DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
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EFFECTS OF PROTEASOME INHIBITORS ON CHONDROGENESIS AND LINEAR BONE GROWTH

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Cover photo: Reflection of growth in the human being.
- From small beginnings come great things -

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Nothing is IMPOSSIBLE, the word itself says "I'M POSSIBLE"!

To my beloved Family ♥

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ABSTRACT

Linear bone growth occurs at the growth plate, a thin layer of cartilage between the epiphysis and metaphysis of long bones. In the growth plate, resting/stem-like chondrocytes divide and generate the highly proliferative chondrocytes, which further differentiate into the enlarged hypertrophic form before being substituted by bone, a process called *endochondral ossification*. A precise balance between different factors affecting chondrocyte proliferation, differentiation/hypertrophy, matrix synthesis, and cell death within the growth plate must exist to ensure normal bone growth. Anti-cancer therapy can interfere with any of these processes, thereby affecting chondrogenesis and bone growth negatively. Proteasome inhibitors (PIs, e.g., MG262 and bortezomib) are a new, novel class of anti-cancer drugs. Bortezomib is approved for the treatment of adult hematologic malignancies, and is currently under clinical trials with pediatric cancers. So far, any undesired secondary side effects are yet unknown in treated children.

The aim of this thesis was to address whether PIs affect linear bone growth and bone homeostasis, and if so, what the underlying cellular mechanisms are, and to find potential ways to protect bone growth during anti-cancer treatment.

In the first study (Paper I), the effect of the non-clinically used PIs, MG262 and lactacystin, were investigated both *in vitro* and *in vivo*. Here we report for the first time that systemic administration of MG262 specifically targets the growth plate, and impairs linear bone growth in treated mice. The effect is linked to increased apoptosis of resting/stem-like chondrocytes in a caspase-dependent and independent manner. Inhibition of p53 and apoptosis-inducing-factor (AIF) were able to partly rescue from MG262-induced chondrocyte apoptosis.

Since bortezomib is in pediatric clinical trials, it is even more important to delineate any possible secondary side effects on linear bone growth and bone homeostasis (Paper II). Our results demonstrate that a clinically relevant dose of bortezomib specifically and efficiently impairs the ubiquitin/proteasome system (UPS). Consequently, young mice display severe growth failure during treatment, as well as after a follow-up period of 6 months post-treatment. This effect was mediated through a local action of bortezomib in the growth plate, causing increased resting/stem-like chondrocyte apoptosis and decreased differentiation. We also show that bortezomib mainly acts via the intrinsic apoptotic pathway, in which p53 and Bax appear to be the key regulators triggering apoptosis. In addition, cultured human growth plate cartilage was confirmed to be highly sensitive to bortezomib.

In an attempt to rescue bone growth during bortezomib treatment, we utilized pharmacological inhibition of Bax by the synthetic peptide analog to endogenous humanin, [Gly^{14}]-Humanin (HNG) (Paper III). We made the novel finding that HNG can rescue bone growth during bortezomib treatment by protecting resting/stem-like growth plate chondrocytes. Importantly, HNG did not interfere with the desired anti-cancer effect of bortezomib as tested and verified in tumor xenograft models as well as several human tumor cell lines. HNG also protected cultured human growth plate cartilage from the cytotoxic effects of bortezomib.

In conclusion, our observations confirmed *in vivo* and *in vitro*, including human growth plate cartilage, suggest that bone growth could potentially be suppressed in children treated with PIs. We hereby propose that bone growth and bone mineralization should be closely monitored in ongoing pediatric clinical trials. In addition, HNG may have the capacity to prevent PI-induced bone growth impairment without interfering with the desired anti-cancer effect.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund: Benets längdtillväxt sker i tillväxtplattan, ett tunt skikt av brosk som återfinns i ändarna av de långa rörbenen. Tillväxtplattan består av 3 unika zoner, "den vilande" innehållande stamcellslika broskceller (kondrocyter) som övergår till den "proliferativa" där cellerna delar sig snabbt för att sedan öka i storlek och ge upphov till "hypertrofa" kondrocyter som slutligen dör och ben bildas. Så länge man växer på längden finns alltså tillväxtplattan kvar, men under den senare delen av puberteten har den helt omvandlats till ben och därmed slutar vi även växa. Cancerbehandling hos unga individer kan störa kondrocyternas utveckling, vilket resulterar i tillväxthämning. Proteasomhämmare (ex. bortezomib) är en ny, lovande klass av cancermediciner som är i kliniska försök på barn, men man vet ännu inte om den har några skadliga effekter på normala vävnader och tillväxtplattans kondrocyter och/eller längdtillväxt.

Frågeställning: Syftet med denna avhandling var att undersöka om/hur proteasomhämmare påverkar benens tillväxt och förbening, utreda de bakomliggande cellulära mekanismerna och att finna möjliga sätt att skydda tillväxten under pågående cancer behandling.

Experimentella modeller: Olika musmodeller, tillväxtbrosk tillvarataget i samband med operation från unga patienter, mellanfotsben från råtta samt odlade broskceller från både människa och råtta och även humana cancerceller.

Resultat: I den första studien (artikel I) har vi studerat effekten av de icke-kliniskt använda proteasomhämmarna, MG262 och lactacystin. Våra resultat visar på att MG262 har en direkt effekt i tillväxtplattan och hämmar tillväxten hos behandlade möss. Effekten är kopplad till ökad celldöd av de stamcellslika broskcellerna. Genom att blockera uttrycket av två regulatoriska proteiner, p53 och AIF, lyckades vi delvis rädda kondrocyterna från MG262-inducerad celldöd.

Eftersom bortezomib är i kliniska prövningar på barn med cancer är det av yttersta vikt att undersöka om den har några biverkningar på benets utveckling och längdtillväxten (artikel II). Våra resultat tyder på att en klinisk relevant dos av bortezomib resulterar i permanent tillväxthämning, både under behandlingen och även efter en uppföljningsperiod på 6 månader efter sista injektionen hos möss. Bortezomib inducerar celldöd i tillväxtplattans stamcellslika kondrocyter, genom att aktivera flera proteiner som är kända för att medverka till att inducera celldöd. Dessa resultat är även i linje med vad vi ser i odlade biopsier från human tillväxtplatta, dvs. ökad celldöd (20%) jämfört med kontroll (obehandlad).

I ett försök att rädda längdtillväxten vid behandling med bortezomib använde vi oss av ett syntetiskt framställt protein vid namn [Gly^{14}]-Humanin (HNG) (artikel III). HNG har visat sig skydda från celldöd. Genom att kombinera bortezomib med HNG kan vi förhindra bortezomib's negativa effekter på kondrocyterna och därmed rädda längdtillväxten. Viktigt nog så interfererar inte HNG med bortezomib's anti-cancer effekt, vilket har bekräftats i flera olika experimentella modeller.

Betydelse: Det är viktigt att barn kan ges nya mediciner med förbättrad anticancereffekt utan att orsaka allvarliga biverkningar i form av extrem kortvuxenhet, något som våra resultat tyder på att HNG kan förhindra. Vi rekommenderar att längdtillväxten övervakas och följas upp noggrant hos behandlade barn i de pågående kliniska prövningarna med proteasomhämmare.

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ADDITIONAL PUBLICATIONS (Not included in the thesis)

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6. Moverare-Skrtic S, Henning P, **Eriksson E**, Sävendahl L, Lerner U and Ohlsson C. The importance of Wnt16 for bone metabolism. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AB/vG	Alcian Blue/van Gieson
AD	Alzheimer’s disease
AIF	Apoptosis inducing factor
ALL	Acute lymphoblastic leukemia
Apaf-1	Apoptotic protease activating factor 1
AR	Androgen receptor
Bax	Bcl-2 associated X protein
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BrdU	5-bromo-2´-deoxyuridine
C5.18	RCJ3.1C5.18
c-FLIP	cellular FLICE inhibitory protein
C-L	Chymotrypsin-like
Ctx	Collagen type 1 cross-linked C-telopeptide
ATP	Adenosine-5'-triphosphate
dATP	Deoxyadenosine triphosphate
DD	Death domain
DISC	Death inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DR	Death receptor
DUB	Deubiquitinating enzymes
DXA	Dual X-ray absorptiometry
E_1	ubiquitin-activating enzyme (UAE)
E_2	ubiquitin-carrier proteins
E_3	ubiquitin-protein ligases
E_4	ubiquitin-chain assembly factor
ECM	Extracellular matrix
EMEM	Eagle's minimal essential medium
ER	Endoplasmatic reticulum
ERα	estrogen receptor α
ERβ	estrogen receptor β
FBS	Fetal bovine serum
FDA	Food and Drug Administration (USA)
FGF	Fibroblast growth factor
FGFR3	Fibroblast growth factor receptor 3
FPRL	Formyl peptide receptor like
Fzd	Frizzled
GC	Glucocorticoid
GFP	Green fluorescent protein
GH	Growth hormone
GHR	Growth hormone receptor
gp130	Glycoprotein 130
GPER1	G protein-coupled estrogen receptor 1

GPOF	Growth plate orienting factor
HNG	[Gly^{14}]-Humanin
IGFBP-3	Insulin-like growth factor-binding protein-3
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-II[(M-6-P)]R	IGF-II mannose-6-phosphate receptor
Ihh	Indian hedgehog
IHC	Immunohistochemistry
IL-6	Interleukin-6
Ip.	Intreperitoneal
IR	Insulin receptor
Iv.	Intrevenous
MBL	Medulloblastoma
MEM	Minimum Essential Medium
MMP	Matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium
NAE	Nedd8 activating enzyme
NBL	Neuroblastoma
NF-κB	Nuclear factor kappa light-chain-enhancer of activated B cells
NK	Natural killer cell
PARP	Poly (ADP-ribose) polymerase
PGPH	Peptidyl-glutamyl peptide hydrolyzing-like
PI	Proteasome inhibitor
PIs	Proteasome inhibitors
PINP	Procollagen type 1 N-terminal
PPR	PTH/PTHrP receptor
Ptc-1	Patched-1
PTHrP	Parathyroid hormone-related protein
pQCT	Peripheral quantitative computed tomography
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
Runx-2	Runt-related transcription factor-2
Sc.	Subcutaneously
siRNA	Small interfering RNA, sometimes known as short interfering RNA or silencing RNA
Smac/DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis- binding protein with low pI
Smo	Smoothend
tBid	Truncated Bid
TNFα	Tumor necrosis factor-α
T-L	Trypsin-like
TR	Thyroid hormone receptor
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-metiated dUTP nick-end labeling
UAE	Ubiquitin activating enzyme

Ub	Ubiquitin
Ub^{G76V}-GFP	$Ubiquitin^{G76V}$-green fluorescent protein
UPS	Ubiquitin/proteasome system
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type MMTV integration site family

1 FOREWORD

This thesis focuses on a specific type of chemotherapy drugs called proteasome inhibitors (PIs), and their effects on linear bone growth. The general aim was to characterize whether PIs may have any eventual negative effects on chondrocytes and, in turn, induce bone growth impairment. Finally, an attempt was made to identify targets and therapies in the prevention of bone growth impairment without interfering with the desired anti-cancer effect of PIs. To address this, a wide range of experimental models were applied including chondrogenic and cancer cell lines, rat metatarsal bones, normal and genetically modified mice, human tumor xenograft mouse models, as well as human growth plate cartilage obtained from adolescent patients.

Stockholm, May 2013

2 INTRODUCTION

The development of more successful anti-cancer agents has increased the population of adult childhood cancer survivors (Smith, Seibel et al. 2010). However, the recent advances in treating childhood cancers with new and progressively more intensive treatment regimens have also led to cancer survivors facing long-term skeletal defects and impaired bone health (Robson, Anderson et al. 1998). It has become increasingly apparent that children grow poorly during and after applied cancer therapy, where osteopenia and osteoporosis often are found in adult survivors, a condition that increases the risk for fractures. Many clinical studies have outlined these problems (Kirk, Raghupathy et al. 1987; Schriock, Schell et al. 1991; Thun-Hohenstein, Frisch et al. 1992), and recently, experimental *in vivo*/*in vitro* studies have started to investigate the direct effects of chemotherapy on linear bone growth, including underlying cellular mechanisms. The notion of these facts make it even more important to evaluate the eventual long-term effects on normal bystander tissues, including linear bone growth, of new therapeutic approaches in childhood cancers, including possible ways to prevent them.

2.1 LINEAR BONE GROWTH

2.1.1 The skeleton

Skeletal growth is one of the most fundamental tasks of childhood development, including an important tool for the assessment of an individual's health status. The skeletal system is multifactorial in that it provides the firm framework and support to the body, serves to protect internal organs, is the primary storage site for minerals, and functions in hematopoiesis. The vertebrate skeleton is separated into two major subdivisions, the axial and appendicular components. The axial skeleton consists of the skull, spine, sternum, and ribs, whereas the appendicular skeleton defines the bones of the extremities. Bone formation of the skeleton is the result of two distinct processes, *intramembranous bone formation* and *endochondral ossification* (Kronenberg 2003). Intramembranous bone formation gives rise to certain flat bones of the skull, pelvis, scapula, parts of the mandible and clavicle, as well as the cortical dense bone of the long bones, and is achieved by direct transformation of condensing mesenchymal cells into bone forming cells (osteoblasts). The axial and appendicular skeleton develops by endochondral ossification through a more complex, multistep process that first requires

formation and degradation of a cartilage structure that then serves as a foundation for the developing bone. This does not only take place during skeletogenesis, but is also a part of the subsequent postnatal growth, remodeling, and fracture repair (Stevens and Williams 1999).

2.1.2 Bone development - limb formation

The formation of the cartilage model and skeletal elements begins during embryogenesis with the migration and subsequent condensation of immature mesenchymal cells (Fig. 1a). The mesenchymal cells differentiate and become chondrocytes that proliferate in a randomly oriented fashion and deposit extra cellular matrix (ECM) rich in collagen type II and the proteoglycan aggrecan that serves as a template for future bones (Fig. 1b). In humans, condensation can be found at 6.5 weeks gestation, whereas the cartilage anlagens have been detected by 8 weeks gestation (Burkus and Ogden 1984; Horton 2003). Comparable structures can be seen in mice at 10.5 days and 11.5 days of the 19 days gestation, respectively (Kaufman 1992). Members of the Sox family of transcription factors, mainly Sox9, are essential for cartilage formation and chondrocyte differentiation, and has been implicated in the production of collagen type II (Bi, Deng et al. 1999). When the cartilage template is formed, chondrocytes in their centers stop proliferating, enlarge in size (hypertrophy), and stop expressing many chondrocyte specific genes such as Sox9 and begin to express genes characteristic of hypertrophic chondrocytes, including collagen type X, VEGF, HIF-1α, and alkaline phosphatase (Fig. 1c and d) (Iyama, Ninomiya et al. 1991; Gerber, Vu et al. 1999; Schipani, Ryan et al. 2001). Hypertrophic chondrocytes in the mid-shaft of the bone direct the mineralization of the cartilage model. Coinciding with these changes, the loose mesenchyme surrounding the cartilage model differentiate into the perichondrium, where bone forming cells (osteoblasts) form the bone collar adjacent to the mid-shaft, hypertrophic region (Fig. 1c and d). Blood vessels, osteoclasts, as well as bone marrow, and osteoblast precursors then invade the cartilage model from the perichondrium and proceed to form the *primary ossification center* (Fig. 1e and f). The primary center expands towards the ends of the cartilage model as osteoclasts, remove cartilage ECM, and osteoblasts deposit bone on the cartilage remnants (Fig. 1g and h). As linear bone growth proceeds chondrocytes in the center of the epiphysis stop proliferating, become hypertrophic and attract vascular invasion along with osteoblasts forming the *secondary ossification centers* at each end of the long bones (Fig. 1i) (Kronenberg 2003). Now, in-between the primary- and secondary

ossification centers, at each end of the long bones, the cartilage that is left is called *growth plate*, which is the tissue responsible for linear bone growth. Growth plates are found in all long bones, and are established around the end of the first trimester in humans and around 15 days of gestation in mice (Horton 2003). Skeletal maturity occurs when the expanding primary center meets the secondary ossification centers, thus eliminating the growth plate. This process is called *endochondral ossification*, from where the cartilage template is replaced by bone that is initiated during fetal life and continues until growth ceases in late puberty/early adulthood.

