INVESTIGATING PROCASPASE-3 AS A POTENTIAL THERAPEUTIC TARGET IN CANINE OSTEOSARCOMA

BY

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THESIS

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ABSTRACT

Apoptosis is a specialized, active form of cell death that is important in both health and disease. It is responsible for maintaining regulated development and tissue homeostasis and participates in the elimination of aged, damaged, or displaced cells. Apoptosis is also an inherent tumor suppressive mechanism, and the ability of cancer cells to evade death is widely acknowledged as one of the hallmarks of cancer development. In tumor cell populations, disruptions in apoptosis commonly occur through modulation of key players in the apoptotic cascade. The central role of capsase-3 as an executioner of apoptosis makes it an ideal drug target for circumventing such upstream disruptions and restoring effective apoptosis in cancer cells. This work investigates the use of PAC-1, a newly developed procaspase-3 activating compound, in the treatment of canine osteosarcoma. Procaspase-3 is expressed in osteosarcoma cells, and furthermore, greater expression is seen in malignant canine osteoblasts than normal osteoblasts. Exposing cells to PAC-1 results in the activation of procaspase-3 to caspase-3, increased caspase-3 catalytic activity, and induction of apoptosis in osteosarcoma cells. The greatest degree of activity is seen after 48 hours of exposure to the PAC-1. Additionally, a large proportion of spontaneous primary and metastatic tumors express procaspase-3 to a varying degree, and the pattern of expression appears to be conserved within patients from primary to metastatic lesions. In conclusion, the study’s findings support future investigation of PAC-1 as a personalized anticancer treatment strategy for osteosarcoma.
CHAPTER 1
INTRODUCTION

Osteosarcoma (OS) is the most common primary bone tumor of dogs, and it is estimated that 10,000 dogs and 1,000 children per year develop OS in the United States. Osteosarcoma is a high grade, aggressive neoplasm whose behavior closely parallels pediatric OS in humans. Morbidity results from pain and decreased limb function as a result of focal skeletal osteolysis and tumor extension from the medullary canal. Currently, both aggressive and palliative treatments directed at the primary tumor are limited by patient and owner specific considerations. Additionally, despite aggressive treatment with surgical resection and adjuvant chemotherapy, most dogs will eventually go on to die of metastatic disease. Characterization of new molecular targets has the potential to lead to the development of novel anti-cancer strategies that could result in better treatment outcomes for dogs and people with OS.

Chapter 2 opens by providing an overview of apoptosis and its general role as a hallmark of cancer development. It begins to set the stage by discussing how restoration of effective apoptosis may be a platform for development of new anti-cancer drugs. The next section of chapter 2 introduces the most crucial members of the apoptotic program—caspases. Caspases, a family of cysteine proteases, are the specialized cellular machinery which propels the cascade of events leading to cell death. This section details the structure and varied roles of caspases as initiators or executioners. It discusses in detail the pathways that lead to caspase activation and the players involved in regulating these pathways. The third section of chapter 3 discusses the role of apoptosis in tumorigenesis and specifically highlights caspase-3 in cancer. Because caspase-3 activation is a pivotal and common endpoint of apoptotic pathways, it is an ideal target for circumventing acquired upstream disruptions in apoptosis. Neoplastic cells have been shown to sequester caspase-3 in its inactive form, and thus, therapies that focus on activating caspase-3 are a promising novel anti-cancer strategy. PAC-1, the first procaspase activating compound, is a small molecule activator of caspase-3 that produces apoptosis of neoplastic cells in vitro. Chapter 2 concludes by discussing the advantages and disadvantages of
local treatment strategies currently available for canine OS, and the rationale for investigating PAC-1 as new local treatment for OS.

In order to justify the use of PAC-1 in OS, we first sought to identify the target procaspase-3 in osteosarcoma cells. Chapter 3 identifies the presence of procaspase-3 in canine, murine, and human osteosarcoma cell lines. It then investigates the effects of exposure of osteosarcoma cells to PAC-1 in vitro. When osteosarcoma cells were exposed to low, biologically relevant concentrations of PAC-1, conversion of the target to active caspase-3 was documented. Additionally, PAC-1 caused an increase in caspase-3 catalytic activity and induced apoptosis in canine osteosarcoma cells in a dose and time dependent fashion. These findings are encouraging, and warrant further investigation of PAC-1 as a treatment for canine OS.

Although procaspase-3 was identified in osteosarcoma cells in vitro, it was important to determine whether the target was also present in spontaneously occurring primary and metastatic canine OS as well. Additionally, we sought to ascertain if malignant osteoblasts had increased expression of the target over normal osteoblasts. Chapter 4 reports on the differential expression between malignant and normal canine osteoblasts. Furthermore, it describes the variation in expression of procaspase-3 in spontaneously occurring tumors. The data highlights procaspase-3 as a viable drug target in canine OS and lends justification for the use of PAC-1 as a personalized anticancer strategy, especially in cases where prescreening procaspase-3 concentrations are found to be high.

Chapter 5 concludes this manuscript by summarizing the author’s findings and discusses future strategies for the clinical application of PAC-1 as a treatment for canine OS.
2.1 Apoptosis, an overview.

Apoptosis or programmed cell death is an innate physiologic process that is indispensable for the maintenance of regulated development and tissue homeostasis. It plays a vital role in the elimination of damaged or displaced cells and cells lacking the appropriate signals for survival, thus allowing the body to actively determine if and when cells should die [1-4]. In contrast, dysregulated apoptosis has been implicated in the development of many pathologic conditions, including neurodegenerative disorders, autoimmune diseases, sepsis and particularly, cancer [1, 3-6]. It is now widely acknowledged that evasion of apoptosis is one of the hallmarks of cancer development [3, 4, 7, 8], and naturally, this discovery has led to a diverse array of scientific explorations into the mechanisms by which apoptosis is disrupted in (or inhibited by) tumor cell populations. With such questions at the forefront of cancer research, the last ten years has brought a wealth of continually emerging insight into the mechanisms by which programmed cell death is executed. The eventual hope is to use this knowledge to identify drug targets and develop compounds that might effectively treat cancer through restoration of the apoptotic program [9-11].

In order to begin to identify potential therapeutics that might re-sensitize cancer cells to apoptosis, a detailed understanding of the apoptotic process and machinery is required. Apoptosis is a specialized form of cell death, which is distinguished from its counterpart, necrosis, by distinct morphological changes, including membrane blebbing, cytoskeletal disruption, nuclear pyknosis, chromatin condensation and genomic fragmentation [1-5, 12, 13]. This ordered process of cellular disassembly allows for the formation of membrane-enclosed vesicles or apoptotic bodies, which are subsequently engulfed by neighboring phagocytic cells without provoking an inflammatory response [2, 3, 7, 14] (Figure 2.1). Induction of apoptosis may be promoted through a variety of external and internal pro-apoptotic stimuli; and in mammals, is initiated via two principle pathways: (1) the extrinsic pathway through ligation of death receptors and (2) the intrinsic pathway by way of cellular stress and mitochondrial
release of cytochrome c [4-8, 12, 15, 16]. However, regardless of the pathway to cellular suicide, all routes converge to share a key and central component—the activation of caspases [5, 6, 8, 12, 15, 17]. Caspases, a family of cellular proteases, are the specialized cellular machinery which propels the cascade of events leading to cellular death. Understanding these vitally important enzymes and the factors which regulate their activation is fundamental for the derivation of strategies to manipulate apoptosis for therapeutic gain [6, 8, 10, 15, 17].

2.2 Caspases

Caspases were implicated as a central feature of apoptosis in 1993 when the *C. elegans* ced-3 gene was demonstrated to encode a cysteine protease analogous to interleukin-1-β-converting enzyme (ICE) in mammals [18, 19]. Overexpression of ICE, which was subsequently renamed caspase-1, was then shown to be adequate for the induction of apoptosis within mammalian cells [19, 20]. These seminal breakthroughs suggested that mammalian caspases may possess crucial apoptotic functions similar to that of the ced-3 gene product, and thus, set in motion the characterization of the mammalian caspases. Currently, 14 caspases are identified in mammals of which caspase-1 through -10 and caspase-14 are found in humans [5, 8, 21, 22]. Caspase-11 and -12 are murine homologues of human caspase-4 and -5, and caspase-13 is a bovine homologue of human caspase-4 [5, 8, 23]. While not all caspases are necessary for apoptosis or solely capable of those functions [24, 25]; within the context of apoptosis, these powerful proteases act as both initiators and effectors of cellular dismantling [5, 15, 17, 22].

2.2.1 Caspase Structure

Caspases are potent cysteine proteases whose functions are tightly linked to their structural makeup [5, 8]. Generally speaking, there is a great deal of similarity among caspases with regards to their amino acid sequence, structure, and the predominant requirement for cleavage of substrates after an aspartic acid residue (Asp) [1, 17, 26, 27]. Caspases contain three structural domains, including an N-terminal prodomain and two conserved catalytic domains, which are comprised of a large subunit (20 kDa) and a small subunit (10 kDa) (Figure
2.2 A). Additionally, in a number of family members, a linker region is present to connect the catalytic subunits [5, 17, 28]. These unstructured regions are often the focus of (auto)proteolysis during maturation or, in some cases, activation [29]. Importantly, cleavage sites within these regions contain Asp residues, emphasizing the ability of caspases to auto-activate or become activated by other caspases as part of the amplification cascade [1].

Caspases, in accordance with other proteolytic enzyme systems, are synthesized as inactive proenzymes (zymogens). Because unregulated caspase activity would be lethal to the cell, these latent precursors (procaspases) require conversion to their active form via distinct conformational changes [30]. Active caspases exist as obligate dimers of identical catalytic units, each composed of one large and one small subunit and containing one active amino acid recognition site (total of two active sites in the proteolytic dimer) [31, 32] (Figure 2.2 B.). Based on crystal structure and studies with synthetic peptide libraries, it has been established that all caspases bind their substrates with similar substrate pockets (S₁, S₂, S₃, and S₄) [33, 34]. While both the large and small subunits donate residues to form the binding cleft, it appears that the major factors that determine substrate specificity are located within the small subunit [34]. Initially, it was thought that all caspases were activated by proteolysis [27], but over the last few years, overwhelming evidence has surfaced that this is only one mechanism responsible for caspase activation (principally pertaining to the executioner caspases) [30, 32]. It appears that while the structures of all active caspases are largely the same, the mechanism of transformation from the inactive to active form varies greatly between initiators and executioners [29] (Figure 2.2 B).

2.2.2 Caspase classification: initiators versus executioners

While several classification schemes have been described, this discussion will focus on distinguishing caspases by their function and position within apoptotic signaling pathways: either as initiators or executioners (effectors) of apoptosis. This method of classification emphasizes key differences in structure, function, activation, and substrate specificity. Discussion of the inflammatory caspases (caspases-1, -5, and -4) will not be included as it is outside the scope of this review.
Initiator Caspases

The importance of caspase structure is highlighted when we consider the different roles of the proapoptotic caspases. Initiator caspases (procaspase-2, -8, -9, -10) are characterized by long prodomains, containing special motifs such as death effector domains (DED, procaspase-8, -10) or caspase recruitment domains (CARD, procaspase-9) that are critical for their activation \textit{in vivo} \([3, 8, 12, 30]\) (Figure 2.3). In the inactive state, initiator procaspases exist as monomers within the cell and require dimerization to undergo activation, a process recently discovered to be independent of cleavage \([35-37]\) (Figure 2.2 B). Dimerization is facilitated by recruitment of initiator caspases to adapter molecules, resulting in high local concentrations. Adaptor molecules are part of larger oligomeric activation assemblies within the cell that convene following an apoptotic signal \([29, 30, 38, 39]\). The DED and CARD are responsible for mediating the homophilic interactions (e.g. CARD-CARD and DED-DED) between procaspases and their adaptor proteins (e.g. APAF-1 with procaspase-9; FADD with procaspase-8), and thus, directing them to their specific activation platforms (e.g. DISC with caspases-8, -10 and apoptosome with caspases-9) \([29, 30, 38, 39]\). Initiator caspases, as their name implies, generally respond to upstream cellular signals in order to set the apoptotic cascade in motion. Their inherent narrow substrate specificity allows them to recognize and activate their downstream targets—the executioner caspases \([30, 40]\).

Executioner caspases

The apoptotic executioner caspases (procaspase-3, -6, -7) are characterized by short prodomains (Figure 2.3) and occur constitutively as inert homodimers \([36]\). The role of this short N-terminal prodomain has yet to be clearly defined; however, it seems that its purpose relates to the efficiency of activation of executioners \textit{in vivo}, perhaps due to sequestration or cellular compartmentalization \([41, 42]\). The proteolytic activity of inactive executioners is restrained by a short linker region, and during activation, cleavage at specific Asp residues divides the amino acid chain into its small and large catalytic subunits \([29, 31, 32]\) (Figure 2.2 B). The crystal structures of zymogen and active caspase-7 provide the greatest evidence elucidating the process of catalytic groove formation during activation \([43, 44]\). Processing of
the linker region allows re-ordering of catalytic and substrate binding residues via mobile loops, and consequently, formation and stabilization of the active site is achieved [29, 30, 32]. As alluded to earlier, cleavage is carried out under most circumstances by an initiator caspase, but this may also occur in limited cases by the serine protease granzyme B [29, 33]. Once activated, the apoptotic executioners are collectively responsible for the degradation of many cellular substrates that bring about the characteristic morphologic changes known as apoptosis [3, 5, 6, 8, 30].

2.3 Caspase activation pathways

As previously mentioned, initiator caspases require some form of signal for a cell to be targeted for destruction. The origin of this message, from either the soup of extracellular signaling molecules or from variations within the intracellular environment, determines the pathway of caspase activation. There are two main, distinct but convergent pathways that trigger apoptosis within the cell—the extrinsic and the intrinsic pathways [3-5, 32, 45]. The extrinsic pathway, also known as the “death receptor pathway”, is initiated by engagement of death receptors at the cell surface [39, 46]. Conversely, the intrinsic pathway, otherwise known as the “mitochondrial pathway,” is triggered by mitochondrial membrane changes and the release of cytochrome c [47-49].

