Peptides 33 (2012) 18–26

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Toxicity study of antimicrobial peptides from wild bee venom and their analogs toward mammalian normal and cancer cells

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ARTICLE INFO

Article history:
Received 29 August 2011
Received in revised form 2 November 2011
Accepted 2 November 2011
Available online 7 November 2011

Keywords:
Antimicrobial peptides
Venom
Hymenoptera
Cancer cells
Toxicity
Confocal microscopy

ABSTRACT

Recently, we have isolated and characterized remarkable antimicrobial peptides (AMPs) from the venom reservoirs of wild bees. These peptides (melectin, lasioglossins, halictines and macropin) and their analogs display high antimicrobial activity against Gram-positive and -negative bacteria, antifungal activity and low or moderate hemolytic activity. Here we describe cytotoxicity of the above-mentioned AMPs and some of their analogs toward two normal cell lines (human umbilical vein endothelial cells, HUVEC, and rat intestinal epithelial cells, IEC) and three cancer cell lines (HeLa S3, CRC SW 480 and CCRF-CEM T). HeLa S3 cells were the most sensitive ones (concentration causing 50% cell death in the case of the most toxic analogs was 2.5–10 μM) followed by CEM cells. For the other cell lines to be killed, the concentrations had to be four to twenty times higher. These results bring promising outlooks of finding medically applicable drugs on the basis of AMPs. Experiments using fluorescently labeled lasioglossin III (Fl-VNWKKILGKIIKVVK-NH_2) as a tracer confirmed that the peptides entered the mammalian cells in higher quantities only after they reached the toxic concentration. After entering the cells, their concentration was the highest in the vicinity of the nucleus, in the nucleolus and in granules which were situated at very similar places as mitochondria. Experiments performed using cells with tetramethylrhodamine labeled mitochondria showed that mitochondria were fragmented and lost their membrane potential in parallel with the entrance of the peptides into the cell and the disturbance of the cell membrane.

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1. Introduction

Venoms of arthropods are a rich source of biologically active compounds, antimicrobial peptides included. New antimicrobial peptides melectin [3], lasioglossins [4], halictines [13] and macropin [14] (for structure and MW, see Table 1) have recently been isolated from the venom reservoirs of wild bees collected in the Czech Republic. These peptides have 12–18 amino acids in the peptide chain and belong to cationic α-helical amphipatic peptides. After identification and structure determination they were prepared synthetically to have enough material for further studies. Their antimicrobial potencies were described in the above-mentioned Refs. [3,4,13,14] along with a structure–activity study using their synthetic analogs. They were found to be microbiocidal and to act on both Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria. The effect of selected compounds was also tested against five types of yeast cells (*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei*) [16]; the compounds were fungicidal and the effect occurred like in the case of bacteria within minutes as has been described for this type of compounds [2]. The peptides showed low or moderate hemolytic activity.

In the literature, also the anticancer activities of some antimicrobial peptides have been described. The Wang database [19] contains about 109 entries of antimicrobial peptides with anticancer activities. Heinen and daVeiga [10] have reviewed the effect of arthropod venom peptides on cancer cells. Fadnes et al. [6] have stressed the fact that the effect of AMPs is unaltered by the multidrug resistance mechanism and by the heparan sulfate on the surface of cancer cells. Membranolytic and non-membranolytic mechanisms of action were proposed for different AMPs [17].

Abbreviations: AMP, antimicrobial peptide; CEM, CCRF-CEM T lymphoblastoid (human acute lymphoblastic leukemia) cells; SW480, human colon adenocarcinoma; HeLa S3, human cervix carcinoma; HUVEC, human umbilical vein endothelial cells; IEC, rat intestinal epithelial cells; HEP G2, human liver hepatocellular carcinoma cells; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; Fl, 5,6-carboxyfluorescein; MEP, melectin; LL, lasioglossin; HAL, halictin; MAC, macropine; MIC, minimal inhibitory concentration; Trit, Triton X-100; TMRE, tetramethylrhodamine.

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doi:10.1016/j.peptides.2011.11.002

Table 1
The structure and molecular weight of natural studied peptides.

peptide	Sequence	M.W.
MEP	H-Gly-Phe-Leu-Ser-Ile-Leu-Lys-Lys-Val-Leu-Pro-Lys-Val-Met-Ala-His-Met-Lys-NH_2	2038.23
LL-I	H-Val-Asn-Trp-Lys-Lys-Val-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2	1722.14
LL-II	H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2	1736.16
LL-III[a]	H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Val-Lys-NH_2	1764.19
HAL-1	H-Gly-Met-Trp-Ser-Lys-Ile-Leu-Gly-His-Leu-Ile-Arg-NH_2	1408.8
HAL-2	H-Gly-Lys-Trp-Met-Ser-Leu-Leu-Lys-His-Ile-Leu-Lys-NH_2	1451.9
MAC-1	H-Gly-Phe-Gly-Met-Ala-Leu-Lys-Leu-Leu-Lys-Lys-Val-Leu-NH_2	1416.0

[a] This analog was also prepared fluorescently labeled as described in the text.

In this paper, we describe the toxicity of three groups of peptides, lasioglossins, halictines and macropins [3,4,13,14] toward four sessile mammalian cell lines and one planktonic one. Two of the cell lines were normal cell lines (HUVEC and IEC) and three were cancer cell lines (HeLa S3, CRC SW480 and CEM). The localization of the peptides within the cells was studied using fluorescent microscopy. The aim of the work was to determine whether the compounds are safe enough to be used as antimicrobial agents as well as whether they have any potential to be used as anticancer drugs.

2. Materials and methods

The tetracycline, clotrimazole, fluconazole, amphotericin B, p-nitrophenyl-N-acetyl-β-D-glucosaminidine, Triton X-100 (Trit), and fluorescent stains propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich and the 5(6)-carboxyfluorescein (Fl) from NovaBiochem. Tetramethylrhodamine (TMRE) was acquired from Invitrogen. The RPMI 1640 medium was from the PAA laboratories, Austria. All of the other reagents were of the highest purity available from commercial sources.

The cell lines used were as follows: CCRF-CEM T lymphoblastoid (human acute lymphoblastic leukemia, CCL 119), human colon adenocarcinoma SW480 (CCL-228), human cervix carcinoma (HeLa S3) and normal rat intestinal epithelial cells (IEC-6, CRL-1592) from ATCC (Manassas, VA, USA), HUVEC (human umbilical vein endothelial cells) from Millipore, Prague, HEP G2 cells were obtained from the European Culture Collection (ECACC85011430).