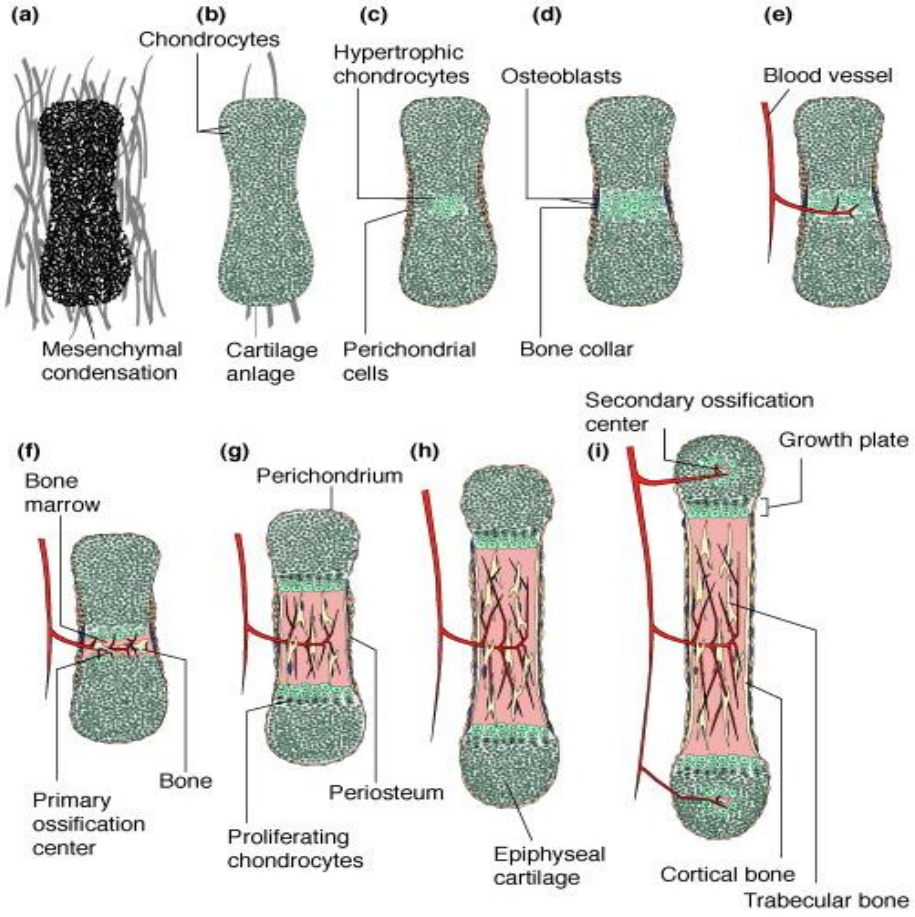

Figure 1. Schematic representation of bone formation and growth. a, **b)** The initial condensation of mesenchymal cells and their differentiation to chondrocytes forms the cartilage anlagen of future bones. **c)** Chondrocytes in the central anlage further differentiate and enlarge in size (hypertrophy). Coincidently, the loose mesenchyme surrounding the cartilage anlage differentiates into perichondrium. **d)** Osteoprogenitor cells in the perichondrium differentiate into osteoblasts and form the bone collar adjacent to the mid-point of the cartilage model, which will become surrounded by the periosteum. This process is followed by vascular and osteoblastic invasion into the central cartilage

anlage **(e)**, and cartilage is replaced by bone (formation of the primary ossification center) **(f)**. This process expands toward the ends of the bone **(g**, **h)**, where secondary ossification centers later form in the epiphyseal cartilage with formation of the mature growth plates **(i)**. Illustration reprinted with permission from Elsevier Copyright (2006) and Horton WA from the paper "FGFs in endochondral skeletal development" by Horton WA and Degnin CR. *Trends in Endocrinology and Metabolism* 2006;20(7):341-348.

2.1.3 Growth plate structure and function

The growth plate, a transient layer of hyaline cartilage that is present only during the growth period is found between the epiphysis and metaphysis at each end of the growing long bones, and is the basic structure for endochondral ossification. The growth plate consists of three distinct zones: the resting zone, the proliferative zone, and the hypertrophic zone (Figure 2). Any imbalance in the different factors regulating chondrocytes in the different zones may result in impaired bone growth. It is the combination of chondrocyte proliferation, chondrocyte hypertrophy, and ECM production that is the major contributor to linear growth: each of them accounting for approximately 10%, 60%, and 30%, respectively (Wilsman, Farnum et al. 1996).

2.1.3.1 *Resting zone*

The resting zone contains immature and undifferentiated chondrocytes, resting/stem-like cells, capable of generating new clones of proliferative zone chondrocytes (Hunziker 1994; Abad, Meyers et al. 2002). The term "stem-like" indicate that they have the capacity to feed daughter cells into the adjacent proliferative layer, but are not a true stem-cell per se with the ability to continuously divide and develop into various other kinds of cells/tissues. Resting/stem-like chondrocytes are nearly spherical in shape, exist as single cells or in pairs separated by large amounts of ECM consisting largely of collagen type II and proteoglycans, and they exhibit a low proliferative rate. In rabbits, it was previously shown that when removing the proliferative- and hypertrophic zones from the growth plate, leaving only the resting zone, this was enough to reestablish a completely new growth plate (Abad, Meyers et al. 2002). The same group also showed that these cells are essential for orientation of the underlying proliferative-zone columns by producing a growth plate-orienting factor (GPOF) (Abad, Meyers et al. 2002). These findings underscore the importance of the resting/stem-like chondrocytes for proper bone growth,

and any disturbances in their activity can therefore have severe harmful effects of chondrogenesis and bone growth.

2.1.3.2 *Proliferative zone*

Chondrocytes in the matrix-rich proliferative zone become larger in size and more discoid/flattened in shape, and line up in columns perpendicular to the long axis of the bone. These cells actively produce large amounts of ECM containing collagen type II and type IX, which help maintain the integrity, function, and shape of the growth plate (Hunziker and Schenk 1989; Nilsson and Baron 2004). The human growth plate grows slowly in comparison to rodents, and the rate of cell division in the proliferating cells of the cartilage columns is low. For example, distal femur growth rate in humans (5-8 years of age) is 35 µm per day with a cell cycle time of approximately twenty days, whereas in a young rat, the growth rate is 200 µm per day with a cell cycle time of 2 days (Kember and Sissons 1976). In the rat, there is a relatively rapid rate of cell division for cells in the central part of the proliferation zone (50-60 % of cells dividing every day), while cells at the end of the columns are dividing more slowly (5-10 % each day). Similar phenomenon is also seen in the human growth plate (Kember and Sissons 1976). Eventually, the chondrocytes in this zone lose their characteristic discoid shape and their capacity to divide; subsequently, they enter the zone of maturation (hypertrophic zone). An interesting observation is that during puberty, when the characteristic growth spurt is obvious, there is no evidence that the number of cells in the proliferative zone increases, and thus it seems likely that proliferating cells divide faster in order to produce the increased growth rate (Kember and Sissons 1976).

2.1.3.3 *Hypertrophic zone*

Growth in this zone is no longer the result of proliferation/cell division, instead the chondrocytes enlarge in size (hypertrophy), take on a round appearance, secrete large amounts of ECM rich in collagen type X, and express vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and fibroblast growth factors (FGFs), which all are important for subsequent bone remodeling (Baron, Klein et al. 1994; Gerber, Vu et al. 1999; Haeusler, Walter et al. 2005). These cells continue to enlarge to the point where they have increased their intracellular volume approximately 10 times (Hunziker, Schenk et al. 1987). Hypertrophy is characterized by an increase in intracellular calcium concentration, essential for the production of matrix vesicles (small membrane-bound

particles that are released from hypertrophic chondrocytes), which contains large amount of annexins that mediate calcium uptake (Anderson 2003). The vesicles secrete calcium phosphatase, hydroxyapetite, and MMPs, resulting in mineralization of the surrounding matrix. The mineralization process together with the low oxygen tension and expression of VEGF attracts blood vessels from the underlying primary ossification center/primary spongiosum, which together are the key mechanisms for attracting bone cells into the hypertrophic cartilage (Gerber, Vu et al. 1999). When all glycogen stores are depleted, the mineralized chondrocytes lastly undergo "death" at the chondro-osseous junction, leaving a platform for new bone formation. There still seems to be a debate as to how chondrocytes are finally removed, and different theories have been proposed, such as programmed cell death (apoptosis) (Zenmyo, Komiya et al. 1996), or a type of aberrant cell death (e.g., necrosis, chondroptosis, autophagy, transdifferentiation, "paralysis"/"limbo" (unable to live or die)) (Roach and Erenpreisa 1996; Erenpreisa and Roach 1998; Meijer and Codogno 2004; Roach, Aigner et al. 2004). What is clear, however, is that chondrocyte removal at the chondro-osseous junction is a part of the normal process of bone elongation, and any disturbances might lead to defective linear bone growth.

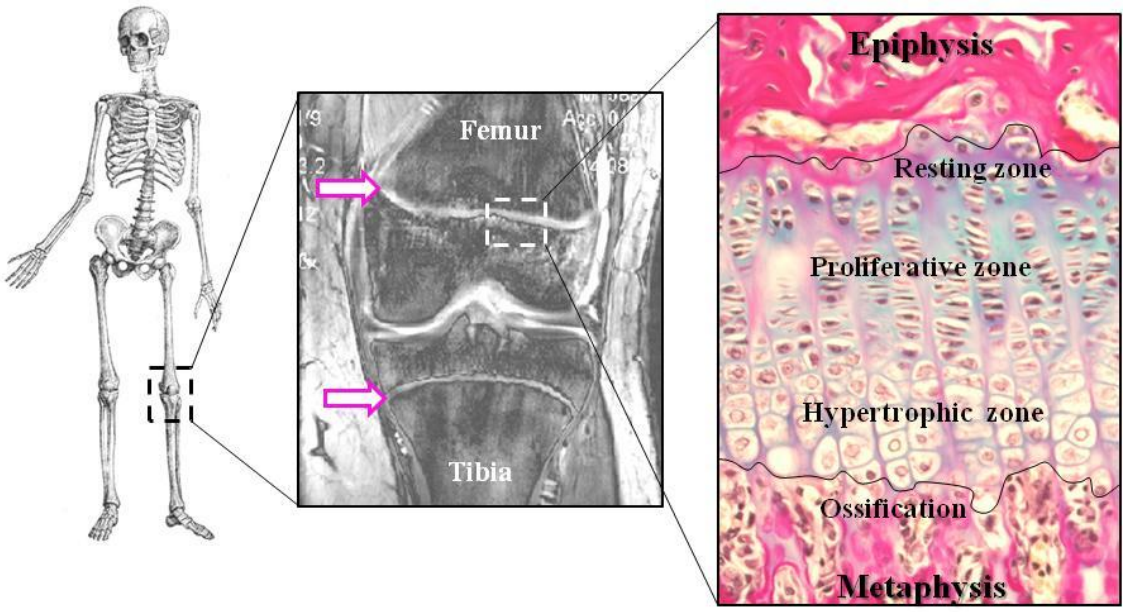

Figure 2. Structural organization of the growth plate cartilage. The growth plate is located in each end of the long bones. The hatched square on the left skeletal image is further clarified by the middle magnetic resonance (MR) picture that indicates the distal femur, knee joint, proximal tibia, and the growth plates (white horizontal line within the

bones indicated by arrows). The right microscopic image shows the schematic cellular orientation of the growth plate located between the epiphysis and metaphysis. The growth plate is divided into three distinct zones that represent histological and functional stages of chondrocyte differentiation: resting (stem-like), proliferative, and hypertrophic zones.

2.1.4 Mediators and regulation of bone growth

Formation of the skeleton and linear bone growth are processes that are critically dependent on the proper homeostasis and balance between different genetic and hormonal factors, growth factors, environment, and nutrition, which may influence the final height of an individual (some of which are further explained below). Intrauterine growth in humans is where the most rapid growth of a lifetime takes place, with a complete fetus of approximately 50 cm in length produced from a single cell in only 9 months. Before birth, the key regulators of growth are believed to be nutrition, IGF-I and -II, and insulin, functioning largely independent of GH (Gluckman 1997). This is based on findings from both knockout experiments in mice, and in congenital GH deficiency in humans were birth length was only mildly diminished, whereas in congenital IGF deficiency, birth size was severely affected (Woods, Camacho-Hubner et al. 1996). Postnatal linear growth in humans is divided into three major phases: Infancy, Childhood, and Puberty (according to the ICP-model), which are strongly reflected by the different hormonal phases of the growth process (Karlberg 1987). The first phase, infancy, is characterized by a high growth rate from birth, with a rapid deceleration up to about three years of age. Childhood, the second phase, sees slow growth during the early age of childhood up to puberty. From birth, GH is an important modulator of longitudinal bone growth (given normal thyroid hormone secretion) together with the IGFs. Consequently, defects in any of these factors results in severe dwarfism (Rosenfeld, Rosenbloom et al. 1994; Gothe, Wang et al. 1999; Lopez-Bermejo, Buckway et al. 2000). The third period, puberty, is associated with an increased growth rate known as the pubertal growth spurt. The spurt itself accounts for approximately 20% of final height, then growth velocity rapidly decreases due to growth plate maturation in the long bones and spine, and thus, subsequently final height for an individual will be achieved. In other mammals, a similar dramatic decline in growth rate occurs, but without a superimposed pubertal growth spurt. Epiphyseal fusion is an active process with its own hormonal control, cellular mechanisms, and structural features (Perry, Farquharson et al. 2008). In both sexes, estrogen is the critical hormone in controlling growth plate acceleration and fusion

(Grumbach 2004). The general idea that bone growth stops has been believed to be because of growth plate fusion (Wilkins 1965). However, this concept has been challenged by the observations that cessation of growth occurs first, followed later by fusion of the growth plate (Roach, Mehta et al. 2003).

2.1.4.1 *Local (autocrine/paracrine) regulation of growth plate cartilage*

SRY (Sex determining region) Y-box 9 (Sox9): Sox9 is a critical factor for all phases of the chondrocyte lineage, from early condensation to the conversion of proliferating to hypertrophic chondrocytes, and also determines the fate of mesenchymal stem-cell (MSC) condensations into collagen type II-expressing chondrocytes (Lefebvre and de Crombrugghe 1998). Sox9 mutation causes the rare condition campomelic dysplasia, characterized by severe dwarfism and skeletal anomalies (Foster, Dominguez-Steglich et al. 1994).

Runt-related Transcription Factor 2 (RUNX2): RUNX2, previously named Cbfa1, is important in the regulation of growth plate cartilage by promoting differentiation of chondrocytes into hypertrophy as well as for its role in osteogenesis (Inada, Yasui et al. 1999).

Indian Hedgehog (Ihh)/ Parathyroid Hormone-related Peptide (PTHrP) signaling: Ihh, produced by prehypertrophic and early hypertrophic chondrocytes, is considered the master regulator of chondrocyte proliferation and differentiation, as well as osteoblast differentiation and ossification of the perichondrium (Vortkamp, Lee et al. 1996). Ihh binds to its receptor, patched-1 (Ptc-1), which leads to activation of the membrane protein, Smoothend (Smo), required for the actions exerted by Ihh on cells. PTHrP, expressed by periarticular perichondrium with its receptor found highly expressed in late-proliferating and early-hypertrophic chondrocytes, plays a crucial role in keeping proliferative chondrocytes in the proliferative stage (Vortkamp, Lee et al. 1996). The orchestrated feedback loop involving Ihh and PTHrP plays key roles in regulating the entry and exit of cells into and out of the columnar zone. Ihh can stimulate the entry of resting/stem-like chondrocytes into the proliferative zone independent of PTHrP (Kobayashi, Soegiarto et al. 2005), or it can stimulate the expression of PTHrP in periarticular cells, thereby regulating the onset of hypertrophic

differentiation. PTHrP in turn signals back to chondrocytes in the proliferative zone by binding to its receptor, inhibiting differentiation into Ihh-expressing prehypertrophic cells, thereby shutting off the production of Ihh by maintaining these cells in the proliferative phase (St-Jacques, Hammerschmidt et al. 1999). The importance of this Ihh/PTHrP feedback loop for normal endochondral bone formation is underscored by the illustrations that disruption of any of the components results in abnormal limb development (St-Jacques, Hammerschmidt et al. 1999).

Bone morphogenetic proteins (BMPs): The family of BMPs is comprised of at least 15 members. BMP signaling is essential for endochondral ossification by promoting the commitment of mesenchymal cells to the chondrogenic lineage, as well as in the regulation of proliferation and hypertrophy of growth plate chondrocytes (Pogue and Lyons 2006).

Fibroblast growth factors (FGFs): The family of FGFs constitutes at least 22 members that interact with at least four receptors (FGFR), and are major regulators of embryonic bone development (Ornitz and Marie 2002). FGFs are mainly produced by cells in the perichondrium, and act in a paracrine manner on FGFRs expressed in proliferative and hypertrophic chondrocytes in the growth plate. Opposite to Ihh/PTHrP and BMP signaling, FGFs provide essential inhibitory signals in the control of chondrocyte proliferation.