2.3.1 The Death Receptor Pathway (Extrinsic)

The extrinsic pathway plays a vital role in the physiological regulation of apoptosis (e.g. immune system education, tumor immunosurveillance), and dysregulation has been implicated in the pathogenesis of cancer and disorders of the immune system [50-55]. Stimulation of the extrinsic pathway is initiated by ligand-bound death receptors (DR) of the tumor necrosis factor receptor superfamily (TNFR), chiefly TNF-TNFR-1, FasL-Fas, and TRAIL (TNF-related apoptosis-inducing ligand/APO2)-DR4 or –DR5 [39, 52, 56, 57]. These death receptors are type I transmembrane proteins, which are characterized by cysteine rich extracellular domains (CRDs) that facilitate ligand binding and cytoplasmic interaction domains such as the death domain (DD) [46, 57, 58]. Of the members of the TNF-receptor family, the Fas and TRAIL (DR4 and DR5)
receptor-mediated signaling paths are best understood with regards to induction of apoptosis. Both Fas and TRAIL are alike in that ligation of receptors induces the formation of the death inducing signaling complex (DISC), which subsequently recruits and activates the initiator procaspase-8 [57, 59] (Figure 2.4). Interestingly, caspase-10 may also be recruited to the DISC, but its action alone is not sufficient to induce apoptosis and its physiologic role remains ambiguous [60, 61].

**Ligation of death receptors and the DISC**

Signal transduction begins when trimerization of receptors within the cell membrane brings the intracellular domains in close proximity with one another [46, 62]. Previously, it was believed that trimerization was the result of ligand binding in a trimeric form; however, the discovery of preassembled receptor clusters on the cell surface challenged this notion [52, 63, 64]. Preassembly has since been shown to occur as a result of a region termed the preligand assembly domain (PLAD), which resides within the death receptors’ extracellular domain [65-67]. The PLAD plays a critical role in the efficiency of ligand binding and consequently receptor activation [67]. Following ligation, an activating conformational change takes place within the receptors to cause recruitment of the adaptor proteins, Fas associated death domain (FADD) or TNF-receptor associated protein with death domain (TRADD). The adaptor proteins act, exactly as the name denotes, to link the extracellular signal (DR ligation) to an intracellular effect (caspase activation). These adaptor proteins interact with the death receptors through homologous death domains (DDs) present on both molecules and similarly, with the initiator caspases via corresponding death effector domains (DEDs) [46, 52, 57].

The ensuing aggregate of proteins is known as the DISC and sets the apoptotic cascade in motion starting with activation of caspase-8 by dimerization [68] (see section 2.2.2). Active caspase-8 goes on to cleave caspase-3, which in turn, leads to cell death [69]. As is typical of many biological systems, the extrinsic pathway is not completely independent of the intrinsic pathway and some degree of “cross-talk” between the two occurs. Caspase-8 also cleaves Bid, a BH3-only member of the Bcl-2 family of proteins, to truncated Bid (tBid), causing changes in mitochondrial membrane permeability and subsequent release of cytochrome c and
Smac/DIABLO [70-72]. This cross stimulation of the intrinsic pathway drives the activation of caspase-9 which in turn cleaves caspase-3. In addition to cellular degradation, caspase-3 activates caspase-8, completing a positive feedback loop that amplifies the apoptotic cascade [53, 73] (Figure 2.4).

**Regulators of the extrinsic pathway**

Notably, several proteins serve to negatively regulate the death receptor pathway at various levels including: decoy receptors, FLICE-inhibitory proteins (c-FLIP), and inhibitors of apoptosis proteins (IAPs) [74]. The decoy receptors (DR-1, -2, -3 and osteoprotegerin [OPG]) are similar to Fas and DR-4/-5 in their extracellular domains but have truncated or absent death domains rendering them incapable of transmitting an apoptotic signal [39, 74-78]. Consequently, competition for FasL/TRAIL binding through decoy receptor expression may work to inhibit ligand-induced apoptosis [52, 77, 79]. Further downstream, c-FLIP (long [c-FLIP\_L], short [c-FLIP\_S], and Raji B-cell line derived [c-FLIP\_R] isoforms [80]) acts to regulate recruitment and activation of the initiator caspases at the DISC [81, 82]. All c-FLIP variants contain two DEDs and can associate with FADD by way of DED-DED interactions, but only c-FLIP\_L additionally contains a caspase-like domain [80, 82] (Figure 2.5). While it is widely accepted that c-FLIP\_S and c-FLIP\_R inhibit death receptor-mediated apoptosis by vying for binding to FADD [46, 82, 83], the function of c-FLIP\_L has long been controversial [84]. Only recently, a clear dual pro- and anti-apoptotic role for c-FLIP\_L has been established that appears to depend on the c-FLIP\_L to caspase-8 concentration ratio [83, 85]. The role of the IAP proteins will be discussed in detail in section 2.4. Briefly, with regards to the extrinsic pathway, IAPs bind and inhibit procaspase-3, thus arresting the cascade of proteolysis and preventing Fas/caspase-8 induced apoptosis [86-88].

**2.3.2 The Mitochondrial Pathway (Intrinsic)**

The intrinsic pathway is triggered principally in response to internal cellular stimuli such as cytokine deprivation, cytotoxic stress, and DNA damage (e.g. ionizing radiation and chemotherapeutic drugs) [32, 47, 89]. The focal point of the pathway is the mitochondria, and
advancement through it revolves around a key event—mitochondrial outer membrane permeabilization (MOMP) [52, 57, 89, 90]. During MOMP, various pro-apoptotic proteins, sequestered in the intermembrane space, such as cytochrome c and Smac/DIABLO, acquire access to other proteins in the cytosol where they promote caspase activation and apoptosis [89, 91-94] (Figure 2.6). In order to clearly comprehend this event, a cursory understanding of mitochondrial structure, function and the factors that regulate them is required.

**Mitochondria**

Mitochondria are the metabolic hub of the cell, and are best known for their role in conversion of dietary nutrients to cellular energy (ATP) through oxidative phosphorylation [90, 95, 96]. Their ability to perform this function relies on the selective permeability of the mitochondrial membranes. The mitochondrial inner membrane (MIM) is normally impermeable to protons and other ions, and this barrier is vital to maintaining transmembrane potential and energy transduction [97-99]. Conversely, the mitochondrial outer membrane (MOM) is permeable to small molecules but impermeable to proteins, which is critical for cell survival [96, 98, 99]. Disruptions in the MIM and MOM may be produced by multiple mechanisms [100, 101]; however, strong evidence points to the Bcl-2 family of proteins, particularly Bax and Bak, as the central regulators of MOMP relevant to apoptotic cell death [96, 102, 103]. Intriguingly, we will see that ATP is not only an absolute requirement for life but also for energy-dependent cell death, emphasizing that the MIM must be left intact during the apoptotic program [104-106].

**Bcl-2 Family Proteins**

The Bcl-2 (B-cell lymphoma-2) family proteins are the key controllers of the mitochondrial pathway, and it is the balance between the members’ opposing activities that determine the ultimate fate of the cell [107]. Classically, three groups of Bcl-2 family members are recognized that share up to four homology domains (BH1-BH4): the pro-apoptotic group (Bax, Bak), the anti-apoptotic group (Bcl-2, Bcl-XL), and the BH3-only group (Bad, Bid, Bim, NOXA, PUMA) [3, 52, 107, 108]. As alluded to earlier, members of the first group, Bax and Bak,
commit the cell to apoptosis by mediating MOMP. Activated Bax translocates to the MOM where activated Bak is already incorporated [109, 110]. Within the MOM, Bax and Bak are thought to undergo conformational changes and form homo-oligomers preceding apoptosis [111, 112]. While the precise mechanism of MOMP execution remains controversial, one model proposes that Bax and Bak oligomers form pores through which intermembrane proteins can pass, thus allowing cytochrome c efflux to the cytoplasm [107, 113].

Conversely, the anti-apoptotic Bcl-2 family members protect the cell from apoptosis and guard mitochondrial integrity. Bcl-2 is an integral MOM protein in all cells and Bcl-XL associates with the MOM in many cell types, usually after an apoptotic signal [114-116]. Localization at the MOM allows Bcl-2/Bcl-XL to sequester and/or inhibit pro-apoptotic counterparts thereby preventing release of cytochrome c [3, 107, 116-118].

The last group, activated BH3-only proteins, promote apoptosis via interactions with each of the core groups of Bcl-2 family proteins [119]. Certain BH3-only proteins (Bid, PUMA, Bad, NOXA) appear to antagonize prosurvival members, whereas others (Bid and Bim) have been shown to directly activate Bax and Bak [119-121]. Two competing models exist regarding the relative importance of these interactions. Briefly, the derepression model suggests that BH3-only proteins must antagonize the steady inhibitory binding of Bcl-XL to Bax/Bak that usually prevents MOMP [116, 122, 123]. Alternatively, the direct activation model suggests that Bcl-XL acts as a sponge, sequestering BH3-only proteins that are direct activators of Bax/Bak (e.g. Bid). In order to cause activation, these proteins must be freed by other BH3-only members via interactions with Bcl-XL [113, 119, 124].

**The apoptosome—“wheel of death”**

Following MOMP, cytochrome c release into the cytoplasm leads to the assembly of the caspase activating complex of the intrinsic pathway—the apoptosome [125, 126]. Cytochrome c complexes with the adaptor protein Apaf-1 (apoptotic protease activating factor-1), which leads to a conformational change and permits exposure of its N-terminal CARD and greatly enhances dATP/ATP binding activity [91, 125]. This binding triggers formation of a seven-spoked wheel, often called the “wheel of death,” which in turn recruits procaspase-9 into its
central hub via CARD-CARD interactions [91, 125] (Figure 2.7). Procaspase-9 is then transformed to its active form and can go on to activate downstream caspases-3 and -7 to execute cell death [127].

2.3.3 The Common Pathway: Caspase-3 in the spotlight

Both the extrinsic and the intrinsic pathways converge upon a common pathway—activation of executioner caspases. Named for their role in cleaving the major cellular substrates during apoptosis, the executioner caspases are vital for producing cell death and amplifying the apoptotic signal [33, 128, 129]. The three executioner caspases-3, -6, and -7 are highly homologous to one another [130], but caspase-3 is the most extensively studied, and thus, its role in apoptosis is more clearly defined [8, 130]. Analysis of apoptosis in cells of caspase-3 knockout animals showed inhibition of certain morphological hallmarks (membrane blebbing, DNA degradation, nuclear fragmentation) but retention of others (nuclear condensation, phosphatidylserine exposure) [130, 131]. Similarly in a cell-free apoptotic system, depletion of caspase-3, but not caspase-6 or caspase-7, resulted in hindrance of many downstream events of apoptosis, such as various substrate cleavages and DNA fragmentation [8, 130, 132]. These findings suggest that caspase-3 may be the most important effector of cellular destruction, but likely does not fully control all aspects of apoptosis [130]. One explanation for this is that caspase-6 and -7 may play specific roles in more minor pathways that activate executioner caspases such as endoplasmic reticulum (ER) stress [33, 133, 134].

The substrates of the effector caspases are numerous and include: cytoplasmic proteins (e.g. actin, β-catenin), nuclear proteins (e.g. laminin-A/B, nuclear mitotic apparatus protein), DNA metabolism/repair proteins (e.g. PARP, DNA-topoisomerases, RNA-polymerase), protein kinases (e.g. PKC, MAPK, ERK, Akt) signal transduction pathway proteins (e.g. pro-interleukin cytokines, phospholipases), cell cycle/cell proliferative proteins (e.g. p21, p27, pRB), apoptosis related proteins (e.g. Bcl-2, Bcl-XL, ICAD), and more [33, 34, 135-137]. Proteolytic cleavage of this vast array of substrates is believed to result in a variety of cellular consequences, many of which contribute to the signature changes observed during apoptosis [138]. Among these, one of the best characterized examples is the degradation of nuclear DNA through caspase-
mediated activity of CAD (caspase activated DNase). Caspase-3 cleaves ICAD (inhibitor of caspase-3-activated DNase) inside the nucleus releasing CAD [139-142]. Thereafter, CAD forms a homodimer and cuts double stranded DNA precisely at nucleosomal linkers [143, 144]. This process occurs early in apoptosis, fragmenting DNA and swiftly halting DNA replication and transcription.

2.4 Inhibitors of Apoptosis (and IAP inhibitors)

Most cell regulatory pathways include a system of checks and balances, and apoptosis is no exception. Caspases are powerful killers that irrevocably enact cellular suicide, and thus, healthy cells must have a way to prevent unwanted activation of these enzymes. Inhibitors of apoptosis proteins (IAPs) are a family of structurally alike proteins that restrain caspase activity under conditions favoring cell survival [145, 146]. They are characterized by 1-3 novel domains of 70-80 amino acids called the baculoviral IAP repeat (BIR) [27, 147, 148], and to date, eight mammalian members have been identified [NAIP (neuronal apoptosis inhibitory protein), c-IAP1, c-IAP2, Survivin, XIAP (cross-linked IAP), Bruce/Apollon, ILP-2 (IAP-like protein-2), and Livin/ML-IAP (melanoma IAP)] [145]. The IAP’s BIR domains are zinc-chelating fingers that directly bind specific caspases and allow obstruction of caspase catalytic grooves by BIR linkers [3, 33]. Generally speaking, the IAPs that contain multiple BIRs, such as XIAP, utilize the BIR1-BIR2 linker to impede caspase-3 and -7 and the BIR3 to inhibit caspase-9 [3, 88, 125, 149] (Figure 2.8). When cellular conditions shift towards those favoring apoptosis, inhibition of caspases must be relieved. This additional tier of control is accomplished by Smac (second mitochondria-derived activator of caspases)/DIABLO, which along with cytochrome c, is released from the mitochondria during MOMP to antagonize IAPs and promote apoptosis [27, 74, 92, 150].

