Comparative analysis of internalisation, haemolytic, cytotoxic and antibacterial effect of membrane active cationic peptides: aspects of experimental setup

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ABSTRACT

Cationic peptides proved fundamental importance as pharmaceutical agents and/or drug-carrier moieties functioning in cellular processes. The comparison of the *in vitro* activity of these peptides is an experimental challenge and a combination of different methods, such as cytotoxicity, internalisation rate, haemolytic and antibacterial effect, is necessary. At the same time several issues need to be addressed as the assay conditions have a great influence on the measured biological effects and the experimental setup need to be optimised. Therefore, critical comparison of results from different assays using representative examples of cell penetrating and antimicrobial peptides was evaluated and optimal test conditions were suggested. Our main goal was to identify carrier peptides for drug delivery systems of antimicrobial drug candidates. Based on the results of internalisation, haemolytic, cytotoxic and antibacterial activity assays, a classification of cationic peptides is advocated. We found eight promising carrier peptides with good penetration ability of which Penetratin, Tat, Buforin and Dhvar4 peptides showed low adverse haemolytic effect. Penetratin, Transportan, Dhvar4 and the hybrid CM15 peptide had the most potent antibacterial activity on *Streptococcus pneumoniae* (MIC lower than 1.2 μM) and Transportan was effective against *Mycobacterium tuberculosis* as well. The most selective peptide was the Penetratin, where the effective antimicrobial concentration on pneumococcus was more than 250-times lower than the HC_{50} value. Therefore, these peptides and their analogues will be further investigated as drug delivery systems for antimicrobial agents.

KEYWORDS

Cationic peptides; cell penetrating peptide; antimicrobial peptide; antibacterial drug carrier; haemolysis; tuberculosis

1. INTRODUCTION

In the design and development of new antimicrobial drug candidates, delivery to target site is one of the major consideration. However, several libraries of synthetic compounds providing activity against a vast spectrum of pathogens have been discovered so far, the general inability to penetrate the target cells seems to be the main obstacles, especially in the case of diseases that are caused by intracellular bacteria (Brown and Wright 2016).

The cellular uptake rate and the bioavailability of drug compounds can be enhanced by covalent attachment to appropriate targeting or carrier peptides. Cationic oligopeptides, including cell penetrating peptides (CPP) and certain antimicrobial peptides (AMP) have the ability to cross the lipid membrane and to access intracellular targets. However, a number of cationic oligopeptides entered clinical trials (Marr et al. 2006; Yeung et al. 2011) lack of cell specificity and extensive cytotoxicity remains a major drawback for further development. To improve the therapeutic effect of these peptides, intracellular trafficking and antimicrobial activity with low cytotoxicity toward human host cells is needed. For the calculation of the *in vitro* selectivity the haemolytic activity toward human red blood cells (RBC) is often used for the comparison to the antimicrobial activity (Chen et al. 2005).

1.1. Experimental Conditions of *In Vitro* Assays

To determine the *in vitro* selectivity of membrane active cationic peptides many types of biological assays are described in the literature. Previous studies have warned that the absence of haemolysis or high HC_{50} values in certain media (i.e. PBS) can give untrue results, when the selectivity index is calculated (Saberwal and Nagaraj 1994). Even in the case of the bee venom Melittin, haemolytic activity can be inhibited if the phosphate concentration is raised (Helmerhorst et al. 1999; Portlock et al. 1990). In general, the membrane activity of many cationic peptides is strongly dependent on the ionic strength and salt content of the used media (Helmerhorst et al. 1999; Herbel and Wink 2016). Raghuraman and colleagues studied the effect of ionic strength on the dynamics and aggregation behaviour of Melittin and they showed, that the peptide undergoes a structural transition from a random coil monomer to an α-helical tetramer at high ionic strength (Raghuraman and Chattopadhyay 2006; Raghuraman et al. 2006).

The most significant differences in the test conditions can be found in the haemolytic assays of peptides. Important variants are as follows: (i) concentration of RBC (0.5% v/v (Hollmann

et al. 2016), 1% (Kobayashi et al. 2000), 2% (Yang et al. 2013), 4% (Lee and Lee 2008; Song et al. 2005), 20% (Chongsiriwatana et al. 2008), 2×10^6 cells/mL (Li et al. 2005) or 2.5×10^8 cells/mL (Dathe et al. 2001; Dathe et al. 1996), 1.2×10^9 cells/mL (Zeitler et al. 2013) etc.); (ii) incubation media (PBS (Chongsiriwatana et al. 2008; Dennison and Phoenix 2014; Hollmann et al. 2016; Lee and Lee 2008; Song et al. 2005), Tris (Dathe et al. 2001; Dathe et al. 1996; Zeitler et al. 2013), RPMI-1640 or HEPES buffer (Li et al. 2005), 0.9% NaCl or normal saline (Wu et al. 2014), etc.); (iii) incubation time (30 min (Hollmann et al. 2016; Wu et al. 2014), 1 hour (Chongsiriwatana et al. 2008; Dennison and Phoenix 2014; Yang et al. 2013), 4 hrs (Li et al. 2005) or 18-24 hrs (Mojsoska et al. 2015)); (iii) used positive control (0.1% Triton-X (Dennison and Phoenix 2014; Lee and Lee 2008; Song et al. 2005), 1% Triton-X (Mojsoska et al. 2015), 10% Triton-X (Wu et al. 2014), distilled water (Hollmann et al. 2016; Yang et al. 2013), 2 % SDS (Zeitler et al. 2013), 0.05% saponin (Davanco et al. 2014). There are differences even in the used wavelength at which the optical density of released haemoglobin is determined (λ=350 nm (Chongsiriwatana et al. 2008), 405-415 nm (Kaushik et al. 2012; Lee and Lee 2008; Song et al. 2005; Zeitler et al. 2013) , 540-550 nm (Davanco et al. 2014; Hollmann et al. 2016; Mojsoska et al. 2015; Yang et al. 2013) or 570 nm (Dennison and Phoenix 2014; Wu et al. 2014)).

Because of the different test conditions, it is questionable whether the results of the haemolytic assays can be properly compared to the antimicrobial activity and/or cytotoxicity that are usually performed in lower ionic strength media or broths (RPMI, HEPES, DMEM, Bouillon, etc.). Therefore we have investigated in the comparative analysis of haemolytic activity, cytotoxicity, cellular uptake and antimicrobial activity of cationic oligopeptides with the aim to clarify the role of experimental conditions such as the cell type, concentration of cells, used media, incubation time, anticoagulant and counter-ion, etc.

1.2. Peptide Selection

In this project, well studied cationic oligopeptides which can be considered as representative CPPs and AMPs, were chosen for critical analysis. Our goal was to identify peptides as promising antibacterial drug carriers with potent penetrating ability, antibacterial effect and suitable selectivity. Criteria of the peptide selection were the following: size (lower than 25 amino acids); cationic character; synthetic considerations, such as bearing suitable conjugation site; literature data on membrane activity (e.g. cell penetration and/or antibacterial effect).

However, in many cases, the exact mechanism of bacterial killing of AMPs is not known, these evolutionarily conserved peptides can permeabilize the phospholipid membrane and provoke a broad spectrum of antimicrobial activity against bacteria, viruses, and fungi (Reddy et al. 2004; Yeaman and Yount 2003). Many antimicrobial peptides were isolated from amphibians, such as Magainin (Zasloff 1987) and Buforin (Park et al. 1996). Granulysin, a 9 kDa protein found in granules of cytotoxic T lymphocytes and natural killer cells, lyses a variety of tumour and bacterial cells *in vitro*, and directly kill *Mycobacterium tuberculosis* (Stenger et al. 1998). GranF2, a 23-mer peptide synthetic derivative, represents a helix-loop-helix region, which is postulated to be the membrane-docking part of Granulysin protein (Andreu et al. 1999). Histatins are salivary histidine-rich cationic peptides found in human parotid secretion. Synthetic derivatives, including Dhvar4, were studied on the ability to adopt helical conformation which is considered to be the key feature to act as membrane-active antimicrobial peptides (Helmerhorst et al. 1997). Crot(1-9,38-42) peptide is representing the *N*- and *C*-terminus of Crotamine, a toxin found in the venom of rattlesnake (Andreu et al. 1992). Recently, chimera peptides were developed to improve the biological activity: CM15 is a linear, hybrid AMP composed of Cecropin A (1-7) and Melittin (2-9) (Radis-Baptista et al. 2008). The major toxin found in bee venom is Melittin, a linear 26-residue peptide, which displays strong lytic activity against bacteria and human cells (Habermann 1972), and it is often used as a positive control in haemolytic assays.

