Targeting Cancer in the Bone with the
Hypoxia Activated Pro-Drug Evofosfamide

This thesis is submitted to Adelaide University as a requirement for the degree of Doctor of Philosophy

By

Vasilios Liapis (BAg.Sc)

June 2017

Discipline of Surgery, School of Medicine,
Faculty of Health Sciences
University of Adelaide
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Apoptosis analysis

DAPI staining of nuclei

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Bibliography
DECLARATION

I, Vasilios Liapis, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Vasilios Liapis
ACKNOWLEDGMENTS

First of all I would like to thank my main supervisor Professor Andreas Evdokiou for his patience, advice and for the confidence he had in me before and throughout my candidature, even with his hectic schedule. I would also like to thank my co supervisor Dr Mark DeNichilo, who gave me sound advice and many ideas about my project which also included the future beyond my candidature. Mark also proof read my papers, my thesis and was patient as well as understanding when checking my grammar and helped me present my work at conferences and seminars, which I avoided during my time as a research assistant before my candidature. I would also like to thank my other co-supervisor Dr Irene Zinonos, who helped me with the animal ethics application, proof reading my papers, conducting the animal studies and for teaching me the special tricks in making power point presentations easier to set up and use.

I would especially like to thank Shelley Hay for her patience and having an exceptional ability to teach others such as myself effectively, particularly on how to work in a laboratory and to write up results comprehensively during my time as a research assistant, which I used as a platform for my PhD candidature. In addition, I would also like to thank Dr Agatha Labrinidis for her continuous support and showing me her technical skills, especially in regards to the animal work which included the intratibial and mammary fat pad injections as well as the analysis. There are also other members of my lab who I would also like to thank which include Aneta Zysk who also helped me with the intratibial injections and data analysis, Bill Panagopoulos, Alexandra Shoubridge and Christopher Difelice who also provided me with support, advice and understanding during my time in the laboratory and while I wrote my three papers and thesis.
I would also like to thank other staff members who I have come to know at the Basil Hetzel Institute and the Hospital Research Foundation from whom I have also learnt about other fields of science, the running and maintenance of an institute and organizing community events. I would also like to thank the University of Adelaide for providing the Division of Health Science scholarship and the Breast Cancer Research Unit supplementary scholarship. Completing my candidature would not have been possible without these.

Finally, this thesis would not have been possible without the support of my family. I would like to thank my wife Luciana, for her support, love and care. I would also like to thank both my children Nico and Eva for providing me with constant entertainment during my candidature. I can’t forget Frankie my dog who is always happy to see me. My mother and father and my in-laws, who are very proud of me for undertaking a PhD. My sister, sister in law and both brother in laws who constantly asked me how my research is going, I love you all. My apology if I have missed anybody else. Having said this, my three year PhD candidature has been filled with many personal events and achievements that are certain to shape my character for future years to come.
ABSTRACT

Tumour hypoxia is widely recognised as a major cause of treatment failure and poor outcome for a variety of malignancies. Tumour hypoxia also results in resistance to conventional anticancer therapies leading to an increase in malignancy and metastases to other sites, in particular, the bone. Bone metastases occur in more than 75% of patients with breast, prostate and lung cancer. Tumours in the bone are often resistant to anticancer therapy due to the hypoxic nature of the bone micro-environment, resulting in their recurrence and metastasis.

Hypoxia also offers treatment opportunities, exemplified by the development of highly active compounds that target hypoxic zones known as Hypoxia Activated Pro-drugs. Evofosfamide is a hypoxia activated pro-drug created by the conjugation of 2-nitroimidazole to bromo-isophosphoramide (Br-IPM). When Evofosfamide is delivered to regions of hypoxia, Br-IPM, the DNA cross linking toxin is released resulting in cancer cell death.

The cytotoxic activity of evofosfamide against osteosarcoma cells was assessed in vitro and its anticancer efficacy as a single agent and in combination with doxorubicin was evaluated in an orthotopic mouse model of human osteosarcoma (OS). In vitro, evofosfamide was cytotoxic to osteosarcoma cells selectively under hypoxic conditions, whereas primary normal human osteoblasts were protected. Animals transplanted with OS cells directly into their tibiae and left untreated developed mixed osteolytic/osteosclerotic bone lesions and subsequently developed lung metastases. Evofosfamide reduced tumor burden in bone and cooperated with doxorubicin to protect the bone from osteosarcoma induced bone destruction, while also reducing lung metastases.
In addition, under hypoxic conditions *in vitro*, evofosfamide cooperated with Pro Apoptotic Receptor Agonists (PARAs) dulanermin and drozitumab, resulting in a dose-dependent increase in cytotoxicity to osteosarcoma cells selectively under hypoxic conditions. In contrast primary normal human osteoblasts under the same hypoxic conditions were resistant. *In vivo*, evofosfamide cooperated with drozitumab, reducing tumor burden in the orthotopic mouse model of human osteosarcoma and protected the bone from osteosarcoma-induced bone destruction while also reducing the growth of pulmonary metastases.

In order to assess the anticancer efficacy of evofosfamide against breast cancer, a panel of human breast cancer cell lines were treated with evofosfamide and shown to be highly cytotoxic under hypoxia. Osteolytic MDA-MB-231-TXSA cells were transplanted into the mammary fat pad or into the tibiae of mice, allowed to establish and treated with evofosfamide, paclitaxel, or both. *In vivo* evofosfamide demonstrated tumor suppressive activity as a single agent and cooperated with paclitaxel to reduce mammary tumor growth. Breast cancer cells transplanted into the tibiae of mice developed osteolytic lesions. Treatment with evofosfamide or paclitaxel resulted in a significant delay in tumor growth and with an overall reduction in tumor burden in bone, whereas combined treatment resulted in a significantly greater reduction in tumor burden in the tibia of mice.

In conclusion the preclinical data presented in this thesis demonstrate that evofosfamide may be an attractive therapeutic agent when used alone and in combination with chemotherapy or PARAs for the treatment of osteosarcoma and breast cancer.
CONFERENCE PRESENTATIONS


Anticancer efficacy of the hypoxia activated prodrug evofosfamide is enhanced in combination with the proapoptotic receptor agonist drozitumab against osteosarcoma.

The Queen Elizabeth Hospital Research Day, Friday 21st October 2016. Poster Presentation.


Anticancer efficacy of the hypoxia activated prodrug TH-302 in osteolytic breast cancer murine models.

Metastasis Research Society, Chengdu, China, Saturday 17th September 2016. Poster Presentation.

Hypoxia-activated prodrug Evofosfamide inhibits tumour growth and cooperates with chemotherapy against osteosarcoma.

*Metastasis Research Society, Chengdu, China, Friday 16th September 2016. Oral Presentation.*


Anticancer efficacy of the hypoxia activated prodrug evofosfamide is enhanced in combination with drozitumab against osteosarcoma.

*Australian Society for Medical Research (ASMR), Wednesday 8th June 2016. Oral Presentation.*


Anticancer efficacy of the hypoxia activated prodrug TH-302 in osteolytic breast cancer murine models.

*The Queen Elizabeth Hospital Research Day, Friday 16th October 2015. Oral Presentation.*

Anticancer efficacy of the hypoxia activated prodrug TH-302 in osteolytic breast cancer murine models.


