Comparison of cytotoxic properties of free and liposomal bisphosphonates in vitro

Master Thesis

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Comparison of cytotoxic properties of free and liposomal bisphosphonates in vitro

Diploma Thesis

by

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TABLE OF CONTENTS

1   ABSTRACT

Anti-cancer drugs were originally developed in order to directly kill tumour cells. As the most different characteristics of cancerous cells from those of normal cells are their accelerated growth, cytotoxic drugs against rapid proliferating cells are common drugs used in cancer treatment. However, these chemotherapeutic agents also cause severe damage to all fast growing non-tumour cells.

With the discovery of the tumour associated macrophages (TAMs), a new therapeutic drug target for tumour treatment has been found as these cells are part of the tumour microenvironment and indirectly support tumour growth and metastasis.

In this project bisphosphonates, agents originally used for the treatment of diseases involving excessive bone resorption, were tested on their selective toxicity on murine peritoneal macrophages *in vitro*. As macrophages are known to have phagocytic activity, also liposomal bisphosphonate preparations were investigated.

The obtained results demonstrate that the bisphosphonates tested have a cytotoxic effect on tumour as well as on endothelial cells. The nitrogen-containing bisphosphonates pamidronate and zoledronate were 3 respectively 6 times more potent than the simple bisphosphonate clodronate. However encapsulation of the drugs into liposomes resulted in a 2 to 4-fold increase of the IC_{50} value. Investigations on the cytotoxicity of liposomal clodronate (Clodrolip) and pamidronate (Pamidrolip) on activated murine peritoneal macrophages showed that at a drug concentration corresponding to the IC_{50} value of macrophages, tumour as well es endothelial cells were not affected. However, a cytotoxic effect could be oberved when treated with the same amount of the free drug. Nevertheless, for activated peritoneal macrophages there was no significant difference between the free drug and the liposomal formulation for both clodronate and pamidronate.

The results obtained in this study show the potential of clodronate and pamidronate liposomes as new antitumour drug as they selectively kill macrophages. As in this therapeutic approach the drug target are not rapidly proliferating cells, unwanted toxic side effects might be reduced.

2 INTRODUCTION

2.1 Angiogenesis in tumour growth

Angiogenesis is a complex and tightly controlled physiological process in which new blood vessels are formed from pre-existing capillaries during normal growth. Apart from the high activity required for the female reproductive cycle, placentation, embryogenesis and wound healing, angiogenesis is a relatively rare event in the healthy adult, and turnover of vascular endothelial cells is generally rather low. However angiogenesis is not only a crucial event in physiological conditions, it is also a prominent pathological feature of many diseases (e.g. diabetic retinopathy, chronic inflammation, and cancer). Especially for tumour expansion angiogenesis has been found to be a crucial process for malignant tumours to grow beyond 2-3 mm^3 in size by providing oxygen and nutrients for further growth and removal of waste products and dead cells [1]. In addition, angiogenesis provides an essential exit route for metastasizing tumour cells from the primary tumour to the bloodstream. Angiogenesis in tumours is a complex multistep process involving the degradation of the extracellular matrix (ECM) around a local venule, the proliferation and migration of capillary endothelial cells, and their differentiation into functioning capillaries [2]. All these steps are regulated by various angiogenic factors that are produced in tumor cells and in the surrounding stroma [3].
As tumour growth is highly dependant on angiogenesis, it represents an interesting target for cancer treatment.

2.2 Tumour associated macrophages (TAMs)

Macrophages are the most important component of the mononuclear phagocyte system (MPS). They are released from the bone marrow as immature monocytes and, after circulating in the blood stream, migrate into tissues where under the influence of the local environment differentiation into various phenotypes (e.g. resident macrophages, Kupffer cells in the liver, alveolar macrophages in the lung, osteoclasts in the bone and peritoneal macrophages) takes place. Macrophages are multifunctional cells that play important roles in tissue morphogenesis during development, tissue repair, inflammation and immunity, including phagocytosis, cytotoxicity and secretion of a wide array of cytokines, growth factors, lysozymes, proteases, complement components, coagulation factors and prostaglandins [2].
Apart from malignant cells, solid tumours are also composed of non-malignant cell types that have the ability to modify the neoplastic properties of the tumour by creating an unique microenvironment. A representative of these non-malignant cells are tumour-associated macrophages (TAMs), a special type of macrophages that are recruited into tumours as immature monocytes by chemotactic factors that are often produced by the tumour cells themselves [3]. TAMs are multifunctional cells with the potential to perform diverse functions including the secretion of anti- as well as pro-tumorigenic factors depending on the cytokine microenvironment in the tumour (*Figure 1*) [3]. Antibodies or macrophage stimulants such as interferon gamma (IFN-γ) or the bacterial product lipopolysaccharide (LPS) activate TAMs to kill tumor cells either directly or indirectly through the presentation of tumour antigens to T-cells [2]. In hypoxic areas of the tumour, TAMs are activated to produce factors

that promote all steps associated with malignancy within tumours. These factors include enzymes that stimulate tumour angiogenesis as well as proteases, various growth factors and mitogens [2]. The coordinated spatial and temporal expression of these molecules leads to angiogenesis, matrix breakdown and tumour-cell motility – all of which are elements of the tumour growth and of the metastatic process [3].
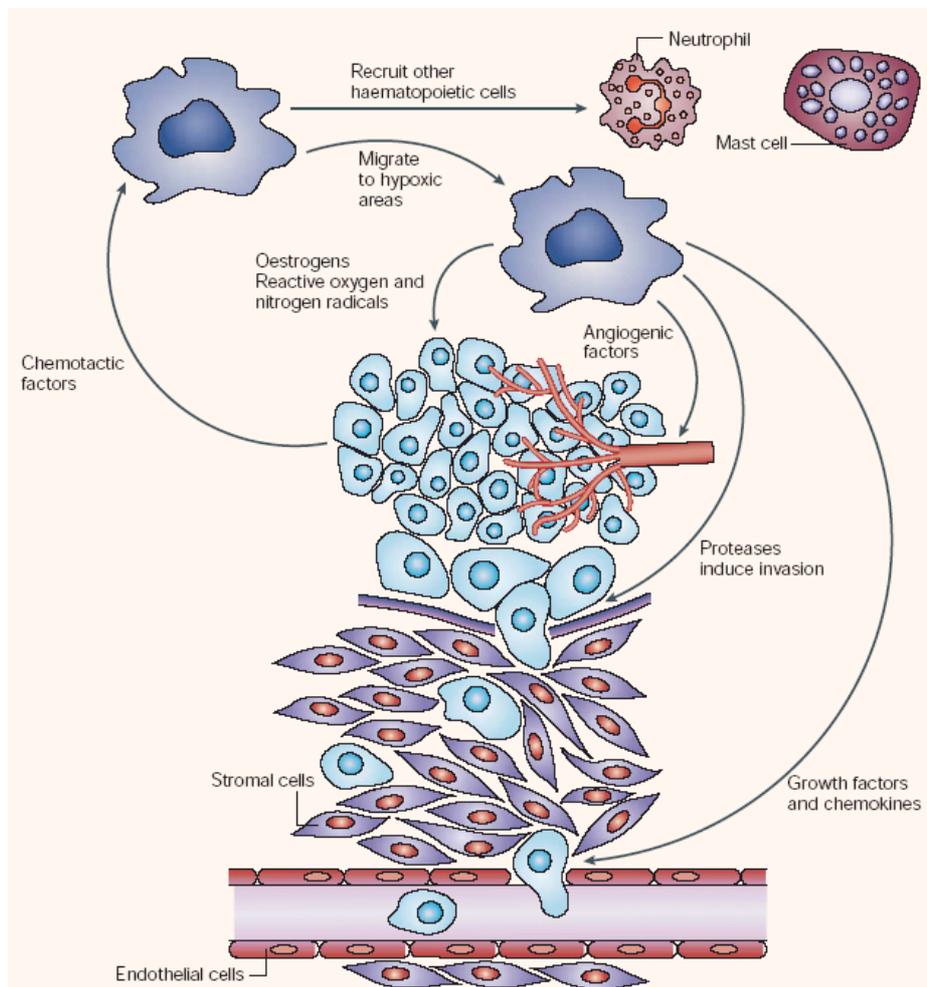
Figure 1   *Pro-tumourigenic functions of TAMs. Macrophages are recruited to hypoxic areas within the tumour by chemotactic factors and provide many tropic functions that promote tumour progression and metastasis. These tropic functions include the secretion of oestrogens, reactive oxygen and nitrogen radicals, angiogenic factors, protease, growth factors and chemokines [3].*

The conventional knowledge about TAM function is that they are recruited to reject the tumour, which has been recognized as foreign because tumours express unique antigens [3]. However clinical studies have shown a strong correlation between an abundance of TAMs and poor prognosis for different types of cancer [3]. In more than 80 % of the clinical studies there is a significant correlation between TAM density and poor prognosis, whereas in less than 10 % of the studies TAM density can be associated with good prognosis [3]. This means that despite being an integral part oft the host immune system, macrophages appear to do little to help to mount an effective immune response against the tumour, in the majority of patients [2]. This could be due to the fact that in some cases the local cytokine milieu in the tumour tends to block the immunological functions of these newly recruited

monocytes – such as antigen presentation and cytotoxicity – towards tumours, and diverts them towards specialized TAMs that are immunosuppressed and trophic [2,3].

The findings that the tumour microenvironment educates non-malignant cells such as TAMs to perform supportive roles that promote tumour progression and metastasis offer new therapeutic drug targets.

2.3 Bisphosphonates

Because of their ability to inhibit osteoclastic activity bisphosphonates were first used as therapeutic agents for diseases involving excessive bone resorption such as postmenopausal and steroid-induced osteoporosis, Paget's disease, tumor-induced osteolysis and hypercalcemia [6]. However, bisphosphonates do not only act on osteoclasts, they can also be used as potent agents to treat bone metastasis by inhibiting the adhesion of tumour cells to mineralized bone as well as tumour cell invasion and proliferation. In addition, as a new property, bisphosphonates have been found to inhibit angiogenesis [7].

The basic structure of bisphosphonates (*Figure 2*) is characterized by two phosphate groups linked to a central carbon atom, which results in high affinity for hydroxyapatite bone mineral surfaces and therefore these drugs are concentrated to sites of osteoclastic bone resorption [9]. Addition of various side chains (R1 and R2) to the geminal carbon atom has generated a wide range of compounds (*Figure 2*) that differ in their ability to bind to bone as well as in their anti-resorptive ability [10]. Depending on the structure of the side chains, bisphosphonates can be grouped into two general classes, the simple bisphosphonates (e.g. clodronate) and the nitrogen-containing bisphosphonates (e.g. pamidronate and zoledronate) that are several orders of magnitude more potent at inhibiting bone resorption in vivo than are the simple bisphosphonates [7].
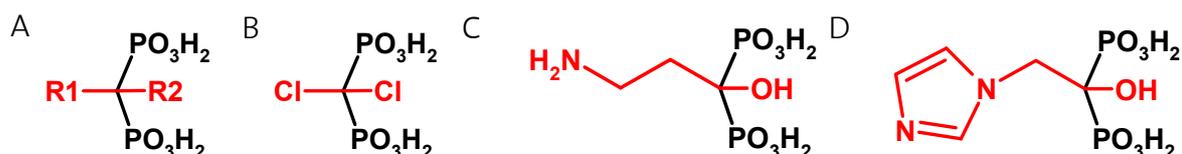
Figure 2   *Structures of bisphosphonates: A) Basic structure, B) Clodronate, C) Pamidronate, D) Zoledronate.*

Both groups of bisphosphonates inhibit bone resorption by induction of osteoclast apoptosis. However, at the molecular level the mechanisms of action differ. While simple bisphosphonates are metabolically incorporated into analogues of adenosine triphosphate (ATP) that are resistant to hydrolysis by ATP-dependent metabolic enzymes, nitrogen-containing bisphosphonates are inhibitors of the mevalonate pathway, thereby preventing prenylation of small GTPase signalling proteins required for osteoclast function [9]. The molecular mechanisms through which bisphosphonates act on tumour and on endothelial cells are presently unknown. However it has become clear that bisphosphonates can act on tumour cells either directly or indirectly by stimulating T-cell cytotoxicity against tumour cells [7].

2.4 Liposomes

Liposomes are nano-sized non-toxic biodegradable vesicles that consist of one or more concentric outer phospholipid bilayers, enclosing an aqueous inner compartment. The structure of liposomes resembles the one of biological membranes that is composed of amphiphilic phospho-lipids. Liposomes form spontaneously when phospholipids, (e.g. phosphatidylcholine molecules) are dispersed in water. Depending on the preparation method and the lipid composition, liposomes have a diameter between 60 nm (SUV, small unilamellar vesicles) (*Figure 3*) to 1 µm (MLV, multilamellar vesicles).
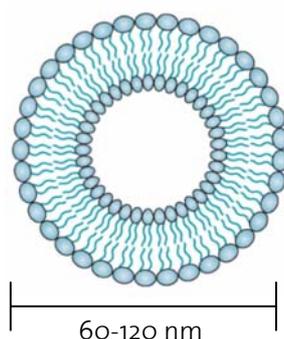

60-120 nm

Figure 3   *Schematic representation of a SUV (small unilamellar vesicle) [13].*

Liposomes can be loaded with a broad selection of drugs as well as with a variety of biologically active substances (e.g. proteins, peptides, DNA, antisense oligonucleotides, RNA and siRNA) [13]. Hydrophilic molecules, DNAs and RNAs are trapped in the aqueous liposome interior, whereas lipophilic molecules are incorporated in the liposomal membrane. In this way the water solubility of hardly soluble substances can be enhanced. Liposomes can carry a high concentration of the drug that can gradually be released to maintain a therapeutic (but non-toxic) drug level in the blood or at the local administration site for prolonged periods of times and in this way can act as depot form [14]. Drugs or biologically active molecules that are encapsulated in liposomes are protected from enzymatic degradation and inactivation or immunological reaction in the blood stream. Conversely, the degree of non-specific unwanted toxicities can be decreased using liposomal formulations [15]. As liposomes can interact with target cells in various ways they are able to promote the intracellular delivery of drug molecules that in their free form (i.e. non-encapsulated) would not be able to enter the cellular interior due to unfavourable physico-chemical characteristics [14].

