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Effect of the mycotoxin destruxin E on osteoclasts and on the bone remodeling in vitro

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List of used abbreviations

1α,25-$(OH)_2$ vitamin D_3	1α,25-dihydroxycholecalciferol
α-MEM	minimum essential medium in α-configuration
ADO	autosomal dominant osteopetrosis
ARO	autosomal recessive osteopetrosis
ATP	adenosine triphosphate
BBMV	brush border membrane vesicles
BCA	biological pest control
BL	basolateral domain
BMD	bone mineral density
BMU	basic multicellular unit
BRC	bone remodeling compartment
cAMP	cyclic adenosine monophosphate
c-Fms	a receptor-type tyrosine kinase
CTR	calcitonin receptor
DAPI	4′,6-Diamidino-2-phenylindole
Des. E	destruxin E
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPO	erythropoietin
FCS	fetal calf serum
FSD	functional secretory domain
HBsAg	hepatitis B viral surface antigen
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC-DAD	high pressure liquid chromatography with diod array detector
HSC	hematopoietic stem cell
MMP-9	matrix metalloproteinase 9

M-CSF	macrophage colony-stimulating factor
NADH	nicotinamide adenine dinucleotide hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
OB	osteoblast
OC	osteoclast
OCL	osteoclast-like multinucleated cell
OPG	osteoprotegerin
op/op mice	macrophage-deficient osteopetrotic mice
PBS	phosphate-buffered saline
PGE_2	prostagladin E2
P/S	penicillin-streptomycin solution
PTH	parathyroid hormone
RANK	receptor activator of nuclear factor kappaB ligand
RANKL	RANK ligand
RB	ruffled border
RL	resorption lacuna
rpm	revolution per minute
SD	standard deviation
SZ	sealing zone
TNF	tumor necrosis factor
TRAP	tartrate resistant acid phophatase
UV	ultraviolet

1. Introduction and aim

Destruxins are mainly known for their insecticidal and phytotoxic activities. But as Nakagawa et al. have already shown, these cyclodepsipeptides have potential for being used as antiresorptive drugs. So the aim of my thesis was to get a deeper insight into the inhibtory mechanism of destruxin E on osteoclast bone resorption and on the osteoclasts-osteoblast interface in vitro.

In a first step a resorption assay was performed to observe the inhibitory effect of destruxin E on the activity of osteoclasts. The determined osteoclasts were derived from osteoclast precursors (bone marrow cells) in coculture with osteoblasts. The effect of destruxin E on the activity as well as on the differentiation of osteoclasts could be examined.

In parallel, the osteoclast marker enzyme tartrate resistant acid phosphatise (TRAP) was stained to get an idea about the osteoclast cell number in the different cultures.

Additionally, the cell viability of osteoblasts and osteoclast precursor cells in presence of destruxin E was tested.

Finally, the morphological structure of osteoclasts (and osteoblasts) and the induction of apoptosis by the test substance were investigated. For this reason the nuclei and the cytoskeleton of the cells were stained.

2. Literature review

2.1 Bone Organization

Bone is a highly specialized form of dense connective tissue. Together with cartilage, it makes up the skeletal system. This system serves three functions: mechanical (muscle attachment for locomotion), protective (for organs and bone marrow) and metabolic (reserve of ions, primarily calcium and phosphate, for maintaining the serum homeostasis). The fundamental constituents of bone are the cells and the extracellular matrix. The latter is abundant in this connective tissue. It is composed of collagen fibers and noncollagenous proteins (Baron, 2003).

2.1.1 Bone matrix

The majority of bone is made of the bone matrix, which has inorganic and organic parts. Type I collagen constitutes about 95 % of organic matrix. The remaining 5 % is composed of proteoglycans and numerous noncollagenous proteins (Marks and Odgren, 2002). The major non-collagenous protein that is produced is osteocalcin. It plays an important role in calcium binding, stabilization of hydoroxyapatite in the matrix and in regulation of bone formation (Hadjidakis and Androulakis, 2006). Crystalline salts deposited in the organic matrix of bone are mainly calcium and phosphate in the form of hydroxyapatite (Marks and Odgren, 2002).

2.1.2 Bone types

Two different bone types are observed in the normal mature human skeleton: cortical and trabecular (Hadjidakis and Androulakis, 2006).

2.1.2.1 Cortical bone

Cortical bone comprises 80 % of the skeleton. It is dense and compact, has a slow turnover rate and a high resistance to bending and torsion and forms the outer part of all skeletal structures (Hadjidakis and Androulakis, 2006). Cortical bone is present in the shafts (diaphyses) of long bones and is surrounding cancellous bone (Khosla et al., 2008). The main part of the cortical bone is calcified, what explains its function to provide mechanical strength and protection (Hadjidakis and Androulakis, 2006).

2.1.2.2 Trabecular bone or cancellous bone

Trabecular bone comprises 20 % of the skeleton. It is less dense, more elastic and has a higher turnover rate than cortical bone. It achieves mainly metabolic functions (Hadjidakis and Androulakis, 2006). Cancellous bone, the "spongy" bone, is present in the vertebrae, pelvis and ends (metaphyses) of long bones (Khosla et al., 2008).

2.1.3 Bone remodeling

Bone is continuously remodeled by osteoblasts and osteoclasts, which synthesize bone matrix and resorb bone, respectively. These two cell types cooperate with each other to maintain homeostasis of bony tissue (Miyamoto and Suda, 2003). Bone remodeling is needed to remove old bone and stress-induced microcracks. So it is possible to insure biomechanical stability and to regulate mineral homeostasis of the whole organism (Vaananen and Laitala-Leinonen, 2008). The balance between the activities of osteoblasts and osteoclasts is essential for normal bone functioning. Several bone diseases, for instance osteoporosis and osteopetrosis, are related to a pathological imbalance between their activities (Roodman, 1996). An important role of osteoclasts in various skeletal diseases has become evident. Specific inhibition of osteoclast function has become a major strategy to treat osteoporosis and many other metabolic bone diseases (Vaananen and Laitala-Leinonen, 2008). See more below.

2.1.3.1 Basic multicellular unit (BMU)

In the basic multicellular unit osteoclasts and osteoblasts closely collaborate in the remodeling process. The organization of the BMUs in the two bone typs differs, but these differences are mainly morphological rather than biological (Hadjidakis and Androulakis, 2006).

The BMU is approximately 1-2 mm long and 0.2-0.4 mm wide. It comprises a cutting cone of osteoclasts in front, a closing cone lined by osteoblasts following behind, a central vascular capillary, a nerve supply and an associated connective tissue. In healthy human adults, 3-4 million BMUs are initiated each year and about 1 million BMUs are operating at any moment (Parfitt, 1994).

The BMU maintains its size, shape and internal organization for many months while it travels through the bone in a controlled direction (Parfitt, 1994). In cortical bones, the BMU travels through the bone, creating and replacing a tunnel. In cancellous bones, the BMU moves across the trabecular surface, creating and replacing a trench. As the BMU advances, cells are recruited at each new cross-sectional location. New osteoblasts do not arrive until the osteoclasts have already moved on (Manolagas, 2000).

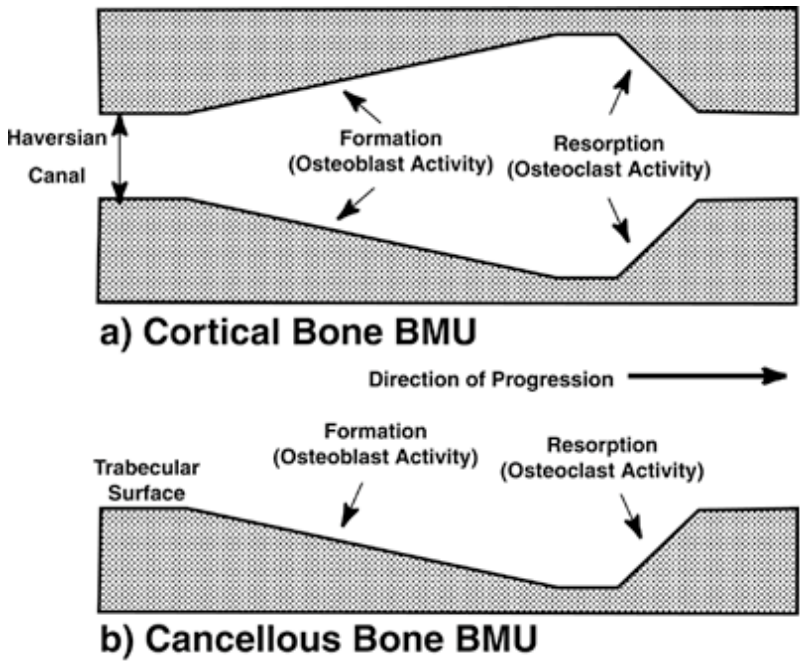


Figure 2.1: The basic multicellular unit (BMU) in cortical and concellous bone.

In cortical bone (a) remodeling takes place in Haversian canals; in concellous bone (b) remodeling takes place on the surface of the trabecula (Hernandez et al., 2000).

The lifespan of the BMU is 6-9 months what is much longer than the lifespan of the executive cells. Therefore, continuous supply of new osteoclasts and osteoblasts from their respective progenitors is essential (Manolagas, 2000).

2.1.3.2 The three phases of bone remodeling

Bone remodeling is occuring in three consecutive phases: initiation, transition and termination of remodeling (figure 1.1). The *initiation phase* includes recruitment of osteoclast precursors, differentiation and activation of osteoclasts as well as the

maintenance of bone resorption. Osteoclast differentiation is induced by osteoblast linage cells that express osteoclastogenic ligands such as RANKL (receptor activator of nuclear factor kappaB ligand). Bone resorption lasts about three weeks in human bone. In the *transition phase* osteoclastic bone resorption is inhibited, osteoclasts undergo apoptosis, and osteoblasts are recruited and differentiate. In the *termination phase* new bone (osteoid) is formed and mineralized. The termination phase lasts much longer than the initiation phase. Osteoblastic bone formation, which lasts about three months, is a much slower process. During the termination phase, osteoclastic differentiation appears to be inhibited (Matsuo and Irie, 2008). A detailed illustration of the occuring processes included would exceed the extent of this work and can be found in the mentioned references.

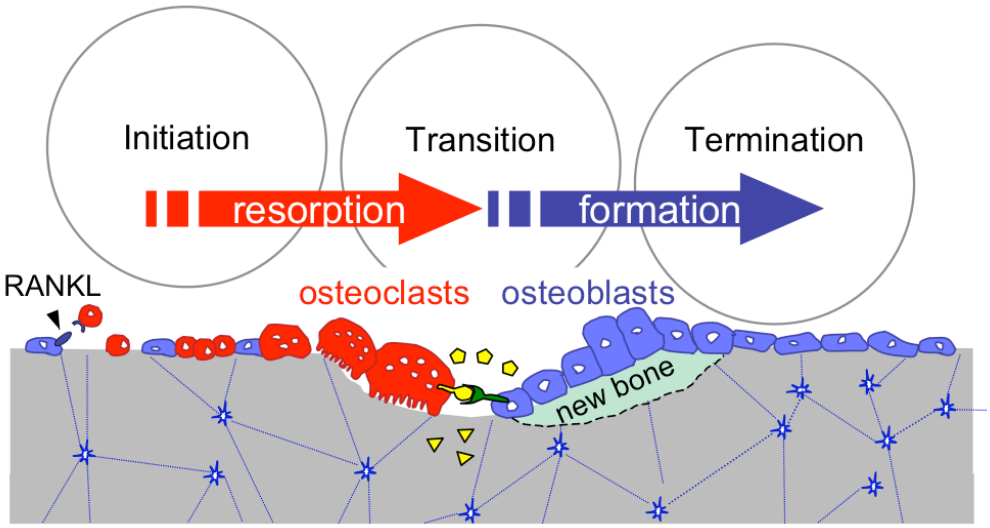


Figure 2.2: The three-phase model of bone remodeling.
Osteoclasts (red) and osteoblasts (blue) are shown. Osteocytes (star-shaped) and canaliculi (blue lines) are shown in bone (gray) (Matsuo and Irie, 2008).

2.1.3.3 Regulation of bone remodeling

The balance of bone seems to be controlled by hormones and other proteins secreted by hemopoietic bone marrow cells and bone cells. There are systemic and local regulations of bone cell function (Hadjidakis and Androulakis, 2006).

2.1.3.3.1 Systemic hormones

Parathyroid hormone

Parathyroid hormone (PTH) is the most important regulator of the calcium homeostasis. It is able to maintain serum calcium concentrations by stimulating bone resorption, increasing renal tubular calcium re-absorption and renal calcitriol production. PTH can lead to catabolic or anabolic effects on bone. It is interesting that PTH stimulates bone formation when given intermittently (in low doses) (Mosekilde et al., 1991; Neer et al., 2001), but stimulates bone resorption when secreted continuously (Parfitt, 1976; Eriksen et al., 1986).

The primary target cell for PTH seems to be the osteoblast (Rodan and Martin, 1981). Isolated osteoclasts do not resorb bone in response to PTH, but they do so when osteoblasts or osteoblastic cell lines are added to the cultures (McSheehy and Chambers, 1986). Furthermore, parathyroid hormone induces RANKL expression by marrow stromal cells (Yasuda et al., 1998).

Calcitriol: 1α,25(OH)$_2$-cholecalciferol

1α,25(OH)$_2$-cholecalciferol is the active form of the prohormone vitamin D$_3$. Metabolites of vitamin D$_3$ are potent stimulators of osteoclastic bone resorption. The most active metabolite is 1α,25(OH)$_2$-cholecalciferol (Roodman, 1999).

Intestinal calcium and phosphorus absorption is enhanced by calcitriol. In this way it promotes bone mineralization (Hadjidakis and Androulakis, 2006).

It is generally recognized that 1α,25$(OH)_2$-cholecalciferol stimulates bone resorption. This is based on the fact that it was found to be a bone-resorbing hormone in a classic experiment using bone organ cultures (Raisz et al., 1972). 1α,25$(OH)_2$-cholecalciferol also induces the expression of the receptor activator of NF-κB ligand (RANKL), which is an essential cytokine for osteoclast differentiation (Yasuda et al., 1998).

Calcitonin

Calcitonin is a peptide hormone and is secreted by the parafollicular cells of the thyroid gland. It is a potent inhibitor of osteoclastic bone resorption (Roodman, 1999). Calcitonin receptors (CTRs) are expressed on both osteoclast precursors as well as mature osteoclasts (Lee et al., 1995).

Calcitonin, in pharmacologic doses, causes loss of the ruffled border, ending of osteoclast motility and inhibition of the secretion of proteolytic enzymes. This effect is mediated through its receptor on osteoclast. However, this effect is limited and its physiologic role is minimal in the adult skeleton (Hadjidakis and Androulakis, 2006).

Glucocorticoids

Glucocorticoids exert stimulatory as well as inhibitory effects on bone cells. They are essential for osteoblast maturation and they also decrease osteoblast activity. Furthermore, glucocorticoids sensitize bone cells to regulators of bone remodeling and they enhance osteoclast recuitment (Weinstein et al., 1998).

Estrogens

Estrogen is considered to be an important steroid hormone. It regulates bone metabolism not only in females but also in males (Smith et al., 1994). A deficiency of

this sex steroid caused by either menopause or removal of the ovaries results in bone loss by increasing osteoclastic bone resorption (Jilka et al., 1992).

Estrogens decrease the responsiveness of the osteoclast progenitor cells to RANKL and so they prevent osteoclast formation (Srivastava et al., 2001). Furthermore, they reduce osteoclast life span (Kameda et al., 1997), stimulating osteoblast proliferation and decreasing their apoptosis (Manolagas, 2000).

Prostaglandins

Prostaglandins can be stimulators of osteoclastic bone resorption in bone organ culture systems as well as stimulators of osteoclast formation in murine marrow cultures (Takahashi et al., 1988). However, prostaglandins (PGE_2) inhibit osteoclastic bone resorption and formation in long-term human marrow sytems (Chenu et al., 1990). But it appears that the effect of prostaglandins on osteoclast formation and osteoclastic bone resorption may be dependent on the dose administered and the assay system used (Hadjidakis and Androulakis, 2006).

2.1.3.3.2 Local regulation

OPG/RANKL/RANK system

see chapter 2.1.6.2

Macrophage colony-stimulating factor (M-CSF)

One of the factors produced by osteoblastic/bone marrow stromal cells that support osteoclast formation is the cytokine M-CSF. The evidence for this observation came from the identification of a mutation in the gene for M-CSF in *op/op* mice

(macrophage-deficient osteopetrotic mice), which have osteopetrosis (Yoshida et al., 1990; Wiktor-Jedrzejczak et al., 1990).

M-CSF binds to its receptor c-Fms (a receptor-type tyrosine kinase) on preosteoclastic cells. Since M-CSF is the primary determinant of the pool of these precursor cells it is necessary for osteoclasts development (Udagawa et al., 1990).

The main role of M-CSF in osteoclast biology seems to be to enhance the proliferation and survival of precursors and also to enhance the survival of mature cells (Chambers, 2000).

It is suggested that M-CSF plays an important role in osteoclast development as well as in osteoclast function (Suda et al., 1997).

2.1.4 Bone cells

Bone consists of four different cell types. Osteoblast, osteoclasts and bone lining cells are present on bone surface. Osteocytes permeate the mineralized interior. Osteoblasts, osteocytes and bone lining cells originate from local osteoprogenitor cells. Osteoclasts on the other hand arise from the fusion of mononuclear precursors, which originate in hemopoietic tissues (Marks and Odgren, 2002).

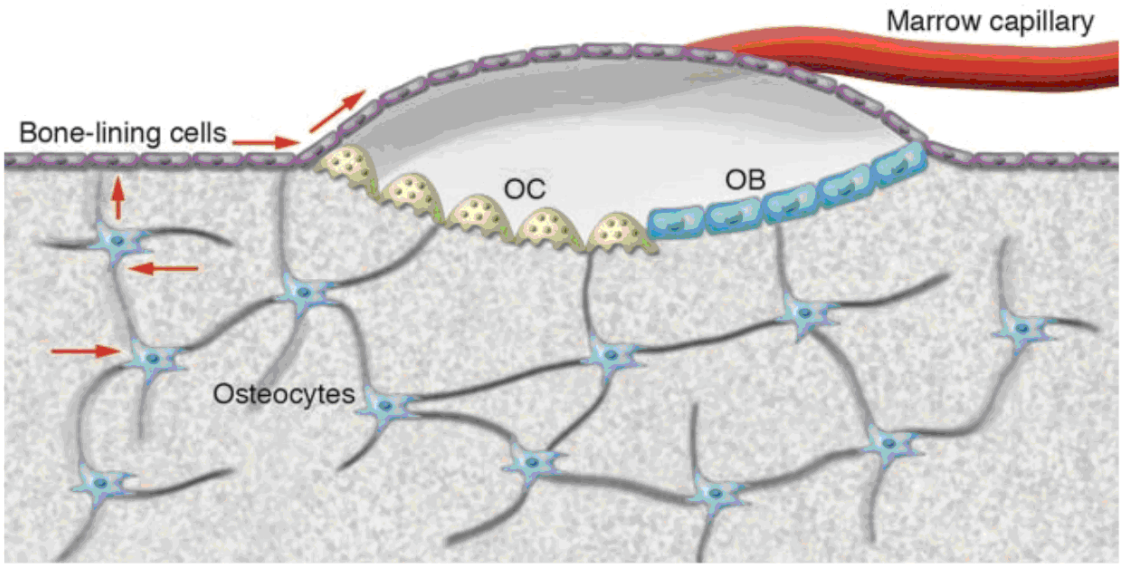


Figure 2.3: Schematic of the BRC (bone remodeling compartment).
All cells in the network are connected with gap junctions. Especially osteoclasts (OCs), osteoblasts (OBs), osteocytes, and bone lining cells can be seen (Khosla et al., 2008).