Anticancer efficacy of the hypoxia activated prodrug TH-302 in osteolytic breast cancer murine models.

Australian Society of Medical Research (ASMR), Wednesday 3rd June 2015. Poster Presentation.


Hypoxia-activated prodrug TH-302 inhibits tumour growth and cooperates with chemotherapy against osteosarcoma.

Hypoxia-activated prodrug TH-302 inhibits tumour growth and cooperates with chemotherapy against osteosarcoma.


Hypoxia-activated pro-drug TH-302 exhibits potent tumor suppressive activity and cooperates with chemotherapy against osteosarcoma.

Australian Society for Medical Research (ASMR), Wednesday 4th June 2014. Oral Presentation.
PRIZES AWARDED

**Best Lay Description.** The Queen Elizabeth 25th Anniversary Research Day Hospital Research Day, Woodville, South Australia. *Friday 24th October 2016.*

**Best Poster Presentation.** The Queen Elizabeth Hospital Research Day, Woodville, South Australia. *Friday 24th October 2016.*


**Young Investigator Travel Award.** Metastasis Research Society, Chengdu, China. *Saturday 17th September 2016.*

**School of Medicine Research Travel Award.** University of Adelaide, Adelaide, South Australia. *Monday 5th September 2016.*

**Ross Wishart Award Finalist.** Australian Society for Medical Research (ASMR), Adelaide, South Australia. *Wednesday 8th June 2016.*


**Poster Prize.** Australian Society for Medical Research (ASMR), Adelaide, South Australia. *Wednesday 3rd June 2015.*


**School of Medicine Prize.** Florey Postgraduate Research Conference, University of Adelaide, Adelaide, South Australia. *Thursday 25th September 2014.*
CO-AUTHORED PUBLICATIONS

Inflammatory peroxidases promote breast cancer progression in mice via regulation of the tumour microenvironment.

Vasilios Panagopoulos, Damian A Leach, Irene Zinonos, Vladimir Ponomarev, Giovanni Licari, Vasilios Liapis, Wendy Ingman, Peter Anderson, Mark O DeNichilo and Andreas Evdokiou


Adoptive transfer of ex vivo expanded Vγ9Vδ2 T cells in combination with zoledronic acid inhibits cancer growth and limits osteolysis in a murine model of osteolytic breast cancer.

Aneta Zysk, Mark O DeNichilo, Vasilios Panagopoulos, Irene Zinonos, Vasilios Liapis, Shelley Hay, Wendy Ingman, Vladimir Ponomarev, Gerald J Atkins, David M Findlay, Andrew C Zannettino and Andreas Evdokiou


Peroxidase enzymes inhibit osteoclast differentiation and bone resorption.

Vasilios Panagopoulos, Vasilios Liapis, Irene Zinonos, Shelley Hay, Damien A Leach, Wendy Ingman, Mark O DeNichilo, Gerald J Atkins, David M Findlay, Andrew Zannettino and Andreas Evdokiou

Peroxidase enzymes regulate collagen biosynthesis and matrix mineralization by cultured human osteoblasts.


Uncovering a novel role for peroxidase enzymes as drivers of angiogenesis.

Vasilios Panagopoulos, Irene Zinonos, Damien A Leach, Shelley Hay, Vasilios Liapis, Aneta Zysk, Wendy Ingman, Mark O DeNichilo and Andreas Evdokiou


Systematic in vitro nanotoxicity study on anodic alumina nanotubes with engineered aspect ratio: Understanding nanotoxicity by a nanomaterial model.

Ye Wang, Gagandeep Kaura, Aneta Zysk, Vasilios Liapis, Shelley Hay, Abel Santos, Dusan Losic, Andreas Evdokiou

CHAPTER 1

INTRODUCTION
**Hypoxia and Cancer**

An important limitation to current cancer therapy is the cellular environment within solid tumours. This microenvironment has subregions of nutrient deprivation, low extracellular pH, high interstitial fluid pressure and hypoxia (Kaisa R Luoto1†, 2013). Hypoxia is a factor which is present within nearly all cancer types and plays a central role in cancer progression. The vasculature within most solid tumours consists of poorly functional and abnormally formed blood vessels that are unable to deliver enough oxygen and nutrients to support the rapidly growing tumour mass. This reduction in oxygen is lethal to some cancer cells resulting in necrotic regions within the tumour, but some cancer cells adapt and survive the low oxygen conditions (Bennewith & Dedhar, 2011).

These hypoxic cancer cells evade most anticancer drugs, because hypoxic cells reside some distance away from blood vessels, therefore conventional chemotherapy, which is administered systemically, cannot reach these cells. Most antitumor agents cannot penetrate more than 50-100 μM from capillaries, which leads to cytotoxic concentrations not being attained in hypoxic regions. Cellular proliferation also decreases partly due to hypoxia and cells in this hypoxic environment have lower sensitivity to p53 mediated apoptosis. In summary, tumours in the hypoxic environment have a more malignant phenotype, have increased rates of mutations and they express genes which are associated with angiogenesis, tumour invasion and anticancer resistance. These tumour cells are also highly metastatic (Graeber et al., 1996). Therefore, the presence of hypoxic regions within the tumour is a major cause of treatment failure and poor outcome for a wide variety of malignancies.

In addition, an important transcriptional factor involved in the resistance of cancer cells in the hypoxic environment is the stabilization of Hypoxia Inducible Factor 1 (HIF-1). HIF-1 is the transcriptional factor that is essential in the response of cells to hypoxia and is a key regulator in oxygen homeostasis (Maxwell, 2004). HIF-1α is the molecule that is constantly present in normoxic conditions and under these conditions, this molecule is regulated post transcriptionally by prolyl hydroxylases via hydrolysis and is constantly degraded. This hydrolysis is inhibited under hypoxia, resulting in the accumulation HIF-1α instantaneously and translocating to the nucleus (Semenza, 2004). Heterodimerization of HIF-1α and HIF-1β (which is constantly expressed in both normoxic and hypoxic conditions) subunits result in the formation of the HIF-1 protein, activating the transcription of specific hypoxia-responsive genes (Ljungkvist, Bussink, Kaanders, & van der Kogel, 2007).
Fig.1. Squamous cell carcinoma (×40) showing hypoxia staining in brown (pimonidazole adducts) and blood vessels in red (factor VIII). N = area of necrosis (Bonn, 2000).
HIF-1 regulates more than 70 genes which are involved in cellular adaptation to hypoxia (Semenza, 2004), including Lysyl oxidase, Carbonic anhydrase 9, Chemokine receptor 4, Facilitated glucose transporter 1 and VEGF. This stabilization of HIF-1 adds to the adaptation capability of cancer cells to the hypoxic tumour environment and also leads to the resistance of cancer cells to conventional chemotherapeutic drugs and radiotherapy.

**The Bone Micro-environment**

Bone is made up of both trabecular (spongy) and cortical (solid) bone and these compositions are constantly being remodelled in order to maintain skeletal integrity. Within these bone types there are two categories of bone cells. The first category of bone cells is made up of osteoblasts, osteocytes and endosteal cells. Osteoblasts are derived from the mesenchymal lineage (Rauner, Stein, & Hofbauer, 2012) and synthesize proteins that form the organic matrix of bone. Osteoblasts control the mineralisation of bone and have receptors for hormones such as vitamin D, estrogen glucocorticoids, leptin and the parathyroid hormone. When osteoblasts finish making new bone they become surrounded with matrix and differentiate into osteocytes or they remain on the surface of new bone and differentiate into lining cells (Rauner, Stein & Hofbauer 2012).

