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International Journal of Pharmaceutics 309 (2006) 208–217
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Pharmaceutical Nanotechnology

Biological properties of low molecular mass peptide dendrimers

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Received 22 April 2005; received in revised form 16 October 2005; accepted 21 October 2005

Abstract

A series of new, low molecular mass, lysine-based peptide dendrimers with varying distribution of cationic and aromatic groups in the structure were synthesized. They expressed antimicrobial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria as well as against fungal pathogens (*Candida albicans*). Their cytotoxic, haematotoxic, and genotoxic effects were studied. It appears that degree of branching and steric distribution and types of hydrophobic (aromatic) groups and cationic centres are important components of dendrimeric structure and influence both antimicrobial potency and toxicity. Such 3D structure of our dendrimers mimics that of the natural antimicrobial peptides and can be achieved by application of dendrimer chemistry.
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Keywords: Antimicrobial dendrimers; Cytotoxicity; Dendrimer; Genotoxicity; Haemolysis; Peptide; Dendrimeric effect

1. Introduction

Dendrimers are a relatively novel class of branched polymers. They consist of a central core and several generations of branches what results in a large number of reactive end groups at the surface (Tomalia et al., 1990). Among them peptide dendrimers attracted serious attention due their potential biomedical applications, such as protein mimics or drug delivery vehicles (Cloninger, 2002; Kim and Zimmerman, 1998). They vary from low molecular mass species to a large, protein-like structures. Purely peptidic dendrimers are often synthesised without a core using lysine, as the most common amino acid branching unit in dendrimer chemistry (Kim et al., 1999; Sadler and Tam, 2002). Poly(L-lysine) dendrimers were applied as gene transfection reagents (Ohsaki et al., 2002; Toth et al., 1999). These dendrimers form stable supramolecular complexes with DNA via electrostatic interactions between anionic nucleic acids and a large number of cationic surface amino groups (Choi et al., 2000). Their efficiency of transfection suggests that they can be the next suitable systems for gene delivery in vivo. It is important that when polypeptides are complexed with DNA their cytotoxicity is low (Shah et al., 2000). The ability to bind DNA increases with molecular mass of poly(L-lysine) dendrimers (Männistö et al., 2002) (i.e. number of positively charged amino groups per one polymer molecule). However, low molecular mass peptides have the advantage of being less immunogenic than high molecular mass dendrimers. Poly(L-lysine) dendrimers have been also found to block herpes simplex virus attachment to a cell membrane (Bourne et al., 2000).

Last 15 years brought discovery of an important group of natural compounds named antimicrobial peptides. A number of 10–50 amino acid residues long, very often cationic peptides, have been identified from both vertebrate and invertebrate sources. They act in a variety of ways against many Gram-negative and Gram-positive organisms. The majority of experiments investigating their mode of action have focused primarily on the interaction of cationic peptides with model membrane systems. According to these studies, the accepted mechanism involves permeation and disruption of the target cell membranes. In the global problem of bacterial resistance to the commonly used antibiotics these properties make cationic

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doi:10.1016/j.ijpharm.2005.10.039

peptides valuable candidates as future therapeutic agents to combat bacterial, viral, and fungal infections.

The opportunity that has been recognized immediately, as a consequence of discoveries of these two groups of compounds, was the use of lysine-based dendrimers as carriers for functional groups taken from antimicrobial peptides (Chen and Cooper, 2002). For example, lysine dendrimers have been used as synthetic scaffolds for attachment of two to eight copies of a tetrapeptide R4 (RLYR) or an octapeptide R8 (RLYRKVYG). Both R4 and R8 have been found in protegrins and tachyplesins — natural peptides with antimicrobial activity (Tam et al., 2002). High potency in antimicrobial assays against 10 organisms in high- and low-salt conditions was found in this group of compounds. Another structural approach in this area was construction of low molecular mass lysine-based peptide dendrimers, designed as branched analogues of the natural, cationic antimicrobial peptides (Janiszewska et al., 2003). They represent a new class of membrane-active dendrimers, where dendrimer tree is used not only for multiplication of active elements but also for spatial distribution of cationic and aromatic (hydrophobic) groups, that is essential for interactions with bacterial membranes. These dendrimeric peptides expressed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. Although they are structurally different that any other of the above types of compounds, it appears that both, linear endogenous antimicrobial peptides and their synthetic, branched cationic analogs have ability to disrupt bacterial membranes. This structural dissimilarity raises several questions about mechanism of their interactions with target membranes and other elements constituting living cells. It is particularly interesting if they combine antimicrobial properties of linear peptides and affinity to the molecules of the genetic system (DNA) as other lysine dendrimers.

In this study, a synthesis and antimicrobial activity of six new lysine-based peptide dendrimers R 121, R 131, R 132, R 124, R 155, and R 169 is reported (Fig. 1). Haematotoxicity, in vitro cytotoxicity and genotoxicity of these compounds were examined and will be discussed in correlation with their molecular structure, i.e. degree of branching, types of amino acids used for their synthesis and character of terminal residues.