An interesting aspect of liposome technology is the possibility to produce liposomes with different surface modifications (*Figure 4*). Polyethyleneglycol (PEG) coated liposomes   for example, show an enhanced systemic circulation time compared to conventional liposomes composed of phospholipids and cholesterol. This is because conventional liposomes are rapidly eliminated from the blood as they are captured by the macrophages of the MPS, primarily in liver and spleen. A further modification is the coupling of targeting ligands (e.g. antibodies or scFvs) resulting in immunoliposomes that have a high selectivity for the target cells (e.g. tumour cells)

and therefore lead to an increased liposomal drug accumulation in the desired tissues or organs [13].
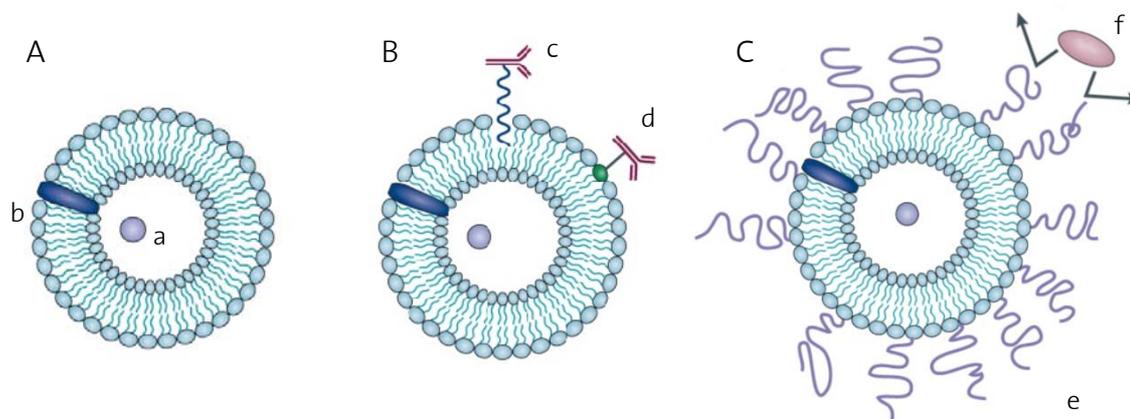

Figure 4 *Schematic illustrations of possible liposome modifications. A) Early traditional phospholipids "plain" liposomes with water soluble drug (a) entrapped into the aqueous liposome interior, and water-insoluble drug (b) incorporated into the liposomal membrane. B) Antibody-targeted immunoliposomes with antibody covalently coupled (c) to the reactive phospholipid in the membrane (d). C) Long-circulating liposomes grafted with a protective polymer (e) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (f) [13].*

Due to the advantages of liposomes as drug delivery systems, several pharmaceutical products based on liposomes have emerged on the market as for example the anti-cancer drugs DOXIL® and DaunoXome®. The advantage of liposomes as drug delivery systems in cancer treatment is that newly formed blood vessels are fragile and highly permeable compared with pre-existing vessels, thus macromolecules such as liposomes are known to passively accumulate in tumour tissue as a reflection of this feature. This results in an enhanced anti-cancer effect of liposomal cancer agents compared to the application of free drug [1].

There are different approaches in liposome production as for example high-shear homogenisation, the "microfluidizer" technique, detergent dialysis, reverse phase evaporation or filter extrusion. Liposomes produced by these techniques have different physico-chemical characteristics. These differences have an impact on their behaviour *in vivo* (disposition) as well as *in vitro* [14]. In this work, liposomes were prepared by filter extrusion.

2.5 The Macrophage as target in liposome-based therapy strategies

Because of the phagocytic activity of macrophages, liposomes can be used as efficient vehicles for the intracellular delivery of drugs. After uptake the phospholipase-mediated disruption of the liposomal phospholipid bilayers and the release of the encapsulated drug lead to an intracellular accumulation of the drug [16]. The selective depletion of macrophages based on this principle has been used in several studies in order to investigate whether macrophages are involved in any particular biological control mechanism [17].

As a result of the high angiogenic activity, tumours are richly supplied with blood that leads to an accumulation of drug containing liposomes in tumour tissue and possibly to a complete depletion of TAMs with low toxic effect to other cell populations.

2.6 Aim of the Thesis

In the first part of this study, the cytotoxic effects of the three different commercially available bisphosphonates clodronate, pamidronate and zoledronate either free in solution or encapsulated into liposomes (only for clodronate and pamidronate), were compared *in vitro* on the human rhabdomyosarcoma cell line A673, the murine teratocarcinoma cell line F9, human endothelial cells (HUVECs) and on peritoneal macrophages, freshly isolated from mice. In addition, *in vitro* analysis of the two non-commercial bisphosphonates bis2 and bis3 were carried out on the cell lines mentioned above.

The second part of this thesis was aimed to analyze the percentage of cytotoxic drug that was encapsulated into liposomes when prepared. For clodronate the stability of the drug containing liposomes was tested under different conditions. In a further step, clodronate liposomes (Clodrolip) were lyophilised and after rehydration of the liposomes their stability was analysed.

Finally, in the third part an analytical method to detect bisphosphonates was developed. As a model, a post-column indirect fluorescence detection method of bisphosphonates after anion-exchange separation described in a paper [18] was used. The method described was adapted to a 96-well plate assay.

3   MATERIALS AND METHODS

3.1   Chemicals, solutions and cells

Buffer salts and other chemicals   Fluka or Sigma, Buchs, CH

3.1.1   Cell culture

Attachment Factor (AF)	Cascade Biologics, Portland OR, USA
Dulbecco's modified Eagles medium (DMEM) with 1000 mg glucose / litre	Sigma-Aldrich, Saint Louis, USA
Endothelial growth medium	PromoCell, Heidelberg, Germany
Female SV 129 mice	Charles River Wiga, Sulzfeld, Germany
Foetal bovine serum (FBS)	Gibco, Basel, CH
Human rhabdomyosarcoma cells A673	American Type Culture Collection (ATCC), Rockville, MD
Human umbilical vein endothelial cells (HUVEC)	PromoCell, Heidelberg, Germany
Lipopolysaccharide (LPS)	Sigma-Aldrich, Saint Louis, USA
Murine teratocarcinoma cells F9	American Type Culture Collection (ATCC), Rockville, MD
Nystatin suspension 10'000 units / ml	Gibco, Basel, CH
Penicillin-Streptomycin, liquid contains 10'000 units of penicillin and 10'000 µg of Streptomycin	Gibco, Basel, CH
Trypsin – EDTA (1 x) in HBSS w/o Ca & Mg	Gibco, Basel, CH

3.1.2   Liposomes

$^{45}CaCl_2$	Amersham Pharmacia Biotech Ltd., Little Chalfont, UK
$CaCl_2$	Fluka, Buchs, CH
Cholesterol (Chol, MW 386.66)	Fluka, Buchs, CH
D,L α–tocopherol (MW 431.00)	Merk, Darmstadt, Germany
Mannitol (MW 182.17)	Sigma, Buchs, CH
Soy phosphatidylcholine (SPC, MW 770.00)	L. Meyer, Hamburg, Germany

3.1.3   Cytotoxic drugs

Clodronate (chlormethylene bisphosphonic acid, $CH_2Cl_2Na_2O_6P_2$ x 4 H_2O, MW 361.01)	Bioindustria L.I.M., Novi Ligure, Italy
Bis2 (MW 306.15)	Prof. H. Schott, Institute of Organic Chemistry, Tübingen, Germany
Bis3 (MW 391.3)	Prof. H.Schott, Institute of Organic Chemistry,Tübingen, Germany
Pamidronate (pamidronic acid, MW 279.07)	Novartis, Basel, CH
Zoledronate (zoledronic acid, MW 290.1)	Novartis, Basel, CH

8

3.1.4　Viability Assay Reagent

Optimem I medium with Glutamax – I	Gibco, Basel, CH
Resazurin Solution (7-Hydroxy-3H-pheoxazin-3- one 10-oxide)	Sigma-Aldrich, Saint Louis, USA

3.1.5　Detection of bisphosphonates

Aluminiumnitrat Nonahydrat ($Al(NO_3)_3 \times 9H_2O$, MW 375.13)	Fluka, Buchs, CH
Morin (2',3,4',5,7-pentahydroxyflavon, $C_{15}H_{10}O_7 \cdot 2H_2O$, MW 338.27)	Sigma, Buchs, CH

3.2　Liposomes

3.2.1　Preparation of bisphosphonate-liposomes by filter extrusion

Bisphosphonate-liposomes were prepared using the filter extrusion method (*Figure 5*) described by Marty *et al*. [19].
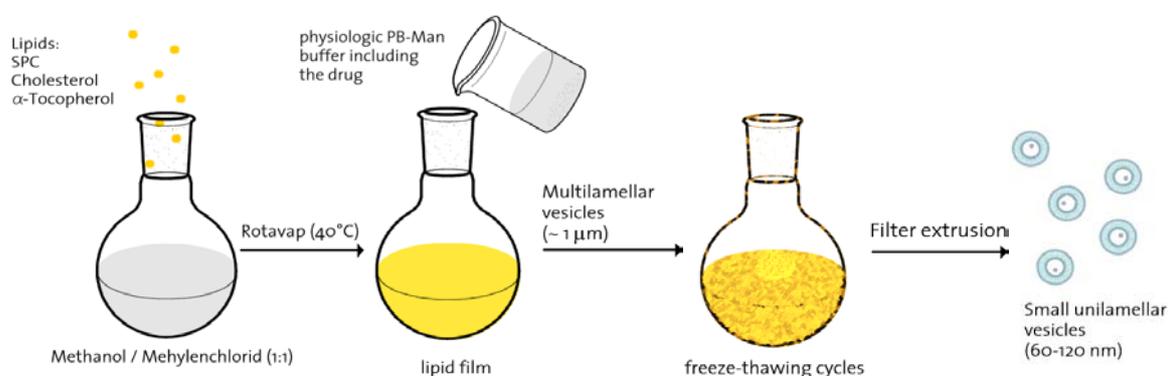

Figure 5　*Flow-chart: Production of bisphosphonate liposomes.*

Briefly, the lipid mixtures listed in *Table 1* were dissolved in methanol / methylenechloride (1:1, v/v). The organic solvents were evaporated under vacuum at 40°C with a Rotavap (Büchi, Flawil, CH). The dried lipid film was dispersed in a physiologic phosphate buffer (20 mM, pH 7.4), supplemented with the corresponding amount of drug and mannitol (230 mM) as cryoprotectant allowing freezing of the liposomes. The resulting milky suspension containing large multilamellar vesicles (MLV) with a size of about 1 µm, was freeze-thawed three times in liquid nitrogen and water of 40°C in order to enhance the trapping efficiency. Small unilamellar vesicles (SUV) with a diameter of about 100 nm were obtained by repetitive (5 x) filter extrusion through Nucleopore™ membranes (Sterico, Dietikon, CH) of 0.4 µm pore diameter using a Lipex™ extruder (Lipex

Biomembranes Inc., Vancouver, Canada). Zoledronate liposomes were additionally extruded to a filter of 0.1 μm pore diameter. Non-encapsulated bisphosphonates were removed by dialysis (Spectrapore tube, 12 – 14000 mol. wt. cut-off) with phosphate-mannitol buffer (PBMan, 20 mM phosphate, 230 mM mannitol, pH 7.4) as dialysis buffer (1 : 100, v/v). The liposomal preparation was sterile filtrated through a 0.45 μm filter and aliquots were stored at –80°C. Several aliquots (0.5 and 1 ml) stored at –80°C were frozen in liquid nitrogen and lyophilized using a Savant Speed Vac® (Fisher Scientific, Wohlen, CH) for 24 h.

Table 1   *Lipid and drug composition used for the preparation of bisphosphonate liposomes.*

Lipid film components	MW [$g/_{mol}$]	Clodrolip		Pamidrolip		Zoledrolip	
		mol [%]	$mg/_{ml}$	mol [%]	$mg/_{ml}$	mol [%]	$mg/_{ml}$
SPC	770	100	80	100	80	100	80
Cholesterol	387	20	8	20	8	20	8
α-Tocopherol	431	1	0.48	1	0.48	1	0.48
Cytotoxic drugs							
Clodronate	361.01	164	66	-	-	-	-
Pamidronate	279.07	-	-	307	89	-	-
Zoledronate	290.1	-	-	-	-	3	1

Size and stability of the liposomes were analyzed at a dilution of 1 : 200 (v/v) in phosphate buffer (PB), by dynamic light scattering with a Nicomp particle sizer (Model 370, Santa Barbara, USA).