Osteocytes exist inside the bone and have long branches to allowing contact with each other and lining cells. Osteocytes secrete various factors that activate, osteoblasts, osteoclasts or lining cells which then remodel bone (Bonewald, 2011). The lining cells cover the surface of the bone and have receptors for hormones and other factors that can initiate bone remodelling (Miller, de Saint-Georges, Bowman, & Jee, 1989), thereby acting as a blood-bone barrier while still having the ability become osteoblasts upon exposure to the parathyroid hormone or mechanical forces (Clarke, 2008).

The second category of bone cells are osteoclasts which are multinucleated cells derived from the hematopoietic lineage that dissolve and resorb bone. Osteoclasts are derived from monocytes which become osteoclast precursor cells and are transformed into osteoclasts when RANK (‘receptor activator of NF-kB’) is activated by RANK-ligand on osteoclast precursor cells. RANK-ligand is a member of the TNF family which is secreted by osteoblasts and stromal cells (Lacey et al., 1998).

Osteoprotegerin (OPG) is also produced by osteoblasts, it binds with and neutralises RANK-ligand and is involved in inhibiting osteoclast activation and function (Simonet et al., 1997). When osteoclasts are finished resorbing bone they undergo apoptosis.
Fig.2. Normal bone remodelling. Osteoblasts and stromal cells produce RANKL which binds to the RANK receptor on pre-osteoclasts, stimulating their differentiation and maturation into functional osteoclasts. However OPG, also produced by osteoblasts binds to RANKL, inhibiting the association between RANKL and RANK, therefore inhibiting osteoclast formation (Kohli & Kohli, 2011).
In addition the bone is a hypoxic micro-environment. Hypoxia regulates normal marrow haemopoiesis and mesenchymal differentiation. This is achieved by hypoxia stimulating the formation and activation of cells which have been generated from marrow precursors, including cells of the monocyte–macrophage lineage. The medullary cavity oxygen pressure in humans is estimated to be 5% O₂ (Asosingh et al., 2005) and the bone tissue has oxygen levels that range between 1-7%. Recent studies have also shown that hypoxia blocks the growth and differentiation of osteoblasts, while stimulating osteoclast formation (Arnett, 2010).

This balance of bone formation and bone resorption resulting from the interaction of these cells in the hypoxic bone environment can be altered due to a variety of bone diseases including osteosarcoma and metastatic breast cancer.

**The Vicious Cycle of Bone Cancer**

Bone is a preferred site for breast and prostate cancer metastasis in patients with late stage disease. Cancer within the bone forms two types of lesions. Osteolytic lesions, which are caused by excessive osteoclast activity in relation to osteoblast bone formation, resulting in net bone loss. The second type, osteosclerotic lesions are caused by an increase of osteoblastic activity in relation to osteoclast resorption. This leads to an increase in disorganised bone formation. In most patients, cancers in the bone are characterised by a combination of both, as the processes of bone resorption and bone formation are linked but distorted in cancer (Mundy, 2002a).

This imbalance of bone remodelling caused by cancer creates a ‘vicious cycle’, where bone resorption is induced by the tumour, causing the release growth factors from the bone matrix. This further promotes tumour growth, leading to increases of pro-resorptive factors, which further break down bone (Ooi, Zheng, Stalgis-Bilinski, & Dunstan, 2011). The vicious cycle is largely osteolytic, leading to a variety of SREs which include bone pain, hypercalcaemia, pathological fractures, spinal cord and nerve compressions, these complications lead to a diminished quality of life and ultimately death in these patients caused by metastases to other sites in the body such as the lung, liver and brain (R. E. Coleman, 1997).

The bone matrix is rich in growth factors such as transforming growth factor-β (TGF-β) and insulin growth factors (IGFs). These growth factors are released during osteolysis caused by osteoclast activity, further stimulating tumour cell proliferation.
Metastatic cells in the bone environment, in particular breast cancer cells secrete parathyroid hormone-related protein (PTHrP). PTHrP increases osteoblast production of RANKL, which stimulates osteoclast formation and activity, resulting in an increase in bone resorption, releasing more growth factors such as TGF-β and IGFs from the bone and ultimately maintaining this vicious cycle. TGF-β also increases hypoxic signalling by inhibiting prolyl hydroxylase 2 resulting in the decrease in HIF-1α degradation which stabilises HIF-1 and results in the transcription of HRE genes.

Bone itself is a hypoxic environment, with oxygen levels ranging between 1-5% O₂ (Asosingh et al., 2005). Cancer cells that survive in this hypoxic bone environment proliferate and participate in this vicious cycle of bone destruction (Kingsley, Fournier, Chirgwin, & Guise, 2007). Hypoxia is usually accompanied by an increase in acidity in the bone which also has significant effects on bone remodelling. Extracellular acidification increases osteoclast activity leading to bone resorption, while osteoblast mineralisation is inhibited by acidic pH (Brandao-Burch, Utting, Orriss, & Arnett, 2005). Bone destruction also leads to increase in calcium levels which also promote tumour growth and the production of PTHrP.

Therefore this bone microenvironment consists of numerous factors, such as hypoxia, acidosis, extracellular calcium, and growth factors which all combine to drive this vicious cycle of bone destruction in the presence of tumour cells in the bone.
**Fig. 3. The Vicious Cycle of Bone Cancer.** Tumour cells produce factors such as PTHrP that promote the formation and activation of osteoclasts. Osteoclast activation results in bone resorption and thus release of factors from the bone matrix, such as TGF-β, IGFs and Ca²⁺ which stimulate tumour cell proliferation. (Ignatiadis & Sotiriou, 2013).
**Osteosarcoma**

Osteosarcoma is a cancer derived from primitive bone-forming mesenchymal cells and is the most common primary bone cancer and the eighth most common tumor in children and adolescents (Ottaviani & Jaffe, 2009). Osteosarcoma can occur in any bone, but the most common sites are the femur, the tibia and the humerus (Chou & Gorlick, 2006). Bone lesions caused by osteosarcoma are characterized on the basis of their radiologic appearance and present as either osteolytic which results in net bone loss, osteoblastic (osteosclerotic) which results in highly disorganised bone formation, or mixed (Mundy, 2002a). Metastatic spread from the bone is preferential to the lungs and is seen in 20% of osteosarcoma patients which is correlated with poor survival (Link et al., 1991; Saeter et al., 1997).

Current treatment of osteosarcoma includes approximately 10 weeks of preoperative chemotherapy comprised of doxorubicin, cisplatin and high dose methotrexate, followed by surgery which involves the removal of the primary tumour and if present, all sites of the metastatic disease. After surgery patients receive 20 weeks of maintenance chemotherapy with the same 3 agents (Stefano Ferrari et al., 2012). Following this treatment, a 5 year survival rate for non-metastatic osteosarcoma is 70% (Akiyama, Dass, & Choong, 2008). However if metastatic osteosarcoma disease is detected at the time of initial diagnosis, then this leads to poor prognosis, with long term survival rates between 10-40% (Kager et al., 2006). Therefore there is a need to develop new anticancer therapies to improve survival in osteosarcoma patients.

