is an Antimicrobial Peptide from *Duttaphrynus melanostictus* and Has Wound-Healing Therapeutic Potential

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ABSTRACT: Antimicrobial peptides (AMPs) are a class of templates with application potential for drug development. Amphibians are important sources of AMPs. *Duttaphrynus melanostictus* is the main source of traditional Chinese medicine "Chansu", which has anti-infection effect while without a clear mechanism. This study aimed to find the cathelicidin peptide in *D. melanostictus* and then investigate the activity *in vivo* and *in vitro*, and an AMP-encoding gene (cathelicidin-DM, GenBank: KJ820824.1) was obtained from the constructed cDNA library of *D. melanostictus*. The MIC test and SYTOX Green uptake were used for the evaluation of the bactericidal capacity and mechanisms. The serum stability tests were used for the evaluation of the application potential. The skin wound infection model and *in vivo* imaging were used for *in vitro* application of possibility evaluation. The results showed that cathelicidin-DM was a 37 amino acid AMP with good bactericidal ability, which was similar to melittin: both can kill bacteria within 15 min. Moreover, cathelicidin-DM exhibits good therapeutic potential in the mouse wound infection model, and it can be enriched to the site of infection for treatment. Thus, cathelicidin-DM could be a new template for antimicrobial drug development given its good antibacterial activity *in vivo* and *in vitro*.
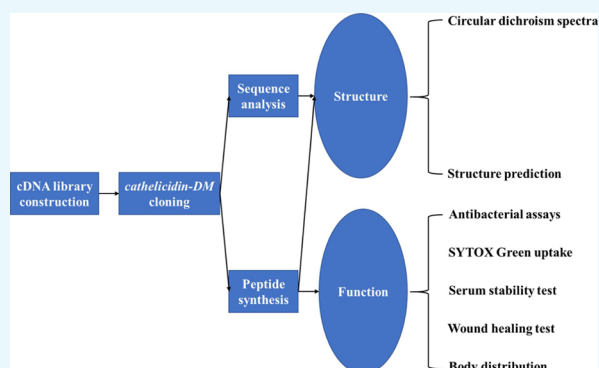
INTRODUCTION

Antibiotics are among the most important discoveries in the history of chemotherapeutic drugs and have greatly reduced mortality rates from infectious diseases.[1−3] However, the abuse of antibiotics has led to the outbreak of drug-resistant bacteria, especially multi-drug-resistant (MDR) and extensively drug-resistant (XDR) bacteria, which seriously threaten the life and health of human beings.[4−7] Thus, novel antibacterial substances that mitigate severe antibiotic resistance are urgently needed. The naturally occurring host defense peptides (HDPs) may be the novel antibacterial substances. HDPs, sometimes called antimicrobial peptides (AMPs) because of their ability to kill microorganisms, are produced by almost all kinds of organisms as components of the innate immune system.[8−10] Their key attributes are positive charge, hydrophobicity, and amphipathic structures linked to the antimicrobial potency of peptide candidates. Since the first cathelicidin AMP, Bac5, was discovered from bovine, a lot of cathelicidins AMPs have been identified from vertebrates, including fish, amphibians, birds, reptiles, and mammals.[8,11−14] AMPs possess a broad spectrum of antibacterial activities and exert strong inhibitory effects on most Gram-positive bacteria, Gram-negative bacteria, and fungi (including MDR and XDR microorganisms).[15−17] Other advantages of AMPs include anti-inflammatory and immunomodulatory activities, virulence

factor neutralization, and slow resistance development. Given these characteristics, AMPs exhibit great potential in the development of anti-infection drugs.

Amphibians have naked, scaleless, and hairless skin and live in wet conditions. Wet conditions are very suitable for microbial reproduction.[18,19] The anatomical characteristics and diverse living environments of amphibians render them capable of facing severe challenges from microorganisms.[20] More than 1400 kinds of AMPs have been found in the skin secretions of amphibians, such as *Paa yunnanensis*, *Limnonectes fragilis*, *Rana catesbeiana*, and *Tylototriton verrucosus*, which cover more than 600 species of frogs.[21−23] Bioactive substances protect amphibians from pathogenic microorganisms.[24−26] However, only few cathelicidin AMPs have been identified in the amphibians. Cathelicidins play critical roles in the innate immune system of most vertebrates and can provide the first line of defense against various infectious factors.[8] The precursors of cathelicidins comprise three parts, namely, N-

A ggattgcaccatcatccaggaa*tataaa*gaaaggaggggggtaatgagaagacatttcct
ctaagacagtccaagatgaggagctggaggctgtctctgctgctggtctctgcagtcaca