2. Materials and methods

2.1. Chemicals

Eagle's minimal essential medium (MEM), L-glutamine, trypsin, newborn calf serum and streptomycin were purchased from Gibco (UK). 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), sodium salt of deoxyribonucleic acid from calf thymus (DNA), ethidium bromide (EB), benzylamine, and dimethyl sulfoxide (DMSO) were obtained from Sigma (USA). Nutrient broth was purchased in Biotest AG (Germany). α- And ε-2-chloro-benzyloxycarbonyl-L-lysine (2-CL-Z-Lys), α- and ε-2-benzyloxycarbonyl-L-lysine (Z-Lys and Lys-Z) and *t*-butyloxycarbonyl-protected (Boc-protected) amino acids were purchased from Nova Biochem and Chem-Impex. All other chemicals were of analytical grade. Water used to prepare solutions was double-distilled.

2.2. Dendrimer synthesis

All tested dendrimers were synthesised in solution step-by-step procedure using commercial Boc-Z-or 2-Cl-Z-protected amino acids (Bodanszky and Bodanszky, 1984). For coupling reactions, *N*,*N*′-dicyclohexylcarbodiimide (DCC) in presence of hydroxybenzotriazol (HOBt)and di-phenylethylamine (DIPEA) was used. Hydrochloric acid in acetic acid has been used for Boc-group deprotection. In the last step of synthesis peptides were converted into an amide form by treating methyl asters by ammonia overnight. Crude peptide dendrimers have been purified using gel filtration on Sephadex LH-20 in methanol, followed by preparative HPLC in water/ethyl acetate system. All dendrimers were confirmed to have corrected amino acid analysis molecular weights and purity by ESI-MS technique analysing both molecular peak and fragmentation ions. A list of ESI-MS spectra with peak assignments for all six compounds has been submitted as Appendix B Supplementary materials.

2.3. Cell culture

Chinese hamster cells (B14 cell line) were obtained from Child Health Centre in Warsaw. Monolayer cultures were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum, essential and non-essential amino acids, with 100 U/ml streptomycin and 2 mmol/l L-glutamine. The cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air with more than 95% humidity.

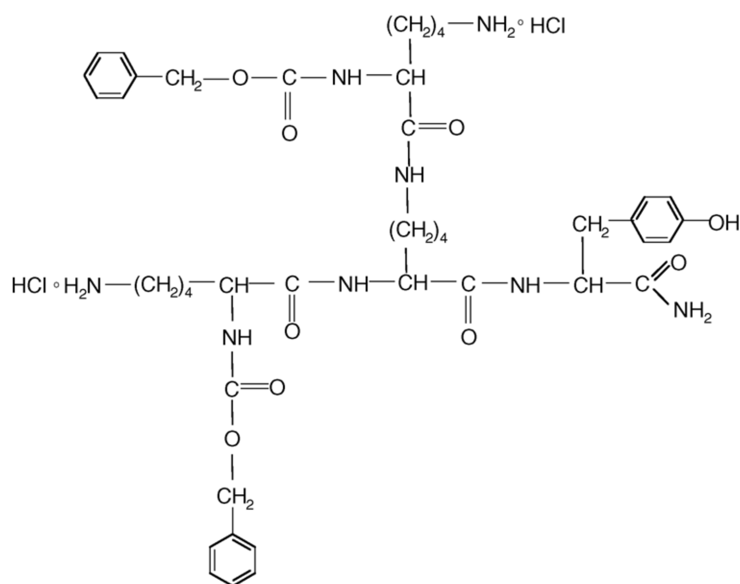
2.4. Cytotoxicity assay

Evaluation of cytotoxicity of dendrimers was assessed with a MTT assay (Hansen et al., 1989). MTT (3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide) is a salt that is oxidized by mitochondrial dehydrogenase in living cells and gives a dark purple formazan product. Damaged or dead cells show reduced or no dehydrogenase activity (Mosmann, 1983).

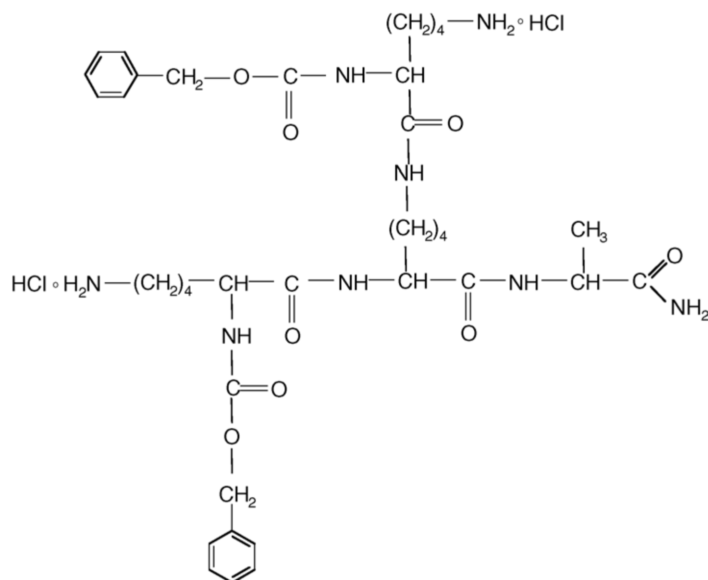
Cells were seeded into 96-well microplates at a density of 3000 cells per well in a growth medium. 24 h after plating, the medium was removed and different concentrations of dendrimers in fresh media were added. Cells were incubated for 1 h with dendrimers. Then the medium was discarded and the cells were washed twice with phosphate-buffered saline (PBS: 150 mM NaCl; 1.9 mM NaH_2PO_4; 8.1 mM Na_2HPO_4; pH 7.4). Next, 50 μmol/l of 5 mg/ml MTT solution in PBS was added to each well. Plates were incubated under cell culture conditions for 4 h. Formazan crystals, formed by living cells, were dissolved by the addition of 100 μl dimethyl sulfoxide (DMSO) to each well. The absorption of samples was measured at 570 nm with a background correction at 630 nm using a microplate reader ELISA. The measurement was performed in triplicate.