**Breast Cancer**

Breast Cancer is the most common malignancy and the leading cause of cancer mortality in women worldwide. It occurs in 1 out of 9 women and accounts for 23% of the total new cases of cancer and 14% of the total cancer deaths in 2008 worldwide (Ferlay et al., 2010; A. Jemal et al., 2011). It is also estimated that worldwide, over 508000 women died in 2011 due to breast cancer (Metin Seker et al., 2014). The incidence of breast cancer is increasing in the developing world due to increase life expectancy, increase urbanization and adoption of western lifestyles (Hery, Ferlay, Boniol, & Autier, 2008). In contrast, breast cancer death rates have been decreasing in developed countries for the past 25 years, largely as a result of early detection through the wide spread use of mammography, an increase in breast cancer awareness and improved treatment by chemotherapy, hormone targeted therapy and more recently drugs that target the cancer’s specific proteins, genes or tissue environment (DeSantis et al., 2015; Ahmedin Jemal, Center, DeSantis, & Ward, 2010).
Treatment depends upon tumor size, the number and location of the lymph nodes involved, the presence or absence of distant metastatic disease and pathologic features such as receptor status and tumor grade (Moulder & Hortobagyi, 2008). Early stage breast cancer is often treated using local therapy. Local therapy can include breast conserving surgery or mastectomy which is followed by radiotherapy. More advanced breast cancer requires systemic therapy that can reach cancer cells anywhere in the body. Types of systemic therapy used to treat breast cancer include the use of chemotherapeutic agents such as paclitaxel, doxorubicin, cyclophosphamide, carboplatin and 5-fluorouracil, which are given to patients in various combinations (Guarneri & Conte, 2004). In addition, the use of hormone therapeutic drugs such as tamoxifen can specifically target breast cancer types that require estrogen and progesterone to fuel their growth, due to their estrogen and progesterone receptor positivity. Advances in targeted therapy have also improved patient outcomes. Therapeutic targeting drugs target specific changes to breast cancer cells which are different from normal cells, such as HER2 targeted therapy, which target breast cancer cells that are HER2 positive (Arteaga et al., 2012).

Current treatment options for the treatment of the early stages of breast cancer have been very effective in prolonging survival and improving the quality of life in women diagnosed with early breast cancer. However despite the significant improvements in detecting and treating the early stages of breast cancer 30% of women diagnosed with early-stage breast cancer, will eventually have locally advanced or metastatic breast cancer.

**Breast Cancer and Bone Metastasis**

Patients diagnosed with advanced breast cancer experience poor prognosis and as a result the 5 year survival rate is reduced to 20% (Beaumont & Leadbeater, 2011). The low survival rate is not due to the cancer at the primary site, but the result of the subsequent metastasis to secondary sites in the body, most common site being the skeleton.

An estimated 80% of patients with advanced breast cancer develop skeletal metastasis (R. E. Coleman, 2006; Siegel, Ward, Brawley, & Jemal, 2011). Bone metastases result in various pathological complications that can result in patients experiencing debilitating skeletal related events (SREs). These SREs include pathological skeletal fractures, hypercalcaemia and spinal cord compression.
SREs such as fractures have become less frequent with bisphosphonate therapy which blocks bone destruction by inhibiting the formation of osteoclasts (Ganz & Stanton, 2015), however, patients still suffer from severe bone pain and loss of mobility which eventually leads to reduction of quality of life and survival (Robert E. Coleman, 2012).

These SREs arise because breast cancer cells that metastasize to the bone disrupt normal bone remodeling which leads to extensive bone destruction. Most patients with late stage metastatic breast cancer receive chemotherapy which includes taxanes (paclitaxel), anthracyclines (doxorubicin), hormone therapy and sometimes surgery (DeSantis et al., 2014). However, due to high toxicity and the development of drug resistance, such anticancer treatments are often discontinued and in patients with advanced disease in particular where the cancer has metastasized to the bone, the treatment becomes only palliative.

**Hypoxia Activated Pro-drugs (HAPs)**

Prodrugs that are enzymatically converted within tumours to active metabolites have been of particular interest for selective cancer therapy. HAPs have been investigated for the treatment of cancer for over 30 years and exploit a generic feature that differentiates tumours from normal tissue, potentially overcoming the resistance that hypoxic tumours have to conventional chemotherapy and radiotherapy. HAPs that have been evaluated in various stages of clinical trials but have been discontinued include Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide; SR 4233) which under hypoxic conditions is bioreduced to a nitroxide-base free radical that removes hydrogen from DNA strands causing DNA damage. When tirapazamine was used in combination with cisplatin and with radiotherapy in phase 3 clinical trials, it did not improve overall survival in patients with advanced head and neck cancer (NCT00094081). In addition these combinations led to an increase in toxicities which caused nausea, diarrhoea, vomiting, myalgia, and muscle spasms (Wu et al., 2012).

PR-104 is a water soluble phosphate ester that gets converted to the corresponding 3,5-dinitrobenzamide mustard prodrug, PR-104A. Nitroreduction of PR104A leads to the formation of the cytotoxic hydroxylamine (PR104H) and amine (PR104M) by crosslinking DNA which is determined by hypoxia, relevant oxidoreductases and the functional status of DNA repair pathways in the cell (Christopher P. Guise et al., 2012). PR-104 has also been discontinued after phase I studies for the treatment of numerous solid tumour types resulted in myelotoxicity in 2 out of 3 patients in the study (NCT00349167). In addition PR-104 as a monotherapy also caused myelotoxicity and grade four thrombocytopenia (NCT00459836) (McKeage et al., 2012).
The HAP AQ4N (banoxantrone; 1,4-bis((2-(dimethylamino)-N-ethyl)amino)-5,8-dihydroxyanthracene-9,10-dione) is nontoxic until bioreduced in hypoxic cells by a number of cytochrome P450 isozymes (CYP) or inducible nitric oxide synthase (NOS2A) in tumours to form the short-term mono-N-oxide intermediate AQ4M, which then becomes the cytotoxic ditertiary cationic amine AQ4 (Manley & Waxman, 2013). AQ4 then intercalates DNA and inhibits topoisomerase 2 (C. P. Guise et al., 2013). Phase I clinical studies investigating the HAP Banoxantrone (AQ4N) against solid malignancies and Non-Hodgkin’s Lymphoma (NCT00090727) have also been terminated. Among the most common adverse events observed by the administration of AQ4N in Phase I clinical trials were fatigue, diarrhoea, nausea, vomiting, and anorexia (Albertella et al., 2008; Papadopoulos et al., 2008). Evofosfamide (TH-302) and Tarloxotinib bromide (TH-4000) which is a prodrug that selectively releases a covalent (irreversible) EGFR tyrosine kinase inhibitor under hypoxic conditions are still in clinical trials, of which evofosfamide is the most advanced as well as having the least amount of toxicities.
**Fig. 4.** Chemical structure of tirapazamine (Francis W. Hunter et al., 2012).

**Fig. 5.** Chemical structures of PR104, its converted form PR-104A and reduced cytotoxic hydroxylamine PR-104H and amine PR-104M (Gu et al., 2009).

