Chapter 6

N-Phosphoamino Acids
as Potential Drug Delivery System

After introducing a new class of amphiphiles, namely \(N\)-phosphoamino acids, and studying the aggregation behavior, we demonstrate in this chapter their potential application as drug delivery system. Towards this goal an investigation of release mechanisms and cytotoxicity was performed. In the latter study the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used demonstrating high biocompatibility of representative \(N\)-phosphoamino acids. Moreover, open vesicles were observed for \(N\)-phosphoamino acids carrying no residue in the \(\alpha\)-position. This degradation of the aggregates, caused by self-activation of the \(N\)-phosphoamino acids, might be used in further studies towards a time-dependent substrate release mechanism.
6.1 Introduction

Liposomes are artificial vesicles which were reported for the first time by Bangham et al. in 1965.\textsuperscript{1} They introduced liposomes as a model for cell membranes for biophysical studies. Moreover, in the 1970s liposomes were employed as artificial transport systems for the first time. As shown in Figure 6.1.1, liposomes can act as hosts for hydrophobic, hydrophilic and amphiphilic guest molecules.\textsuperscript{2}

![Figure 6.1.1: Schematic representation of a liposome carrying hydrophilic, hydrophobic and amphiphilic compounds.](image)

The first generation of the host-guest systems shown in Figure 6.1.1 was based on naturally occurring phospholipids that show high biocompatibility.\textsuperscript{3,4} However, these carrier systems did not allow a controlled release of the transported compound, since they were detected rapidly by the immune system resulting in immediate release of the guest molecules (Figure 6.1.2).

Nowadays, liposomes are common drug carrier systems. However, depending on the indication of the transported drug an immediate or delayed release is necessary. As shown in Figure 6.1.2, both strategies result in high plasma drug concentrations, preferably lower than the minimal toxic concentration (MTC), and short life times. However, to allow a treatment over time constant plasma drug concentrations above the minimal effective concentration (MEC) and below MTC are essential. This can be obtained by a continuous or controlled release mechanism (Figure 6.1.2).

In order to influence the life time of liposomes and control the drug release several studies were performed over the last half century.\textsuperscript{6-12} It was found that the clearance of drug delivery systems is caused by the mononuclear phagocyte system (MPS) of the immune system, which is triggered by surface charge, saturation, size and composition of the liposome.\textsuperscript{6-8} To understand the influence of these parameters different studies were performed demonstrating that the circulation time is strongly dependent on the size of the liposomes. In this regard, a decrease of the life time of the carrier was observed with increasing size of the liposome. In contrast, small carriers were able to pass the endothelium leaving the blood stream or were taken up in shorter time by hepatocytes (liver cells).\textsuperscript{6,7} Based on these results an optimal size of about 100 nm in diameter was determined, allowing longer circulation time and delayed release of the drug (Figure 6.1.2).\textsuperscript{9,10}
Another important parameter influencing the circulation times of transport systems are the composition of liposomes and their surface charge\textsuperscript{11-15}. It was shown that negatively charged liposomes are detected by the MPS more rapidly than positive charged liposomes\textsuperscript{11-13}. However, in further studies longer circulation times were also observed for negatively charged transport systems\textsuperscript{14,15}. This was achieved by changing the molecular structure of the lipid and shielding of negative charges on the liposome. For example, the latter strategy was achieved by coating the negatively charged liposome surface with hydrophilic polymer structures, such as poly(ethylene glycol) (PEG) or palmitoylglucuronide\textsuperscript{16,17}.

In 1991 Papahadjopoulos \textit{et al.} demonstrated the prolongation of the circulation time and the decreased uptake of PEGylated liposomes (stealth liposomes)\textsuperscript{18}. During the following year Lasic \textit{et al.} encapsulated successfully the cancer drug doxorubicin applying stealth liposomes\textsuperscript{19}. This research lead to the first by the FDA approved PEGylated liposome as drug delivery system for doxorubicin in 1995 (DOXIL in US and Caelyx in Europe).