2.1. Peptide synthesis

The peptides used in this study were prepared by the solid-phase method as described in the literature [3,4,13,14,16]. Briefly, the synthesis was performed in 5 ml polypropylene syringes with a bottom Teflon filter using the Nα-Fmoc chemistry protocol on a Rink Amide MBHA resin (100 mg) with a 0.7 mmol/g substitution. The protected amino acids were coupled in four-fold excess in DMF as the solvent and DIPC (7 equiv.)/HOBt (5 equiv.) as coupling reagents. The peptides were deprotected and cleaved from the resin with a mixture of TFA/H_2O/TIS (95:2.5:2.5) or TFA/EDT/thioanisol/H_2O/TIS (90:2.5:3:2.5:2) in the case of methionine-containing peptides for 3.5 h and precipitated with tert-butyl methyl ether. The crude peptides were purified by preparative RP-HPLC using a Vydac C-18 column (250 mm × 10 mm) at a 3.0 ml/min flow rate. Their identity was checked by MS and their purity by analytical HPLC (>97%). The fluorescein-labeled peptide Fl-LL-III (see Table 1) was prepared by the labeling of resin-bound LL-III on its N-terminus

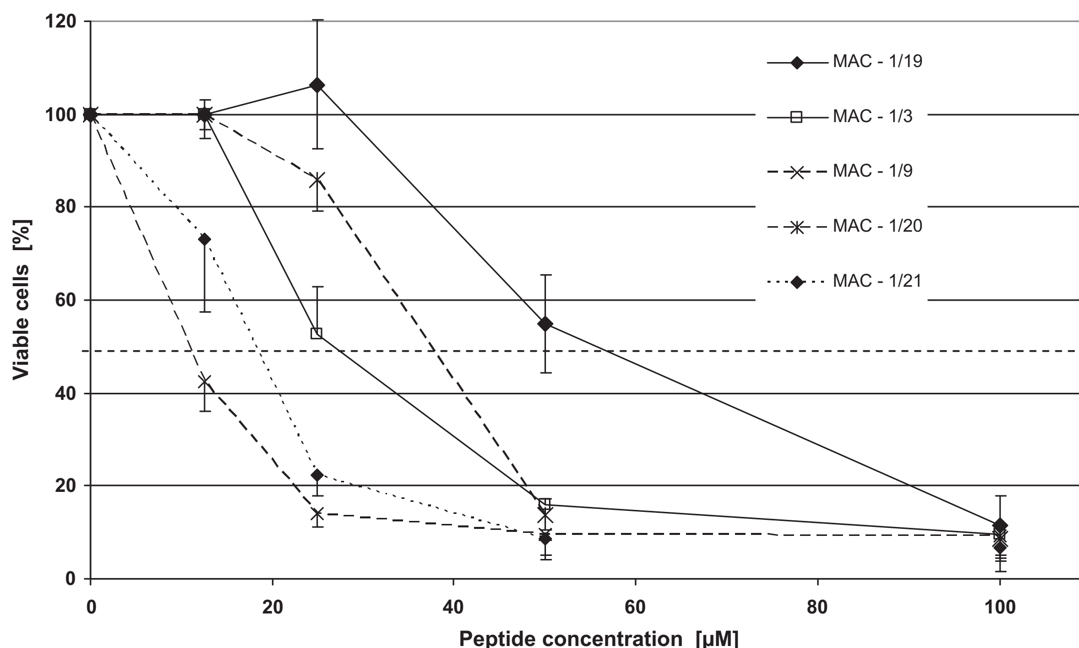

Fig. 1. An example of a graph from a typical toxicity experiment. The dependence of the viable cell quantity (CEM cells) expressed in percentage on the concentration of peptide in the incubation mixture. The concentration of peptide causing a 50% reduction in cell viability is taken as IC_{50}. Each point is an average of triplicate determination. Most peptides were tested in 2–5 such experiments.

Table 2
The toxicity of lasioglossin III and its analogs (average ± SD).

	Sequence[a]	Toxicity[b], IC_{50} [μM]					Hemolysis[c] LC_{50} [μM] rat erythrocytes
		HUVEC	IEC	HeLa	SW	CEM	
LL-III[d]	VNWKKILGKIIKVVK-NH$_2$	15 ± 4	19 ± 1	4 ± 1	18 ± 4	5 ± 3	>200
LL-III/1	VNWKKIL**A**KIIKVVK-NH$_2$	10 ± 2	18 ± 5	3 ± 1	12 ± 3	3 ± 1	>200
LL-III/2	**NV**WKKILGKIIKVVK-NH$_2$	n.t.	18 ±	n.t.[e]	11	7 ± 2	>200
LL-III/3	VNWKKIL**K**KIIKVVK-NH$_2$	20 ± 1	22 ± 4	n.t.	>40	n.t.	>200
LL-III/4	VNWKKILGKI**K**KVVK-NH$_2$	>80	>100	7 ± 1	>40	18	>200
LL-III/6	VNWKKIL**P**KIIKVVK-NH$_2$	>100	>100	>40	n.t.	6 ± 3	>200
LL-III/8	VNWKKILGKIIKVVK-**OH**	80±	>100	12 ± 1	n.t.	9	>200
LL-III/9	VNWKKILGKIIKVVK-**OMe**	30 ± 2	18 ± 3	3 ± 1	25 ± 5	n.t.	79.3
LL-III/10	**K**NWKKILGKIIKVVK-NH$_2$	35 ± 11	≥80	9 ± 4	n.t.	9	>200
LL-III/11	VNWKK**I**ILGKIIKVVKNH$_2$	50±	≥80	8 ± 1	48	4 ± 1	>200
LL-III/12	*VNWKKILGKIIKVVK-NH$_2$* (all-D-LL-III)	17 ± 3	17 ± 7	6 ± 2	20	5 ± 2	>200
LL-III/15	VN**F**KK**L**LGK**LL**KVVK-NH$_2$	≥70	≥100	7 ± 1	42	9 ± 3	>200
LL-III/16	VN-**Nal**-KKLLGKLLKVVK-NH$_2$	15 ± 1	18 ± 1	6 ± 1	n.t.	n.t.	71.3
LL-III/17	VNW**RR**ILG**R**II**R**VV**R**-NH$_2$	11 ± 4	18 ± 1	9	n.t.	n.t.	39.9
LL-III/18	**K**NWKKILKKIIKVVK-NH$_2$	22 ± 8	22 ± 5	3	n.t.	7 ± 2	>200
LL-III/19	**V**NWKK-**Aib**-LGK-**Aib**-IK-**Aib**-VK-NH$_2$	38 ± 6	57 ± 8	3 ± 1	28	8 ± 2	>200
LL-III/22	**K**NWKK-**Aib**-LKK-**Aib**-IK-**Aib**-VK-NH$_2$	>100	>100	11 ± 7	>100	25 ± 4	>200
LL-III/23	VNWKK**L**LGK**LL**KVVK-NH$_2$	12 ± 3	15 ± 6	4	15	6	71.5
LL-III/24	VNW**OO**ILG**O**II**O**VV**O**-NH$_2$	20 ± 4	25 ± 10	3 ± 1	20	5 ± 2	>200
LL-III/25	*VNWKK**L**LGK**LL**KVVK-NH$_2$* (all-D-LL-III/23)	12 ± 3	13 ± 4	4 ± 2	20	4	120
LL-III/26	V**Y**WKKILGKIIKVVK-NH$_2$	18 ± 1	24 ± 11	5 ± 1	30	6	190
LL-III/27	VNWKK**V**LGK**VV**KVVK-NH$_2$	>100	>100	13 ± 2	>100	18	>200
LL-III/34	**N**KWKKILGKIIKVVK-NH$_2$	52 ± 7	40 ± 5	5 ± 2	37	5 ± 3	>200
LL-III/36	VNWKKIL**A**KIIKVVK-NH$_2$	n.t.	23 ± 4	8	n.t.	n.t.	>200
LL-III/37	VNWKK**V**L**A**KIIKVVK-NH$_2$	18 ± 2	17 ± 3	5 ± 1	13	4 ± 1	>200