3.2.2   Quantification and stability of bisphosphonates in liposomes

In order to determinate the efficiency of bisphosphonate encapsulation into liposomes and to carry out the stability tests, the liposomal preparation were trace labelled with $^{45}CaCl_2$ and 1mM $CaCl_2$ as carrier.

3.2.2.1   *Dialysis*

Dialysis cells (Diachema dialysis membrane 5000 mol. wt. cut-off), containing each 1 ml of a 1 : 20 (v/v) dilution of Clodrolip containing 20 mg clodronate / ml or Pamidrolip containing 18 mg pamidronate / ml in PB-Man were dialysed against 12 l of PBMan (1 : 1600, v/v) at 4°C. After different time points three probes (one per dialysis cell) were collected and the dialysis was continued in the same buffer. The concentration of remaining bisphosphonates in the probes was measured with a beta counter (Tri-Carb 2250 liquid scintillation counter, PerkinElmer Life and Analytical Sciences, Inc., USA).

3.2.2.2   *Size Exclusion Chromatography*

Thawed samples of Clodrolip containing 20 mg clodronate / ml were diluted 1 : 1 (v/v) with PB-Man, 20% FBS or 100 % FBS. Samples with FBS were incubated for 1 h at 37°C. In addition, a sample of lyophilized Clodrolip was reconstituted in demineralised water and diluted 1 : 1 (v/v) with PB-Man. 0.5 ml of the diluted samples

were loaded on a size exclusion chromatography column consisting of a pipette of 25 ml filled with Biogel P4. As elution buffer PBMan was used. Collected fractions were measured by liquid scintillation using a beta-counter. Before incubation and loading on the column, the size of the liposomes was analyzed at a dilution of 1 : 200 (v/v) in phosphate buffer, by dynamic light scattering with a Nicomp particle sizer.

3.3   Cells

3.3.1   Cell culture

Murine teratocarcinoma cells F9, human rhabdomyosarcoma cells A673 and macrophages were cultivated in Dulbecco's modified Eagle medium (DMEM) with 1000 mg glucose / litre whereas human umbilical vein endothelial cells (HUVECs) were maintained in endothelial growth medium, supplemented with a supplement mixture. DMEM was additionally supplemented with 10 % foetal bovine serum (FBS) and antibiotics (100 U / ml Penicillin, 50 U / ml Nystatin, 10 μg / ml Streptomycin). All cells were maintained at 37 °C, in a humid atmosphere containing 5 % CO_2 and they were splitted at a ratio 1:20 to 1:5, when they were confluent.

3.3.2   Isolation of peritoneal macrophages

Macrophages were isolated from female SV 129 mice by injection of 5 ml ice-cold phosphate buffer (PB) into the abdominal cavity of the anaesthetised mouse. After gently massaging the anterior and lateral walls of the abdomen, the peritoneum washes were collected and placed on ice. The cell suspension was centrifuged at 500 rpm for 5 min. The cell pellet was resuspended in DMEM supplemented with 10 % FBS and antibiotics (100 U/ml Penicillin, 50 U/ml Nystatin, 10 μg/ml Streptomycin). For cell viability assays, cells were directly seeded into 96-well plates and kept under culture conditions. After three days the medium was changed and after five days macrophages were completely attached to the bottom of the plate and ready to use for experiments.

3.3.3   Activation of peritoneal macrophages

Macrophages were activated by adding 10 ng / ml of lipopolysaccharide (LPS). After incubation for 16 h at 37 °C, in a humid atmosphere containing 5 % CO_2, cell viability assays were performed.

3.3.4   Cell viability assay

In order to measure the cytotoxicity of the different test compounds, cells were seeded at different densities (F9 and A673 10'000 cells/well, HUVECs 15'000 cells / well and macrophages 20'000 cells / well) into 96-well plates and grown under culture conditions in 200 μl of the corresponding medium per well.
After 24 h, the growth medium was replaced by 100 μl sterile filtered test compound solution in different concentrations that were obtained by diluting a stock solution with growth medium. Stock solutions of each compound were prepared in phosphate buffer (PB, 67 mM, pH 7.4). For clodronate and Clodrolip, cells were

incubated for 4 h, 6 h and 24 h, whereas for all other test compounds 4 h incubation experiments were performed. After incubation the cells were washed and recovered in 200 µl medium for 24 h. In order to measure the cell viability the medium was removed and 100 µl resazurin solution (resazurin reagent in Optimem I medium 1:10, v/v) were added per well. The fluorescent product that appeared through the metabolisation of resazurin, was measured with a Tecan reader (Ultra Evolution, Tecan, Männedorf, CH) at a wavelength of 590 nm using an excitation wavelength of 560 nm. The IC_{50} was determined by subtracting the background (cell free wells) and taking the positive control (cells incubated with medium) as 100 %. The IC_{50} value was calculated with the curve equation of the survival curve. All values were measured in triplicates.

3.4 Quantitative determination of clodronate by indirect fluorescence detection (IFD)

The method used for the detection of bisphosphonates was taken from a paper on post-column indirect fluorescence detection in combination with anion-exchange separation of bisphosphonates and related analytes [18]. Briefly, highly fluorescent solutions of the Al^{3+}-morin complex were prepared by combining different amounts of stock solutions of $Al(NO_3)_3$ (2mM) and morin (2mM) in ethanol-acetate-buffer (37 mM, pH4.5, 80 : 20, v/v). According to the paper, an Al^{3+} - morin ratio of 1 : 4 to 1 : 5 and a concentration of 2 – 200 µM of Al^{3+} was used in the experiments. The Al^{3+}-morin complex solutions were stored overnight prior to use, in order to ensure formation of the complex. After addition of different amounts of bisphosphonates taken from a stock solution of clodronate (10 mM) in ethanol-acetate-buffer (37 mM, pH4.5, 80 : 20, v/v) fluorescence decreased in proportion to the amount of bisphosphonate added, because of the formation of the nonfluorescent Al^{3+}-clodronate complex (*Figure 6*). The clodronate stock solution was diluted to solutions of different concentrations that were used as test samples and to create the calibration curves. The pH of all solutions was adjusted to 4.5 with acetic acid.

$$Al^{3+}\text{-morin} + clodronate \rightleftharpoons Al^{3+}\text{-clodronate} + morin$$

Figure 6 *Equilibrium between the fluorescence Al^{3+}-morin complex and the nonfluorescent Al^{3+}-clodronate complex.*

As test sample, Clodrolip containing 20 mg clodronate / ml (formerly determined by trace labelling with $^{45}CaCl_2$) was diluted to different concentrations to the range of the calibration curve. Aliquots (200 µl) of the mixtures to be measured were pipette into a 96-well plate and fluorescence of the Al^{3+}-morin complex was measured with a Tecan reader at a wavelength of 510 nm using an excitation wavelength of 430 nm.

4 RESULTS

4.1 Characteristics of liposomes

As liposome properties *in vitro* and *in vivo* strongly depend on their size, lamellarity and composition, the homogeneity and diameter of liposomes was determined as production quality control. Directly after preparation as well as after storage at -20°C and thawing at room temperature (RT) all bisphosphonate liposomes had a relatively homogenous size distribution as shown in *Table 2*. However, after thawing a decrease of the mean diameter of 20% for Clodrolip (clodronate liposomes) respectively 27% for Zoledrolip (zoledronate liposomes) could be observed while there was no significant change in the case of Pamidrolip (pamidronate liposomes).

Table 2 *Mean diameter of clodronate liposomes (Clodrolip), pamidronate liposomes (Pamidrolip) and zoledronate liposomes (Zoledrolip) directly after preparation or after storage at −20°C and thawing at room temperature (RT).*

Liposome type	Mean diameter [nm] after preparation	Mean diameter [nm] after thawing
Clodrolip	132.4 ± 63.5	107.7 ± 58.4
Pamidrolip	107.6 ± 54.5	104.8 ± 55.9
Zoledrolip	106 ± 56.4	77.4 ± 56.7

4.2 Quantification and stability of liposome encapsulated clodronate and pamidronate

In order to be able to compare the cytotoxic effect of liposomal bisphosphonate preparations with the free drug, the percentage of drug that is incorporated into liposomes needs to be determined. In addition, the stability of encapsulation is essential for therapeutic applications.

The initial concentration of the drug solutions used for liposome preparation, the concentration after removal of non-encapsulated drug by dialysis and the entrapment efficiency are listed in *Table 3*.

Table 3 *Initial concentration of the drug solutions used for liposome preparation, final concentration after removal of non-encapsulated drug and entrapment efficiency of clodronate liposomes (Clodrolip), pamidronate liposomes (Pamidrolip) and zoledronate liposomes (Zoledrolip).*

Liposome	Concentration of drug solution for preparation		Final drug concentration		Entrapment
	[mg / ml]	[mM]	[mg / ml]	[mM]	[%]
Clodrolip	66	182.8	20	55.4	33.5
Pamidrolip	89	318.9	18.3	65.6	20.6
Zoledrolip	2.7	9.3	1	3.4	36

4.2.1   Dialysis

The stability of liposomal clodronate and pamidronate was analyzed by dialysis. As shown in *Figure 7* the stability of both liposome types was about the same. For Clodrolip the loss of clodronate after one day was about 30 % of the originally encapsulated drug, for Pamidrolip it was 35 %. The further time course of dialysis up to 168 h did not show any considerable leakage.
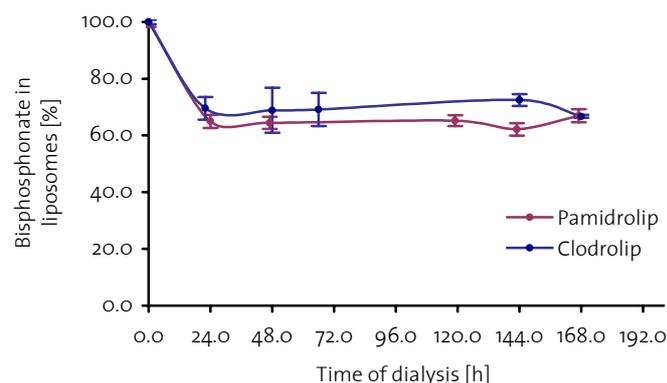

Figure 7 *Relative amounts [%] of encapsulated clodronate and pamidronate in liposomes, after different time points of dialysis in PBMan (1 : 1600, v/v), analyzed by trace labelling with Ca^{45} and performed in triplicates (mean ±S.D.).*

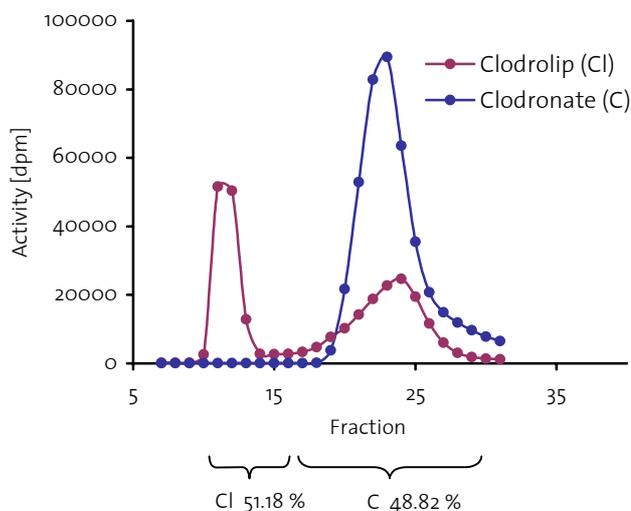### 4.2.2   Size exclusion chromatography

Figure 8 *Size exclusion chromatogram showing the relative loss of clodronate [%] from Clodrolip, diluted (1 : 1, v/v) in PBMan, analyzed by trace labelling with Ca^{45}. (all other chromatograms are shown in appendix I).*

In order to test whether a part of the clodronate lost by dialysis is adsorbed to the outer liposome surface, size exclusion chromatography was performed, as this method can be used for the separation of small solutes from liposomes. When used for *in vitro* as well as *in vivo* experiments, liposomes are in a different environment compared to the one in physiologic PBMan (*Figure 8*). In order to imitate the physico-chemical situation in culture medium and in human plasma, samples of Clodrolip containing 10 % respectively 50 % FBS were analyzed as well.

Incubation of liposomes with FBS either 10 % (Appendix I, *Figure 16*) or 50 % (Appendix I, *Figure 17*) resulted in an decrease of the liposome mean diameter for about 20 % (*Table 4*). In both cases the loss of clodronate after passage through the column was 70 %. However in comparison with PBMan as dilution medium, the samples that were incubated with FBS-PBMan showed an 1.5-fold higher loss of clodronate.

Table 4 *Relative loss of clodronate from Clodrolip diluted (1 : 1, v/v) in PBMan or FBS (final concentrations: 10 % and 50%) determined by size exclusion chromatography. Probes containing FBS were analysed after one hour of incubation at 37°C. The mean diameter of liposomes (analysed by dynamic laser scattering) was measured before and after incubation. All samples were measured by trace labelling with Ca^{45}.*

Dilution medium	loss of clodronate [%]	Mean diameter [nm] of liposomes before incubation	Mean diameter [nm] of liposomes after 1h incubation at 37°C
PBMan	48.82	140.9 ± 62.2	-
10 % FBS	71.30	128.0 ± 60.9	162.8 ± 63.5
50 % FBS	72.46	137.7 ± 63.1	157.9 ± 65.9

4.2.3   Stability of lyophilised Clodrolip

One of the practical diffi-culties with liposomes is that they are relatively unstable for long-term storage at room temperature (RT). As lyophi-lised liposomes are known to be more stable for long-term storage, the loss of clodronate of lyophilised Clodrolip after rehydration was tested.