Results were demonstrated as a percent of absorbance relative to untreated control cells. The results of the cytotoxicity assay

R 121



R 131



R 132

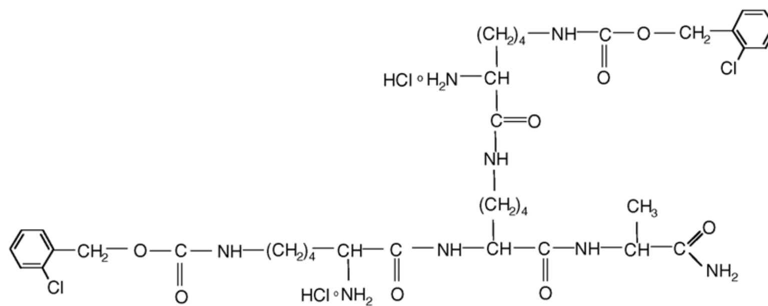
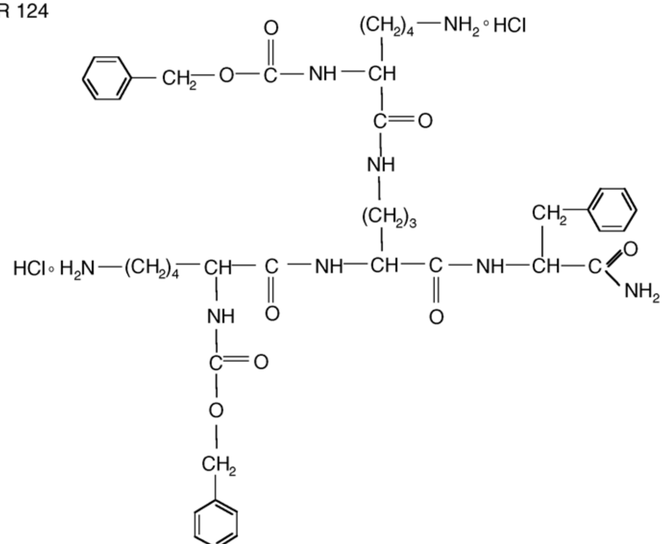
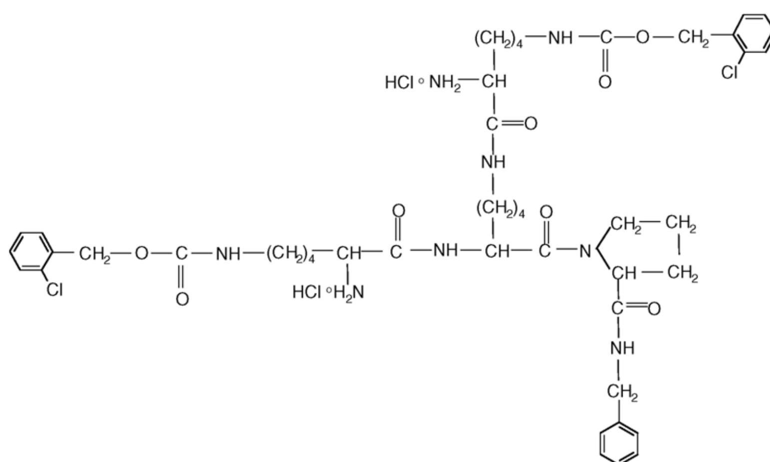


Fig. 1. Structures of dendrimers.





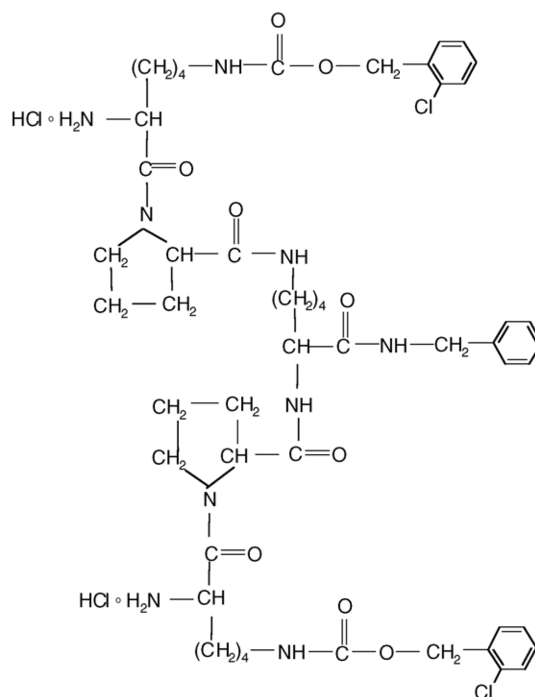


Fig. 1. (*Continued*).