    M R S W R L S L L L V S A V T

ttacacggctgtctctctgaccctgcagagcctgaggtccaagatggaagatctatagaa

 L H G C L S D P A E P E V Q D G R S I E

gatgtcatcgacctctacaaccagagggagggggtcacatacttatataaatccctggac

 D V I D L Y N Q R E G V T Y L Y K S L D

cagctgccccctgttccaatggaggaggatgagaatccgaacagaagaggctttatcatg

 Q L P P V P M E E D E N P N R R G F I M

aaagagaccgtgtgcctcaaatccgagaatcctgatttaacccagtgtgatttcaagcct

 K E T V C L K S E N P D L T Q C D F K P

gacggagatgtgaagatctgttctctggatttggggatgaggatcctgaggatatcatg

 D G D V K I C S L D L G D E D P E D I M

tgcttcagtctgaacaaggaggtccgtatgaagcggtccagcagaaggaaaccatgcaag

 C F S L N K E V R M K R | S S R R K P C K

gggtggctctgcaagctgaagctaagaggaggttatactcttatcggcagtgctacaaac

 G W L C K L K L R G G Y T L I G S A T N

ctaaatagacctacctacgtgagggcataaagatctgtagtcgacggtggaaccagatct

 L N R P T Y V R A | ***

gagattgtggcttatgacgagcacattactgttatataaacctttattagttattattatga
ggcacgaggtggcacattgtgatccctcctcacttctcctgacctctaccatcttcactc
tggtccctgaaggagagatttcaggactttcagcttgctatgagatcagccatgttctgt
ctgtggaacgttctgaatggatacaatgttaca*aataaa*catagcaacaaataacattgt
gtggttacttcctggaaagtcatggtgaggatgatgaatgaagagggaaataattacttc
aattgcggat

B

Xenopus tropicalis **Cath-2**	MESWVTVRLLLTVGAIFNSLSLVQNISIPDKELTQKAADLYNEQENVAYIFRPAENHPER
Cathelicidin-DM	MRSWRLSLLLVSAVTLHGCLSDPAEPEVQDGRSIEDVIDLYNQREGVTYLYKSLDQLPPV
Limnonectes fragilis **Cath-1**	MKIWQCVLWL-SAVT--LARSQF-----LDQDGWIREALDLYNQREDGEYFFKVLSDVPAD
Rana catesbeiana **Cath-1**	MKIWQCVVWL-CAITLEVAHSQS-----PDREGWITEALDLYNQREDGEFLFKLLSELPAP
Rana catesbeiana **Cath-2**	MKIWQCVVWL-CAITLEVAHSQS-----PDREGWIREALDLYNQREDGEFLFKLLSELPGP
Limnonectes fragilis **Cath-2**	MKIWQCVLWL-STVT--WARSQS-----PDQDGWVREALDLYNQREDGEFYFKLLSDLPAA
Nanorana yunnanensis **Cath**	MKVWQCVLWI-SALTLQMARSQS-----PDQDGWIREALDLYNQREDGEFFFKFLSDLPDV
	*. * : . : . * .. ****::* : :: .: *

Xenopus tropicalis **Cath-2**	DLYLEENPPLVRFIIKETECLRAETNTDADCSFKPDGETKLCTAFISDQT-PVEVTCNPL
Cathelicidin-DM	PMEEDENPNRRGFIMKETVCLKSENPDLTQCDFKPDGDVKICSLDLGDED-PEDIMCFSL
Limnonectes fragilis **Cath-1**	LLQEGEDSPEVAFLIKETECLKSEDNELARCDYKNDGEVKACGLYLEEGEASGTLKCVSL
Rana catesbeiana **Cath-1**	LLEEEGDSPAIGFLIKETDCPKSEENDLEGCDYSKDGEVKVCALHREDE----DVKCVSL
Rana catesbeiana **Cath-2**	LLEEEGDSPAIGFLIKETDCPKSEEIDLERCDYSKDGEVKVCALHQEEQ----DVKCVSL
Limnonectes fragilis **Cath-2**	LLQEEGDSPAVGFLIKETECRKSEAGDPEQCDYRQDGEVKVCALYREEE----EVKCVSL
Nanorana yunnanensis **Cath**	LLEEEGDSPAIGFLIKETDCPKSEDCDLEKCDYRKDGEVKVCTLYREEE----DVKCVSL
	: : *::*** * ::* *.: **:.* * : : * *

Xenopus tropicalis **Cath-2**	PSEPQVNSKY-SRTKRSTKTKKCKTSGCRFTGAG------SAIAGVKPLQSIG-----
Cathelicidin-DM	NKE--------VRMKRSSRRKPCKGWLCKLKLRGGYTLIGSATNLNRPTYVRA-----
Limnonectes fragilis **Cath-1**	TKN--------PRMKRANRRPPCRGIFCRRV--------GSSSAIARPGKTLSTFITV
Rana catesbeiana **Cath-1**	TAE--------NSRSKRSSN-KKCG-FFCKLKNKL--KSTGSRSNIAAGTHG-GTFR-V
Rana catesbeiana **Cath-2**	TEN--------SRSKRSSKKKCK-FFCKVKKKI--KSIGFQIPIVSIPFK-------
Limnonectes fragilis **Cath-2**	TEN--------SRIRRANKKGKCN-VLCQLKQKL--RSIGSGSHIGSVVLP-RG----
Nanorana yunnanensis **Cath**	SKN--------SRTRRAGTKRKCN-FLCKLKEKL--RTVI-TSHIDKVLRP-QG----
	: * :* : * *:

Figure 1. Sequence analysis of cathelicidin-DM. (A) Nucleotide sequence encoding cathelicidin-DM and the deduced amino acid sequence. The putative TATA box, signal peptide, and polyadenylation signal are marked by italic, underline, and dashed underline, respectively. The mature peptide is boxed. The stop codon TAA is indicated by asterisks. (B) Alignment of cathelicidins identified in amphibians. The mature peptide of known amphibian cathelicidins is underlined. (*) Indicates positions which have a single, fully conserved residue. (:) Indicates conservation between groups of strongly similar properties. (.) Indicates conservation between groups of weakly similar properties.

terminal signal peptide (30 residues), highly conserved cathelin domain (99−114 residues), and C-terminal mature peptide (12−100 residues) with remarkable structural variety.[27] In 2011, the first amphibian cathelicidin AMP (cathelicidin-AL)
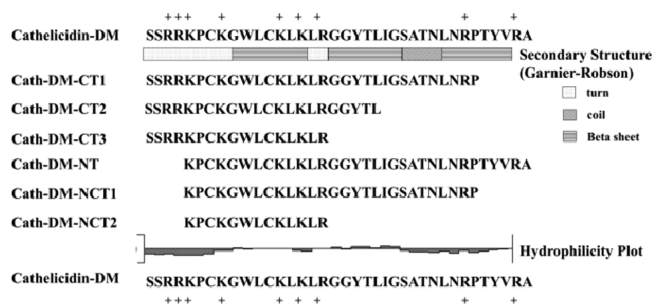
was identified from *Amolops loloensis*.[28] Several amphibian cathelicidin AMPs were then identified from different amphibians, including BG-CATH37 from *Bufo bufo gargarizans Cantor*,[16] cathelicidin-OA1 from *Odorrana andersonii*,[29] cathelicidin-NV from plateau frog *Nanorana ventripunctata*,[30] Lf-CATH1 from *L. fragilis*,[21] and Temporin-1Ta from the European red frog *Rana temporaria*.[31]

In 2018, WHO included traditional Chinese medicine in the International Classification of Diseases; this event brought great opportunities for the development of traditional Chinese medicine materials.[32] The traditional Chinese medicine material "Chansu" was a white serous substance secreted from the posterior ear and skin glands of Duttaphrynus melanostictus or *Bufo bufo gargarizans Cantor*; this substance has functions in detumescence and exerts analgesic, anti-inflammatory, and detoxification effects.[33−35] Thus far, studies on the active components of "Chansu" have focused on small molecular compounds, but the research on protein/polypeptide bioactive substances is not enough.[36,37] In 2010, researchers found that the skin secretions of *D. melanostictus* have strong bacteriostatic activity against Gram-negative and Gram-positive bacteria, although this activity may stop after trypsin treatment. Thus, the skin secretions of *D. melanostictus* may contain some peptides or proteins.[38]

In the present study, an AMP encoding gene (cathelicidin-DM) was isolated from the cDNA library of *D. melanostictus* dorsal skin. Then, the function of the synthesized cathelicidin-DM and the truncated peptides were determined.

■ RESULTS

Molecular Cloning and Characterization of Cathelicidin-DM. Total RNA was successfully extracted from the dorsal skin of *D. melanostictus* and synthesized to cDNA library. A cathelicidin-encoded cDNA was identified and designated as cathelicidin-DM. The bioinformatics analysis of cathelicidin-DM is shown in Figure 1. The complete nucleotide sequence of cathelicidin-DM (GenBank accession number KJ820824) and the deduced amino acid sequence are shown in Figure 1A. The open reading frame was composed of 164 amino acid residues (aa), including a predicted signal peptide, conserved cathelin domain, and variable cationic C-terminus.[27] The multisequence alignment of amphibian cathelicidins indicates that cathelicidin-DM is similar to other amphibian cathelicidins, including *Xenopus tropicalis* Cath-2, *L. fragilis* Cath-1 and Cath-2, *Rana catesbeiana* Cath-1 and Cath-2, and *Nanorana yunnanensis* Cath in the structure (Figure 1B). Elastase, which was generally considered to be responsible for the release of mature cathelicidins,[43,44] was not observed in amphibian cathelicidins. The precursors of amphibian AMPs often possess a typical dibasic cleavage site for trypsin-like proteases.[45] Moreover, a conserved dibasic cleavage site was found in all amphibian cathelicidins. The mature cathelicidin-DM was predicted as SSRRKPCKGWLCKLKLRGGYTLIG-SATNLNRPTYVRA by Translate Tool, which contains 37 amino acids including nine basic amino acids (Arg + Lys) and has no acidic amino acid (Asp + Glu). The theoretical molecular weight is 4.1659 kDa, and the isoelectric point is 11.05. The secondary structure of cathelicidin-DM was predicted at Predicprotein (http://www.predicprotein.org) (Figure 2A), and the 3D structure was predicted at the Quark server (http://zhanglab.ccmb.med.umich.edu/QUARK/)[12] (Figure 2B). The predicted structure of cathelicidin-DM contains three antiparallel β sheets combined

Figure 2. Predicted secondary structure and three-dimensional structure of cathelicidin-DM. (A) Predicted secondary structure of cathelicidin-DM and (B) de novo structure prediction of cathelicidin-DM. The model was produced by the QUARK server. Visualization of the structure was accomplished using RASMOL.

with the random coil (Figure 2B) and is consistent with the result of the CD spectra (Figure S1). The physicochemical properties of the peptide showed that cathelicidin-DM is a stable polypeptide.