2.5 P53 in Apoptosis

P53 is undoubtedly one of the most famous of the tumor suppressors due to the fact that it is functionally impaired in the vast majority of human tumors [151]. It is a highly complex, multifunctional protein that acts as a crucial responder to a variety of stress signals
encountered during malignant transformation and progression, for example genotoxic damage, oncogene activation, loss of normal cell contacts, and hypoxia [152, 153]. The central mechanism of p53’s anticancer activity has been attributed to its ability to induce apoptosis, and it does so in both a transcriptional-dependent and independent manner [57, 153, 154]. As a transcription factor, p53 stimulates expression of a number of pro-apoptotic gene products involved in the mitochondrial pathway, such as Bax, PUMA, NOXA, and Apaf-1, and represses other anti-apoptotic gene products such as Bcl-XL and survivin [153, 155-158]. In the death receptor pathway, it induces Fas expression in certain tissues [159] and activates TRAIL receptor DR5 in response to DNA damage [160, 161]. Aside from transcriptional activation and repression, p53 provokes apoptosis in a transcriptional-independent manner via direct interactions with MOMP inducers at the level of the mitochondria [154, 162-164]. While the details of this mechanism are still emerging, p53 appears to cause Bax and Bak oligomerization at the MOM and antagonize anti-apoptotic actions of Bcl-2 and Bcl-XL through physical interactions [156, 165].

### 2.6 Apoptosis in Tumorigenesis

Acquired resistance to apoptosis is one of the hallmarks of all types of cancer, and over 50% of neoplasms display defects specifically in their apoptotic machinery [3, 4, 7, 8, 52]. Moreover, such defects may confer tumor cells resistance to many of the anti-cancer therapies currently in use. Disruptions of both the extrinsic and intrinsic pathways may be involved in tumorigenesis and, in general, this can occur either via inactivation/downregulation of pro-apoptotic elements or overactivation/upregulation of anti-apoptotic elements [166-168].

Within the extrinsic pathway, evasion of death receptor signaling may occur by decreased receptor expression or inactivation. For example, downregulation of Fas (CD95) is observed in hepatocellular carcinomas, neoplastic colon epithelium, melanomas, and drug-resistant leukemias [169-173]; and CD95 gene mutations have been reported in hematological malignancies as well as a variety of solid tumors [174-178]. Additionally, deletions or mutations in TRAIL receptors, resulting in a loss of both copies, have been demonstrated in a small proportion of some cancers, such as osteosarcoma and breast carcinoma [179, 180]. A second
mechanism to evade death receptor induced apoptosis is aberrant expression of decoy receptors. In glioblastomas and some carcinomas, overexpression or genetic amplification of decoy receptors has been identified [181, 182] and may help predict stage in gastric carcinoma [183]. Aside from interfering with death receptors, signal transduction through the extrinsic pathway can be blocked at the level of the DISC. One strategy seen in carcinomas is overexpression of c-FLIP, resulting in competitive inhibition of procaspase-8 recruitment at the FADD [184]. Furthermore, caspase-8 may be impaired by genetic or epigenetic mechanisms. Alternative splicing of caspase-8 mRNA in leukemia and neuroblastoma generates caspase-8L, a variant lacking the catalytic site [185, 186]. Also, caspase-8 inactivation can be achieved via hypermethylation of regulatory gene sequences in various pediatric tumors (e.g. rhabdomyosarcoma, medulloblastoma, and retinoblastoma) [187, 188].

Within the intrinsic pathway, MOMP may be impeded by abnormal expression of anti-apoptotic Bcl-2 family proteins or inactivation of Bax or BH3-only proteins. One well documented example is overexpression of Bcl-2 in human follicular lymphoma, caused by translocation of the bcl-2 gene onto the immunoglobulin gene locus [189, 190]. In addition, mutated Bax, either via nucleotide substitutions or frameshift mutations has been identified in colon cancer as well as hematopoietic malignancies [191, 192]. Caspase-9 inactivation is another theoretical mechanism by which cancer cells may evade apoptosis. Strong evidence demonstrating an association between caspase-9 mutations and cancer risk is currently minimal; however, some studies have reported that certain gene polymorphisms were associated with a significantly increased risk of lung cancer [8, 193]. Finally, aberrant expression of inhibitor of apoptosis proteins may also confer tumor cells resistance to apoptosis. A notable example of this is deregulation of survivin. Survivin, in contrast to its low or undetectable expression in most normal human adult tissues, was found to be expressed at high levels in the majority of cancers [194] and predicted an unfavorable prognosis in neuroblastomas, acute myeloid leukemia, and non-Hodgkin lymphoma [195-197].
2.6.1 Caspase-3 in Cancer

Since both intrinsic and extrinsic apoptotic pathways lead to the common pathway, any upstream disruption in apoptosis causing inefficient effector caspase activation may be hypothesized to lead to increased concentrations of the inactive procaspase-3 within tumor cells. Indeed, cells from certain cancer cell lines and cancerous tissues have been confirmed to have elevated procaspase-3 concentrations when compared with noncancerous controls. Recently, the National Cancer Institute performed a systematic evaluation of 60 cell lines and found that concentrations of procaspase-3 varied from undetectable to greatly elevated, particularly in lung, melanoma, renal and breast cancers [198]. In addition, a study evaluating isolates from individuals with colon cancer demonstrated a six-fold average elevation when compared to nearby noncancerous tissue controls [199]. Other notable examples of elevated procaspase-3 expression include a proportion of neuroblastomas [200], lymphomas [201], leukemias [202], hepatocellular carcinomas [203], and melanomas [204]. Moreover, procaspase-3 elevation appears to be clinically relevant in certain tumors. In one study of patients with acute myeloid leukemia, a high level of uncleaved caspase-3 was correlated with a significantly decreased survival, whereas, a high level of cleaved caspase-3 denoted an improved survival [202].

Aside from procaspase-3 sequestration, caspase-3 mutations may occasionally occur in tumors. Mutated caspase-3 has been reported in the MCF-7 breast cancer cell line [205]. Also, in one report by Soung et al which investigated caspase-3 mutations in various cancers, a small proportion of colon cancers and one case each of lung, stomach adenocarcinoma, hepatocellular carcinoma, and multiple myeloma were found to have a mutated caspase-3 gene. These mutations were concluded to have arisen somatically, and consisted of 6 missense mutations either involving the large (17kD) or small (12kD) protease subunit. The remainder of the mutations were silent or in non-coding or untranslated regions [206]. Another study evaluating squamous cell carcinoma of the head and neck (SCCHN) detected an association between certain caspase-3 polymorphisms and increased risk of SCCHN, suggesting a possible role of caspase-3 mutations in tumorigenesis [207].
2.7 PAC-1

Induction of apoptosis has recently been recognized as a powerful anticancer strategy. Because caspase-3 activation is a common end-point of all activating pathways, it may be an ideally suited target for circumventing many possible upstream disruptions of apoptosis. Furthermore, evidence of procaspase-3 elevation in cancer cells in combination with the relative infrequency of inactivating caspase-3 mutations suggests that pharmacological caspase-3 activation may be an effective strategy to direct apoptosis within tumor cells. In 2006, a novel small molecule called PAC-1 was identified as the first procaspase activating compound from a library of structurally diverse small molecules screened in vitro [208]. PAC-1 preferentially activated procaspase-3 in a dose- and time-dependent fashion as assessed by caspase-3 substrate cleavage activity and western blotting. PAC-1 also demonstrated powerful proapoptotic effects, such as phosphatidylserine exposure, chromatin condensation, PARP-1 cleavage, and cellular blebbing. These effects were observed in cancer cell lines in a manner strongly correlated with procaspase-3 concentrations [208]. Additionally, promising anticancer properties were observed in primary colon cancer isolates and mouse xenograft models as a result of direct activation of procaspase-3. Conversely, noncancerous cell types were less susceptible to the proapoptotic effects of PAC-1, suggesting a degree of selectivity for cancer cells [208]. In 2009, Peterson et al. elucidated the mechanism of action of PAC-1 in vitro; PAC-1 activates procaspase-3 through the sequestration of inhibitory zinc ions, allowing procaspase-3 to autoactivate itself to caspase-3. These investigators demonstrated that the catalytic activity of procaspase-3 is inhibited by zinc and that PAC-1 binds strongly to zinc, relieving this inhibition (Figure 2.9). They hypothesized that once caspase-3 is generated, it can activate more procaspase-3, causing caspase-3 substrate cleavage and apoptosis [209]. In further work, they showed that fluorescently labeled PAC-1 co-localizes with sites of caspase-3 activity in cancer cells, bolstering the evidence supporting a direct mechanism of action for PAC-1 [210].

An in vivo study by Lucas et al. investigating the pharmacokinetics of PAC-1 in healthy dogs has been recently reported [211]. The purpose of this research was to evaluate whether concentrations and exposure durations of PAC-1 shown to induce cytotoxicity in lymphoma cell lines in vitro were achievable in healthy dogs through a constant rate infusion (CRI) intravenous
delivery strategy. The investigators concluded that the oral bioavailability of PAC-1 was variable and relatively low, but that maintenance of predicted steady state concentrations in normal dogs was safely attained via intravenous CRI lasting for 24 or 48 hours in duration. Using a pharmacologically derived dosing regimen in 4 healthy dogs, plasma concentrations of 10.7 ± 2.0 μM were achieved after 6 hours of infusion and steadily maintained to 36 hours [211].

2.8 Canine Osteosarcoma

Osteosarcoma (OS) is the most common primary bone tumor of dogs, accounting for approximately 85% of malignancies arising in the skeleton [212-215]. It is a high grade, biologically aggressive neoplasm of mesenchymal origin that closely parallels human OS [216]. It is estimated that 10,000 dogs and 1,000 children per year develop OS in the United States [214, 217, 218]; the majority of children diagnosed are in their second decade of life [216]. The peak incidence of canine OS occurs primarily in middle-aged to older animals, with a median age of 7 years; although, a bimodal age distribution is reported with a second small peak at 18 to 24 months [219]. Approximately 75% of OS occurs in the appendicular skeleton [213, 220]. Analogous to humans, the metaphysis of long bones is the most common primary location, with the forelimbs affected twice as commonly as the rear limbs [221]. The most frequent anatomical sites are the distal radius (35%) and proximal humerus (18%) followed by the distal femur, proximal tibia, and distal tibia [213, 221]. Osteosarcoma is typically a cancer of large and giant breed dogs with only 5% of tumors occurring in dogs weighing less than 15 kilograms, the majority of which originate in the axial skeleton [222]. The precise etiology of both canine and human OS is unknown. Genetic predispositions have been documented in humans, such as Li-Fraumeni, Rothmund-Thomson and Bloom syndromes, and suspected in dogs, based on breed and size prevalence [214, 216]. Additionally, exposure to ionizing radiation and sustained microtrauma (ex. repetitive weight bearing stresses, metallic implants) has been proposed as possible risk factors in dogs for OS development [223-227].

Canine OS is a locally aggressive and invasive neoplasm that causes osteolysis, aberrant bone production, or both processes concurrently [214]. Dogs affected with appendicular OS
typically present with progressive lameness and a firm swelling at the primary site. Pain can be a significant problem and is likely induced by disruption of the periosteum due to lysis of cortical bone and tumor extension from the medullary canal [214]. Pathologic fracture may occur and is usually associated with acute non-weight-bearing lameness [228]. The pulmonary parenchyma is the most frequent site of metastasis [219], and regrettably, despite local control, most dogs (90%) will go on to eventually die of metastatic disease [213, 229].

2.8.1 Current Local Therapy for Osteosarcoma

The local effects of OS have a significant impact on patient mobility and quality of life, and thus, addressing the primary tumor is one of the major goals of OS therapy. Effective local therapy for canine OS necessitates the removal or killing of malignant osteoblasts [214] and various treatment modalities have been employed to this end. Additionally in the palliative setting, therapies aimed at counteracting local consequences, such as malignant osteolysis and pain, play a major role in disease management. The following discussion will focus on the advantages and disadvantages of current local therapies for canine OS.

Surgery

Surgical resection of the primary tumor followed by either a platinum- or doxorubicin-based chemotherapy protocol generally results in the longest median survival times [230-234]. For appendicular OS, surgical options include amputation or limb-sparing procedures. High amputation of the affected limb is the standard local treatment, and most dogs function well after this procedure, retaining good mobility and quality of life [214, 235]. An advantage of amputation is that it usually ensures complete local tumor removal. However, in cases where severe preexisting conditions exist, such as obesity, orthopedic or neurological disease, limb amputation may not be a viable option [214]. Additionally, this surgery may be undesirable to owners for cosmetic or psychological reasons. In select cases, a limb-sparing surgery may be an alternative to amputation, in which the affected bone is resected and replaced by a normal bone allograft, metal endoprosthesis, or other less common methods [214, 236]. Overall, outcome has been acceptable following limb salvage, with approximately 80% of dogs
experiencing good to excellent limb function [237]. A disadvantage of limb-sparing surgery is the relatively high rate of local complications including recurrent disease, construct failure, and post-operative infection [236]. In dogs, infections rates ranging from 40-60% have been reported, although quality-of-life and limb functionality were typically acceptable when infection was adequately controlled with systemic antibiotics [236-238]. Other drawbacks include the necessity for stringent case selection (i.e. distal radius, less than 50% bone involvement) to produce good results, and that access to this procedure is limited by surgical expertise, financial resources, and owner dedication [214].