Vascular endothelial growth factor (VEGF): VEGF appears to be a key factor for vascularization of the growth plate, and a critical step for successful bone formation. During chondrocyte hypertrophy, ECM surrounding the hypertrophic cells becomes calcified, which triggers the invasion of blood vessels from the underlying metaphyseal bone. This is preceded by the expression of VEGF in hypertrophic chondrocytes (Gerber, Vu et al. 1999). Thus, VEGF is an essential coordinator of chondrocyte death, extracellular matrix remodeling, angiogenesis, and bone formation in the growth plate.

Wingless-type MMTV integration site family (Wnts): At least 19 Wnts comprise a family of secreted cysteine-rich glycoproteins that interact with several receptors called Frizzled (Fzd). Wnts are expressed in the surrounding tissue of the early mesenchymal condensations that will become the cartilage template of the new bone (Day, Guo et al.

2005). Low Wnt signaling allows for chondrogenesis and subsequent endochondral ossification, whereas high Wnt signaling enhances ossification of mesenchymal cells.

2.1.4.2 *Hormonal regulators of growth plate cartilage*

Growth hormone (GH): GH is believed to be the key endocrine regulator of linear bone growth, together with a coordinated network of IGFs, IGF-I and IGF-II, and their receptors. Enhanced GH secretion caused by a pituitary adenoma in childhood cause gigantism (Sotos 1996), while any defects leads to severe dwarfism (Wit, Drayer et al. 1989; Rosenfeld, Rosenbloom et al. 1994; Lopez-Bermejo, Buckway et al. 2000). Systemic actions of GH are thought to be mediated by IGF-I, formerly known as "sulfation factor" or "somatomedin" (Salmon and Daughaday 1957), which is produced systemically by the liver or locally by chondrocytes (Le Roith, Bondy et al. 2001). Interestingly, double knockout of GHR and IGF-I results in mice that are smaller than single gene knockouts, indicating that GH and IGF-I co-interact positively by stimulating bone growth (Lupu, Terwilliger et al. 2001). However, a direct effect of GH in chondrocytes has been suggested, as the growth hormone receptor is detected in all zones of the growth plate (Parker, Hegde et al. 2007). This concept is supported by the finding that local GH injection into the tibia growth plate accelerated linear growth compared to the unilateral bone (Isaksson, Jansson et al. 1982). Furthermore, GH may act directly on resting/stem-like chondrocytes to stimulate proliferation, as well as indirectly, through IGF-I to promote chondrocyte hypertrophy (Wang, Zhou et al. 2004). However, these observations do not discard the possibility that some of the effects are mediated by local production of IGF-II.

Insulin-like Growth Factors (IGFs): This family includes three ligands (IGF-I, IGF-II, and insulin), their cell surface receptors (IGF-IR, IGF-II/[M-6-P]R, and IR), and six high-affinity binding proteins (IGFBP-1 to -6) which prolong the half-life of the IGFs and modulate their bioavailability and activity (Le Roith, Bondy et al. 2001). IGF-I plays an important role during both embryonic and postnatal growth, indicated by severe growth failure in mice carrying null mutations in the IGF-I gene (Liu, Grinberg et al. 1998). IGF-I is produced by chondrocytes in the proliferative zone, and increased expressions are found upon stimulation with GH, suggesting that IGF-I has a specific role in the

differentiation of chondrocytes through autocrine/paracrine mechanisms (Nilsson, Isgaard et al. 1986). IGF-I and -II receptors are expressed throughout the growth plate, but was found to decrease with age coincident with a period of rapid decline in growth velocity (Parker, Hegde et al. 2007). IGF-II was found at high levels in the growth plate, especially in resting and proliferative chondrocytes, suggesting a role in proliferation (Parker, Hegde et al. 2007). IGF-II is a positive regulator of prenatal growth; however, its role during postnatal growth remains unclear.

Thyroid hormones: Thyroid hormones, triiodothyronine (T_3, the active form of thyroid hormone) and thyroxine (T_4, the pro-hormone) are crucial for normal bone maturation. They act through thyroid hormone receptors (TRs) expressed in the resting and proliferative zones to regulate chondrocyte proliferation, differentiation, and vascular invasion at the growth plate (Robson, Siebler et al. 2000). Part of these effects appear to be mediated by modulating local GH and/or IGF-I actions (Williams, Robson et al. 1998).

Glucocorticoids (GCs): Prolonged GC therapy in various clinical conditions is associated with decreased bone volume as well as growth retardation (Bello and Garrett 1999). In contrast, familial GC deficiency is associated with tall stature (Elias, Huebner et al. 2000), suggesting that GC is a potent negative regulator of chondrogenesis. Evidence for a direct effect of GC in the growth plate came from a study in which local dexamethasone infusion was found to reduce tibia growth compared with the contralateral vehicle-injected leg (Baron, Klein et al. 1994). GC-receptors are expressed in the proliferating and hypertrophic zones, and GC-induced growth inhibition is most likely explained by reduced chondrocyte proliferation and matrix synthesis in combination with increased apoptosis of hypertrophic chondrocytes (Chrysis, Ritzen et al. 2003).

Estrogens: Estrogen is the main determinant for the puberty-associated phenomena related to longitudinal growth and bone quality, including growth plate fusion in boys and girls (Grumbach 2000), probably by accelerating chondrocyte proliferation, and thus advancing chondrocyte senescence (exhaustion of the proliferative capacity). Much of the growth acceleration due to estrogen is mediated by estrogen-induced stimulation of the GH/IGF-I axis. The local action of estrogens in the growth plate is mainly

supported by the expression of the two nuclear receptors, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), and also by the more recently identified membrane-bound G protein-coupled estrogen receptor 1 (GPER1, formerly known as GPR30) (Nilsson, Chrysis et al. 2003; Chagin and Savendahl 2007).

***Androgens**:* Androgens also contribute to the pubertal growth spurt, although to a lesser extent than estrogens, by mechanisms not fully understood. Most of the androgen effects on linear bone growth are probably due to aromatization into estrogen in peripheral tissues, possibly in the growth plate as well. This hypothesis is supported by findings of chondrocyte expression of aromatase P450 (CYP19), which converts testosterone into estrogen (Oz, Millsaps et al. 2001). However, androgens may also have a direct effect as androgen receptor (AR) expression has been detected in rat and human growth plate cartilage (van der Eerden, van Til et al. 2002; Nilsson, Chrysis et al. 2003).

Leptin: Leptin is a hormone secreted primarily by white adipose tissue, regulates food intake and body weight. Leptin deficiency in mice impairs linear bone growth, while treatment of these mice with leptin injections increased bone growth (Steppan, Crawford et al. 2000). In contrast, in the few humans described with leptin deficiency or leptin-receptor deficiency, skeletal growth appeared normal (Ozata, Ozdemir et al. 1999). Leptin receptors are expressed in chondrocytes, and leptin-treatment was found to stimulate chondrocyte proliferation and differentiation as well as IGF-I-receptor expression (Maor, Rochwerger et al. 2002).

2.1.4.3 Environmental factors

Besides genetic control, many lifestyle/environmental factors including exercise, nutrition and medical treatments also play important roles in regulation of bone growth and remodeling. Adequate physical exercise and loading are important for normal bone growth, bone mass accumulation, and bone strength (Khan, McKay et al. 2000).

2.2 CELL DEATH

Cell death can occur by either of two distinct mechanisms: apoptosis or necrosis. In addition, autophagy is considered yet another mode of cell death, as is cytotoxicity by certain chemical compounds that can combine the aspects of deaths mentioned above.

2.2.1 Apoptosis

Apoptosis (type I cell death), "normal" or "programmed" cell death, was first described in the literature in 1972 (Kerr, Wyllie et al. 1972), and the term *apoptosi*s, from the Greek word for the "falling off" of leaves from trees is used to describe the process in which a cell actively participates in its own destructive process. Cell death is a normal physiological and highly controlled process that occurs during embryonic development, in the maintenance of tissue homeostasis, and also includes the death of differentiated hypertrophic chondrocytes to facilitate linear bone growth. The apoptotic program is characterized by certain morphological features such as cell shrinkage, loss of membrane symmetry (blebbing), condensation of the cytoplasm and chromatin within the nucleus, and DNA cleavage (biochemical hallmark of apoptosis), an irreversible event that commits the cell to die. Thus, organelle structures are usually preserved intact. In the final stages, the dying cells become fragmented into "apoptotic bodies", which are rapidly eliminated by phagocytotic cells or macrophages without inducing any inflammatory response. On the other hand, inappropriate induction of apoptosis, either too much or too little, has pathological implications. Many cancer therapeutics (including PIs) exert their effects through initiation of apoptosis, and even cancer progression itself seems sometimes to depend upon a selective, critical failure of apoptosis. In mammalian cells, two major apoptotic signaling pathways exist, the *extrinsic pathway* that is dependent on death receptors (DRs) on the cell surface, and the *intrinsic pathway*, which is dependent on the mitochondria (Figure 3).

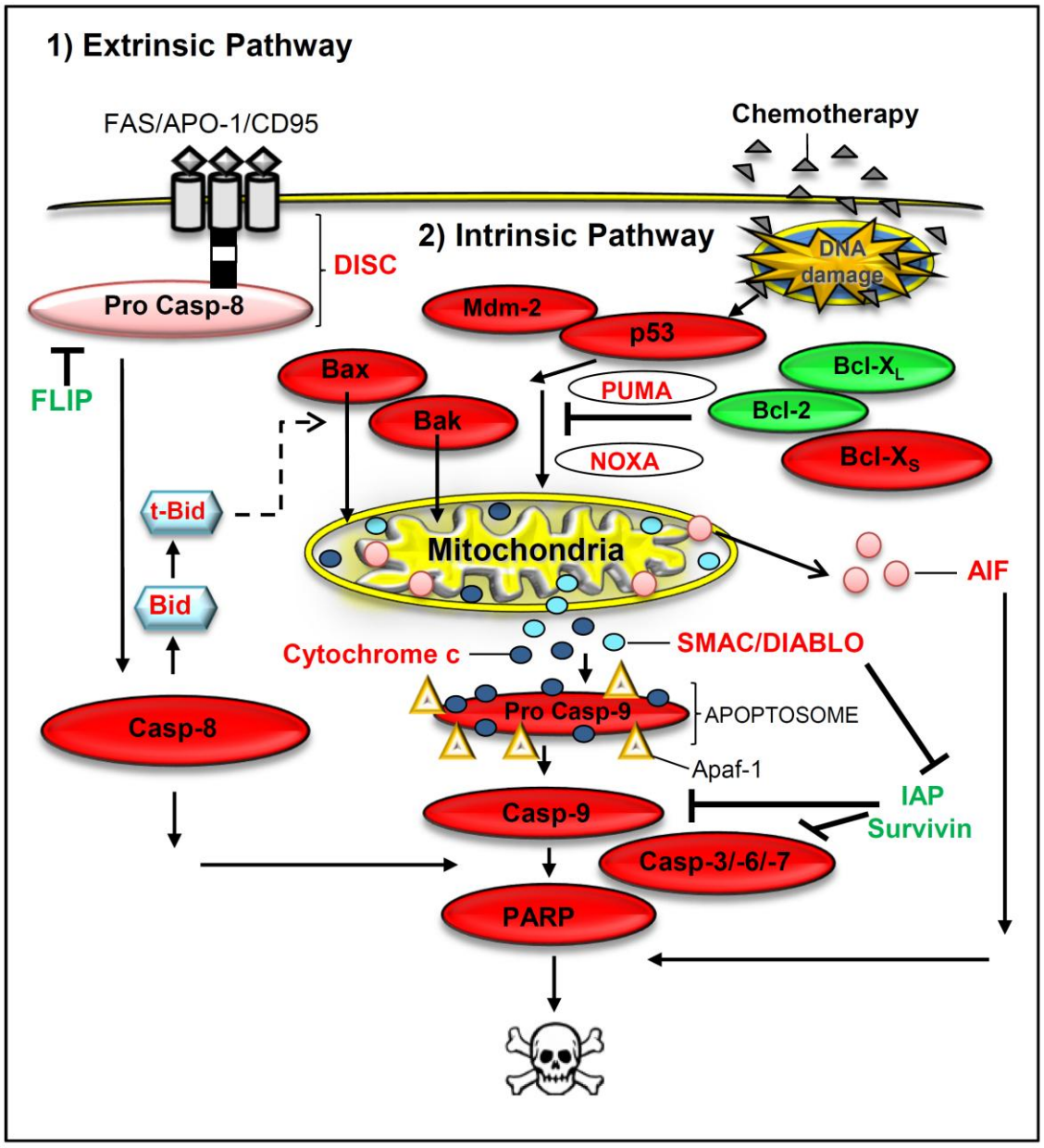

Figure 3. Extrinsic/Intrinsic apoptotic pathways. (1) Extrinsic/death receptor (DR) pathway: Upon death, stimuli/ligand binding by Fas to its specific extracellular DR (Fas/APO-1/CD95), formation of the death inducing signaling complex (DISC) takes place, which results in recruitment and activation of caspase-8. Caspase-8 can cleave effector caspases such as caspase-3, resulting in a caspase cleavage cascade to induce apoptosis, and thus caspase-8 is an important pro-apoptotic protein for the extrinsic apoptotic pathway. **(2)** In comparison, the intrinsic/mitochondrial apoptotic pathway initiates from within the cell. A number of different stimuli such as DNA damage can induce transcription of p53, which can modulate transcription of a number of members of the Bcl-2 family BH3-only proteins such as Bak, Bax and Bid, for example. These proteins translocate to the mitochondria where they promote the release of cytochrome c and/or inhibit anti-apoptotic Bcl-2/Bcl-X_L. Cytochrome c then binds to Apaf-1, which further complexes with pro caspase-9 to form the apoptosome, promoting further cleavage of downstream effector caspases. FLIP, Bcl-2, Bcl-X_L, survivin, and IAP are the key anti-apoptotic proteins within the extrinsic and intrinsic apoptotic pathways. Crosstalk between pathways occurs at the caspase level. Caspase-8 can cleave cytosolic

Bid to truncated Bid (tBid), whereby tBid promotes cell death via activation of Bax and/or Bak. Cleavage of effector caspases (by either pathway) results in apoptosis induction and its associated phenotype (DNA fragmentation, membrane blebbing, cell shrinkage, and the formation of apoptotic bodies). Mitochondrial dysfunction can also result in caspase-independent apoptosis, regulated via apoptosis-inducing factor (AIF). Proteins indicated in red are pro-apoptotic and those in green are anti-apoptotic.

2.2.1.1 *Extrinsic apoptotic pathway*

Activation of apoptosis is initiated by the binding of specific protein ligands to cell surface transmembrane DRs that will transduce pro-apoptotic signals from the extracellular space into the intracellular milieu (see Figure 3). The DRs consist of 6 members: TNF-R1, Fas, DR3, DR4, DR5, and DR6, which all have an extracellular cysteine-rich domain, which is required for ligand binding, and an intracellular death domain (DD), which is required for apoptotic signal transduction (Rossi and Gaidano 2003). When the specific ligand binds its respective DR, a trimerization of the receptor occurs, which is essential for the downstream apoptotic signaling events, with subsequent formation of the Death Inducing Signaling Complex (DISC) and recruitment of procaspase-8. Next, procaspase-8 is proteolytically activated to caspase-8 with subsequent activation of effector caspases such as caspase-3 and/or -7, leading to apoptosis by digestion of proteins (Thorburn 2004). The apoptotic signal can be amplified through the mitochondria (Luo, Budihardjo et al. 1998) or suppressed by the endogenous inhibitor, c-FLIP, that competes with procaspase-8 for binding to the DISC (Irmler, Thome et al. 1997). The extrinsic and intrinsic apoptotic pathways are thereby intimately connected.

2.2.1.2 *Intrinsic apoptotic pathway*

This pathway is induced by direct damage to the cell from a wide range of factors, such as cellular stress, irradiation, lack of growth factors and chemotherapeutic agents that may cause mitochondrial damage (see Figure 3). Mitochondria are triggered to release proteins into the cytoplasm, such as cytochrome c, AIF, and/or second mitochondria-derived activator of caspases (Smac)/DIABLO. Released cytochrome c interacts with the caspase adaptor molecule, Apaf-1, procaspase-9, and dATP to form the apoptosome complex (Li, Nijhawan et al. 1997). This complex dimerizes and activates caspase-9, which then promotes effector caspases, caspase-3, -6, and -7, resulting in cell death by activation of the executioner protein in the apoptotic cascade, Poly (ADP-ribose) polymerase (PARP). Cytochrome c release and subsequent activation of caspase-9 and the downstream events

are controlled by the Bcl-2 family of proteins, which are important in preventing and promoting apoptosis (Danial and Korsmeyer 2004). The pro-apoptotic protein, Bax, belonging to the Bcl-2 family, has been shown to play an essential role during intrinsic/mitochondria- mediated apoptosis where, upon activation, it translocates to mitochondria and causes apoptogenic protein release (Elmore 2007). Previous observation suggests that the ratio of Bcl-2 to Bax determines survival or death following an apoptotic stimulus (Oltvai, Milliman et al. 1993).