Expression Profile of Cathelicidin-DM. The expression profiles of cathelicidin-DM in healthy *D. melanostictus* tissues were evaluated by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis (Figure 3). All

Figure 3. Tissue expression profile of cathelicidin-DM. The sample from lane 1−6 was skin, small intestine, large intestine, liver, ovarium, and spleen, respectively. *β*-Actin as the internal reference.

of the tested tissues expressed cathelicidin-DM genes, including the small intestine, large intestine, liver, spleen, ovary, and skin. Cathelicidin-DM was highly expressed in the spleen and large intestine and had low expression in the small intestine, ovary, liver, and skin.

Analysis of Antimicrobial Activity and SYTOX Green Uptake of Cathelicidin-DM. The antimicrobial activity of cathelicidin-DM and its derivatives (Cath-DM-CT1, Cath-DM-CT2, Cath-DM-CT3, Cath-DM-NT, Cath-DM-NCT1, and Cath-DM-NCT2; >95% purity; Figure 4) against the tested bacteria were determined as minimal inhibitory concentration (MIC). Cathelicidin-DM and its derivatives (except Cath-DM-NT and Cath-DM-NCT1) exhibited potent antimicrobial activity against common pathogens, including MDR and XDR clinical isolates (Table 1). The MIC of cathelicidin-DM against *Staphylococcus haemolyticus* (CI 1410970016), *Enterococcus faecalis* (MDR 14U0445), and *Staphylococcus aureus* ATCC25923 was 12 μg/mL. The MIC of

Cathelicidin-DM	+++ + + + + + +
Cathelicidin-DM	SSRRKPCKGWLCKLKLRGGYTLIGSATNLNRPTYVRA
Cath-DM-CT1	SSRRKPCKGWLCKLKLRGGYTLIGSATNLNRP
Cath-DM-CT2	SSRRKPCKGWLCKLKLRGGYTL
Cath-DM-CT3	SSRRKPCKGWLCKLKLR
Cath-DM-NT	KPCKGWLCKLKLRGGYTLIGSATNLNRPTYVRA
Cath-DM-NCT1	KPCKGWLCKLKLRGGYTLIGSATNLNRP
Cath-DM-NCT2	KPCKGWLCKLKLR

Secondary Structure (Garnier-Robson): □ turn, ▨ coil, ▨ Beta sheet

Hydrophilicity Plot

Cathelicidin-DM: SSRRKPCKGWLCKLKLRGGYTLIGSATNLNRPTYVRA
+++ +   + + +   +   +

Figure 4. Cathelicidin-DM and its truncated peptides.

cathelicidin-DM against *Escherichia coli* (MDR 13A10022), *E. coli* (MDR 13U1780), *E. coli* ATCC25922, and *Citrobacter freundii* (CI 1410UR0112) was 12 µg/mL, while that for *Staphylococcus paratyphoid A* (CI 1410UR0112), *Klebsiella pneumoniae* (MDR 13U1752), *K. pneumoniae* (MDR 13A11923), and *K. pneumoniae* (XDR 13A13361) was 6 µg/mL. All the derivatives except Cath-DM-NT and Cath-DM-NCT1 showed similar antimicrobial activity against partially tested strains, such as *S. haemolyticus* (CI 1410970016) and XDR *K. pneumoniae* but lost such activity against *E. faecalis* (MDR 14U0445) and two *E. coli* strains. The derivatives lost antimicrobial activity against the strains inhibited by cathelicidin-DM.

The action mode of cathelicidin-DM against the tested bacteria can be related to membrane permeabilization. This supposition was supported by the results of the SYTOX Green uptake experiment. SYTOX green is an excellent nuclear dye with green fluorescence, which can penetrate the fragile cell walls of dead cells. Thus, it is a useful indicator of dead cells. The bactericidal effect of cathelicidin-DM is consistent with that of melittin[44] (Figure 5), an AMP that can lyse cell membranes. The bactericidal rate of cathelicidin-DM is also similar to that of melittin, killing more than 60% of *E. faecalis* (ATCC29212) and *E. coli* (ATCC35218) within 10 min and 80% of *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) within 10 min. After mixing with a blood serum for 12 h, cathelicidin-DM lost its inhibitory activity against Gram-positive bacteria but retained its activity against Gram-negative bacteria (Table 1).

Therapeutic Effect of Cathelicidin-DM on Mouse Skin Infection Model. A mouse model of skin infection was constructed by daubing *E. coli* ATCC25922 (100 µL, 2×10^5 CFU/mL) in the 1 cm² wound. One hour after model construction, the mice in the three groups were intravenously injected with cathelicidin-DM (10 mg/kg), gentamycin (5 mg/kg), or PBS (pH 7.4) as the control for 5 days. The mice were photographed every 24 h. The wound in the mouse that received cathelicidin-DM (Figure 6) healed slightly faster than the wounds in the control and gentamycin groups. Two days after the administration of cathelicidin-DM, the wound-healing effect was significantly different.