Radiation Therapy

Radiation therapy (RT) for OS management may be utilized in the setting of palliative- or curative-intent therapy. The goal of palliative radiation therapy (PRTT) is to decrease pain and lameness associated with OS, while inducing minimal to no radiation-induced adverse effects [239, 240]. Although the exact mechanism is unknown, alleviation of clinical signs is proposed to be the result of disruption of inflammatory cells, decreased progression of tumor-induced osteolysis, and reduction of tumor size [239, 241]. In dogs that are not good surgical candidates (i.e concurrent disease or advanced stage) or in which surgery is declined, PRTT is achieved with radiation protocols delivering multiple large fractions to the primary tumor. Various reported protocols, delivering anywhere from 2-4 fractions and a total dose of 16-30 Gray (Gy) with or without concurrent chemotherapy, have resulted in similar short term outcomes with response rates and median onset to pain relief in the range of 74-93% and 11-21 days, respectively [242-248]. Unfortunately, the duration of response to PRTT is limited, lasting a median 53-103 days, and survival tends to be restricted by patient comfort (approximately 2-3 months) [242-248].

Curative-intent radiation therapy for the management of canine OS has also been described in a number of settings including curative intent fractionated external beam protocol (CI-F), extracorporeal intra-operative irradiation (IORT), stereotactic radiosurgery (SRS), and the gamma knife [249-252]. CI-F radiation therapy has been reported only twice with limited success, offering no substantial improvement in outcome over palliative protocols [251, 253]. Limb-sparing with extra-corporeal IORT allows for a single 70 Gy fraction to be safely delivered
to the tumor during surgery while sparing adjacent normal and radiosensitive soft tissue structures [250]. This technique has been reported for distal radius, proximal humerus, and distal tibia [250, 254], and results in clinical improvement with good to excellent limb function in 77% of dogs [250]. When combined with chemotherapy, survival was comparable to other definitive strategies (MST- 298 days); however, post-operative complications were common (69-100%), including infection, fracture, and implant failure [250, 254]. SRS and the gamma knife are types of RT that utilize multiple, focused beams of radiation to deliver an entire dose in 1-3 treatments while minimizing damage to the surrounding tissues. A major advantage of these techniques are their extreme accuracy of delivery and steep dose gradient, resulting in a possibly greater biological effect in a single dose, all while remaining well-tolerated [239, 249, 252]. When combined with chemotherapy, overall survival times appear similar to standard of care therapies (MST 363 days), although a small number of dogs have been treated [249, 252].

Major disadvantages of SRS and the gamma knife include the need for highly specialized equipment/ personnel and careful case selection, as dogs with metastatic disease, advanced osteolysis, and large tumor volumes (> 5 cm in diameter) are not good candidates [239]. Additionally in theory, patients may be prone to bone fracture as radiation therapy is not selective for tumor cells and normal osteoblasts and stromal elements would also be affected.

Bone-seeking radioisotopes are another method of radiation delivery to the tumor. The most commonly reported radioisotope used in canine and human OS is samarium-153-lexidronam [255], which is targeted to areas of increased bone metabolism by the ligand lexidronam [255]. In canine OS patients, tumor doses equivalent to 20 Gy may be deposited using low to moderate doses, and the tumor to surrounding tissue dose ratio is favorable. While modest pain relief and some delay in tumor growth have been documented, treatment outcome is not predictable [256-258]. Additionally, cost and availability may limit its use.

**Chemotherapy**

Chemotherapy agents that have demonstrated efficacy in the treatment of OS include the platinum agents and doxorubicin [214]. While chemotherapy is primarily used in the management of canine OS for the purpose of delaying onset of metastasis, it may also be
employed in local therapy as a pretreatment to amputation or limb salvage. In pediatric OS, neoadjuvant chemotherapy is used to reduce tumor volume in order to facilitate limb-sparing surgery [259] and also to assess in vivo treatment response by change in size and histologic evaluation. As assessed by percent tumor necrosis (Huvos score), histologic response to neoadjuvant chemotherapy has been shown to be an independent prognostic factor in both OS and Ewing’s sarcoma [260-265]. In veterinary medicine, studies that evaluated dogs receiving intra-arterial (IA) cisplatin prior to limb spare surgery found that cisplatin IA with or without radiation therapy induced a significantly greater percent tumor necrosis when compared with dogs receiving no pretreatment, and that percent tumor necrosis was strongly predictive of local tumor control [266, 267]. An alternative method is isolated limb perfusion (ILP), which allows for delivery of high concentrations of chemotherapy locally, and is used in people with sarcomas as the sole treatment or to downstage local disease prior to limb sparing [214]. Finally, a local polymer chemotherapy implantation can be used at the time of surgery and may delay onset of local recurrence [238, 268]. A clear disadvantage of chemotherapy used for local treatment is the potential for systemic off-target toxicities.

Pharmacologic palliation

In addition to radiation therapy, palliation of bone pain may be achieved through the use of analgesics and bisphosphonates. Aminobisphosphonates are drugs which both impede osteoclastic activity and induce osteoclast apoptosis, and therefore, are ideally suited for treating malignant bone pain and delaying progression of bone lesions [269, 270]. In canine appendicular OS, pamidronate and zoledronate have been evaluated as sole agents or in combination with radiation therapy [271-273]. In dogs receiving pamidronate alone, 28% of dogs had pain alleviation for greater than four months, lasting a median of 321 days [273]. In these dogs, significant increases in primary tumor bone mineral density were seen [273]. When combined with radiation and standardized analgesic therapy, adjuvant pamidronate did not clearly improve pain alleviation; however, a subset of dogs experienced decreased focal bone resorption in the local tumor environment [271]. On the whole, bisphosphonate drugs appear to be safe and may provide pain relief and/or delay onset of fracture in a subset of dogs [271-
More recently, concerns have arisen that pamidronate may be associated with a decreased survival time in canine OS patients; however, definitive conclusions are yet to be made [274].

2.8.2 Molecular and genetic abnormalities: evidence for aberrant apoptosis in canine OS

Recent experimental evidence suggests that aberrations in specific molecular pathways likely play a role in the pathogenesis of OS and that dogs and humans probably share common genomic imbalances [216, 275]. Many of these molecular changes result in dysfunction of upstream regulators of apoptosis, and one can begin to recognize how this may contribute to disruptions in the efficiency of the apoptotic pathway and to tumor development. In humans, alterations affecting the tumor suppressor genes, \textit{p53} and \textit{Rb} (retinoblastoma), are among the most frequently identified gene mutations in OS tumorigenesis [214, 216, 276, 277], the overall frequencies being 15-30% and 60%, respectively [278, 279]. Likewise, cytogenetic abnormalities have been demonstrated in dogs that are consistent with those previously reported in human OS, including gene dosage imbalances of known oncogenes and tumor suppressor genes [275].

Tumor Suppressor Genes

As discussed previously, the P53 protein acts as a crucial regulator of cell replication and an initiator of apoptosis through transcriptional activation of specific genes (Figure 2.10). Thus, mutations in P53 can lead to unregulated replicative capacity and promote tumor development [280]. Similar to humans, P53 mutational inactivation has been described in both spontaneous OS and \textit{in vitro} models in the dog [281-284]. The \textit{p53} gene is reported to be mutated and overexpressed, resulting in elevated levels of P53 protein in 84% of appendicular and 56% of axial cases of canine OS [285, 286]. In one study population, \textit{p53} mutations were identified in 40% of OS from all skeletal sites [287]. Additionally, mutation status correlated with significantly shorter survival times, as well as with known poor prognostic indicators, such as elevated alkaline phosphatase concentration and tumor histological grade [287].
PTEN (phosphatase and tensin homolog deleted on chromosome 10) is another important tumor suppressor gene that is mutated in a wide range of human malignancies [288]. PTEN prevents tumorigenesis through multiple mechanisms, including antagonism of the PIK3/AKT survival pathway, maintenance of genome stability, and regulation of cell migration; but also plays a role in tumor invasion and angiogenesis [289-292] (Figure 2.11). PTEN expression has been evaluated in canine OS cell lines, and four out of five expressed high levels of the phosphorylated form of AKT (an indirect indicator of aberrant PTEN expression) while three out of five were essentially devoid of PTEN protein [293]. Recently, deletion of the PTEN gene was identified as the most common copy number loss (41%) in a study of OS cases in the dog looking for breed associated genetic abnormalities [275].

Oncogenes

In addition to alterations in the tumor suppressor genes, many proto-oncogenes have been implicated in OS, although the role of these in the pathogenesis of OS remains unclear. Notable examples of oncogenes demonstrated to be overexpressed in canine and/or human OS with varying frequencies include \textit{erbB2/HER-2} (epidermal growth factor receptor 2) [294-297], \textit{c-myc} [298], \textit{c-met} [299], \textit{c-sis/PDGF} (platelet derived growth factor) [298, 300], and \textit{c-kit} [301]. Additionally, insulin like growth factor-1 (IGF-I) and IGF binding proteins (IGFBPs) have been shown to contribute to the malignant phenotype in canine and human OS [302]. IGFs are the most abundant growth factors in normal bone, and have significant effects on bone remodeling and the regulation of osteoblasts [303]. OS cells in culture have been shown to express IGF-I receptors, proliferate in response to IGF-I, and display an anti-apoptotic phenotype \textit{in vitro} [216, 304, 305]. However, in a study of dogs receiving a long acting analogue of somatostatin (OncoLAR) in addition to carboplatin, no differences were observed in primary tumor necrosis, apoptosis, or survival over chemotherapy alone, despite suppression of serum IGF levels [306].

While the complete picture regarding the role of molecular and genetic alterations in OS tumor development is still being developed, it is clear that multiple complex pathways appear to be involved [307]. Many of these pathways, when disrupted, have the potential to confer tumor cells the ability to avoid programmed cell death, and thus evidence exists that
resensitizing OS to apoptosis is a reasonable therapeutic strategy. This may be achieved by targeting the specific molecular alterations or circumventing deregulated apoptosis by reactivating downstream effectors of cell death.

2.9 Novelty of PAC-1 as a potential local treatment for OS

PAC-1 as a local treatment for OS may have theoretical advantages over currently available treatment options. For example, in cases where a patient is neither a good candidate for amputation nor limb-salvage surgery for reasons described previously, effective induction of apoptosis in malignant osteoblasts by PAC-1 could represent an alternative “curative-intent,” limb-sparing strategy. Based on evidence presented earlier, PAC-1 has several probable advantages. First, in contrast to targeted radiotherapies, such as stereotactic radiation and radioisotopes, evidence suggests that PAC-1 is selective for cancer cells [208]. Sparing of normal osteoblasts and supportive bone stromal cells would have the obvious benefit of preserving maximum bone strength, thereby minimizing the incidence of local complications such as pathologic fracture. Additionally, delivery of PAC-1 to dogs with OS is not likely to be limited by tumor location or stage or the need for specialized equipment, facilities, or expertise. Because of this, PAC-1 may be a good local treatment option for dogs regardless of confounding patient and owner limitations. A third theoretical advantage is that PAC-1 may be chemically manipulated to specifically target bone, thus limiting off-target toxicities and increasing the concentration and efficacy of drug within the OS tumor microenvironment.

2.9.1 Bone specific drug delivery: a potential strategy for prolonging bone tumor exposure to PAC-1

Bone is a unique tissue in that it is mainly composed of hydroxyapatite. In fact, two-thirds of the mineral mass in our bodies is estimated to be present in bone tissue [308, 309]. Therefore, molecules that display a preferential affinity for hydroxyapatite have the potential to seek and concentrate in bone tissue, providing a distinctive opportunity for bone-targeted therapies [309]. In reality, there are very few therapeutics that exhibit a strong natural affinity for hydroxyapatite. Thus, current approaches to designing bone-specific therapies have relied
on engineering a therapeutic agent with a bone seeking molecule—the former delivers the desired effect while the latter achieves the intended localization [309]. While multiple strategies have been investigated, approaches involving bisphosphonate conjugation appear to be the most promising and will be the primary focus here.

Bisphosphonates are a class of synthetic compounds that are structurally similar to pyrophosphate, an endogenous regulator of calcium homeostasis [310]. Because a portion of their chemical structure (the P-C-P bond) has a high affinity for calcium crystals, bisphosphonates are selectively trapped in calcified tissues, thereby inherently targeting bone tissue [308, 311]. Bisphosphonate conjugation via chemical linkage is an ideal technique for bone-specific drug delivery for the following reasons: 1) preferential binding to bones that have high-turnover rates suggests selectivity for areas of active bone remodeling (i.e. malignant osteolysis), 2) resistance to chemical and enzymatic hydrolysis imparts a long half-life and sustained release, and 3) acidic and water-soluble properties limit distribution to soft tissues, except for the kidney where excretion occurs [308, 310, 312, 313]. A major pharmacokinetic drawback to bisphosphonate conjugates is the propensity for formation of colloids or precipitates, which may lead to changes in pharmacokinetic properties resulting in possible soft tissue disposition and damage [308, 314]. Consequently, it is important that dose, mode of administration, and type of linkage be optimized. Another important consideration when designing bisphosphonate conjugates is the lipophilicity of the conjugate compound, as work in rats suggests that increased lipophilicity decreases the fractional dose of the bisphosphonate compound delivered to the skeleton after intravenous injection [315, 316]. Notably, development of nanoparticle technology has provided a cutting-edge alternative method of encompassing and “coating” a desired therapeutic agent with a bisphosphonate [317], and this may circumvent some of the aforementioned challenges in the future.

Bone-specific drug delivery systems using bisphosphonate conjugates in preclinical or clinical studies include estradiol [318-320], prostaglandin E2 [321], fluoroquinolones [322], cisplatin [323], melphalan [324], methotrexate [325], taxanes [326] and others [327, 328]. One interesting application aggressively investigated in clinical trials for palliation of bone metastases, is bisphosphonate coupled radiopharmaceuticals [329-334]. $^{153}$Sm-EDTMP is a
bone-seeking tetraphosphonate, which has been approved by the Food and Drug Administration for the treatment of painful osseous metastases in people [335]. New bisphosphonate conjugates are currently under investigation, for example 188Re(CO)3-dipicolylamine-alendronate, which has been shown to accumulate in areas of high metabolic bone activity while having low soft-tissue uptake [332]. The proposed conjugation of PAC-1 to a bisphosphonate is a rational strategy for delivering high doses of PAC-1 to bone in order to direct apoptosis within the OS microenvironment.