**Fig. 6.** Chemical structures of AQ4N its intermediate AQ4M and reduced cytotoxic amine AQ4 (Loadman, Swaine, Bibby, Welham, & Patterson, 2001).

**Fig. 7.** Chemical structure of tarloxotinib bromide and its 1e- reduction resulting in the EGFR/HER2 tyrosine kinase inhibitor being released under hypoxic conditions (Stephen V Liu & Stew Kroll, 2016).
**Evofosfamide**

Evofosfamide is a 2-nitromidazole-linked prodrug of a brominated version of the cytotoxin bromo-isophosphoramide mustard (Br-IPM) developed by Threshold Pharmaceuticals. The 2-nitromidazole functional group of evofosfamide is attracted to hypoxic conditions and acts as a hypoxia activated trigger under these conditions, releasing Br-IPM into the hypoxic environment. Br-IPM acts as a DNA crosslinking agent which is able to kill both dividing and non-dividing cells in this hypoxic environment.

Evofosfamide also has a ‘bystander effect’, where once activated evofosfamide can also diffuse into normoxic areas to act against neighbouring cells in the normoxic regions within the tumour. Hypoxia activation of evofosfamide occurs when the 2-nitromidazole portion of evofosfamide undergoes electron rearrangement forming a radical anion under hypoxia and then fragments releasing the toxic bromo-isophosphoramide mustard which diffuses into the hypoxic tumour (Meng et al., 2012). However under normoxic conditions, evofosfamide is converted into the radical anion and then back oxidized into its pro-drug form in a futile cycle generating superoxides from the reduction of oxygen (Fig.6). These superoxides react with free hydroxyl radicals which act as oxidants that can also damage DNA (Keyer & Imlay, 1996).

Evofosfamide showed encouraging data in phase II clinical trials for the treatment of metastatic or locally advanced unresectable soft tissue sarcoma with doxorubicin and for the treatment of pancreatic ductal adenocarcinoma with gemcitabine. Unfortunately phase III studies did not achieve primary overall endpoints in combination with chemotherapy for the treatment of soft tissue sarcoma (NCT01440088) and metastatic pancreatic adenocarcinoma (NCT0174679). These phase III results have led to the termination and withdrawal of a number of phase II trials such as the treatment of oesophageal cancer (NCT02598687), non-squamous non-small cell lung cancer (NCT02093962), liver cancer (NCT01497444), soft tissue sarcoma (NCT02255110) and pancreatic cancer (NCT02047500, NCT02496832).

However evofosfamide is still being investigated in phase II trials for multiple myeloma (NCT01522872), high grade astrocytoma (NCT01403610), metastatic neuroendocrine pancreatic tumours (NCT02402062), metastatic melanoma (NCT01864538) and advanced biliary tract cancer (NCT02433639). Phase I trials include solid tumours (NCT02020226, NCT01485042) and hepatocellular carcinoma (NCT01721941).
The strategy of hypoxia-targeted treatment with evofosfamide in the hypoxic bone marrow has been tested in preclinical studies of multiple myeloma (Jinsong Hu et al., 2010; J. Hu et al., 2013) and acute myeloid leukaemia (Benito et al., 2015).

These preclinical studies investigating the anticancer efficacy of evofosfamide against multiple myeloma have provided support for the currently active phase II clinical trial to evaluate evofosfamide and dexamethasone, with or without bortezomib, or pomalidomide for the treatment of patients with relapsed/refractory multiple myeloma (NCT01522872). The preclinical data investigating evofosfamide for the treatment of acute myeloid leukaemia also provides support for the future clinical assessment of evofosfamide in the treatment of various leukaemia types, following the recent completion of the phase I study (NCT01149915), which determined the maximum tolerated dose, dose limiting toxicity, safety and tolerability of evofosfamide in patients with various types of advanced leukaemias (Badar et al., 2016).

To date, there has been no investigation of evofosfamide or any other HAP for the treatment of solid tumours that originates or metastasizes to the bone. These previous studies, which investigate the role of evofosfamide against multiple myeloma and leukaemia provide supporting evidence that targeting the hypoxic bone microenvironment niche with the HAP evofosfamide, is a useful and novel strategy for the treatment of cancer in bone.

Fig.8. Chemical structure of evofosfamide and its 1e- reduction resulting in the bis-alkylator warhead (Br-IPM) being released under hypoxic conditions (Meng et al., 2012).
The Apoptotic Pathways

Apoptosis is the major mechanism to eliminate damaged cells via programmed cell death. It can be stimulated by a variety of factors including developmental cues, cellular stress or damage to cellular components caused by radiation, heat shock, infection, oncogenic transformation and cytotoxic drugs (Fadeel, Orrenius, & Zhivotovsky, 1999). The deficiency in apoptosis is one of the key hallmarks in cancer (Hanahan & Weinberg, 2000). Two pathways induce apoptosis in mammalian cells, firstly the intrinsic pathway, which is activated by intracellular processes and depends on the mitochondrion releasing proapoptotic factors. Secondly, the extrinsic pathway, which receives signals through the binding of extracellular protein ligands to proapoptotic death receptors which are located on the cell surface (Gonzalvez & Ashkenazi, 2010).

Both pathways stimulate pro-apoptotic caspases which are a family of cysteine proteases that exist as procaspases. Activation of these procaspases is known as ‘the caspase cascade’. Apoptosis is firstly stimulated by the initiator caspases (caspase-8,-9,-10) which then activate the effector caspases (caspase-3,-6-7) by proteolytic processing. These effector caspases then process cellular proteins, which lead to the unique features of apoptosis such as plasma membrane ‘blebbing’, cell shrinkage, chromatin condensation and DNA fragmentation (Ashkenazi, 2008b).
Fig. 9. The intrinsic and extrinsic apoptotic pathways. These two pathways are also known as the “death receptor” and the “mitochondrial pathway” respectively. Caspase 8 is the initiator caspase of the death receptor pathway, and cleavage of bid results in cytochrome c being released from the mitochondrion. Both pathways activate the effector caspases (Torok & Gores, 2004).
Pro-Apoptotic Receptor Agonists (PARAs)

ProApoptotic receptor Agonists dulanermin and drozitumab can induce apoptosis selectively in cancer cells in vitro and in vivo and as a result have demonstrated anticancer efficacy in numerous preclinical studies. Both dulanermin (Apo2L/TRAIL/TNFSF10) and drozitumab target the extrinsic apoptotic pathway of cancer cell death, whereas most conventional anticancer therapies stimulate apoptosis via the intrinsic pathway. Inactivation of p53 in this intrinsic pathway is the most common mutation that allows cancer cells to become resistant to apoptosis (Ashkenazi & Herbst, 2008). By focusing on the extrinsic pathway these new compounds may overcome cancer cell resistance to chemotherapy.

Dulanermin activates apoptosis through death domain containing receptors DR4 (TNFRSF10A/TRAILR1) and DR5 (TNFRSF10B/TRAILR2). In addition, dulanermin also interacts with decoy receptors known as DcR1 (TNFRSF10C/TRAILR3), DcR2 (TNFRSF10D/TRAILR4) and Osteoprotegerin (TNFRSF11B/OPG) which do not transmit apoptotic signals. However the expression of DcR1 and DcR2 determines which cells undergo apoptosis (Pan, Ni, Yu, Wei, & Dixit, 1998). Dulanermin selectively triggers apoptosis in tumor cells over normal cells, which highlights its potential as a therapeutic drug in cancer treatment.