were used for the calculation of cell viability after incubation with dendrimers:

$$\text{viability}\,[\%] = \frac{x}{x_C} \times 100\%,$$

where x is the absorbance in a well containing a particular dendrimer concentration and x_C is the absorbance for untreated control cells.

IC_{50} values (concentration at which 50% inhibition of mitochondrial dehydrogenase activity was measured) were determined.

2.5. Haemolysis test

Blood from healthy donors was obtained from Central Blood Bank in Lodz. Blood was anticoagulated with 3% sodium citrate. Erythrocytes were separated from blood plasma and leukocytes by centrifugation (5000 × *g*, 5 min) at 4 °C and washed three times with phosphate-buffered saline (PBS). Erythrocytes were used immediately after isolation. To study the effect of dendrimers on erythrocyte haemolysis, red blood cells were suspended in dendrimers solutions at a haematocrit of 1% and incubated 0.5 h at room temperature (20 °C). Next, the suspension was centrifuged (1000 × *g*, 5 min). To avoid the influence of mechanical lysis for every series the control samples were made. For reference, red blood cells were treated with double-distilled water, which corresponds to 100% haemolysis. The percentage of haemolysis was determined on the basis of released haemoglobin in supernatants and measured spectrophotometrically from the absorbance at 540 nm:

$$\text{haemolysis}\,[\%] = \frac{y - y_C}{y_{100\%} - y_C} \times 100\%,$$

where y is the absorbance of the samples incubated with dendrimers, y_C, the absorbance for control samples, and $y_{100\%}$ is the absorbance of the reference.

The effect of dendrimers' absorbance at 540 nm did not exceed 1% of the total absorbance.

2.6. Ethidium bromide intercalation assay

Ethidium bromide (1 μg/ml) and DNA (3 μg/ml) were dissolved in 0.05 mol/l Tris–HCl buffer with 50 mmol/l NaCl (pH 7.0).

The fluorescence spectra of ethidium bromide (EB) in the presence of DNA before and after addition of dendrimers were taken with a Perkin-Elmer LS-50B spectrofluorometer. Excitation wavelength of 477 nm was used. The emission spectra were recorded from 490 to 850 nm. The excitation and emission slit widths were set to 10.0 and 5.0 nm, respectively. Samples were contained in 1 cm path length quartz cuvettes and were continuously stirred.

Before examining the fluorescent properties of the DNA–EB–dendrimer aggregate, it was checked that dendrimers do not interact with EB.

2.7. Antimicrobial activity measurements

Antimicrobial activity was assayed against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *Candida albicans* ATCC 20231. To determine the minimum inhibitory concentration (MIC), i.e. the concentration at which 100% inhibition of bacterial growth is observed, the microdilution broth method was used. This is the concentration at which 100% inhibition of bacterial growth is observed.

Cells of each bacterial strain were collected in the logarithmic phase of growth and suspended in nutrient broth. The concentration of colony-forming units (CFU) per milliliter was quantified by measuring absorbance at 600 nm ($A = 0.2$). Dendrimer samples were dissolved in nutrient broth (pH 7.0) and diluted serially. The sample solution (100 μl) was mixed with the diluted bacterial suspension (100 μl). Mixtures containing 105 bacterial CFU and from 1 to 0.003% of tested dendrimers were incubated for 24 h at 37 °C. For microbiological study, indolicidin, Ile–Leu–Pro–Trp–Lys–Trp–Pro–Trp–Trp–Pro–Trp–Arg–Arg–NH_2 has been used as a reference compound (Kowalska et al., 2002) (Table 1).

3. Results

3.1. Antimicrobial activity

Tested dendrimers showed an antimicrobial activity towards used microorganisms. The obtained results are given in Table 1. Depending on the type of dendrimer, the minimum inhibitory concentration (MIC) varied significantly. Usually, for the same type of dendrimer its potency to inhibit different species was similar.

3.2. Cytotoxicity

The MTT assay was chosen to investigate changes in the cell viability on dendrimer addition. The ability of cells to reduce MTT indicates the mitochondrial activity, which in turn may be interpreted as a proof of cell viability.

The strongest influence on cell viability was observed for R 169 and R 155 dendrimers. R 121 Dendrimer was the least cytotoxic. R 131 Dendrimer induced only a moderate decrease in cell viability (Fig. 2).

For all dendrimers the IC_{50} values (concentrations corresponding to 50% inhibition of cell growth) were calculated from semi-logarithmic plot (Table 2).