2.10 Conclusion

Resistance to apoptosis is one of the hallmarks of cancer development, and thus, treatments aimed at inducing apoptosis have recently been recognized as powerful anticancer tools. Because caspases are at the heart of the apoptotic process and caspase-3 is a requirement for the cleavage events that lead to cellular death, therapies directed at activating executioner caspase proenzymes may be capable of circumventing upstream disruptions and provoking apoptosis within tumor cell populations. PAC-1 is the first identified procaspase activating compound and has demonstrated promising proapoptotic and anticancer effects in vitro and in vivo. Additionally, preliminary pharmacokinetic data in dogs suggests that achievement and maintenance of a therapeutically relevant dose of PAC-1 is feasible via intravenous CRI [211]. Canine OS is a common bone cancer of dogs that is biologically similar to pediatric OS. Molecular and genetic evidence suggests that disruption in upstream regulators of apoptosis may contribute to the pathogenesis of canine and human OS. Therefore, a molecule directed at induction of apoptosis, such as PAC-1, may be a viable local treatment strategy. Methods for prolonging tumor exposure to effective drug concentrations include constant rate infusion and bone specific drug delivery. Bone specific delivery of PAC-1 via conjugation with a bisphosphonate would be expected to produce greater local tumor concentration and efficacy in addition to less off-target toxicity, and may provide advantages over current local treatment strategies. Additionally, there may be a role for PAC-1 as a combination therapy as low doses could theoretically sensitize tumor cells to apoptotic cell death by other anticancer drugs such as chemotherapy.
From a broader prospective, bisphosphonate linked PAC-1 could have a more global application in human cancer as well, particularly with regards to the treatment of malignant osteolysis and pain caused by bone metastases. It is estimated that more than 350,000 people in the United States die with bone metastasis each year and the number of affected people increases when we consider those currently living with the condition, as many patients live greater than 1 year [336]. Breast, prostate, lung, renal, and thyroid carcinoma are most likely cancers to participate in osseous metastasis, making up over 90% of the metastatic lesions seen in people [337]. Interestingly, evidence presented previously shows that certain breast, lung, and kidney cancers also have elevated concentrations of procaspase-3, and thus, should be susceptible to the anticancer activity of PAC-1. In conclusion, bone specific delivery of PAC-1 is a promising therapy, either used alone or as an adjunct to traditional treatments, for primary bone tumors and osseous metastases in people and dogs, although its safety and efficacy are not known at this time.
2.11 Figures

**FIGURE 2.1:** Final stage of Apoptosis. Note the formation of apoptotic bodies, which are then recognized and consumed by neighboring macrophages. ([http://ghr.nlm.nih.gov](http://ghr.nlm.nih.gov))

**FIGURE 2.2:** A. Caspase organization. A prodomain precedes the catalytic domain, composed of two covalently linked subunits. Sites for (auto)proteolysis at Asp residues are indicated. B. Activation mechanisms. Initiators are monomers that activate by prodomain-mediated dimerization. Executioners are dimers that activate by cleavage of intersubunit linkers. Following activation, additional proteolytic events mature the caspases to more stable forms, prone to regulation [29]. ([http://www.jbc.org/content/284/33/21777/F1.expansion.html](http://www.jbc.org/content/284/33/21777/F1.expansion.html))
FIGURE 2.3: Schematic diagram of the structure of mammalian caspases. Three major groups of caspases are presented based on a common classification scheme. Group I: inflammatory caspases; group II: apoptosis effector caspases; group III: apoptosis initiator caspases. Note the CARD, the DED, and the large (p20) and small (p10) catalytic subunits [30].

(http://www.biochemj.org/bj/384/0201/bj3840201f01.htm)
FIGURE 2.4: Binding of TRAIL to TRAIL receptor (TRAILR/DR) or Fas-L to Fas results in receptor trimerization and recruitment of FAS-associated protein with death domain (FADD) and caspase-8 to form the death-inducing signaling complex (DISC). Upon DISC formation, initiator caspase-8 is cleaved and activated. In turn caspase-8 can cleave and activate caspases-3 and BID. Active BID (tBID) binds to pro-apoptotic BAX and BAK, resulting in mitochondrial membrane permeabilization and release of cytochrome c and DIABLO (cross-talk). Caspase-3 goes on to cleave intracellular targets resulting in apoptosis and can also activate caspase-8, thereby amplifying the apoptotic signal [53]. Adapted from [http://www.nature.com/nrc/journal/v8/n10/fig_tab/nrc2465_F1.html](http://www.nature.com/nrc/journal/v8/n10/fig_tab/nrc2465_F1.html)

FIGURE 2.5: Death Effector Domain (DED) containing c-FLIPs are compared to FADD and caspase-8/10. Structural homology domains are indicated. Note the pseudo-caspase domain of c-FLIP, [83].
FIGURE 2.6: Mitochondrial Pathway. The core of the pathway is the process of mitochondrial outer membrane permeabilization (MOMP), mediated by the proapoptotic Bcl-2 family members, Bax and Bak, and inhibited by the anti-apoptotic Bcl-2 family proteins. The BH3-only proteins regulate MOMP by promoting Bax and Bak and by antagonizing anti-apoptotic Bcl-2 proteins. As a result of MOMP, proteins from the intermembrane space gain access to the cytosol. Cytochrome c triggers activation of APAF-1, leading to apoptosome formation and activation of caspase-9. Caspase-9, in turn, cleaves and activates executioner caspases 3 and 7 to orchestrate apoptosis. IAPs inhibit caspase activation; this inhibition can be reversed by IAP antagonists (e.g. Smac and Omi) also released from the mitochondria upon MOMP [89]. (http://www.sciencemag.org/cgi/content/full/310/5745/66/FIG1)
**FIGURE 2.7**: Apoptosome formation. Cytochrome c binding induces an ATP dependent conformational change in Apaf-1 that exposes its CARD domain. The CARD domains of seven Apaf-1 molecules interact to form a wheel-like heptameric structure where procaspase-9 will be recruited by CARD/CARD interactions to the hub of the apoptosome (Adapted from Colin et al. 2009) [338].

**FIGURE 2.8**: XIAP interacts with caspase-3 and -9. XIAP is depicted with BIR1 at the N-terminus, and the RING domain at the C-terminus. The caspases are shown in their dimeric structure, with large subunits in grey, small subunits in blue, and active-site substrate pockets (S-designation) as yellow dots. Inhibition of caspase-3 (and caspase-7) is achieved mainly by docking of the 'hook' and 'sinker' regions between the BIR1 and BIR2 domain. The BIR3 domain is used to inhibit caspase-9 [146].

(\url{http://www.nature.com/nrm/journal/v3/n6/fig_tab/nrm830_F3.html})
FIGURE 2.9: Mechanism of Action of PAC-1. PAC-1 activates procaspase-3 and induces apoptotic cell death through the chelation of inhibitory zinc ions (scs.uiuc.edu/~phgroup/research1.html).

FIGURE 2.10: P53 expression leads to growth inhibition and apoptosis. Following DNA damage, p53 expression induces the cyclin kinase inhibitor p21, resulting in cell cycle arrest, and bax, which induces apoptosis. The p53 pathway is linked to Rb through p21, which inhibits cyclin dependent kinase 4 and promotes cellular proliferation through inactivation of Rb [152].
FIGURE 2.11: PTEN negatively regulates signal transduction and cancer development. A. PTEN negatively regulates PI3K activity in the AKT signaling pathway, preventing excessive cell proliferation. B. In the absence of PTEN, increased activity of AKT leads to activation of mTOR and a subsequent increase in cell division. Checkpoints that would usually halt division of aberrant cells are overridden, leading to an accumulation of mutations and possibly malignant transformation (Nature 2006) [339].
CHAPTER 3  
INVESTIGATING A PROCASPASE ACTIVATOR, PAC-1, IN CANINE, MURINE, AND HUMAN OSTEOSARCOMA CELL LINES

3.1 Introduction

Apoptosis is a specialized, active form of cell death that is important in both health and disease. It is responsible for maintaining regulated development and tissue homeostasis and participates in the elimination of aged, damaged, or displaced cells [1-4]. Apoptosis is also an inherent tumor suppressive mechanism, and the ability of cancer cells to evade death is widely acknowledged as one of the hallmarks of cancer development [3, 4, 7, 8]. In tumor cell populations, disruptions in apoptosis commonly occur through modulation of key players in the apoptotic cascade—particularly inactivation or downregulation of pro-apoptotic proteins and overactivation or upregulation of anti-apoptotic proteins [167-169].

Classically, there are two main pathways which trigger apoptosis, both of which revolve around the activation of a key family of cysteine proteases, called caspases, which cleave cellular substrates after C-terminal aspartate residues [3-5, 32, 45]. The extrinsic pathway is initiated by ligation of death receptors, which leads to engagement of adaptor proteins and formation of the death inducing signaling complex (DISC) [39, 46]. The result is recruitment and activation of the initiator caspase-8 [68]. The intrinsic pathway, also known as the mitochondrial pathway, is activated by cellular stress and mediated by pro-apoptotic Bcl-2 family members’ induction of mitochondrial outer membrane permeabilization (MOMP) [52, 57, 89, 90]. Following MOMP, cytochrome c release into the cytoplasm leads to assembly of the apoptosome, which in turn recruits and activates the initiator caspase-9 [91, 125, 126]. Ultimately, both pathways converge at the cleavage of procaspase-3 to caspase-3, the key “executioner” caspase that cleaves hundreds of cellular substrates, leading to cell death [69, 127-129].

Caspases exist in the cell as zymogens, where the proteolytic activity of inactive executioners is restrained by a linker region. During activation, cleavage at specific Asp residues divides the amino acid chain into active p12 and p17 catalytic subunits [29, 31, 32].
Cancer cells often have upstream defects in apoptotic machinery that prevent efficient procaspase activation [3, 4, 7, 8, 30, 52]. Because the activation of caspase-3 is a committed and shared pathway for apoptosis, compounds that directly activate procaspase-3 have the potential to bypass these defects and restore effective apoptosis. Additionally, procaspase-3 levels are elevated in some cancers suggesting theoretical selectivity for tumor over normal cells [198-204, 208]. This, along with the pivotal and downstream location of procaspase-3 in the apoptotic cascade, makes the direct activation of procaspase-3 with a small molecule an attractive, novel anti-cancer therapy [208, 340].

The discovery of Procaspe-Activating Compound 1 (PAC-1), an ortho-hydroxy N-acyl hydrazone, was reported in 2006 by Putt et al. [208, 210]. PAC-1 enhances the enzymatic activity of procaspase-3 in vitro by chelation of inhibitory zinc ions from the “labile zinc pool” allowing auto-maturation of procaspase-3 to its active form [208-210]. It also has been demonstrated to induce apoptosis in cancer cells and shown efficacy in multiple murine tumor models [208]. PAC-1 has been safely administered to mice and research dogs at doses that give serum concentrations of ~10 μM for 48 hours; however, neuronal hyperexcitation is dose-limiting and becomes a significant concern at higher serum concentrations [211, 341, 342]. Because of this undesirable side effect, administration of native PAC-1 as a sole agent is problematic, and targeting strategies which generate high concentrations of PAC-1 within the immediate tumor microenvironment or PAC-1 derivatives with attenuated neuronal hyperexcitation characteristics are likely to be more viable therapeutic options. Other potential strategies include increased duration of exposure to lower, tolerable doses of PAC-1 and use of procaspase activating compounds as a low dose, sensitizing agent for other chemotherapeutics. Increasing duration of exposure may be achieved through constant rate infusion or hydroxyapatite binding (bone delivery) strategies. Recently, a sulfonamide containing derivative, S-PAC-1, was investigated in mice and in pet dogs with spontaneously occurring lymphoma, and found to be well tolerated and effective at reducing or stabilizing tumor growth in 4 out of 6 patients without induction of neurotoxicity [341]. These findings support the investigation of targeted procaspase-3 activating strategies, which may provide greater therapeutic benefits with less off-target toxicity.
Osteosarcoma (OS) is the most common primary bone tumor of dogs, accounting for approximately 85% of malignancies arising in the skeleton [212-215]. Malignant osteolysis is the major presenting complaint and cause of patient morbidity [214], and surgical removal of the tumor is not always a viable option due to patient and owner considerations [214]. Effective palliative strategies for bone pain are limited. For example, stereotactic radiosurgery (SRS) is highly effective but costly and requires specialized equipment [239, 249, 252]. Response to bone seeking radioisotopes is unpredictable and its use limited by availability [257, 258, 343], and palliative radiation and bisphosphonates, while widely available, are constrained by the relatively short durability of clinical responses [242-248, 273]. Thus, novel localized therapies are clinically justified for canine OS, making this a reasonable model to investigate a targeted procaspase-3 activating drug.

The primary goal of the present study was to investigate PAC-1’s pro-apoptotic effects in canine, murine, and human OS cell lines. Our study hypothesis was that biologically tolerable concentrations of PAC-1 could induce apoptosis in OS cells in a manner consistent with the known mechanism of action of PAC-1.

3.2 Materials and methods
3.2.1 Cell lines

Four canine OS cell lines (HMPOS, Abrams, Cos31 and D17), two murine OS cell lines (K7M2 and DLM8), and two human OS cell lines (MG63 and 143B) were used for experimentations. The MCF7, a human breast cancer cell line known to be negative for the expression procaspase-3, was used as a negative control. The cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and plasmocin (Invitrogen, Carlsbad, CA). All adherent cell cultures were grown in cell culture plates (Corning, Inc., Corning, NY) and were maintained at 37°C in 5% CO₂ and passaged twice weekly or more frequently if needed to maintain cell viability. When needed for experimentation, cells were harvested and counted with a hemacytometer. A trypan blue dye exclusion test was performed on each respective cell line
prior to experimentation to ensure high viability (> 90%) (Hazleton Research Products, Denver, PA).