The size of liposomes 1 h after rehydration (104.7 ± nm) was equal to the one of freshly thawed liposomes. However as illustrated in *Figure 9* only 15.05 % of the originally entrapped or adsorbed clodronate was retained in rehydrated vesicles previously freeze-dried, resulting in a 36.1 % higher loss compared to freshly thawed Clodrolip.
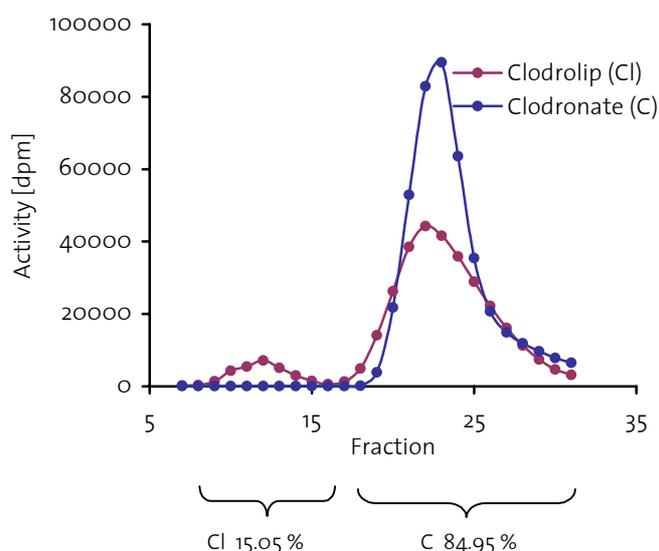

Figure 9 *Size exclusion chromatogram showing the relative loss of clodronate [%] of lyophilized Clodrolip after rehydration with sterile H_2O and diluted (1 : 1, v/v) in PBMan, analyzed by trace labelling with Ca^{45}.*

4.3   Cytotoxic activity of bisphosphonates in vitro

As *in vivo* experiments do not give any clue on the specific toxicities of drugs on different cell types, *in vitro* analyses have to be done prior to mouse experiments. In the following experiments the cytotoxicities of bisphosphonates on human and murine tumour cells, endothelial cells and mouse peritoneal macrophages were determined.

4.3.1   Comparison of free bisphosphonates on tumour cells and HUVECs

The relative cytotoxicity of the five bisphosphonates clodronate, pamidronate, zoledronate, bis2 and bis3 was determined by analysing the cytotoxic effect on F9, A673 and HUVE cells. *Figure 10* illustrates the IC_{50} values after 4 h of incubation and 24 h recovery.
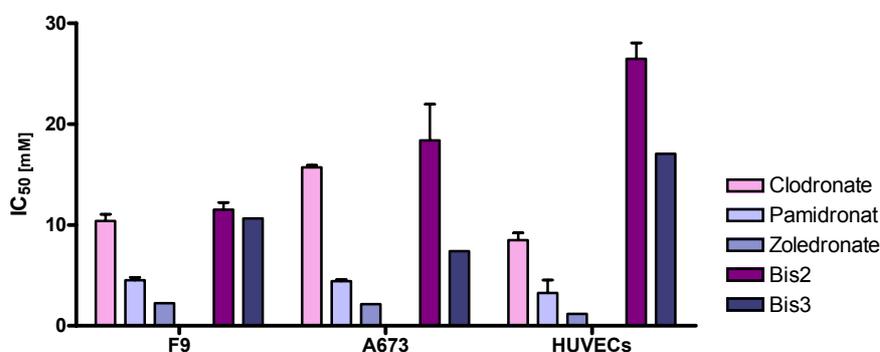

Figure 10   *In vitro cytotoxicity of free clodronate, pamidronate, zoledronate, bis2 and bis3 on F9, A673 cells and HUVECs after 4 h of incubation and 24 h recovery. Each value is the mean ±S.D., n = 2 (expect bis3 and zoledronate n = 1).*

In all cases the most effective drug was zoledronate with an IC_{50} value of about 2 mM for F9 and A673 cells respectively 1 mM for HUVECs, followed by pamidronate that was only half as toxic as zoledronate. For F9 and HUVE cells the IC_{50} value of clodronate was 2.5 fold higher than the one of pamidronate, for A673 cells the factor was even 3.5. The cytotoxicity of bis2 and bis3 did not show an uniform pattern. While in the case of F9 and A673 cells the IC_{50} value of bis2 was slightly higher than the one of clodronate, the cytotoxicity of clodronate compared to the one of bis2 was three times higher when tested on HUVECs. Bis3 was almost as toxic as bis2 in the case of F9 cells, for A673 cells the IC_{50} value of bis3 was 2.5-fold lower and for HUVECs 1.5-fold lower than the one of bis2. The exact IC_{50} values of all cytotoxic assays performed are listed in Appendix II *Table 6*.

4.3.2   Comparison of free and liposomal clodronate and pamidronate

In a second set of experiments the cytotoxic effect of free and liposomal bisphosphonates (clodronate and pamidronate) were compared. As the bisphosphonates bis2 and bis3 were not as efficient as the commercial ones, no liposomal formulations of these drugs were prepared.
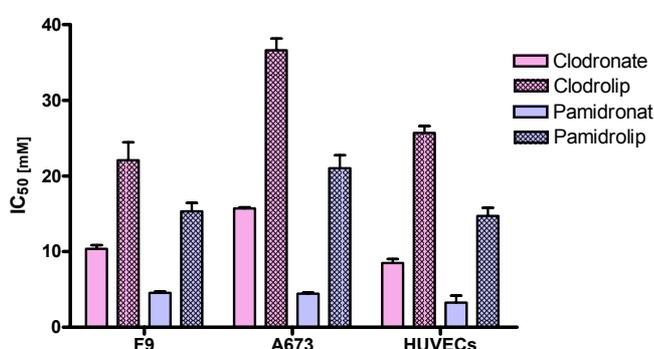

Figure 11   *In vitro cytotoxicity of liposomal and free drug (clodronate and pamidronate) on F9, A673 and HUVE cells after 4 h of incubation and 24 h recovery. Each value is the mean ±S.D., n = 2.*

Figure 11 indicates that encapsulation of drug into liposomes decreased the cytotoxicity on all cells tested compared to free drug. The ratios (liposomal drug : free drug) of the IC_{50} values shown in *Table 4* indicate that the relative toxicity of Clodrolip is slightly but not significantly higher compared to Pamidrolip.

Table 5 *Overview of the ratios of the IC_{50} values of liposomal preparations and free drug tested on F9, A673 and HUVE cells after incubation of 4 h and 24 h recovery.*

Cell line	Cytotoxic drug	
	Clodrolip : Clodronate	Pamidrolip : Pamidronate
F9	2.1	3.4
A673	2.3	4.7
HUVEC	3.0	4.5

In order to investigate whether the cytotoxicity of liposomal bisphosphonate preparations is time dependent, further experiments with prolonged incubation times (6 h and 24 h) were performed (*Figure 12*).
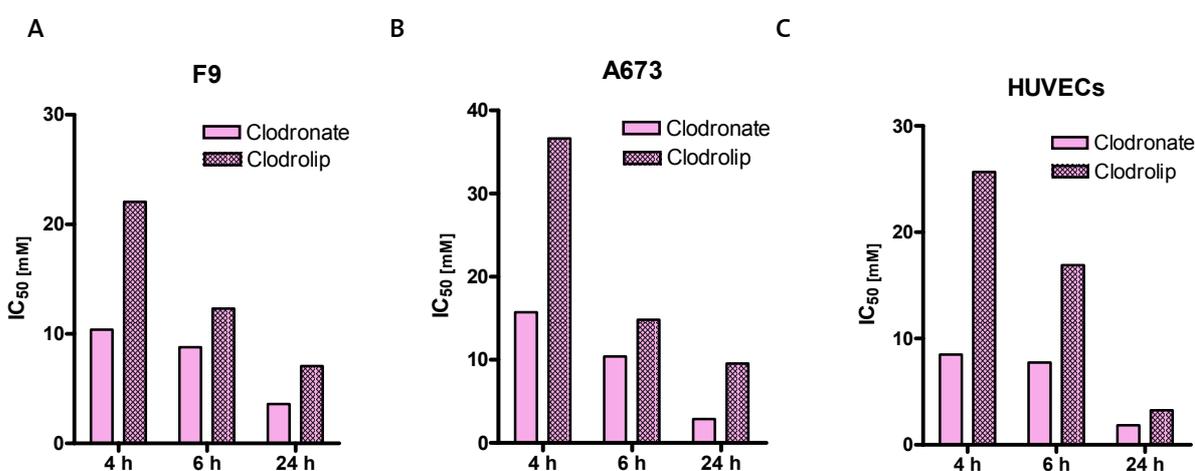

Figure 12   *Cytotoxic effect of Clodrolip (clodronate liposomes) and the free drug after different incubation times (4, 6, and 24 h) on A) F9, B) A673 and C) HUVE cells.*

According to *Figure 12* the cytotoxicity of Clodrolip and the free drug increased with prolonged incubation times. For F9 cells the IC_{50} value for free clodronate decreased by 65 % respectively by 68 % for liposomal clodronate, when incubation time was prolonged from 4 to 24 h. A673 cells showed a decrease of the IC_{50} value of 82 % for clodronate and 74 % for Clodrolip. Also for the HUVECs the decrease of the IC_{50} value was not significantly different for clodronate (78 %) and Clodrolip (87 %).

4.3.3 Comparison of the cytotoxic effect of liposomal and free clodronate and pamidronate on activated and non-activated macrophages

In vivo, macrophages are usually activated by natural stimuli. As the environment of cells *in vitro* differs from the one *in vivo,* an artificial activating stimulus needs to be added. Only then the *in vivo* situation can be partially imitated. In the following experiments macrophages were activated with the bacterial product lipopolysaccharide (LPS, 10 ng / ml).

For both clodronate and pamidronate activation of macrophages resulted in enhancement of the cytotoxicity of both free and liposomal drug. For clodronate the decrease of the IC_{50} value of LPS activated peritoneal macrophages was 43 % for the free drug and 34 % for the liposomal form. For pamidronate a decrease of the IC_{50} value of 86 % for pamidronate respectively 95 % for Pamidrolip was determined. Pamidronate tested on activated macrophages was three times more potent than clodronate, for the liposomal forms a 4-fold higher activity of Pamidrolip was determined compared to Clodrolip.
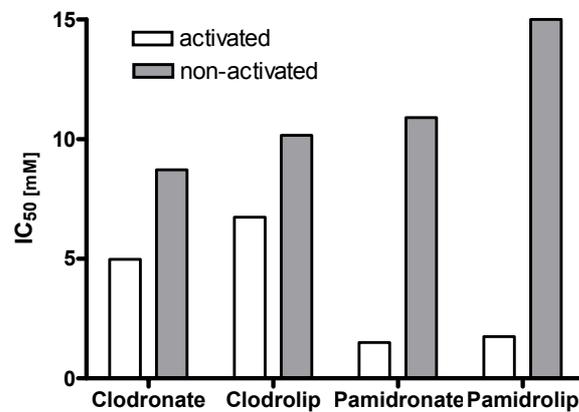

Figure 13   *In vitro cytotoxicity of free and liposomal clodronate and pamidronate on non-activated and activated (10 ng LPS / ml) murine peritoneal macrophages after 4 h of incubation and 24 h recovery.*
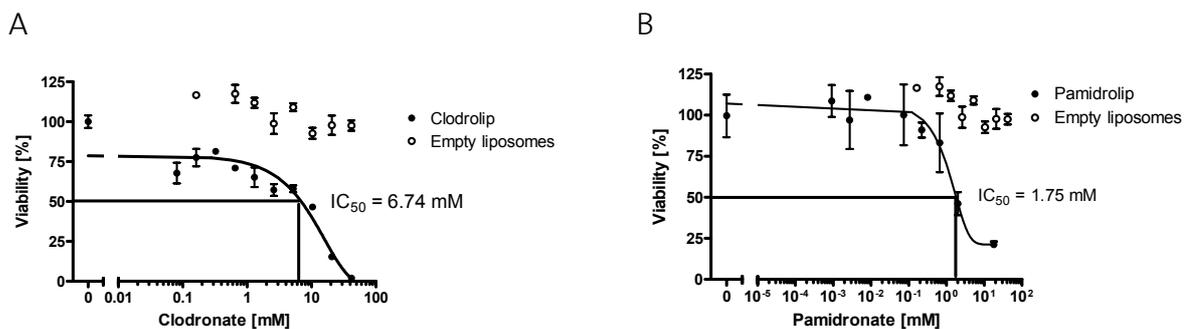

Figure 14   *Cytotoxic effect of A) Clodrolip and B) Pamidrolip on murine peritoneal macrophages activated with 10 ng LPS / ml. Each value is the mean ± S.D., n = 3.*