3.3. Haematotoxicity

Dendrimers impact on human red blood cell haemolytic fragility was checked. The results, presented as the percentage of released haemoglobin, are shown in Fig. 3. The strongest effect was observed for R 155 dendrimer. R 169 and R 132 dendrimers also expressed the haemolytic activity. The rest of tested dendrimers did not cause a loss of membrane integrity, even for high concentrations.

Table 1
Antimicrobial activity of tested dendrimers

Dendrimer		MIC (μmol/l) *S. aureus* ATCC 25923	MIC (μmol/l) *E. coli* ATCC 25922	MIC (μmol/l) *C. albicans* ATCC 20231
R121	Z-Lys(HCl) / Z-Lys(HCl)-Lys-TyrNH_2	662	165	82
R131	Z-Lys(HCl) / Z-Lys(HCl)-Lys-AlaNH_2	368	368	368
R132	HClLys(2-Cl-Z) / HClLys(2-Cl-Z)-Lys-AlaNH_2	40	85	40
R124	Z-Lys(HCl) / Z-Lys(HCl)-Orn-PheNH_2	85	85	85
R155	HClLys(2-Cl-Z) / HClLys(2-Cl-Z)-Lys-Pro-NH-$CH_2C_6H_4$	75	75	75
R169	HClLys(2-Cl-Z)-Pro / HClLys(2-Cl-Z)-Pro-Lys-NH-$CH_2C_6H_4$	68	244	303
Indolicidin		1.5	3.0	–

Values are means of three experiments, error has been estimated as less than 20%, except of for compound R 155 that reached 40% in tests against *C. albicans*.

Table 2
IC_{50} in MTT assay

Dendrimer	IC_{50} (mmol/l)[a]
R 121	1.59 ± 0.18
R 131	1.07 ± 0.15
R 132	0.63 ± 0.10
R 124	0.58 ± 0.14
R 155	0.49 ± 0.16
R 169	0.28 ± 0.08

[a] Results are expressed as means ± S.D. of three experiments.

3.4. Genotoxicity

The ability of dendrimers to interact with DNA was assessed by ethidium bromide displacement assay. There are three main models, which describe binding into DNA: electrostatic binding, groove binding and intercalative binding (Cao and He, 1998). Ethidium bromide (EB) is the most widely used fluorescent probe to study the intercalation. EB is a planar aromatic dye which immediately intercalates between the base pair of DNA and emits intensive fluorescence. It has been reported that the EB fluorescence can be quenched by the addition of a second molecule which competes with EB for the intercalation process (Liu et al., 2002). The extent of quenching is used to determine the ability of the second molecule to intercalate into DNA.

The emission spectra of EB bound to DNA in the absence and in the presence of R 169 dendrimer are shown in Fig. 4. The addition of dendrimer to DNA pretreated with EB caused significant decrease in emission intensity, indicating that dendrimer competes with EB for binding to DNA. Moreover, the reduction of emission intensity was accompanied by the red shift of emission maximum from 605 to 615 nm for the highest con-

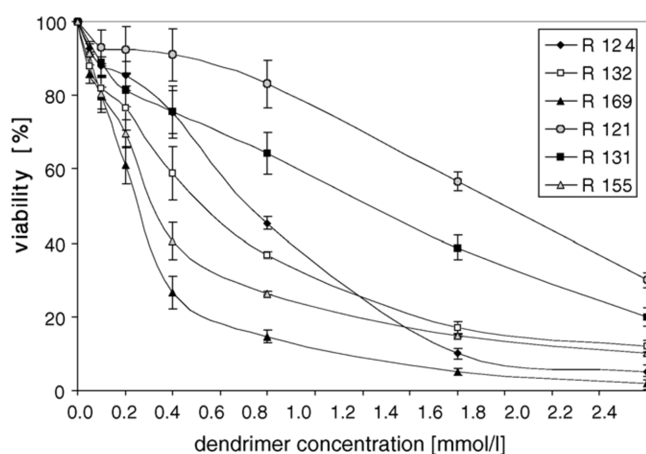


Fig. 2. Effect of dendrimers on the cell viability.

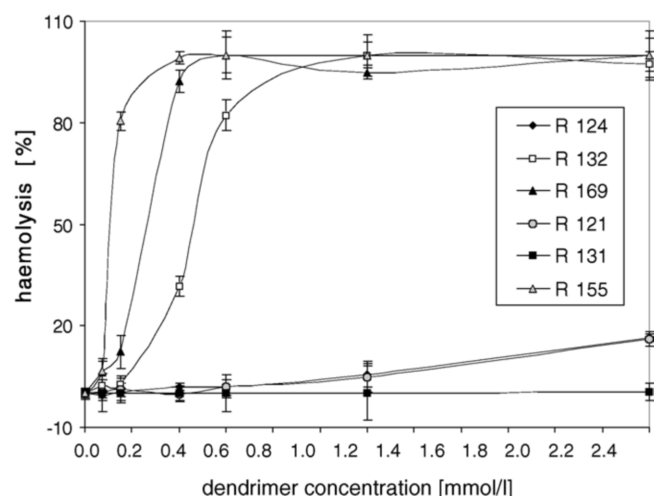


Fig. 3. Dendrimer-induced haemolysis.