2.1.4.1 Bone lining cells

Bone lining cells are flat, elongated, inactive cells. They cover bone surfaces that are undergoing neither bone formation nor bone resorption. Since these cells are inactive, they exhibit few cytoplasmic organelles (Marks and Odgren, 2002). Bone lining cells are thinly extended over bone surface. They have flat or slightly ovoid nuclei and connect to other bone lining cells via gap junctions. They send cell processes into surface canaliculi. Activation of bone remodeling occurs on inactive bone surface. That means that bone lining cells may be involved in the propagation of the activation signal that initiates bone resorption and bone remodeling (Miller et al., 1989). Still little is known regarding the function of these cells. But it has been speculated that bone lining cells can be precursors for osteoblasts (Marks and Odgren, 2002).

2.1.4.2 Osteoblasts

The osteoblast produces the bone matrix constituents. Since osteoblasts do not function individually, they are found in clusters along the bone surface. They line on the layer of bone matrix that they are producing (Hadjidakis and Androulakis, 2006). They originate from multipotent mesenchymal stem cells. This mesenchymal stem cells can differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts, or fibroblasts (Bianco et al., 2001).

Toward the end of the matrix-secreting period, 15 % of mature osteoblasts are entrapped in the new produced bone matrix and so they differentiate into osteocytes (as mentioned above). There are also some cells that remain on the bone surface and become flat lining cells. Bone formation occurs in three successive phases: the production of osteoid matrix, the maturation of osteoid matrix and finally the mineralization of the matrix. Initially, osteoblasts produce osteoid by rapidly depositing collagen. This causes an increase in the mineralization rate to equal that of collagen synthesis. Finally, the rate of collagen synthesis decreases, but the mineralization

process continues until the osteoid becomes fully mineralized (Hadjidakis and Androulakis, 2006).

2.1.4.3 Osteocytes

Ostoecytes derive from osteoblasts. The bone forming cells (osteoblasts) are of mesenchymal origin. Osteoblasts secrete non-mineralized bone matrix (osteoid) and finally become incorporated as osteocytes in mineralized bone matrix. Despite the fact that osteocytes are the most abundant cellular component of mammalian bone, little is known about the process of osteoblast-to-osteocyte transformation (Franz-Odendaal et al., 2006).

An osteocyte is a mature osteoblast within the bone matrix and is responsible for its maintenance (Buckwalter et al., 1996).

Osteocytes extend enormously and long cell processes connect with each other and with lining cells and osteoblasts on the bone surface. Thereby they form a syncytial network throughout the skeleton. Osteocytic cell processes travel through small tunnels within mineralized bone that are called osteocytic canaliculi. These tunnels are filled with extracellular fluid. Thereby they provide an immense surface for the transport and exchange of ions and other bioactive substances (Ikeda, 2008).

During differentiation from osteoblasts to mature osteocytes the cells lose a large part of the cell organelles (Aarden et al., 1994). Osteocytes are long living cells. They exist inside bone for 10-20 years, compared to the extremely short lifespan of osteoclasts, which perform bone resorption for less than 2 weeks, and osteoblasts, that are occupied with bone formation for a period of several months (Ikeda, 2008).

Osteocyte functional activity and morphology varies according to the age of the cell. A young osteocyte exhibits most of the structural characteristics of the osteoblast, but has decreased cell volume and capacity of protein synthesis. An older osteocyte is located

deeper within the calcified bone, has a further decrease in cell volume and an accumulation of glycogen in the cytoplasm (Hadjidakis and Androulakis, 2006). Furthermore, the osteocytes are phagocytosed and digested during osteoblastic bone resorption (Elmardi et al., 1990).

2.1.4.4 Osteoclasts

The definition of osteoclasts is "bone resorbing cells" (Miyamoto and Suda, 2003). Bone resorption involves both dissolution of bone mineral and degradation of organic bone matrix. Osteoclasts are highly specialized cells and can perform both of these functions. Many of the molecular meachanisms that are needed for the resorption process have recently been clarified (Vaananen and Laitala-Leinonen, 2008).
Tartrate-resistant acid phosphatase (TRAP) activity and the calcitonin receptor (CTR) are available markers for osteoclasts, other than their bone resorbing activity (Miyamoto and Suda, 2003).

Osteoclasts exhibit abundant Golgi complexes, mitochondria, and transport vesicles loaded with lysosomal enyzmes (Hadjidakis and Androulakis, 2006).

2.1.4.4.1 Differentiation of Osteoclasts

Osteoclasts are derived from hematopoietic stem cells (HSCs) through monocyte lineage progenitor cells (Miyamoto and Suda, 2003). The differentiation pathway of osteoclasts is common to that of macrophages and dendritic cells (Vaananen et al., 2000). Mononuclear osteoclasts tightly adhere to bone and then fuse with each other to form multinuclear osteoclasts. The multinucleation is the most characteristic feature of osteoclasts (Miyamoto and Suda, 2003). The differentiation of osteoclasts is regulated by a number of other cells and their products, especially by RANK and M-CSF (Vaananen and Laitala-Leinonen, 2008).

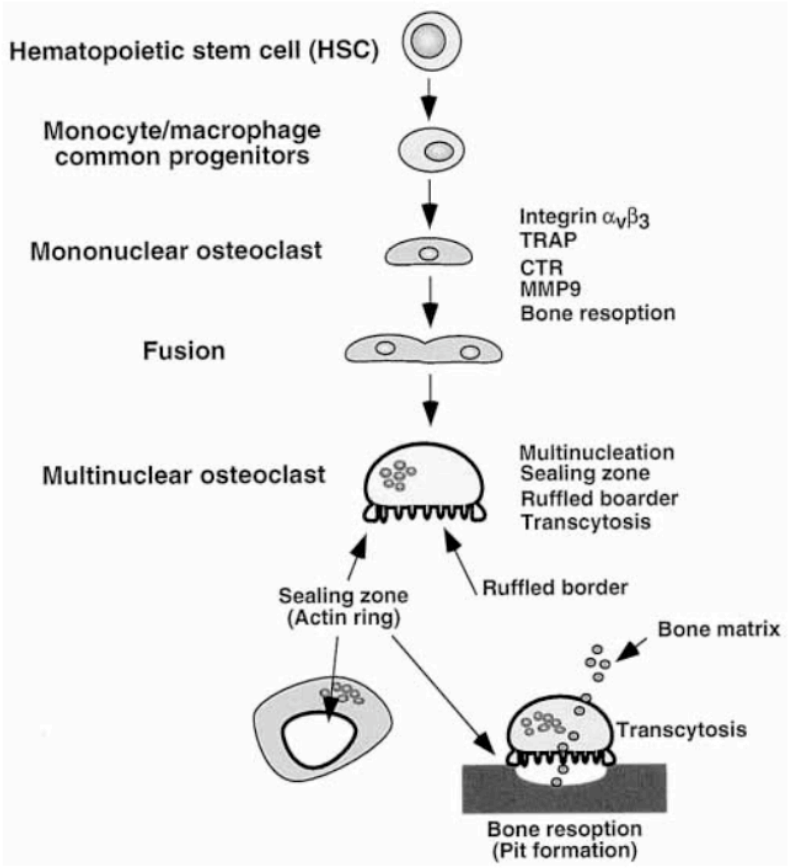


Figure 2.4: Development of osteoclasts from hematopoietic stem cells.
TRAP, tatrate resistant acid phophatase; CTR, calcitonin receptor; MMP9, matrix metalloproteinase 9 (Miyamoto and Suda, 2003).

Osteoclast formation is restricted to the bone surface, suggesting that bone provides a suitable environment for osteoclastogenesis (Miyamoto and Suda, 2003). During the resorption process osteoclasts are tightly sealed to the bone surface and so the resorption area is isolated from the extracellular fluid (Vaananen and Horton, 1995).

2.1.4.4.2 Resorption cycle

The sum of cellular events needed for bone resorption is called the resorption cycle. The term resorption cycle covers neither the differentiation pathway of osteoclasts nor the cellular activities needed for the fusion of mononuclear precursors to form the multinuclear mature osteoclasts (Vaananen et al., 2000).

Osteoclasts have several unique ultrastructural characteristics, for instance multiple nuclei surrounded by a well-developed Golgi apparatus, abundant pleomorphic mitochondria and many vacuoles and lysosomes (Takahashi et al., 2007).
Resorbing osteoclasts are highly polarized cells. They achieve this polarization by reorganizing the cytoskeleton and from special areas such as a sealing zone (Miyamoto and Suda, 2003).

Sealing zone/clear zone
After migration of the osteoclast to the resorption site, a specific membrane domain, called sealing zone, forms on the apical side of the osteoclast (Vaananen et al., 2000). The sealing zone, defined as a unique thick band of actin, serves for the attachment of osteoclasts to the bone surface. In this way they isolate the resorption area, called "Howship´s lacuna", from the surroundings (Takahashi et al., 2007). By tight sealing of the surrounding plasma membrane to the bone matrix the "proton-impermeable" resorption lacuna is formed. This is necessary to create conditions where bone resorption can take place. This tight sealing of plasma membrane to the bone matrix is visualized at the ultrastructural level as the clear zone of osteoclasts (Vaananen and Horton, 1995).
The molecular interactions between the plasma membrane of osteoclasts and the bone matrix at the sealing zone are still unknown. It appears that integrins play an important role in early phases of the resorption cycle (Vaananen et al., 2000).

Attachment occurs via dynamic structures called podosomes. Through their continuous assembly and disassembly they allow osteoclasts to move across the bone surface and let bone resorption proceed (Hadjidakis and Androulakis, 2006).

Resorbing osteoclasts contain not only the sealing zone but also three other specialized membrane domains: a ruffled border, a functional secretory domain and a basolateral membrane (Vaananen et al., 2000).

Ruffled border

The ruffled border is the actual resorbing organ which faces the resorption lacuna (Vaananen and Horton, 1995).

Acidification of the cavity between the cell membrane and the bone surface, and secretion of proteolytic enzymes into it, occurs through this specialized organelle (Mulari et al., 2003). This activity causes local dissolution of the mineral and digestion of the collagen matrix, thereby forming a "resorption pit" (Baron, 1989). The low extracellular pH is generated via an active secretion of protons by V-type proton pumps/vacular type proton-ATPases in the ruffled border membrane and in intracellular vacuoles (Blair et al., 1989; Vaananen and Horton, 1995; Sundquist et al., 1990). Proteolytic enzymes such as cathepsin K, matrix metalloproteinase 9 (MMP-9), and lysosomal enzymes are secreted from the ruffled border into the resorption lacuna (Takahashi et al., 2007).

Bone resorption involves both dissolution of bone mineral and degradation of organic bone matrix (Vaananen and Laitala-Leinonen, 2008). The first process during bone matrix resorption is the mobilization of the hydroxyapatite crystals that is determined by digestion of their link to collagen. Then the residual collagen fibers are digested by either cathepsins (which are secreted from the ruffled border) or by activated collagenases (Hadjidakis and Androulakis, 2006). Matrix degradation products are endocytosed from the central portion of the ruffled border. They are packaged into

transcytotic vesicles, transported to a functional secretory domain in the basolateral membrane and finally secreted through exocytosis (Takahashi et al., 2007).

Basolateral membrane and functional secretory domain

Basolateral membrane is the non-bone facing membrane. It is divided into two distinct domains. A specific membrane domain, called functional secretory domain (FSD), is formed in the center of the basolateral membrane (Salo et al., 1997).

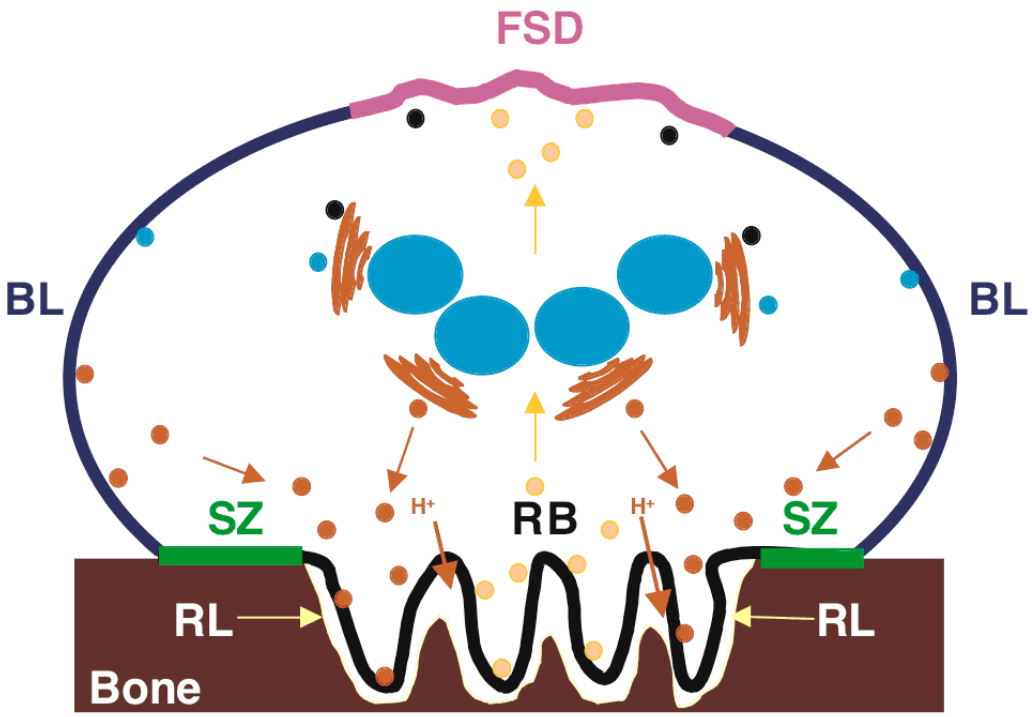


Figure 2.5.: Scheme of a bone-resorbing osteoclast.
BL, basolateral domain (blue); FSD, functional secretory domain (rose); SZ, sealing zone (green); RB, ruffled border (black); RL, resorption lacuna (white) (Vaananen et al., 2000).

Cathepsin K

Cathepsin K is a cysteine proteinase with the unique ability to degrade the triple helix of native collagen at an acid pH. It is the major acid hydrolase responsible for matrix degradation (Garnero et al., 1998). The enzyme is selectively expressed in osteoclasts, while other cathepsins are virtually absent (Saftig et al., 1998).

Organization of the cytoskeleton during the resorption cycle

The change in the polarization of the osteoclast during activation is caused by re-organization of the actin cytoskeleton (Teti et al., 1991; Turksen et al., 1988).

When the osteoclast is "transformed" from the non-resorbing to the resorbing stage, three clearly distinct formations of F-actin and five patterns of vinculin distributions are observed (figure 1.6). In the first stages of the resorption cycle actin and vinculin are distributed throughout the developing punctate structures (podosomes) on the bone matrix. Towards the resorption stage these podosome-like structures coalesce to the specific area of the osteoclasts. Actin and vinculin dissociate from each other and actin forms a dense continuous band around the future resorptive area. Furthermore, the podosome type dot-like appearance is lost. This accumulation of actin filaments roughly corresponds to the morphological clear zone and is delimited by a double ring of vinculin and talin (Vaananen and Horton, 1995). The organization of F-actin into a belt or ring-like structure (actin ring) is typical only for resorbing osteoclasts. Therefore it can be used as a marker for resorbing cells. This characteristic changes in the molecular organization of the cytoskeleton in osteoclasts during the resorption cycle (Lakkakorpi and Vaananen, 1996).

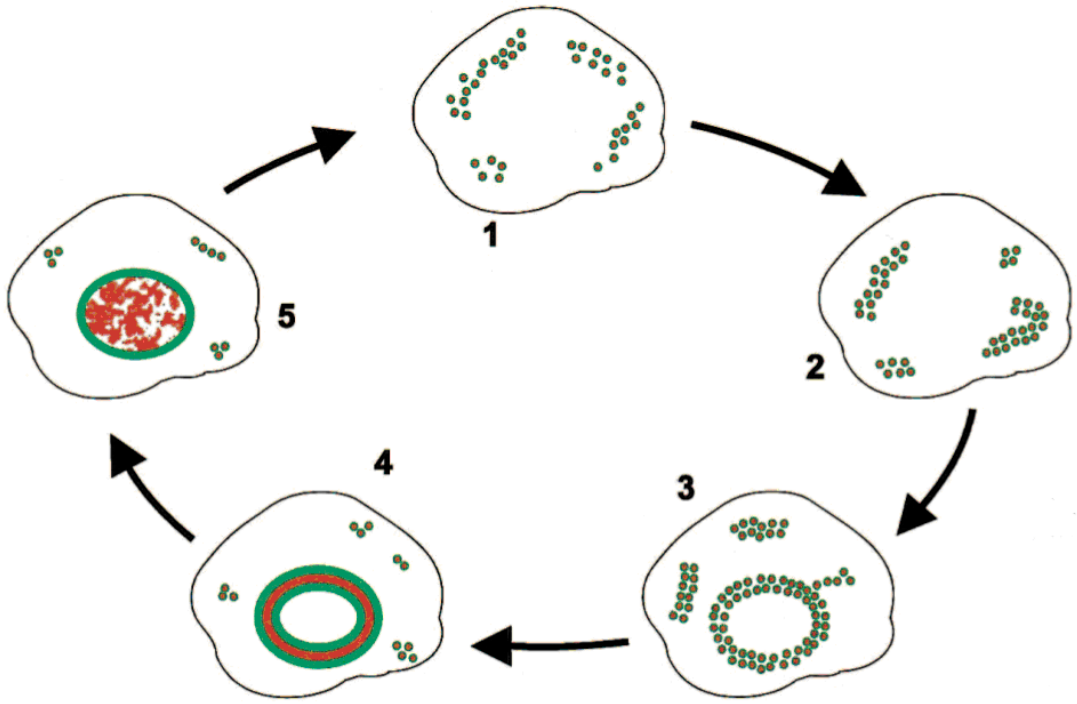


Figure 2.6: Five different patterns of vinculin (green) and three different formations of actin (red) in osteoclasts during the resorption cycle. Stage 1 to 3 represent pre-resorptive events, stage 4 is a resorbing cell and stage 5 represents the post-resorptive stage (Vaananen and Horton, 1995).

2.1.5 Death of bone cells by apoptosis

The average lifespan of human osteoclasts is about 2 weeks while that of osteoblasts is 3 months (Parfitt, 1994). After osteoclasts have eroded, they die and are quickly removed by phagocytes (Hughes et al., 1996). The majority (65 %) of the osteoblasts that originally gathered at the remodeling site also die. The remaining osteoblasts are converted to lining cells. These lining cells then cover quiescent bone surface or are entrapped in the mineralized matrix as osteocytes (Jilka et al., 1998). Osteoclasts as well

as osteoblasts die by apoptosis. Apoptosis is a morphologically distinct form of programmed cell death and is a process common to several regenerating tissues (Steller, 1995). It is an important regulatory program in which activation or suppression of many factors, including oncogenes, tumor suppressor genes, growth factors, cytokines and integrins, can determine a cell´s fate (Steller, 1995).