[a] Amino acid replacements are in bold; D-amino acids are in italics; O is ornithine; Aib is 1-amino-isobutyric acid; Nal is 3-(1-naphthyl)alanine.
[b] The values without SD were determined only once in triplicate.
[c] LC_{50} means lytic concentration, i.e. concentration at which 50% of erythrocytes in the assay are lysed.
[d] IC_{50} value for HEP G2 cells amounts to 28.5 μM.
[e] n.t. means not tested.

by 5(6)-carboxyfluorescein using DIPC/HOBt coupling reagents as described by others [21].

2.2. Toxicity

2.2.1. Cell incubation

The cells were grown at 37 °C and in a 5% CO_2 atmosphere in the appropriate growth media (IEC-6 cells in DMEM, SW 480, HeLa S3 and CCRF-CEM T cells in RPMI 1640, and HUVEC cells in EndoGro medium) supplemented (with the exception of the HUVEC cells) with inactivated fetal bovine serum (iFBS), glutamine and penicillin/streptomycin solutions and in the case of the IEC-6 cells also with an insulin solution. For the experiments, the sessile cells were detached from the bottom of the vessels using a trypsin solution, centrifuged and diluted to a concentration of 25,000 cells per ml. The cells were seeded into 96-well plates at 5000 cells per well in 0.2 ml of media and incubated under standard conditions for 40–48 h. Subsequently, all the media were removed and 0.1 ml of fresh media with different concentrations (0.1–100 μM) of peptides was added to the cells (final peptide concentration

Table 3
The toxicity of macropins and their analogs (average ± SD).

	Sequence[a]	Toxicity[b], IC_{50} [μM]					Hemolysis[c] LC_{50} [μM] human erythrocytes
		HUVEC	IEC	HeLa	SW	CEM	
MAC1	GFGMALKLLKKVL-NH$_2$	28 ± 6	27 ± 7	10 ± 4	23 ± 4	12 ± 6	165
MAC2	GTGLPMSERRKIMLMMR-NH$_2$	>100	>100	>100	>100	32	>200
MAC1/1	*GFGMALKLLKKVL*-NH$_2$ (all-D-Mac-1)	21 ± 5	25 ± 4	12 ± 4	19 ± 2	11	152
MAC1/2	**A**FGMALKLLKKVL-NH$_2$	30 ± 4	29 ± 7	16 ± 3	16	17	>200
MAC1/3	**L**FGMALKLLKKVL-NH$_2$	27 ± 5	20 ± 5	9 ± 2	11 ± 1	22	53
MAC1/4	GFGM***A***LKLLKKVL-NH$_2$	56 ± 2	72 ± 5	15 ± 5	49 ± 9	29	>200
MAC1/6	G***F***GMALKLLKKVL-NH$_2$	21 ± 5	28 ± 2	8 ± 2	29 ± 3	15	177
MAC1/9	GF**K**MALKLLKKVL-NH$_2$	17 ± 2	19 ± 3	8 ± 1	13 ± 3	14 ± 4	199
MAC1/10	GF***K***MALKLLKKVL-NH$_2$	23	32 ± 4	15 ± 6	n.t.[d]	16	>200
MAC1/16	GFGMALKL***L***KKVL-NH$_2$	87 ± 3	>100	32 ± 2	≥100	70	>200
MAC1/19	GFG***M***ALKLLKKVL-NH$_2$	52 ± 15	75	21 ± 4	68 ± 2	43 ± 11	>200
MAC1/20	GFGMAL**O**LL**OO**VL-NH$_2$	33 ± 3	43 ± 5	10 ± 1	35 ± 4	25 ± 3	>200
MAC1/21	GFGMAL**R**LL**RR**VL-NH$_2$	17 ± 2	23 ± 6	9 ± 3	17 ± 2	22 ± 4	72
MAC1/24	GFGMALKL(**AC$_6$C**)KKVL-NH$_2$	15 ± 3	20 ± 2	8 ± 2	16 ± 1	11	88
MAC1/25	GFGMALK(**AC$_6$C**)LKKVL-NH$_2$	13 ± 2	19 ± 1	7 ± 1	14 ± 1	12 ± 2	88
MAC1/26	GFGMA(**AC$_6$C**)KLLKKVL-NH$_2$	16	19 ± 2	8 ± 2	18 ± 1	9 ± 2	93

[a] Amino acid replacements are in bold; D-amino acids are in italics; O is ornithine; AC_6C is cyclohexyl-1-carboxylic acid.
[b] The values without SD were determined only once in triplicate.
[c] LC_{50} means lytic concentration, i.e. concentration at which 50% of erythrocytes in the assay are lysed.
[d] n.t. means not tested.