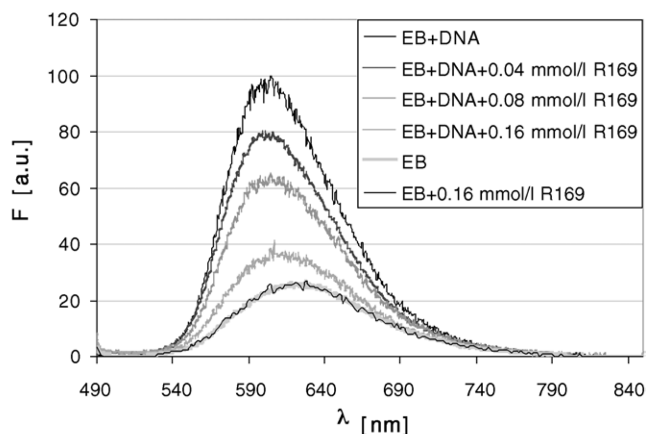


Fig. 4. Emission spectra of ethidium bromide.

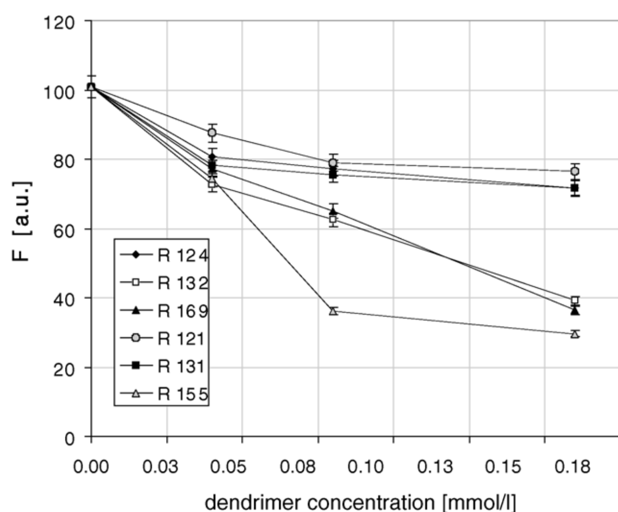


Fig. 5. Ethidium bromide exclusion assay.

centration. The positions of the fluorescence maximum for EB before and after addition of DNA were 625 and 605 nm, respectively. Thus, the observed red shift indicates that, in the presence of dendrimer, the unbound EB had the bigger contribution to the total fluorescence. To exclude the interactions between EB and dendrimers, it was checked that dendrimers, even for the highest used concentrations, did not affect the shape of the EB spectrum.

The same studies were performed for other dendrimers. The obtained results showed that the intercalation to DNA was the strongest for R 155 dendrimer, followed by R 169 and R 132 dendrimers. The rest of dendrimers had only a slight ability to exclude EB from DNA (Fig. 5).

4. Discussion

Over the last years commonly used antibiotics have been progressively demonstrating a decreased efficacy. This stimulated a worldwide search for new types of antimicrobial therapies. Currently observed increased interest in peptide antibiotics was stimulated by the discovery of natural antimicrobial peptides. All higher organisms naturally produce a large number of linear peptides acting as non-specific support of their defence systems (Papo and Shai, 2003). Many of these peptides are positively charged due to presence of basic aminoacids — lysine and arginine, in topographical relations with hydrophobic (aromatic) aminoacids. Although their role is not yet fully understood, they act by disrupting negatively charged bacterial membranes to which they are attracted by electrostatic and hydrophobic forces (Lien and Lowman, 2003). According to the proposed mechanism, after binding to the external surface of a cell membrane, they can form channels, micelles or planar aggregates (“carpet mechanism”) (Powers and Hancock, 2003). It leads to a leakage of cytoplasmic molecules and cell death (Hancock, 1997).

A small library of branched peptides was designed with varying spatial arrangement of cationic and aromatic centres. The tested peptide dendrimers expressed ability to inhibit growth of both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria, as well as a fungal pathogen (*C. albicans*). We observed that dendrimers showed similar potency towards both types of bacteria, except for R 121 dendrimer that was more active against Gram-negative bacterium (*E. coli*). Gram-negative bacteria differ from Gram-positive ones by possessing an outer membrane in addition to a common cytoplasmic membrane. It makes them more resistant to most antibiotics. These observations are in a good agreement with earlier results stating that cationic peptides often act stronger against Gram-negative bacteria than Gram-positive ones (Sawyer et al., 1988).

If clinical applications of peptide dendrimers is considered, the lack of systemic toxicity is as important as a broad-spectrum of antimicrobial activity. Therefore, cytotoxic, haematotoxic, and genotoxic effects of the tested dendrimers were studied. The decrease in a metabolic activity was chosen as an indicator of cell viability. Dendrimers induced variable impacts on cell viability. Usually, the results were consistent with the antimicrobial activity. The higher antimicrobial activity was expressed, the stronger cytotoxic effect was observed. The most promising behaviour was observed for R 121 that showed low cytotoxicity even at higher concentrations. The cell viability decreased sharply with increasing concentration for R 169, R 155, and R 132 dendrimers. Nevertheless, most dendrimers caused only a slight (ca. 20%) decrease in cell viability for minimum inhibitory concentrations (Fig. 6).