Current data provide sufficient evidence to support a role for apoptosis in the growth plate as a developmentally normal process during bone elongation (Burdan, Szumilo et al. 2009), including the regulation of different anti-apoptotic (e.g., Bcl-2 and Bcl-X_L) and pro-apoptotic (e.g., Bax, Bad, Bcl-X_S, and caspases) proteins (Amling, Neff et al. 1997; Chrysis, Nilsson et al. 2002). Interestingly, Bcl-2 was shown to be widely expressed in proliferative and prehypertrophic chondrocytes, but markedly decreased in late hypertrophic chondrocytes (Amling, Neff et al. 1997). The opposite pattern was observed for Bax protein expression, with undetectable levels in proliferative cells, and a progressive increase towards hypertrophic chondrocytes. This imbalance of anti- and pro-apoptotic proteins, including TUNEL-positive cells, indicates that apoptosis is a process of normal chondrogenesis. Chrysis and co-workers further concluded that apoptosis is developmentally regulated during normal growth in rats by the detection of Bcl-2, Bcl-X, p53, Bax, and caspase-3 and -6 (Chrysis, Nilsson et al. 2002). They also reported that in older rats that show decreased growth rate and growth plate height, apoptosis is increased in terminal hypertrophic chondrocytes. The importance of Bcl-2 in the growth plate was further demonstrated in mice lacking Bcl-2, which showed accelerated apoptosis and bone growth impairment (Amling, Neff et al. 1997). Moreover, in both PTHrP knockout mice (Amizuka, Henderson et al. 1996) and in mice having an active mutation in FGFR3 (Legeai-Mallet, Benoist-Lasselin et al. 1998), increased apoptosis of chondrocytes was demonstrated. In summary, these studies and others point to the importance of apoptosis for normal development and regulation of linear bone growth.

2.2.2 Necrosis

Necrosis, "accidental" cell death, is a pathological process in which the cell has no active role (Kerr, Wyllie et al. 1972). The cellular characteristics are swelling of cells, loss of

membrane integrity, and total cell lysis with subsequent provoking of an inflammatory response.

2.2.3 Autophagy

Autophagy (type II cell death), a type of self-degradation has been reported as the final fate for hypertrophic chondrocytes (Shapiro, Adams et al. 2005). The discovery of autophagy was first described in 1966 (De Duve and Wattiaux 1966), and the term autophagy in 1973 (Schweichel and Merker 1973): it is characterized by double-membrane autophagic vacuoles (autophagosomes), which are organelles that are used for "eating" itself by use of its own proteins and lipids as nutrients. In 1996, Roach and Erenpreisa described hypertrophic chondrocytes that exhibited unusual ultramicroscopic structures with condensed chromatin, although the morphology was different from both apoptosis and necrosis (Erenpreisa and Roach 1998). Later, they observed an increase in the amount of both the endoplasmic reticulum and Golgi apparatus, and termed the type of death observed as *chondroptosis* (Roach, Aigner et al. 2004). This term was later revised by reassessment of the terminal hypertrophic chondrocytes that showed a death that resembled the characteristics of autophagy (Shapiro, Adams et al. 2005). Recently, genes known to trigger autophagy were found to be expressed in the growth plate (Watanabe, Bohensky et al. 2008) as well as the cartilage microenvironment, where low protein, glucose, and oxygen levels further support a trigger of the autophagic response.

2.3 CHEMOTHERAPY

The development of increasingly intense and successful chemotherapy regimens has appreciably produced a growing population of childhood cancer survivors (Smith, Seibel et al. 2010). Chemotherapy drugs can be divided into several groups based on how they work, their chemical structure, and their relationship to other drugs. The main chemotherapeutic drug classes include *alkalyting agents* (DNA-damaging), *antimetabolites* (interfering with DNA and RNA synthesis), *anti-tumor antibiotics* (anthracyclines, interfering with enzymes involved in DNA replication), *topoisomerases* (inhibiting of topoisomerase enzymes), *mitotic-inhibitors* (interfering with cell replication), *corticosteroids/GCs* (slowing growth, and killing of cancer cells), *miscellaneous chemotherapy drugs/targeted therapies* (e.g., proteasome inhibitors). The main aim of chemotherapy is to target cancer cells that by definition are quickly

growing cells with high proliferative rates. However, in children, normal cells in some tissues such as cartilage also grow, proliferate, and differentiate relatively fast during certain periods in life, and may thereby be targeted by these drugs as well. Consequently, skeletal defects and impaired bone health during childhood cancer treatment is a common problem, and its etiology is often multifactorial, resulting from the disease itself, the intensity and duration of chemotherapy, other types of therapies applied to enhance the cure, and malnutrition. It has become increasingly apparent that children grow poorly during and after the cancer therapy, and osteopenia and osteoporosis are also often found in adult survivors, leading to a higher risk for fractures (Schriock, Schell et al. 1991; Athanassiadou, Tragiannidis et al. 2005). Many clinical studies have outlined these problems, and recently, *in vivo* and *in vitro* experimental studies have started to delineate the effects by which these agents target chondrocytes, and in turn affect linear bone growth. Hence, the question arises whether the reported growth impairment of chemotherapy is due to a direct effect on cartilage/bone tissue, or via a systemic imbalance of essential hormones for bone growth (e.g., GH/IGF-I). We, and others have demonstrated a direct effect on growth plate chondrocytes and linear bone growth, without any systemic alterations by drugs such as 5-fluorouracil (commonly used for treatment of solid tumors), topoisomerase inhibitor, etoposide, and the alkylating agent cyclophosphamide (Wu and De Luca 2006; Xian, Cool et al. 2006; Xian, Cool et al. 2007; Zaman, Menendez-Benito et al. 2007; Eriksson, Zaman et al. 2012). So far, any unwanted effect on linear bone growth has to our knowledge not yet been reported in children treated with PIs. Nevertheless, in preclinical models, we, and others, have reported that PIs have severe negative effects on chondrogenesis and linear bone growth (Wu and De Luca 2006; Xian, Cool et al. 2006; Xian, Cool et al. 2007; Zaman, Menendez-Benito et al. 2007; Eriksson, Zaman et al. 2012) (discussed further in 2.4.5). In summary, it is of great importance to increase our understanding of the underlying cellular mechanisms involved, and finally to determine how the growth potential of individuals might be maintained during treatment for childhood cancers.

2.3.1 Malnutrition

Adequate nutritional intake is also essential for optimal skeletal development and growth in children, as the most common cause of growth retardation, worldwide, is malnutrition. In most cases, when food consumption is corrected, spontaneous catch-up growth occurs: however, reaching a final height depends upon several factors: the amount

of growth that is completed before starvation, the growth that is left, and the duration of the starvation period (Acheson and Macintyre 1958). Consequently, catch-up growth is not always complete, leading to growth deficits. Vitamin D and Calcium, both necessary for normal bone growth, are taken up in sufficient amounts by securing an adequate diet. The importance of vitamin D for skeletal growth has been demonstrated by vitamin D deficiency, leading to delay of linear bone growth, bone abnormalities, and increased fracture risk in adulthood (Holick 2007). Calcium is a fundamental nutrient for bone mineralization, formation, and maintenance of both the structure and stiffness of the skeleton (Bueno and Czepielewski 2008).

2.3.2 Catch-up growth

Catch-up growth may occur following remission of diverse growth-retarding conditions (e.g., Cushing syndrome, hypothyroidism, celiac disease, anorexia nervosa/malnutrition, and GH deficiency). The phenomenon of catch-up growth was first described by Prader et al. (Prader, Tanner et al. 1963), in which they noted an accelerated height velocity that exceeds the normal growth rate for the particular age. As a result, final height is improved, although this recovery of height may or may not be complete. Two principal hypotheses have been proposed to explain the mechanism of catch-up growth. Tanner postulates that catch-up growth is regulated by a "time tally" mechanism that exists in the brain that compares the actual body size with an age-appropriate set point and adjusts the growth rate accordingly (Tanner 1963). This neuroendocrine hypothesis has been challenged by recent studies, suggesting that catch-up growth is due to intrinsic factors in the growth plate (Baron, Klein et al. 1994; Gafni and Baron 2000). According to the intrinsic model, the mechanism explaining catch-up growth may be that a maximum number of cell divisions exist for each chondrocyte within the growth plate. Growth-inhibiting conditions decrease chondrocyte proliferation, and when remission takes place, these cells have a greater proliferating potential, explaining the increased growth rate. However, these studies have all been performed in animals, in which the pattern of catch-up growth is quite different from that of humans. For example, in a child who catches up, height velocity can be four times that of normal growth, whereas in rodents and rabbits the growth velocity increment is minimal (van der Eerden, Karperien et al. 2003). Additional studies are needed to address the process of catch-up growth in humans.

2.4 THE UBIQUITIN/PROTEASOME SYSTEM (UPS)

2.4.1 Proteasome – structure and function

In cells, two major destruction pathways exist, involving either the lysosome or the ubiquitin/proteasome system (UPS). This thesis focuses on the latter system.

Proteasomes are large (2000 kDa), multimeric protease complexes residing inside all eukaryotes, archaea, and in some bacteria. In eukaryotes, they are located in the nucleus and the cytoplasm (Peters, Franke et al. 1994). The main function of the proteasome is to maintain cellular homeostasis by degrading unwanted or misfolded proteins, thereby making it essential for many cellular processes including cell proliferation, regulation of gene expression, cell death, signal transduction, and immune surveillance.

In structure, the eukaryotic 26S proteasome is a cylindrical complex composed of the two outer 19S regulatory cap subunits (700 kDa) situated at each end of the 20S proteolytic core (see Figure 4). The two outer 19S regulatory subunits consist of six ATPase active sites, and approximately eight non-ATPase subunit ubiquitin binding sites: it is these structures that recognize the polyubiquitinated proteins, unfold them and transfers them into the catalytic 20S core were they become degraded into peptide fragments. The 20S proteolytic core of the proteasome is well conserved between species. It resembles a hollow, barrel shaped structure, consisting of four stacked heptameric rings composed of a total 28 subunits. The outer two rings in the stack consist of seven α subunits each, whose function is to maintain a "gate" through which proteins can enter the barrel, as well as to block unregulated access of substrates into the interior core. The inner two rings each consist of seven β subunits and contain the protease active sites that perform the proteolysis reactions. Three distinct proteolytic active sites within the β subunits have been identified: chymotrypsin-like (C-L, β5, cleavage after hydrophobic residues), trypsin-like (T-L, β2, cleavage after basic residues) and caspase- or peptidyl-glutamyl peptide hydrolyzing-like (PGPH, β1, cleavage after acidic residues) (Cardozo 1993).

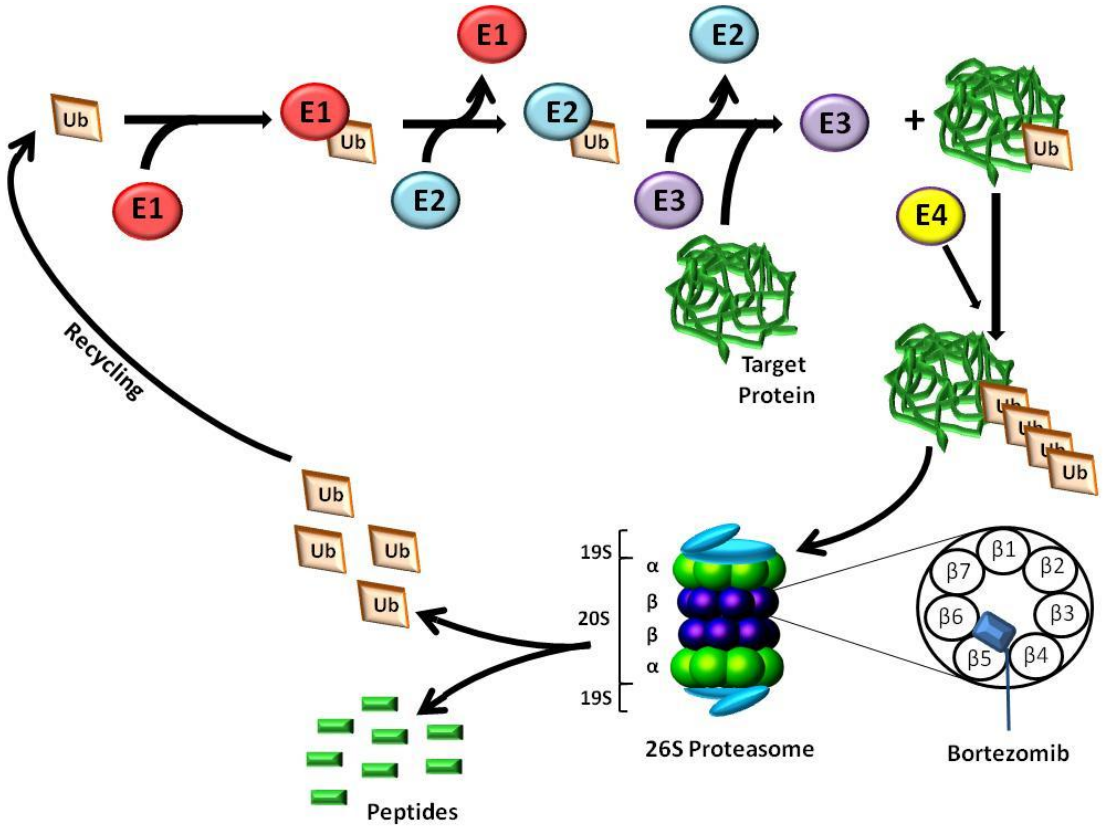

Figure 4. The ubiquitin/proteasome system (UPS). The ubiquitination of target proteins is mediated by ubiquitin-activating enzyme (UAE, E1), ubiquitin-carrier proteins (E2), ubiquitin-protein ligases (E3), and ubiquitin-chain assembly factor (E4). Polyubiquitinated substrate proteins are recognized and unfolded by the 19S cap and then degraded into peptide fragments within the 20S catalytic core of the 26S proteasome. Bortezomib reversibly inhibits the C-L, β5-site of the proteasome. This figure is adapted with minor modifications by me with permission from: D. Chen, M. Frezza, S. Schmitt, J. Kanwar and Q. P. Dou, Bortezomib as the First Proteasome Inhibitor Anticancer Drug: Current Status and Future Perspectives. *Current Cancer Drug Targets*, 2011;11(3): 239-253.

2.4.2 Degradation by the proteasome - Ubiquitination and targeting

Aaron Ciechanover, Awram Hershko, and Irwin Rose's work from the late 1970s and early 1980s received the Nobel Prize in Chemistry in 2004 for the identification of proteolytic degradation inside cells (Hershko, Ciechanover et al. 1981) and the role of ubiquitin in proteolytic pathways (Hershko, Ciechanover et al. 1980).

Proteins destined for proteasomal degradation are first recognized and tagged with the 76 amino acid polypeptide ubiquitin (highly conserved from yeast to mammals), which binds to lysine residues on the targeted protein (see Figure 4). The tagging reaction is catalyzed by sequential action of key *ubiquitin ligases*, consisting of four different sets

of enzymes: E1 ubiquitin-activating enzyme (UAE), E2 (ubiquitin-carrier proteins), E3 (ubiquitin-protein ligases), and E4 (ubiquitin-chain assembly factor). In the first step of the ubiquitin conjugation cascade, ubiquitin is activated by E1 in an ATP-dependent manner. Following activation, activated ubiquitin is transferred to E2, which either directly shuttles ubiquitin to a protein substrate or does it in cooperation with E3. E4 enzymes function as mediators of ubiquitin chain elongation. Once the protein substrate is mono-ubiquitinated, a polyubiquitin chain is formed through the same cascade as described above. The cooperative action of the ubiquitin ligases results in specific tagging and subsequent degradation of intracellular proteins. The degradation process within the 20S proteasome yields peptides of about seven to eight amino acids long, which can then be further degraded into shorter amino acid sequences and used in synthesizing new proteins.

2.4.3 Proteasome inhibitors (PIs)

Due to the fact that the UPS is involved in a diverse number of critical cellular processes, defects of various components within this complex system are associated with pathological disorders including malignancies, neurodegenerative diseases (e.g., Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, and Creutzfeld–Jakob disease), and genetic diseases (e.g., Cystic fibrosis and Angelman's syndrome) (Ciechanover 1998).