As in other tissues, bone cells undergoing apoptosis are recognized by condensation of chromatin, the degradation of DNA into fragments and the formation of plasma and nuclear blebs (Manolagas, 2000).

2.1.6 Cross-talk among bone cells

Bone cells "talk" to each other to maintain bone integrity and they communicate in order to regulate the balance between bone resorption and bone formation. Recent findings have suggested that the bone remodeling compartment is critical for osteoclast-osteoblast communication during bone remodelling process. Differentiation, activation and apoptosis of the different bone cells are often dependent on the status of other types of bone cells. Bone cells in different lineages achieve intercellular communication not only by ligand-receptor interactions but also by molecules and ions that travel in the extracellular space or across gap junctions (Matsuo, 2009).

2.1.6.1 Osteoclast-osteoblast communication

Osteoclast-osteoblast communication occurs in the BMU (basic multicellular unit). There are at least three types of osteoclast-osteoblast communication. Osteoclasts and osteoblast can have direct contact where they allow membrane-bound ligands and receptors to interact and initiate intracellular signalling. They can also form gap junctions. This allows the passage of small water-soluble molecules between the two cell types. Their communication can also occur through diffusible paracrine factors,

such as growth factors, cytokines, chemokines and other small molecules secreted by either cell type and acting on the other via diffusion (Matsuo and Irie, 2008).

2.1.6.2 OPG/RANKL/RANK system

Around 1997, several laboratories identified the essential osteoclastogenic ligand RANKL (also known as OPGL, ODF, or TRANCE). This is a transmembrane glycoprotein belonging to the tumor necrosis factor (TNF)-α superfamily and produced as a trimer on the surface of pre-osteoblastic/stromal cells. Osteoclast precursors, on the other hand, express RANK (receptor activator of nuclear factor kappaB), the receptor for RANKL (Matsuo and Irie, 2008). The RANKL/RANK interaction results in activation, differentiation, and fusion of hematopoietic cells of the osteoclast lineage. Then they can begin the process of resorption. Furthermore, this interaction also prolongs osteoclast survival because the process suppresses their apoptosis (Hsu et al., 1999). The expression of RANKL is relatively limited in osteoblasts. But it is also expressed in other cells, such as T cells and spleen cells (Miyamoto and Suda, 2003).

The effects of RANKL can be blocked by osteoprotegerin (OPG), a secretory dimeric glycoprotein belonging to the TNF receptor family. OPG is a soluble receptor and acts as an antagonist for RANKL. This decoy receptor is mainly produced by cells of the osteoblast lineage, but can also be produced by the other cells in the bone marrow (Hofbauer and Schoppet, 2004; Simonet et al., 1997). By inhibiting the final differentiation and activation of osteoclasts and by inducing their apoptosis OPG regulates bone resorption. Since OPG is not incorporated into the bone matrix, its effects on bone resorption are fully reversible (Hadjidakis and Androulakis, 2006).

Estrogen deficiency, glucocorticoid exposure, T-cell activation (e.g. rheumatoid arthritis) and skeletal malignancies (e.g. myeloma, metastases) enhance the ratio of RANKL to OPG. This fact promotes osteoclastogenesis, accelerates bone resorption and so induces bone loss. RANKL blockade (using OPG, RANK fusion proteins or RANKL antibodies) has prevented bone loss caused by osteoporosis, chronic

inflammatory disorders and malignant tumors in some animal models. Therefore, RANKL blockade can emerge as a therapy in humans (Hofbauer and Schoppet, 2004).

Denosumab

In the last few years densumab was studied as a treatment for postmenopausal osteoporosis and bone destruction due to rheumatoid arthritis or metastatic cancer (Pageau, 2009). Denosumab is a human antibody to RANKL. It prevents the interaction of RANKL with RANK on osteoclasts and their precursors, thereby blocking the formation, function, and survival of osteoclasts. Hence to that, densosumab decreases bone resorption, and increases bone density. Denosumab offers an alternative approach to the treatment of osteoporosis (Cummings et al., 2009).

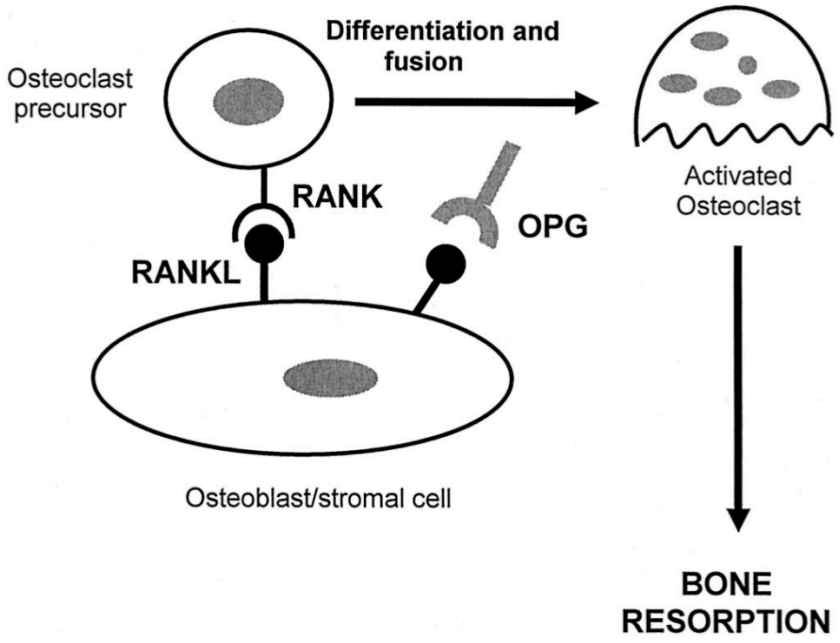


Figure 2.7: Interaction of RANKL, RANK and OPG (Coetzee and Kruger, 2004).

2.2 Skeletal disorders

2.2.1 Osteopetrosis “marble bone disease”

Osteopetrosis comprises a clinically and genetically heterogeneous group of conditions that have the characteristic of increased bone density. The increase in bone density results from abnormalities in osteoclast differentiation or function and mutations in at least 10 genes in humans (Stark and Savarirayan, 2009).

These conditions are quite rare. Autosomal recessive osteopetrosis (ARO) has an incidence of 1 in 250,000 births and autosomal dominant osteopetrosis (ADO) has an incidence of 1 in 20,000 births (Stark and Savarirayan, 2009).

The different subforms are classified into three major groups on the basis of inheritance, age of onset, severity and secondary clinical features: autosomal recessive infantile malignant osteopetrosis, autosomal recessive intermediate mild osteopetrosis and autosomal dominant adult onset benign osteopetrosis (Stoker, 2002).

The increased bone mass can cause phenotypic changes such as macrocephaly and altered craniofacial morphology. But more importantly it can effect other organs and tissues, notably the bone marrow and nervous systems (Stark and Savarirayan, 2009).

2.2.2 Osteoporosis

Osteoporosis is one of the major public health problems that is associated with aging. It is defined as a skeletal disorder characterized by decreased bone strength increasing the risk of fracture for the respective person. Bone strength is influenced by bone density as well as by bone quality (Sipos et al., 2009).

A BMD (bone mineral density) value between 1 and 2 SD (standard deviation) below the mean value for young adults is described as osteopenia. A BMD value more than 2.5 standard deviations below the adult mean value is defined as osteoporosis (Kanis et al., 1994).

It is well known that osteoporosis is primarily a consequence of estrogen deficiency. But there are also many additional factors that contribute to the pathogenesis of osteoporosis such as genetic and life-style factors, nutrition and the intake of medications (Sipos et al., 2009).

In the early 1980s Riggs and Melton proposed the existence of two types of involutional osteoporosis: postmenopausale (type 1) and senile (type 2) osteoposrosis. They stated that postmenopausal women experience both types of osteoporosis, whereas men undergo mainly type 2 osteoporosis. Furthermore, Riggs and Melton believed that postmenopausal osteoporosis is caused by estrogen deficiency and senile osteoporosis is due to aging processes including osteoblast dysfunction (Riggs and Melton, 1983).

In 1998 the type 1/type 2 model of Riggs and Melton was revised and from now on termed “unitary model of osteoporosis in postmenopausal women and aging men”. In the unitary model, estrogen deficiency was suggested to be the main cause of both phases of bone loss in postmenopausal women and osteoporosis in elderly men (Riggs et al., 1998).

2.3 Destruxin

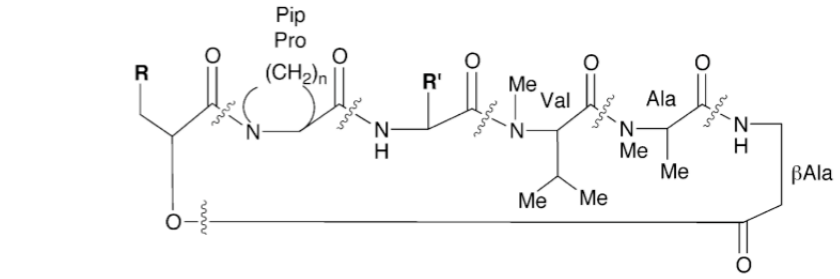
Destruxins are secondary metabolites produced by different soil fungi (Pedras et al., 2002). Most of the more than 35 characterized congeners have been described from *Metarhizium anisoplia*, an entomopathogenic fungus that is used as a biological pest control (BCA) (Wang et al., 2004).

2.3.1 Nomenclature

The name "destruxin" is derived form the word "destructor". This term derives from the species *Oospora destructor*, the entomopathogenic fungus from which these metabolites were first isolated. Later on, *Oospora destructor* was re-named *Metarhizium anisoplia*, but the compounds´ trivial names were retained. The trivial names of destruxins include "destruxin" followed by a single capital which may contain a subscript number, as shown in figure 1.8 (Pedras et al., 2002). Destruxin A, B and E are predominating in quantitative terms (Wang et al., 2004).

2.3.2 Chemical structure

Destruxins are cyclic hexadepsipeptides composed of an α-hydroxy acid and five amino acid residues joined by peptide and ester bonds. Individual destruxins differ on the hydroxyl acid, the N-methylation and the R group of the amino acid residues. The configurations of the amino acid residues are S and those of the hydroxy acids are R (Pedras et al., 2002).



n = 3 Pro; n = 4 Pip

A series	B series	C series	D series	E series
R = –CH=CH$_2$	R = –CHMe$_2$	R = –CHMeCH$_2$OH	R = –CHMeCOOH	R = –CHCH$_2$ (O)
R' = –CH(Me)–CH$_2$Me	R' = –CH(Me)–CH$_2$Me	R' = –CH(Me)–CH$_2$Me	R' = –CH(Me)–CH$_2$Me	R' = –CH(Me)–CH$_2$Me

Figure 2.8: Chemical structures of destruxins.
A-E (*n*=3, R′=–CHMeCH$_2$Me), A$_1$–E$_1$ (*n*=4, R′=–CHMeCH$_2$Me), A$_2$–E$_2$ (*n*=3, R′=–CHMe$_2$) (Pedras et al., 2002).

2.3.3 Synthesis

Cyclic peptides are normally synthesized by coupling intact amino acid and hydroxy acid residues. This coupling is followed by intramolecular cyclization of the resulting linear peptide chain (Pedras et al., 2002).

2.3.4 Biological activity

2.3.4.1 Insecticidal activities

The insecticidal activity of destruxins was tested on a large variety of insects. These toxins were administered by topical application, forced ingestion, immersion or

injection to larvae or adult insects. Apparently, destruxins cause an initial tetanic paralysis, which at lethal doses, can lead to the death of insects. The tetanic paralysis appears to cause muscle depolarization by direct opening of the Ca^{2+} channels in the membrane (Pedras et al., 2002). Moreover, Dumas et al. found out that destruxin E induced Ca^{2+} influx and phosphorylation of intracellular proteins in insect cells. They tested this effect in vitro on lepidopteran cell lines (Dumas et al., 1996).

Structure-activity relationship studies showed that the presence of the ester bond is essential for biological activity of destruxins (Cavelier et al., 1998). Tests on the variation of the side chain of the hydroxy acid residue showed that the presence of a hydrophilic group decreased the insecticidal activity. Compounds with an electron-rich side chain (destruxin A) showed a greater effect than those with a fully saturated side chain (destruxin B). But overall, destruxin E seemed to be the most potent of all compounds (Cavelier et al., 1997).

Dumas et al. found out that destruxin, especially destruxin E and A, exhibited a cytotoxic effect on insect cells (Dumas et al., 1994).

Destruxin A, destruxin B and destruxin E were shown to exhibit antiviral and immunodepressant activity in insect cells (Pedras et al., 2002).

2.3.4.2 Phytotoxic activities

Destruxins, mainly destruxin B, produced by *Alternaria brassicae* were shown to be phytotoxic to a variety of plants. This toxicity is manifested by chlorosis and necrotic spots on the leaf surface. The degree of sensitivity of different *Brassica* species to destruxin B correlated with their degree of susceptibility to *Alternaria brassicae*. This suggestes that the toxin is host-specific (Pedras et al., 2002).

2.3.4.3 Immunosuppressive effects on the hepatitis B viral surface antigen

Chen et al. have shown the strong suppressive effect of destruxin B on hepatitis B viral surface antigen (HBsAg) gene expression in human hepatoma Hep3B cells. Destruxin B and homodestruxin B were suggested as potential candidates for development of new antihepatitis agents (Chen et al., 1997).

2.3.4.4 Cytotoxic effects on leukemia cells

Odier et al. investigated the antitumor effect of destruxin A, B and E on P388 leukemic cell line cells in vitro by flow cytometry on growth, cell viability and cell cycle perturbation 48 hours after destruxin exposure. Destruxin A, B and E inhibited P388 leukemic cell proliferation. Destruxin E showed greater antiproliferative activity than destruxin A and destruxin B (Odier et al., 1992).

2.3.4.5 Inhibition of the growth of certain cancer cell lines

Kobayashi et al. found out that destruxin E inhibited anchorage-independent growth of v-Ki-ras-expressed pMAM-ras-REF cells. Anchorage independence is a specific characteristic of malignant tumor cells. Inhibitors of anchorage-independent growth of tumor cells, such as destruxin E, could be useful for the treatment of cancer (Kobayashi et al., 2004).

Also see: cytotoxic effect on leukemic cells (Odier et al., 1992)

2.3.4.6 Effect on the contraction of isolated rat heart tissue

Destruxin A and Destruxin B exhibited a positive inotropic effect with negative chronotropy on isolated rat cardiac tissue in vitro. Furthermore, these effects are independent of cAMP levels in the heart muscle tissue. Other positive inotropic agents in contrast, such as digitalis and phosphodiesterase inhibitors, were shown to increase cyclic AMP in heart muscles thus inducing pathological changes in the myocardium (Tsunoo and Kamijo, 1999).

2.3.4.7 Induction of erythropoietin production

Erythropoietin (EPO) is the primary hormone that regulates the proliferation and differentiation of immature erythroid cells. An alternative to the human erythropoietin, as an intravenous drug, in the treatment of patients with anemia, would be an orally active drug that induces endogenous EPO production. Cai et al. stated that destruxin-A_4 is able to induce gene expression and marked secretion of erythropoietin in cultured cells of the epo-3 line (Cai et al., 1998).

2.3.4.8 Inhibition of vacuolar ATPase activity

Destruxin B was shown to be a specific, dose dependent and reversible inhibitor of vacuolar-type ATPase, which maintains the acidity in the vacuolar organelles such as Golgi apparatus and lysosomes (Muroi et al., 1994).
Bandani et al. studied the effect of destruxins on the hydrolytic activity of a vacular type ATPase that they had identified from *Galleria mellonella* midgut columnar cell brush border membrane vesicles (BBMV) by its cation and pH dependence and sensitivity to proton pump inhibitors. They found that destruxins had little effect on this ATPase, whereas destruxin B was the most effective of all tested destruxins (Bandani et al., 2001).

2.3.4.9 Influence of calcium flux dependent processes

Destruxins also modify calcium-dependent processes like the modulation of calcium balance in Lepidopteran cell lines and the induction of calcium-dependent morphological changes in insect epithelial cells from *Leptidoptera sp.* (Dumas et al., 1996).

2.3.4.10 Formation of trans-membrane ion channels

It has been suggested that the bioactivity of destruxin (respectively of cyclohexadepsipeptides) are linked to ionophoric properties. Computer-aided molecular modeling shows the potential for destruxin A to form a coordination complex with calcium. In this complex the divalent cation is bound at the center of a sandwich formed by two molecules of destruxin A (similar to that observed for the enniatins). Hinaje et al. demonstrated an ionophoric capability for destruxin A by detecting a movement of calcium ions across the liposomal barrier by externally applied destruxin A (Hinaje et al., 2002).

2.3.4.11 Inhibition of the bone-resorbing activity of osteoclast-like multinucleated cells

Nakagawa et al. tested destruxin B and E on their anti-resorptive activity. They stated that the substances inhibit pit formation without affecting osteoclast differentiation and survival. Destruxins block the formation of actin rings, prominent clear zones and ruffled borders in osteoclast in a dose-dependent manner (Nakagawa et al., 2003).

During my research on the biological activity of destruxins I found out that there are only a few recent studies available about these compounds. This field offers a wide range of opportunities for further research.

3. Material and methods

3.1 Equipment

Equipment	Name of the company
Autoclave	SX-300E, Tomy Digital Biology, Japan
Bone saw + Saw blade	Isomet Low speed saw, Buehler, USA Diamond wafering blade/series 15 HC diamond, Buehler, USA
Cell counter	Cell counter + analyzer Casy®1 Model DT, Schärfe System, Germany
Centrifuge	Hermle Z323K, Bartelt, Austria
Computers	Power Macintosh G4 (Apple) PC (Windows)
Digital camera	Coolpix 995, Nikon, Japan Color View III, Olympus, Japan F-601, Nikon, Japan
ELISA-reader	Multiscan MS reader, Labsystem, Finland
Fluorescence high pressure lamp	Nikon, Japan
Incubator	Model 500, Memmert, Germany HERAcell® 240, Kendro, USA
Laminar airflow workbench	Ehret, Austria
Light source for microscope	KL 1500 electronic, Schott, North America
Magnetic stirrer	Ikamag® RCT, Janke & Kunkel, Germany
Microscopes	Diaphot 300, Nikon, Japan Optiphot-2-UD, Nikon, Japan (fluorescence) TMS, Nikon, Japan BX51, Olympus, Japan
pH-meter	Metrohm, Austria

Scales	LC 621 P, Sartorius, Austria MC 210 P, Sartorius, Austria
Shaker	Swip, Edmund Bühler, Germany
Sterilisator	WTC, Binder, Germany
Ultrasound bath	Transsonic 570, Elma, Austria
Vortex	Vortex Genie 2^{TM}, Bender & Hobein AG, Switzerland
Water bath	GFL-1086, GFL, Germany

Table 3.1: List of all used equipment.

3.2 Materials

Materials	Name of the company
Adjustable micropipette	Eppendorf, Germany Thermo-Labsystem, Finland Gilson, France
Centrifuge tube: 15 ml, 50 ml	Becton Dickinson, USA Greiner Bio-One, Germany
Cover slips	La Fontaine, Germany
Cryo vials	Becton Dickinson, USA
Eppendorf tube	Eppendorf, Germany
Glass pipette: 5 ml, 10 ml, 25 ml	Brand, Germany
Injection needle: 27G3/4, 0,4 * 19, Nr. 20	Becton Dickinson, USA
Microscope slide	La Fontaine, Germany
Parafilm “M”	American National Can Group, USA
Pasteur pipette	Copan, Italy
Petri dish	Iwaki, Japan Sterilin, Bibby Sterilin Ltd., U.K.
Pipetboy	Integra Biosciences, Switzerland
Polystyrene tube	BD Biosciences, USA
Silicon	GE Bayer Silicones, USA
Syringe	Terumo Europe, Belgium
Well plate	Iwaki, Japan Nunc, Denmark Costar, USA Greiner Bio-One, Germany

Table 3.2: List of all used materials.