Although there are plenty of mechanisms of killing cells, two main targets are the most important on the cellular level: a membrane and a nucleus. An erythrocyte is a good model for studying alterations in membrane conditions. The loss of the integrity of red blood cell membrane is accompanied by a leakage of haemoglobin. The strongest haemolytic effect was observed for R 155 dendrimer. The concentration causing 50% haemolysis was even lower than MIC. It can make R 155 dendrimer not suitable for in vivo applications. On the other hand, three of the tested dendrimers were not haemolytic in a broad concentration range. It inclined us to check dendrimers' impact on DNA. First, the comet assay was employed to study their genotoxic effects. Comet assay allows measuring DNA damage by analysing DNA migration upon electrophoresis (Singh et al., 1988). The tail moment, calculated by multiplying of the tail length and the percentage of DNA in the tail, is a good indicator of DNA damage. We observed a significant increase in the tail

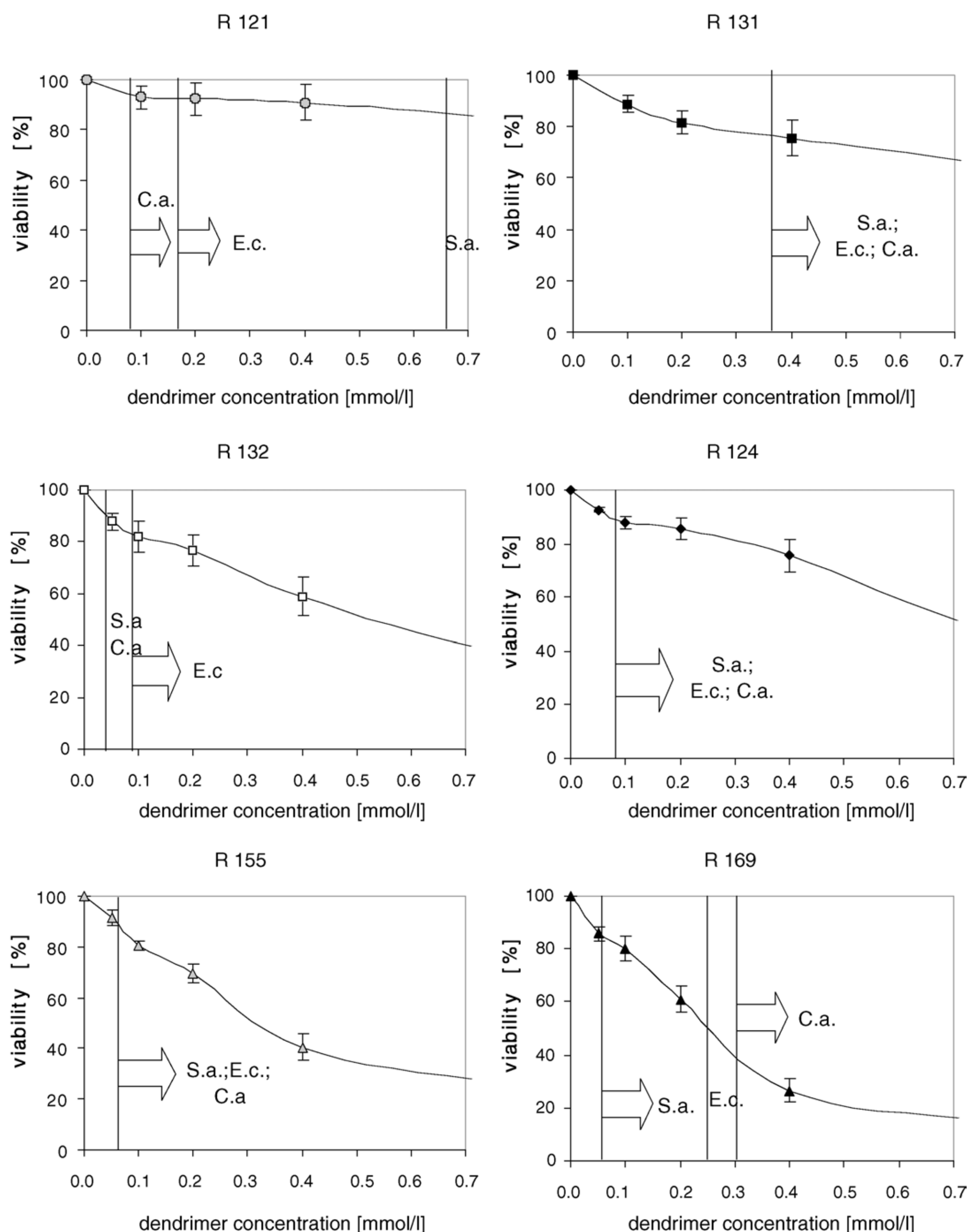


Fig. 6. Comparison of dendrimers impact on the cell viability estimated by MTT assay and the minimum inhibitory concentrations shown as vertical lines. C.a.: *C. albicans*, E.c.: *E. coli*, S.a.: *S. aureus*.

moment after incubating cells with dendrimers in comparison with the untreated control cells (results not shown). It means that dendrimers interacted with DNA and caused its fragmentation. However, because dendrimers are large molecules, they can change efficiency and speed of the migration of DNA fragments upon electrophoresis. Thus, electrophoretic methods were not suitable for quantitative assay of DNA damage.