Furthermore, the importance of this cellular system has also attracted great interest into the development of inhibitors against it as an attractive target for therapeutic intervention of different human diseases such as cancer and stroke. PIs are broadly categorized into two groups: natural inhibitors and synthetic inhibitors. Natural product PIs include: linear peptide epoxyketones, peptide macrocycles, γ-lactam thiol ester, and epipolythio-dioxopiperazine toxin (Myung, Kim et al. 2001). Six main chemical classes of synthetic PIs exist: peptide benzamides, peptide α-ketoamides, peptide aldehydes, peptide α-ketoaldehydes, peptide vinyl sulfones, and peptide boronic acids (Myung, Kim et al. 2001). These agents were generally not considered for clinical development due to their lack of specificity and potency for the proteasome, and the fact that they were metabolically unstable. Most of today's available PIs are targeted against the 20S catalytic core of the proteasome, thereby blocking general proteolysis, and ultimately resulting in the accumulation of a wide variety of proteins destined for degradation by

the proteasome, thus likely contributing to toxicity. To date, among all tested and available PIs, bortezomib (Velcade™) and Carfilzomib (Kyprolis™) have been approved by the US Food and Drug Administration (FDA) for the treatment of multiple myeloma (Kane, Bross et al. 2003; Kortuem and Stewart 2013), and bortezomib for mantle cell lymphoma as well (Kane, Dagher et al. 2007). The efficacy of bortezomib as a single agent or in combination with other drugs is also being extensively studied in different types of malignancies (Cvek 2012). Currently, there are about 200 active clinical trials of bortezomib for various kinds of cancers listed in ClinicalTrials.gov. Several new and promising compounds have entered into clinical trials such as CEP-18770 (phase I), MLN9708 (phase I), marizomib (NPI-0052, Salinosporamide A, phase I), and ONX-0912 (phase I) (Cvek 2012). The knowledge and interest of specific upstream inhibitors of the UPS aims to improve specificity by limiting protein targets within the cell, hopefully resulting in fewer adverse effects with similar anti-cancer activity. Novel targeted inhibitors of the enzymes within the UPS are in development and testing, including inhibitors of: UAE (PYZD-4409, PYR-41), Nedd8 activating enzyme (NAE, MLN4924), E3 (nutlins, R7112, MI-219, MI-43), deubiquitinating enzymes (DUBs, b-AP15) (Cvek 2012). The work within this thesis has tested two different classes of PIs: the natural inhibitor lactacystin, and the two synthetic peptide boronic acids, MG262 and bortezomib (Velcade™).

2.4.3.1 *Lactacystin*

An organic compound that is a *Streptomyces lactacystinaeus* metabolite that was discovered due to its ability to induce neurite outgrowth in a murine neuroblastoma cell line (Omura, Fujimoto et al. 1991). Later, Fenteany and colleagues showed that lactacystin (376.43 g/mol) also targets the 20S proteasome by irreversible blocking of all three β-subunits (Fenteany, Standaert et al. 1995), where lactonization into its active component, clasto-lactacystin β-lactone, is necessary for inhibition of the proteasome.

2.4.3.2 *MG262*

MG262 (Z-LLL-Boronate, 491.4 g/mol) is a highly potent and selective cell permeable synthetic peptide boronic acid inhibitor of the 20S proteasome, where it binds and blocks the C-L activity in the β5 subunit of the proteasome. MG262 significantly inhibits the growth of most cultured cells with a concentration of less than 100nM.

2.4.3.3 *Bortezomib*

Bortezomib (Pyrazylcarbonyl-PheLeu-Boronate, 384.24 g/mol) was originally synthesized in 1995 and termed MG-341 (Myogenics Company). After promising results from *in vitro* and *in vivo* studies, it was tested in a small phase I clinical trial on multiple myeloma patients and then named PS-341. In 1999, Millennium Pharmaceuticals bought the rights to it and performed extensive clinical trials. Preclinical studies demonstrated that bortezomib was very potent against a broad range of cancer cell lines *in vitro* (Adams, Palombella et al. 1999) and in various animal xenograft models (Adams 2002). In 2003, seven years after the initial synthesis, it was approved in the US by the FDA with the name of bortezomib (brand name Velcade™) for treatment of multiple myeloma (Kane, Bross et al. 2003; Adams and Kauffman 2004), and has recently been approved for mantle cell lymphoma (Kane, Dagher et al. 2007).

Bortezomib is a low molecular weight, water-soluble synthetic dipeptide boronic acid that shows high inhibitory potency and degree of target selectivity for the 20S core of the proteasome, and disassociates slowly (Adams, Palombella et al. 1999). Pharmacokinetic studies revealed that bortezomib quickly distributes into tissues from the plasma within 10 minutes, where its half-life is more than 40 hours (Schwartz and Davidson 2004). Bortezomib is primarily metabolized through intracellular oxidative deboronation mediated by several cytochrome P450 isoenzymes into inactive enantiomers that are further processed and eliminated, mainly in the bile (66%), with the remainder excreted in the urine (Adams, Palombella et al. 1999). Bortezomib binds and blocks the C-L activity in the β5 subunit of the proteasome. Maximum proteasome inhibition occurs within 1 hour and recovers close to baseline within 72 to 96 hours after administration (Schwartz and Davidson 2004). In addition, the measurement of proteasome inhibition is the clinical marker for a targeted effective dose and should be within the 50–80% range (Adams and Kauffman 2004). Because of promising results of bortezomib as an anti-cancer drug in adults and in pre-clinical studies of pediatric cancers, clinical trials in children are ongoing. Recent phase I studies of bortezomib in pediatric patients with solid tumors or acute lymphoblastic leukemia (ALL) demonstrated that the drug is well tolerated with promising therapeutic activity (Blaney, Bernstein et al. 2004; Horton, Pati et al. 2007; Messinger, Gaynon et al. 2010; Muscal, Thompson et al. 2013).

2.4.4 Malignant cells vs. normal cells - side effects associated with PIs

The most frequent adverse effects (incidence > 30%) in the patients treated with bortezomib include asthenic conditions (e.g., fatigue, generalized weakness), gastrointestinal events (e.g., nausea, constipation, diarrhea, vomiting, poor appetite), hematological effects (e.g., low platelet and erythrocytes counts), and peripheral neuropathy (Chen, Frezza et al. 2011). It has been demonstrated that malignant cells harbor elevated proteasome activity compared with normal cells (Arlt, Bauer et al. 2009; Ma, Kantarjian et al. 2009). Furthermore, expression of proteasome and mRNA levels is increased in malignant human cell lines compared with lymphocytes and monocytes from healthy adults (Kumatori, Tanaka et al. 1990). Accordingly, it seems widely accepted to say that cancer cells are more dependent on their proteasome activity for survival than normal cells. Hideshima et al. reported that myeloma cell lines or patient-derived myeloma cells were at least 170-fold more sensitive to bortezomib compared with peripheral blood mononuclear cells from healthy volunteers (Hideshima, Richardson et al. 2001). Bortezomib has shown multiple targets in malignant cells including: *i)* activation of extrinsic and intrinsic apoptotic pathways, depending on cell type and dose, coupled with decreased levels of anti-apoptotic proteins; *ii)* suppression of the growth and survival factor, Nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) signaling pathway; *iii)* inhibition of angiogenesis; *iiii)* induction of endoplasmatic reticulum (ER) stress and generation of reactive oxygen species (ROS) (Adams 2004). Thus, given the fact that bortezomib is able to target multiple pathways and networks important for cell survival, it is not surprising that normal cells may also be targeted. Cytotoxic effects of bortezomib on immunocompetent cells have been observed, suggesting an important role of the proteasome in these cells, and that the immune system may be defenseless in treated patients. It was recently demonstrated that bortezomib induces apoptosis in resting natural killer (NK) cells (Wang, Ottosson et al. 2009), and bortezomib treatment has been shown to enhance the susceptibility to viral infections by altering antigen processing (Basler, Lauer et al. 2009). PIs have also been shown to impair protein synthesis and induce cell death in rat neurons due to mitochondrial damage (Cavaletti, Gilardini et al. 2007). In pigs, chronic proteasome inhibition is associated with increased coronary artery oxidative stress and early arthrosclerosis (Herrmann, Saguner et al. 2007).

2.4.5 Proteasome inhibition and side effects on chondrocytes

It has been reported that articular cartilage chondrocytes undergo apoptosis after treatment with the peptide aldehyde PIs, PSI and MG132, an effect related to induction of the extrinsic apoptotic pathway with CD95 and caspase-8 activation (Kuhn and Lotz 2001). On the contrary, proteasome inhibition has also been implicated as a potential chondroprotective modality in rat experimental osteoarthritis by increasing intracellular HSP70 expression, which was able to protect the chondrocytes from OA-induced cytotoxicity (Grossin, Etienne et al. 2004). To date, except for our own studies, there is only one additional study showing a link between linear bone growth and proteasome inhibition (Wu and De Luca 2006). In that study, they used the peptide aldehyde proteasome inhibitor I (PSI), and showed that it reduces linear bone growth in cultured fetal rat metatarsal bones. This effect was linked to suppressed growth plate chondrocyte proliferation and hypertrophy/differentiation, together with increased chondrocyte apoptosis. They also showed that treatment with PSI in chondrocyte cell cultures increases the expression of β-catenin (negative regulator of chondrogenesis) and reduces the DNA binding of the transcription factor NF-κB (known to stimulate chondrogenesis). In line with this, we have recently shown that proteasome inhibition, by MG262 and bortezomib causes severe growth retardation both *in vitro* and *in vivo* (Papers I-III in this thesis). We observed growth failure to be associated with increased apoptosis mainly in resting/stem-like chondrocytes: in addition, detailed characterization of the underlying apoptotic pathways revealed that several pro-apoptotic proteins were up-regulated (e.g., p53 and Bax) with induction of mitochondrial damage, whereas the anti-apoptotic proteins were down-regulated (e.g., Bcl-2 family of proteins). These finding underscore the importance of proper follow-up studies in treated children, both during and after PI-treatment.

2.5 GROWTH RESCUING THERAPY

Our mechanistic data in growth plate chondrocytes supports a role for both p53 and Bax during PI-induced chondrocyte apoptosis. In order to prevent the undesired effect on chondrogenesis and linear bone growth caused by PIs, we aimed to test genetic and pharmacological targeting against these pro-apoptotic proteins as potential growth rescuing therapies. However, complete characterization of the apoptotic pathways after

proteasome inhibition will also help us to identify the molecular players involved, and to be targeted, for the prevention of undesired bone growth-effects.

2.5.1 Inhibition of p53

To investigate the role of p53 during PI-induced apoptosis, a small molecule p53-inhibitor (pifithrin-α) was employed for suppression via the siRNA technique. Pifithrin-α has been shown to suppress p53-mediated apoptosis, and to protect mice from lethal doses of gamma-radiation (Komarov, Komarova et al. 1999), suggesting that pharmacological suppression of p53 may be a therapeutic approach to reduce the side effects of cancer treatment. Occurrence of p53-dependent apoptosis is likely to be one of the major players associated with side-effects in normal cells during cancer treatment (Komarova and Gudkov 1998), therefore, therapeutic suppression of p53 could help reduce the damage to normal tissues. Obviously, this approach is only applicable in tumors where p53 is mutated and/or absent, and the anti-cancer treatment applied is known to kill cancer cells independent of p53. PIs can induce both p53-dependent and independent apoptosis (Pandit and Gartel 2011). Accordingly, a combination of PIs and p53-inhibitors should therefore not interfere with the ability to induce apoptosis in p53-mutated cancers, but instead, rescue normal p53-dependent cells.

2.5.2 Humanin

Another approach that we used was through pharmacological inhibition of Bax, with a relatively new peptide called humanin. Approximately 10 years ago, humanin (MTRNR2) was discovered by a group in Japan (Hashimoto, Niikura et al. 2001). Humanin was discovered during a search for neurosurvival factors in unaffected areas of an AD patient's brain. The question of the origin of endogenous humanin still needs to be resolved, due to two peptide isoforms of 21 or 24 amino acids being known, depending on cytoplasmic or mitochondrial translation respectively (Guo, Zhai et al. 2003). Additionally, there is still an open question whether humanin is translated in the cytoplasm or mitochondria, as both of them showed similar biological activity (Guo, Zhai et al. 2003). Humanin is also the first peptide discovered within the mitochondrial genome since its complete sequencing in 1981 by Anderson et al., and it is encoded by an open reading frame (ORF) found within the 16s rRNA gene. The synthetic analog to humanin, [Gly^{14}]-HNG (HNG), where serine at position 14 is replaced by a glycine residue, was shown to increase the neuroprotective effects by 1000-fold (Hashimoto, Niikura et al.

2001) (Figure 5). Humanin and its analogs have shown promising therapeutic effects, thus so far only in experimental models, mainly in neurodegenerative diseases such as memory impairment (Mamiya and Ukai 2001), dementia/AD (Hashimoto, Niikura et al. 2001; Tajima, Kawasumi et al. 2005), and stroke (Xu, Chua et al. 2006). Humanin has also been shown to protect during myocardial infarction (Muzumdar, Huffman et al. 2010), and during conditions of oxidative stress and/or ischemia (i.e., oxygen deprivation due to reduced blood flow) (Bachar, Scheffer et al. 2010). Humanin levels are also known to decrease with age in both rats and humans, suggesting that humanin may play a role in the aging process (Bachar, Scheffer et al. 2010; Muzumdar, Huffman et al. 2010). Although all studies with humanin and its analogs show protective effects, the mechanisms by which they act is not yet understood. However, humanin has been shown to activate G-coupled formyl peptide receptor like 1 and 2 (FPRL1 and FPRL2) (Harada, Habata et al. 2004), STAT (Hashimoto, Suzuki et al. 2005), and glycoprotein 130 (gp130) (Hashimoto, Kurita et al. 2009). Thus, the findings above were observed in different cell types and not consistent for all cells, which stress the fact that different cell types may employ different receptors or mechanisms to respond to humanin. Humanin has shown both *extracellular* as well as *intracellular* rescuing activities. Extracellular activities by humanin have been shown by its possibility to increase insulin/IGF-I signaling assessed by AKT-1 phosphorylation in mouse neurons (Zou, Ding et al. 2003), to be a potent insulin sensitizer (Muzumdar, Huffman et al. 2009), and protect neuronal cells by interacting with insulin-like growth factor-binding protein 3 (IGFBP-3) (Ikonen, Liu et al. 2003). Humanin's intracellular activities were indicated by its interaction with pro-apoptotic proteins (e.g. Bax, Bid, Bak and BimEL) (Guo, Zhai et al. 2003; Luciano, Zhai et al. 2005; Zhai, Luciano et al. 2005). The anti-apoptotic effect of humanin was specific to Bax-dependent apoptosis, as apoptosis by Bax-independent stimuli was not suppressed. Humanin has also been shown to have anti-inflammatory properties by decreasing cytokine levels, such as TNF-α and IL-6 (Miao, Zhang et al. 2008). Interestingly, the ubiquitin-protein ligase, TRIM11, was found to bind and ubiquitinate humanin for proteasomal degradation, thus adding another level to its regulation (Niikura, Hashimoto et al. 2003). In summary, humanin has shown beneficial anti-apoptotic and cell-protective properties during episodes of toxicity, serum starvation, hypoxia, and stress conditions, for example, both *in vitro* and *in vivo* in different cell-types, and possibly acts as a mitochondrial autocrine, paracrine and endocrine signal.

Figure 5. Amino acid sequence of humanin and HNG. The 24-amino acid peptide sequence of endogenous humanin (top), and of the synthetic peptide analog, HNG (bottom), where a single amino acid substitution at position 14 with a glycine (G) for serine (S) increases the biologic potency by ~1000-fold.

3 PROJECT RATIONALE

Current treatment programs for pediatric cancers provide more than an 80% survival rate. However, adult survivors of childhood malignancies frequently suffer from secondary long-term complications after previous life-saving treatments (Oeffinger and Hudson 2004; Oeffinger, Mertens et al. 2006), experiencing consequences of short stature, skeletal morbidity such as osteopenia, as well as bone fractures as well-known recorded long-term sequelae (Robson, Anderson et al. 1998; van der Sluis, van den Heuvel-Eibrink et al. 2002). Increasing survival rates and progressively more intensive treatment regimens make it even more important to evaluate the long-term effects on normal bystander tissues. Despite the prevailing clinical evidence, there have been few investigations into the direct effects of anti-cancer agents and potential ways to rescue linear bone growth during treatment for childhood cancers. New therapeutic options specifically targeting cancer cells and limiting the toxic side effects are needed. The proteasome is a potential target for such a novel cancer therapy (Rajkumar, Richardson et al. 2005).