3.3 Substances

Substances	**Name of the company**
α-MEM	Gibco BRL, USA
1,25-$(OH)_2$-Cholecalciferol	Hoffmann-La Roche, Switzerland
4´,6-Diamidino-2-phenylindole (DAPI)	Sigma, USA
Acetone	Riedel-de Haen, Germany
Alexa Fluor®488 phalloidin	Molecular Probes, USA
$CaCl_2$ (calcium chloride)	Sigma, USA
CellTiter 96® AQ_{ueous} One Solution Reagent	Promega, Germany
Collagenase 0,1 %	Sigma, USA
Diethyl ether	Riedel-de Haen, Germany
Dispase 0,2 %	Boehringer Mannheim, Germany
DMSO (dimethyl sulfoxide)	Sigma, USA
EDTA (ethylenediaminetetraacetic acid)	Sigma, USA
Ethanol absolute	Sigma, USA
Fast-Red-Violet-Salt	Sigma, USA
Fetal calf serum	PAA Laboratories, Austria
Formaldehyde	Sigma, USA
HCl (hydrogen chloride)	Merck, Germany
HEPES	Sigma, USA
Isopropanol 70 %	Riedel-de Haen, Germany
KCl (potassium chloride)	Merck, Germany
KH_2PO_4 (monopotassium phosphate)	Merck, Austria
Methanol	Merck, Germany
$MgCl_2.6H_2O$ (magnesium chloride hexahydrate)	Sigma, USA
Mounting medium (FluorSave)	Calbiochem-Novabiochem, USA
N-N-dimethylformamide	Sigma, USA
$Na_2C_4H_4O_6$ (sodium tartrate)	Sigma, USA
$Na_2HPO_4.2H_2O$ (disodium phosphate dihydrate)	Merck, Germany

NaCl (sodium chloride)	Sigma, USA
NaOH (sodium hydroxide)	Merck, Germany
$NaCH_3CO_2$ (sodium acetate)	Sigma, USA
Naphtol AS-MX-phosphate	Sigma, USA
Penicillin	Gibco BRL, USA
PGE_2 (prostaglandin E2)	Cayman, USA Pfizer, USA
Silicon grease	Wacker-Chemie, Germany
Sodium borate	Sigma, USA
Streptomycin	Gibco BRL, USA
Toluidine blue	Sigma, USA
Trypsin	Boehringer Mannheim, Germany

Table 3.3: List of all used substances.

3.4 Solutions

Phosphate Buffered Saline Solution (PBS) 10x:

2,00 g ..... KCl (potassium chloride)
2,00 g ..... KH_2PO_4 (monopotassium phosphate)
80,00 g ..... NaCl (sodium chloride)
27,07 g ..... $Na_2HPO_4.2H_2O$ (disodium phosphate dihydrate)

KCl (2,6 mM), KH_2PO_4 (1,4 mM), NaCl (140 mM) and $Na_2HPO_4.2H_2O$ (15,2 mM) was diluted in 1000 ml double distilled water.

The solution in use was PBS 1x. For this purpose, the PBS 10x was diluted 1:10 with double distilled water.

Phosphate Buffered Saline Solution (PBS) with Ca^{2+} and Mg^{2+} 10x:

2,00 g ..... KCl (potassium chloride)
2,00 g ..... KH_2PO_4 (monopotassium phosphate)
80,00 g ..... NaCl (sodium chloride)
14,342 g ..... $Na_2HPO_4.2H_2O$ (disodium phosphate dihydrate)
1,00 g ..... $CaCl_2$ (calcium chloride)
1,00 g ..... $MgCl_2.6H_2O$ (magnesium chloride hexahydrate)

KCl (2,6 mM), KH_2PO_4 (1,4 mM), NaCl (140 mM), $Na_2HPO_4.2H_2O$ (8 mM) and $MgCl_2.6H_2O$ (0,5 mM) were diluted in a approximately 100 ml double distilled water. Then the pH was adjusted to a value of 2-3 with 5N HCl. At this pH-value $CaCl_2$ is dissolved best. $CaCl_2$ (0,9 mM) was added and the solution was filled up with double distilled water to 1000 ml.

The solution in use was PBS with Ca^{2+}/Mg^{2+}1x. For this purpose, PBS with Ca^{2+}/Mg^{2+} 10x was diluted 1:10 with double distilled water. Finally the pH was adjusted to the value of 7,2 (almost neutral) with 5N NaOH (sodium hydroxide).

TRAP-buffer solution:

544 mg .....$NaCH_3CO_2$ (sodium acetate)
230 mg .....$Na_2C_4H_4O_6$ (sodium tartrate)

$NaCH_3CO_2$ (40 mM) was dissolved in 100 ml double distilled water. After adjusting the pH-value to 5 with 1N HCl, $Na_2C_4H_4O_6$ (10 mM) was added.

TRAP-staining solution:

30 mg .....fast red violet salt
5 mg.....naphtol AS-MX-phosphate
500 µl.....N-N-dimethylformamide

Fast red violet salt (1,59 mM) was dissolved in 50 ml TRAP-buffer solution. Naphtol AS-MX-phosphat (0,24 mM) was dissolved in N-N-dimethylformamide (10 µl/ml). Finally these two solutions were combined.

Toluidine blue staining solution 1 %:

1,00 g .....sodium borate
1,00 g .....toluidine blue O

Sodium borate and toluidine blue O were suspended in 100 ml double distilled water.

Fixing Solution:

A 37 % formaldehyde solution was diluted 1:10 with PBS with Ca^{2+}/Mg^{2+} 1x to obtain a 3,7 % formaldehyde solution to fix the cells (OCs).

4´,6-Diamidino-2-phenylindole (DAPI)-dye:

DAPI stock solution (5 µg/ml) was diluted 1:50 with PBS with Ca^{2+}/Mg^{2+}. This means that 200 µl of the DAPI stock solution was diluted in 10 ml PBS with Ca^{2+}/Mg^{2+}.

Phalloidin-rhodamine-dye:

200 units (6,6 µM) of a phalloidin rodamine stock solution have been diluted in 1 ml of methanol (solvent). Therefore, methanol was let evaporate at room temperature before use. For the solution used (0,165 µM), the stock solution was diluted 1:40 with PBS with Ca^{2+}/Mg^{2+}. This means that 5 µl of the phalloidin rodamine stock solution were diluted in 200 µl PBS with Ca^{2+}/Mg^{2+}.

Enzyme-solution for isolation of the osteoblasts:

0,10 g ..... collagenase 0,1 %
0,20 g ..... dispase 0,2 %

Collagenase and dispase were dissolved in 100 ml of α-MEM without serum and then the solution was filtrated through a 0,2 µm filter.

Trypsin-EDTA solution.10x:

0,155 g ..... EDTA (ethylendiaminetetraacetic acid)
0,50 g ..... trypsin
0,90 g ..... NaCl (sodium chloride)

EDTA was dissolved in approximately 90 ml of double distilled water. Then the pH was adjusted to a value of 7,4 - 7,6. Trypsin and NaCl were added and the solution was restocked to 100 ml of double distilled water. Then the pH was adjusted again, to a value of 7,7 with 5N NaOH and the solution was sterile filtrated.

To obtain trypsin-EDTA solution 1x, 1 ml of trypsin-EDTA solution 10x was added to 9 ml of PBS without Ca^{2+}/Mg^{2} 10x.

3.5 Culture medium

The culture medium used for the coculture was Minimal Essential Medium, α-configuration (α-MEM) by Harry Eagle. It contains amino acids, salts (choline chloride, calcium chloride, potassium chloride, sodium chloride, magnesium sulphate and sodium dehydrogenate phosphate), glucose and vitamins (folic acid, thiamine/vitamin B_1, riboflavin/vitamin B_2, niacin/vitamin B_3, pantothenic acid/vitamin B_5, pyridoxal/vitamin B_6).

Powdered α-MEM (reference number: 11900-073) was solved in 10 liters of sterile water and 2,2 g sodium bicarbonate for each liter of final volume of the medium. The pH of the medium was adjusted to 7,1 by using 1N HCL or 1N NaOH. Finally, the solution was sterilized by filtration through a membrane with porosity of 0,2 µm.

The medium in use contains fetal calf serum (FCS), which provides growth factors for the cells to survive, grow and divide. For the coculture α-MEM with 10 % FCS was used. For the pre-incubation of the surface and for the seeding of osteoblasts heat inactivated FCS was used. By the time bone marrow cells were added to the culture, regular FCS (not heat inactivated) was used. Heat-inactivation is achieved by warming up the serum to 56°C in a water bath for 30 minutes under mild shaking. In the experiments with isolated rabbit osteoclasts α-MEM + 10 % heat inactivated FCS was only used for pre-incubation. As soon as the osteoclasts were seeded α-MEM + 5 % heat inactivated FCS was used. To control microbial contamination in cell culture antibiotics (penicillin-streptomycin solution – P/S) were added to the medium. The concentration of penicillin and of streptomycin in the medium was 100 U/ml and 100 µg/ml respectively.

3.6 Test substance

The cyclohexadepsipeptide destruxin E was investigated in this thesis. The substance was applied by the University of Innsbruck; Department of Pharmacognosy (Dr. Christoph Seger).

Destruxin E had a purity of 90 % (according to HPLC-DAD). The molecular weight is 593 g/mol. The used concentrations of destruxin E were $1*10^{-6}$ M, $1*10^{-7}$ M, $1*10^{-8}$ M, $3*10^{-9}$ M and $1*10^{-9}$ M.

The substance was dissolved in 100 % methanol. The concentration of the stock solution was 2,58 mM. This stock solution was diluted in methanol to gain the used standards with the concentration $1*10^{-3}$ M, $1*10^{-4}$ M, $1*10^{-5}$ M, $3*10^{-6}$ M and $1*10^{-6}$ M destruxin E. These standards were used in the experiments in a dilution of 1:1000 in the culture medium as indicated in the experimental description. To make sure that the solvent methanol does not harm the cells, a group with culture medium alone was always used in the experiment to be compared with the methanol group.

Destruxin E was stored dark at -20°C.

3.7 Isolation of osteoblasts

Osteoblasts were isolated from the calvariae of 12-48 hour old mice. The calvariae were used for the isolation because they contain a big number of osteoblasts.

The calvariae were removed and washed with cold PBS without Ca^{2+}/Mg^{2+}. For the enzymatic digestion the calvariae were treated with an enzyme-solution (0,1 % collagenase, 0,2 % dispase, 10 mM HEPES, α-MEM) for 10 minutes in a shaking incubator at 37°C. This step was repeated 5 times and all fractions were collected in an ice-cold centrifuge tube. The first fraction was discarded because it contains a lot of other cells beside osteoblasts. After collection of all fractions in the centrifuge tube they were centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was extracted and the cell pellet was re-suspended in α-MEM + 10 % FCS. The suspension was put in Petri dishs and incubated for 24 hours. Then the medium was changed to remove contaminating cells and incubated for 24 more hours so that the cells became confluent.

Splitting

The cell surface was detached with a trypsin-EDTA solution 1x. The detached osteoblasts were transferred into a 50 ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was extracted and the cell pellet was re-suspended in culture medium and seeded on a new culture plate with a splitting rate of 1:4 (2 ml of the re-suspension were added to 8 ml of α-MEM + 10 % FCS).

Freezing

The cell surface was detached with a trypsin-EDTA solution 1x again. Then the detached osteoblasts were transferred into a 50 ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was extracted and the cell pellet was re-suspended in culture medium. The cells were then counted with a Casy® cell counter and analyzer system, centrifuged (1500 rpm for 5 minutes at 4°C) and suspended in the freezing solution (50 % FCS hi, 40 % α-MEM, 10 % DMSO/dimethyl sulfoxid). 1,5 ml cell suspension was put in each vial containing approximately 4 million cells ($4*10^6$ cells). The vials were stored overnight in a Nalgene freezing container

(containing isopropanol) at -80°C. So the cells could be frozen continuously at 1°C/hour. Finally the osteoblasts were stored in a liquid nitrogen tank at –196°C until they were needed for the experiments.

3.8 Methods

3.8.1 Resorption assay ("pit assay")

3.8.1.1 Preparation of bone slices

The bone used as a substrate for the resorption of the osteoclasts in the resorption assay was isolated from cattle obtained from a local slaughterhouse. The long bones were cleaned off muscles and the ends were cut off with a metal saw. After the bone marrow was removed the bones were dried for 24 hours at 50°C and cut into smaller pieces. Finally they were stored in the refrigerator (4°C).

At first a piece of bovine long bone was cut in slices with a thickness of 300-350 µm using a low speed saw with a diamond blade. The slices were then cut into small equal squares (ca. 5 * 5 mm) with a scalpel. To clean the bone slices they were washed 3 times with double distilled water and once with 70 % ethanol in an ultrasonic bath for 5 minutes each time. Then they were put in ethanol absolute and stored overnight. After that the bone slices were sterilized with a 15-minute UV-light treatment for each side in a laminar airflow workbench. The sterilized bone slices were then glued to the well surface of a 48-well culture plate with sterile silicone (4 bone slices per well) and pre-incubated in a α-MEM + 10 % FCS for at least 2 hours (or overnight).

3.8.1.2 Coculture

Seeding of Osteoblasts

First osteoblasts isolated from murine calvariae were seeded on 48-well culture plates. The frozen cells (stored in 1,5 ml vials with approximately 4 million cells) were taken

from the liquid nitrogen tank and thawed in a water bath until there was only a small ice cube left. The cells were then diluted in heat inactivated α-MEM + 10 % FCS. The whole medium used for pre-incubation was removed with a glass Pasteur pipette and a vacuum pump. Finally 2-4 million osteoblasts were seeded on one culture plate (48-well culture plate: 42.000 – 83.000 cells/cm^2 area of growth). The culture plates were then incubated (37°C, 5 % CO_2) for 24 hours to give the cells time to attach to the bone or plate surface.

Seeding of Bone Marrow Cells

Femora and tibiae of several week old mice were isolated. 8 bones were used for one culture plate. The mice were killed by cervical dislocation and their hind feet were cut off with scissors or a scalpel. The legs were then placed in a PBS without Ca^{2+}/Mg^{2+} filled Petri dish that was set on ice. Under the laminar airflow bench the muscles were removed from the bones with a knife, tweezers and by hand (with kitchen roll paper). The cleaned bones were put into another Petri dish filled with PBS without Ca^{2+}/Mg^{2+} and settled on ice again. Then the upper ends of the bones were cut off. The bone marrow cells were flushed out of the bone with α-MEM + 10 % FCS, using a hypodermic needle (0,4 * 19 mm) and a 1 ml syringe. The cell suspension was transferred to a 50 ml centrifuge tube and then centrifuged for 5 minutes, at 1500 rpm and 4°C. After the centrifugation, the supernatant medium was removed from the tube and the remaining pellet was re-suspended in medium (6 ml medium for 2 bones). Finally the medium in the wells of the culture plate was removed and the bone marrow cells were added to the culture. For that purpose 400 µl of the bone marrow cell suspension and 100 µl of medium with 1,25-$(OH)_2$-vitamin-D_3 and PGE_2 was added to each well of a 48-well culture plate. The stock solution of 1,25-$(OH)_2$-vitamin-D_3 was $1*10^{-5}$ M, which was added to the cell suspension to reach the final concentration of $1*10^{-9}$ M. The stock solution of PGE_2 was $1*10^{-3}$ M, which was added to the suspension to reach the final concentration of $1*10^{-6}$ M. Then the well plate was incubated again.

The medium was changed every 48 hours. The test substance was added according to the aim of the respective experiment (day 0 – day 3/day 0 – day 5/day 3 – day 5). The coculture was ended after 5-6 days when osteoclasts could be observed.

3.8.1.3 Staining of the bone slices

At the end of the culture time the bone slices were taken out of the wells of the culture plate and placed in polypropylene tubes. To clean them from remaining medium, silicon and osteoclasts, the bone slices were then washed with 70 % isopropanol in an ultrasound bath for 15 minutes. Finally the slices were stained with a 1 % toluidine blue solution for 4 minutes on a shaker and then washed 3 times with double distilled water to rinse off the remaining dye. The stained bone slices were placed in a marked 96-well plate until pit area measurement.

3.8.1.4 Pit area measurement

The resorption areas were visualized with an Olympus BX51 reflected-light microscope with a 10x magnification. For each bone slice 2 images, one in the upper left corner and the other one in the lower right corner, were taken with an Olympus Color View III digital camera, which was connected to the microscope. The 2 images of one bone slice were merged in an imaging software program called cell^F to one picture (figure 3.1) which could be analyzed in the same program. The resorption areas could be marked with this program because of the variable color intensity of the pits compared to the rest of the bone slice.

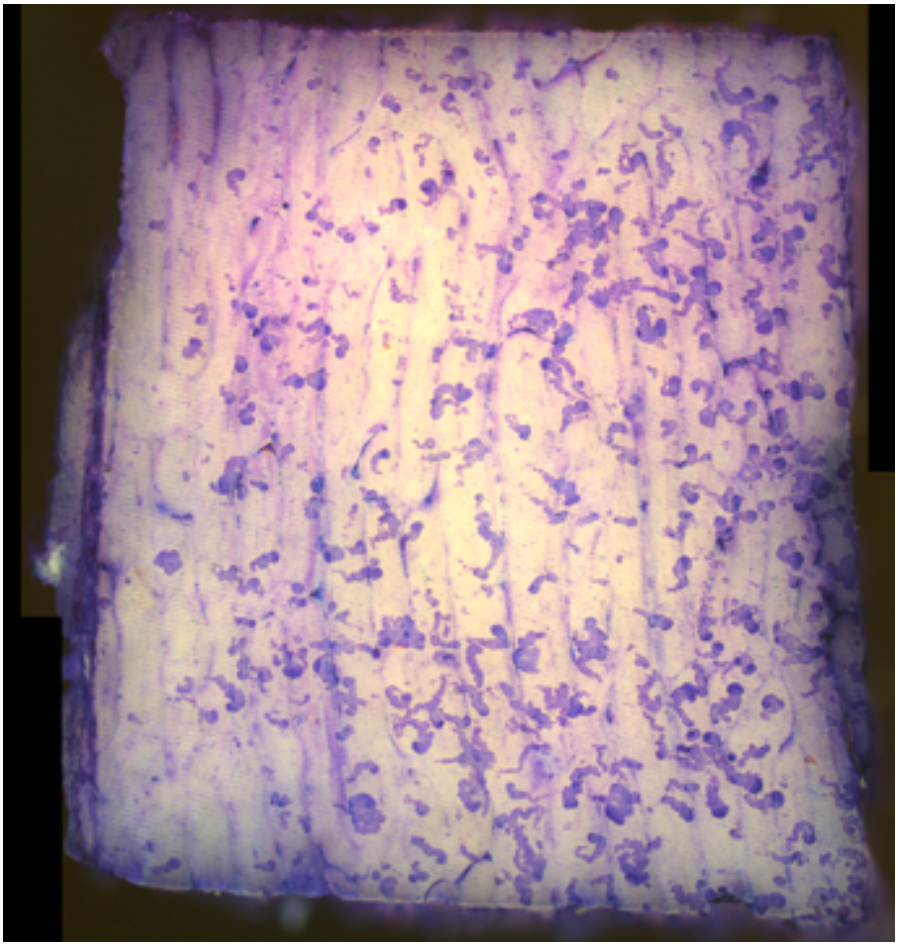


Figure 3.1: Stained bone slice with resorption pits in the imaging software cell^F.