Cell penetrating peptides could have a great pharmaceutical potential as *in vitro* and *in vivo* delivery vectors for a wide range of bioactive cargos. CPPs are of different sizes, amino acid sequences, and charges but all published CPPs have the ability to translocate the plasma membrane and facilitate the delivery to the cytoplasm (El-Andaloussi et al. 2005; Hudecz et al. 2005; Morris et al. 2008; Vives et al. 2008). During the last two decades several CPPs were described. Tat (transactivator of transcription) and Penetratin, the two most studied CPPs, were derived from the sequence of HIV transactivator protein (Green and Loewenstein 1988) and the third helix of the homeodomain of *Drosophila Antennapedia* protein (Derossi et al. 1994). Transportan is a hybrid peptide, constructed from a 6-residue sequence of the neuropeptide Galanin (GWTLNSAGYLLGPHAVGNHRSFSDKNGLTS) with a peptide toxin from wasp venom (mastoparan, INLKALAALAKKIL) connected *via* a lysine (Langel et al. 1996).

Tuftsin is a receptor binding peptide produced by the enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G. Based on the canine tuftsin sequence, $[TKPKG]_4$ (OT20) peptide was developed in our laboratory (Bai et al. 2008; Horvati et al. 2012; Mezo et al. 2004). OT20 peptide, a tetramer derivative of tuftsin, represents comparable chain length (20-mer), net charge (9+) and hydrophilicity therefore it was used as negative control in this study.

2. MATERIALS AND METHODS

Materials

N,N'-diisopropylcarbodiimide (DIC), triisopropylsilane (TIS) and Amberlit IRA-400 anion-exchange resin were purchased from Fluka. The amino acid derivatives were obtained from Reanal or from IRIS Biotech. 1-hydroxybenzotriazole (HOBt) and trifluoroacetic acid (TFA) was also from IRIS Biotech. Fmoc-Rink Amide MBHA resin was purchased from NovaBiochem. Acetonitrile and dimethyl sulfoxide (DMSO) were from Merck. *N,N*-dimethylformamide (DMF) and dichloromethane (DCM) were from Reanal. 5(6)-carboxyfluorescein (Cf) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were obtained from Sigma-Aldrich.

For the *in vitro* assays RPMI-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum (FCS), nonessential amino acids, 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), Löwenstein-Jensen medium base were obtained from Sigma-Aldrich. Sula medium, PBS buffer (10 mM sodium phosphate in 150 mM NaCl, pH 7.4), Tris buffer (0.1 M tris(hydroxymethyl)aminomethane, pH 7.4, adjusted with HCl), and HPMI buffer (9 mM glucose, 10 mM $NaHCO_3$, 119 mM NaCl, 9 mM HEPES, 5 mM KCl, 0.85 mM $MgCl_2$, 0.053 mM $CaCl_2$, 5 mM $Na_2HPO_4 \times 2H_2O$, pH 7.4) were prepared in our laboratory using components obtained from Sigma-Aldrich. 0.9% NaCl was a Fresenius infusion solution.

2.1. Peptide Synthesis and Purification

Peptides were produced on 100 mg Fmoc-Rink Amide MBHA (capacity = 0.67 mmol/g) resin in an automated peptide synthesizer (Syro-I, Biotage) using standard Fmoc/^{t}Bu strategy with DIC/HOBt coupling reagents. Peptides were cleaved from the resin with TFA/H_2O/TIS (9.5 : 2.5 : 2.5 v/v) mixture (2 hrs, RT). After filtration the compounds were precipitated in cold

diethyl ether, centrifuged (4000 rpm, 5 min) and freeze-dried from water. Crude products were purified by RP-HPLC on a semipreparative C-18 Phenomenex Jupiter column (250×10 mm) using gradient elution, consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water = 80/20 (v/v) (eluent B).

For the cellular uptake studies *N*-terminus of the peptides were labelled with 5(6)-carboxyfluorescein (Cf) using DIC/HOBt coupling method. Cleavage, work-up and purification of Cf-peptides were performed in the same way as mentioned above.

2.2. Peptide Characterization

Purified peptides were analysed by RP-HPLC on an analytical C-18 Eurospher-100 (5 μm, 250×4 mm) column using gradient elution with the above mentioned eluent A and B (flow rate was 1 mL/min, UV detection at λ=220 nm).

Molecular mass of peptides was determined by using a Bruker Esquire 3000+ ESI mass spectrometer. Peptide samples were dissolved in a mixture of acetonitrile/water = 1/1 (v/v) containing 0.1% acetic acid and introduced by a syringe pump with a flow rate of 10 μL/min.

2.2.1. Determination of Peptide Content by Amino Acid Analysis

The peptide content was determined by amino acid analysis using a Sykam Amino Acid S433H analyser equipped with an ion-exchange separation column and postcolumn derivatization. Prior to analysis, samples were hydrolysed with 6 M HCl in sealed and evacuated tubes at 110°C for 24 h. For post-column derivatization the ninhydrin-method was used.

2.2.2. Counter Ion Replacement and the Determination of Fluorine Content

Acetate-exchange of the purified peptides was performed using Amberlit IRA-400 (20-50 mesh, Cl^- form) anion-exchange resin. First the resin was washed with water, three times with 0.2 M NaOH, washed with water, and treated with 20 v/v% acetic acid. After washing with water, 2 g resin was mixed with the peptide solution (26.5 mg / 4 ml of water). The peptide-resin suspension was stirred for 1 h, then filtered and washed with 20 v/v% acetic acid and water. After freeze-drying, peptides were analysed by analytical RP-HPLC, ESI MS and amino acid analysis. To calculate the anion-exchange rate, the Fluorine content of the peptides was determined by using modified Schöniger method (Rogers and Yasuda 1959). The

exchange rate was calculated by dividing the percentage of the Fluorine content of acetate-exchanged peptide by the percentage of the Fluorine content of the original peptide.

2.2.3. Circular dichroism (CD) spectroscopic measurements and secondary structure estimation

Peptide samples were dissolved in deionized water and in 10 mM, pH 7.3 potassium phosphate buffer (0.05 M Na_2SO_4). Far-UV CD curves were acquired on a JASCO J-715 spectropolarimeter at 25 ± 0.2 °C in a 0.1 cm path-length rectangular quartz cuvette (Hellma, USA). Temperature control was provided by a Peltier thermostat. The CD data were monitored in continuous scanning mode between 185 and 260 nm at a rate of 50 nm/min, with a step size of 0.1 nm, response time of 2 sec, three accumulations, 2 nm bandwidth. The CD curves were corrected by spectral contribution of blank water or buffer solution.

CD spectra were plotted in mean residue molar CD units (Δε / residue) calculated by the following equation:

$$\Delta\varepsilon = \Theta / (32982cl)$$

where Θ is the measured ellipticity as a function of wavelength (nm), *c* is the molar concentration of the peptide, and *l* is the optical path length (cm).

The secondary structure content of CM15 was estimated from the CD spectra using the MS Excel version of the PEPFIT program developed originally by Reed and Reed (Amon et al. 2008; Reed and Reed 1997). It calculates the percentage of secondary contents by fitting experimental data to reference secondary structure spectra. The best fit is defined by the R^2 value, where an $R^2 = 1$ corresponds to a perfect fit. Before secondary structure analysis, the CD spectra were smoothed with a convolution width of 17 using the Means-Movement method (JASCO Spectra Analysis software, version 1.53.00).