During the development of a new drug to be used in clinical practice, it is important to investigate any potential adverse effect(s) the drug might have on normal bystander tissues. The proteasome is an enzyme that is central to many processes required for cell survival, and questions regarding complications with side effects towards normal cells when targeting this vital cellular system arise, especially in young individuals. In this thesis, the focus has been directed toward a new class of anti-cancer drugs, PIs, which are thought to signal a new era of novel drugs. My main investigation has been on bortezomib, which is routinely used in the adult clinic for hematological malignancies (Kane, Bross et al. 2003; Kane, Dagher et al. 2007), and is currently in clinical trials of pediatric cancers (Blaney, Bernstein et al. 2004; Messinger, Gaynon et al. 2010; Muscal, Thompson et al. 2013). However, so far, any unwanted secondary side effects on chondrogenesis and linear bone growth in treated children are unknown. Therefore, all my efforts have been directed toward finding out if there might be any potential risks for chondrocytes and on linear bone growth by the use of this drug, and, if so, can we prevent these without interfering with the desired anti-cancer effect in young, treated individuals?

Often it is difficult to address the contribution of a specific drug/agent and its relative effect on chondrocytes and linear bone growth from clinical studies, since the disease

itself, the well-being of the patient, the nutritional status, and the different agents applied to enhance the cure, will have an effect on linear bone growth. For that reason, different experimental models can be of great help in trying to differentiate these effects, including mechanisms of action, and preventative strategies. Accordingly, we have employed a number of different model systems and methods to address our specific aims, all of which are discussed in the coming sections.

4 PROJECT AIMS

The thesis focuses on the preclinical evaluation of proteasome inhibitor (PI) treatment and its effects on chondrogenesis and linear bone growth. The aims also encompassed characterization of new therapeutic approaches to prevent bone growth failure after previous life-saving treatment.

The specific aims of the thesis were:

- To investigate the effects of the non-clinically approved PIs, MG262 and lactacystin, on chondrogenesis and linear bone growth both *in vitro* and *in vivo*.

- To investigate the effects of the clinically used PI, bortezomib, on chondrocytes, linear bone growth, and bone remodeling including the underlying molecular mechanism both *in vitro* and *in vivo*.

- To explore if [Gly14]-Humanin (HNG, a 24-aminoacid synthetic anti-apoptotic peptide), can prevent bortezomib-induced bone growth impairment without interfering with the desired anti-cancer effect of bortezomib both *in vitro* and *in vivo.*

5 METHODOLOGY

5.1 Proteasome Inhibitors (PIs):

The drugs used and included in this thesis include Lactacystin (Sigma-Aldrich, Schnelldorf, Germany), and MG262 (Bimol International, SMS-gruppen), which both were pre-diluted in dH2O, of which the latter was diluted in DMSO for *in vivo* studies. Bortezomib (Velcade™, formerly known as PS-341, LDP-341 and MLM341, Millennium Pharmaceuticals, Cambridge, MA) was dissolved in 3.5 ml saline (0.9%) to a final concentration of 1 mg/ml (2.6 mM). The synthetic peptide analog of endogenous humanin, [Gly14]-humanin (HNG) (Sigma-Aldrich, Schnelldorf, Germany) was diluted in saline (0.9%) to a final concentration of 0.5 mg/ml or 1 mM. All drugs were aliquoted and stored at -80 ºC until use.

5.2 MODEL SYSTEMS

The perfect animal model and/or method might not exist, and each of them has its own advantages and drawbacks. It is up to the investigator to choose the best model/method to be used that best represents and has the best ability to determine and illustrate the features of the current investigation. The model/method chosen should also be assessed upon whether its labor, technical, and/or financial demands are properly met.

5.2.1 Cell lines

5.2.1.1 *HCS-2/8 (Paper I):*

The availability of primary human chondrocytes is very limited, and therefore we chose to use the human clonal chondrocytic cell line, HCS-2/8. These cells derive from a well-differentiated type of human chondrosarcoma that best resembles the *in vivo*-like phenotype (Takigawa, Tajima et al. 1989). It is well characterized and widely used to study chondrocyte proliferation/differentiation. The cells grow slowly, with a doubling time of 3-4 days, and their morphology resembles that of primary chondrocytes. Moreover, they maintain the important markers for a chondrocytic phenotype, such as collagen type II (marker for proliferative chondrocytes), and following differentiation, they start to express proteoglycans and collagen type X (i.e., a marker of hypertrophic chondrocytes), including three-dimensional nodule formation.

5.2.1.2 *RCJ3.1C5.18 (C5.18) (Papers I, II, and III):*

In this thesis, the non-transformed clonal rat chondrogenic C5.18 cell line was generally used. This is a mesenchymal stem cell system that has been established through sequential steps of subcloning of isolated fetal rat calvaria cells (Grigoriadis, Heersche et al. 1988). The C5.18 cell line spontaneously and sequentially undergoes chondrocyte differentiation and terminal differentiation, displaying cartilage phenotypic stability without requiring biochemical or oncogenic transformation (Grigoriadis, Heersche et al. 1996; Lunstrum, Keene et al. 1999). Even though this system is based upon an *in vitro* situation, the morphology, cartilage specific histochemical markers, and the acquisition of the chondrocytic phenotype in this cell system is identical to the chondrogenic process that occurs *in vivo* (Lunstrum, Keene et al. 1999). These facts make it ideal and unique for studying chondrocytic cellular and molecular regulation, in addition to suggesting that our findings are relevant to the *in vivo* process. The C5.18 cell line was used and cultured as previously described (Spagnoli, Hwa et al. 2001). Briefly, after reaching confluence (resting phase; 4 days), cells were treated with fresh Eagle's minimum essential medium (MEM) alpha supplemented with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate. After 4-7 days of culture, the cells acquire markers of early chondrocytic differentiation (i.e., collagen type II and proteoglycan synthesis), and progressively acquire markers of terminal differentiation (e.g., collagen type X and alkaline phosphatase activity) at 10-14 days of culture. The cultures were monitored over a total period of 12 days and cultures were supplemented with fresh MEM alpha every 3 days.

5.2.1.3 *Human cancer cell lines (Paper III):*

In order to verify that HNG does not interfere with the anti-cancer effect of bortezomib, we investigated several different human cancer cell lines with different phenotypic and genotypic characteristics. The cell lines include (and are further described in Paper III); six neuroblastoma cell lines (SH-SY5Y, SK-N-BE(2), SK-N-AS, SK-N-SH, SK-N-DZ and IMR32); six medulloblastoma (MBL)/supratentorial primitive neuroectodermal cell lines (D283, D324 (also called DAOY), D425, D458, UW228-3, and PFSK-1), prostate cancer PC3 (ATCC CRL-1435), non-small cell lung carcinoma A549 (ATCC CRL-2271), colon cancer (e.g., HCT116), breast cancer (e.g., MCF-7), and acute lymphatic leukemia (ALL; CCRF-CEM).

5.2.2 Organ cultures of metatarsal bones (Papers I, II, and III):

In vitro cultures of both fetal (embryonic day 20 (E20)) rat metatarsal bones (Papers I-III), and postnatal (day 8 (P8)) rat metatarsal bones (Paper II) have been used to screen and characterize the local/direct effects of PIs on linear bone growth. Organ cultures, in comparison to cell cultures provide an environment that more closely reflects the *in vivo* situation. This *in vitro* model system enables maintenance of important factors, such as the cell-cell interactions, cell-matrix contacts, three-dimensional growth, and the possibility to follow linear growth over time. An advantage, though also a possible disadvantage not reflecting the actual *in vivo* situation, is the fact that these bones are cultured under serum-free conditions, are lacking blood supply and thereby are absent of the systemic factors and influence, which in turn facilitate the investigation of the direct effects of added drugs. Detailed description of the culture conditions and treatments can be found in the individual papers. Briefly, metatarsal bones were cultured in serum-free medium that was changed every 2-3 days, and cultured for a maximum of 12 days. Digital images were captured throughout the culture period, allowing the assessment of linear bone growth.

5.2.3 Animal models

All animal studies were performed with permission from the relevant Animal Ethical Board, and in strict accordance with the Swedish National Board for Laboratory Animals (SFS 1988:541). All efforts were made to minimize animals from suffering.

5.2.3.1 *Normal mice (Papers I and II):*

Evidently, time-patterning and regulation of linear bone growth are different in humans and rodents. For instance in humans, there is the characteristic pubertal growth spurt, and by the end of puberty the growth plates disappear and are remodeled into bone (Kember and Sissons 1976). While in rodents, closure of the growth plate does not occur, the growth plate persists as an inactive structure (Roach, Mehta et al. 2003). We used young mice that best represent the rapid growth and great growth potential as seen in children. Mice grow relatively quickly during the first weeks, and then slower growth is observed up to approximately 13 wks of age when they reach a plateau and growth is almost arrested. Furthermore, mice have been extensively studied with regards to both linear bone growth, and pharmacokinetics/pharmacodynamics of PIs. Two different

mouse strains were used: the inbred C57B strain, and the outbred NMRI strain, in order to delineate any strain-specific effects of PI treatment.

5.2.3.2 *Genetically modified mice (Paper I):*

For evaluation of the *in vivo* effect of proteasome inhibition in different tissues, we used a reporter mouse model for the UPS (Lindsten, Menendez-Benito et al. 2003). These mice express the ubiquitin^{G76V}-green fluorescent protein (Ub^{G76V}-GFP) that is constitutively targeted for ubiquitin-dependent proteasomal degradation. Tissues from these mice display low GFP fluorescence unless the cells fail to degrade the Ub^{G76V}-GFP protein as a consequence of functional impairment of the UPS. Low doses of the PI, MG262 (0.2 µmol/kg), were administered to male Ub^{G76V}-GFP mice at 5 wks old. Mice were treated on day 1, 3, and 5, and then killed 48 hrs after the last injection, with tissues then removed for subsequent analyses of GFP accumulation under a fluorescent microscope.

5.2.3.3 *Tumor xenograft mouse models (Paper III):*

Most pediatric preclinical *in vivo* testing has involved tumor xenografts as the model. We implanted the tumor cells subcutaneously (sc.) in our xenograft models which have the advantage of being well described, fast, easy to handle, cheap, as well as allowing tumor-host interaction, which makes it possible to study angiogenesis. Xenograft tumor growth and response to therapy can be directly followed by observation and tumor burden evaluated by volume calculation using caliper measurement, without being invasive and with minimal stress to the animal. The main drawback of the xenograft model is the site/place of the tumor with regards to environmental cues from which the tumor cells actually originally originate (brains, in this case). Furthermore, in contrast to engrafting primary cells, human tumor cell lines have generally been cultured over several months to years, and there are concerns that they no longer represent the characteristics of the original modeled human tumor. The establishment of human tumors cells requires mice that are immunosuppressed, and we therefore used NMRI nude (athymic) mice for our study. These mice lack T-cells, but have a compensatory increase in natural killer (NK) cells, which can also limit tumor growth and prevent metastasis (Habu, Fukui et al. 1981). For all xenograft studies, animals were xenografted at an age of 4-5 weeks by the method described in other cited research

(Morton and Houghton 2007), and treatment procedures, housing, measurement of tumor- and linear bone growth etc., are described in Paper III.

5.2.4 Human growth plate cartilage (Papers II and III):

Most of our *in vitro* assays on the sensitivity of growth plate chondrocytes to apoptosis are obtained from rat and mouse tissues/cells. To verify our rodent results in non-transformed human chondrocytes, we had the advantage of using intact human growth plate tissue. Biopsies were obtained from the proximal tibia and distal femur growth plates from pubertal children undergoing epiphyseal surgery for different medical conditions (e.g., constitutional tall stature or leg length discrepancy). Growth plate biopsies were collected with a bone marrow biopsy needle, transferred to tubes containing culture medium DMEM-high glucose, and placed directly on ice. In the lab, biopsies were cut into ½-1 mm thick slices under an inverted microscope, transferred into individual 24-well plates, and cultured for 24 hrs in 1 ml of supplemented culture medium including the drug of interest. After the culture period, samples were fixed in 4% formaldehyde for 24 hrs, decalcified in 10% EDTA pH 7.8 for 24 hrs. Samples were embedded in paraffin, cut into 4 µm thick slices, and mounted on glass-slides for further analyses.

5.3 ANALYSES OF BONE GROWTH AND STRUCTURE

5.3.1 Quantitative histomorphometrical growth plate analyses (Paper II):

Growth plate histology (i.e., height of the growth plate, proliferative zone height, number of columns, number of cells per column, and size of terminal hypertrophic chondrocytes) was assessed by examining proximal tibia Alcian blue/van Gieson (AB/vG)-stained sections under a light microscope connected to a digital camera and a computer. Terminal hypertrophic chondrocytes were considered as the last chondrocyte in the intact lacuna closest to the chondro-osseus junction. All histological measurements were performed in the central two thirds of the growth plate by a person blinded to the experimental groups.

5.3.2 Growth rate determination by Calcein labeling (Paper I):

The use of fluorochromes is a relatively simple technique to considerably increase insights into the dynamics of *in vivo* bone formation (van Gaalen, Kruyt et al. 2010). Except for bone length measurement with calipers that only provides an end-point

measurement, we utilized fluorochrome-labeling by calcein. Calcein is a fluorescent marker that is incorporated into newly formed bone tissue, allowing determination of bone mineralization that is commonly used to evaluate bone elongation (Turner 1994). Calcein was injected 7 days before, and 1 day before sacrifice. Non-calcified femurs were subsequently embedded in blocks of methylmethacrylate and examined under a fluorescent microscope. The distance between the two calcein bands, which corresponded to the bone growth that took place over the 6 days, was determined by using image analyses.

5.3.3 Dual X-ray Absorptiometry (DXA) (Paper II):

To be able to follow and measure bone growth longitudinally during the *in vivo* experiment, we utilized DXA. DXA is a non-invasive procedure that is commonly used to determine bone mineral density (BMD) in clinical settings. This system distinguishes between hard tissue (bone) and soft tissue based on how these different tissues absorb the two low-energy X-ray beams that the machine sends out. The denser the tissue is, the less X-rays get through to the detector. By using two different X-ray sources rather than one, it greatly improves the accuracy. DXA analyses were performed on lightly anesthetized mice by using the Norland pDEXA Sabre and Sabre research software (Version 3.6; Norland Medical Systems, Fort Atkinson, Wsconsin, USA). Bone lengths were determined from these scans by measuring the length from the proximal part to the distal part of femurs.

5.3.4 Radiographic imaging (Papers II and III):

Radiographic imaging by X-ray is the true evaluation of bone structure, and was thereby used for evaluation of *in vivo* bone growth in Papers II and III. Bones contain much calcium which, due to its relatively high atomic number, absorbs x-rays efficiently. X-ray analyses were performed on lightly anesthetized mice, and their backbones were fixed in a flat position for optimal imaging. The bones were visualized at a distance of 1.0 m with the settings 50 kV and 2.5 mAs by employing the GE AMX-4 (GE Healthcare, USA). Mean bone length per animal was calculated by measuring the length from the proximal part to the distal part of femurs using computerized software (Sectra Image Display System 5).

5.3.5 Peripheral quantitative computed tomography (pQCT) (Paper II):

pQCT is a useful tool for determination of bone mass, but due to the relatively high dose of radiation it delivers, its routine application for use in human clinical procedures is limited. Tomographic bone measurements were performed by using the Stratec XCT Research M (software version 5.4B; Norland Medical Systems), adapted especially for examination of small bones as previously described (Tivesten, Moverare-Skrtic et al. 2004), using a rotating X-ray tube that moves around the object of interest at a fixed distance, allowing independent evaluation of parameters associated with trabecular and cortical bones. In our study, we evaluated trabecular BMD in the metaphyseal region of the tibia, and cortical BMD, and area and thickness in the diaphyseal region.

5.3.6 Mechanical testing of bone strength by 3-point bending (Paper II):

The mechanical properties of the femur shafts were tested with the 3-point bending method using a universal mechanical testing device (Avalon Technologies, Rochester, MI, USA). Each femur was compressed in the diaphyses (middle part) at a constant rate of 0.155 mm/s until breakdown. Mechanical parameters, including ultimate strength (maximal load in N) and energy absorbed by the bone tissue representing structural toughness (area under the load deformation curve, $Nm \times 10^{-3}$), were calculated.