The total number of resorption pits, their total area and the average pit area were calculated by the program as well as the total area of the whole bone slice visible on the picture. These data were then copied to Microsoft Excel for calculation of the resorption (%).

- Total number of pits = sum of all pits on the whole picture
- Total area of pits = sum of the areas of all pits
- Total area of bone slice

- $\text{Average pit area} = \frac{\text{total area of pits}}{\text{total number of pits}}$

- $\%\ \text{Resorption} = \frac{\text{total area of pits} * 100}{\text{total area of bone slice}}$

This method was carried out with 8 samples for each group (8 bone slices in 2 wells/group).

Finally the graphical presentation was performed with the program Prism 5.0.

3.8.2 Quantification of TRAP-positive cells

TRAP-positive cells were stained with a red staining solution and were then visible under a light microscope with a 10x objective. Only TRAP-positive multinucleated cells with 3 or more nuclei were counted as OCs. With this method the number of osteoclasts in each group (different concentrations of treatment or control) could be determined.

3.8.2.1 Coculture

Done as described in detail in chapter 3.8.1.2.

3.8.2.2 Fixation

OC-like cells were fixed with 3,7 % formaldehyde solution (3 minutes, and 10 minutes). Before and after the fixation the cells were washed several times with PBS with Ca^{2+}/Mg^{2+}.

3.8.2.3 Staining

The cells were pre-incubated with a TRAP-buffer (containing 40 mM sodium acetate and 10 mM sodium tartrate) for 30 minutes. Then the cell membranes were made permeable with a treatment with an acetone/ethanol 1:1 solution for 30 seconds. Thereafter the wells with the cells were dried for 2 minutes before the cells were treated with the TRAP staining solution. This solution contained dimethyl formamide, naphtol AS-MX phosphate and Fast-Red-Violet-Salt. The cells were then incubated for 5-8 minutes in the incubator. Finally, washing the cells 3 times with double distilled water stopped the staining. The cells were stored in double distilled water at 4°C until further analysis.

This method was carried out with 3 samples for each group (3 wells/group).

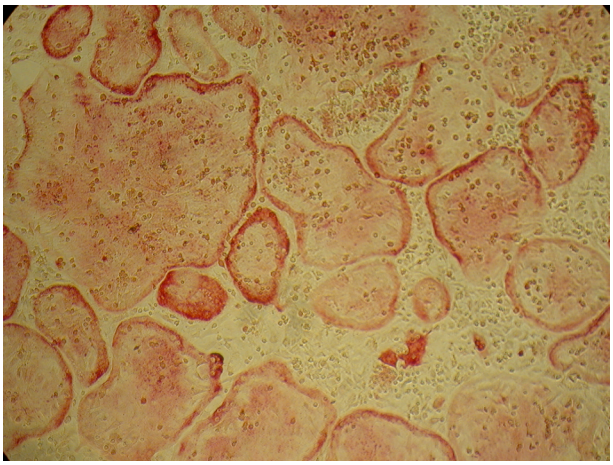


Figure 3.2: TRAP-positive cells of a coculture with osteoblasts and bone marrow cells (control-group) under the light microscope.

Finally, the TRAP-positive cells with 3 or more nuclei were counted under a light microscope. The graphical presentation was done with the program Prism 5.0

3.8.3 Morphological analysis

To examine the morphology of osteoclasts and of osteoblasts the cytoskeleton and the nuclei of these cells were stained.

Fluorescence labeled phalloidin was used to lable and to observe the cytoskeleton under the fluorescence microscope. Phalloidin binds at the interface between F-actin subunits with high affinity. By labeling phalloidin with fluorescent analogs, like alexa fluor®488, the distribution of F-actin in cells (in this case osteoclasts and osteoblasts) could be investigated. The conjugated dye, alexa fluor®488, emits green light. This way it was possible to visualize the actin rings of the active osteoclasts and the widespread cytoskeleton of the osteoblasts.

The nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI). This fluorescent dye (blue) easily enters cells and binds strongly to DNA because it forms complexes with AT-rich regions of double-stranded DNA, mainly at the minor groove.

3.8.3.1 Preparation of the cover slips

The cover slips were cleaned with ethanol absolute overnight. Then they were sterilized at 180°C for about 2 hours. The sterilized cover slips were then pre-incubated in α-MEM + 10 % FCS for at least 2 hours (or overnight) before the experiment.

3.8.3.2 Rabbit osteoclasts

Isolation of rabbit osteoclasts

Long bones of 1-4 day old rabbits with an average weight of 100 g were isolated. Under the laminar airflow bench the bones were cut into slices with a scalpel and the slices were put into a centrifuge tube filled with 6 ml α-MEM + 5% FCS (for the long bones of one rabbit). The bone pieces were cut with scissors for about 2 minutes, before 7 ml of α-MEM + 5 % FCS was added. With a transfer pipette the supernatant cell suspension (OCs) was transferred to a second centrifuge tube set on ice. This process was repeated twice. 13 ml of α-MEM + 5 % FCS (first 5 ml, then another 5 ml and finally 3 more ml) were added to the hackled bone in the first centrifuge tube and the cell suspension was vortexed for 30 seconds. After 2 minutes of sedimentation, the supernatant was also transferred to the first centrifuge tube on ice with the other suspension. The collected cell suspension was centrifuged for 5 minutes, at 600 rpm and 4°C. After the centrifugation the supernatant medium was removed from the tube and the remaining pellet was re-suspended in the right amount of new medium (15 ml α-MEM + 5 % FCS for the long bones of one rabbit).

Seeding of rabbit osteoclasts

100 µl of the osteoclast cell suspension was put on each cover slip of a 6-well culture plate. The cell suspension should not flow down of the cover slips. After incubating (37°C, 5 % CO_2) the plates for 1,5 – 2 hours, the medium was removed and replaced by new medium (2 ml for each well of the 6-well culture plate) including the respective concentration of test substance or vehicle (control). Finally the well plates were incubated (37°C, 5 % CO_2) for 24 or 48 hours depending on the experimental design.

3.8.3.3 Fixation of the cells

At the end of the culture, the medium was removed and the cover slips were washed with 1x PBS with Ca^{2+}/Mg^{2+}. The osteoclasts were then fixated on the cover slips with 1 ml 3,7 % formaldehyde solution per well, first for 3 minutes and then for 10 minutes.

Finally the cover slips were washed again with PBS with Ca^{2+}/Mg^{2+} (3 times) and stored at 4°C in PBS with Ca^{2+}/Mg^{2+} in addition of NaN_3 (500x) for further analysis.

3.8.3.4 Alexa Fluor/Phalloidin-DAPI staining

The cover slips were washed once more with PBS with Ca^{2+}/Mg^{2+}. Then they were treated with 3 ml of pre-cooled (-20°C) acetone per well for 3 minutes in suitable glass plates. This step is required to make the cell membranes permeable.

Since the Alexa Fluor/Phalloidin dye was dissolved in methanol, the solvent had to evaporate (at room temperature or through aeration with nitrogen). 5 µl Alexa Fluor/Phalloidin dye were diluted in 200 µl PBS with Ca^{2+}/Mg^{2+} for staining one cover slip. The cover slips were put in a light free metal box and each one was treated with 200 µl of the staining solution for 30 minutes.

To stop the Alexa Fluor/Phalloidin staining, the cover slips were washed with PBS with Ca^{2+}/Mg^{2+} again. 40 µl of DAPI stock solution (50x) were diluted in 2 ml PBS with Ca^{2+}/Mg^{2+} to stain one cover slip. Each cover slip was treated with 2 ml of this solution and incubated for 10 minutes at 37°C. To stop the staining, the cover slips were washed 3 times with PBS with Ca^{2+}/Mg^{2+} and in order to avoid salt formation they were also washed 3 times with double distilled water.

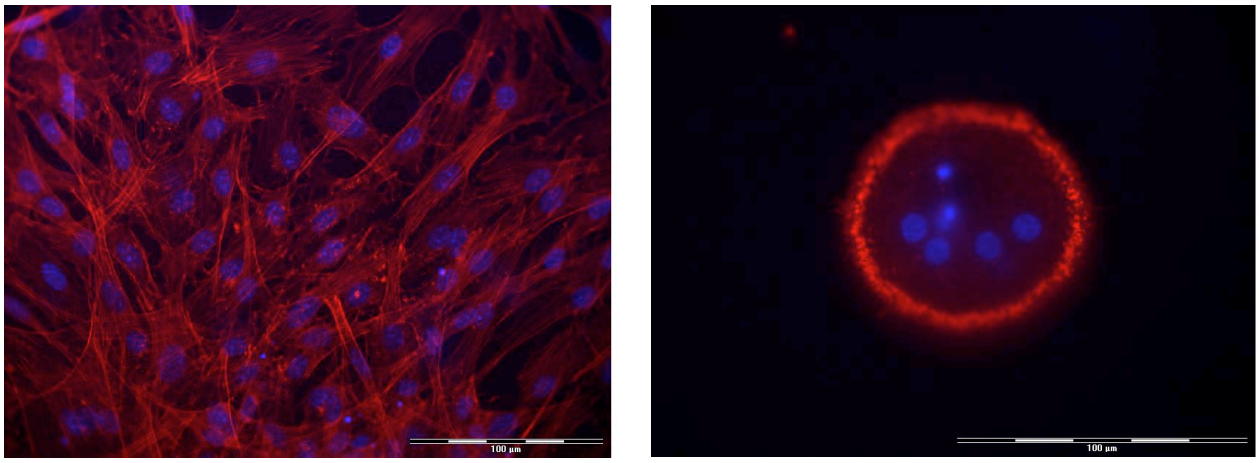
Finally the cover slips with the fixed and stained cells were placed on an object slide with the cell sight upside down on a mounting fluid. The edges of the cover slips were glued to the object slide so that the cells would not dry out. For further analysis the cover slips were stored at 4°C.

Notice that the staining solution always had to be protected against light.

3.8.3.5 Analysis

The cells of each experiment were examined with a Nikon Optiphot-2-UD fluorescence microscope with a 40x or 60x magnification, using filters for EGFP and DAPI. For each cover slip around 10-15 images were taken with an Olympus Color View III digital camera, which is connected to the microscope. The images were then saved in the cell^F program, which displays the nuclei blue and the cytoskeleton red. The total number of nuclei and apoptotic cells was counted and the differences between the cells in the control group and the cells of the treated groups were estimated. Osteoclasts were analyzed in regard of the actin ring and cytoskeleton oranisation.

Each treatment was carried out on 2 cover slips with cells.



A) **B)**

Figure 3.3: Alexa fluor®488/phalloidin and DAPI staining.
The nuclei are stained blue and the cytoskeleton red.; scale bar indicates 100 µm.
A) Example for osteoblast (control)
B) **Example for osteoclast (control):** with an actin ring shown around the cell, clear cytoplasm and smooth periphery.

3.8.4 MTS-test

The MTS-test is a colorimetric method for determining the number of viable cells in cytotoxicity assays. For this assay a CellTiter 96® AQ_{ueous} One Solution Reagent is used. It contains a tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine ethosulfate; PES).

The MTS tetrazolium compound is bioreduced by cells (for instance by osteoblasts or RAW 264.7 cells) into a colored formazan product. The conversion is dependent on the existence of NADPH or NADH, which are produced by dehydrogenase enzymes in metabolically active cells. The quantity of the produced formazan is directly proportional to the number of living cells in the tested culture. This was measured with the absorbance at 490 nm, using an ELISA plate reader.

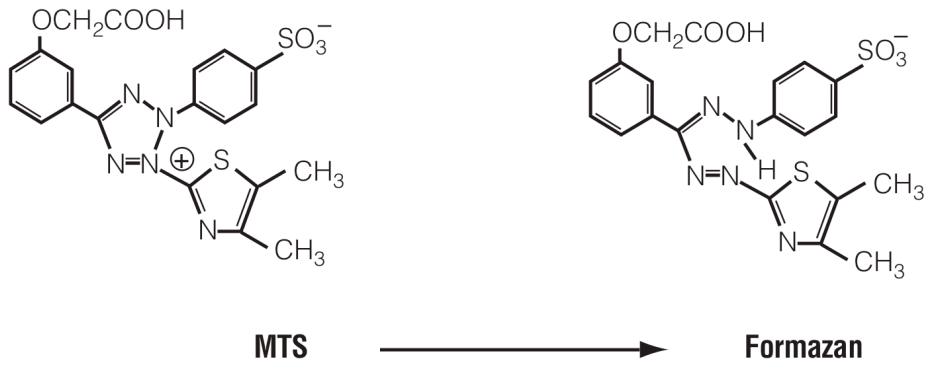


Figure 3.4: Conversion of MTS tetrazolium in its formazan product (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega Technical Bulletin Part # TB245 – according to the manufacturer´s instruction).

For the assay, about 4 million osteoblasts, respectively 8 million RAW 264.7 cells were seeded on one 96-well plate in a volume of 100 µl medium per well. Then the cells were incubated for 24 hours (37°C, 5 % CO_2, humidified atmosphere) before the treatment with the test substance took place. After 24 hours additional incubation time 20 µl of the CellTiter 96® AQ_{ueous} One Solution Reagent, that contained already 100 µl medium with treatment, was added per well. The well plates were then incubated with the reagent for 2 more hours (total incubation time with treatment was 26 hours). At the end, the absorbance was measured and recorded at 490 nm with an ELISA 96-well plate reader.

The graphical presentation was done with the program Prism 5.0. The number of viable cells/absorbance of the wells treated with the test substance (Destruxin E in different concentrations) was compared to the wells that were only treated with the solvent of Destruxin E. The results were evaluated as percentage of viable cells in treated wells compared to the wells of the control.

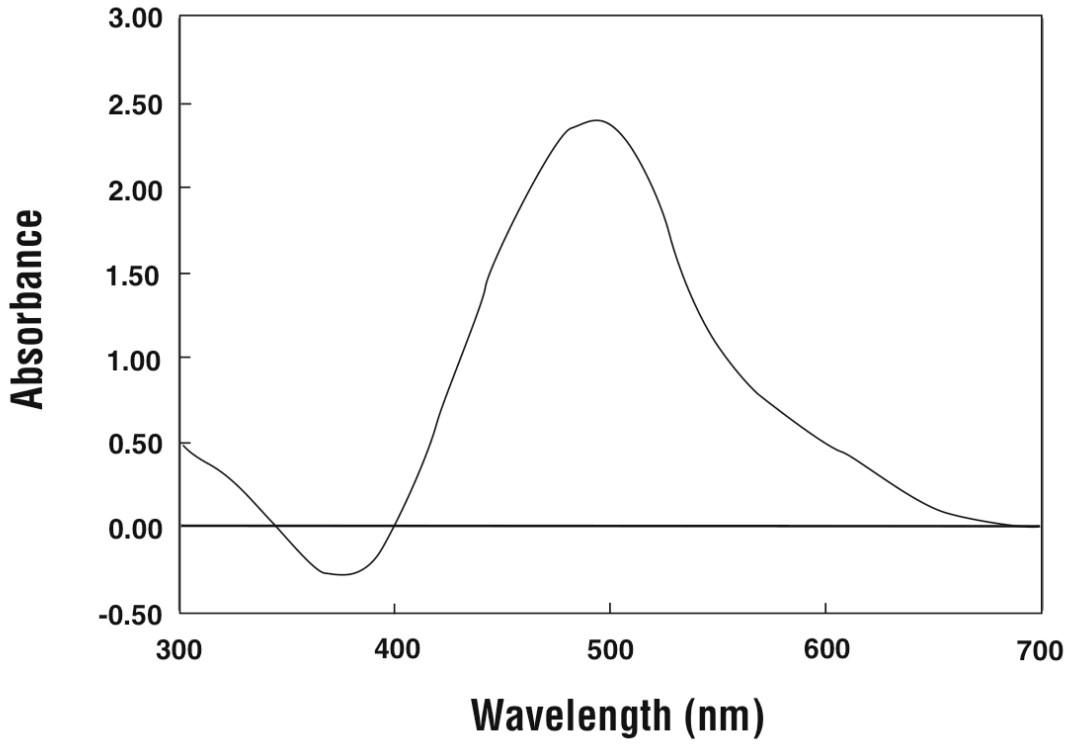


Figure 3.5: **Absorbance spectrum of MTS/formazan** (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega Technical Bulletin Part # TB245 - according to the manufacturer´s instruction).

This method was carried out with 8 samples for each group (8 wells/group).

With this test I tested the viability of osteoblast and RAW 264.7 cells. RAW 264.7 cells are a murine macrophage cell line. Their viability under treatment with destruxin E reflects the effect of this substance on osteoclast precursors, which again affects the number of mature resorbing osteoclasts. Also osteoblasts were tested to see at which

concentration the test substance has an effect, which is not wished for these cells (death of the cells).

3.8.5 Statistical analysis

Statistical significance was defined by using analysis of variance (ANOVA). Statistical differences between the respective groups were computed using Fisher's least significant difference (LSD) test. Data are presented as the mean ± standard error of mean (SEM). Significant differences were compared to the control group (solvent = methanol) as $p<0,05$; $p<0,01$ or $p<0,001$.

4. Results and discussion

4.1 Resorption assay

For the resorption assay the used concentrations of destruxin E were $1*10^{-7}$ M, $1*10^{-8}$ M and $1*10^{-9}$ M. In preliminary tests we also analyzed the concentration of destruxin E of $1*10^{-6}$ M, but since it exhibited no resorption at all like the next higher concentration ($1*10^{-7}$ M), it is not shown in the results above. Destruxin E was found to inhibit the formation of pits in a resorption assay in a dose-dependent manner. Not only the number of pits, but also the size of pits decreased with increasing concentrations. The main subject of interest was at which stage of the coculture the test substance has which effect on the osteoclasts. Therefor three different groups were tested. For group 1 (effect of destruxin E on the activity of OCs) the test substance was added on day 3 of the coculture until its end. For group 2 (effect of destruxin E on the differentiation of OCs) the test substance was added from the beginning of the coculture till day 3. For group 3 (effect of destruxin E on the differentiation and the activity of OCs) the test substance was added from the beginnung of the coculture until its end.

4.1.1 Effect of destruxin E on activity and differentiation of coculture-derived osteoclasts

4.1.1.1 Effect of destruxin E on the activity of osteoclasts (Group 1)

Figure 4.1 shows that destruxin E had a clear inhibitory effect on bone resorption. Destruxin E, at a concentration of $1*10^{-7}$ M and $1*10^{-8}$ M, significantly inhibited OC function. Since the substance in this group was added on day 3 of the coculture

(beginning of OC appearance) until the end of it, it appears that destruxin E has a strong effect on the activity of the osteoclast (which is the resorption of bone material).