Dulanermin has been tested in phase I and II clinical trials in patients with range of cancer types including advanced solid and hematologic tumours, alone or in combination with traditional chemotherapy. Dulanermin was well-tolerated by all patients, and most of the studies reported some partial responses or stable disease (Amarante-Mendes & Griffith, 2015). However, dulanermin did not demonstrate significant clinical efficacy when it came to complete responses. One possible explanation is the short bioavailability of 30 minutes (Ashkenazi et al., 1999) and its inability to bind to death-inducing TRAIL receptors, preferring to bind with the decoy TRAIL receptors in various cancer types.

As an alternative to dulanermin, drozitumab is a fully human agonistic monoclonal antibody that specifically binds to and activates DR5 in the same manner as Apo2L/TRAIL (I. Zinonos et al., 2009). Drozitumab has a half-life ranging from several days to weeks and has been developed to specifically target DR5 (Ashkenazi, 2008a) and not the TRAIL decoy receptors. In addition, circulating Fragment Crystalline Gamma (Fcγ) receptors expressed on the surface of various immune cells, crosslink with drozitumab which leads to enhanced antibody-dependent, cell-mediated cytotoxicity (ADCC) (Wilson et al., 2011), resulting in immune cell activation leading to recruitment of other Fcγ receptor expressing cells to the tumor microenvironment.
The apoptotic tumor cells are then phagocytosed by the activated Fcγ receptor expressing immune cells (Takeda et al., 2004), further enhancing the cytotoxic activity of drozitumab against cancer.

Drozitumab has undergone phase I and II clinical trials for the treatment of colorectal cancer and advanced tumours (Lemke, von Karstedt, Zinngrebe, & Walczak, 2014). Drozitumab was well tolerated with minimal adverse events. Despite the favourable safety profile, patients receiving drozitumab in clinical trials receive only minor benefit from treatment (Micheau, Shirley, & Dufour, 2013).

**PARAs in bone**

In the context of bone related malignancies, the anticancer efficacy of PARAs has been evaluated in bone cancer therapy. Preclinically, Apo2L/TRAIL and drozitumab reduce tumour burden in bone and limit cancer-induced bone destruction in murine intratibial models of metastatic breast cancer and multiple myeloma without compromising normal bone metabolism (Agatha Labrinidis et al., 2009; Thai et al., 2006; I. Zinonos et al., 2009). However some tumours exhibited or acquired resistance to PARAs-induced apoptosis and prolonged treatment failed to completely eradicate tumours from the bone, giving rise to late recurrence in both a myeloma and a breast cancer model. The basis for this resistance is not well understood, with multiple mechanisms proposed (Ashkenazi, 2015; Ashkenazi & Herbst, 2008; Bouralexis et al., 2003; Agatha Labrinidis et al., 2009).

Therefore additional agents such as HAPs can potentially resensitize cancer cells in the bone to PARAs and as yet, there have been no clinical trials investigating PARAs in the treatment of primary or metastatic bone tumours (Picarda, Trichet, Teletchea, Heymann, & Redini, 2012).

Conventional chemotherapeutic drugs have a significant effect on bone health and patients who have received certain chemotherapeutics are at an increased risk for developing osteoporosis due to the toxicities to cells of the bone marrow. Preclinical studies show that PARAs have no effect on normal bone metabolism (A. Labrinidis et al., 2008). Therefore, the combination of HAPs and PARAs should provide great efficacy in the elimination of cancer in the bone and metastasis, with the additional advantage that this combination should be non-toxic and safe.
**Hypothesis**

Selective targeting of hypoxic tumour cells with the Hypoxia Activated Pro-drug evofosfamide in combination with conventional chemotherapeutic agents and Pro-Apoptotic Receptor Agonists (PARAs) is an effective approach to eliminate cancer in bone, with minimal toxicity to normal bone metabolism.

**Aims**

**Aim 1:** To evaluate the anticancer efficacy of the hypoxia activated pro-drug evofosfamide, alone and in combination with doxorubicin against osteosarcoma using both in vitro and in vivo systems.

**Aim 2:** To evaluate the anticancer efficacy of the hypoxia activated pro-drug, evofosfamide alone and in combination with paclitaxel against breast cancer using both in vitro and in vivo systems

**Aim 3:** To evaluate the anticancer efficacy of the hypoxia activated pro-drug, evofosfamide, alone and in combination with Pro-Apoptotic Receptor Agonists against osteosarcoma using both in vitro and in vivo systems

**Significance and contribution to the discipline**

Bone metastases occur in more than 75% of patients with late stage breast, prostate and lung cancer. Cancer in bone is often associated with bone destruction, which causes considerable morbidity and reduced quality of life (R. E. Coleman, 1997). Bone destruction caused by primary and metastatic cancer leads to bone pain, paralysis due to spinal cord compression, hypercalcaemia, fractures and the need for extensive orthopaedic surgical intervention (Mundy, 2002a). The cost of this morbidity is enormous, especially since patients with cancer in the bone often survive for a longer time than patients with visceral metastases.

Data from this thesis will raise the possibility that HAPs may be potential therapeutic targets in combination with conventional therapeutic strategies and PARAs against tumour progression which gives hope of eradicating the tumour completely. Adding to this, the thesis will provide important information for future clinical trials of evofosfamide and PARAs in the treatment of osteosarcoma and metastatic breast cancer.
CHAPTER 2

HYPOXIA ACTIVATED PRO-DRUG TH-302 EXHIBITS POTENTIAL TUMOR SUPPRESSIVE ACTIVITY AND COOPERATES WITH CHEMOTHERAPY AGAINST OSTEOSARCOMA
HYPOXIA-ACTIVATED PRO-DRUG TH-302 EXHIBITS POTENT TUMOUR SUPPRESSIVE ACTIVITY AND COOPERATES WITH CHEMOTHERAPY AGAINST OSTEOSARCOMA

Vasilios Liapis¹, Agatha Labrinidis¹, Irene Zinonos¹, Shelley Hay¹, Vladimir Ponomarev², Vasilios Panagopoulos¹, Mark DeNichilo¹, Wendy Ingman³, Gerald J Atkins⁴, David M Findlay⁴, and Andreas Evdokiou¹

¹Discipline of Surgery, Breast Cancer Research Unit, Basil Hetzel Institute and Centre for Personalised Cancer Medicine, University of Adelaide Woodville, South Australia, Australia
²Department of Radiology, Memorial Sloan-Kettering Cancer Centre, New York, USA
³Discipline of Surgery, School of Medicine at The Queen Elizabeth Hospital, University of Adelaide, Woodville, Australia and Robinson Research Institute, University of Adelaide, Australia
⁴Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, South Australia, Australia

Address correspondence to: Professor Andreas Evdokiou
Breast Cancer Research Unit
Level 1, Basil Hetzel Institute
Queen Elizabeth Hospital
28 Woodville Road, Woodville,
South Australia, AUSTRALIA
Fax: 618 8222 7451
E-mail: andreas.evdokiou@adelaide.edu.au