In order to use the N-phosphoamino acids 1 introduced in chapter 5 as drug delivery systems several aspects have to be explored. Therefore in this chapter experimental results are reported demonstrating the high biocompatibility of the amphiphiles and introducing possible drug release mechanisms. However, considering the negative charge of these compounds and the above mentioned literature\textsuperscript{11-15} it is also of high interest to investigate the mixing behavior as well as possible modifications of the liposome surface. Approaches, demonstrating simple modification methods of N-phosphoamino acids 1 are explored in Chapter 7 giving excess to more stable negatively charged dipeptides as head group on one hand and showing simple modification methods on the other hand.
6.2 Results and Discussion

6.2.1 Biocompatibility of \(N\)-Phosphoamino Acids

One of the most important requirements for drug delivery systems is the biocompatibility of the amphiphile. Therefore, not only the surfactant itself but also its metabolic products need to be considered. In this regard, \(N\)-phosphoamino acid 1 seems to be promising class of amphiphiles, since they consist only of naturally occurring building blocks and their degradation results in amino acids 2, phosphates 3 and alcohols 4 (Scheme 6.2.1).

![Scheme 6.2.1: Metabolic degradation of \(N\)-phosphoamino acids 1.](image)

To study the biocompatibility of \(N\)-phosphoamino acids 1 we employed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In 1953, MTT 5 was used for the first time by Black and Speer for chemosensitivity tests (Scheme 6.2.2).\(^{20}\) Thirty years later Mosmann reported the application of MTT 5 in a colorimetric assays to study cellular growth and survival in presence of different compounds reflecting their cytotoxicity.\(^{21}\) During the following decades a number of reports have been published confirming the correlation of the results obtained by MTT assays with clinical studies.\(^{21-23}\)

As shown in Scheme 6.2.2, this toxicity test is based on an enzymatic modification of MTT 5 in living cells. Hereby, a reduction of the yellow MTT 5 by a reductase takes place resulting in the water insoluble and blue formazan 6 (Scheme 6.2.2). Further, the amount of formazan 6 formed by the reductase is proportional to the number of metabolically active cells and can be determined through UV/Vis-spectroscopy (absorbance at 570 nm).

![Scheme 6.2.2: Enzymatic reduction of MTT 5 resulting in formazan 6.](image)

To investigate the cytotoxicity of \(N\)-phosphoamino acids 1, HeLa (Henrietta Lacks) cells were employed. For this study, amphiphiles 1d, 1i and 1m were chosen carrying alanine, aspartic
acid and tryptophan as amino acid head group, respectively. *HeLa* cells were incubated together with each *N*-phosphoamino acid 1 for 16 hours at 37 °C. Subsequently, MTT 5 was added and the resulting solution was incubated for an additional four hours. Finally, to determine the cell viability the formed amount of water insoluble formazan 6 was dissolved in DMSO and the absorbance was measured at 570 nm. In Figure 6.2.1, the results of the MTT assay is shown using of *N*-phosphoamino acid 1d with a concentration ranging from 0.25 µM to 400 µM. Like expected a high cell viability ranking from 94% to 100% was observed in the presence of compound 1d. The graph (Figure 6.2.1) shows high biocompatibility for amphiphile 1d up to a concentration of 100 µM. Corresponding results were obtained for *N*-phosphoamino acids 1i and 1m showing a cell viability of more than 80% at concentrations of up to 100 µM. (Figure 6.2.2).

![Figure 6.2.1: MTT assay using *N*-phosphoamino acid 1d. Cell viability is given for 1d at concentrations between 0.25 µM and 400 µM. Each experiment was performed three times.](image)

![Figure 6.2.2: MTT assay using *N*-phosphoamino acid 1i (left) and 1m (right). Cell viability is given for both compounds, 1i and 1m, at concentrations of 50 µM and 100 µM. Each experiment was performed three times.](image)
6.2.2 Potential Drug Release Mechanism