At a concentration of Clodrolip corresponding to the IC_{50} value of activated peritoneal macrophages (*Figure 14*), HUVE, F9 and A673 cells were not affected (*Figure 15*). However treatment with clodronate at the same concentration decreased cell viability by 12 % (F9), 18 % (A673) and 33 % for HUVECs. Also for Pamidrolip no

cytotoxic effect could be observed on tumour as well as on endothelial cells at a concentration corresponding to the IC_{50} value of activated peritoneal macrophages. When treated with the same amount of free drug, cell viability decreased by 37 % for HUVECs, 6 % for F9 and 15 % for A673 cells.
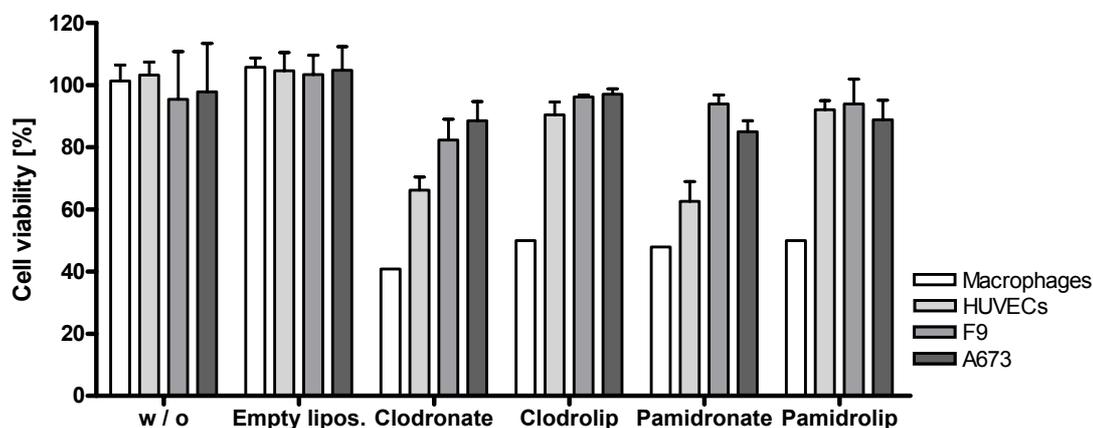

Figure 15   *In vitro cytotoxic effect of Clodrolip (6.74 mM) and Pamidrolip (1.75 mM) on murine peritoneal macrophages (activated with 10 ng LPS / ml), HUVE, F9 and A673 cells. Each value is the mean ±S.D., n = 2 (expect for cytotoxicity assays on activated macrophages n = 1).*

4.4 Quantitative determination of clodronate by indirect fluorescence detection (IFD)

The method for the detection of bisphosphonates described by Lovdahl and Pietrzyk [18] is an alternative to the radioactive trace labelling with $^{45}CaCl_2$.

Figure 6 shows that replacement of morin in the fluorescent Al^{3+}-morin complex by clodronate forms the stronger nonfluorescent Al^{3+}-clodronate complex. The decrease in fluorescence is proportional to an increasing amount of added clodronate. In the 96-well plate assay developed, a linear calibration curve (Appendix II) with an r^2 of 0.9967 was obtained in a range of 0 to 440 nM clodronate. The Al^{3+}-morin ratio was found to be best at 1 : 5 using a concentration of 10 µM Al^{3+}, respectively 50 µM morin. For the calibration curve 12.5 % of the available well volume (200 µl) was filled with Al^{3+}-morin complex solution. The remaining 87.5 % of the volume were occupied by clodronate stock solution (500 nM) diluted with different amounts of ethanol-acetate-buffer (37 mM, pH4.5, 80 : 20, v/v) resulting in a clodronate concentration range from 0 – 500 nM.

The concentration of clodronate from a Clodrolip sample, diluted 1 : 100'000 was determined to be 23.3 ± 0.001 mg/ml whereas a concentration of 25 mg/ml was measured by trace labelling with $^{45}CaCl_2$ resulting in a difference of 7.6 %.

5 DISCUSSION AND PERSPECTIVES

Bisphosphonates, originally used as therapeutic agents for diseases involving excessive bone resorption, have been found to be potent agents to treat bone metastasis. In this project it could be shown that due to the phagocytic uptake, liposomal clodronate (Clodrolip) and pamidronate (Pamidrolip) selectively kill murine peritoneal macrophages. Therefore bisphosphonates might serve as potent anti-cancer drugs by selectively killing tumour associated macrophages (TAMs).

5.1 Characteristics, quantification and stability of bisphosphonate liposomes

Quality control of liposomes showed that immediately after preparation as well as after thawing of aliquots stored at –20°C, all bisphosphonate liposome types showed a homogenous distribution of the liposome mean diameter. In addition, no aggregation or break down of liposomes could be observed. However, for Clodrolip and Pamidrolip a decrease of the mean diameter of 20%, respectively 27% could be observed after thawing, while there was no change for Pamidrolip. This difference in behaviour can be explained by the fact that the physicochemical properties of liposomes strongly depend on the enclosed or adsorbed drug.

In all liposomal bisphosphonate preparations the trapping efficiency was about 20 - 30 %. This percentage is quite low as the freeze-thawing cycles performed during preparation should result in higher incorporation rates. The low trapping efficiency is not satisfactory and not economic as a large portion of drug gets lost during preparation. Therefore, the rate of encapsulation should be enhanced, for example by increasing the density of vesicles of the liposomal preparations that means to enhance the total available vesicle volume per ml of liposomes. In this study only hydrophilic bisphosphonates that can not be incorporated into the liposomal lipid bilayer were used. Therefore, another possible approach to optimize the entrapment rate could be the use of lipophilic bisphosphonates carrying a long alkyl-chain, so that they could be incorporated into the lipid bilayer. The lipophilic bisphosphonates bis2 and bis3 were synthesized by Prof. H. Schott (Institute of Organic Chemistry, Tübingen, Germany) with the goal to get a higher incorporation rate. However, as they showed a rather low cytotoxic effect, no liposomal formulations were prepared. The reason for the low activity could be due to trapping of these lipophilic drugs into the phospholipid bilayer of the cell membrane and therefore they were not able to act as cytotoxic agents. This means that it should always be analysed whether lipophilic bisphosphonates still have a cytotoxic activity or not. In order to enhance the drug load of liposomes, it should be tested whether hybrid bisphosphonate liposomes could be produced carrying a lipophilic bisphosphonate in the lipid bilayer and a hydrophilic one in the liposome interior.

Dialysis experiments showed that once incorporated into liposomes, clodronate and pamidronate are entrapped in a very stable way. For the liposomal bisphosphonates (Clodrolip and Pamidrolip) tested, leakage of only about 30 % occurred during the first 24 h, the further time course up to 168 h did not show any significant leakage. It should be considered that the dialysis volume of 12 l used in the experiments

represents a rather unnatural situation as in humans the plasma volume is only about 3 litres.

Size exclusion experiments performed as a second way to test the stability showed a 19 % higher loss of clodronate compared to dialysis experiments. The reason for this enhanced loss could be due to the fact that bisphosphonates adsorbed to the liposome surface could be detached as they were attracted by the beads used. This means that a part of the assumed entrapped clodronate may be adsorbed to the outer liposome surface but does not get lost during dialysis. This assumption could be tested by performing size exclusion chromatography experiments using positively charged beads, as for example CM-Biogel beads. If the amount of lost clodronate during passage through the column is increased, it is probable that a large part of the drug is adsorbed to the outer liposome surface. As a consequence the adsorbed drug molecules might bind to plasma proteins *in vivo,* resulting in a depot effect or possibly in pharmacokinetic interactions.

The size exclusion chromatography experiments performed with Clodrolip containing 10 % respectively 50 % FBS showed that after passage through the column only 30 % of the originally entrapped or adsorbed clodronate was still present in the liposomes. This increased loss of drug supports the assumption that a large part of the supposed entrapped clodronate is adsorbed to the outer liposome surface as these drug molecules can be adsorbed by plasma proteins.

Nevertheless, as stability of drug containing liposomes is essential for therapeutic approaches, it should be investigated whether the stability of the liposomal bisphosphonate preparations could be further improved. As the rigidity and permeability of the bilayer strongly depends on the type and amount of lipids used, the stability of the liposomal bisphosphonate preparations could be further enhanced using different lipid compositions. For example HSPC, (a phospholipid with saturated fatty acid side chains) or DPPE (a saturated, positively charged phospholipid) lipids could be added as further lipid bilayer components. Using DPPE lipids, possibly more drug molecules adsorb to the surface of liposomes. By increasing the percentage of cholesterol, the rigidity of the lipid bilayer could be improved as well. Altogether, in order to be able to enhance the stability of clodronate liposomes further investigations should be done to get a better understanding of the interaction of liposomal lipids and the drug molecules.

Freeze-drying of liposomes could result in stable formulations for long term storage at room temperature (RT). Therefore, the stability of reconstituted freeze-dried Clodrolip was analysed. After reconstitution lyophilised Clodrolip showed a mean diameter and a homogeneity comparable to the one of non lyophilised liposomes stored at –20°C, however during rehydration leakage occurred, resulting in a loss of a rather large amount of the encapsulated clodronate. The problem of leakage of freeze-dried liposomes upon rehydration has been reported in several studies to be a critical step in the reconstitution process of lyophilised liposomal drugs. It has been suggested that repacking processes of the lipid bilayer are responsible for the enhanced permeability [20]. This experiment shows that size homogeneity, mean diameter and a lack of visible change in morphology do not give any clue on the stability of drug encapsulation. Nevertheless, it is worth doing further investigations to enhance the stability of freeze-dried bisphosphonate liposomes as a lyophilised formulation would be of great advantage. As there is already a cryoprotectant (mannose) present in the analysed liposome formulation, the only option to reduce leakage would be to modify the lipid composition.

5.2   Cytotoxicity of bisphosphonates

The experiments performed in this study show, that the bisphosphonates clodronate, pamidronate and zoledronate have a cytotoxic effect on endothelial as well as on human and mouse tumour cells, all representing components of the tumour stroma. The IC_{50} values of all bisphosphonates were in the mM range.

Clodronate belonging to the group of simple bisphosphonates showed the lowest efficiency in all cases (Appendix II, *Table 6*). The nitrogen-containing bisphosphonates pamidronate and zoledronate had a 2-fold respectively 6-fold higher activity. HUVECs showed the highest sensibility for all bisphosphonates tested, especially for zoledronate the cytotoxic activity was twice as high as for tumour cells. The enhanced cytotoxicity of zoledronate on HUVECs compared to other bisphosphonates has already been reported in context with anti-angiogenic activity of zoledronate [21]. The difference in activity of simple and nitrogen-containing bisphosphonates results from the different way of action already described in chapter 2.3. Clézardin *et al.* [7] have reported the different potencies of bisphosphonates. However, the different efficacies were not mentioned in context with cytotoxicity on tumour and endothelial cells but in context of adhesion of tumour cells to bone and tumour cell invasion. Therefore, the ratios of potency were not the same as determined in this study.

Liposomal formulations of bisphosphonates reduced their potencies 2 to 4-fold. Extension of incubation time showed an increased cytotoxic effect of both free and liposomal clodronate at a comparable magnitude. Therefore, tumour and endothelial cells do not show an increased cell uptake of Clodrolip compared to the free drug even after 24 h.

The non commercial drugs bis2 and bis3 were synthesized with the aim to get a higher incorporation rate due to their lipophilic properties. Despite being lipophilic, they could be dissolved in physiologic phosphate buffer. The activity of the simple bisphosphonate bis2 was in the range of clodronate or even lower. Bis3 a representative of the group of nitrogen-containing bisphosphonates showed a greater cytotoxic effect compared to bis2 but it did not reach the potency of pamidronate and zoledronate. Because of the rather low activity no liposomal formulations of bis2 and bis3 were prepared.

Firstly, cytotoxicity experiments with freshly isolated peritoneal macrophages were performed without activation. As no phagocytic activity could be observed, LPS was added for activation. The data obtained clearly show that the cytotoxicity of liposomal clodronate and pamidronate resulted in a significantly higher activity on macrophages, compared to the cytotoxic effect on tumour and endothelial cells (*Figure 15*). For Pamidrolip a 4-fold higher toxicity compared to Clodrolip could be determined. However, there was no significant difference of cytotoxicity on macrophages between free and liposomal drug. This data does not correspond to the fact that phagocytosis of drug containing liposomes enhances the amount of drug taken up. The reason for this inconsistency could be due to the fact that clodronate liposomes are unstable in culture medium as the instability of Clodrolip in 10 % FBS could be demonstrated. This means that phagocytosis of bisphosphonate liposomes does not result in a higher drug uptake as liposomes only contain little drug. However, in order to understand this inconsistency further experiments with varied cytotoxicity assay parameters (e.g. LPS concentration, incubation time, recovery time) need to be performed. In addition, further

investigations on the interaction of free as well as liposomal bisphosphonates with macrophages should be done.

The attempt to monitor the phagocytic uptake of liposomes by macrophages using fluorescence imaging posed some problems. Firstly macrophages did not attach to the cover slips so well, even though they were coated with polylysine. Therefore, it should be tested whether adsorption of macrophages could be enhanced by coating the cover slips with fibronectin. In order to monitor the phagocytic uptake, internalisation of fluorescently labelled liposomes was analysed by fluorescence imaging. However, as macrophages are the most autofluorescent cells [22], a high background occurred, that made it impossible to measure the fluorescence from liposomes.