Table 4
The toxicity of halictines and their analogs (average ± SD).

	Sequence[a]	Toxicity[b], IC_{50} [μM]					Hemolysis[c] LC_{50} [μM] rat erythrocytes
		HUVEC	IEC	HeLa	SW	CEM	
HAL-1	GMWSKILGHLIR-NH_2	48 ± 19	38 ± 2	11 ± 2	44 ± 8	49 ± 9	82
HAL-1/22	*GMWSKILGHLIR*-NH_2 (all-D-HAL-1)	42 ± 7	40 ± 10	15 ± 3	≥40	n.t.[d]	52
HAL-1/6	GMWSKILGHLI**K**-NH_2	≥40	>100	15 ± 1	>100	n.t.	132
HAL-1/4	GMWSKILGHL**K**R-NH_2	n.t.	>100	>100	n.t.	n.t.	>200
HAL-1/9	GMWSKILG**K**LIR-NH_2	≥40	≥50	9 ± 1	>50	30 ± 10	143
HAL-1/18	GMWSKIL**K**HLIR-NH_2	20 ± 4	30 ± 12	4 ± 2	18 ± 2	n.t.	45
HAL-1/5	GMW**K**KILGHLIR-NH_2	49 ± 16	≥40	9 ± 1	44 ± 5	>40	93
HAL-1/12	G**K**WSKILGHLIR-NH_2	>100	>100	43 ± 14	>100	>40	>200
HAL-1/17	**K**MWSKILGHLIR-NH_2	>100	85 ± 4	35 ± 3	>100	n.t.	>200
HAL-1/10	GMW**K**KILG**K**LIR-NH_2	63 ± 13	>100	13 ± 2	>60	n.t.	>200
HAL-1/20	G**K**WSKILG**K**LIR-NH_2	>100	>100	22 ± 3	>40	>40	>200
HAL-1/19	G**K**W**K**KILGHLIR-NH_2	>100	n.t.	>100	>100	n.t.	>200
HAL-1/21	G**K**W**K**KILG**K**LIR-NH_2	>100	>100	30 ± 3	>100	n.t.	>200
HAL-1/29	GMWS***K***ILGHLIR-NH_2	>100	n.t.	>100	n.t.	n.t.	>200
HAL-1/15	GMWSK**L**LGHL**L**R-NH_2	26 ± 6	37 ± 7	14 ± 2	>40	>40	62
HAL-2	GKWMSLLKHILK-NH_2	23 ± 9	40 ± 5	12 ± 1	35 ± 3	34	78.1
HAL-2/22	*GKWMSLLKHILK*-NH_2 (all-D-HAL-2)	22 ± 7	40	12	>100	n.t.	54
HAL-2/4	GKWMSLLK**K**ILK-NH_2	>100	n.t.	>40	38 ± 6	n.t.	126
HAL-2/2	GKWM**K**LLKHILK-NH_2	40 ± 4	36 ± 4	6 ± 1	35 ± 5	>40	87.4
HAL-2/1	GKW**K**SLLKHILK-NH_2	>100	>100	14 ± 2	>100	>40	>200
HAL-2/8	GK***W***MSLLKHILK-NH_2	52 ± 3	≥40	15 ± 3	>40	n.t.	99
HAL-2/18	GKWMSLLKHI**W**K-NH_2	56 ± 9	≥40	14 ± 2	>40	n.t.	87
HAL-2/19	GKWMSLLKH**W**LK-NH_2	40 ± 1	78 ± 10	16 ± 4	>40	n.t.	106
HAL-2/20	GKWMSL**W**KHILK-NH_2	73 ± 3	≥100	30 ± 5	>40	n.t.	>100
HAL-2/24	GK**F**MSLLKHILK-NH_2	74 ± 10	n.t.	34	>40	n.t.	>200
HAL-2/6	GKWMS**F**LKHILK-NH_2	55 ± 9	35 ± 10	23 ± 3	>40	n.t.	69
HAL-2/13	GKWM**T**LLKHILK-NH_2	60 ± 12	56 ± 13	13 ± 3	>60	n.t.	86
HAL-2/11	GKW**L**SLLKHILK-NH_2	42 ± 3	40	14 ± 3	40	n.t.	65

[a] Amino acid replacements are in bold; D-amino acids are in italics.
[b] The values without SD were determined only once in triplicate.
[c] LC_{50} means lytic concentration, i.e. concentration at which 50% of erythrocytes in the assay are lysed.
[d] n.t. means not tested.

0.1–100 μM). After a 90-min incubation of the cells with the peptides, the quantity of viable cells was determined using the MTT method. The incubation time used for routine testing (90 min) was determined in preliminary experiments, when the cells were incubated with the peptides for 10 min, 30 min, 90 min, 2 h, 6 h, 24 h and 48 h and the IC_{50} value determined remained the same.

In the case of floating cells, the cells were incubated for 40–48 h in 96-well plates (5000 cells in 0.2 ml/well) and after this time the peptides were added directly to the cells in the wells without the change of the medium in a 20 μl volume (final concentration 0.1–100 μM). After further 24 h incubation an XTT test was performed to determine the percentage of viable cells. Wells without cells and wells with cells but no peptide added served as 0 and 100% of the viable cell number, respectively.

2.2.2. MTT test

After incubation with peptides as described above, 10 μl of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (5 mg/ml in physiological solution) were added to the cells in the wells and the plates were incubated for a further 30–120 min. The medium was then carefully removed and the generated blue formazan crystals were dissolved in 200 μl of DMSO. The absorbance in the wells was determined on a TECAN spectrophotometer (Switzerland) at 540 nm. Zero viability and 100% viability were determined by the absorbance of DMSO in the wells without cells and with cells incubated without peptides, respectively.

2.2.3. XTT test

After incubation with peptides as described in Section 2.2.1, 50 μl of the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) reagent (mixture of 1 mg/ml of XTT and 0.05 mM of N-methyl dibenzopyrazine methyl sulfate in a physiological solution prepared immediately before the test) were added to the cells in the wells and the plates were incubated for a further 30–180 min. After this time, the absorbance in the wells was determined at 450/650 nm. Wells without cells and wells with cells but no peptide added served as the 0 and 100% of the viable cells, respectively.