After high biocompatibility of \( N \)-phosphoamino acids 1 was successfully demonstrated the potential release mechanisms for aggregates formed by these amphiphiles was investigated. First, pH-dependent aggregation of 1 was investigated by cryo-TEM. Dependent of the pH value a different protonation state of the acid moiety of the head group of 1 can be expected resulting in changes of the aggregation behavior or even clearance of the aggregate. Therefore, it is necessary to understand the aggregation behavior of \( N \)-phosphoamino acids 1 at pH-values close to the physiological pH of blood, i.e. 7.4.\(^{24}\) Considering additionally the pK\(_a\)-values of the amphiphiles 1 which are located between 6.1 and 8.9 for the carboxylic acid group (Chapter 5.2.2), aggregation studies were performed at pH 1, 6.5, 8.5 and 11 (Table 6.2.1). Whereas one can expect full protonation and loss of the amphiphilic character under slightly acidic condition (<7), full deprotonation is given in basic conditions (pH 11). For these aggregation studies we employed \( N \)-phosphoamino acids 1a, 1i and 1m exhibiting L-phenylalanine, L-aspartic acid and L-tryptophan as amino acid head group, respectively, as representatives.

As shown in Table 6.2.1, no aggregation was observed for any of those amphiphiles 1 under very acidic conditions, i.e. pH 1. This is based on the full protonation of the acidic moiety resulting in the loss of the amphiphilic character of the amphiphile. In contrast, aggregates were observed for all \( N \)-phosphoamino acids, i.e. 1a, 1i and 1m, at higher pH (Table 6.2.1). As shown in Figure 6.2.3a and Table 6.2.1, amphiphile 1a forms between pH 6.5 and 11 the same aggregates, namely vesicles. However, 1m form additionally sheets at pH 6.5, while at higher pH exclusively vesicles were observed (Figure 6.2.3b). This can be traced back to the fact that 1m has a slightly higher pK\(_a\) value compared to 1a giving rise to a lower number of charges per amphiphile. Consequently, the head group of 1m owns a smaller effective head group area (a\(_0\)) and therefore, has almost a cylindrical shape resulting also in sheets at pH 6.5.

<table>
<thead>
<tr>
<th>amphiphile</th>
<th>Amino acid</th>
<th>pK(_a)</th>
<th>pH 1</th>
<th>pH 6.5</th>
<th>pH 8.5</th>
<th>pH 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>L-Phe</td>
<td>6.66</td>
<td>-</td>
<td>vesicle</td>
<td>vesicle</td>
<td>vesicle</td>
</tr>
<tr>
<td>1m</td>
<td>L-Trp</td>
<td>6.94</td>
<td>-</td>
<td>vesicle,sheets</td>
<td>vesicle</td>
<td>vesicle</td>
</tr>
<tr>
<td>1i</td>
<td>L-Asp</td>
<td>6.83 and 8.76</td>
<td>-</td>
<td>vesicle</td>
<td>wormlike micelles</td>
<td>bicelles</td>
</tr>
</tbody>
</table>

All samples were prepared using the thin layer technique followed by hydration with 20 mM buffer solution and three freeze thaw cycles. Aggregates observed from an aqueous buffered solution at room temperature.

The largest changes in aggregation, depending on the pH value, were observed for \( N \)-phosphoamino acid 1i carrying two carboxylic acid moieties at the head group. As shown in Table 6.2.1 and Figure 6.2.3c, unlike 1a and 1m amphiphile 1i forms at pH 8.5 and 11 micelle-based aggregates. The reason is the presence of a number of charges higher than one per
molecule 1i resulting in a bigger $a_0$ than for 1a and 1m and a cone shaped amphiphile. In contrast, at a pH of 6.5 vesicles for 1i were observed (Table 6.2.1 and Figure 6.2.3d). Under slightly acidic conditions one carboxylic acid group ($pK_a$ 8.8) is fully protonated, while the other ($pK_a$ 6.8) is only partly deprotonated. This protonation state results in almost the same number of charges per molecules as for 1a and 1m. Hence, at this pH all three amphiphiles have a truncated cone shape resulting in vesicles (Table 6.2.1).