2.3. Haemolytic Activity Assay

Peripheral blood from healthy volunteers was collected in vacuum tubes containing heparin (Li-heparin LH, VenoSafe) as anticoagulant. Tubes were centrifuged (1000 rpm, 5 min) and the pellet was washed 3 times with RPMI-1640 (culturing media without phenol red). To the pellet RPMI media was added to yield a final 4, 1, 0.25 or 0.0625 v/v% RBC suspension. Peptides were dissolved in the same media and three-fold serial dilution series were prepared (final concentration: 0.1 – 200 μM). RBC suspension (100 μL/well) were placed into a 96-

well cell culture plate and mixed with 100 μL peptide solution. The plates were incubated for 4 hrs at 37°C. After centrifugation (1500 rpm, 5 min), 100 μL of the supernatant was transferred to a flat-bottom microtiter plate and absorbance was measured at 414 nm using an ELISA plate reader (iEMS Reader, Labsystems). The percentage haemolysis was compared to 0.1 % Triton-X treated RBC and the concentration of peptide at which 50% haemolysis (HC_{50} value) was determined.

To study the time dependence of haemolysis, RBCs were incubated for 30 min or 4 hrs or 24 hrs with CM15 peptide solution at 37°C using the same conditions as described above. To compare the effect of different anticoagulants on the haemolysis evoked by CM15 peptide, three types of vacuum tubes were used: heparin (Li-heparin LH, VenoSafe), EDTA (K_3-EDTA, VenoSafe), citrate (Na_3-citrate 9NC, VenoSafe). The influence of different buffers was tested using either PBS, TRIS buffers, Bouillon broth, DMEM, RPMI (without phenol red) media or Fresenius 0.9% NaCl infusion solution. Always the same media was used for washing, culturing the erythrocytes and for dissolving the peptide samples.

2.4. Cell Culturing, Cytotoxicity Assay and Cell Morphology

Peripheral blood mononuclear cells (PBMC) were prepared from peripheral blood of healthy volunteers (purchased from Hungarian National Blood Transfusion Service, HNBTS) using Ficoll-Hypaque density gradient centrifugation method. PBMC were cultured in complete medium prepared from RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine and 160 μg/mL gentamycin at 37°C in 5% CO_2 atmosphere. Twenty-four hours prior to treatment, PBMC cells were plated into a 96-well round bottom plate (250.000 cell/100 μL complete medium).

MonoMac6 human monocytic cell line (DSMZ no.: ACC 124) and HepG2 human hepatocellular liver carcinoma cell line (ATCC HB-8065) were maintained under conditions described above. For cytotoxicity assay, cells were distributed on a 96-well flat bottom tissue culture plate (5000 cell/100 μL complete RPMI-1640 medium).

SH-SY5Y human neuroblastoma cell line (ATCC CRL-2266) was cultured in DMEM medium containing 10% FCS, 2 mM L-glutamine, 160 μg/mL gentamycin, 1 mM pyruvate and 1% nonessential amino acids. SH-SY5Y cells (10.000 cell/100 μL complete DMEM medium) were plated into a 96-well flat bottom tissue culture plate.

Prior to treatment, cells were washed with serum-free RPMI-1640 (PBMC, MonoMac6, HepG2) or DMEM (SH-SY5Y) medium. Peptides to be tested were dissolved in serum-free medium and added to the cells to achieve 0.15 μM – 300 μM final concentration. Cells were incubated with the peptides for 4 hrs, then the cell viability was tested using MTT assay (Liu et al. 1997; Mosmann 1983; Slater et al. 1963). Briefly, 45 μL MTT solutions were added to each well (2 mg/ml, solved in serum-free medium). Following 4 hrs of incubation, plates were centrifuged at 2000 rpm for 5 minutes, and the supernatant was carefully aspirated with a G30 needle. The precipitated purple crystals were dissolved in 100 μL DMSO, and after 10 minutes agitation, the absorbance was determined at λ = 540 nm and 620 nm using ELISA plate reader (iEMS Reader, Labsystems). Cytotoxicity, expressed in percentage as the function of peptide concentration was represented graphically and IC_{50} values were determined.

To visualize cell morphology after peptide treatment, microscopic images of MonoMac6 cells were captured. MonoMac6 cells were plated in a 96-well flat bottom tissue culture plate (5000 cell/100 μL RPMI-1640 medium without phenol red), then treated with the peptides for 4 hrs at 1, 10 and 100 μM final concentration. Microscopic images of the adherent cells were captured using an Olympus CKX41 microscope.

2.5. Cellular Uptake Studies by Flow Cytometry

The measurement of cell penetration of the compounds was evaluated on MonoMac6 human monocytic cell line by using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) with 488 nm (Coherent Sapphire, 22 mW) laser. Cells were harvested in the logarithmic phase of growth and plated on a 24-well tissue culture plate (10^5 cells/1 mL medium/well) 24 hours prior to the experiment. Cf-labelled peptides were dissolved in serum free RPMI medium and added to the cells at 20, 10 and 5 μM final concentrations. Cells were incubated with compounds for 2 hrs (37 °C, 5% CO_2 atmosphere). After centrifugation (1000 rpm, 5 min) and washing with RPMI medium, supernatant was removed and 100 μL 1 mM trypsin was added to the cells. After 5 min incubation at 37 °C 0.8 mL 10% FCS/HPMI medium was added than cells were washed and re-suspended in 0.5 mL HPMI. The cell viability was assessed using 10 μg/mL propidium iodide (PI) solution (for the gating strategy see Supporting Information FIGURE S1). The intracellular fluorescence intensity of the cells

was measured on channel PE LP550 (emission at λ = 550 nm) and data were analysed with FACSDiva 5.0 software. All measurements were performed in triplicates. Parallel with flow cytometry measurements microscopic image of the cells were captured with an Olympus CKX41 microscope.

2.6. *In Vitro* Antibacterial Activity Assay

In vitro antibacterial activity of the compounds was determined against *Streptococcus pneumoniae* (ATCC 49619) by serial dilution method in Bouillon medium, which was prepared in-house. Compounds were added to the medium as 10 μL DMSO solutions in duplicates (range of final concentrations was between 1.2 and 300 μM). Each tube was inoculated with 0.5 Mcfarland bacteria and the minimal Inhibitory concentration (MIC) was determined after incubation at 37 ^{o}C for 24 hrs. MIC was the lowest concentration of a compound at which no visible growth of the bacteria occurred.

Antitubercular effect of the compounds was also tested on *Mycobacterium tuberculosis* H_{37}Rv (ATCC 27294) in Sula semisynthetic medium (prepared in-house) (Sula 1963; Sula and Sundaresan 1963; Vinsova et al. 2006) using previously described method (Baranyai et al. 2015; Horvati et al. 2015). Compounds were dissolved in DMSO and added to the medium at ten various concentrations (c = 0.5 – 500 μM). MIC value was determined after incubation at 37 ^{o}C for 28 days. In order to confirm the growth inhibition colony forming unit (CFU) was determined by subculturing from the Sula medium onto drug-free Löwensten-Jensen solid medium. Samples were further incubated for 28 days. Experiments were repeated at least two times.

Selectivity indices were calculated from the concentration value where 50% of human RBCs were lysed (HC_{50}) by the peptides divided by the minimal inhibitory concentration (MIC) determined against *Streptococcus pneumoniae*. Both *in vitro* measurements were performed in Bouillon broth using 24 hrs incubation time.