2.2.4. The IC_{50} determination

The IC_{50} value, i.e. concentration of peptide leading to the 50% decrease of viable cell number, was established graphically from the dependence of the quantity of life cells in per cent on the peptide concentration, see Fig. 1. Routinely, 5–6 compounds in 4 different concentrations in triplicates were tested on one 96-well plate. The values given in Tables 2–4 are averages from 2 to 5 independent experiments performed in triplicates ± SD with the exception of some analogs and SW480 and CEM cells, see the tables.

2.3. Uptake of the fluorescein-labeled analog Fl-LL-III

The cells grown for 48 h on cover glasses in twenty-four well plates (seeded 1 ml of a cell suspension of 25,000 cells/ml) were incubated with Fl-LL-III (1–2 μM) for different time intervals in the absence or presence of an unlabeled peptide LL-III or MAC-1 (5–100 μM). After the incubation, the media were removed from the wells, the cells on the cover glasses were washed by PBS (1 ml) and then fixed by a formaldehyde solution (20 min). Subsequently, the cells were washed twice by PBS and the cover glasses lifted out and attached to microscope glasses by a mowiol solution (4 μl) containing DAPI.

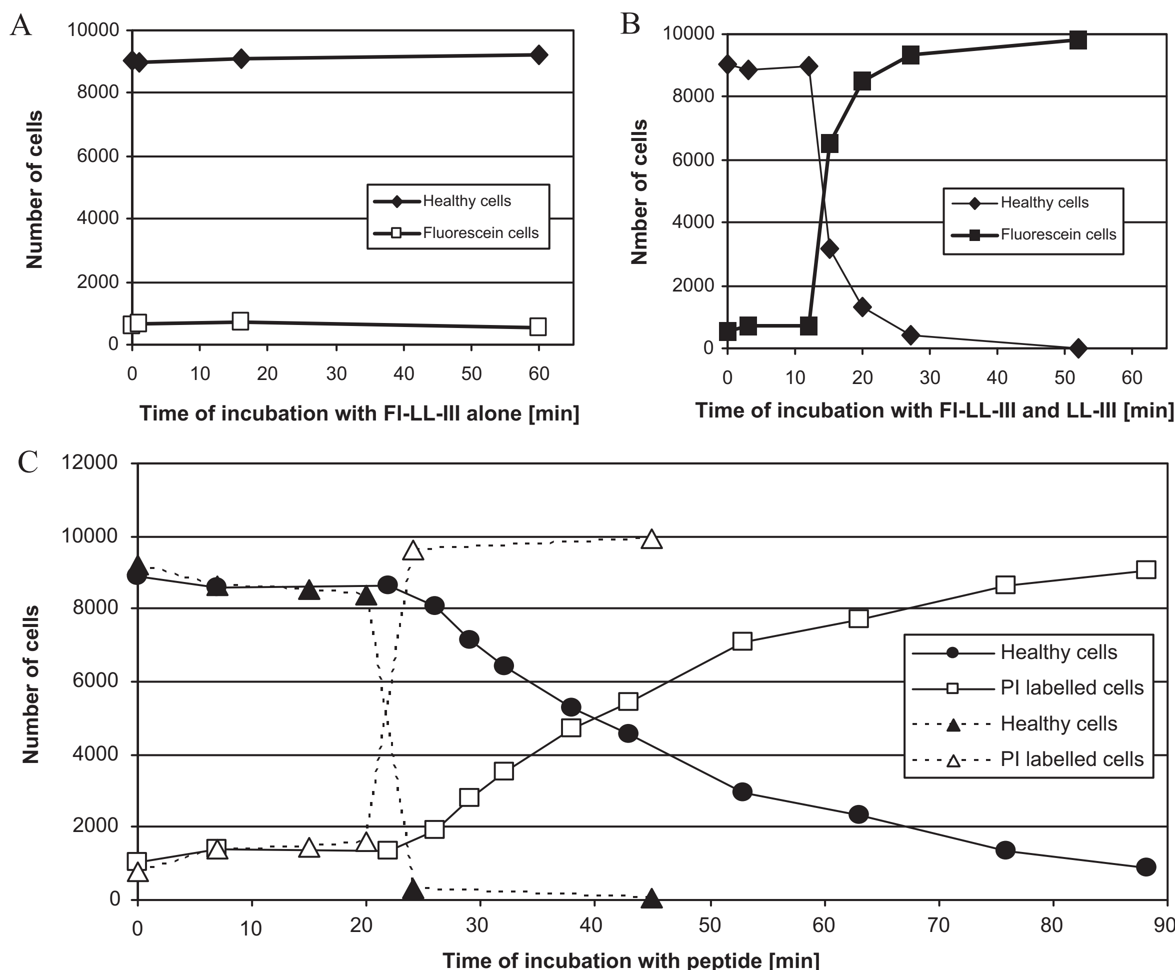

Fig. 2. The effect of LL-III on CEM cells determined by flow cytometry. The effect of a 60-min incubation of CEM cells with 1 μM of Fl-LL-III alone (A) and 1 μM of Fl-LL-III with 20 μM LL-III added at min 12 (B). The effect of 5 μM (●) and 20 μM (▲) concentration of LL-III on the entrance of PI into CEM cells (C). The peptide was added to cells at min 22.

2.4. Uptake of tetramethylrhodamine

The cells were grown for 48 h on cover glasses in 6-well plates. Before the experiments, the cover glass was carefully removed and placed into the sample chamber of the confocal microscope. In the sample chamber, 1 ml of 20 nM TMRE solution was added and the uptake followed. Roughly after 1 min the TMRE solution was removed and changed for fresh medium.

2.5. Fluorescent microscopy of fixed cells

The fixed cells (see Sections 2.3 and 2.4) were examined using the Olympus IX81 microscope (Olympus C&S s.r.o., Prague, Czech Republic). In the case of carboxyfluorescein, TMRE, and DAPI, the excitation/emission filters 460-490/505/510 nm (U-MWIB2), 510-550/570/590 nm (U-MWG2), and 330-385/400/420 (U-MWU2), respectively, were used.