Figure 6.2.3: (a) Cryo-TEM image of 1a at pH 6.5. (b) Cryo-TEM image of 1m at pH 6.5. (c) Cryo-TEM image of 1i at pH 11 (white arrow indicate bicelles) and schematic representation of a bicelle. (d) Cryo-TEM image of 1i at pH 6.5.
The pH-dependent aggregation and the fact that at pH 1 no aggregation for \(N\)-phosphoamino acids \(1\) can be observed and offers the possibility for fast drug release under acidic conditions, i.e. endocytosis. During endocytosis the pH value drops from 6.5 to pH 4.5\(^{25}\) which would cause full protonation of the amphiphiles \(1\) head group resulting in clearance of the liposome and drug release.

A second potential release mechanism is based on the formation of open vesicles of \(N\)-phosphoamino acids \(1\). As shown in Figure 6.2.4a-c, for amphiphiles \(1b, 1c\) and \(1d\) carrying glycine, beta-alanine and alanine as head group, respectively, open vesicles were observed.

![Figure 6.2.4: Cryo-TEM image of \(1b\) (a), \(1c\) (b) and \(1d\) (c) showing open vesicles. (d) Schematic representation of the aggregation of single and double tailed amphiphiles leading to open vesicles (left) and the formation of open vesicle which proceeds through partial hydrolysis of double tailed amphiphiles caused by self-activation (right). White arrow indicates opening.](image)

As reported by Stuart et al.,\(^{26}\) open vesicles can be obtained via mixing single-tailed and double-tailed amphiphiles (Figure 6.2.4d, left). Hereby, the double-tailed surfactant owning a truncated cone shape forms the lipid bilayer, while the amphiphile exhibiting only one hydrophobic tail and a cone shaped structure causes an end-capping of the bilayer, as shown in Figure 6.2.4d (left). Considering the packing parameters for \(1b, 1c\) and \(1d\) (Chapter 5.2) it is expected that those amphiphiles have a truncated cone shape and form only closed vesicles. However, \(N\)-phosphoamino acids exhibiting a small residue at the amino acid head group can degrade to single-tailed amphiphiles, what could result in opening of vesicles as observed for \(1b, 1c\) and \(1d\) (Figure 6.2.4a-c). As described by Ni et al. in 2009,\(^{27}\) hydrophobic tails can be cleaved stepwise from \(N\)-phosphoamino acids \(1\) by a self-activated hydrolysis. As shown in Scheme 6.2.3, the first aliphatic alcohol \(7\) is cleaved via cyclization of \(1\) resulting in a mixed anhydride \(8\). Then, by subsequent hydrolysis of \(8\) the single-tailed \(N\)-phosphoamino acid \(9\) is obtained owing a cone shaped structure giving rise to end-capped bilayers (Figure 6.2.4a-c). Repetition of both steps results in cleavage of the second tail giving an additional alcohol \(7\) and the
phosphoamide 11 via cyclic intermediate 10 as products (Scheme 6.2.3). While the first cleavage of 7 will lead to open vesicles the subsequent formation of 10 would lead to clearance of the liposome.

Both cleavage steps shown in Scheme 6.2.3 would result in a drug release from liposomes formed by 1b, 1c and 1d.

Scheme 6.2.3: Self-activated cleavage of hydrophobic tails from N-phosphoamino acids 1 resulting in the formation of lauryl alcohol 7 (ROH=C12H25OH).

The formation of lauryl alcohol 7 from N-phosphoamino acid 1d was proven by NMR-spectroscopy (Figure 6.2.5). Hereby, the liposomes formed by 1d were stored at room temperature in an aqueous buffered solution (pH 8.5) for two weeks and the formed alcohol 7 was extracted from the aqueous solution after acidification. The comparison of the NMR (Figure 6.2.5a and b) spectra of 1d and the extracted compound confirms the cleavage of 7 under the applied conditions for aggregation. As shown in Figure 6.2.5 (2), no N-phosphoamino acid 1d is present in the extracted mixture suggesting the formation of phosphoamide 10 (Scheme 6.2.3). The formation of lauryl alcohol was confirmed against independent sample of this compound and by comparison with literature data.