2.7. Statistical Analysis

In vitro tests were performed in triplicates and the results were expressed as mean ± Std. deviation. For the evaluation of haemolysis and cytotoxicity and for the determination of HC_{50} and IC_{50} values of the peptides dose-response curves were plotted with non-linear regression analysis using GraphPad Prism 5.0 software. The effects of different experimental

conditions (incubation time, media, percentage of RBC, anticoagulant and counter ion) were analysed by one-way ANOVA test by using GraphPad Prism 5.0 software. If means were significantly different ($P < 0.05$), than ANOVA test was followed by a t-test (unpaired, two-tailed, confidence intervals: 95%).

3. RESULTS AND DISCUSSION

Representative examples of cationic oligopeptides, listed in TABLE 1, were critically analysed and compared in well-defined *in vitro* assays. Our aim was to find good future peptide candidates for antimicrobial drug delivery. Cytotoxicity, haemolytic and antimicrobial activity of membrane-active peptides is often used to estimate their therapeutic applicability (Helmerhorst et al. 1999; Maher and McClean 2006). In this paper, we show that the calculated selectivity index is strongly dependent on the conditions (i.e. culturing media) of the *in vitro* assays. Therefore, systematic studies were performed to establish proper test conditions for accurate comparison. All 11 peptides were then tested on PBMC and MonoMac6 cells, against two bacterial strains (*S. pneumoniae* and *M. tuberculosis*) and on human erythrocytes in different incubation media. As positive control, Melittin was used which has strong lytic activity against microbes and human cells. OT20 was the negative control in this study. OT20 is a non membrane-active, receptor binding peptide, which represents comparable cationic character.

TABLE 1. Origin and classification of cationic oligopeptides used in this study

peptide	*classification*	*origin*	*ref.*
Tat(47-57)	CPP	HIV transactivator protein	(Green and Loewenstein 1988)
Penetratin	CPP	*Drosophila Antennapedia* protein	(Derossi et al. 1994)
Transportan	hybrid CPP	constructed from Galanin and Mastoparan proteins	(Langel et al. 1996)
Magainin	AMP	peptide from *Xenopus laevis* skin	(Zasloff 1987)
Buforin II (5-21)	AMP	peptide from the stomach tissue of *Bufo bufo garagrizans*	(Park et al. 1996)
GranF2	AMP	peptide derived from Granulysin protein	(Andreu et al. 1999; Stenger et al. 1998)
Dhvar4	designed AMP	human salivary Histatin derivative	(Helmerhorst et al. 1997)
Crot(1-9,38-	designed AMP	Crotamine, a toxin of *Crotalus durissus*	(Andreu et al. 1992)

42)		*terrificus*	
CM15	hybrid AMP	from Cecropin A and Melittin sequences	(Radis-Baptista et al. 2008)
Melittin	positive control (strong lytic activity)	venom of *Apis mellifera*	(Habermann 1972)
OT20	negative control peptide	tetramer derivative of tuftsin sequence	(Bai et al. 2008)

3.1. Synthesis and Characteristics of Cationic Peptides

Cationic peptides, listed in TABLE 2, were synthesized, purified and carefully characterized by analytical RP-HPLC, ESI MS and amino acid analysis.

Throughout experimental design it is important to note that the peptide content of a lyophilised material can vary according to the sequence and the used synthetic procedures. Generally, the content of residual salt and water, which are the main impurities in a lyophilized peptide, can be up to 40-50%, especially in the case of high number of positively charged amino acids in the sequence. For more reliable comparison, precise determination of peptide content was evaluated by amino acid analysis. We found that the higher the average hydrophilicity (*H*) was, the lower peptide content was measured. The average peptide content was 50%, the lowest value was 41% for Tat(47-57) peptide (9+ charge in 11 amino acids), the highest was 58% for Magainin (4+ charge in 23 amino acids) (TABLE 2).

TABLE 2. Analytical characteristics of cationic peptides

peptide	*sequence*	*Z* [a]	M_{av} [b] *calcd / found*	R_t [c] *(min)*	*H* [d]	*peptide content* [e] *%*
Tat(47-57)	YGRKKRRQRRR	9+	1558.9/1559.2	15.5	+2.0	41
Penetratin	RQIKIWFQNRRMKWKK	8+	2245.7/2245.6	25.9	+0.5	49
Transportan	AGYLLGKINLKALAALAKKIL	5+	2181.8/2181.9	29.1*	-0.3	56
Magainin	GIGKFLHSAKKFGKAFVGEIMNS	4+	2465.9/2466.0	33.0	-0.1	58
Buforin II (5-21)	RAGLQFPVGRVHRLLRK	6+	2002.4/2002.5	26.8	+0.2	52
GranF2	VCRTGRSRWRDVCRNFMRRYQSR	8+	2988.5/2988.7	27.7	+0.6	45
Dhvar4	KRLFKKLLFSLRKY	7+	1839.4/1839.6	29.6	+0.3	48
Crot(1-9,38-42)	YKQCHKKGGKKGSG	6+	1504.8/1504.9	11.3	+0.8	48
CM15	KWKLFKKIGAVLKVL	6+	1770.3/1770.4	32.0	-0.1	52
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	6+	2846.5/2846.6	39.6	-0.2	57
OT20	TKPKGTKPKGTKPKGTKPKG	9+	2063.5/2063.5	14.8	+1.1	49

C-terminus of the peptides was amidated.

[a] *Z*: net charge at neutral pH. Calculated by the number of (K+R)-(E+D). Positive charge at the *N*-terminus increases *Z* by 1 unit.

[b] Measured average molecular mass by Bruker Esquire 3000+ ESI-MS.

[c] Analytical RP-HPLC, gradient: 5% B, 5 min; 5-60% B, 35 min. [*] gradient: 10% B, 5 min; 10-80% B, 35 min.

[d] *H*: hydrophilicity is calculated from the average of hydrophilicity values of each amino acids (Hopp and Woods 1981).

[e] Peptide content was determined by amino acid analysis using freeze-dried final product.

3.1.1. Counter-Ion Replacement

After standard solid phase synthesis and purification, cationic peptides are often isolated as trifluoroacetate salts (Roux et al. 2008; Vemuri 2005). In the case of lysine and arginine rich peptide, the high amount of TFA can modify the biological and physicochemical properties (i.e. by modifying the conformation of the peptide, acidifying the media, and lowering the peptide content of the lyophilized powder). The removal of excess TFA and change trifluoroacetate counter-ion into acetate, which is less acidic, less toxic and has lower molecular mass, was performed using Amberlit IRA-400 anion-exchange resin. Anion-exchange rate was monitored by elemental analysis. The Fluorine content of the peptide after acetate-exchange was decreased dramatically and the calculated exchange rate was up to 80% (F% = 13.42 (unchanged peptide); 2.88 (acetate-exchanged)).

3.1.2. Circular Dichroism Spectroscopic Investigation of the Secondary Structure of CM15 Peptide

Far-UV CD spectra of peptides represent the summation of signals from the optically active n-π^* and π-π^* transitions of the amide bonds (Toniolo et al. 2012). α-Helical structures display a negative n-π^* CD band at ~220 nm and two, higher intensity negative-positive peaks at ~207 and ~190 nm, respectively. This couplet is attributable to chiral exciton interaction of the helically arranged amide chromophores. The β-sheet CD pattern consists of a negative n-π^* band near 217 nm and two π-π^* exciton components below 200 nm. Unordered peptides and proteins show a strong negative peak centered in the 195-200 nm region and substantially weaker, positive or negative signals above 215 nm. Taking these into consideration, the CD curve of CM15 measured in water (pH ~ 6) is characteristic to the dominant contribution of the unordered or random coil structure (FIGURE 1) (Pistolesi et al. 2007). In full concordance with this, quantitative analysis of the CD data yielded 62% random coil and 26% β-sheet with

no α-helical content. Interestingly, a small fraction of type III β-turn (Reed and Reed 1997) was also required to achieve the best fit between the experimental and calculated data (FIGURE 1). In relation to water, the negative CD band is red shifted above 200 nm in buffer solution together with the development of a prominent shoulder above 215 nm. According to the secondary structure estimation, these spectral changes can be associated to a β-turn → α-helix conformational conversion (FIGURE 1). These results suggest that the pH of the medium where CM15 acts, *e.g.*, normal or acidic inflamed tissues, may provoke a slight, but significant modification of its secondary structure.