5.4 CELL VIABILITY AND DNA SYNTHESIS ASSAYS

5.4.1 MTT-assay (Paper III):

We used the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) assay (Mosmann 1983). This method is suitable to assess cell viability, proliferation or toxicity. This *in vitro* model system of measuring metabolic/mitochondrial activity of viable cells is widely used for screening purposes of drug activity, and well suited for defining interactions between drugs when having many different cell-types and drugs to analyze. It is non-radioactive, convenient (i.e., performed entirely in a microplate), quantitative, and allows samples to be analyzed rapidly and simultaneously. The principle of this method is the cleavage of the tetrazolium salt by the mitochondrial "succinate-tetrazolium reductase" that is active only in viable cells to a colored water-insoluble formazan salt, which must be solubilized before spectrophotometric measurement. After its solubilization, the formazan can easily and rapidly be quantitated in an ELISA reader. However, one has to keep in mind that during *in vitro*

culture conditions, changes in pH and nutrients in the medium might also affect to the metabolic state of the cells and thus give variation in the final readout. Briefly about the MTT test, cells were cultured and treated in a 96-well microplate before incubation with MTT solution for 2 hrs (viable cells convert MTT to a water-insoluble formazan dye), followed by solubilization of the formazan by acidic isopropanol, and quantitation of the colored product by an ELISA reader (spectophotometrically at 595 nm).

5.4.2 5-bromo-2´-deoxyuridine (BrdU) labeling (Paper II):

To study DNA synthesis *in vivo,* we employed the BrdU labeling technique. BrdU labeling was developed as an alternative approach for determining the proliferative index of tumors (Hoshino, Nagashima et al. 1989), and was introduced for studying cell proliferation in chondrocytes in 1993 (Farnum and Wilsman 1993). This technique offers the advantage of studying cell proliferation in individual cells. BrdU is a synthetic nucleoside (modified uridine), and an analog to thymidine. The principle underlying this method is the incorporation of BrdU into newly synthesized DNA of replicating cells during the S phase of the cell cycle (i.e., before cellular division into 2 daughter cells), substituting for thymidine during replication. This method of assessing cell proliferation has the advantage over the traditional method of ^{3}H-thymidine incorporation, in that it does not require handling of radiolabeled material, it is reliable, fast, and susceptible for quantification. However, one should also keep in mind that this method is also associated with some cell-toxicity and teratogenicity, and may not exclusively detect dividing cells, but also cells undergoing DNA repair: it is also noted that BrdU can increase proliferation and decrease differentiation by itself (Taupin 2007). BrdU-solution was intraperitoneally (ip.)-injected twice into mice at 16, and 2 hrs before autopsy. To enable quantification of the BrdU-incorporated, paraffin-embedded tissue sections were incubated with a BrdU-specific monoclonal antibody, followed by an FITC-conjugated secondary antibody, and then counterstained with DAPI. The number of BrdU-positive (FITC, green color) chondrocytes per growth plate was determined by digital automatic cell counting as described below.

5.5 CELL DEATH ANALYSES

5.5.1 TUNEL assay (Papers I, II, and III):

The terminal deoxynucleotidyl transferase (TdT)-metiated dUTP nick-end labeling (TUNEL) method (Gavrieli, Sherman et al. 1992) was utilized for quantification of apoptosis in growth plate chondrocytes obtained from animals, cultured metatarsals, and human growth plate tissues. The TUNEL assay has been further optimized for use in growth plate tissue in our laboratory (Chrysis, Nilsson et al. 2002). Briefly, proteinase K digestion was reduced from 20 min as recommended by the manufacturer to 5 min, and the concentration of enzyme was reduced from 20 ng/ml to 10 ng/ml, in order to minimize false positive detection of apoptotic cells. Thus, this protocol was undertaken in all of the studies. The assay works on the principle that nuclear DNA strand breaks (single or double) that occur during apoptosis can be identified by labeling the free 3'-hydroxyl (OH)-termini. Biotin-labeled nucleotides, dUTP, are polymerized to these 3'-OH groups in a reaction catalyzed by the TdT enzyme. The incorporated biotin-labeled nucleotides were then detected by a streptavidin-conjugated secondary antibody (Alexa Fluor-546) before mounting in DAPI-containing media. The number of TUNEL-positive chondrocytes in relation to the total cell number per growth plate was determined by digital automatic cell counting as described below. This is a fast, relatively sensitive, and quantitatible method. It must be kept in mind that the TUNEL assay may also detect DNA damage associated with non-apoptotic events such as necrotic cell death due to extensive DNA degradation induced by exposure to toxic compounds, for instance (Ansari, Coates et al. 1993), and it has also been reported to stain cells undergoing active DNA repair (Kanoh, Takemura et al. 1999). To distinguish apoptosis from necrosis, we performed additional analyses that included the expression of pro-apoptotic proteins, including active/cleaved caspases and PARP, the cell death ELISA, and studies of mitochondrial damage as discussed below.

5.5.2 Cell death ELISA (Paper I):

For quantification of apoptosis in cultured chondrocyte populations, we used the commercially available Cell Death ELISA kit. This immunoassay detects cytoplasmic low molecular histone-associated DNA fragments (mono- and oligonucleasomes) of apoptotic cells. This assay is based on the fact that DNA fragmentation without loss of the plasma membrane integrity is the hallmark of apoptosis. The test principle is a one-step sandwich immunoassay, were cells first are lysed, centrifugated and an aliquot of

the supernatant (DNA, singel- and double- stranded) is transferred to a streptavidin-coated 96-well microplate, an addition of monoclonal antibodies is made (anti-histone biotin labeled and anti-DNA peroxidase conjugated), and the sample is incubated with peroxidase substrate before determining the amount of colored product spectrophotometrically. This method is quantitative, convenient (non-radioactive) and reproducible, also allowing a large sample number. Further, the antibodies in the kit are not species-specific and can thereby be used for a wide variety of species. Moreover, this method can also measure necrosis, by using the supernatant where the oligonucleasomes will be released into: thus, the assay provides the ability to distinguish from apoptosis and necrosis. Accordingly, under our experimental conditions we excluded the occurrence of necrosis since no mono- or oligonucleasomes were present in the supernatant of cell cultures.

5.5.3 Caspase-3 fluorometric assay (Paper I):

Activation of caspases (cysteine proteases) plays a key role in mediating the early stages of apoptosis. The activation involves specific proteolyses of individual caspases. We chose to analyze the activity of the executioner caspase in the apoptotic cascade, caspase-3, by using the specific peptide substrate, DEVD, conjugated with a fluorogenic 7-amino-4-methylcoumarin enzyme, which can be detected fluorometrically following cleavage and release. Fluorescence values were converted to picomoles of AMC release by using a standard curve generated with free AMC, and the maximum rate of AMC release (pmol/min) was then estimated for each sample. This assay is described to show proportional caspase-3 activity to the percentage of apoptotic cells.

5.5.4 Analyses of mitochondrial membrane potential (Paper II):

To assess loss of the mitochondria membrane potential ($\Delta\Psi m$), which is indicative of apoptosis, we used tetramethylrhodamine (TMRE) ethyl ester to quantify the time-dependent damage of mitochondria after drug treatment (Ehrenberg, Montana et al. 1988). TMRE is a cell permeable, positively-charged, red-orange dye that rapidly accumulates in active mitochondria due to their relative negative charge. It also exhibits low cytotoxicity, and is relatively photostable with low tendency to bind other intra-cellular organelles. When the $\Delta\Psi m$ collapses in apoptotic cells, mitochondria fail to incorporate TMRE that become dispersed into the cytoplasm, and cellular fluorescence drops dramatically. This event can easily be detected by fluorescence microscopy or

quantified by flow cytometry. This technique can distinguish between healthy/vital cells, and the ones transitioning into an apoptotic state, as further discussed in Paper II.

5.5.5 Digital automatic cell counting (Papers I, II, and III):

Quantification of the number of proliferative and apoptotic cells of *in vivo*/*in vitro* treated growth plates/bone rudiments, was performed respectively by this method. Digital images of BrdU-positive cells (FITC, green color) or TUNEL-positive cells (by Alexa-546 antibody, red color) were captured using an Olympus DP70 digital camera. For each visual field to be quantified, three images were captured: a phase-contrast/light image, a Dapi image (detection of all cells, emission at 449 nm, blue color), and an FITC (emission at 530 nm) or Alexa-546 (emission at 573 nm) image. Automatic counting was then performed using Image-Pro® Plus software (Media Cybernetics Inc, Bethesda, USA) within the growth plate (containing resting, proliferative, and hypertrophic zones). It was identified based on the light image, and the selections were applied to the corresponding Dapi- and FITC or Alexa-images. The percentage of positive cells was calculated as the ratio of bright objects in the FITC (proliferation)- or Alexa-546 (apoptosis)-image over the total number of bright objects in the corresponding Dapi image.

5.6 DETERMINATION OF PROTEIN EXPRESSION

5.6.1 Immunohistochemistry (IHC) (Papers I, II, and III):

The procedure utilizing specific antibodies to identify, localize, and to a certain extent, quantify protein expression in the cells of a tissue section is referred to as IHC. IHC was utilized to detect the expression of different proteins (antigens) in formalin-fixed growth plate- and tumor tissue sections. Furthermore, cartilage-tissue sections, specifically obtained from *in vivo* studies are difficult to work with since they easily detach from the slides. Consequently, we therefore used saline-coated Superfrost +/+ glass-slides, which improved the attachment of the tissue to the slides. Fixation of tissue is an important step before subsequent analyses to preserve the cellular components and proteins intact. Cross-linking of proteins by formaldehyde may mask their binding-sites (epitopes), making it inaccessible to detection antibodies, which could result in false-negative results. To minimize false-negativity, the step of antigen-retrieval was added for all stainings. Antigen-retrieval was, in our case, achieved either by heating of the tissue in citrate buffer

(pH 6.0) for 15 min at 93-98°C, or by enzyme treatment. Furthermore, antigen-retrieval enhances the sensitivity of the immunostaining procedure, making it possible to decrease the concentration of the primary antibody, and thereby decrease the risk for non-specific staining (false-positivity). Before the addition of primary antibody, sections were incubated using normal serum from the host of the secondary antibody to reduce unspecific staining. The next step of choosing the antibody with greatest specificity and sensitivity against the particular antigen in the specific specie of interest is probably the most important issue connected with IHC, and it requires proper controls to be included in order to support the validity of the staining and identify experimental artifacts. Appropriate controls to be included are tissue samples that are known to express (or not express) the protein/epitope of interest. A negative control can also be obtained by pre-incubation of the primary antibody with the appropriate immunogen, replacement of the primary antibody with non-immune immunoglobulin of the same isotype (when using monoclonal antibodies), and/or by omitting the primary antibody. Detailed description for each protein is further described in the Material and Method section of the individual papers.

5.6.2 Western Immunoblot (Papers I, II, and III):

This technique is well-established, reproducible, allows separation of proteins by their molecular weight, and offers the ability to quantify the expression level of proteins. We applied this technique to study the expression levels of both anti-apoptotic (e.g., Bcl-2, Bcl-X_L) and pro-apoptotic (e.g., Bax, Bcl-X_S, AIF, caspases, and PARP) proteins, including the transcription factor and its regulatory protein, p53 and Mdm-2 respectively, in the cell extracts. Loading of equal protein amounts were based on quantitation using the Bradford assay (Bio-Rad Laboratories AB, Sundbyberg, Sweden). Proteins were separated on Tris-HCl or gradient acrylamide gels, and then transferred onto a Hybond-P polyvinylidene difluoride-transfer membrane, which allows detection of low protein levels (nanogram). Blocking of non-specific binding before incubation with primary antibody was achieved by placing the membrane in a dilute solution of protein: in our case, we always used 5% non-fat dry milk. The secondary antibodies used were linked to horseradish peroxidase (HRP) enzyme that will cleave the added chemiluminescent agent, the reaction product emitting light in proportion to the amount of protein, creating an image (dark band) of the antibodies bound to the blot. The resulting bands were confirmed by comparing the size of the protein in the cell extract

with known molecular weight markers. The densities of the protein-bands of interest were analyzed in relation to the house-keeping gene (GAPDH) to evaluate the relative amount of protein between samples. Finally, the extensive data in the literature concerning Western Immunoblotting and on the regulation of the different proteins were very useful for validation of our findings.

5.7 BLOOD ANALYSES

5.7.1 Proteasome activity analyses (Papers I, II, and III):

Proteasome activity was measured in whole blood collected at different time-points from treated mice. Blood was collected in tubes containing Heparin (100IE/KY/ml, LEO Pharma, Malmö, Sweden), and chymotrypsin-like (C-L, subunit β5) proteasome activity was measured using the synthetic fluorometric substance method (Lightcap, McCormack et al. 2000). Briefly, the assay involves hydrolysis of exogenous added LLVY-AMC by the chymotrypsin-like activity of the 20S proteasome. AMC is a highly fluorescent molecule, and its release was measured in a spectrophotometer. This assay offers a unique method of measuring proteasome activity that is sensitive, accurate, and reproducible. In addition, the measurement of proteasome inhibition is the clinical marker for a targeted effective dose, and it should be in the 50-80% range (Adams and Kauffman 2004).

5.7.2 Serum IGF-I levels (Papers I, II, and III):

To investigate if there was any interference with the IGF-I system in treated mice, and try to delineate if the observed bone growth impairment was more a systemic or local effect, serum IGF-I levels were analyzed. Serum concentrations of IGF-I were measured by radioimmunoassay, using a commercially available kit (IGF-R20) purchased from Mediagnost, Tuebingen, Germany. The sensitivity of the assay is 0.02 ng/mL. The assay was performed following the instructions from the manufacturer with the exception that a sample volume of 3 µL was used, based on previous experiences. All samples were analyzed in duplicate.

5.7.3 Analyses of bone biomarkers (Paper III):

To assess the activity of osteoblasts (bone formation) and osteoclasts (bone degradation), a commercial sandwich enzyme-linked immunosorbent assay (ELISA)

was utilized according to the manufacturer instructions for quantification of serum levels of procollagen type 1 N-terminal (PINP) and collagen type 1 cross-linked C-telopeptide (Ctx) respectively. The propeptide, PINP is released into the blood circulation during bone formation (collagen I synthesis), and is the most sensitive and specific marker for measurement of bone formation (Hale, Galvin et al. 2007). On the contrary, during osteoclastic bone resorption, Ctx is one of the degradation products of collagen type I. Briefly about the assay, a microtiter plate is coated with a capture antibody, and a sample is added before the addition of the detecting antibody that will bind the antigen (PINP or Ctx) in the sample. Thereafter, an enzyme-linked secondary antibody is added that binds the detecting antibody, and finally the chromogen that will be converted by the enzyme into a color that is quantified spectrophotometically is added**.**

6 RESULTS

6.1 EFFECTS OF PROTEASOME INHIBITION ON CHONDROGENESIS AND LINEAR BONE GROWTH (PAPERS I AND II)

Proteasome Inhibition Up-regulates p53 and Apoptosis-Inducing Factor in Chondrocytes Causing Severe Growth Retardation in Mice (Paper I)

In this study, we mostly focused on the non-clinically used PI, MG262. By using the transgenic reporter mouse model for the UPS, Ub^{G76V}-GFP mice (Lindsten, Menendez-Benito et al. 2003), we show that systemic administration of a clinically relevant dose of MG262 (0.2 μmol/kg), tissue-specifically impairs the UPS in growth plate chondrocytes. The impairment of the UPS was accompanied by the induction of chondrocyte apoptosis in growth plate cartilage of treated mice. Furthermore, this effect resulted in severe linear bone growth impairment, observed both 48 hrs, as well as 45 days post treatment with MG262, compared to vehicle-treated animals. The bone length differences (femur and tibia) after the 45 day follow-up period was not as striking as 48 hrs after the last injection, suggesting that some catch-up occurred, although this 45-day follow-up period was not enough to fully catch-up in growth, and we do not know what happens beyond this period.

The underlying mechanistic studies revealed that MG262-induced growth failure was mainly caused by a severe thinning in the height of the resting zone, which was followed by chondrocyte apoptosis of the resting/stem-like and proliferative chondrocytes. Caspase inhibitory experiments in organ cultures of metatarsal bones and human- and rat chondrocytic cell lines confirmed that MG262 triggered both caspase-dependent and independent apoptosis of chondrocytes. Accordingly, protein expression of the transcription factor, p53, was also found to be increased in growth plate cartilage of MG262-treated mice. In addition, the regulator of caspase-independent apoptosis, apoptosis inducing factor protein expression, also appeared to be highly up-regulated in chondrocytes after MG262 treatment, both *in vitro* and *in vivo*.