Figure 6.2.5: (1) 1H-NMR of 1d. (2) 1H-NMR of 1d after storing for 3 days under basic aqueous condition showing a mixture of 1d and lauryl alcohol 7. Solvent impurity: Ethanol: m represents the CH2 group at 3.72 ppm. (3) 1H-NMR of 1d after storing for 2 weeks under basic aqueous condition showing mainly lauryl alcohol 7.
In contrast to amphiphile 1b, 1c and 1d no open vesicles were observed during cryo-TEM measurements for freshly preparation sample of N-Phosphoamino acids 1a, and 1e - r carrying a larger residue at the α-position. However, $^1$H-NMR as well as $^{13}$C-NMR studies show clearly the degradation of these compounds after longer storing under inert atmosphere ($N_2$) and low temperatures (4 °C) (Figure 6.2.6). Again lauryl alcohol 7 was observed as the main product pointing to a similar hydrolysis mechanism. This suggests a slower hydrolysis based on self-activation resulting in more stability of the observed aggregates. Hence, further studies need to be performed to understand the time- and temperature-dependent self-activation of $N$-phosphoamino acids 1. This investigation could reveal impressive ways to tune release rates by choosing the right amino acid head group.

Figure 6.2.6: (1) $^1$H-NMR of 1m. (2) $^1$H-NMR of 1m after storing for approximately three month under inert atmosphere ($N_2$) and 4 °C. Solvent impurity: Ethanol: n represents the CH$_2$ group at 3.72 ppm.
6.3. Conclusion

In this chapter, the potential applicability of \( N \)-phosphoamino acids 1 as drug delivery systems was investigated. First, it was demonstrated that these newly introduced amphiphiles show high biocompatibility. Employing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays high HeLa cell viability was shown in the presence of three \( N \)-phosphoamino acids carrying aliphatic, aromatic and negatively charged residues at the amino acid head group. It needs to be emphasized that all tested amphiphiles own high biocompatibility using concentrations up to 100 \( \mu \)M allowing full cell viability. The low toxicity is based on the fact that \( N \)-phosphoamino acids 1 exhibiting only natural occurring building blocks, i.e. amino acid, phosphate and aliphatic alcohols.

Second, we demonstrated different potential release mechanisms for \( N \)-phosphoamino acids 1. The first strategy is based on the protonation of the negatively charged head group of surfactant 1 resulting in the loss of the amphiphilic character and the clearance of the liposome under acidic conditions. This behavior suggests a fast drug release during endocytosis. A more interesting release mechanism was observed for \( N \)-phosphoamino acid 1 carrying small amino acids, i.e. glycine, alanine and beta-alanine, as head group. Employing cryo-TEM and NMR studies it was shown that these \( N \)-phosphoamino acids 1 degrade leading to open vesicles. This degradation is based on a self-activated cleavage of the amphiphilic tail resulting in mono-tailed \( N \)-phosphoamino acids allowing opening of the liposome.

Considering the facile synthesis, the high biocompatibility and the potential release mechanism, we can conclude that \( N \)-phosphoamino acids 1 could be promising candidates for future drug delivery systems.
6.4 Experimental Section

6.4.1 Materials and Methods

All chemicals and reagents were purchased from commercial suppliers (Acros and Sigma-Aldrich) and used without further purification. All buffers were prepared using double distilled water. 

\textsuperscript{1}H- and \textsuperscript{31}P-NMR- spectroscopy were recorded on an Agilent 400 using CDCl\textsubscript{3}.

A pH-meter (Hanna Instruments pH 209) equipped with a glass combination electrode was used for pH adjustments of the reaction buffers.

Cryo TEM images were taken on an FEI Tecnai T20 transmission electron microscope and on a Philips CM12 cryo transmission electron microscope operating at 200 and 120 kev, respectively. Images were recorded on a slowscan CCD camera under low-dose conditions. Synthesis and characterization of all amphiphiles used here are given in chapter 5.

Cryo-TEM

Sample preparation: After dissolving N-phosphoamino acid in chloroform the solvent was carefully evaporated under a slow stream of nitrogen and continuing rotation. The resulting thin film was stored for 30 min under high vacuum to assure the complete removal of organic solvents. Next an aqueous buffered solution was added to a final concentration of 3 mg/mL. The resulting sample was vortexed to obtain a homogenous suspension. Finally three freeze-thaw cycles were performed to enable aggregate formation. The resulting turbid solution was used for cryo-TEM measurements.