Detection of Cathelicidin-DM Distribution in the Target Site. The hair was removed from two mice groups (skin infection and control groups, three female mice/group). After 1 h postinoculation of *E. coli* (ATCC25922), all the two groups received 10 mg/kg of FITC-labeled cathelicidin-DM by tail vein injection. Then, the mice were immediately anesthetized before they were transferred to the IVIS LT imaging system (IVIS spectrum) and measured at 0, 5, 10, 30, 60, 90, and 120 min. Significant fluorescence was observed in

Table 1. MICs of Cathelicidin-DM and Its Derivatives[a]

bacterial strains	cathelicidin-DM	cath-DM-CT1	cath-DM-CT2	cath-DM-CT3	cath-DM-NT	cath-DM-NCT1	cath-DM-NCT2	Amp	cathelicidin-DM after incubation in 50% serum
					MICs (µg/mL)				
Gram-Positive Bacteria									
S. haemolyticus (CI 1410970016)	12			3			3	3	
E. faecalis (MDR 14U0445)	12							2	
S. aureus ATCC25923	12	12	6	6				0.15	
Gram-Negative Bacteria									
E. coli ATCC25922	12							0.3	
E. coli (MDR 13U1780)	12	12	12					32	
E. coli (MDR 13A10022)	12							32	
S. paratyphi A (CI 1410BL0513)	6	6		12			6	32	6
C. freundii (CI 1410UR0112)	12	6		6			12		12
K. pneumoniae (MDR 13U1752)	6			6				32	6
K. pneumoniae (MDR 13A11923)	6							32	6
K. pneumoniae (XDR 13A13361)	6	6		3			6	32	6

[a]Note: CI, clinically isolated strain; MDR, multi-drug-resistant clinical isolate; XDR, extensive drug-resistant clinical isolate; (-) no detectable activity at a dose of 48 µg/mL.
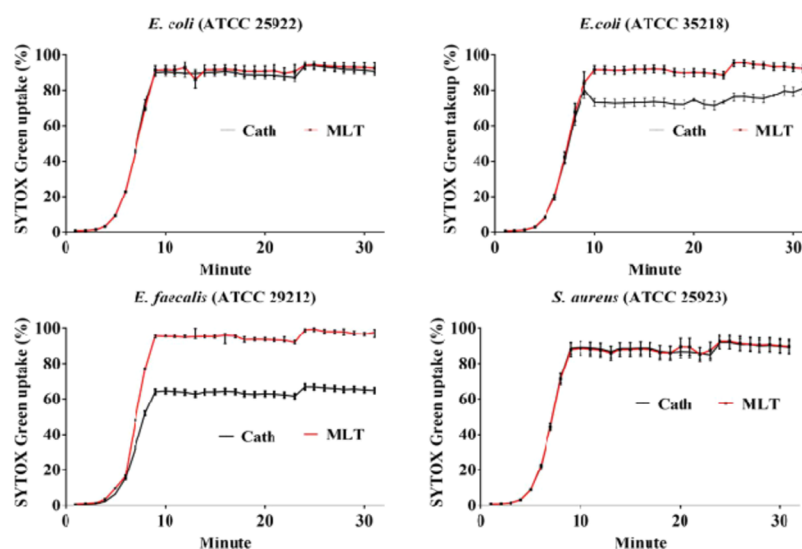
Figure 5. SYTOX green uptake of cathelicidin-DM and melittin. Four tested bacteria including *E. coli* (ATCC 25922), *E. coli* (ATCC 35218), *E. faecalis* (ATCC 29212), and *S. aureus* (ATCC 25923) were diluted to 2×10^5 CFU/mL. After the addition of peptides to the final concentrations corresponding to their respective MIC, they were mixed with a final concentration of 50 mM SYTOX Green (Invitrogen) for 15 min in the dark and finally detected in wavelengths 485 and 523 nm filters for excitation and emission.

Figure 6. Wound healing of mice after 5 days of continuous administration. All the three mice groups (three female mice/group) were administered with cathelicidin-DM (10 mg/kg), gentamycin (5 mg/kg), or PBS as control lasted for 5 days by tail vein injection. The mice were photographed every 24 h.
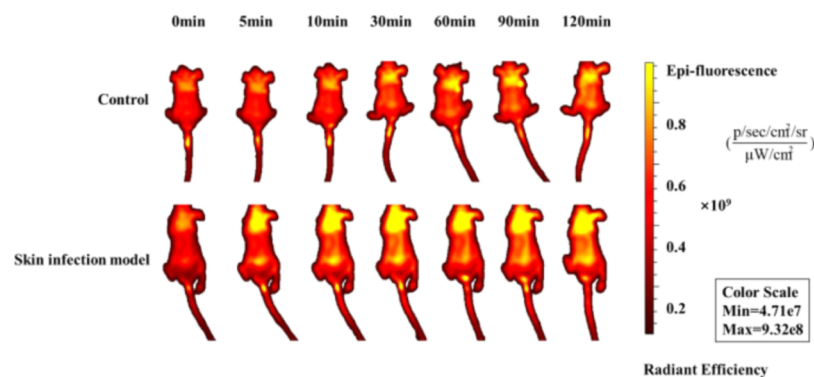
Figure 7. *In vivo* imaging of FITC-labeled cathelicidin-DM. All the two mice groups (three female mice/group) were administered with cathelicidin-DM (10 mg/kg) and gentamycin (5 mg/kg) as control lasted for 5 days by tail vein injection. The mice were photographed every 24 h.

different regions, such as the chest, abdomen, and brain (Figure 7). FITC-cathelicidin-DM was abundant in infected areas. Hence, cathelicidin-DM was distributed to different tissues and exhibited enrichment effect on bacterial infection sites.