Suppression of p53 expression by employing the siRNA technique resulted in a 35% decrease in MG262-induced chondrocyte apoptosis. This finding supports a role for p53

during PI-induced chondrocyte apoptosis. Furthermore, in support of a role for AIF-mediated chondrocyte cell death, suppression of AIF by siRNA decreased apoptosis of chondrocytes by 41%. These data support an important role for the UPS in growth plate chondrocytes, and by impairing this system, it results in deleterious effects on growth plate chondrocytes, followed by growth impairment.

Bortezomib Is Cytotoxic to the Human Growth Plate and Permanently Impairs Bone Growth in Young Mice (Paper II)

In this study we extended our investigations to the clinically used PI, bortezomib that is currently in clinical trials of pediatric cancers (Blaney, Bernstein et al. 2004; Messinger, Gaynon et al. 2010; Muscal, Thompson et al. 2013). However, so far, any undesired secondary side effects in fast-growing individuals have, to our knowledge, not yet been described. Because of the alarming data of the non-clinically used PIs on chondrogenesis and induction of bone growth impairment from us and others (Wu and De Luca 2006; Zaman, Menendez-Benito et al. 2007; Zaman, Fadeel et al. 2008), we decided to elucidate any potential risks of bortezomib treatment on linear bone growth, and bone metabolism, including the underlying molecular mechanisms. The studies were performed both *in vivo*, in two different strains of young mice (which best represents the rapid growth of a child), and *in vitro*, in cultured metatarsal bones and a chondrocytic cell line. Finally, we also used pubertal human growth plate cartilage to assess and verify the toxicity of bortezomib.

Our results indicate that bortezomib efficiently blocks the UPS, with a similar degree of proteasome inhibition as seen in treated humans, that is to say, within the 50–80% range (Adams and Kauffman 2004). By using a clinically relevant dose of bortezomib (1 mg/kg) along with a similar dosing regimen as in the clinic, we confirmed that one 2-week cycle (2 injections/wk) causes permanent growth failure in treated mice, when followed for up to 6 months post-treatment. This effect was mainly due to induction of apoptosis in resting/stem-like chondrocytes. Previous studies suggest that it is the resting/stem-like cells that influence the growth plate structure and function (Gafni, Weise et al. 2001; Schrier, Ferns et al. 2006), and that this cell population serves as the pool for generating the columnar clones of the underlying proliferative zone (Abad,

Meyers et al. 2002). Together, these studies indicate the importance of the resting/stem-like chondrocytes for maintenance of the normal growth potential and thus, any disturbances and/or depletion of it might therefore result in incomplete growth. Treatment with bortezomib in fetal and postnatal cultured rat metatarsal bones resulted in a dose-dependent growth inhibitory effect. Interestingly, bortezomib treatment for only 24 hrs in fetal metatarsals was enough to permanently inhibit bone growth, further suggesting irreversible growth failure. To identify what cells in the metatarsal bones that were targeted, we analyzed the bones by using the TUNEL method. Bortezomib dose-dependently increased chondrocyte apoptosis, an effect mainly observed in resting/stem-like chondrocytes. Metatarsal bones were also stained with Alcian Blue/van Gieson (AB/vG) to detect changes of matrix components such as glucosaminoglycans and collagens. Indeed, bortezomib decreased the levels of matrix components, indicated by the low levels of AB/vG-staining. We further confirmed our results in cultured human growth plate cartilage, which was found to be highly sensitive to bortezomib after 24 hrs of treatment. Again, mainly the resting/stem-like chondrocytes, and to some extent also the early proliferative chondrocytes, were targeted, as quantified by the TUNEL method.

Our data support a local action of PIs, selectively targeting resting/stem-like growth plate chondrocytes, leading to decreased bone growth. This concept is supported by the findings in the Ub^{G76V}-GFP mouse model, and measurement of serum IGF-I levels that were not different from vehicle-treated mice, together with the growth inhibitory effect in cultured metatarsal bones.

The sensitivity of chondrocytes to bortezomib treatment was further verified in the rat chondrocytic cell line, C5.18, by utilizing the cell viability assay, MTT. The cells were treated for 24 hrs and 48 hrs with bortezomib (0-100 nM), which resulted in a time- and dose-dependent decrease in cell viability. Again, the resting/stem-like cell population was found to be the most sensitive one, in contrast to both proliferative- and hypertrophic chondrocytes.

In an attempt to delineate the underlying molecular mechanisms regulating bortezomib-induced apoptosis, protein expression profiles (using the Western immunoblot approach) of several pro- and anti-apoptotic proteins were investigated in resting/stem-

like C5.18 chondrocytes. These cells were exposed to bortezomib (1000 nM) for 3, 6, 12, and 24 hrs. Our results indicated that bortezomib induced early activation of p53 and Bax, as early as 3 hrs after treatment, suggesting key roles for these proteins in the regulation of bortezomib-induced chondrocyte apoptosis. We also observed subsequent cleavage of caspases (-9, -8, and -3), and finally also of poly-ADP-ribose polymerase (PARP) in exposed chondrocytes.

Skeletal morbidity such as osteopenia and osteoporosis, including increased risk for bone fractures are common long-term side effects associated with childhood anti-cancer treatment (Siebler, Shalet et al. 2002). However, the impact of PIs on bone metabolism and bone strength in children are still unknown. To investigate this, we performed analyses of serum bone biomarkers, tomographic trabecular, and cortical bone measurements (by pQCT), and mechanical bone strength assessment (by 3-point bending test) in treated mice. Our results showed no significant effects of bortezomib on the bone biomarkers (PINP and Ctx), and neither on BMD, nor on bone biomechanical properties, such as cortical content, cortical thickness or bone strength. Previous studies have shown that PIs such as proteasome inhibitor-1, epoxomicin and bortezomib may enhance bone formation and BMD in 5-week-old Swiss ICR white mice (Garrett, Chen et al. 2003) and in 7-week-old C57B/6 mice (Mukherjee, Raje et al. 2008). Moreover, a recent study provides convincing and promising results of bortezomib on bone formation through stimulation of vitamin-D receptor signaling (Kaiser, Heider et al. 2013). Bortezomib has also been shown to suppress osteoclast activity (von Metzler, Krebbel et al. 2007) and increase osteoblast activity (Zangari, Esseltine et al. 2005) by activating Runx2 (Mukherjee, Raje et al. 2008) or inhibiting Dickkopf-1 (DKK1), an inhibitor of osteoblast function (Oyajobi, Garrett et al. 2007). However, bortezomib had no effect on femur BMD in a myeloma model of 15-week-old CB.17/Icr-SCID mice (Pennisi, Li et al. 2009), which is in line with our data. These conflicting results may suggest that regulation of mouse bone remodeling by the UPS is influenced by age, mouse strain, dose, duration of treatment, and/or immune function. Furthermore, we did not observe any positive effect of bortezomib treatment on bone strength when biomechanical testing was performed. Bortezomib might therefore not offer the same level of benefit to bone health in fast growing individuals as earlier reported in adults with multiple melanoma (Zangari, Terpos et al. 2012).

6.2 PREVENTIVE STRATEGIES TO RESCUE BONE GROWTH IN PI-TREATED INDIVIDUALS (PAPER III)

Humanin prevents bortezomib-induced bone growth impairment without interfering with the desired anti-cancer effect (Paper III)

The present study was designed to investigate the potential for HNG to rescue from bortezomib-induced bone growth impairment without interfering with the desired anti-cancer effect of bortezomib. To test this, we applied an array of different *in vivo* and *in vitro* models, including human tumor xenograft models of childhood neuroblastoma (NBL) and medulloblastoma (MBL), cultures of human growth plate cartilage, fetal rat metatarsal bones, and chondrogenic- and cancer cell lines.

First we used the organ culture model of fetal rat metatarsal bones as a screening tool to assess any rescuing effect by HNG from bortezomib-induced bone growth impairment. Our results indicate a partial rescue of metatarsal bone growth, when combining HNG (100nM) with bortezomib (25nM). This promising result led us to further verify if this was also true *in vivo*, with the aim to also rule out any potential interference of HNG with the anti-cancer effect of bortezomib. To test this, young nude mice were first established with either NBL or MBL childhood tumors before receiving treatment with either one 2-week cycle (2 injections/wk) of bortezomib, HNG, the combination of HNG/bortezomib, or the vehicle. Our results indicate that intravenous (iv.) injections of bortezomib (0.8 mg/kg) efficiently blocked the proteasome (approximately 60%), an effect that was not disturbed by the addition of HNG. However, intraperitoneal (ip.) injections of bortezomib (1.0 mg/kg) resulted in a less efficient proteasome inhibition in these mice. Despite this, bortezomib treatment resulted in significant bone growth failure, irrespective of intravenous or intraperitoneal administration. Combination treatment with HNG could almost completely rescue bone growth, close to the growth rate observed in vehicle-treated mice.

Histomorphometrical analyses of the mouse growth plates revealed that combination treatment with HNG could restore growth plate height to a similar level as observed in vehicle treated mice. This rescuing effect was mainly due to a prevention of resting/stem-like chondrocyte apoptosis. We also confirmed our observations in cultured human

growth plate cartilage, where HNG prevented bortezomib-induced chondrocyte apoptosis to a similar extent as seen *in vivo*. The chondrocyte rescuing effect of HNG was coupled to a suppressive effect of the pro-apoptotic proteins, Bax and the downstream executioner protein, PARP. Our data are in line with previous studies in other cell types where humanin and its analogs were found to bind Bax, preventing its activation, and thereby protect the cells from apoptosis (Guo, Zhai et al. 2003; Zhai, Luciano et al. 2005). An interesting observation that we made, was that bortezomib increased Bax accumulation in chondrocytes, but not in human NBL cells, suggesting that the chondrocyte rescuing effect of HNG might be linked to a Bax-dependent effect.

The key-question if administering HNG to individuals with cancer is the risk of any potential interference with the anti-cancer effect of bortezomib, and hence, a rescue of the tumor cells. To investigate this we used several different human NBL and MBL tumor cell lines, including tumor cells from some of the most common human cancer diagnoses (i.e., lung, prostate, colon, and breast cancer), and also the human tumor xenograft mice models of NBL and MBL. Our *in vivo* data demonstrate that HNG does not diminish the anti-cancer effect of bortezomib, but instead potentiates it. Interestingly, HNG by itself showed an anti-tumor effect as documented with the highest HNG concentration tested in three tumor cell lines, as well as in the two different NBL tumor xenograft experiments, where the effect was linked to decreased angiogenesis and increased tumor-cell apoptosis. Our *in vitro* and *in vivo* result also confirms an anti-cancer effect of bortezomib that is in line with previous reports (Brignole, Marimpietri et al. 2006; Hamner, Dickson et al. 2007; Yang, Jove et al. 2012). We observed a clear delay of tumor growth in response to bortezomib, but no apparent evidence of tumor regression, also consistent with previous reports (Michaelis, Fichtner et al. 2006; Houghton, Morton et al. 2008).

7 CONCLUDING REMARKS

Using an array of different *in vitro* and *in vivo* models of chondrogenesis and linear bone growth, the current study revealed that clinically relevant doses of PI-treatment specifically target the growth plate and damage normal chondrogenesis, which in turn is reflected by bone growth failure. Histology and cellular analyses further demonstrated pronounced reduction of growth plate height, associated with a suppressed height of all zones in the growth plate, reduced size of terminal hypertrophic chondrocyte, and we also observed that bone matrix deposition was severely decreased after proteasome inhibitor-treatment. PI-induced impairment of the chondrogenesis process and failure of linear bone growth was probably found mainly due to the induction of resting/stem-like chondrocyte apoptosis, and accordingly, GFP reporter accumulation was evident mainly in these cells. The stem-like cells in the resting zone have a finite proliferative capacity that is gradually depleted (Schrier, Ferns et al. 2006), and any disturbances to this might result in growth disturbances (Abad, Meyers et al. 2002). Consistent with this hypothesis, we speculated that PI-treated animals would not be able to catch-up. Some tendencies of catch-up growth were observed, specifically after MG262-treatment, however, it was not complete. Bortezomib-treated mice were followed up to 6 months after cessation of treatment after administration of one 2-wk cycle of a clinically relevant dose of bortezomib, and still they were found growth retarded as compared to their weight-matched and pair-fed, vehicle-treated mice. When investigating the underlying molecular/apoptotic pathways after PI-treatment, we found early accumulation and activation of p53, Bax, and AIF, cleavage of caspases, and the executioner protein in the apoptosis cascade, PARP, while the anti-apoptotic proteins were found to be down-regulated (e.g., Bcl-2 and Bcl-X_L). Moreover, mitochondrial dysfunction was also observed, which has previously been implicated as being a key mechanism involved in apoptosis (Susin, Zamzami et al. 1997). These outcomes therefore emphasize the need of finding preventive strategies to protect chondrocytes and maintain normal bone growth during PI-treatment in young individuals without interfering with the desired anti-cancer effect of PIs. In an attempt to test this, we used the synthetic peptide analog to endogenous humanin, [Gly^{14}]-Humanin (HNG), which has been shown to be a wide-spectrum survival molecule in different cell-types and diseases (Xu, Chua et al. 2006; Hoang, Park et al. 2010) with the ability to bind Bax, preventing its activation, and thereby protect the cells from apoptosis (Guo, Zhai et al. 2003). To address our question, human tumor xenograft mouse models, *in vitro* cultures

of human growth plate cartilage, rat metatarsal bones, and both chondrogenic and cancer cell lines were applied. Here, we made the novel finding that HNG can rescue from PI-induced bone growth impairment. Importantly, HNG did not interfere with the desired anti-cancer effect of bortezomib. The cytoprotective effect of HNG was associated with a protection of resting/stem-like chondrocytes from bortezomib-induced apoptosis, an effect mediated through interference with the pro-apoptotic protein, Bax. We also confirmed that HNG has the ability to protect cultured human growth plate cartilage from the cytotoxic effects of bortezomib.

In conclusion, we strongly recommend linear bone growth and bone mineralization to be closely monitored in the current pediatric clinical trials of PIs, and for the future, HNG supplementary treatment may be a potential therapy for preventing any undesired effects associated with PI treatment in children.

8 FUTURE PERSPECTIVES

Overall, it appears that inhibition of proteasome function in chondrocytes specifically induces apoptosis, linking the UPS of protein degradation with the regulation of apoptotic cell death in chondrocytes and, in turn, with negative consequences on linear bone growth. Our observations indicate a more local effect of PIs in the growth plate, although we cannot exclude that other systemic factors other than IGF-I may play a role, so this should be further investigated. Consequently, observations from this study suggest that bone growth could be suppressed in young individuals treated with PIs. However, it should be emphasized that so far, any side effects on linear bone growth in treated children are unknown, and one should be cautious when extrapolating pre-clinical data to the clinical arena. In accordance, our finding needs to be confirmed in ongoing pediatric clinical trials.

We showed that resting/stem-like chondrocytes are the main target of PIs, and that no complete catch-up growth occurred after PI-treatment, which emphasizes the importance of the resting zone chondrocytes, and that loss of them may lead to loss of growth potential, which is also supported by previous studies (Abad, Meyers et al. 2002; Schrier, Ferns et al. 2006). However, the fact that we found the "quiescent"/slowly-proliferative cells to be most sensitive to PI-treatment is in contrast to other studies that have reported that rapidly proliferating cells are most susceptible to PIs (Kisselev and Goldberg 2001; Voorhees, Dees et al. 2003). The different sensitivity in terms of chondrocytes in the growth plate might be due to a cell-type-specific effect with, for example, variable dependence on intact proteasomal function, sensitivity to changes in normal protein composition, more efficient PI-uptake, or slower inactivation of PIs. This finding , therefore, warrants further investigation.

Due to the skeletal morbidities associated with PI-treatment found during this study, it is important to develop strategies that will minimize the risk of complications while still maintaining high cure rates. Here we show that HNG-supplementary treatment has the ability to reverse the negative effects induced by PIs on bone growth by protecting the growth plate, mainly by preventing resting/stem-like chondrocyte apoptosis. Importantly, the rescuing effect of chondrocytes by HNG did not interfere with the desired anti-cancer effect of bortezomib. Hence, humanin and its analogs are novel, potential cell-survival

peptides under substantial investigation in different conditions: there is still a lot to explore and learn about them, in other words, knowledge of their stability/half-life, cell-interaction and binding-sites, full dose- and time response, complete interaction of the apoptotic pathways and other factors still needs to be gathered. Furthermore, the long-term effects of HNG remain to be elucidated, as well as any long-term interference with the anti-cancer effect, and/or potential cancer-cell rescue. The observed dual roles of HNG acting both as a chondrocyte-protective factor, as well as suppressor of tumor growth, are remarkable and need to be further investigated in other tumor models. Moreover, our findings of a bone growth rescuing effect by HNG may also have wider implications as disturbed growth has been linked to many different types of anti-cancer treatments.

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