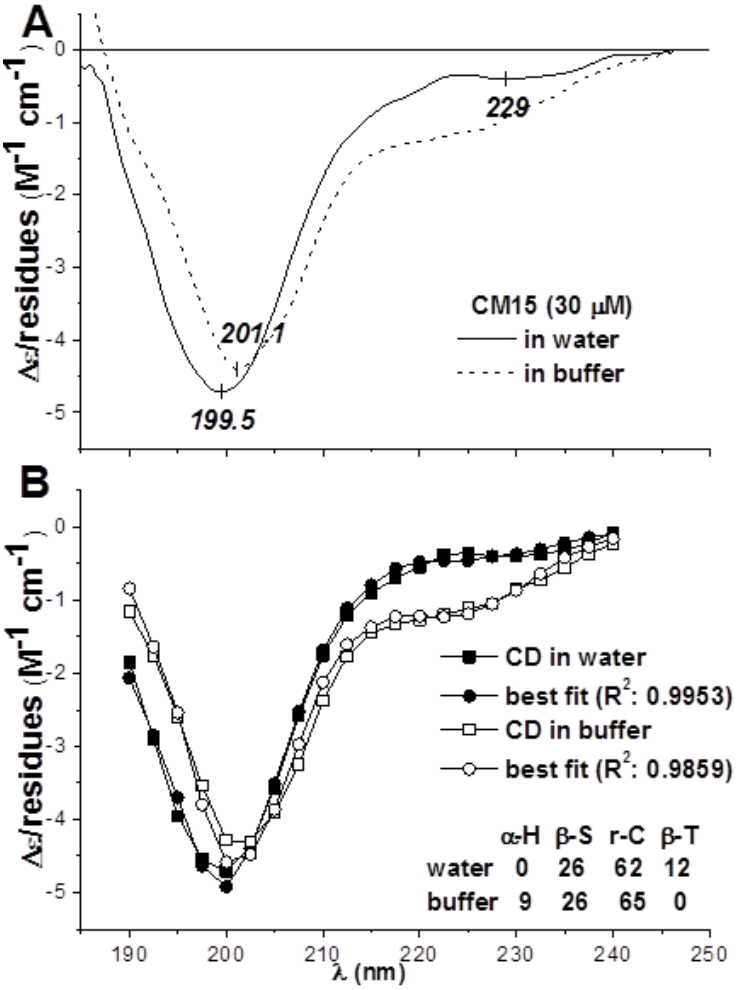

FIGURE 1. Far-UV CD spectra of CM15 peptide. CM15 peptide (30 μM) was measured in deionized water (pH ~ 6, 0.05 M Na_2SO4) and in 10 mM, pH 7.3 potassium phosphate buffer (0.05 M Na_2SO4), panel **A**. Panel **B** represents the results of the curve-fitting procedure made by using the PEPFIT analysis program (α-H: α-helix; β-S: β-sheet; r-C: random coil; β-T: type III β-turn). The inset shows the estimated secondary structure percentages of the peptide.

3.2. Haemolytic Activity of Peptides: Effect of Experimental Conditions

Published methods to determine haemolytic activity of cationic peptides often use different test conditions that can strongly influence the results. Therefore the effect of time, incubation buffer, concentration of RBC, identity of anticoagulant and counter-ion on haemolytic activity of cationic peptides was investigated. To establish proper test conditions CM15 peptide was chosen because of its average characteristics (charge and size) and medium haemolytic activity.

3.2.1 Incubation Time and Media

First, the time dependence of haemolysis evoked by cationic peptides was studied. Erythrocytes were incubated for 30 min, 2 hrs, 4 hrs or 24 hrs with CM15 peptide. Half an hour was enough to reach the maximum haemolysis; no further haemoglobin release was detected (FIGURE 1 A, B). Comparing the means of the HC_{50} values no significant difference was observed ($P = 0.4449$).

In contrast, the type of incubation media strongly influenced the haemolytic activity of CM15 peptide. The resulted means of HC_{50} values showed significantly difference ($P < 0.0001$) in the one-way ANOVA test. In the measurement of haemolysis, isotonic conditions are required to prevent erythrocytes from spontaneous lysis. Most commonly PBS and TRIS buffers are used in haemolytic assays however, for antimicrobial and cytotoxic assays these are considered to be high salt buffers (Helmerhorst et al. 1999). RPMI, DMEM and other cell culturing media contain amino acids, vitamins, glucose as well as additional supplementary components to be optimal for cultivation of certain cell types and are formulated to have the final osmolality in the range of 290 to 310 mOs/kg. It was found that the haemolytic activity of CM15 peptide is significantly lower ($P < 0.0001$) in PBS (28.2 ± 1.22 μM) than in cell culturing DMEM media (DMEM: 6.70 ± 0.50 μM). Haemolysis in RPMI, DMEM and Bouillon broth were in the range of 6.70 to 15.32 μM (FIGURE 2 C, D). For other cationic peptides, same results were found namely lower HC_{50} values were measured in PBS and Tris buffers than cell culturing media and broths (data not shown).

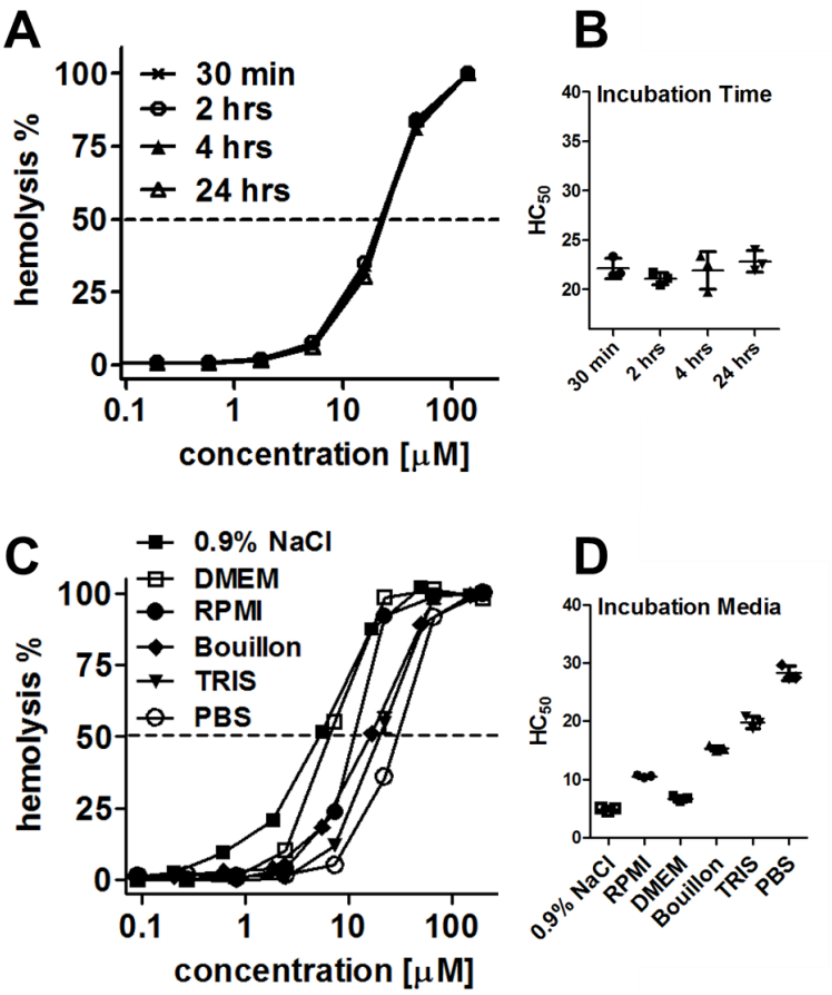

FIGURE 2. Effect of incubation time (A, B) and media (C, D) on the haemolytic activity of CM15 peptide. Human erythrocytes were treated with CM15 peptide at various concentrations using different experimental conditions and the haemolysis was determined by reading the absorbance of the supernatant at λ=414 nm. Haemolysis of the erythrocytes reached the maximum after 30 min treatment and no further haemoglobin release was measured. The haemolytic activity of CM15 peptide is strongly dependent on the used incubation buffer: in PBS significantly lower HC_{50} value was measured than in low ionic strength media or broth. (One-way ANOVA test, means are significantly different, $P < 0.0001$).