Cancer Letters, Volume 357, Issue 1, 1st February 2015, Pages 160–169

¹ Agatha Labrinidis Present Address: Adelaide Microscopy, the Centre for Electron Microscopy and Microstructure Analysis, University of Adelaide, Adelaide, South Australia, Australia
Statement of Authorship

| Title of Paper | Hypoxia-activated pro-drug TH-302 exhibits potent tumor suppressive activity and cooperates with chemotherapy against osteosarcoma |
| Publication Status | ✓ Published | □ Accepted for Publication |
|                   | □ Submitted for Publication | □ Unpublished and Unsubmitted work written in manuscript style |

Principal Author

| Name of Principal Author (Candidate) | Vasilios Liapis |
| Contribution to the Paper | Performed invitro experiments and animal studies, analysed all samples, wrote the manuscript and acted as corresponding author. |
| Overall percentage (%) | 80% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date | 7th November 2016 |

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.
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<tr>
<td>Agatha Labrinidis</td>
<td>Performed the intratibial injections, generated the micro CT and bioluminescent data, evaluated the manuscript.</td>
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<td>7/11/16</td>
</tr>
<tr>
<td>Irene Zinonos</td>
<td>Assisted with animal Ethics, manuscript evaluation and submission</td>
<td></td>
<td>10th November 2016</td>
</tr>
<tr>
<td>Shelley Hay</td>
<td>Assisted with the invitro work and evaluated the manuscript.</td>
<td></td>
<td>8/11/16</td>
</tr>
<tr>
<td>Vladimir Ponomarev</td>
<td>Provided the triple reported construct (SFG-NES-TGL).</td>
<td></td>
<td>10th November 2016</td>
</tr>
<tr>
<td>Vasilios Panagopoulos</td>
<td>Assisted with the invitro work.</td>
<td></td>
<td>7/11/16</td>
</tr>
<tr>
<td>Mark DeNichilo</td>
<td>Data interpretation and manuscript evaluation.</td>
<td></td>
<td>7th November 2016</td>
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<tr>
<td>Name of Co-Author</td>
<td>Wendy Ingman</td>
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<th>Gerald J. Atkins</th>
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<th>David M. Findlay</th>
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<th>Name of Co-Author</th>
<th>Andrew C. W. Zannettino</th>
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<th>Andreas Evdokiou</th>
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Hypoxia-activated pro-drug TH-302 exhibits potent tumor suppressive activity and cooperates with chemotherapy against osteosarcoma

Vasilios Liapis 1, Agatha Labrinidis 1, Irene Zinonos 3, Shelley Hay 2, Vladimir Ponomarev 1, Vasilios Panagopoulos 4, Mark DeNichilo 1, Wendy Ingman 5, Gerald J. Atkins 6, David M. Findlay 4, Andrew C.W. Zannettino 6, Andreas Evdokiou 1,6

1 Discipline of Surgery, Breast Cancer Research Unit, Basil Hetzel Institute and Centre for Personalised Cancer Medicine, University of Adelaide Woodville, South Australia, Australia
2 Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, USA
3 Discipline of Surgery, Haematology – Oncology, Breast Biology Cancer Unit, Basil Hetzel Institute, University of Adelaide, Adelaide, South Australia, Australia
4 Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, South Australia, Australia
5 School of Medical Sciences, Myeloma Research Laboratory Cancer Theme, South Australian Health and Medical Research Institute (SAHMRI), Faculty of Health Science, University of Adelaide, Australia

ARTICLE INFO

Article history:
Received 17 September 2014
Accepted 11 November 2014

Keywords:
Hypoxia
TH-302
Osteosarcoma
Metastasis
Chemotherapy

ABSTRACT

Tumor hypoxia is a major cause of treatment failure for a variety of malignancies. However, tumor hypoxia also offers treatment opportunities, exemplified by the development compounds that target hypoxic regions within tumors. TH-302 is a pro-drug created by the conjugation of 2-nitroimidazole to bromo-isophosphoramide (Br-IPM). When TH-302 is delivered to regions of hypoxia, Br-IPM, the DNA cross linking toxin, is released. In this study we assessed the cytotoxic activity of TH-302 against osteosarcoma cells in vitro and evaluated its anticancer efficacy as a single agent, and in combination with doxorubicin, in an orthotopic mouse model of human osteosarcoma (OS). In vitro, TH-302 was potently cytotoxic to osteosarcoma cells selectively under hypoxic conditions, whereas primary normal human osteoblasts were protected. Animals transplanted with OS cells directly into their tibiae and left untreated developed mixed osteolytic/osteosclerotic bone lesions and subsequently developed lung metastases. TH-302 reduced tumor burden in bone and cooperated with doxorubicin to protect bone from osteosarcoma induced bone destruction, while it also reduced lung metastases. TH-302 may therefore be an attractive therapeutic agent with strong activity as a single agent and in combination with chemotherapy against OS.

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Introduction

Osteosarcoma (OS) is the most frequent primary malignancy of the skeleton, developing in children and adolescents and accounts for 20% of all primary osseous neoplasms [1,2]. Metastatic spread, preferentially to the lungs compared with other sites, is seen in 20% of presenting patients and is correlated with poor survival [3,4]. Treatment of OS has undergone considerable changes over the past 20 years, with the efficacy of chemotherapy significantly improving long-term survival. Response to chemotherapy depends on the type and combination of drugs used, the doses given and the sensitivity/resistance of the tumor cells. However, despite recent advances, the development of drug resistance to chemotherapeutic agents remains a problem [5].

Bone lesions caused by OS are characterized on the basis of their radiologic appearance and present as either osteolytic, osteoblastic (osteosclerotic), or mixed [6]. Osteolysis is a common appearance associated with OS, even within predominantly osteoblastic lesions, and is mediated primarily by the osteoclasts and their bone resorbing activity [7,8]. Factors released from the bone stimulate tumor growth and in turn tumor cells produce factors that stimulate osteoclast differentiation and activity, resulting in the establishment of a mutually beneficial relationship, often termed “the vicious cycle” due to its progressively bone destructive nature [9]. Conversely, tumor cells associated with osteoblastic lesions stimulate osteogenesis [10,11].
Tumor Hypoxia in OS and in most solid tumors is a major cause of treatment failure and poor outcomes. Within most solid tumors, there are significant areas of hypoxia, which contain cancer cells that are resistant to chemotherapy and radiotherapy and this predisposes to tumor recurrence and metastasis. Low oxygen levels found in these tumor sub-regions are rarely observed in normal tissues. Therefore, tumor hypoxia offers treatment opportunities, exemplified by the development of highly active compounds that selectively target hypoxia and in groups within solid tumors and cancer as the basis for selective cancer therapy. This is exemplified by the development of highly active compounds that selectively target hypoxic regions within solid tumors [12]. TH-302 is a newly developed hypoxia-activated DNA cross-linking pro-drug that displays potent hypoxia-dependent cytotoxicity both in vitro and in preclinical cancer models [13–15]. TH-302 is currently being evaluated in phase I/II clinical trials for the treatment of solid tumors as a single agent and in combination with conventional therapies [16]. TH-302 is an inactive pro-drug created by the covalent conjugation of 2-nitroimidazole as an oxygen sensor to bromo-isophosphoramide (Bri-IPM). While inactive under aerobic conditions, when TH-302 is delivered to hypoxic regions the imidazole sensor moeity undergoes irreversible reduction and the Bri-IPM moiety, which is the basis of the DNA cross linking toxin in the pro-drug, is released. TH-302 overcomes some of the limitations of the earlier HAPs, including the ability to be activated in severe hypoxia unlikely to be present in non-pathologic tissues in the body. Importantly, due to the stability of the Bri-IPM cytotoxic, the drug exerts “ bystander” effects, not only killing hypoxic cells but also diffusing into the surrounding normoxic zones, thus treating both the hypoxic and normoxic components of the tumor [17].