Sample preparation:

pH 1 was adjusted by using a 1 M aqueous HCl solution to a 4 mg/ mL solution of the N-phosphoamino acid in water. The resulting solution was diluted with water to a final concentration of 3 mg/ mL.

Buffer preparation:

e pH 6.5 (20 mM Phosphate buffer): 0.76 mL of a 1 M aqueous K\textsubscript{2}HPO\textsubscript{4} solution was added to 1.24 mL of a 1 M aqueous KH\textsubscript{2}PO\textsubscript{4} solution. The pH and concentration of the resulting solution was adjusted by adding a 1M aq. KOH solution and dilution to a total volume of 100 mL.

pH 8.5 (20 mM TRIS buffer): 1.21 g of tris(hydroxymethyl)aminomethane was dissolved in 500 mL water and the pH was adjusted by adding 1M aqueous HCl solution. Afterwards water was added to obtain a total volume of 500 mL.

pH 11 was adjusted by using a 1 M aqueous NaOH solution to a 4 mg/mL solution of N-phosphoamino acid in 10 mM aqueous NaOH (20 mM NaOH for 1i). The resulting solution was diluted with water to a final concentration of 3 mg/ mL.
6.4.2 Cytotoxicity assay

Cytotoxicity assay: MTT assay\(^2\)

DMEM: Commercial available Dulbecco’s Modified Eagle’s Medium (DMEM) was supplemented with 10% fetal calf serum, penicillin (100 units/ mL), streptomycin (100 µg/ mL) and L-glutamine (2mM).

Sample preparation: The sample was prepared according to the procedure for cryo-TEM to ensure the aggregate formation. Instead of a buffered aqueous solution DMEM was used for the stock solution. All concentrations were obtained diluting a fresh prepared 3 mM stock solution with DMEM.

Cell line: HeLa cells were grown in DMEM and cultured at 37 °C, 5% CO\(_2\) and 95% relative humidity.

MTT assay: HeLa cells were dispensed in a sterile 96-well plate at a cell density of 10,000 cells/ well and were incubated for 8 hat 37 °C, 5% CO\(_2\) and 95% relative humidity. Afterwards, the N-phosphoamino acid solution was added to the cells at various concentrations and was incubated for 16 h. Then MTT was added to a final concentration of 0.5 mg/ mL in each well and the resulting solution was incubated for additional 4 h. Finally, all medium was removed and 100 µL of DMSO was added to dissolve the created formazane. The absorbance of the DMSO solution was measured at 570 nm using a microplate reader (SynergyMX, BioTek). Cell survival was expressed as a relative viability of cells compared to control cultures that were incubated with medium only.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>0.25 mM*</th>
<th>2.5 mM*</th>
<th>10 mM*</th>
<th>25 mM*</th>
<th>50 mM*</th>
<th>100 mM*</th>
<th>200 mM*</th>
<th>400 mM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>L-Phe</td>
<td>99.10</td>
<td>96.27</td>
<td>-</td>
<td>107.59</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10.23)</td>
<td>(4.09)</td>
<td></td>
<td>(9.49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>L-Ala</td>
<td>102.70</td>
<td>99.03</td>
<td>108.62</td>
<td>109.27</td>
<td>94.51</td>
<td>105.82</td>
<td>53.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.85)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1i</td>
<td>L-Asp</td>
<td>110.17</td>
<td>109.46</td>
<td>-</td>
<td>102.06</td>
<td>101.59</td>
<td>84.28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.10)</td>
<td>(11.74)</td>
<td></td>
<td>(15.37)</td>
<td>(18.48)</td>
<td>(16.67)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12.68)</td>
<td>(10.68)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1m</td>
<td>L-Trp</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100.48</td>
<td>99.00</td>
<td>-</td>
</tr>
</tbody>
</table>

Cell survival given in % compared to control samples that were incubated only with DMEM. All given data are the averages of three separate experiments. Standard deviation is given in brackets. * Final concentration during the 16 h of incubation.
6.5 References