2.6. In vivo confocal fluorescent microscopy

Cells in *in vivo* were examined using a Leica SP2 AOBS confocal inverted fluorescent microscope with a PL APO 100×/1.40–0.70 oil immersion objective (a pinhole of 1 Airy unit) in a termostated sample chamber and preserving atmosphere having 5% CO_2 to mimic cultivation conditions. After the incubation of the HEP G2 cells with tetramethylrhodamine (TMRE, 20 nM) the medium was changed and the cells were incubated with Fl-LL-III (1 μM) or LL-III (5–40 μM) alone or with a mixture of Fl-LL-III and LL-III. The pictures were taken after the addition of the peptides usually for ten to sixty subsequent minutes for every 12 s.

2.7. Flow cytometry

The cells in suspension (CEM, 10^6 per ml in the incubation medium) were incubated with Fl-LL-III (1–2 μM) or PI (0.5 μg/ml) for different time intervals in the absence or presence of different concentrations of unlabeled peptide LL-III (5–40 μM). At different intervals, the fluorescence of the cells and their shape were monitored in the flow cytometer (BD FACSAria II, BD Biosciences, NJ, USA).

3. Results and discussion

3.1. Toxicity

The values of the toxicity of the three groups of compounds – lasioglossins, halictines and macropines – for the five cell lines tested are summarized in Tables 2–4. A typical example of the graphs from which the IC_{50} values were determined is given in Fig. 1. For toxicity determination, such analogs were chosen whose antimicrobial potency was high, i.e. the MIC for *B. subtilis*, *S. aureus* and *P. aeruginosa* was lower than 2, 15 and 50 μM, respectively. For

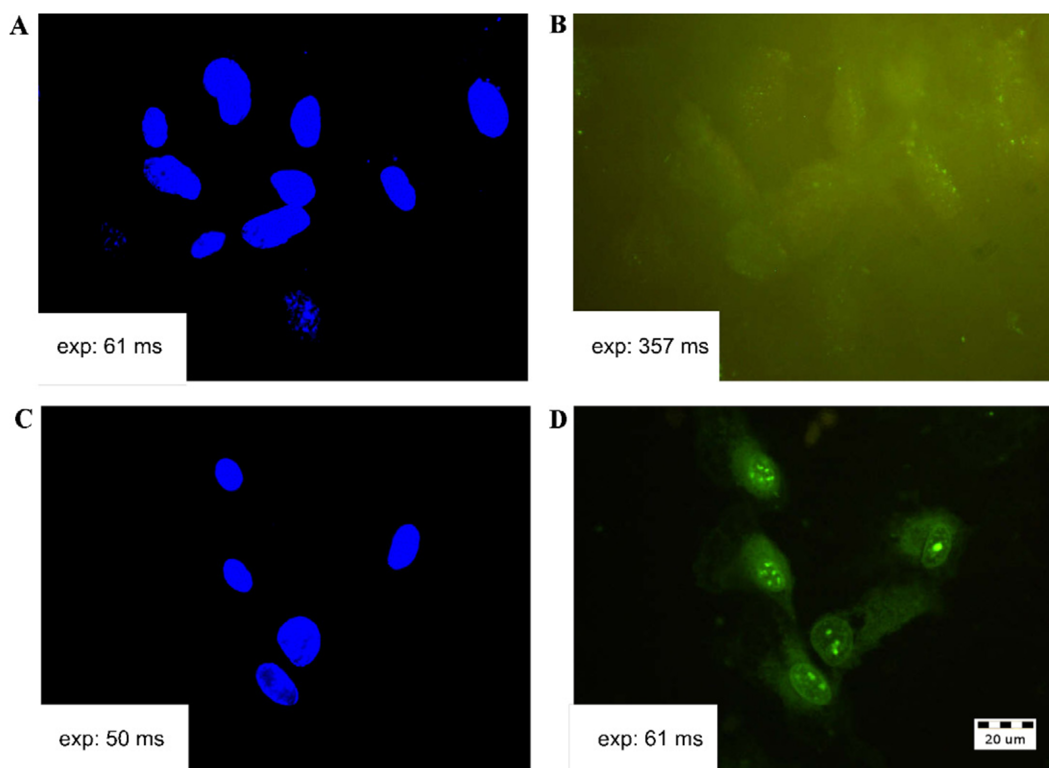

Fig. 3. A picture of HUVEC cells incubated with 2 μM of Fl-LL-III alone (A and B) and with a mixture of 2 μM Fl-LL-III and 20 μM of LL-III (C and D). The cells were incubated with the peptides for 90 min and fixed as described in Section 2. The accumulation of the Fluorescein fluorescence in the cytosol with an especially high density in the nucleoli can be seen in (D). (A) Nuclei colored by DAPI, exposition 61 ms (in UV light); (B) fluorescein fluorescence, exposition 357 ms (in blue light). (C) Nuclei colored by DAPI, exposition 50 ms (in UV light); (D) fluorescein fluorescence, exposition 61 ms (in blue light). The scale bar in the images represents 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

testing toxicity against CEM cells, mostly only those analogs that exhibited low hemolytic activity were chosen, i.e. those whose LC_{50} (concentration causing the lysis of 50% erythrocytes in the test) was higher than 100 μM [3,4,13,14]. The toxicity determination toward the normal mammalian cells showed that the concentration which was able to kill half of the normal cells was always higher than the concentration which was detrimental to the bacteria. Selected compounds (e.g. LL-III or LL-III/12) might thus be promising to become antimicrobial drugs and should be further tested, as only an *in vivo* experiment would confirm the safety. Anyway, in *in vivo* experiments with candidiasis in mice [11], the lasioglossin III showed to be active and non-toxic.

As can be seen from the Tables, the peptides were the most toxic for the HeLa S3 cells followed by CEM cells. The toxicity of the natural compounds and their analogs toward these sensitive cancer cells was generally about 3–5 times higher than toward the cells of the normal lines. The effect may be called semi-selective for cancer cells [8]. The toxic concentration of the most active compounds was lower than 5 μM (IC_{50}), which is comparable to or lower than that described in the literature for the peptides from arthropod venom or other peptides [5–7,9,10,12,17,19]. There are, however, also exceptionally selective compounds among our analogs, e.g. LL-III/19 or LL-III/24. As can be seen in Table 2, toxicity of these two compounds was ten times and six times higher for HeLa S3 and CEM cancer cells, respectively, than for the normal cells. From the three groups of compounds, those derived from lasioglossin-III [4] showed the best parameters. The reason why especially the HeLa S3 cells are the most sensitive to the peptides is not clear however we speculate that the HeLa S3 cells might have slightly different composition of cell membrane and different membrane potential than the other cells and also the majority of the cells in the culture might be in such cell cycle which is more sensitive to the peptides. Thus this is the matter for further study. As far as the difference in toxicity between the normal, non-cancer, cells and cancer cells is concerned, generally higher anionicity and lower rigidity of the cell membrane is described in literature for cancer cells. These parameters are believed to influence the interaction of antimicrobial peptides with the cell membrane.