In published methods there are differences even in the used wavelength at which the absorbance of the supernatant is measured. Percentage of haemolysis is calculated by dividing the absorbance measured for the peptide by the absorbance measured for the positive control (both values are corrected with the background). Therefore, the resulted % haemolysis values are independent from the exact absorbance values. However, better results can be obtained if we measure at the maximum wavelength of the released haemoglobin. We found that the

highest values can be measured if we monitor the absorbance at 414 nm or 405 nm (TABLE 3).

TABLE 3. Absorbance of the released haemoglobin at different wavelength

Wavelength	*Absorbance* [a]	± *Sd* [a]
340 nm	2.034	0.001
405 nm	4.456	0.016
414 nm	4.576	0.120
450 nm	1.190	0.018
492 nm	0.440	0.003
540 nm	1.047	0.006

Peptide treated erythrocytes were centrifuged and the supernatants were transferred to a flat-bottom microtiter plate. The absorbance was measured at various wavelengths using an ELISA plate reader.

[a] Means and standard deviations of two measurements.

3.2.2. Concentration of Red Blood Cells, Anticoagulant and Counter Ion

The percentage or cell number of RBC used in haemolytic assays differs in a wide concentration range. Therefore, to clarify the effect of RBC content, haemolysis of CM15 peptide was studied at 4%, 1%, 0.25% or 0.0625% RBC concentration. No difference was measured ($P = 0.2862$) for 4%, 1%, 0.25%, while at 0.0625% RBC concentration significantly lower HC_{50} value was measured ($P = 0.0004$) (FIGURE 3A, B)

Although, published methods for analysis of haemolytic activity of cationic peptides mentioned different anticoagulant containing vacuum tubes for cupping, no systematic study was evaluated on the effect of anticoagulants. Therefore, most frequently used anticoagulant containing vacuum tubes were used to compare the effect of citrate, heparin and EDTA on the haemolytic activity of CM15 peptide. The results clearly showed that lysis of the red blood cells by CM15 cationic peptide was independent of the used anticoagulant ($P = 0.1210$) (FIGURE 3C).

In the case of lysine and arginine rich peptides, the high amount of TFA could alter the biological activity. Therefore, excess TFA and trifluoroacetate counter-ion was removed and changed to acetate counter-ion and the haemolytic activity of trifluoroacetate and acetate

counter-ion containing CM15 peptide was assayed. No marked effect of counter-ion identity could be observed in case of CM15 peptide treated RBC (P = 0.4106) (FIGURE 3D).

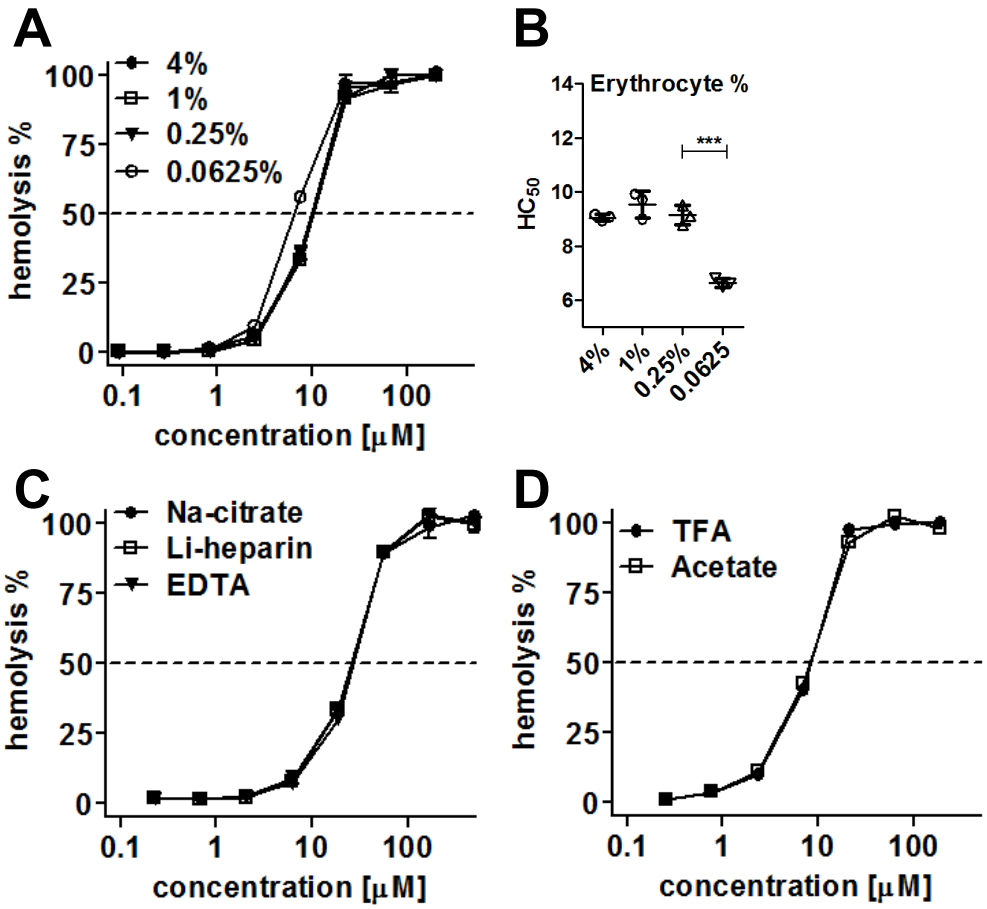

FIGURE 3. Effect of erythrocyte concentration (A, B), identity of anticoagulant (C) and counter-ion (D) on the haemolytic activity of CM15 peptide. Human RBCs were treated with CM15 peptide using different test conditions and the haemolysis was determined by reading the absorbance of the supernatant at λ = 414 nm. Haemoglobin release of 4%, 1%, 0.25% v/v RBCs evoked by the CM15 peptide showed no significant difference, while at 0.0625% RBC concentration significantly lower HC_{50} value was measured (A and B). No significant differences in the resulted HC_{50} values were detected after using three different anticoagulants (C). Counter-ion replacement has no significant effect on the haemolytic activity of CM15 peptide. (One-way ANOVA test, *** means P < 0.0005).

3.3. Cytotoxicity of Cationic Peptides

Cytotoxicity of cationic peptides was studied on freshly prepared human PBMC in RPMI media (incubation time was 4 hrs) using MTT test. PBMC are isolated human cells which can be used to study general toxicity of a compound. The following order of cytotoxicity was found: (i) Melittin, Transportan, CM15 and Dhvar4 peptides have IC_{50} value lower than 50 µM; (ii) modest cytotoxicity (50 – 300 µM) was measured for GranF2, Magainin and

Penetratin; (iii) Tat(47-57), Buforin II, Crot(1-9,38-42) and OT20 peptides were not cytotoxic up to 300 μM (TABLE 4).

When measuring the haemolytic activity of the peptides employing the same experimental conditions as for the cytotoxic assay (RPMI media, 4 hrs of incubation), almost the same order of activity was found, namely the highest HC_{50} values were measured for Melittin, Transportan, CM15, and Transportan. Dhvar4 and GranF2 peptides were found to be cytotoxic to PBMC cells but not for red blood cells (HC_{50} > 300 μM).