5.3 Quantitative determination of clodronate by indirect fluorescence detection (IFD)

The method of quantitative determination of bisphosphonates using indirect fluorescence detection transferred to an assay on a 96-well plate can be used as an alternative method to avoid the use of radioactive substances. However, even though the assay allowed the determination of clodronate concentration in Clodrolip quite accurately, it is not a convenient assay for the routine determination of concentration for large numbers samples. Firstly, the assay is very time consuming as the drug solutions have to be diluted to concentrations that exactly correspond to the linear range of the calibration curve. If the approximate concentration of the drug solution to be tested is not known, several measurements need to be performed to find the dilution ratio that results in a concentration that lies in the measurable range. Secondly, as solutions contain 80 % ethanol, evaporation from a volume of only 200 µl is quite high resulting in large errors. Altogether, the method of indirect fluorescence detection is sensitive and good, however transfer of the method to a 96-well plate assay is only useful for the concentration determination of small sample numbers. As an alternative, indirect fluorescence detection can be used in combination with HPLC.

5.4 Perspectives

In this project the selective toxicity of clodronate and pamidronate liposomes on murine peritoneal macrophages has been demonstrated *in vitro*. However, *in vivo* bisphosphonate liposomes will encounter a different environment. Therefore mouse experiments need to be performed. Special attention should be taken to the effect caused on all other macrophage types in the animal. For example, the effect on the macrophages of the immune system should be analysed when infection occurs.
As zoledronate showed a low IC_{50} value compared to clodronate and pamidronate when tested on tumour and endothelial cells, liposomal zoledronate should be

tested on peritoneal macrophages. In order to enhance the specificity of liposomes to macrophages, liposomes could be coated with molecules that are recognized by macrophage receptors such as the mannose receptor [23]. However, it should be analysed whether the uptake of liposomes by macrophages of the RES is also enhanced which would result in a faster blood clearance and unwanted toxic side effects.

6 ABBREVIATIONS

AF	Attachment factor
A673 cells	Human rhabdomyosarcoma cells
°C	Temperature in degree Celsius
Chol	Cholesterol
DMEM	Dulbecco's modified Eagle medium
DPPE	1,2-dippalmitoyl-sn-glycero-3-phosphoethanolamine
F9 cells	Murine teratocarcinoma cells
FBS	Fetal bovine serum
g	Gram
HSPC	Hydrated soy phosphatidylcholine
h	Hour
HUVECs	Human umbilical vein endothelial cells
IC_{50}	50 % growth inhibition concentration
IFD	Indirect fluorescence detection
IFN-γ	Interferon gamma
kD	Kilo Dalton, 10^3 Dalton
l	Litre
LMV	Large multilamellar vesicles
LPS	Lipopolysaccharide
M	Molar, $\left[^{mol}/_l\right]$
min	Minutes
ml	Milli litre, $[10^{-3}$ litre]
mM	Millli molar, $[10^{-3}$ molar]
MW	Molecular weight
MPS	Mononuclear phagocyte system
nM	Nano molar $[10^{-9}$ molar]
PB	Phosphate buffer
PBMan	Phosphate-mannitol buffer
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PEG	Polyethylenglycol
rpm	Rounds per minute
RT	Room Temperature
SPC	Soy phosphatidylcholine
SUV	Small unilamellar vesicles
TAMs	Tumor associated macrophages
U	Units

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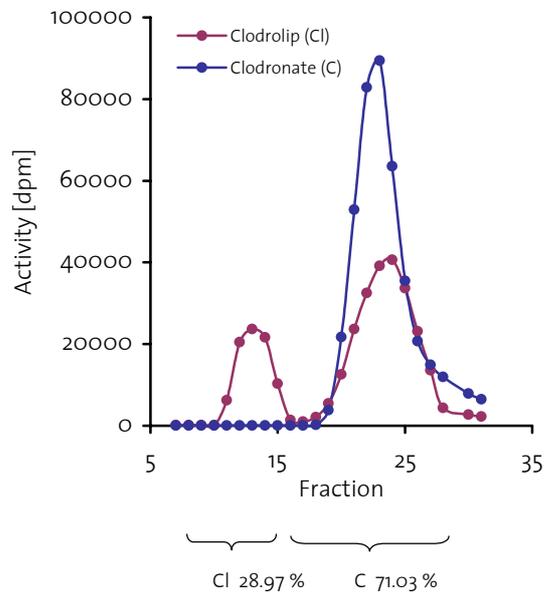
8 APPENDIX

Appendix I

Figure 16 *Size exclusion chromatogram showing the relative loss of clodronate [%] from Clodrolip, diluted (1 : 1, v/v) in PBMan 10% FBS, analyzed by trace labelling with Ca⁴⁵.*
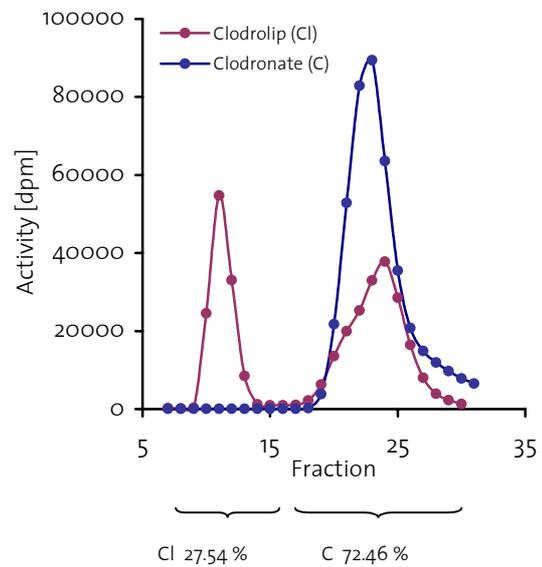

Figure 17 *Size exclusion chromatogram showing the relative loss of clodronate [%] from Clodrolip, diluted (1 : 1, v/v) in PBMan 50% FBS, analyzed by trace labelling with Ca⁴⁵.*
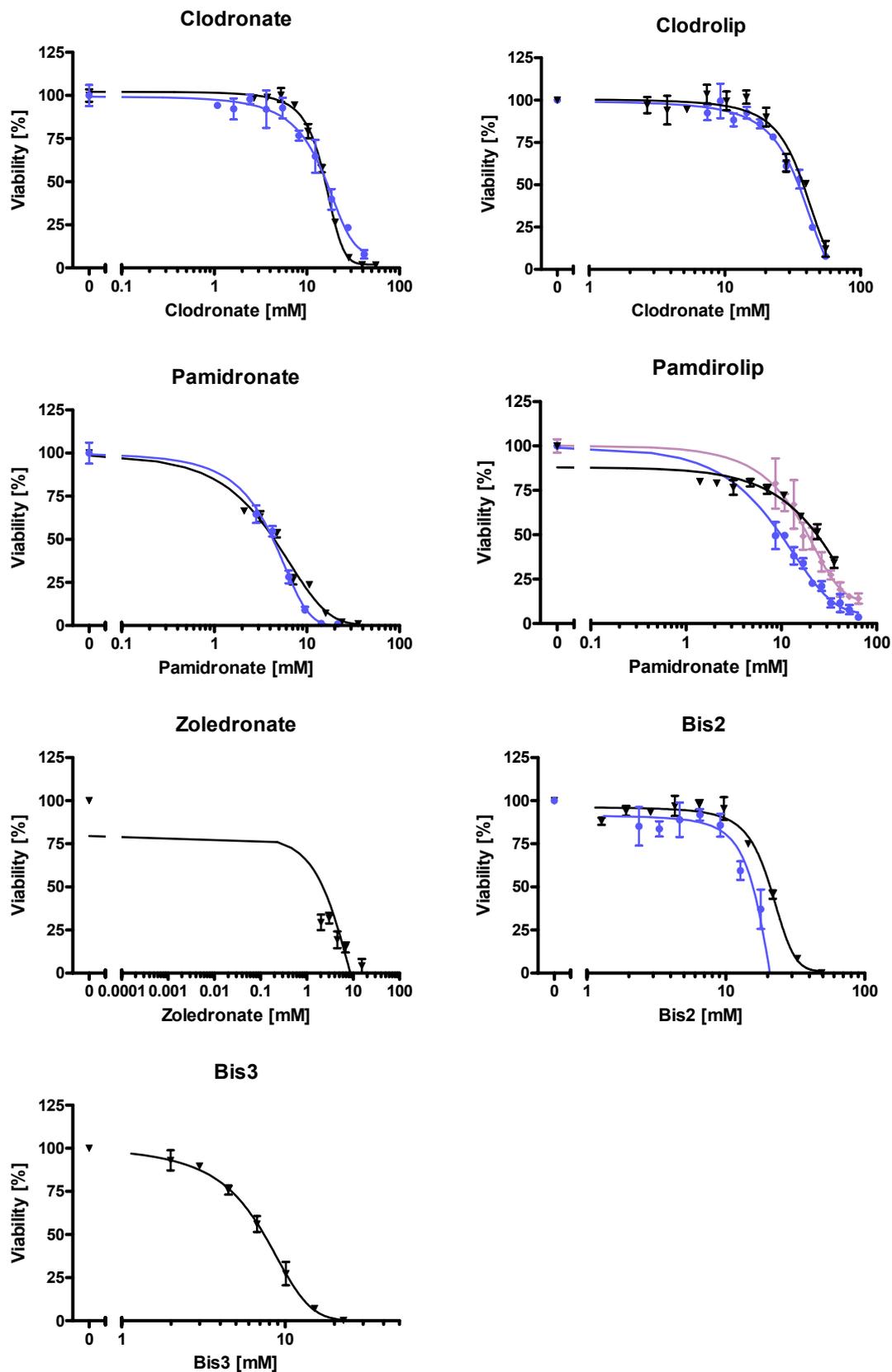
Appendix II