■ DISCUSSION

The emergence of MDR and XDR pathogens is an urgent public problem to be solved all over the world, especially in intensive care units.[46] Given that many patients have serious illnesses and lacked access to effective treatment conditions,

Table 2. Primers Used in This Study

primer	sequence (5′–3′)	size (bp)
CDS III/3′ primer	ATTCTAGAGGCCGAGGCGGCCGACATGd(T)30N-1N-3′(N = A, C, G or T; N-1 = A, G or C)	27
SMARTTM IV oligonucleotide	AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG	39
CDS III/5′-primer	AAGCAGTGGTATCAACGCAGAGT	23
S1	CCYGCCACYSCAGARRTICA	20
S2	CAAGTNGTBGCHGGRDIVA	19
M13 F	CGCCAGGGTTTTCCCAGTCACGAC	24
M13 R	GAGCGGATAACAATTTCACACAGG	24
R2	GGACCTCCTTGTTCAGACTG	20
cath-F	GCCTGAGGTCCAAGATGGA	19
cath-R	GGCTTGAAATCACACTGGGTT	21
actin-F	AAATGGCTACAGCTGCTTCCT	21
actin-R	AAGGCTGGAAGAGGGCTTCT	21

mortality related to MDR and XDR pathogens is extremely high.[47] Thus, novel antimicrobial agents are urgently needed. The skin of amphibians is exposed and moist, and the mucus it secretes contains many antibacterial substances with special molecular structures and complex functions.[48] AMPs are defensive peptides produced by the immune defense system to resist the pathogenic effects of exogenous pathogens. AMPs contain 10−50 amino acid residues, most of which are rich in basic amino acids, such as lysine and arginine, and have positive charges as a whole. Many studies showed that the main mechanism of AMPs is to destroy membrane integrity. AMPs interfere with some important cellular processes and thus play a bactericidal role. Given that AMPs mainly target cell membrane structures, AMPs result in a generally low drug resistance and thus considered substitutes and synergists for antibiotics and used in addressing increasingly serious problem of bacterial drug resistance. AMPs affect the pathogens of some cash crops and are expected to become human or animal antimicrobial agents. *D. melanostictus* is an important traditional Chinese medicine in China, and its "Chansu" has important medicinal value.[49] However, *D. melanostictus*, which is widely distributed in Yunnan, Guizhou, Sichuan province, has not yet attracted enough attention. Therefore, we attempted to screen cDNA-encoding AMPs from the cDNA library of the *D. melanostictus* skin. A cathelicidin AMP was identified and named cathelicidin-DM, which is the first identified cathelicidin AMP from *D. melanostictus*.[50−52]

According to the RT-PCR analysis results, the expression pattern of cathelicidin-DM is similar to the expression patterns of other amphibian cathelicidins.[21−23,28] Notably, the expression of cathelicidin-DM in the skin is not high compared with that in other tissues. This finding suggests that cathelicidin-DM has other important functions in *D. melanostictus* in addition to its direct bactericidal effect. Especially, the high expression in the large intestine and kidney indicates that cathelicidin-DM may play an important role in some processes of digestion and processes in the urinary and endocrine systems.

Although the C-terminal mature peptide region of cathelicidin-DM significantly differed from those of other amphibian cathelicidins, it still possesses potent antibacterial activities. Six truncated peptides were designed from cathelicidin-DM according to its charge, hydrophilicity, and hydrophobicity for the evaluation of the potential of cathelicidin-DM as a template for the development of novel peptide antibiotics. Cathelicidin-DM has strong antibacterial activity against Gram-positive strains and Gram-negative

strains (including clinically isolated MDR and XDR strains). After cathelicidin-DM mixed with serum for 12 h, it lost its antibacterial activity in some tested strains. The human serum is rich in a variety of proteases, such as thrombin and matrix metalloproteinases, which hydrolyze cathelicidin-DM. Whether or not affected by serum, cathelicidin-DM showed good activity against clinically isolated *K. pneumoniae* (MDR 13U1752), *K. pneumoniae* (MDR 13A11923), and *K. pneumoniae* (XDR 13A13361).

In the mouse models of skin infection, cathelicidin-DM showed stronger effect than gentamicin and significantly accelerated the healing of the wound. Using a small animal imaging system, we intuitively observed that cathelicidin-DM was distributed in many places in the body, especially in the brain and heart. In the case of trauma, enrichment was observed. This event promoted the wound-healing ability of cathelicidin-DM. Moreover, cathelicidin-DM can enter the blood−brain barrier into the brain, as shown in the *in vivo* imaging of FITC-labeled cathelicidin-DM. Hence, cathelicidin-DM is a potential drug carrier that targets the brain in the future.

■ CONCLUSIONS

Cathelicidin-DM was identified in the present work by molecular cloning from *D. melanostictus* and was used to treat skin wound infections. Cathelicidin-DM possesses potent antibacterial activities against many pathogens including MDR and XDR strains. It has a good therapeutic effect in the wound infection model. All the characteristics suggest that cathelicidin-DM from traditional Chinese medicine "Chansu" may be an ideal template peptide antibiotic in the development of novel antimicrobial agents.