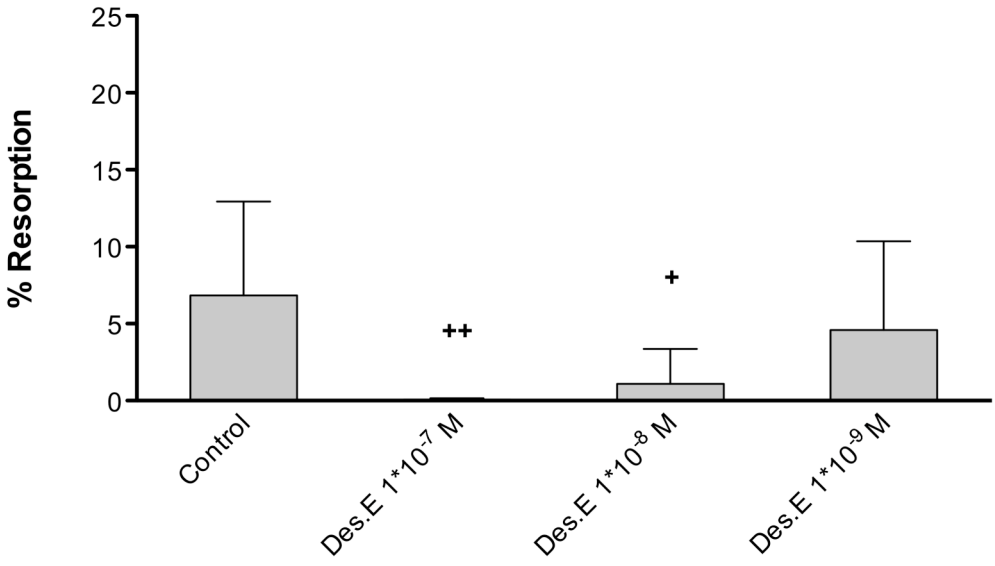


Figure 4.1: Effect of destruxin E on the resorption of murine osteoclasts on bovine bone slices.

The total culture time was 5 days; the substance was added from day 3 to day 5 of the coculture; time of treatment was 48 hours.

Inhibition was 99 % for Des.E $1*10^{-7}$ M, 84 % for Des.E $1*10^{-8}$ M and 32 % for Des.E $1*10^{-9}$ M.

++ $p<0,01$ vs. control; + $p<0,05$ vs. control

	% Resorption							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
Control	13.12	0.59	6.55	0.80	7.13	3.85	4.46	18.25
Des.E $1*10^{-7}$ M	0.00	0.02	0.17	0.01	0.00	0.14	0.18	0.00
Des.E $1*10^{-8}$ M	1.21	0.00	6.60	0.10	0.00	0.84	0.00	0.02
Des.E $1*10^{-9}$ M	5.33	0.01	0.05	0.73	6.47	0.53	16.94	6.61

Table 4.1: Resorption in percentage on the individual bone slices.
Y1-Y8 stands for the different samples of each group.

	% Resorption		
	Mean	SEM	N
Control	6.844	2.154	8
Des.E $1*10^{-7}$ M	0.065	0.029	8
Des.E $1*10^{-8}$ M	1.096	0.803	8
Des.E $1*10^{-9}$ M	4.584	2.043	8

Table 4.2: Resorption on the individual bone slices.
This is stated with the mean of 8 samples and the standard deviation.

4.1.1.2 Effect of destruxin E on the differentiation of osteoclasts (Group 2)

Figure 4.2 does not show inhibition on differentiation at a concentration of destruxin E of $1*10^{-7}$ M and $1*10^{-8}$ M compared to the control. The inhibitory effect was 99 % for destruxin E $1*10^{-7}$ M. At a concentration of destruxin E of $1*10^{-9}$ M there was a significant difference of $p<0,05$ compared to the control. Since at this concentration of destruxin E the resorption was greater than in the control, it rather shows an irregularity in the method. Taken together, destruxin E has a stronger effect on the activity of osteoclasts than on the differentiation of osteoclasts.

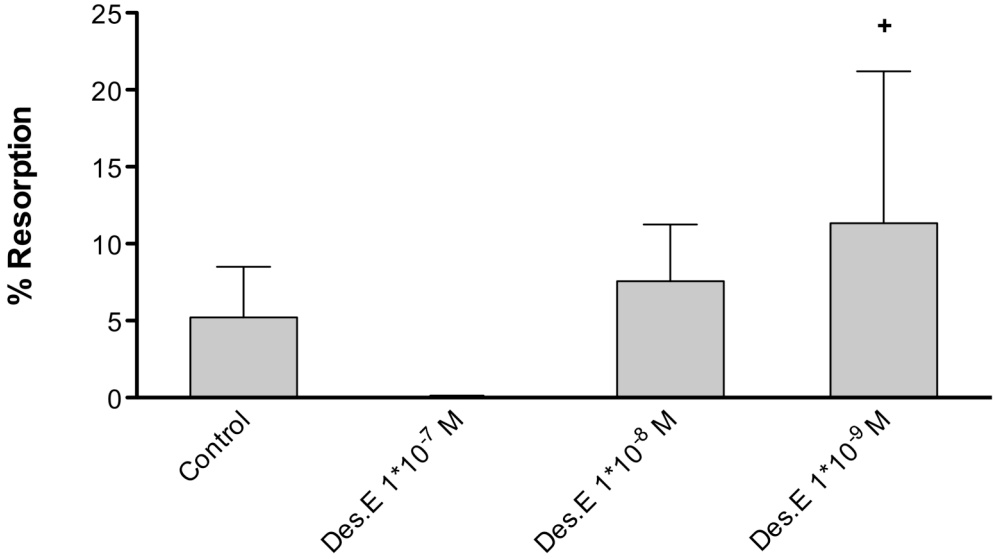


Figure 4.2: Effect of destruxin E on the resorption of murine osteoclasts on bovine bone slices.

The total cultutre time was 5 days; the substance was added from day 0 to day 3 of the coculture; time of the treatment was 72 hours.

Inhibition for Des.E $1*10^{-7}$ M was 99 %.

+ $p<0,05$ vs. control

	% Resorption							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
Control	6.09	10.99	4.43	5.14	0.00	5.39	7.39	2.29
Des.E $1*10^{-7}$ M	0.00	0.00	0.13	0.00	0.18	0.07	0.00	0.11
Des.E $1*10^{-8}$ M	4.46	10.30	4.04	2.74	8.23	6.96	13.20	10.61
Des.E $1*10^{-9}$ M	0.19	2.99	20.00	24.85	9.82	2.58	7.33	22.93

Table 4.3: Resorption in percentage on the individual bone slices.

Y1-Y8 stands for the different samples of each group.

	% Resorption		
	Mean	SEM	N
Control	5.215	1.159	8
Des.E $1*10^{-7}$ M	0.061	0.025	8
Des.E $1*10^{-8}$ M	7.568	1.300	8
Des.E $1*10^{-9}$ M	11.336	3.487	8

Table 4.4: Resorption on the individual bone slices.

This is stated with the mean of 8 samples and the standard deviation.

4.1.1.3 Effect of destruxin E on the activity and on the differentiation of osteoclasts (Group 3)

As shown in figure 4.3, the greatest inhibitory effect on bone resorption by destruxin E was achieved when the test substance was added during the whole period of the coculture (day 0 – day 5). Destruxin E at all 3 concentrations (destruxin E $1*10^{-7}$ M, $1*10^{-8}$ M and $1*10^{-9}$ M) exerted significant inhibition ($p<0,001$ compared to the control).

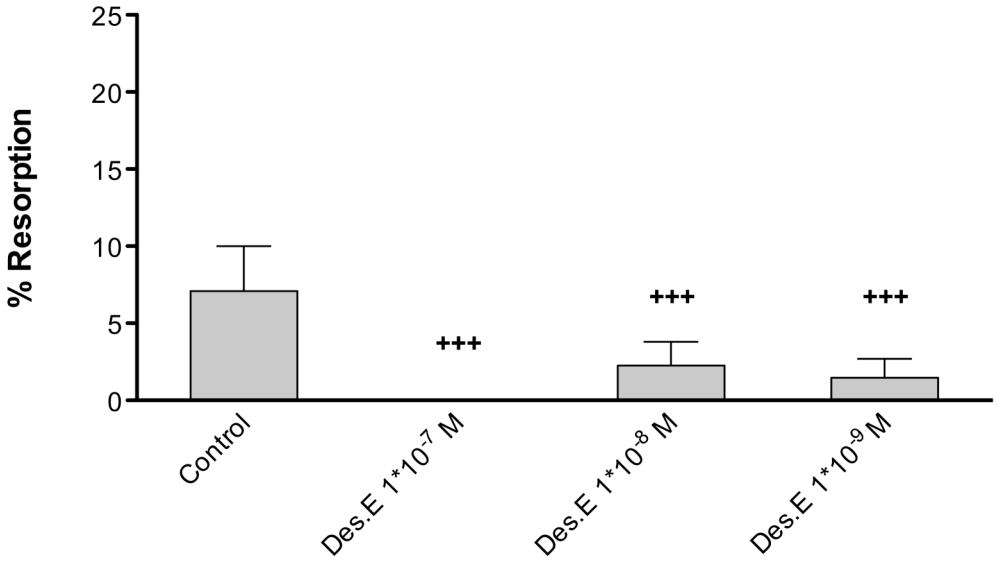


Figure 4.3: Effect of destruxin E on the resorption of murine osteoclasts on bovine bone slices.

The total culture time was 5 days; the substance was added from day 0 to day 5 of the coculture; time of the treatment was 120 hours.

Inhibition was 100 % for Des.E $1*10^{-7}$ M, 68 % for Des.E $1*10^{-8}$ M and 79 % for Des.E $1*10^{-7}$ M.

+++ $p<0,001$ vs. control

	% Resorption							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
Control	5.58	4.89	6.50	9.87	10.97	10.16	5.94	2.84
Des.E $1*10^{-7}$ M	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.02
Des.E $1*10^{-8}$ M	1.39	0.36	2.02	1.63	3.57	1.12	5.15	2.91
Des.E $1*10^{-9}$ M	0.06	1.94	3.50	1.67	0.06	0.93	0.93	2.72

Table 4.5: Resorption in percentage on the individual bone slices.

Y1-Y8 stands for the different samples of each group.

	% Resorption		
	Mean	SEM	N
Control	7.094	1.027	8
Des.E $1*10^{-7}$ M	0.010	0.008	8
Des.E $1*10^{-8}$ M	2.269	0.545	8
Des.E $1*10^{-9}$ M	1.476	0.433	8

Table 4.6: Resorption on the individual bone slices.

This is stated with the mean of 8 samples and the standard deviation.

4.2 Quantification of TRAP-positive cells

For these experiments destruxin E was tested in the concentrations $1*10^{-7}$ M, $1*10^{-8}$ M and $1*10^{-9}$ M. In preliminary tests destruxin E in the concentration of $1*10^{-6}$ M was also analyzed, but since it completely eliminated TRAP-positive cells, it is not shown in the results below. As shown in figure 4.4, the test substance decreased the number of TRAP-positive cells, but not in a clear dose-dependent matter. $1*10^{-7}$ M destruxin E showed a significant difference at a level of $p<0,01$ compared to the control group. There was no significant difference at a concentration of destruxin E of $1*10^{-8}$ M and of $1*10^{-9}$ M. Noticeable is that the inhibitory effect was, with 18 %, smaller at a concentration of destruxin E of $1*10^{-8}$ M than, with 34 %, at a concentration of destruxin E of $1*10^{-9}$ M. This result does not correlate with the dose-dependent result of the resorption assay (see figure 4.1). Based on the results of the resorption assay there should be more resorbing cells (OCs) at a concentration of destruxin E of $1*10^{-9}$ M than at a concentration of destruxin E of $1*10^{-8}$ M, which is not the case in figure 4.4.

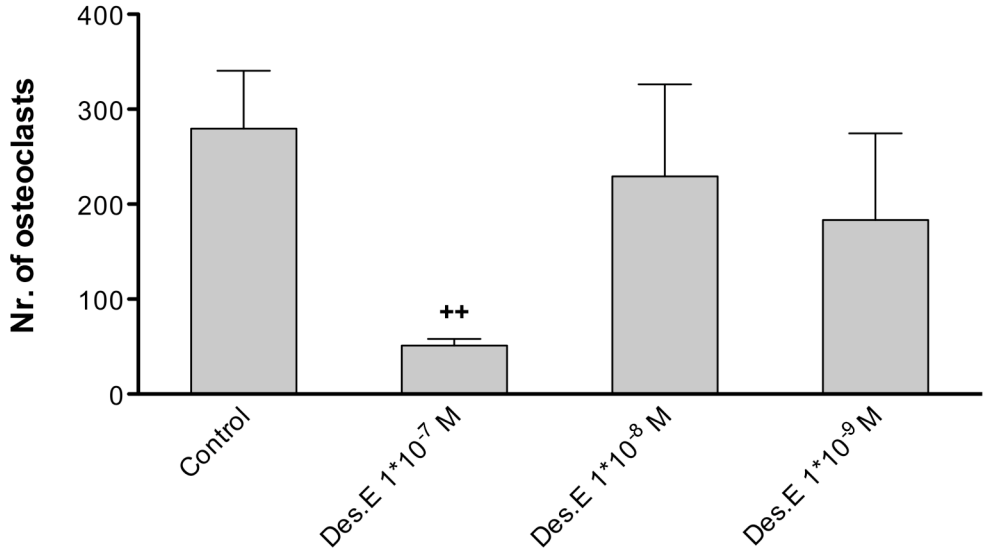


Figure 4.4: Effect of destruxin E on the number of TRAP-positive cells with 3 or more nuclei (osteoclasts).

The total culture time was 5 days; the substance was added on day 3 of the coculture; time of treatment was 48 hours.

Inhibition was 82 % for Des.E $1*10^{-7}$ M, 18 % for Des.E $1*10^{-8}$ M and 34 % for Des.E $1*10^{-7}$ M.

++ $p<0,01$ vs. control

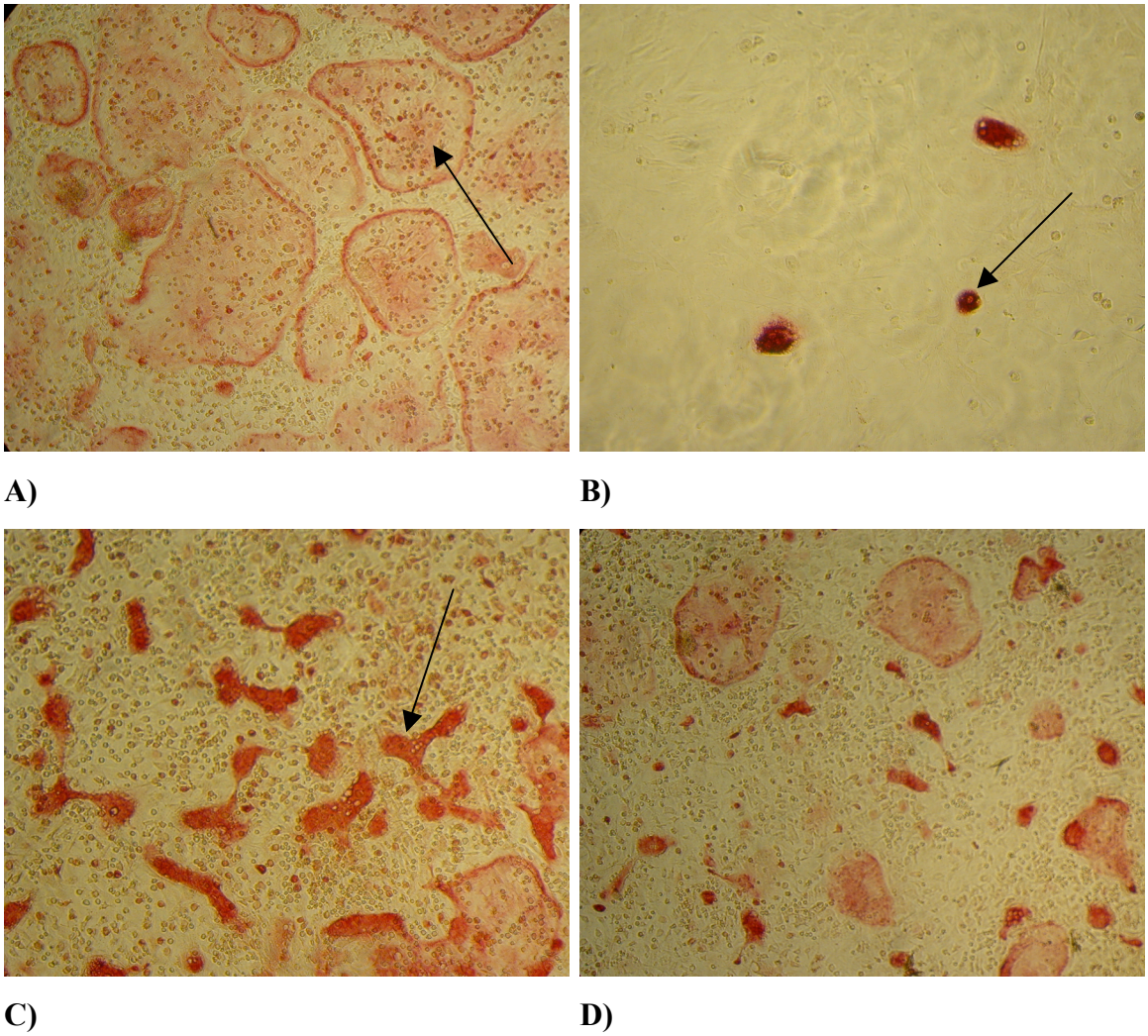
	Nr. of osteoclasts		
	Y1	Y2	Y3
Control	337.00	216.00	286.00
Des.E $1*10^{-7}$ M	53.00	57.00	43.00
Des.E $1*10^{-8}$ M	308.00	259.00	121.00
Des.E $1*10^{-9}$ M	176.00	96.00	278.00

Table 4.7: Number of osteoclasts in the individual wells.
Y1-Y3 stands for the different samples of each group.

	Nr. of osteoclasts		
	Mean	SEM	N
Control	279.667	35.073	3
Des.E $1*10^{-7}$ M	51.000	4.163	3
Des.E $1*10^{-8}$ M	229.333	55.983	3
Des.E $1*10^{-9}$ M	183.333	52.667	3

Table 4.8: Number of osteoclasts in the individual wells.
This is stated with the mean of 3 samples and the standard deviation.

Under the light microscope it could be clearly seen that there were different kinds of TRAP-positive multinucleated cells present in the culture under these conditions. On the one hand there were big, round cells with an outspread cytoplasm (figure 4.5, A, arrow) and on the other hand there were small, mostly star-shaped cells with a concentrated cytoplasm (figure 4.5, C, arrow). The big, round cells could be determined easier. To distinguish the small cells was much harder. They were stained in a darker red (because of the concentration of the cytoplasm) and so the number of nuclei was harder to analyse. But also the star-shaped cells could be distinguished from the mononuclear monocytes (figure 4.5, B, arrow).



A) **B)**

C) **D)**

Figure 4.5: Stained TRAP-positive cells of a coculture under the light microscope.

A) Control: mainly big, round cells (OCs), stated by the arrow.

B) Destruxin E, $1*10^{-7}$ M: almost no cells at all; just small TRAP-positive cells; arrow states a mononuclear monocyte.

C) Destruxin E, $1*10^{-8}$ M: mostly small, star-shaped cells (OCs), stated by the arrow, and a few big ones.

D) Destruxin E, $1*10^{-9}$ M: about same amount of big, round and small, star-shaped TRAP-positive cells with 3 or more nuclei.

For further analysis the two different cell populations were analyzed separately. The statistical outcome and the appendent graphics are shown below.