Figure 18   *Cytotoxicity assays with different free and liposomal bisphosphonates on A673 cells (10'000 cells / well), 4 h incubation time, 24 h recovery.*
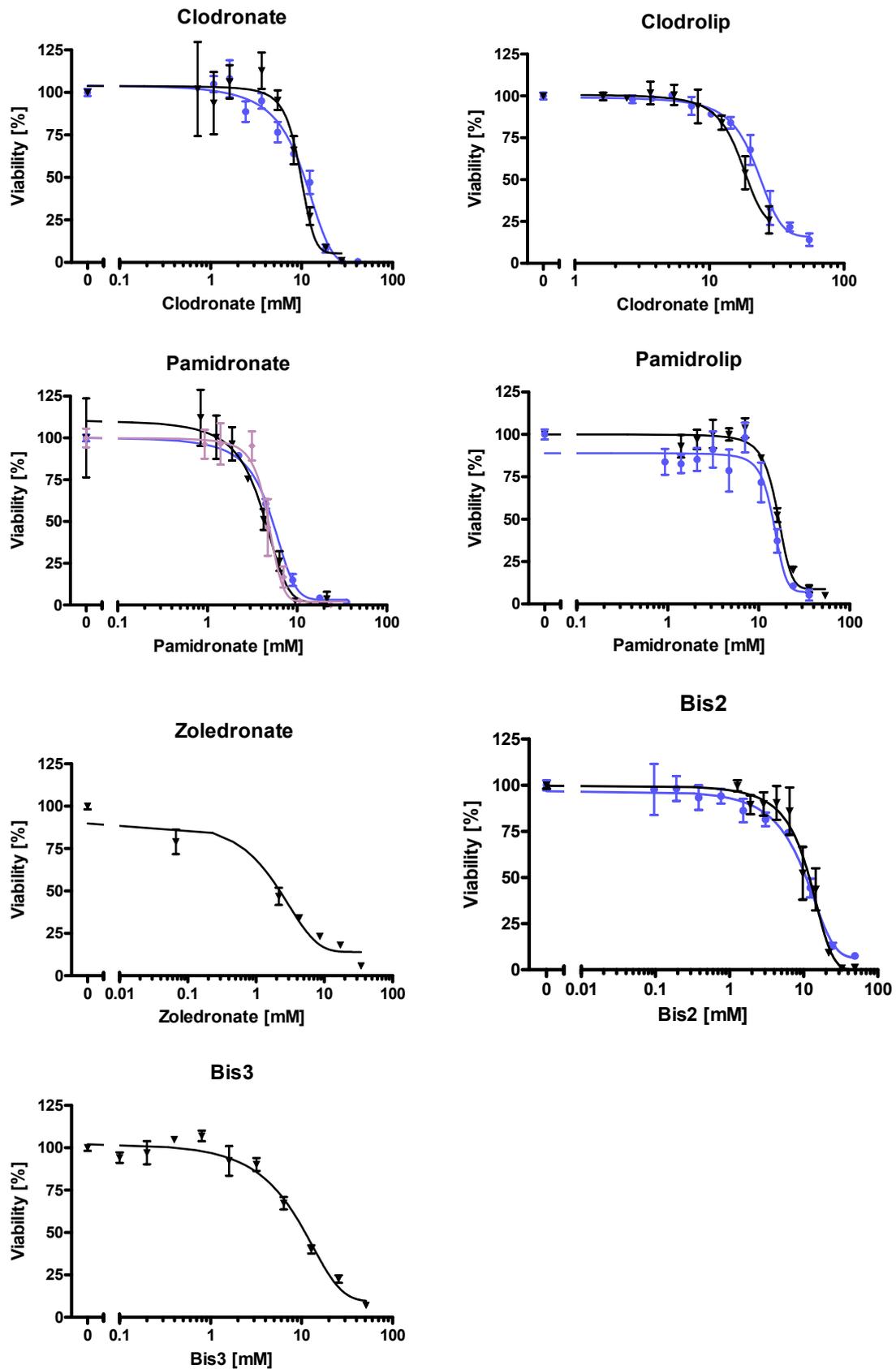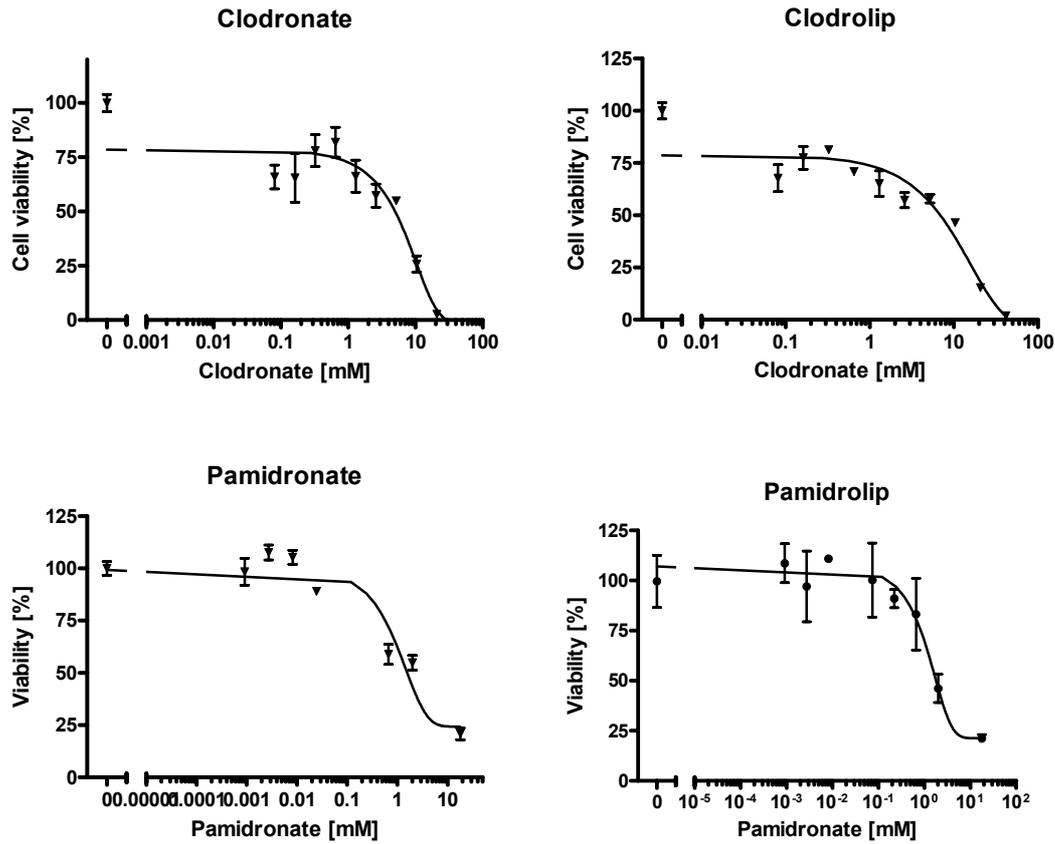
Figure 19   *Cytotoxicity assays with different free and liposomal bisphosphonates on F9 cells (10'000 cells / well,) 4 h incubation time, 24 h recovery.*
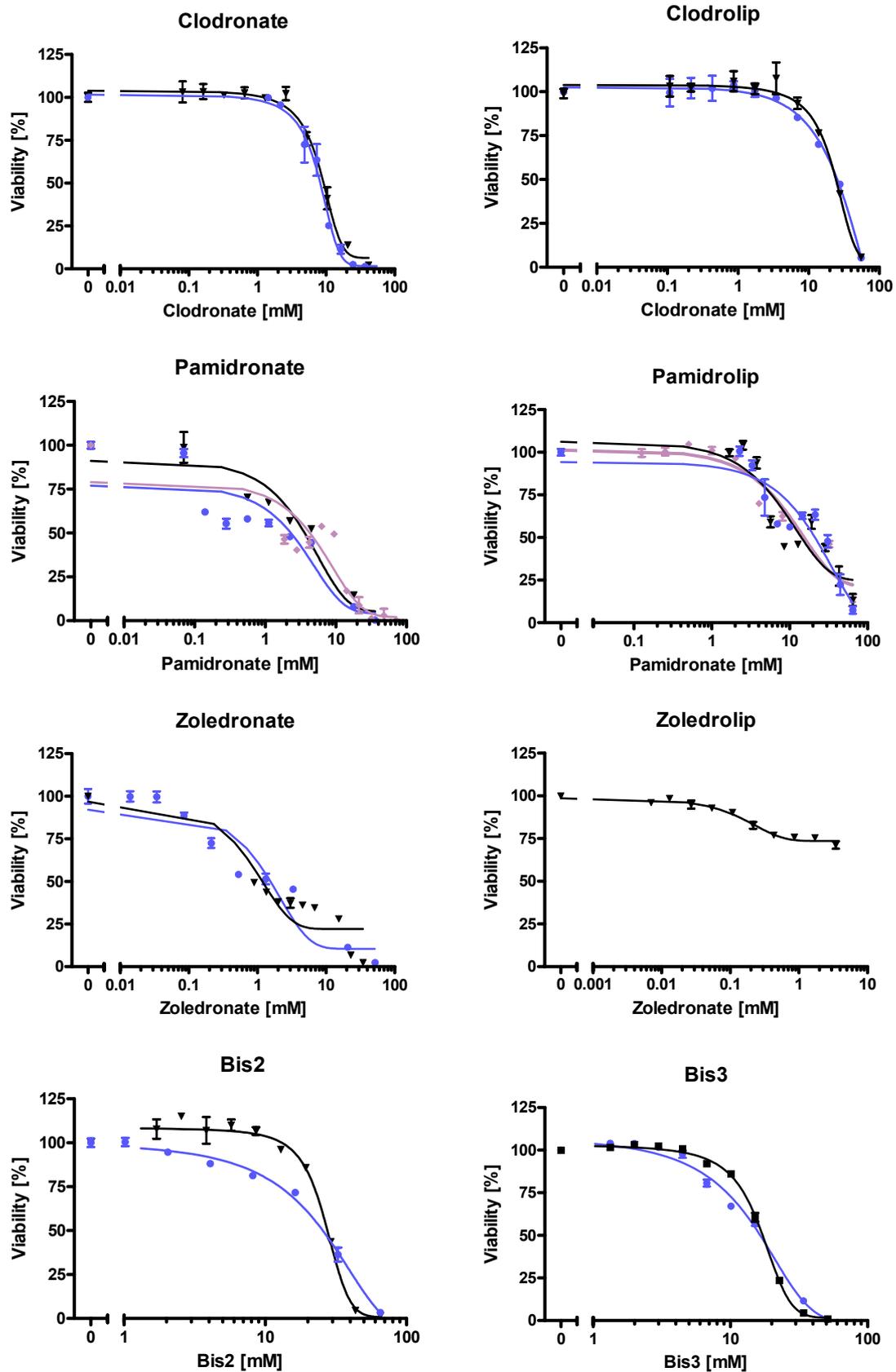
Figure 20 *Cytotoxicity assays with different free and liposomal bisphosphonates on HUVECs (15'000 cells / well), 4 h incubation time, 24 h recovery.*
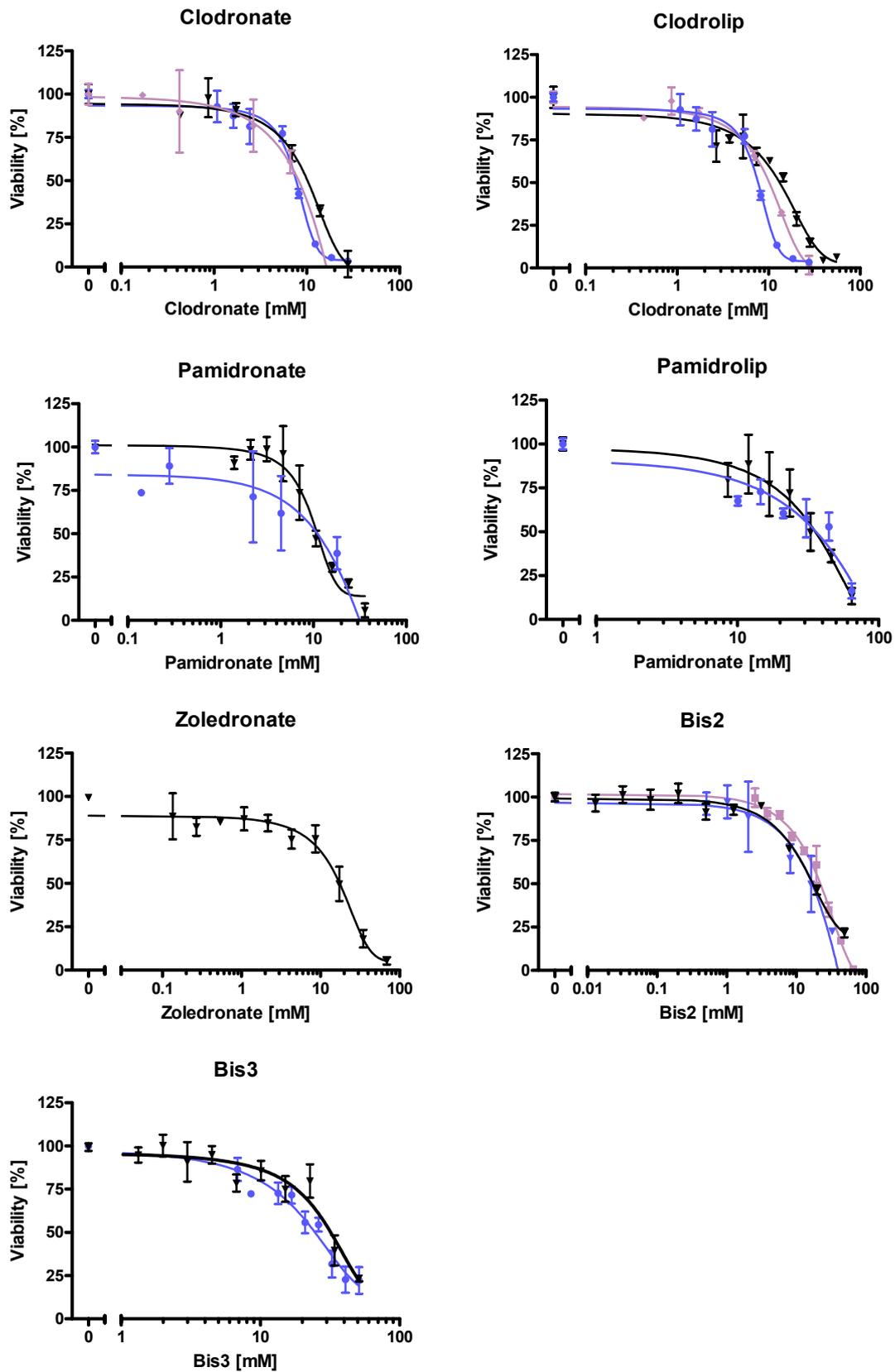
Figure 21 *Cytotoxicity assays with different free and liposomal bisphosphonates on non-activated macrophages (20'000 cells / well), 4 h incubation time, 24 h recovery.*
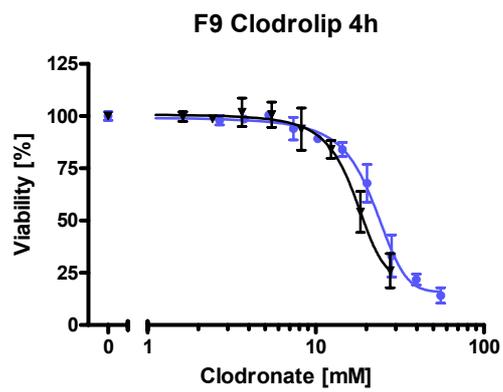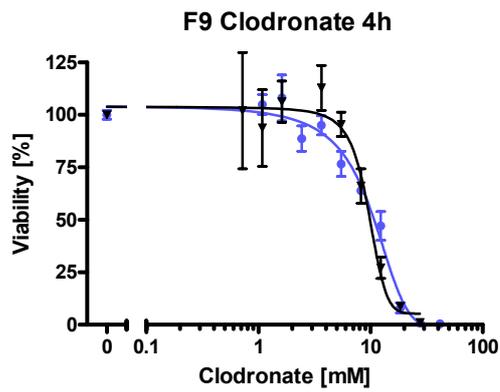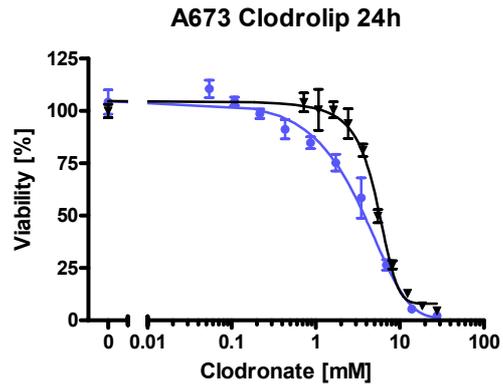
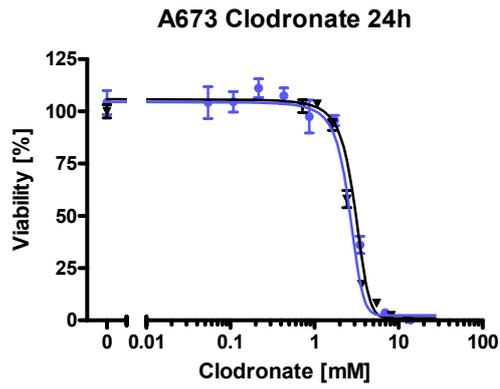*Figure 22* *Cytotoxicity assays with different free and liposomal bisphosphonates on activated (10 ng lipopolysaccharide / ml) macrophages (20'000 cells / well) 4 h incubation time, 24 h recovery.*
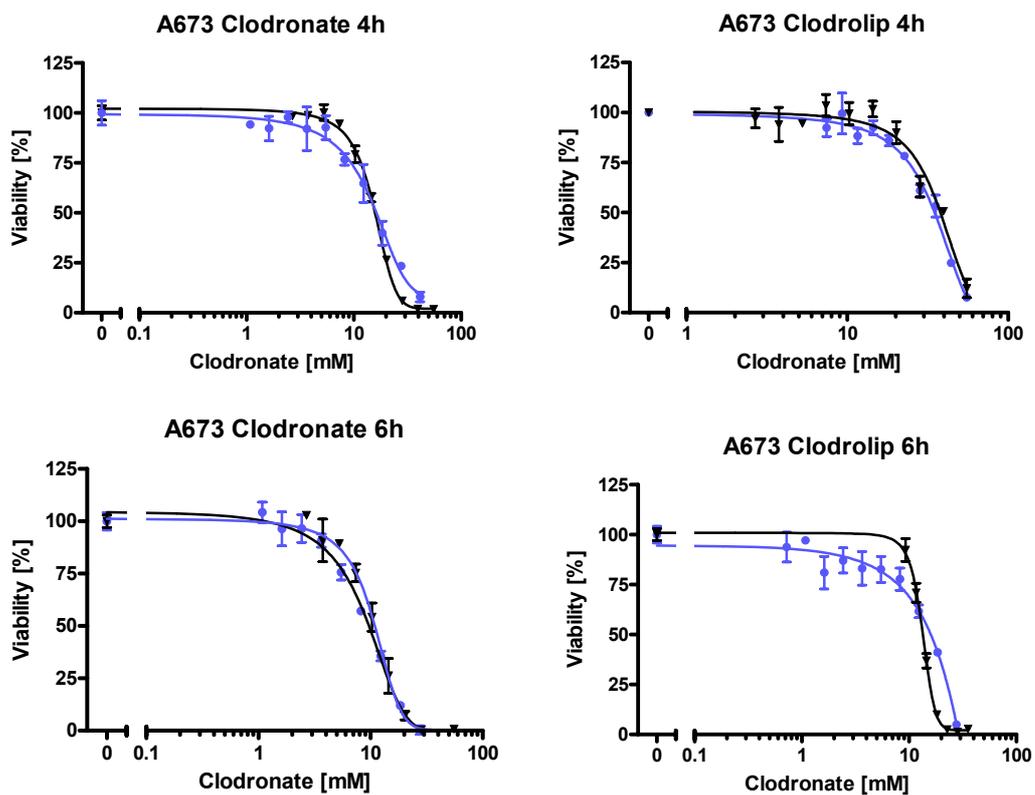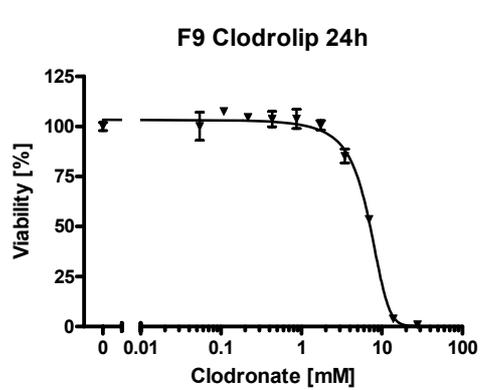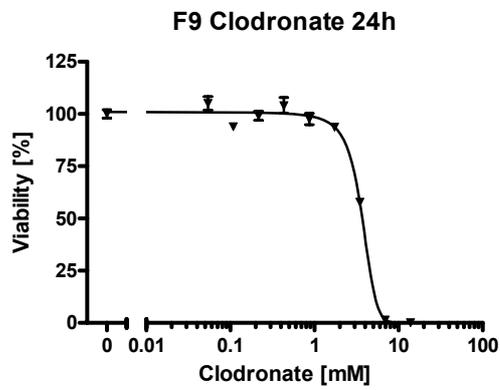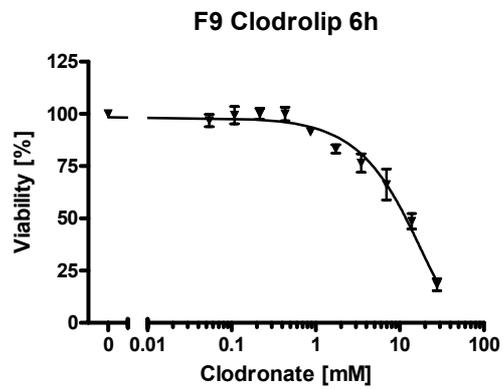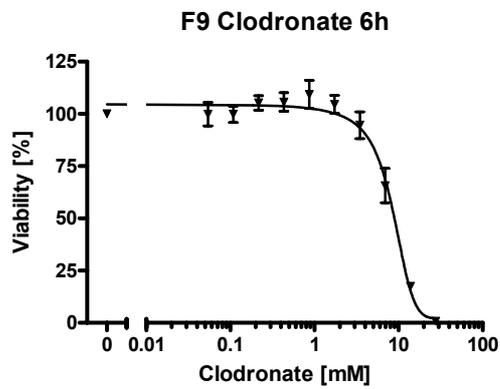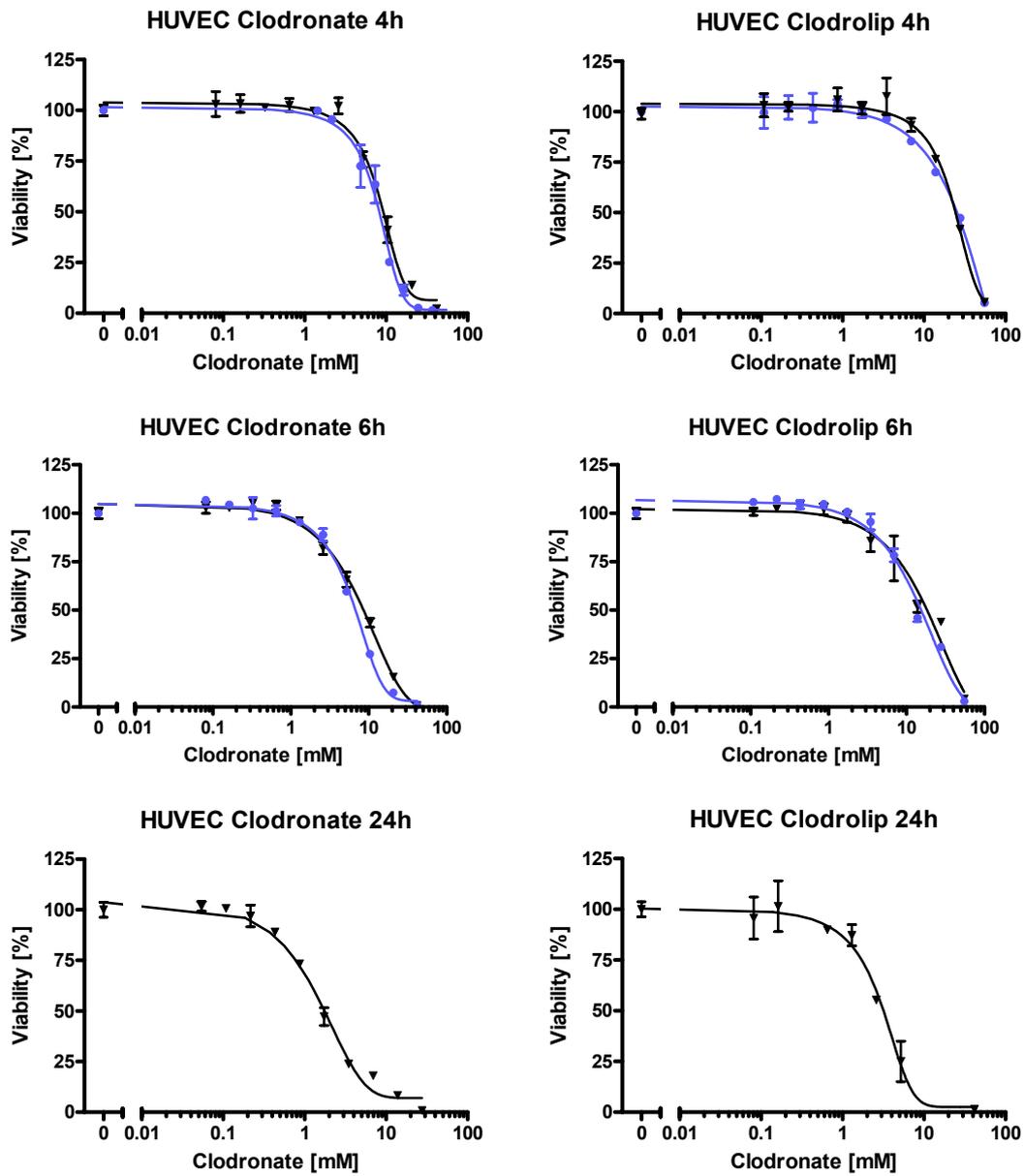
Figure 23   *Cytotoxicity assays with free and liposomal clodronate on A673, F9 (10'000 cells / well) and HUVE cells (15'000 cells / well) after 4 h, 6h and 24 h incubation time and 24 h recovery.*