TABLE 4. Cytotoxic, haemolytic and antibacterial effect of cationic oligopeptides

peptide	*IC_{50} [a] (μM)*	*HC_{50} [b] (μM)*	*MIC [c] (μM)*	*HC_{50} [d] (μM)*	*Selectivity index [e]*	*MTB MIC [f] (μM)*
Tat(47-57)	>300	>300	300	>300	1	>300
Penetratin	136±25.6	>300	1.2	>300	250	>300
Transportan	8.49±1.39	38.0±3.79	1.2	25.2±3.29	21	80
Magainin	132±23.8	>300	33	>300	9	>300
Buforin II (5-21)	>300	>300	>300	>300	1	>300
GranF2	80.1±18.5	>300	100	269±7.11	3	>300
Dhvar4	38.6±5.76	>300	3.7	>300	81	>300
Crot(1-9,38-42)	>300	>300	>300	>300	1	>300
CM15	12.9±2.51	18.9±1.71	1.2	13.9±0.456	12	>300
Melittin	0.941±0.237	0.339±0.0854	1.2	0.681±0.0542	0.6	>300
OT20	>300	>300	>300	>300	1	>300

[a] Mean IC_{50} ± standard deviation. Cytotoxicity of peptides on human PBMC measured in RPMI media (4 hrs incubation time).

[b] Mean HC_{50} ± standard deviation. HC_{50} is a concentration at which 50% haemolysis of human RBCs occurs in RPMI (without phenol red) media (4 hrs incubation time).

[c] Minimal inhibitory concentration on *Streptococcus pneumoniae* strain determined in Bouillon broth (24 hrs of incubation).

[d] Mean HC_{50} ± standard deviation determined in Bouillon broth with 24 hrs of incubation.

[e] Selectivity index was calculated from HC_{50}/MIC (both measured in Bouillon broth).

[f] Minimal inhibitory concentration on *Mycobacterium tuberculosis* H_{37}Rv strain determined in Sula media.

For samples, where MIC, IC_{50} or HC_{50} was higher than 300 μM, a value of 300 μM was assigned.

To estimate the cell-type dependence of cationic peptides four different human cells and cell lines were used. HepG2 human hepatocytes and SH-SY5Y human neuroblastoma cells are frequently used as hepatotoxicity and neurotoxicity model. MonoMac6 human monocytic cell

line is a macrophage model which is suitable to bear intracellular bacteria. Cells were treated with Transportan at various peptide concentrations and the cytotoxicity was determined by MTT assay. Mean IC_{50} values showed significant difference ($P < 0.05$) however, all IC50 were in the same concentration range: 4.70±1.02 μM (MonoMac6), 6.78±0.488 μM (HepG2), 8.28±0.573 μM (SH-SY5Y), 9.81±0.473 μM (PBMC) (FIGURE 4).

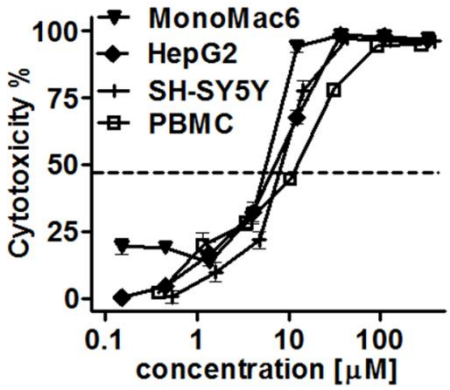

FIGURE 4. Cell-type dependence in the cytotoxicity assay. Freshly isolated PBMC, MonoMac6 human monocytes, HepG2 human hepatocytes and SH-SY5Y human neuroblastoma cells were treated with the peptide for 4 hrs at various concentrations and the cytotoxicity was determined by MTT assay. Comparing the IC_{50} values in one-way ANOVA test, significant difference was calculated however, the resulted IC50 values were at the same concentration range (between 4.70 μM and 9.81 μM).

MonoMac6, which are adherent cells, were chosen to visualize the changes in the cell morphology caused by cationic peptides (FIGURE 5). Microscopic images of the peptide treated cells were captured using an Olympus CKX41 microscope. After treatment with Buforin II (5-21), Crot(1-9,38-42) and OT20 peptides, intact MonoMac6 cells were observed. Melittin, Transportan, CM15, Dhvar4 and GranF2 peptides caused severe damage on the cell membrane and provoked dramatic changes in the cell morphology. Penetratin, Magainin and Tat(47-57) peptides effected the cell membrane integrity just at the highest (100 μM) concentration. These microscopic observations are in accordance with the result of the MTT assay.

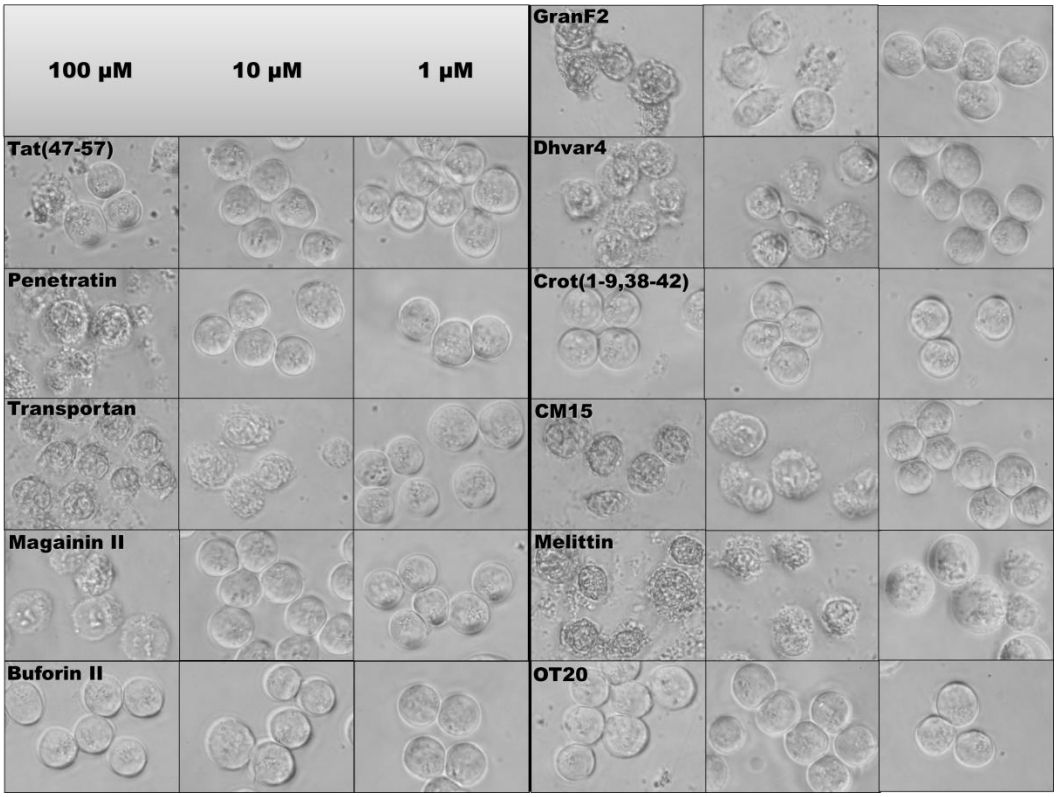

FIGURE 5. Microscopic images of cationic peptide treated MonoMac6 cells. Cells were incubated with the peptides for 4 hrs at 1, 10 and 100 µM final concentration. Dramatic changes in the cell morphology were captured in the case of Melittin, Transportan, Dhvar4, CM15, and GranF2 peptides, which results are in accordance with the MTT assay.