■ EXPERIMENTAL SECTION

Ethical Approval. This study was carried out in accordance with the recommendations of the Animal Care and Utilization Committee of the Kunming University of Science and Technology, which also approved the study protocol.

cDNAs Synthesis and Cathelicidin-DM Cloning. Adult *D. melanostictus* (collected from Xishuangbanna, Yunnan Province, China) was housed (at 25 °C with a 12/12 h light/dark cycle and access to food and water ad libitum), maintained, and cared for in the Animal Experiment Center of Kunming University of Science and Technology. The cDNA library of the dorsal skin was constructed according to the instructions of SMARTer cDNA library construction kit

(Clontech, USA), which was then used in the screening of cDNAs encoding cathelicidin-DM. The first strand was synthesized by CDS III/3′ primer and SMARTTM IV oligonucleotide. The CDS III/5′ primer provided by the kit was used in the synthesis of the second strand. Two oligonucleotide primers, S1 and S2, in the sense of direction were designed according to the conserved sequences of cathelicidins from other amphibians. Nested PCR was performed with CDS III/3′ primer in the antisense direction. The PCR conditions were as follows: 1 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C, and final extension at 72 °C for 10 min. The PCR products of the second round were amplified by the primer S2 and the CDS III/3′ primer, inserted into the pMD 19-T simple vector, and transformed into $DH5\alpha$ competent cells. Sequencing primers M13F and M13R were used for positive clone selection. Another primer, R2, was designed according to the sequenced nucleotide sequence above and combined with the CDS III/5′ primer to amplify the 5′ ends of the cDNA. Sequencing was performed again as described above, and the cathelicidin-DM CDS sequence was finally obtained, which was used for the designing of specific primers (cath-F/R). All the primers used in this study are shown in Table 2.

Sequence Analysis. The cathelicidin-DM nucleotide sequence was analyzed by BLAST and Translate Tool (http://web.expasy.org/translate/) for the deduction of amino acid sequences.[39] Multiple sequence alignment was performed using Clustal Omega (https://predictprotein.org/) and then manually curated.[40] Secondary structure was predicted at Predictprotein (http://www.predicprotein.org), and 3D structure was predicted at the Quark server (http://zhanglab.ccmb.med.umich.edu/QUARK/).[41]

Expression Profile Analysis by RT-PCR Assay. The RT-PCR assay was performed to analyze the gene expression of cathelicidin-DM in different tissues of *D. melanostictus*, including the skin, spleen, liver, large intestine, small intestine, and ovary. After the tissues were removed, they were immediately placed in liquid nitrogen, and the total RNA extraction of each tissue and organ was performed with Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen, USA), which was used as the template for PCR amplification with specific primers cath-F/R of cathelicidin-DM. The total RNA was used as the template, and oligo dT(18) as a primer. The β-actin-specific primer actin-F/R was designed on the basis of the β-actin gene in the GenBank database, which was used as the RT-PCR internal reference (Table 2).

Peptide Synthesis. Cathelicidin-DM, FITC-labeled cathelicidin-DM, and six truncated cathelicidin-DMs (Cath-DM-CT1, Cath-DM-CT2, Cath-DM-CT3, Cath-DM-NT, Cath-DM-NCT1, and Cath-DM-NCT2) based on the deduced amino acid sequence are synthesized by the peptide synthesizer (APEX396, AAPPTec) in Phtd peptides Co., Ltd. (Zhengzhou, China) and used for bioassays. All the six peptides were purified with C18 RP-high-performance liquid chromatography (HPLC) column and 0.1% (v/v) trifluoroacetic acid/acetonitrile as an elution solution with a linear gradient from 30 to 100% acetonitrile in 20 min. The purity of the final product was higher than 95% [the HPLC and mass spectrometry (MS) report of the synthesized peptides were supplied in the Supporting Information]. The peptide was

dissolved in sterilized ultrapure water and stored at −20 °C with a storage concentration of 2 mg/mL.

Antibacterial Assays. The synthetic peptides were tested for MICs against strains, including *S. haemolyticus* (CI 1410970016), *E. coli* (MDR 13A10022), *E. coli* (MDR 13U1780), *E. coli* ATCC25922, *Salmonella Paratyphi A* (CI 1410BL0513), *E. faecalis* (MDR 14U0445), *C. freundii* (CI 1410UR0112), *K. pneumoniae* (MDR 13U1752), *K. pneumoniae* (MDR 13A11923), *K. pneumoniae* (XDR 13A13361), and *S. aureus* (ATCC25923), as shown in Table 1 (all the tested bacteria were provided by the Clinical Laboratory of First People's Hospital of Yunnan Province. The drug resistance information of the tested clinical strains is shown in Table S1). The strains were inoculated on a Luria−Bertani (LB) solid medium and cultured in a 37 °C incubator. The single colony was selected and cultured in the LB liquid medium in a 37 °C incubator to logarithmic growth phase. Then, the concentration of bacterial solution was detected with an ultraviolet spectrophotometer. According to 1 OD600 = 1×10^9 CFU/mL, the bacterial solution was diluted to 2×10^5 CFU/mL with an LB liquid medium. Then, 100 μL of the LB liquid medium was added to each well of aseptic 96-well plate, and then, 100 μL of the AMP solution (192 μg/mL) was added to the first well. After mixing, we transferred 100 μL of the solution from the first well to the second well to perform serial dilutions. The diluted bacterial solution (100 μL) was added to each well and mixed and cultured at 37 °C for 16 h. The absorbance at 600 nm was determined. Each peptide was repeated three times. The MIC was defined as the lowest peptide concentration that fully inhibited the bacterial growth.