Table 6 *IC$_{50}$ values of all cytotoxic assays performed: A) Human rhabdomyosarcoma cells (A673), B) murine teratocarcinoma cells (F9), C) human umbilical vein endothelial cells (HUVECs), D) non-activated and activated mouse peritoneal macrophages.*

A)

Drug / cells	A673						F9			
Experiment	I	II	I	II	I	II	I	II	I	I
Incubation time	4h		6h		24h		4h		6h	24h
Clodronate	15.85	15.57	9.73	11.04	2.57	3.2	9.91	10.85	8.79	3.6
Clodrolip	35.08	38.17	13.47	16.14	3.87	5.7	19.68	24.45	12.31	7.07
Pamidronate	4.32	4.55					4.32	4.73		
Pamidrolip	19.33	22.74					14.22	16.43		
Zoledronate	2.14						2.25			
Zoledrolip										
Bis2	20.91	15.85					12.02	11.04		
Bis3	7.39						10.65			
Empty liposomes	-						-			

B)

Drug / cells	HUVEC				
Experiment	I	II	I	II	II
Incubation time	4h		6h		24h
Clodronate	7.98	9	6.91	8.58	1.84
Clodrolip	24.74	26.6	15.29	18.53	3.27
Pamidronate	2.34	4.16			
Pamidrolip	13.62	15.8			
Zoledronate	1.18	1.51			
Zoledrolip					
Bis2	25.35	27.58			
Bis3	17.04	16.24			
Empty liposomes	-				

C)

Drug / cells	Macrophages				Macrophages activated
Experiment	I	II	III	I	I
Incubation time	4h			6h	4h
Clodronate	7.42	7.84	9.91	6.78	4.98
Clodrolip	7.56	9.91	12.99	11.45	6.74
Pamidronate	10.85	10.85			1.5
Pamidrolip	35.94	35.08			1.75
Zoledronate	17.57				
Zoledrolip	2.02				
Bis2	17.04	16.83	21.94		
Bis3	31.48	24.16			
Empty liposomes	-				

Appendix III

The equation shown on the plot:
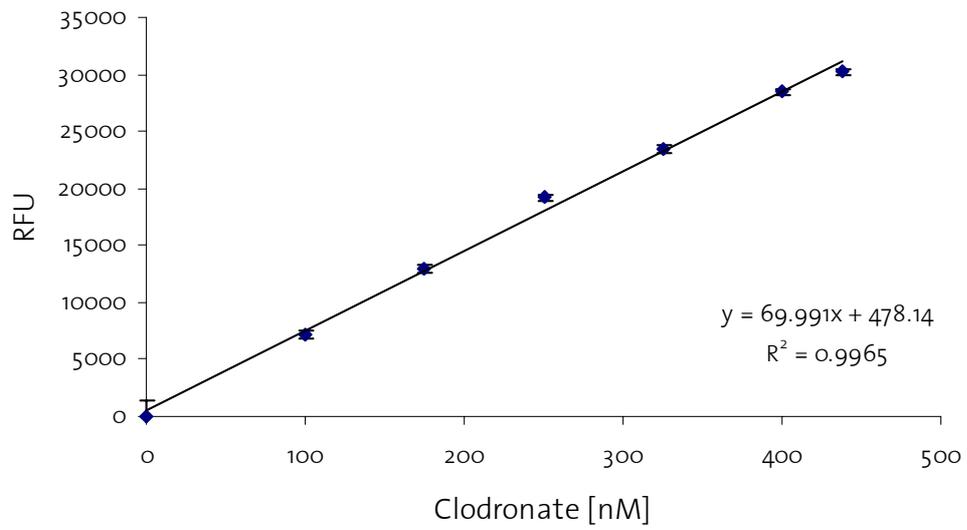$$y = 69.991x + 478.14$$
$$R^2 = 0.9965$$

Figure 24 *Calibration curve for the quantitative determination of clodronate by indirect fluorescence detection (IFD) measured with a Tecan reader at a wavelength of 510 nm using an excitation wavelength of 430 nm*