3.2. Mechanism of action

The speed of entering the cells and killing was studied using different methods and different fluorescently labeled substances in all the cell types for which the cytotoxicity was determined and in addition by means of confocal fluorescent microscopy *in vivo* with the HEP G2 cells which are often used for the study of mitochondria [15]. Experiments with all types of cells used point to the same fact that the peptides enter the cell in higher quantities and quickly only after they reach a concentration close to IC_{50} and higher and that they disturb the cell membrane and the membrane systems in the cell.

We have found using flow cytometry that if the cells were incubated with low concentrations of LL-III (1–2 μM) in the presence of Fl-LL-III (1–2 μM), there was neither an enhancement of the fluorescence adsorbed to the cell membrane, nor an entrance into the cell (Fig. 2A). After further addition of LL-III to concentration which reached the IC_{50} concentration or higher, the entrance of the peptide into the cell was quick and massive (see Fig. 2B). The cells were permeabilized as was demonstrated by the entrance of PI into the cells (Fig. 2C) when similar experiments were performed in the presence of PI instead of Fl-LL-III. PI dye generally does not penetrate healthy cells with an undisturbed cell membrane, but if the cell membrane is disturbed, it enters the cells. Flow cytometry has recently been used by other authors also for the differentiation

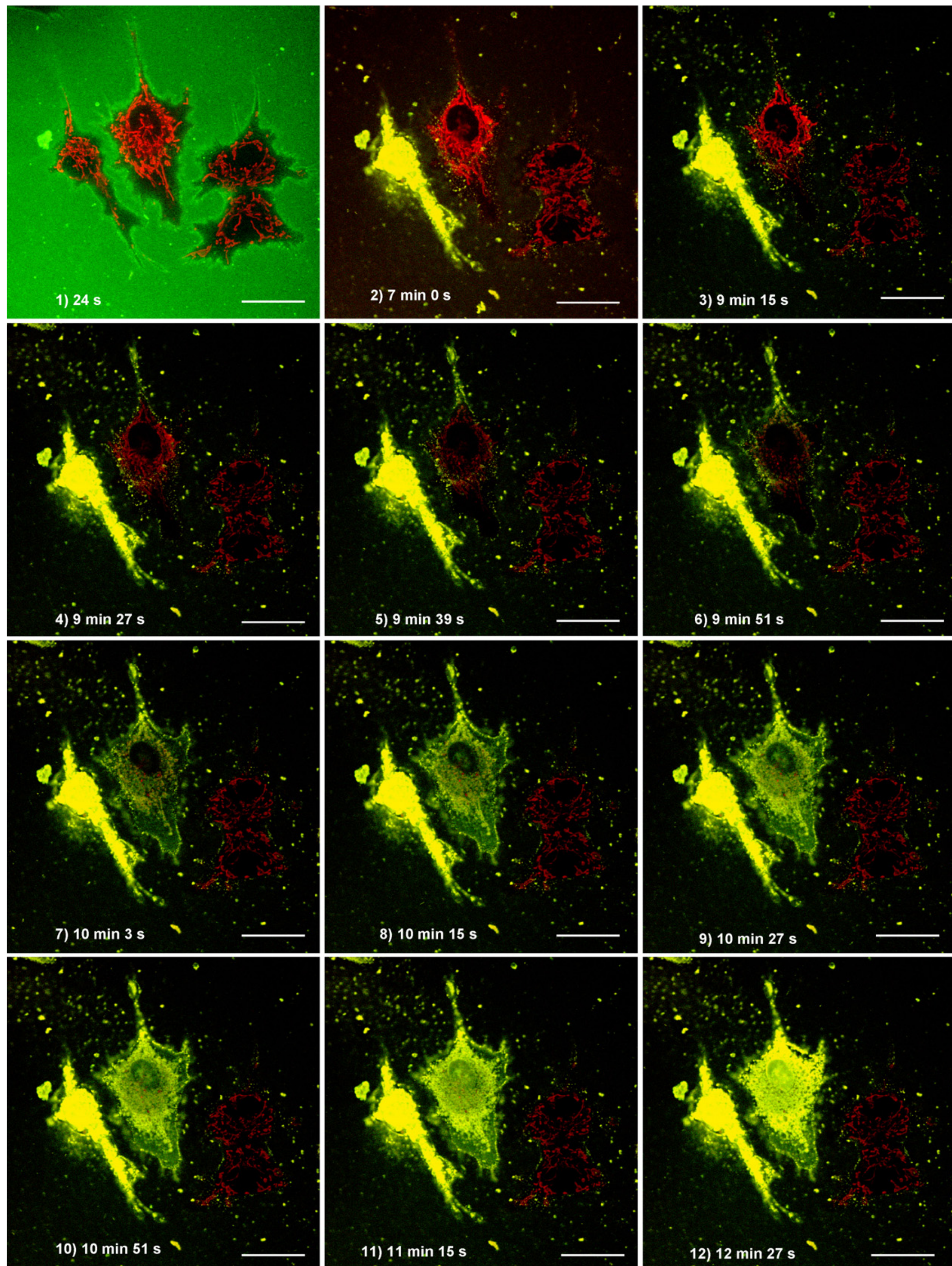

Fig. 4. A series of pictures illustrating the entrance of Fl-LL-III into the Hep G2 cells in time. Green color – Fl-LL-III (the very high concentration of Fl-LL-III is however yellow), red color TMRE containing mitochondria. At time 0, 1 μM of Fl-LL-III was added, at time 5 min, 30 μM of LL-III were added. Our focus was the central cell. Mitochondrial function was impaired shortly before the massive entrance of Fl-LL-III occurred. The time which passed between the first entrance of the peptide into the cell and the moment when the cell was full of fluorescence was approximately 5 min. The peptides did not enter the cells in the cell culture at the same time. Meanwhile the left cell was already full of Fl-LL-III, the central cell started to be attacked by the peptide. However after some 20 min from the moment of addition of the LL-III, all cells were yellow as the left and the central cells, i.e. they were full of the AMP (not shown). The scale bar in the images represents 30 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