We decided to characterise the interactions between dendrimers and DNA by using a fluorescent probe — ethidium bromide (EB). EB intercalates to DNA. This association involves an insertion of planar aromatic rings between DNA base pairs. Intercalation destroys the regular helical structure of DNA. This is the first step that leads to DNA damage and many antitumour drugs act according to this mechanism. They stop cancer cell proliferation by disrupting DNA (Song et al., 2002). After addition of dendrimers release of EB from DNA was observed. Moreover, addition of EB to already formed DNA–dendrimer complex gave the same absorbance. It means that dendrimers competed with EB for the same binding sites in DNA molecule and that EB was partially substituted by dendrimers. It is worth noticing that peptide dendrimers are bigger molecules than EB, so probably partial intercalation via aromatic rings was accompanied by electrostatic interactions and hydrogen bond formation. In fact, the most toxic in all tests were deriva-

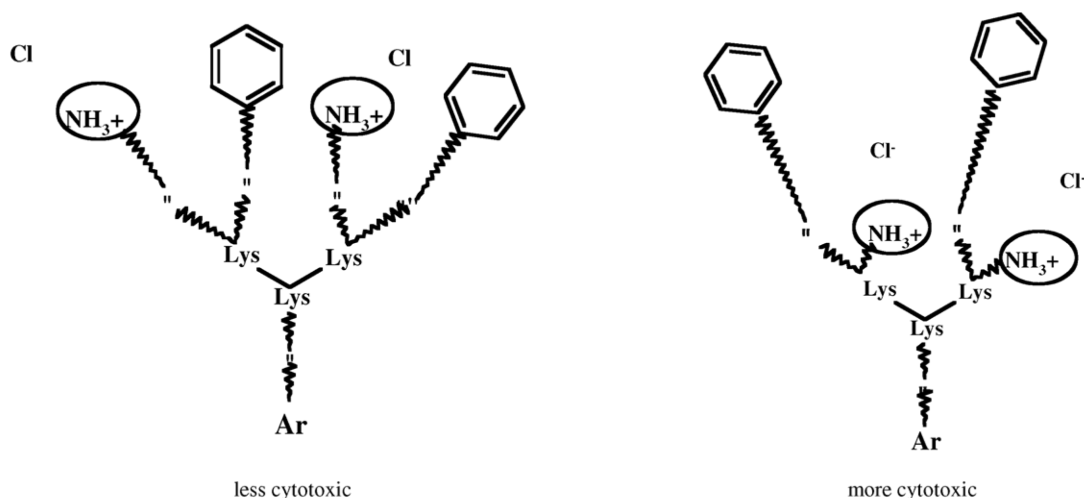


Fig. 7. Molecular simulation for compounds R 131 (α-amino group protection) and R 132 (ε-amino group protection).

tives with protected ε-amino groups, particularly these with 2-chlorobenzyloxycarbonyl group. Derivatives with α-amino group protected by benzyloxycarbonyl (Z) were much less toxic. Molecular simulation performed for compound R 131 and R 132 showed that aromatic rings responsible for interactions with lipophylic components of a bacterial membrane are better exposed in derivatives protected at ε-amino groups (Fig. 7).

Results obtained from several independent assays allow for concluding that tested dendrimers can be divided into two groups. The R 169, R 155 and R 132 dendrimers are more toxic (ε-protection), whereas R 131, R 124 and R 121 dendrimers show only a slight toxicity (α-protection). Table 1 shows schematic structures of all dendrimers. It is also possible that quite strong toxicity of R 169 and R 155 dendrimers is due to a presence of L-proline residue. Further in vivo studies are needed to estimate the biocompatibility of tested dendrimers. To summarise, dendrimers showed different level of cytotoxicity, haematotoxicity, and genotoxicity. The observation that the cationic peptides induced haemolysis or were cytotoxic is not surprising as the toxicity of polycations is well documented (Carreno-Gomez and Duncan, 1997; Malik et al., 2000). However, in our case the presence of amino groups does not seem to be the main reason of the toxicity, because all dendrimers had similar number of protonated lysine amino groups (and charge). It appears that steric distribution and type of hydrophobic (aromatic) groups and cationic centres are important components of dendrimeric structure and influence both antimicrobial potency and toxicity. Such 3D structure of our dendrimers mimics that of the natural antimicrobial peptides interacting with biological membranes and can be achieved by application of dendrimer chemistry.

Acknowledgements

Authors thanks Ms. Hanna Rybak for an excellent technical assistance. Synthesis and purification of the tested compounds were supported by the State Committee for Scientific Research, Grant No. 3T09A 149 19. The work was supported by University of Lodz, Grant No. 505/440.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2005.10.039.

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