SYTOX Green Uptake. Four tested bacterial strains (50 μL, 2×10^5 CFU/mL), namely, *E. coli* (ATCC25922), *E. coli* (ATCC35218), *E. faecalis* (ATCC29212), and *S. aureus* (ATCC25923), were cultured as previously described and then centrifuged. The supernatant was discarded, and the precipitate was washed with aseptic PBS three times and resuspended with 100 μL of aseptic PBS (the tested bacteria were provided by the Clinical Laboratory of First People's Hospital of Yunnan Province). After the addition of the peptides to the final concentrations corresponding to their respective MIC, the suspension was mixed with the SYTOX Green (Invitrogen, USA) to a final concentration of 50 mM and static for 15 min in the dark. The fluorescence intensity was monitored continuously in the Multiskan Sky Microplate Spectrophotometer (ThermoFisher Scientific, USA) for 30 min at 37 °C with 485 and 523 nm wavelength filters for excitation and emission. Melittin was used as the positive control.[42] The strongest fluorescence intensity was used as the denominator, and the others were used as numerators for the evaluation of bactericidal effect.

Serum Stability Test. The MIC method was used for serum stability test. Each blood serum (collected from the healthy volunteers and provided by the Clinical Laboratory of First People's Hospital of Yunnan Province) was mixed with cathelicidin-DM to a final concentration of $2 \times$ MIC. The mixture was cultured at 37 °C for 12 h. The tested strains included Gram-positive and Gram-negative bacteria, which were the same as those used in the MIC assay.

Application of Cathelicidin-DM in a Mouse Skin Infection Model. Gentamicin is an aminoglycoside antibiotic, which is mainly used to treat bacterial infections, especially those caused by Gram-negative bacteria. Gentamicin can bind to the 30 s subunit of the bacterial ribosome and block the

synthesis of bacterial proteins. Moreover, gentamicin is one of the few thermostable antibiotics, so it is widely used in the treatment. Thus, gentamicin was used in this study as the positive control. An overnight culture of *E. coli* ATCC25922 in LB liquid medium was subcultured in a fresh LB liquid medium to log phase, and then, the bacterial solution was centrifuged at 3000g for 10 min. The precipitate was washed three times with aseptic PBS buffer and then resuspended with aseptic PBS to a final concentration of 2×10^5 CFU/mL. Nine 6 week old female Kunming mice (weighing 27−30 g) were obtained from the Laboratory Animal Center of Kunming Medical University (Kunming, Yunnan) and housed (at 25 °C with a 12/12 h light/dark cycle and access to food and water ad libitum), maintained, and cared for in the Animal Experiment Center of Kunming University of Science and Technology. The mice were randomly divided into three groups (three mice/group). Hair at the back of each mouse was carefully shaved, and then, the back skin was scrubbed with warm water, dried, and wiped with alcohol. A 1 cm² wound was generated with an incision using a surgical knife aseptically. Then, 100 μL 2×10^5 CFU/mL of *E. coli* (ATCC25922) liquid was daubed in the wound. All the mice were intravenously injected with cathelicidin-DM (10 mg/kg), gentamycin (5 mg/kg), or PBS as control 1 h postinoculation. The same dose was administered daily for 5 days. The wound-healing rate of the mice was measured every day.

In Vivo Imaging of FITC-Labeled Cathelicidin-DM. Six 6 week old female Kunming mice (weighing 27−30 g) were randomly divided into two groups (skin infection and control groups) for the evaluation of the tissue distribution of intravenously injected FITC-cathelicidin-DM. The hair of each mouse was removed, and the skin infection group was constructed as previously described. The control group had normal growth. One-hour postinoculation of *E. coli*, the two groups received 10 mg/kg of FITC-labeled cathelicidin-DM through tail vein injection. The mice were immediately anesthetized before transferring to the IVIS LT imaging system (IVIS spectrum) and then measured at 0, 5, 10, 30, 60, 90, and 120 min. The IVIS Lumina LT imaging system showed a region with strong yellow luminescence and low red luminescence. Increasing bioluminescence indicates elevated FITC-labeled cathelicidin-DM concentration. The images were an overlay of photographic images and bioluminescence generated by a computer-generated color scale.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00189.

> Circular dichroism spectra and drug resistance information of the tested clinical strains, and HPLC and MS report of the synthesized peptides (PDF)

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Y.Q.S. and C.L. are contributed equally to this work. Y.Q.S. and C.L. performed the experiments and data analysis. M.W., Z.J.C., and Y.L. assisted in collection and molecular cloning; and X.S.X., Y.Z.S., A.Z., and Y.S. conceived and designed the study. Y.Z.S., A.Z., and Y.S. conceived and supervised the project and prepared the manuscript. All authors read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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