4.2.1 Detection of small, star-shaped multinucleated TRAP-positive cells

It could be demonstrated that the number of small, star-shaped multinucleated TRAP-positive cells does not correlate with the dose-dependent results of the resorption assay (see figure 4.1). Figure 4.6 shows that there was no significant difference at any used concentration of destruxin E, compared to the control group, on the number of small, star-shaped multinucleated TRAP-positive cells (OCs). The only inhibitory effect could be seen at the concentration of $1*10^{-7}$ M of destruxin E with 50 %. It is remarkable, that destruxin E at a concentration of $1*10^{-8}$ M even induces an increase in the number of cells. Cultures with destruxin E in a concentration of $1*10^{-8}$ M exhibit almost double the amount of small cells than cultures without destruxin (control group). At the lowest concentration of destruxin E, $1*10^{-9}$ M, the number of small cells is about the same as in the control group.

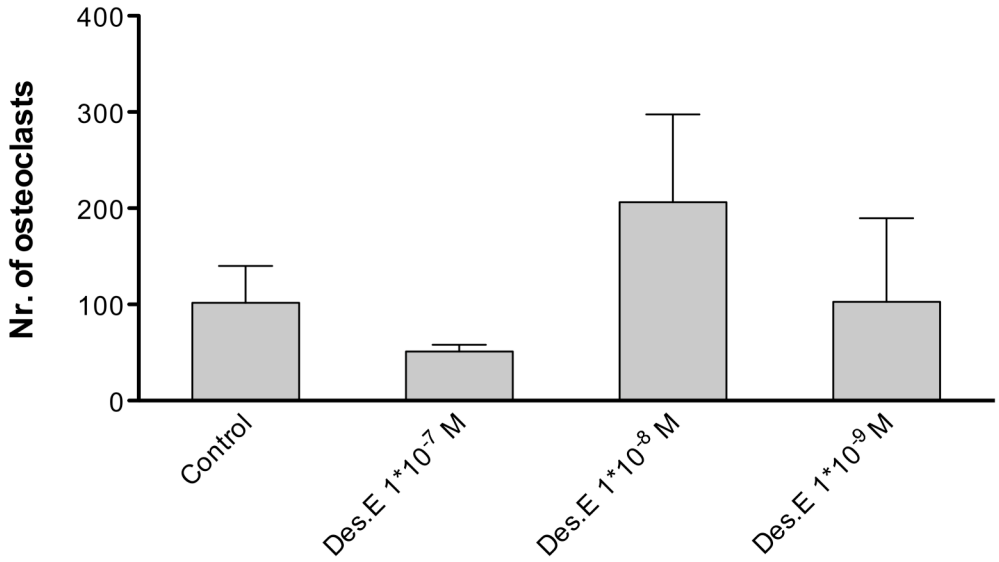


Figure 4.6: Effect of destruxin E on the number of small, star-shaped TRAP-positive cells with more than 3 nuclei (osteoclasts).

The total culture time was 5 days; the substance was added on day 3 of the coculture; time of treatment was 48 hours.

Inhibition was 50 % for Des.E $1*10^{-7}$ M.

	Nr. of osteoclasts		
	Y1	Y2	Y3
Control	133.00	59.00	113.00
Des.E $1*10^{-7}$ M	53.00	57.00	43.00
Des.E $1*10^{-8}$ M	271.00	246.00	102.00
Des.E $1*10^{-9}$ M	51.00	54.00	203.00

Table 4.9: Number of osteoclasts in the individual wells.

Y1-Y3 stands for the different samples of each group.

	Nr. of osteoclasts		
	Mean	SEM	N
Control	101.667	22.101	3
Des.E $1*10^{-7}$ M	51.000	4.163	3
Des.E $1*10^{-8}$ M	206.333	52.664	3
Des.E $1*10^{-9}$ M	102.667	50.174	3

Table 4.10: Number of osteoclasts in the individual wells.
This is stated with the mean of 3 samples and the standard deviation

4.2.2 Detection of big, round multinucleated TRAP-positive cells

Figure 4.7 shows that destruxin E had a clear dose-dependent inhibitive effect on the number of big, round multinucleated TRAP-positive cells (OCs). At the concentration of $1*10^{-7}$ M and $1*10^{-8}$ M destruxin E there was a significant difference at a level of $p<0,001$ compared to the control group. This result correlates perfectly with the result of the resorption assay (see figure 4.1). The inhibition by destruxin E, $1*10^{-9}$ M, reached 55 %, compared to the control (significant difference at a level of $p<0,01$). This inhibiton is comparable to the inhibitory effect of 32 % in the resorption assay (see figure 4.1) at the same concentration.

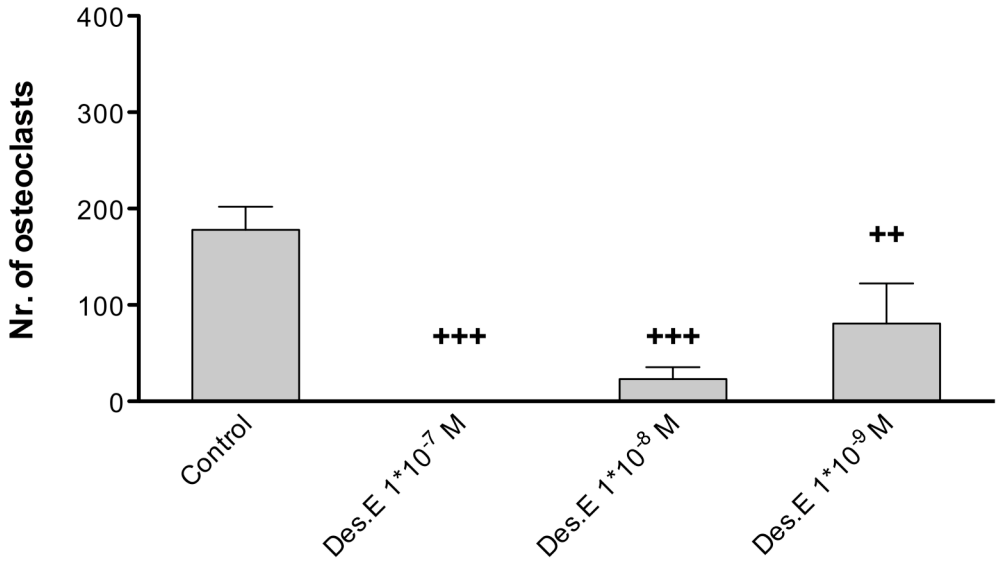


Figure 4.7: Effect of destruxin E on the number of TRAP-positive cells with more than 3 nuclei (osteoclasts).

The total culture time was 5 days; the substance was added on day 3 of the coculture; time of treatment was 48 hours.

Inhibition was 100 % for Des.E 1*10^{-7} M, 87 % for Des.E 1*10^{-8} M and 55 % for Des.E 1*10^{-9} M.

++ $p<0,01$ vs. control; +++ $p<0,001$ vs. control

	Nr. of osteoclasts		
	Y1	Y2	Y3
Control	204.00	157.00	173.00
Des.E 1*10^{-7} M	0.00	0.00	0.00
Des.E 1*10^{-8} M	37.00	13.00	19.00
Des.E 1*10^{-9} M	125.00	42.00	75.00

Table 4.11: Number of osteoclasts in the individual wells.

Y1-Y3 stands for the different parallels of each group.

	Nr. of osteoclasts		
	Mean	SEM	N
Control	178.000	13.796	3
Des.E $1*10^{-7}$ M	0.000	0.000	3
Des.E $1*10^{-8}$ M	23.000	7.211	3
Des.E $1*10^{-9}$ M	80.667	24.127	3

Table 4.12: Number of osteoclasts in the individual wells.

This is stated with the mean of 3 parallels and the standard deviation.

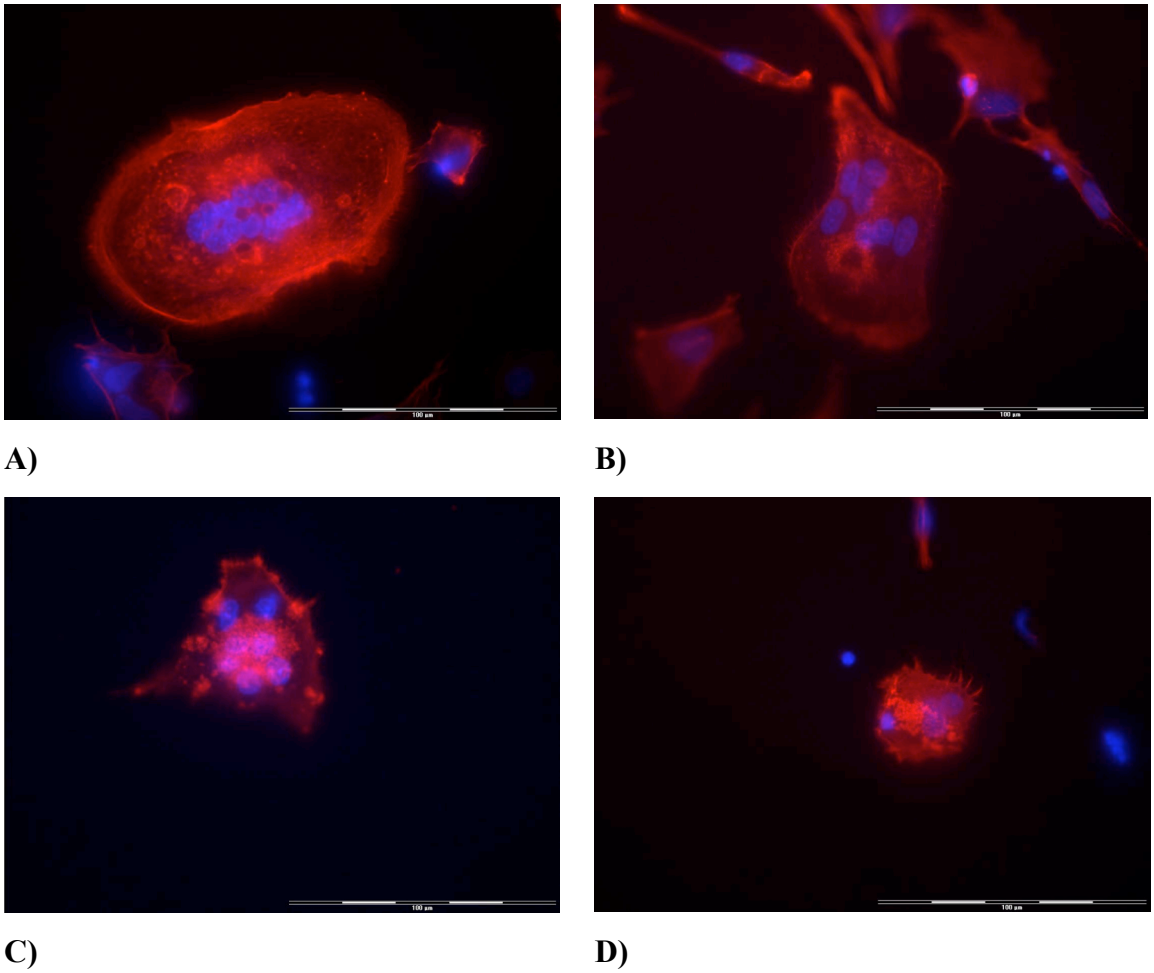
4.3 Morphological effects on bone cells

For these experiments, concentrations of destruxin E used were $1*10^{-6}$ M, $1*10^{-7}$ M, $1*10^{-8}$ M and $1*10^{-9}$ M.

4.3.1 Effect on bone resorbing rabbit osteoclasts

Actin rings are a marker of polarized osteoclasts and polarization is essential to initiate bone resorption. The phalloidin-labelled actin rings can be recognized as bright red-stained belts surrounding the cells (figure 4.8, A). I found that destruxin E induces morphological changes in rabbit osteoclasts. It causes disruption of the actin ring and spreading of F-actins throughout the cytoplasm of the cells. The cytoplasm of osteoclasts was observed as "foamy" rather than clear, and the cell periphery was irregular rather than smooth. Destruxin E also induced apoptosis in the osteoclasts. Bone cells undergoing apoptosis show condensation of chromatin and degradation of DNA into fragments and the formation of plasma and nuclear vesicles.

In untreated cultures mainly large multinucleated cells with well-described actin rings and no sign of apoptosis (figure 4.8, A) could be detected. Adding destruxin E in a concentration of $1*10^{-9}$ M, resulted in no difference compared to the control group. But the grade of apoptosis and disruption of the actin rings increased gradually towards higher concentrations. Osteoclasts treated with destruxin, E $1*10^{-8}$ M, (figure 4.8, B) exhibited mostly ring-like actin structures, but with first signs of disruption. Some cells showed already apoptotic nuclei, mainly by condensation. In cultures with destruxin E at the two highest concentrations, $1*10^{-7}$ M (figure 4.8, C) and $1*10^{-6}$ M (figure 4.8, D), osteoclasts did not form actin rings at all. F-actin was distributed throughout the cytoplasm and the cell periphery was irregular. Furthermore, the general number of cells was highly reduced (osteoclasts, but also other bone marrow cells), the osteoclasts were small in size and all detected cells showed signs of apoptosis.



A) **B)**

C) **D)**

Figure 4.8: Effect of destruxin E on the cytoskeleton and on the apoptosis of rabbit osteoclasts.

The nuclei were stained with DAPI (blue) and the cytoskeleton is stained with Alexa fluor®488/phalloidin (red); on cover slip; under fluorescence microscope; 60x magnification; scale bar indicates 100 µm.

A) Control: large multinucleated osteoclasts; actin rings; no apoptosis

B) Destruxin E, $1*10^{-8}$ M: ring-like actin structures with signs of disruption

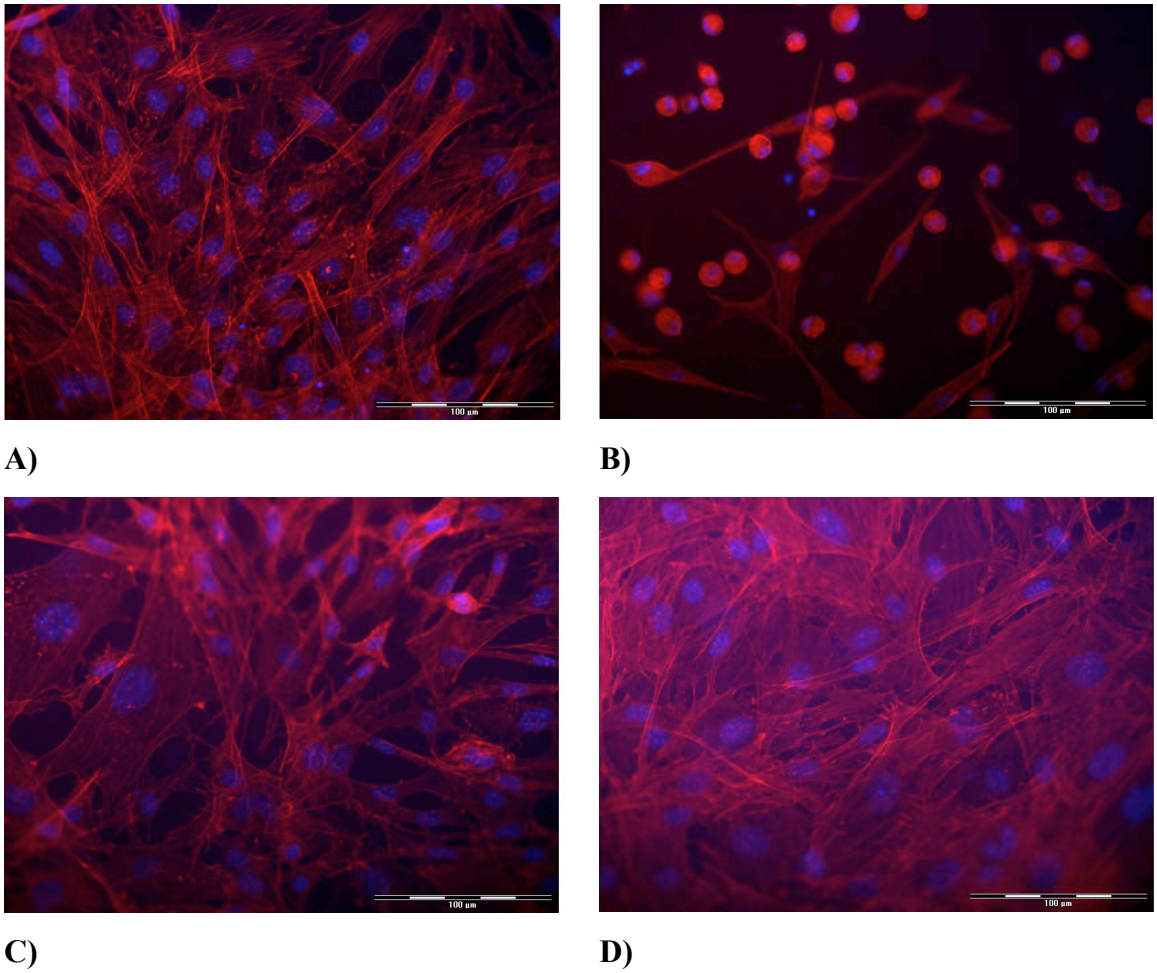
C) Destruxin E, $1*10^{-7}$ M: no actin rings; small apoptotic cells

D) Destruxin E, $1*10^{-6}$ M: no actin rings; small apoptotic cells

4.3.2 Effect on bone forming osteoblasts

Since bone is a living tissue and is continuously remodelled, it is also important to look at the effect of the test substance on the osteoblasts, to consider destruxin E as a potential anti-resorptive drug against osteoporosis.

As shown in figure 4.9, the test substance also induced morphological changes in osteoblasts. In the control group (figure 4.9, A) there were almost no apoptotic cells and their cytoskeleton was outspread over the whole substrate surface (cover slip). At a concentration of destruxin E of $1*10^{-6}$ M (figure 4.9, B) all cells were found to be apoptotic and their cytoskeleton was agglomerated and hardly detectable. At a concentration of destruxin E of $1*10^{-7}$ M (figure 4.9, C) the osteoblasts were fewer in number and their cytoskeleton was not that distinctive compared to the control group. Some apoptotic osteoblasts could be observed at this concentration of destruxin E. There was no noticeable difference in the number of cells, their cytoskeleton and the number of apoptotic cells at a concentration of destruxin E of $1*10^{-8}$ M (figure 4.9, D) and $1*10^{-9}$ M compared to the control group.



A) **B)**

C) **D)**

Figure 4.9: Effect of destruxin E on the cytoskeleton of osteoblasts and on their apoptosis.

The nuclei were stained with DAPI (blue) and the cytoskeleton is stained with Alexa fluor®488/phalloidin (red); on cover slip; under fluorescence microscope; 40x magnification; scale bar indicates 100 µm.

A) Control: outspread cytoskeleton; no apoptotic cells

B) Destruxin E, $1*10^{-6}$ M: agglomerated cytoskeleton; just apoptotic cells

C) Destruxin E, $1*10^{-7}$ M: fewer number of OBs; some apoptotic cells

D) Destruxin E, $1*10^{-8}$ M: outspread cytoskeleton, no apoptotic cells

4.4 Effects of destruxin E on cell viability

For the MTS-test destruxin E was used in the concentrations of $1*10^{-6}$ M, $1*10^{-7}$ M, $1*10^{-8}$ M and $1*10^{-9}$ M. Figure 4.10 shows that destruxin E had no inhibitory effect on the viability of osteoblasts at almost all concentrations. Only at a concentration of destruxin E of $1*10^{-6}$ M there was a significant difference at a level of $p<0,05$ compared to the control. All other concentrations of destruxin E showed no significant difference.