3.2.2 Reagents and antibodies

A monoclonal rabbit anti-caspase-3 (8G10) antibody (Cell Signaling Technology, Danvers, MA), which recognizes both intact procaspase-3 and large fragment (p19/17) of caspase-3, was used as a primary antibody for western blotting. As a secondary antibody, a donkey anti-rabbit IgG:HRP antibody (GE Healthcare Amersham Piscataway, NJ) was used in conjunction with a commercially-available Enhanced Chemiluminescent detection kit (Pierce, Rockford, IL). PAC-1 was synthesized and generously provided by the Hergenrother Laboratory (University of Illinois, Department of Chemistry). For in vitro experimentations, PAC-1 was dissolved in 99.5% DMSO (Sigma, Saint Louis, MO) at a 10 mM stock concentration and kept frozen at -20°C until needed.

3.2.3 In vitro analysis of procaspase-3 expression

Western blot was performed for detection of the target, procaspase-3, in canine, murine, and human OS cell lines. After incubation, OS cells were harvested from cell culture at 60-70% confluence. Media was discarded; cells were washed once in neutral phosphate buffered saline (PBS) and incubated with 1 ml 0.25% Trypsin (Thermo Scientific, Logan, UT) for 5 minutes. Fresh medium was added and cell suspensions were collected in conical tubes, centrifuged, and washed once with neutral PBS. For protein extraction, cells were re-pelleted, incubated with an appropriate quantity of Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL) and protease inhibitor cocktail (Thermo Scientific, Rockford, IL) (100:1) and shaken at room temperature for 15 minutes. Following incubation, cell lysates were centrifuged and the supernatant was collected. Protein quantification was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions.

For analysis by SDS-PAGE electrophoresis, 50 µg of protein from each sample was boiled in Laemmli’s sample buffer with 2-mercaptoethanol reduction at 95°C for 5 minutes. The samples were then briefly placed on ice and loaded in 4–15% Mini-PROTEAN® TGX™ Precast
polyacrylamide gels (BioRad Hercules, CA) in SDS-PAGE electrophoresis buffer. Precision Plus Protein™ WesternC™ (BioRad Hercules, CA) was used for the protein standard, and gels were run at 80V until completion. Proteins were then transferred to the Millipore PVC membrane using the Mini Trans-Blot (BioRad Hercules, CA) at 53V for 45 minutes. For detection of procaspase-3 protein, the membrane was blocked in 5% milk (in TBS-T) for 1 hour, washed three times with TBS-T, and then incubated with the primary antibody at a 1:500 dilution at room temperature for 1 hour. The membrane was then washed and incubated with secondary antibody at a 1:1000 dilution for 1 hour and digitally developed using a BIO-RAD ChemiDoc XRS+ System.

3.2.4 In vitro PAC-1 target modulation studies

Cells were initially cultured in cell culture plates (Corning, Inc., Corning, NY) for target modulation assays. When cells reached 50-60% confluence, the media was discarded and the cells were washed once with neutral PBS. Fresh medium containing varying concentrations of PAC-1 was added to cell culture plates at 0, 3, or 10 µM of PAC-1 for either 12, 24, or 48 hours of exposure at 37°C in 5% CO₂. For analysis of target modulation, protein was collected and western blot was performed as indicated previously to qualitatively determine changes in full length procaspase-3 and cleaved caspase-3 expressions.

3.2.5 In vitro caspase-3 activation

A caspase-3 activation study was performed exposing the HMPOS cell line to vehicle control or 10 µM of PAC-1 for 24 and 48 hours at 37°C in 5% CO₂. In experiments, cells that were not exposed to PAC-1, but were exposed to DMSO, served as a vehicle control population. 200,000 cells per time point were analyzed and all tests were performed in triplicate. The evaluation of active caspase-3 was analyzed using a caspase-3 colorimetric assay (R & D Systems, Minneapolis, Minnesota). The assay was performed according to manufacturer’s instructions. Briefly, once the cells were exposed to the PAC-1 and centrifuged, the media was discarded and the cell pellet was lysed with a provided lysis buffer. The cell lysate was incubated on ice for 10 minutes, centrifuged, and the supernatant preserved. The supernatant
was aliquoted to a 96 well microplate (Fisher Scientific, Pittsburgh, Pennsylvania) with 50 µL in each well, with 5 µL caspase-3 colorimetric substrate (DEVD-pNA) and a reaction buffer added. The plate was incubated at 37°C for 2 hours. The plate was read on a Bio-Tek EL-800 microplate reader using a 405 nm wavelength. All values were expressed as a fold increase over vehicle-treated control cells.

3.2.6 In vitro PAC-1 cytotoxicity studies

For cytotoxicity assays, HMPOS cells were also initially cultured in cell culture plates. After incubation, the cells were harvested and seeded into 12-well plates at 50,000-75,000 cells/well, and allowed to adhere overnight. Fresh medium containing varying concentrations of PAC-1 was added to respective wells at 0, 1, 3, and 10 µM of PAC-1 for 48 hours of exposure at 37°C in 5% CO₂. All conditions were performed in triplicate. For analysis of cytotoxicity, an Annexin V-FITC PI apoptosis detection kit (BD Pharmingen, San Diego, California) was used according to the manufacturer’s directions. Briefly, once the cells had incubated under their respective conditions, the cells were harvested and washed twice in previously reconstituted cold high calcium binding buffer (Annexin Binding Buffer, BD Pharmigen, San Diego, California). The analysis was performed in triplicate with 100,000 cells per flow tube. Next, 5 µL of FITC, 5 µL PI, and 100 µL of high calcium binding buffer were added to each flow tube and gently vortexed. The cells were then allowed to incubate at 4°C in the dark for 15 minutes. 400 µL of cold high calcium binding buffer was added to each flow tube and the cells were analyzed via a Coulter flow cytometry within one hour.

3.2.7 Statistical analysis

Differences in caspase-3 enzymatic activity in HMPOS cells treated with vehicle or PAC-1 at 10 µM for either 24 or 48 hours were compared using the Student’s t-test. Analyses were compared based upon treatment agent (vehicle or PAC-1) for the 2 different incubation durations (24 and 4 hours). The effect of PAC-1 concentration (1-10 µM) for 48 hours of incubation on HMPOS cell viability was compared with a 1-way ANOVA test. If groups were identified as being different, a Dunnett’s post hoc test was performed to identify which
concentration of PAC-1 (1-10 µM) differed from the vehicle treated group. Significance was defined as p < 0.05.

3.3 Results

3.3.1 Procaspase-3 is expressed in osteosarcoma cell lines

Immunoblotting analysis showed that the canine (Abrams, HMPOS, Cos31, D17), murine (K7M2, DLM8) and human (143B, MG63) OS cell lines all express procaspase-3, indicating that the commercial antibody for procaspase-3 is species cross-reactive and that there is homologous protein conservation of the procaspase-3 target among the species in question. Expression of the 35 kDa protein was detected in all cell lines, but not in the negative control MCF7 (Figure 3.1). Notably, in the canine cell lines, procaspase-3 appeared as a double band rather than a single band, as is identified for human and murine procaspase-3.

3.3.2 Incubation with PAC-1 results in modulation of the target procaspase-3 to cleaved caspase-3

The concentration of PAC-1 assessed in vitro was chosen based on unpublished maximum-tolerated dose data from canine studies performed in the Cancer Care Clinic through the conductance of a Phase I study in dogs with multicentric lymphoma. From these in vivo studies, serum concentrations of PAC-1 maintained below 15 µM for 24-48 hours were biologically tolerable. Thus, 12, 24 or 48 hour incubations were performed with 3 or 10 µM concentration of PAC-1, and target modulation assessed in 3 representative species cell lines (human-143B, murine-K7M2, and canine-HMPOS). Subsequently, western blotting was used to detect the expression of procaspase-3 and its active, cleaved counterpart, caspase-3, following PAC-1 exposure. Incubations with both 3 and 10 µM concentrations of PAC-1 resulted in modulation of the target procaspase-3 in all cell lines and automaturation to its active form (Figure 3.2). This was supported by decreasing procaspase-3 band intensity with increasing length of incubation and concentration of PAC-1. Simultaneously, increasing intensity of the fully processed 17 kDa fragment of cleaved caspase-3 was also seen in a dose and time dependent manner (Figure 2.2). These changes were not noted in OS cell lines incubated with
media alone or DMSO vehicle. Of note, the detection of the 17 kDa fragment of cleaved caspase-3 was less intense at 48 hours in comparison with 24 hours in both murine and human OS cell lines exposed to 10 µM of PAC-1.

3.3.3 Incubation with PAC-1 increases caspase-3 activity in HMPOS cells

To confirm that the modulation of procaspase-3 to caspase-3 observed by western blot analysis produced proteolytically active caspase-3 in the HMPOS cell line, an enzymatic methodology to detect changes in cytosolic caspase-3 activity was used. HMPOS cells were incubated with either DMSO vehicle or PAC-1 at 10 µM for 24 and 48 hours, and cytosolic fractions collected and mixed with a colorimetric caspase-3 substrate. Following 24 hours of incubation, there was no significant difference in percent fold increase of cytosolic caspase-3 activity between vehicle control and PAC-1 treated HMPOS cells (100 ± 9.5% and 93.0 ± 12.2%, respectively; p=0.59). However, upon 48 hours of exposure, PAC-1 at 10 µM resulted in a significant percent fold increase in cytosolic caspase-3 activity (~50%) compared to vehicle control (153.4 ± 6.9% versus 100 ± 0.4%, respectively; p=0.008) (Figure 3.3). The demonstration of augmented caspase-3 activity after 48 hours of exposure to PAC-1 at 10 µM in comparison with vehicle control treated cells is complementary to the findings demonstrated by western blot analysis showing the conversion of procaspase-3 to the 19 kDa subunit of caspase-3 after 48 hours of PAC-1 exposure (Figure 3.2).

3.3.4 Incubation with PAC-1 induces apoptosis in the canine HMPOS cell line

Given the observed processing of procaspase-3 to caspase-3 by western blot analysis, in conjunction with the identification of increased caspase-3 enzymatic activity induced by PAC-1, the biologic consequences of procaspase-3 activation by PAC-1 was investigated in the canine HMPOS cell line. Specifically, it was to be determined if procaspase-3 activation by PAC-1 demonstrated by western blot and enzymatic activity studies would result in cell death via the induction of apoptosis. Experimentally, HMPOS cells were exposed to varying low concentrations of PAC-1 ranging from 1-10 µM for 48 hours, and the percentage of cells categorized as viable and early apoptotic were quantified. Viable cells were defined as being
negative for both Annexin V FITC and propidium iodide staining, while early apoptotic cells were defined as being positive for Annexin V FITC staining, but negative for propidium iodide staining. Viability of HMPOS cells remained above 92% for cells incubated with media only, vehicle, and PAC-1 at 1 and 3 µM, with no statistical differences identified (Figures 3.4 and 3.5). However, when HMPOS cells were exposed to PAC-1 at 10 µM for 48 hours, a dramatic reduction in cell viability percentage was observed with an average viability of 9.9 ± 1.0% ($p<0.001$ when compared with media or vehicle control treated cells).

The loss of cell viability was further characterized as being most consistent with the early stages of apoptosis based upon the identification of HMPOS cells which stained positively for Annexin V FITC, but negative for propidium iodide (Figures 3.4 and 3.6). Exposure to PAC-1 at 10 µM for 48 hours exerted a significant and potent pro-apoptotic effect on HMPOS cells, with 87.7 ± 1.6% cells staining positive for Annexin V FITC only, in comparison with basal apoptosis percentage identified in HMPOS cells incubated with media alone, 1.3 ± 1.1%; $p<0.01$.

In addition, a significant increase in apoptotic cells was also observed with PAC-1 at 3 µM for 48 hours, with 3.7 ± 0.6% of HMPOS cells labeling with only Annexin V FITC (Figure 3.6).

### 3.4 Discussion

This is the first report to specifically investigate procaspase-3 expression in OS cells and the action of PAC-1, a procaspase activating compound, on these cells. The present study confirms the expression of procaspase-3 in canine, murine, and human OS cells and demonstrates that PAC-1 is capable of converting procaspase-3 to caspase-3 and subsequently inducing apoptosis in canine OS cell lines. This occurs as a result of increased caspase-3 activity as expected based on the reported mechanism of action of PAC-1.

Prior to investigating the use of PAC-1 as a viable anticancer strategy against canine OS, it was important to first demonstrate the presence of the target procaspase-3 in OS cell lines. All OS cell lines evaluated (canine, murine, and human) expressed the target procaspase-3, indicating homologous protein conservation among the species investigated. To the author’s knowledge, no studies have specifically examined the expression of procaspase-3 in OS cells. However, our results support the findings of other investigations that have suggested caspase-3
mediated induction of apoptosis in OS cell lines following exposure to a pro-apoptotic compound [344, 345].

Notably, the present findings indicate that the relative cellular quantities of procaspase-3 as subjectively determined by western blot analysis (band darkness) is greater in human and murine cell lines when compared to canine cell lines. It is possible that this apparent difference in cellular target is due to suboptimal antibody cross-reactivity against canine procaspase-3. Alternatively, procaspase-3 might truly be reduced in canine OS cell lines in comparison with murine and human cell line counterparts. If the latter is true, this could suggest that procaspase-3 activating strategies might be more effective in the treatment of OS developing in humans and mice, than in dogs. Furthermore, our studies consistently identified the presence of a double band for procaspase-3 in all canine OS cell lines. Plausible reasons for the observed protein doublet include a variance in antibody specificity that allows cross-reactivity with another protein of a similar molecular weight or the existence of an alternate procaspase-3 splice variant in dogs.