3.4. Internalisation of Cationic Peptides

Cellular uptake of MonoMac6 human monocytic cells was measured by flow cytometry. For that purpose, peptides were labelled with 5(6)-carboxyfluorescein (Cf) and measured at 20, 10 and 5 µM final concentrations (FIGURE 6). Based on the internalisation ability, three groups of peptides were created: (i) *superior penetration* was defined for peptides where the percentage of FITC positive cells was higher than 80% at 5 µM concentration – Penetratin, Transportan, Dhvar4 and Melittin; (ii) *good penetration* for peptides where the percentage of FITC positive cells was higher than 50% at 10 µM concentration – Tat, GranF2, CM15 and Buforin II; and (iii) *modest penetration* where the percentage of FITC positive cells was higher than 50% at 20 µM concentration – Crot(1-9,38-42), Magainin II and OT20. It is important to note, that peptides with *superior penetration* shows the highest cytotoxicity,

especially Melittin, Transportan and Dhvar4. However, Penetratin, Tat and Buforin II peptides bear good internalisation property but low cytotoxicity.

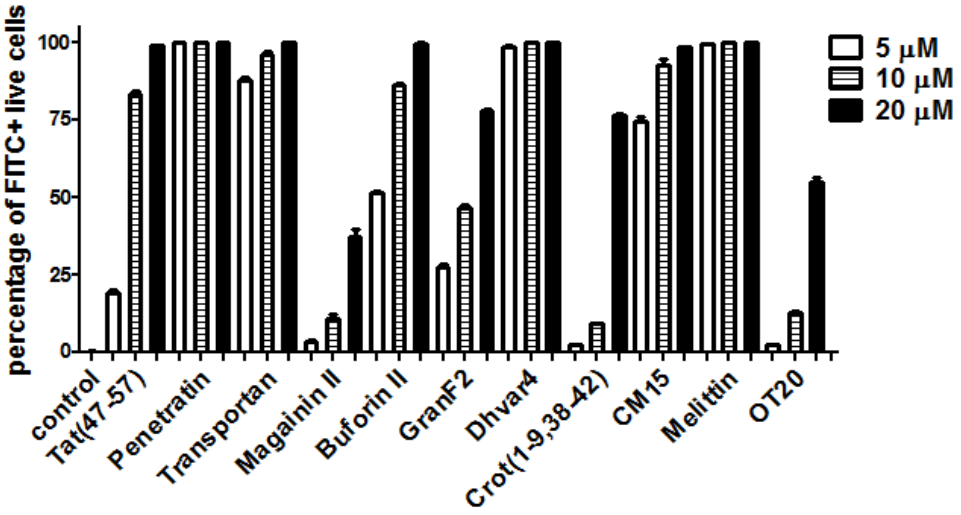

FIGURE 6. Internalisation of cationic peptides. Cellular uptake of MonoMac6 cells were measured by flow cytometry using 5(6)-carboxyfluorescein (Cf)-labelled peptides at 5, 10 and 20 µM concentration. The penetration ability was ranked according to the resulted percentage of FITC positive cells. *Superior penetration*: Penetratin, Transportan, Dhvar4 and Melittin; *good penetrations*: Tat, GranF2, CM15 and Buforin II; *modest penetration*: Crot(1-9,38-42), Magainin II and OT20.

3.5. Antibacterial Activity against *S. pneumonia.*

Antimicrobial efficacy of cationic peptides was measured against *Streptococcus pneumoniae*, a Gram-positive bacterium which is one of the most significant human pathogen (TABLE 4). Buforin II, Crot(1-9,38-42), Tat and OT20 control peptide were not effective against *S. pneumonia.* Magainin and GranF2 showed modest activity; CM15 and Dhvar4 peptides had potent antibacterial activity (less than 4 µM) just as Melittin. Interestingly, Penetratin and Transportan, which are classified as cell penetrating peptides, showed relevant antibacterial activity against *Streptococcus pneumoniae*. In the case of Penetratin, these findings are in accordance with a previous data, which suggested that the membrane induced α–helical structure makes this peptide similar in activity to AMPs (Magzoub et al. 2002; Zhu and Shin 2009).

3.6. Transportan Is Effective against *M. tuberculosis*

Antitubercular effect of all peptides was tested also on *M. tuberculosis* $H_{37}Rv$ strain. *M. tuberculosis*, the causative agent of tuberculosis, can survive in the host phagocytes for years or even decades and it is estimated that more than one-third of the world's population are infected with these pathogen (Lienhardt et al. 2012). Our results demonstrated that none of the peptides were effective against *M. tuberculosis* up to 300 μM concentration, except Transportan. The MIC of Transportan peptide was 80 μM. No published data were found recently on the antitubercular effect of Transportan.

3.7. *In Vitro* Selectivity of Cationic Peptides

We found, that the haemolytic activity of the tested peptides is significantly lower in PBS than in RPMI or in Bouillon broth. Therefore, selectivity indices (given in TABLE 4) were calculated from the results of experiments using the same buffer and incubation time. Based on the comparison of antitubercular effect and haemolytic activity, most selective peptides (where the selectivity indices were higher than 20) were Penetratin, Dhvar4 and Transportan. Among these peptides, Penetratin showed the best selectivity, because the MIC value of Penetratin was 250 times lower than its HC_{50} value. Modest selectivity was found for Magainin II, GranF2 and CM15 peptides. Up to the highest concentration (300 μM) no antibacterial and haemolytic activities were observed for Buforin II, Crot(1-9,38-42), Tat and OT20 peptides (TABLE 4). In the case of the positive control peptide Melittin, the MIC value was higher than the HC_{50} value.

4. CONCLUSION

Here we report the comparative analysis of cationic oligopeptides to identify potent antibacterial carriers for drug delivery. Peptide synthesis and purification was followed by accurate characterization of the product (molecular mass, retention time, peptide content). Haemolytic activity and cytotoxicity on isolated human blood cells and different cell lines have been systematically studied and compared to their penetrating ability and antibacterial activity against *Streptococcus pneumoniae* (pneumococcus) and *Mycobacterium tuberculosis.* We also clarified the role of the used media, incubation time, RBC concentration, type of anticoagulant and counter-ion on the haemolytic activity of the peptides. We found, that the lytic activity of cationic peptides strongly depends on the used incubation media, which observation is in accordance with the results of the circular dichroism measurement. Therefore, it is uncertain whether the results of haemolytic assays, that are conducted in high

salt buffers such as Tris od PBS, can be compared properly with the results of antibacterial assays, that are conducted in low ionic strength buffers or broths. Because of this observation, the same incubation media was applied both for the antibacterial assay and the haemolytic assay. We also found that the haemolytic activity of cationic peptides was almost independent from the other conditions like incubation time and type of anticoagulant. For a proper haemolytic assay, performed on human erythrocytes, at least 0.25% v/v RBC concentration is required, which is equal to approximately 1 - 1.25 × 10^7 cells / mL (counting with the reference range of RBC of a male or female blood). When measuring the cytotoxicity of cationic peptides on different human cells and cell lines, almost the same order of activity was found than in the haemolytic assay.

As a summary of the performed assays we can conclude the following: (i) eight peptides showed superior or good penetration of which Tat, Penetratin, Dhvar4 and Buforin peptides have low haemolytic activity. Therefore, these peptides can be considered as good carriers for different drug molecules without causing severe damage on human cells. (ii) Transportan was found to be effective against *M. tuberculosis* H_{37}Rv bacteria. However, Transportan was cytotoxic at this concentration range. (iii) Dhvar4 peptide showed superior antibacterial effect and penetration ability although, this peptide was cytotoxic to human PBMC but not to human erythrocytes. (iv) Penetratin was identified as promising *antibacterial drug carrier* with potent antibacterial effect (MIC lower than 1.2 μM on pneumococcus) and superior penetration ability with low *in vitro* toxicity on human cells. Conjugation of an antibacterial drug to an antibacterial peptide is proposed to intensify the efficacy by multiple mode of action: including bacterial cell lysis, inhibiting bacterial enzymes, interacting directly with the cytoplasmic membrane and inhibiting intracellular targets. In addition, the consequence of the multiple mechanism of action is the low potential to induce microbial resistance. Therefore, Penetratin and its analogues with remarkable antibacterial activity and high selectivity will be further investigated as drug delivery systems for antibacterial agents.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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