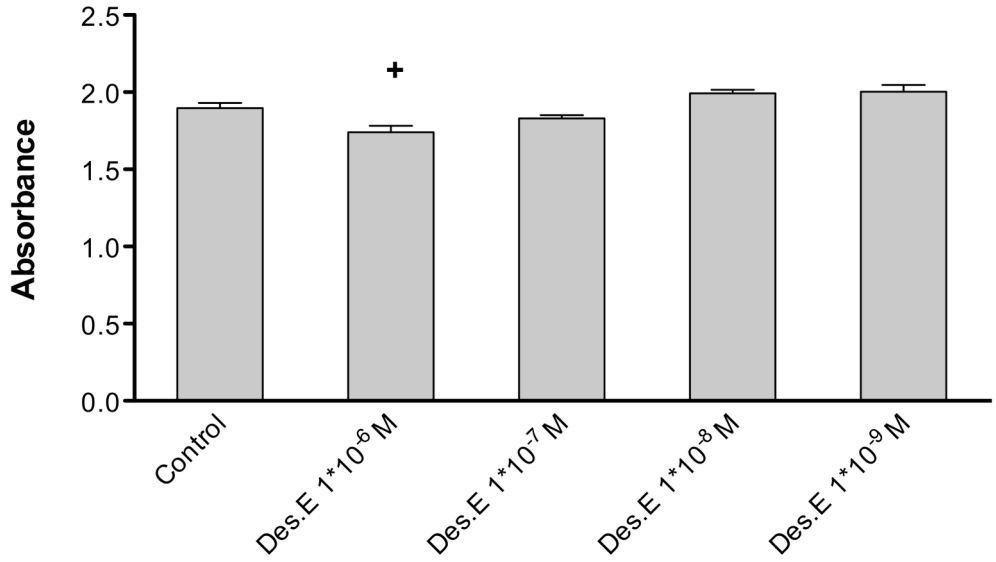


Figure 4.10: Effect of destruxin E on osteoblast cell viability.
Time of treatment was 48 hours.
Inhibition was 8 % for Des. E $1*10^{-6}$ M and 4 % for Des. E $1*10^{-7}$ M.
+ $p<0,05$ vs. control

	Absorbance							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
Control	1.910	1.920	1.740	1.770	1.960	1.960	2.010	1.900
Des.E $1*10^{-6}$ M	1.750	1.860	1.720	1.740	1.870	1.560	1.580	1.840
Des.E $1*10^{-7}$ M	1.800	1.740	1.830	1.770	1.860	1.910	1.820	1.910
Des.E $1*10^{-8}$ M	2.040	2.030	2.010	1.880	2.020	2.030	1.920	2.020
Des.E $1*10^{-9}$ M	2.020	2.060	2.230	1.810	1.900	2.010	2.020	1.980

Table 4.13: Effect of destruxin E on the viability of osteoblasts.

This is stated with the absorbance of formazan within the individual groups. Y1-Y8 stands for the different samples of each group

	Absorbance		
	Mean	SEM	N
Control	1.896	0.033	8
Des.E $1*10^{-6}$ M	1.740	0.042	8
Des.E $1*10^{-7}$ M	1.830	0.022	8
Des.E $1*10^{-8}$ M	1.994	0.021	8
Des.E $1*10^{-9}$ M	2.004	0.043	8

Table 4.14: Effect of destruxin E on the viability of osteoblasts.

Figure 4.11 shows the effect of destruxin E on RAW 264.7 cells. The test substance had an inhibitory effect on the viability of these osteoclast precursor cells. At the concentration $1*10^{-6}$ M and $1*10^{-7}$ M of destruxin E, the reduction of viability was highly significant at a level of $p<0,001$.

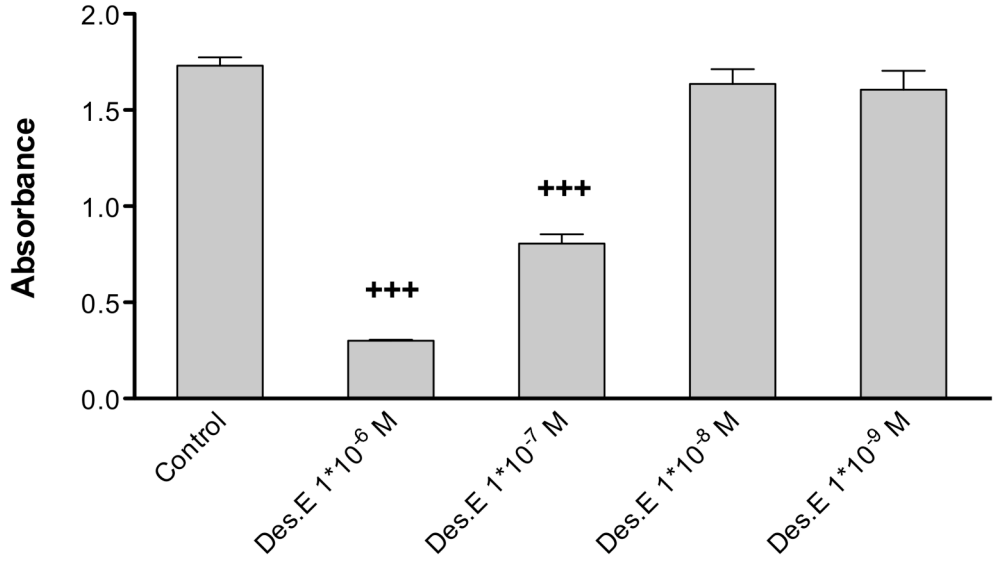


Figure 4.11: Effect of destruxin E on RAW 264.7 cell viability.
Time of treatment was 48 hours.
Inhibition was 83 % for Des. E $1*10^{-6}$ M and 54 % for Des. E $1*10^{-7}$ M.
+++ $p<0,001$ vs. control

	Absorbance							
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8
Control	1.770	1.580	1.740	1.940	1.790	1.760	1.540	1.720
Des.E $1*10^{-6}$ M	0.310	0.290	0.310	0.300	0.310	0.320	0.280	0.290
Des.E $1*10^{-7}$ M	0.660	0.910	0.860	0.970	0.920	0.570	0.750	0.800
Des.E $1*10^{-8}$ M	1.650	1.300	1.730	1.520	1.380	1.870	1.840	1.800
Des.E $1*10^{-9}$ M	1.350	1.410	1.530	1.340	1.650	2.190	1.730	1.650

Table 4.15: Effect of destruxin E on the viability of RAW 264.7 cells.

This is stated with the absorbance of formazan within the individual groups. Y1-Y8 stands for the different samples of each group.

	Absorbance		
	Mean	SEM	N
Control	1.730	0.044	8
Des.E $1*10^{-6}$ M	0.301	0.005	8
Des.E $1*10^{-7}$ M	0.805	0.049	8
Des.E $1*10^{-8}$ M	1.636	0.076	8
Des.E $1*10^{-9}$ M	1.606	0.098	8

Table 4.16: Effect of destruxin E on the viability of RAW 264.7 cells.

4.5 Discussion

This thesis demonstrates that the mycotoxin destruxin E inhibits osteoclast function and differentiation in vitro.

Destruxin E has a dose-dependent inhibitory effect on the activity of osteoclast (figure 4.1) as well as on the differentiation (figure 4.2). The performed resorption assays clearly show that the inhibitory effect on the activitiy of osteoclasts is stronger than the inhibitory effect on the differentiation. When destruxin E was present during the whole period of the coculture hardly no resorption could be observed (figure 4.3). This effect can be explained by the combined inhibition of destruxin E on the differentiation and activity of osteoclasts.

An assay for cell viability on osteoclast precursor cells (RAW 264.7 cells) and on osteoblasts was carried out to determine whether or not the inhibitory effects of destruxin E resulted from cytotoxicity. In the performed experiments no cytotoxicity of destruxin E on osteoblasts in the concentration range at which the compound inhibits pit formation could be observed (figure 4.10). But the reduction of viability of osteoclast precursor cells at high concentrations of destruxin E ($1*10^{-6}$ M and $1*10^{-7}$ M) was highly significant compared to the control group (figure 4.11). From this can be concluded that the inhibitory effect of high concentrations of destruxin E on the pit formation is, at least partly, due to a cytotoxic effect of the substance on osteoclasts precursors.

There is already some evidence of the cytotoxic effect of destruxins. Odier et al. demonstrated the inhibitory effect of destruxins on leukemic cell proliferation (Odier et al., 1992). Dumas et al. found out that destruxin, especially destruxin E and A, exhibited a cytotoxic effect on insect cells (Dumas et al., 1994). Destruxins were shown to be phytotoxic to a variety of plants (Pedras et al., 2002).

Different kinds of TRAP-positive multinucleated cells could be detected in the cultures treated with destruxin E. On the one hand big, round cells with an outspread cytoplasm and on the other hand small, mostly star-shaped cells with a concentrated cytoplasm were distinguished. For this reason the two different cell populations were analyzed separately. This is supported by the evidence of two different types of osteoclasts (Yuasa et al., 2007).

The only inhibitory effect on the number of small, star-shaped multinucleated TRAP-positive cells (OCs) detectable was at the highest concentration of destruxin E ($1*10^{7}$ M). It is remarkable, that destruxin E at a concentration of $1*10^{-8}$ M even induced an increase in the number of cells (figure 4.6). What we have to keep in mind is that for this data only the small cells were counted and not all of the potential resorbing cells.

It could be displayed in this work that destruxin E has a clear dose-dependent inhibitory effect on the number of big, round multinucleated TRAP-positive cells (figure 4.7). This result correlates perfectly with the result obtained from the resorption assay. More precisely, destruxin E inhibits the differentiation of progenitor cells into (big, round) multinucleated osteoclasts, at the same concentrations at which it inhibits pit formation.

Based on these results, I suppose that just the big, round multinucleated TRAP-positive cells are actually active osteoclasts that are able to resorb bone material. The small, star-shaped ones are also considered to be osteoclasts, but without their typical resorbing function. Maybe they are already apoptotic or their functional structure is not fully developed. As a conclusion, it is advisable to consider just the big, round multinucleated TRAP-positive cells to get an idea about the number and nature of the active osteoclasts.

The performed experiments on the quantification of TRAP-positiv cells suggest that destruxin E not only inhibits the activity of osteoclasts, but also reduces the total number of cells. This fact confirms the cytotoxic effect of destruxin E demonstrated in

this study also on osteoclast precursors. The reduction of cells is most likely an effect of induced apoptosis.

Destruxin E affected morphological features in osteoclasts in the performed experiments. Disruption of actin rings increased gradually towards higher concentrations of destruxin E. (See chapter 2.1.4.4.2 for details about polarized morphological features of osteoclasts.)

In cultures treated with the concentration of destruxin E that inhibits pit formation completely ($1*10^{-7}$ M) all actin rings in osteoclasts were disrupted. This result suggests that destruxin E directly induces disruption of the cytoskeletal actin rings in osteoclasts, thereby inhibiting their bone-resorbing activity. Furthermore, a clear reduction in the general number of cells (osteoclasts and also other bone marrow cells) could be observed at the same concentration of destruxin E that showed complete impairment of actin rings. Moreover, the osteoclasts were much smaller in size (compared to the untreated osteoclasts) and all cells showed signs of apoptosis. It appears that the inhibitory effect on the bone resorption (100 %) at this concentration of destruxin E is based on a combination of the toxicity of destruxin E and on the morphological changes it causes in osteoclasts.

Research on the effect of destruxins or related cyclodepsipeptides on the bone is still in the early stages. So far only one paper has been puplished concerning this field of research. Nakagawa et al. reported the inhibitory effect of destruxins (E and B) on the bone-resorbing activity of osteoclasts for the first time. At the moment, further publications on the effect of the related cyclodepsipeptides enniatin and beauvericin on bone are in progress. These compounds seem to have similar effects on bone as destruxins.

The osteoclasts used in the study of Nakagaw et al. were also derived from cocultures of osteoblasts and osteoclast precursors in the presence of 1,25-$(OH)_2$-vitamin-D_3 and

PGE_2 in α-MEM. The pit formation assay was performed on sperm whale dentin slices (in contrast to bovine bone slices in this study).

Destruxin E inhibits pit formation by incducing morphological changes (disruption of the actin rings) in osteoclast without affecting osteoclast differentiation and survival (at a concentration of $1*10^{-8}$ M destruxin E). This conclusion can be confirmed by Nakagawa et al. But disagreements in the affecting concentrations of destruxin E could be noticed, since Nakagawa et al. observed this correlation at a higher concentration of destruxin E, $1*10^{-7}$ M. But, in this study it can be demostrated that a concentration of destruxin E of $1*10^{-7}$ M induces bone resorption mainly by directly reducing the number of TRAP-positve multinucleated osteoclasts.

According to the here stated results it is advisable to take a concentration of $1*10^{-8}$ M destruxin E under consideration for anti-resorptive use.

The changes in morphological structures and the thereby induced inhibition of the bone-resorbing activity of osteoclasts by destruxin E were shown to be reversible if cultivated in fresh medium (Nakagawa et al., 2003). This reversibilty have not been investigated in this study. Since the inhibitory effect at a concentration of $1*10^{-7}$ M destruxin E is partly due to cytotoxicity, the activity of osteoclasts could probably not be regained completely. But, at a concentration of $1*10^{-8}$ M destruxin E the reversibility of the changes in the actin ring structure could regain the activity of osteoclasts to almost full extent.

Further research will be necessary to clarify the detailed mechanism of the inhibitory effect of destruxin on the bone-resorbing function of osteoclasts. Especially the examination of transcription factors involved in the osteoclastogenesis may be revealing. This could be performed with western blot. Also to investigate the effect of destruxins in vivo will be worthwhile.

5. Conclusion

This work was performed to investigate the effect of destruxin E on osteoclasts, on their morphology and their activity, and on bone remodeling with the intention to investigate new therapeutic approaches for the treatment of bone diseases with excessive bone loss, mainly osteoporosis.

Destruxin E was found to inhibit the bone resorbing ability of osteoclasts in a dose-dependent manner. This effect of the test substance was more due to the inhibition of the activity of osteoclasts than to their differentiation from precursor cells. Osteoblasts are not as sensitive to destruxin E as osteoclasts, but a cytotoxic effect could be observed at the highest concentration of the test substance ($1*10^{-6}$ M).

Destruxin E, at the highest concentrations ($1*10^{-6}$ M and $1*10^{-7}$ M), also seems to be cytotoxic to osteoclasts. The anti-resorptive effect derives at least to some extent from the absence of the bone resorbing cells. That could be shown with the reduction of TRAP-positive multinucleated cells. But it may mainly be assumed that destruxin E inhibits bone resorption by disrupting actin rings (a highly polarized morphological feature) that are necessary for functional osteoclasts.

However, further work is necessary before this substance can come into consideration for being used as a pharmaceutical agent in the treatment of bone disorders like osteoporosis.

6. Abstract

Bone is continuously remodeled by osteoblasts (synthesize bone matrix) and osteoclasts (resorb bone). Several bone diseases, most notably ostoeporosis, are related to a pathological imbalance between their activities. Osteoporosis is responsible for millions of fractures annually. Therefore the search for new anti-resorptive agents is a reasonable field of study.

Destruxins are cyclic hexadepsipeptides secreted by the entomopathogenic fungus *Metarhizium anisspliae*. These mycotoxins are best known for their insecticidal and phytotoxic activities. Destruxin E is the most active compound of this family. There is evidence that this mycotoxin inhibits the bone-resorbing activity of osteoclasts (see Nakagawa et al., 2003).

I investigated the effect of destruxin E on osteoclasts and bone remodeling in vitro. Main focus was the inhibition of the bone resorbing activity of osteoclasts, inhibition of osteoclast differentiation, changes in morphological structure, apoptosis and cytotoxicity. Additionally, the effect of destruxin E on the cytoskeleton of osteoblasts, on their apoptosis and its cytotoxicity was investigated.

In the experiments performed destruxin E was used in the concentrations of $1*10^{-9}$ M, $1*10^{-8}$ M, $1*10^{-7}$ M and $1*10^{-6}$ M. Destruxin E was found to inhibit the bone resorbing ability of osteoclasts in a dose-dependent manner. At all concentrations a significant inhibitory effect on the number of big round multinucleated TRAP-positive cells/osteoclasts could be observed. Destruxin E had no effect on the viability of osteoblasts at most concentrations, but had a significant inhibitory effect on the viability of osteoclast precursors at $1*10^{-7}$ M and $1*10^{-6}$ M. The test substance was also shown to cause impairment of actin rings in osteoclasts (markers of active osteoclasts) and induction of apoptosis. At $1*10^{-6}$ M and $1*10^{-7}$ M of destruxin E the actin rings were completely disrupted and only apoptotic cells were found. Additionally, an effect on the cytoskeleton of osteoblasts and on apoptosis at the same concentration of the destruxin E could be found.

7. Zusammenfassung

Der Knochen wird fortlaufend von Osteoblasten und Osteoklasten umgebaut. Etliche Knochenkrankheiten, am beachtenswertesten Osteoporose, stehen in Beziehung mit einem pathologischen Ungleichgewicht zwischen der Aktivität dieser Zellen. Osteoporose verursacht jährlich mehrere Millionen Knochenbrüche. Deshalb ist die Suche nach neuen antiresorptiven Wirkstoffen ein sehr sinnvolles Forschungsgebiet.

Destruxine sind cyclische Hexadepsipeptide, die von dem entomopathogenen Pilz *Metarhizium anisspliae* sezerniert werden. Diese Mykotoxine sind vor allem für ihre insektizide und phytotoxische Wirkung bekannt. Destruxin E ist die wirksamste Verbindung dieser Stoffklasse. Eine Studie deutet schon darauf hin, dass dieses Mykotoxin die knochenresorbierende Wirkung der Osteoklasten hemmt (Nakagawa et al., 2003).

In dieser Diplomarbeit wurde die Wirkung von Destruxin E auf Osteoklasten und auf das Knochenremodeling in vitro untersucht. Das Hauptaugenmerk galt der Hemmung der Substanz auf die knochenresorbierende Wirkung von Osteoklasten, die Hemmung der Zellzahl, sowie Veränderungen in der morphologischen Struktur und Einflüsse auf Apoptose und die Zytotoxizität. Weiters wurde die Zytotoxizität der Substanz auf Osteoblasten, sowie die Wirkung auf Zytoskelett und Apoptose untersucht. In den durchgeführten Versuchen wurde Destruxin E in den Konzentrationen $1*10^{-9}$ M, $1*10^{-8}$ M, $1*10^{-7}$ M und $1*10^{-6}$ M eingesetzt. Es konnte gezeigt werden, dass Destruxin E die knochenresorbierende Wirkung von Osteoklasten dosisabhängig hemmt. Bei allen Konzentrationen zeigte sich ein signifikanter Unterschied in der Zellzahl von großen, runden Osteoklasten zu der Kontrollgruppe. Destruxin E hatte keine Auswirkung auf die Viabilität von Osteoblasten (außer bei $1*10^{-6}$ M), aber es hatte eine signifikante hemmende Wirkung auf die Viabilität von Osteoklastenvorläuferzellen (bei $1*10^{-6}$ M und $1*10^{-7}$ M). Die Substanz verursacht Schädigungen des Aktinring von Osteoklasten und induziert deren Apoptose, vor allem bei Konzentration $1*10^{-6}$ M and $1*10^{-7}$ M. Weiters konnten bei diesen Konzentrationen auch Auswirkungen auf das Zytoskelett von Osteoblasten und deren Apoptose festgestellt werden.

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