Following demonstration of the target, we investigated the ability of PAC-1 to induce the conversion of procaspase-3 to its active form, cleaved caspase-3. Our findings indicate that exposure to low, biologically relevant concentrations of PAC-1 (3 and 10 µM) promote the automaturation of procaspase-3 to caspase-3 in all cell lines investigated (HMPOS, K7M2, 143B). Western blot analysis supported both a time and dose dependent effect of PAC-1 on target modulation, as demonstrated by greater decreases in procaspase-3 with concurrent increases in cleaved caspase-3 following longer durations of exposure and higher concentrations of PAC-1. Importantly, the findings from this study suggest that for lower concentrations of PAC-1 to exert biologic effects, a concurrent increase in exposure time is necessary to activate procaspase-3. The relationship between exposure duration and concentration, in context with PAC-1’s neuroexcitatory potential, emphasizes the requirement for delivery strategies that will result in prolonged exposure durations with low concentrations of PAC-1 within the bone tumor microenvironment for the safe and effective management of primary bone OS.
Although a time-dependent effect of PAC-1 on procaspase-3 modulation was identified between 12 and 24 hours, an exception to this time-dependent trend was observed following 48 hour incubation with PAC-1, in which lesser amounts of both the partially (p19) and fully processed (p17) large subunit of cleaved caspase-3 were observed. Although these findings appeared counter-intuitive, several potential explanations exist for this observation. The first is ubiquitin-mediated proteasome degradation of caspase-3 following PAC-1 activation. Inhibitor of apoptosis proteins (IAPs) can mediate this degradation by directly binding caspases and acting as ubiquitin E3 ligases, and evidence suggests that this might be enhanced in cancer cells, thus contributing to apoptotic resistance [346, 347]. An alternative explanation for this observation is the temporal depletion of procaspase-3 as a substrate for conversion to caspase-3, which could be due to either decreased viability of the cells at latter time points (48 hours) or procaspase-3 substrate exhaustion.

Another interesting finding of the present study was that the canine HMPOS cell line appeared more resistant to PAC-1 activation in comparison with murine (K7M2) and human (143B) OS cells. This supposition is based on the predominance of the 19 kDa fragment rather than the 17 kDa fragment of caspase-3 identified by western blot analysis in HMPOS cells, but not in either K7M2 or 143B cells. The reduced capacity of PAC-1 to process procaspase-3 to the fully activated 17 kDa caspase-3 fragment in HMPOS cells could be explained by greater inherent expressions of counter-regulatory IAP in comparison with the murine K7M2 and human 143B OS cell lines.

Because western blot only detects the presence of protein and not its enzymatic functionality, it was necessary to confirm that the modulation of procaspase-3 to caspase-3 observed by western blot coincided with production of proteolytically active caspase-3. We found that 48 hours of exposure to PAC-1 at 10 µM in HMPOS cells resulted in a significant increase in cytosolic caspase-3 activity (~50%) compared to vehicle control. In contrast, there was no significant difference in cytosolic caspase-3 activity after a 24 hour incubation of 10 µM PAC-1 in HMPOS cells. Our findings suggest that the induction of catalytic activity requires the achievement of minimal time- and concentration-dependent thresholds in order for PAC-1 to activate procaspase-3. Because high concentrations of PAC-1 are associated with neuronal
hyperexcitation, viable therapeutic strategies necessitate the institution of low, but temporally sustained (48 hours) PAC-1 dosing protocols. This requirement will likely impact the therapeutic institution of PAC-1 clinically. Dogs would either need to be hospitalized for 48 hours for constant rate infusion of PAC-1 or a bone-targeted delivery strategy would need to be developed to prolong residence of the PAC-1 within the bone tumor microenvironment.

Given the observed processing of procaspase-3 to caspase-3 in conjunction with the identification of increased caspase-3 enzymatic activity induced by PAC-1 at 48 hours, we sought to determine if procaspase-3 activation by PAC-1 would result in cell death via induction of apoptosis in the HMPOS cell line. When cells were incubated with PAC-1 for 48 hours, a clear dose dependent effect was observed. A dramatic decrease in viability was noted at the 10 µM concentration confirming the significant and strong proapoptotic effects of PAC-1 at this concentration. Notably, the loss of cell viability at 10 µM was accompanied by nearly all HMPOS cell staining positively for Annexin V FITC and negatively for propidium iodide. The appearance of nearly all of the cells within the Annexin V FITC gate suggests that the major mechanism of cell death for PAC-1 is apoptosis, not necrosis. A small but significant increase in apoptotic cells was also observed at the 3 µM concentration, but there was almost no effect at concentrations at or below 1 µM.

In summary, the present findings indicate that procaspase-3 is expressed in canine OS cells and that the target is conserved among the species investigated. When OS cells were exposed to low, biologically relevant concentrations of PAC-1, conversion of procaspase-3 to active caspase-3 was observed. Furthermore, PAC-1 induced caspase-3 catalytic activity and apoptosis in canine HMPOS cells, a finding which was most pronounced after 48 hours of exposure to PAC-1. Based on these results, further investigations of the use of PAC-1 as a treatment for canine OS are warranted. Future studies should focus on delivery strategies that would allow for prolonged exposure of the bone tumor microenvironment to PAC-1 in vivo.
FIGURE 3.1: Procaspase-3 is expressed by human, canine, and murine osteosarcoma cell lines. Lane 1 represents MCF7, a human negative control. Lanes 2-3 are human osteosarcoma cell lines, 4-7 are canine osteosarcoma cell lines, and 8-9 are murine osteosarcoma cell lines. Note the band appearing at the characteristic molecular weight for procaspase-3 for all cell lines, and the double band for all canine cell lines.
FIGURE 3.2: Procaspase-3 target is modulated by PAC-1 in human, murine, and canine osteosarcoma cell lines. Human: The 143B osteosarcoma cell line was incubated with vehicle control (VC) media, or PAC-1 at 10 µM for 12, 24, and 48 hours. A time dependent decrease was seen in the band intensity of procaspase-3 at this dose. The fully processed 17 kDa (large fragment) of cleaved caspase-3 peaked at 24 hours and then declined again at 48 hours. Murine: The K7M2 osteosarcoma cell line was treated as described for 143B. A similar trend was seen with a dose dependent decrease in procaspase-3 and a peak in the large fragment of cleaved caspase-3 at 24 hours. Canine: The HMPOS osteosarcoma cell line was incubated with vehicle control (VC) media or 3 or 10 µM PAC-1 for 24 and 48 hours. A small decrease in procaspase-3 and an increase the large fragment of cleaved caspase-3 was seen at 24 hours for the 10 µM dose and 48 hours for both the 3 and 10 µM doses.
FIGURE 3.3: PAC-1 at 10 µM concentration is capable of promoting procaspase-3 activation to enzymatically-active caspase-3 in HMPOS cells after 48 hours of exposure, but no difference in caspase-3 activity is detected between vehicle or PAC-1 treated cells at shorter incubation times (24 hours).

FIGURE 3.4: Flow cytometry of HMPOS cells exposed to varying concentrations of PAC-1 (1-10 µM) for 48 hours. Dot plots demonstrate that viable cells are retained in the lower left hand quadrant, and early apoptotic cells shift to the lower right hand quadrant. Significant decrease in cell viability observed with HMPOS cells are exposed to PAC-1 at 10 µM. A significant increase in early apoptotic cells is identified when HMPOS cells are incubated with PAC-1 at 3 and 10 µM for 48 hours. Significance defined as $p<0.05$. 
Figure 3.5: Bar graph represents PAC-1’s concentration dependent properties for reducing HMPOS cell viability. Significant reduction in cell viability exerted by PAC-1 at 10 µM for 48 hours, *** $p<0.001$.

Figure 3.6: Bar graph represents PAC-1’s concentration dependent pro-apoptotic effects in HMPOS cells exposed to PAC-1 for 48 hours. Significant increase in early apoptotic cells following exposure to PAC-1 at both 3 and 10 µM for 48 hours; *$p<0.05$ and **$p<0.01$. 
4.1 Introduction

Apoptosis is a crucial physiologic process important for normal cellular differentiation and development [1-4] and is characterized morphologically by cell shrinkage, cytoplasmic membrane blebbing, nuclear fragmentation and condensation, and DNA cleavage [5]. Dysregulated apoptosis contributes to the development of many diseases, including neurodegenerative disorders, autoimmune diseases, and is widely recognized as one of the hallmarks of tumor development [1, 3-7]. Apoptotic cell death is triggered by a variety of cellular stresses which tumor cells endure both during the process of oncogenesis and as a result of anti-cancer therapy [348]. It entails a complex cascade of proteolytic events that involves a family of aspartic-specific cysteine proteases called caspases as well as the activators and inhibitors of these of these proteases [34, 73, 166].

Active caspases are generated by successive cleavage of their zymogen or pro-forms, which occurs following delivery of an apoptotic signal to the cell. Initiation of the cascade can occur either through the ligation of death receptors of the tumor necrosis factor (TNF) receptor family and formation of the death inducing signaling complex (DISC; extrinsic pathway) or following the induction of mitochondrial outer membrane permeability (MOMP; intrinsic pathway) by Bcl-2 family members [1, 2]. All caspase dependent pathways ultimately converge on the activation of a common executioner caspase, procaspase-3, a downstream and crucial event that leads to cleavage of hundreds cellular substrates and cellular dismantling [128, 129, 131]. The active form of caspase-3 is generated after cleavage of the procaspase-3 at specific aspartic acid residues to generate two subunits with a molecular mass of 17 and 12 kDa which associate with each other to form active caspase-3 [32].

Since both intrinsic and extrinsic apoptotic pathways lead to the activation of caspase-3, any upstream disruption in apoptosis causing inefficient effector caspase activation may be hypothesized to lead to increased concentrations of procaspase-3 within tumor cells.
Investigations have shown that certain human cancer cell lines have elevated procaspase-3 concentrations when compared with noncancerous controls, including lung, melanoma, renal and breast cancer cell lines [198]. In addition, tumor samples from individuals with spontaneously occurring neuroblastomas [200], lymphomas [201], leukemias [202], hepatocellular carcinomas [203], and melanomas [204] have been demonstrated to have procaspase-3 elevation. In a recent study of canine lymphoma, it was also shown that canine lymphoma cells expressed procaspase-3 and that those with a higher degree of expression were more susceptible to a procaspase activating drug (PAC-1) [211].

To the author’s knowledge, the relative expression of procaspase-3 has not been studied in canine primary and metastatic osteosarcoma (OS). Therefore, the objective of this investigation was to characterize the expression of procaspase-3 in canine OS cell lines, as well as non-malignant primary canine osteoblasts, and spontaneously occurring primary and metastatic canine OS tumor samples. The study hypothesis was that canine OS cells would have greater procaspase-3 expression than normal canine osteoblasts and that procaspase-3 would be expressed to varying degrees in spontaneous canine OS.

4.2 Materials and Methods

4.2.1 Cell lines

Six canine OS cell lines (HMPOS, POS, Abrams, Cos31, D17, and Molly) were utilized for procaspase-3 quantification. Three non-malignant primary canine osteoblast cell lines were either purchased or generated de novo, including the OB934 canine osteoblast cell line provided by William Kisseberth (The Ohio State University), CnOB a commercially-available canine osteoblast cell line (Cell Applications San Diego, CA), and UIUCOB2 generated at the University of Illinois from canine trabecular bone as described below. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and plasmocin (Invitrogen, Carlsbad, CA). All adherent cell cultures were grown in cell culture plates (Corning, Inc., Corning, NY) and were maintained at 37°C in 5% CO2 and passaged twice weekly or more frequently if needed to maintain cell viability. When needed for experimentation, cells were harvested and counted.
with a hemacytometer. A trypan blue dye exclusion test was performed before each use to check for viability (Hazleton Research Products, Denver, PA).

4.2.2 Reagents and Antibodies

A monoclonal rabbit anti-caspase-3 (8G10) antibody (Cell Signaling Technology, Danvers, MA), which binds both the full length and large fragment (p19/17) of caspase-3, was used as a primary antibody for western blotting. As a secondary antibody, a donkey anti-rabbit IgG:HRP antibody (GE Healthcare Amersham Piscataway, NJ) was used in conjunction with a commercially-available ECL kit.

4.2.3 Canine osteoblast isolation

Trabecular bone samples were harvested under sterile conditions from canine proximal humeri within 12 hours of death or amputation using an 8-11 gauge Jamshidi biopsy needle. The forelimb was clipped and prepped, a skin incision was made, and the muscle and fascia was sharply dissected to expose bone. Using the Jamshidi needle, trabecular bone samples were obtained and placed directly into DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Following harvest, the bone was cut into 1-2 mm$^3$ pieces using a sterile scalpel blade (#10) and washed once in PBS. Bone pieces were then incubated for 1 hour in 4 mL of collagenase solution (2 mg of collagenase/mL of sterile filtered DMEM) at 37°C in a shaking incubator to remove soft tissue. After 1 hour, the solution was shaken vigorously by hand and then incubated an additional hour. Then, 4 mL of complete culture medium (cCM-DMEM with 10% FBS, 1% Penicillin/ Streptomycin) was added to inhibit collagenase, and the bone fragments were rinsed three times with cCM. Approximately twenty bone pieces were then transferred to a 100 mm dish and incubated in cCM. Media was changed three times per week until osteoblasts migrated off of the bone fragments and onto the dish (about 1 week). Once cells migrated onto the dish, media was replaced with osteoblast differentiation medium (DMEM with 10% FBS, 1% Penicillin/ Streptomycin, 0.2 mM ascorbic acid, 10 mM beta-glycerol phosphate, and 1 µM dexamethasone) and changed three times per week until harvest.
4.2.4 *In vitro* analysis of differential expression between canine normal osteoblasts and osteosarcoma

Western blot was performed for detection of the target, procaspase-3, in canine normal osteoblast and OS cell lines. After incubation, cells were harvested from cell culture at 60-70% confluence. Media was discarded; cells were washed once in neutral phosphate buffered saline (PBS) and incubated with 1 ml 0.25% Trypsin (Thermo Scientific, Logan, UT) for 5 minutes. Fresh medium was added and cell suspensions were collected in conical tubes, centrifuged, and washed once with neutral PBS. For protein extraction, cells were re-pelleted, incubated with an appropriate quantity of Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL) and protease inhibitor cocktail (Thermo Scientific, Rockford, IL) (100:1) and shaken at room temperature for 15 minutes. Following incubation, cell lysates were centrifuged and the supernatant was collected. Protein quantification was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions.