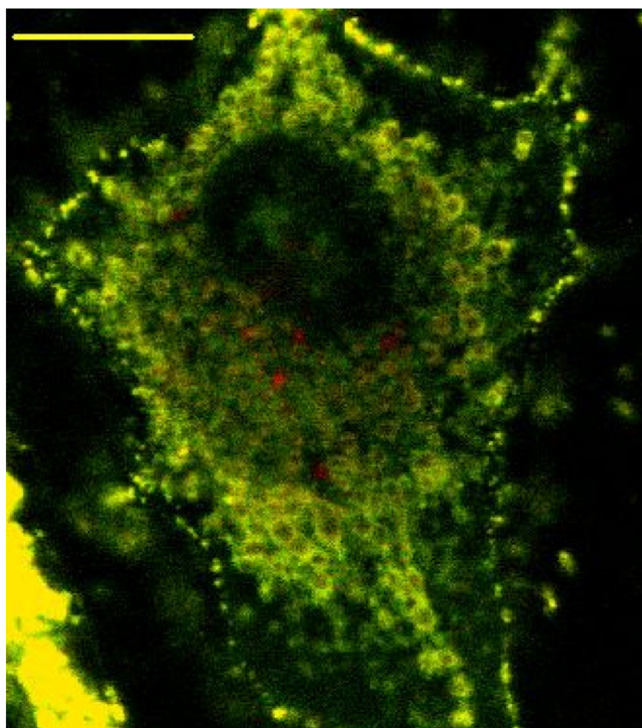

Fig. 5. Detail of Hep G2 cell with accumulated Fl-LL-III. The image shows the Hep G2 cell with accumulated Fl-LL-III just before the mitochondrial remnants stopped working (cells were incubated with 1 μM of Fl-LL-III and 30 μM of LL-III for 10 min). The peptide accumulated in the vicinity of most membrane systems. As can be seen, the green to yellow color circumscribed the disintegrated mitochondria which were alive for a while but died after a few seconds latter – the red color was no longer visible. The scale bar in the image represents 15 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

between cell-penetrating peptides and cell-permeating peptides into bacteria [1].

An examination of the fixed cells by means of fluorescent microscopy showed the same effect; after an incubation of the cells with peptides in a concentration higher than that corresponding to IC_{50}, it was possible to monitor the accumulation of fluorescein fluorescence in nucleoli and some particles which were situated at places where mitochondria were expected to be localized (Fig. 3). The effect was the same in all 4 sessile cell lines and using both, LL-III and MAC-1, peptides.

Using confocal microscopy *in vivo* of HEP G2 cells having mitochondria labeled with tetramethylrhodamine (TMRE) we observed that at low concentrations of the active peptide LL-III the mitochondria were working properly and looked healthy [15] for more than 60 min. However, after the addition of higher concentrations of the peptides, there could be seen the rapid, massive entrance of peptides into the cells and the mitochondria immediately disintegrated and lost their membrane potential. This is illustrated in Figs. 4 and 5 using TMRE labeled mitochondria (TMRE is a red fluorescent dye which accumulates in mitochondrial matrix and its fluorescence dependents on the membrane potential). To follow the entrance of the AMP into the cell, fluorescein labeled peptide was used. There was not an overlap of fluorescein signal and TMRE signal. The problem was only in the case of very high concentrations of the fluorescein accumulated in the cells when the fluorescein emitted light could be detected in both channels. This happened after massive and fast entrance of fluorescein into the cell. We were unable to change settings of channels due to described quick entrance. The peptides did not disturb the membrane of the cells in the cell culture at the same time. The peptides penetrated into some cells earlier (e.g. the left cell which just finished division in Fig. 4, 2nd shot) and into some cells later (see the right cell in Fig. 4, 12th shot). However after some 20 min from the moment of the addition of the AMP all cells were yellow as the left and the central cells (Fig. 4, last shot), i.e. they were full of the AMP (not shown). The yellow color means the highest concentration of AMP. We explain this observation by the fact that the cells in the culture were in different stage of cell cycle. According to literature, cells in different cycle stage have different qualities of the cell membrane, e.g. in addition to other factors they may have different rigidity and different membrane potential [8,18,20]. These parameters are important for the action of antimicrobial peptides on the cells. This point would deserve further study which is however beyond this manuscript.

The peptides did not seem to accumulate in the mitochondrial matrix but they appeared to be localized in the vicinity of mitochondrial surface (Fig. 5) or on the surface of the inner mitochondrial membrane. Therefore, mitochondria did not seem to be their primary target. We hypothesize that the peptide was attached to cytoplasmic particles that corresponded to damaged tubular systems of the cell. This proposed mechanism is different from that described by Ghavami et al. [9] for the smallest defensin family member brevinin-2R. They determined the changes in caspase activities, mitochondrial potential, reactive oxygen species production, ATP concentration, and different markers for mitochondria and lysosomes. They hypothesized that lysosomes are the primary target of brevinin-2R and lead to a sequence of events resulting in cell death. The defensin enters the cells via endosomes, and hence no permeabilization of the cell membrane occurred.

4. Conclusion

In this paper, we have tested more than sixty amphipatic α-helical AMPs for toxicity toward normal and cancer cell lines. The qualities of some of the analogs – selective toxicity toward some cancer cell lines – do support the possibility of preparing compounds that would have strong antimicrobial qualities and would be non-toxic to normal mammalian cells. Even selective antitumor compounds might be prepared through an appropriate modification of the mother compound, e.g. lasioglossin III. We have shown that the mechanism of action of the peptides tested involves the permeabilization of the cell membrane and that after entering the cells the peptides disturb membrane systems of the cell. Specific markers, e.g. for endoplasmatic reticulum, might bring further prove of this.

Acknowledgements

The work was supported by the Czech Science Foundation, grant no. 203/08/0536 and the research project of the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic, v.v.i., No. Z40550506. We also thank Mr. S.M. and Mrs. K. Miller for their language corrections of our text.

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