For analysis by SDS-PAGE electrophoresis, 50 µg of protein from each sample was boiled in Laemmli’s sample buffer with 2-mercaptoethanol reduction at 95˚C for 5 minutes. The samples were then briefly placed on ice and loaded in 4–15% Mini-PROTEAN® TGX™ Precast polyacrylamide gels (BioRad Hercules, CA) in SDS-PAGE electrophoresis buffer. Precision Plus Protein™ WesternC™ (BioRad Hercules, CA) was used for the protein standard, and gels were run at 80V until completion. Proteins were then transferred to the membrane using the Mini Trans-Blot (BioRad Hercules, CA) at 53V for 45 minutes. For detection of procaspase-3 protein, the membrane was blocked in 5% milk (in TBS-T) for 1 hour, washed three times with TBS-T, and then incubated with the primary antibody at a 1:500 dilution at room temperature for 1 hour. The membrane was then washed and incubated with secondary antibody at a 1:1000 dilution for 1 hour and developed. Membranes were stripped and reprobed for β-actin. Band intensities for procaspase-3 and β-actin were determined by measuring the number of pixels/mm² and subsequently expressed as a ratio (procaspase-3/β-actin) to allow for quantitative comparisons among different cell lines.

4.2.5 Immunohistochemical detection of Procaspsase-3 in primary and metastatic OSA tissue
Tissue sections were created from archival material of primary appendicular OS (n=20) and metastatic OS (n=6). Formalin fixed, decalcified, paraffin-embedded tissues were sectioned 3-5 microns thick and captured on charged slides using routine methods. Hematoxylin and eosin (HE) stained slides were used to confirm the diagnosis of OS in all cases. Immunohistochemistry (IHC) for procaspase-3 was performed using a rabbit monoclonal antibody (E61; Epitomics, Burlingame, CA). Heat-induced epitope retrieval was performed using an automated pressure cooker in citrate buffer (pH 6.0). Endogenous peroxidases were blocked using 3% hydrogen peroxide applied to all tissue sections for 10 minutes. Primary antibody was applied at a 1:1000 dilution and immunodetection was performed utilizing Dako Envision+Rabbit polymer and a Dako Autostainer (Dako Corporation, Carpinteria, CA) staining system. Visualization of antibody binding was obtained via DAB+ chromogen, (a 3, 3-diaminobenzidine solution) for 5 minutes; tissue section counterstaining was performed using hematoxylin for 5 minutes, followed by progressive alcoholic dehydration and placement of a coverslip. Normal canine lymph node and human tonsil were utilized as positive controls, while commercially available rabbit non-immune serum (Biogenex) was used as a negative control for each experiment.

All slides were evaluated by one pathologist (LB) using light microscopy to assess for specific labeling of neoplastic osteoblasts. Each section was scored for labeling intensity (1=light, 2=moderate and 3=intense) and the percent of labeled neoplastic cells was estimated. Tumor samples were assigned numbers according to the patient from which they were derived. All primary OS tumor samples derived from bone and all metastatic OS tumor samples were derived from lung tissue. In all patients which metastatic tumors were evaluated, a primary tumor from the same patient was also available for review. A semi-quantitative immunohistochemical (IHC) method was used to determine the level of procaspase-3 expression. The intensity of procaspase-3 staining was scored from 0 to 3, and the extent of staining scored from 1 to 4 (0-24%-1, 25-49%-2, 50-74%-3, 75-100%-4). The scores were then multiplied together and the final scores classified as follow: 0, negligible staining; 1–4, weak staining; 5-8, moderate staining; and 9–12, strong staining.
4.2.6 Statistical analysis

Differences in procaspase-3/β-actin ratio between normal (n=3) and malignant osteoblast (n=6) cell lines were evaluated with a non-parametric Wilcoxon-rank sum test. Significance was defined as \( p < 0.05 \).

4.3 Results

4.3.1 Procaspase-3 is differentially expressed in canine osteoblasts and canine osteosarcoma cell lines

Immunoblotting analysis showed that the canine (HMPOS, POS, Abrams, D17, COS31, and Molly) OS cell lines and normal canine osteoblasts express procaspase-3 as evidenced by a 35 kDa band (Figure 4.1 A). When normalized to β-actin as a ratio, the median band intensity (pixel value/area) for malignant osteoblasts (0.97; range 0.94-1.2) was significantly greater than the median band intensity for normal osteoblasts, (0.27; range 0.19-0.73) (Figure 4.1 B).

4.3.2 Procaspase-3 is expressed in primary and metastatic spontaneously occurring canine osteosarcoma

Twenty primary OS and six metastatic OS samples were analyzed. Among the samples analyzed, 73% demonstrated negligible (11%) or weak (62%) procaspase-3 expression and 27% showed moderate (23%) or strong (4%) procaspase-3 expression (Table 1). Typical examples are shown in Figure 4.2. Overall, 90% of primary and 86% of metastatic tumors demonstrated some degree of positive procaspase-3 immunostaining. Of these, 25% of primary tumors and 42% of metastatic tumors had moderate or strong procaspase-3 immunoreactivity. Notably, paired primary and metastatic tumors derived from the same patient tended to have similar procaspase-3 immunoreactivity, suggesting that procaspase-3 expression does not serve as a selective pressure during metastatic OS progression. Most importantly, there was wide variability among different OS specimens regarding their respective procaspase-3 immunoreactivity, indicating that tumors derived some patients may be more suitable for procaspase-3 activating therapies than others (Figure 4.3).
4.4 Discussion

The present study demonstrated differential expression between canine OS cell lines and normal canine osteoblasts and confirmed the expression of procaspase-3 in canine spontaneously-occurring primary and metastatic OS tissue samples.

To determine if canine OS cells may be more susceptible to procaspase activating therapies than normal canine osteoblasts, we investigated the differential expression between normal and malignant osteoblasts. Our results indicate that while normal and malignant osteoblasts express procaspase-3 by western blot analysis, the median band intensity for malignant osteoblasts was significantly greater than that for normal osteoblasts. This data corresponds with previous studies that have demonstrated a difference between normal and malignant cells. For example Putt et al. found that resected colon tumors had elevated procaspase-3 concentrations when compared with adjacent non-cancerous colon tissue from the same patient in all cases [208]. Additionally, investigations have revealed that some human cancer cell lines have elevated procaspase-3 concentrations when compared with noncancerous controls, including lung, melanoma, renal and breast cancer cell lines [198]. Preferential expression of procaspase-3 in OS cell lines over normal osteoblasts may allow selective targeting of cancerous osteoblasts with procaspase activating therapies, thereby minimizing off-target effects to normal bone.

To the author’s knowledge, this is the first report to specifically characterize the pattern and relative expression levels of procaspase-3 in primary and metastatic canine OS. A recent study evaluating immunohistochemical expression of several apoptosis regulators in canine appendicular OS found diffuse procaspase-3 cytoplasmic staining in all cases (100%) but differences in expression among tumors were not reported [349]. Our data agree with this report in that a high proportion of primary (90%) and metastatic (86%) tumors stained positively for procaspase-3 via immunohistochemistry. However, the present findings indicate a wide variability in procaspase-3 expression among tumor samples. This variability suggests that PAC-1 treatment might be more suitable and effective for dogs whose tumors express aberrantly high levels of procaspase-3 and less so for patients with lower expressing procaspase-3 tumors. Therefore, in order for PAC-1 to be rationally implemented as an
anticancer agent in canine OS, the authors recommend the prescreening of OS biopsy samples for relative procaspase-3 expression and subsequent rationale institution of PAC-1 centric therapies for patients with highest procaspase-3 expression. This recommended assertion is also supported by the findings of Putt et al. which indicate that assessment of procaspase-3 concentrations in cancer biopsies is simple and rapid and can be used to predict PAC-1 efficacy [208]. Our conclusions emphasize the concept of personalized cancer medicine, where only the dogs that are most likely to benefit from the drug would be treated with a procaspase-3 activating strategy.

Another interesting finding is that when primary OS lesions were paired with metastatic lesions derived from the same patient, paired tumors shared the same pattern of procaspase-3 expression with respect to overall percent positivity and staining intensity. This suggests that the procaspase-3 genotype is conserved between primary and metastatic cells, and there does not appear to be downregulation of the target following metastasis. This finding lends justification for the use of PAC-1 for the treatment of both primary OS as well as metastatic lesions. Although a trend was subjectively identified between primary and metastatic OS lesions with regards to procaspase-3 expression, strong conclusions cannot be stated as the current study only evaluated a limited number of metastatic tumors (n=6). Future investigations should seek to determine if this pattern of conserved procaspase-3 expression is consistent in a larger number of paired primary and metastatic samples.

In summary, the data presented herein indicate that canine malignant osteoblasts have higher levels of procaspase-3 expressions than normal osteoblasts and suggest a theoretical selectivity of procaspase-3 activating strategies for malignant cells. Furthermore, procaspase-3 is variably expressed in canine OS samples and the degree of expression appears to be conserved between paired primary and metastatic lesions derived from the same patient. These study results underscore procaspase-3 as a viable drugable target in canine OS and lends justification for the use of PAC-1 as a personalized anticancer strategy, especially in OS lesions demonstrating robust procaspase-3 expressions.
4.5 Figures

**Figure 4.1:** A) Both malignant and normal osteoblasts express procaspase-3. Western blot was performed on canine osteosarcoma and normal osteoblast cell lines, and revealed a band at the characteristic molecular weight for procaspase-3 (35 kDa). B) The pixel value/area was normalized to β-actin (ratio). The median band intensity ratio (procaspase-3/β-actin) was found to be significantly greater for malignant than normal canine osteoblasts, $p=0.02$. 
Table 1: Relative procaspase-3 immunoreactivity in primary and metastatic OS lesions

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**TABLE 1**: Procaspase-3 immunoreactivity scores for spontaneously occurring primary and metastatic osteosarcoma samples by patient number. Note that patients 1-5 have both primary and metastatic tumors that are scored as weakly immunoreactive while patients 6 and 7 have both primary and metastatic tumors that are scored as moderately immunoreactive.
Figure 4.2: Graduation of staining canine osteosarcoma tumor samples. A. Weak staining for procaspase-3 (score 1-4). B. Moderate staining for procaspase-3 (scores 5-8). C. Strong staining for procaspase-3 (scores 9-12).
Figure 4.3: Paired OSA primary and lung metastases samples. Primary and metastases #3 display weak immunoreactivity for procaspase-3; whereas primary and metastases #7 display moderate immunoreactivity for procaspase-3. Overall, 90% of primary tumors were positive for procaspase-3 staining whereas 86% of metastatic tumors were positive for procaspase-3 staining.
CHAPTER 5
CONCLUSIONS

Procaspase-3 is expressed in osteosarcoma (OS) cells and the target is conserved across species. It is possible that there is a decreased degree of procaspase-3 expression in canine OS lines in comparison with other immortalized OS cell lines derived from both human and murine origin. However, further investigation is needed to determine if this is a true finding or related to differences in antibody cross-reactivity. If this is a true difference, it may indicate that procaspase-3 activating strategies will be more effective for human OS than canine OS. Additionally, exposure of OD cells to biologically relevant concentrations of PAC-1 (10 µM) in vitro results in a dose- and time- dependent conversion of the target procaspase-3 to active caspase-3. This is accompanied by an increase in catalytic activity of caspase-3 and significant cell death by apoptosis at the 48 hour time point. These findings support further studies investigating the therapeutic application of PAC-1 in canine OS. Such investigations should focus on using a delivery strategy that results in at least 48 hours of bone tumor exposure to PAC-1.

Procaspase-3 is expressed to a significantly greater extent in malignant versus normal canine osteoblasts. This is clinically important because it suggests that procaspase-3 activating strategies would be selective for malignant cells. In the case of OS, this could result in decreased off-target effects to normal bone, minimizing the risk of decreased bone strength or fracture following treatment. Additionally, procaspase-3 is expressed in a high percentage of spontaneously-occurring primary and metastatic OS. However, inter-patient variability of procaspase-3 expression is wide and pretreatment biopsies with procaspase-3 screening would be recommended for optimizing the rational therapeutic institution of PAC-1 in vivo. Notably, primary and metastatic tumors derived from the same patient trended to have a conserved pattern of expression suggesting that procaspase-3 is not downregulated during metastatic OS progression, and thereby might provide a conserved drugable target for managing both primary and metastatic OS lesions.
The results derived from the current investigation supports future study of targeted procaspase-3 activating strategies for the treatment of OS. Forthcoming investigations should focus on demonstrating a therapeutic response in dogs with OS and determining if that response is greater in dogs with higher levels of procaspase-3 expression. Ideally, a specialized delivery strategy would be used that prolongs PAC-1 exposure, yet minimize the likelihood for off-target toxicity including neuronal hyperexcitation. For the management of primary bone OS lesions, this may be best achieved by bone seeking strategies such as direct conjugation to a bisphosphonate or encapsulation in a bone seeking nanoparticle. For metastases, increased duration of exposure may be achieved by constant rate infusion intravenously or by delivery of PAC-1 as an inhalation therapeutic. Although the current investigation focuses on the justification of PAC-1 as a single agent for treatment OS, additional future studies are planned to assess the ability of PAC-1 to synergize with conventional cytotoxic agents; thereby broadening the potential therapeutic applications of procaspase-3 activating compounds for the management of cancer.
REFERENCES