In this study we investigated the cytotoxic activity of TH-302 against human OS cells in vitro and evaluated its anticancer efficacy as a single agent and in combination with the chemotherapeutic agent doxorubicin, using clinically relevant orthotopic mouse models of osteolytic and osteosclerotic OS and evaluating its effect on the development of subsequent pulmonary metastases.

Materials and methods

Cells

The human OS cell lines, K-HOS, MG-63, SAOS-2, S114-1 BTX-143, were obtained from ATCC (Manassas, VA, USA) and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 2 mM glucose, 100 U/mL penicillin, 100 U/mL streptomycin, 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), and in a 5% CO2-containing humidified atmosphere.

Normal human osteoblasts (NHOB) were obtained from trabecular bone of osteoarthritic patients at joint replacement surgery or from needle aspirates from the iliac crest of normal healthy donors and grown in αMEM (SIGMA, St. Louis, Missouri, USA) containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and L-aspartic acid 2-phosphate (NovoChen, Victoria, Australia). The medium was replaced at 4 day intervals. Cells were then subcultured by treatment with a 0.1% (v/v) mixture of collagenase and dispase. Cells from the first passage were used in all experiments. The use of all normal human donor-derived cell was approved by the ethics committee of the University of Adelaide (Approval No. VHE 091011).

Drugs

TH-302 was provided by Threshold Pharmaceuticals and dissolved in sterile saline at a concentration of 11.2 mM Doxorubicin (Dox) was purchased from Ebepe Pharma (A-4806 Vienna, Austria).

Retrieval expressing of OS cells with the triple reporter gene construct SFC-NES-TGL

Luciferase expressing BTX-143 and K-HOS cells were generated using the reentrance expression vector SFC-NES-TGL, which gives rise to a single fusion protein encoding herpes simplex virus thymidine kinase (TK), green fluorescence protein (GFP) and firefly luciferase (Luc). Virus particle-containing supernatants were harvested and filtered to remove any cellular debris and then used to infect cells [20]. The renally transduced cells were grown as bulk cultures for 48–h subculture.

Please cite this article as: Vyas N. L本土, et al., Hypoxia-activated pro-drug TH-302 inhibits potent tumor suppressing activity and cooperates with chemotherapy against osteosarcoma, Cancer Letters (2014), doi: 10.1016/j.canlet.2014.11.020

The authors acknowledge the Australian Government for financial support through the National Health and Medical Research Council and the National Breast Cancer Foundation. The authors also thank Dr. Liu, Chief Investigator at the University of Queensland, for providing the expression constructs and the experimental protocol for the transduction.

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Animals

Female athymic nude mice at 4 weeks old (Institute of Medical and Veterinary Services Division, Gilles Plains, SA, Australia) were acclimatized to the animal housing facility for a minimum period of 1 week prior to the commencement of experiments. The general physical well-being and weight of animals were monitored continuously throughout the experiments. All of the experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research and were approved by the Animal Ethics Committee of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, SA, Australia.

Intra-tibial injections of osteosarcoma cells

Four week old female Balb/c Nu/Nu mice were housed under pathogen free conditions, in accordance with the guidelines approved by the Institute of Medical and Veterinary Science animal research committee. The OS cell lines K-HOS-TLG and BTX-143-TLG were cultured as described above until they reached 70–80% confluence. Cells were removed from flasks with 2 mM EDTA and resuspended in 1 × PBS at 1 × 10^6 cells/ml and kept on ice in an Eppendorf tube. The left tibia was wiped with 70% ethanol and a 27 gauge needle coupled to a Hamilton syringe was inserted through the tibial plateau with the knee flexed and 1 × 10^6 K-HOS-TLG or BTX-143-TLG cells resuspended in 0.1 ml of PBS were injected in the marrow space. All animals were injected with PBS in the contralateralibia as the control. Mice were assigned randomly into groups of 8 animals and drug dosing started 7 days after cancer cell transplantation. TH-302 was administered at 50 mg/kg body weight via i.p. injection once a day for 5 days followed by 2 days of rest, whereas Dox was administered at 4 mg/kg i.e. once weekly until the end of the experiment.

In vitro bioluminescence imaging (BLI)

Non-invasive, whole body imaging to monitor luciferase-expressing OS cell lines K-HOS-TLG and BTX-143-TLG in mice was performed weekly using the IVIS 100 Imaging system (Xenogen, Almedena, CA). Mice were injected i.p. with 100 μl of the D-Luciferin solution at a final dose of 3 mg/ml/g mouse body weight (Xenogen Almedena, CA) and then gas-anesthetized with isoflurane. Imaging was performed for OS-10 seconds (representative images are shown at 1 second) from the side angle and the photon emission transmitted from mice was captured and quantitated in photons/sec/cm² using Xenogen Living image (lgtv Pro version 2.5) software.

Micro-computed tomography en vivo imaging analysis

Limits for μCT analyses were surgically resected and scanned using the SkyScan-1174 high-resolution μCT Scanner (SkyScan, Belgium). The Scanner was operated at 80 kV, 120 μA, rotation step 0.075, 0.5 mm Al filter and scan resolution of 5.2 μm pixel. The cross sections were reconstructed using a cone-beam algorithm (software ConeRec, SkyScan). Using the 2D images obtained from the μCT scan, the growth plate was identified and 400 sections were selected starting from the growth plate tibial interface and moving down the tibia. For quantification, 3D evaluation was performed on all data sets acquired by selecting total bone of the proximal tibia, to determine 3D bone morphometric parameters (software CTAn, SkyScan). The cross sections were reconstructed using a cone-beam algorithm (software ConeRec, SkyScan). Files were then imported into CTAn software (SkyScan) for 3D analysis and 3D image generation. All images were viewed and edited using ANT visualization software.

Data analysis and statistics

Experiments were performed in triplicate, and data presented as mean ± SE. All statistical analysis was performed using SigmaStat for Windows version 3.0 (Systat Software, Inc., Point Richmond, CA) using the unpaired Student’s t-test. Measures of association between two variables were assessed using the Spearman Rank correlation coefficient. Comparisons between groups were assessed using a one-way ANOVA test. In all cases, p < 0.05 was considered statistically significant.

Results

TH-302 exhibits potent hypoxia-selective cytotoxicity in human OS cell lines

As a prelude to testing the anticancer efficacy of TH-302 in animal models of osteosarcoma, we first examined the cytotoxicity of TH-302 against BTX-143 and K-HOS human osteosarcoma cells in vitro under normoxic (air atmosphere) and hypoxic (N2 atmosphere) conditions. Under normoxic conditions (21% O2), TH-302 exhibited only minimal cytotoxicity, whereas the cytotoxicity of TH-302, measured as a ratio of IC50 values under varying conditions of hypoxia (0%, 0.5%, 1.0%, 5.0% oxygen), increased significantly and was greater than 200-fold during extreme hypoxia of <1.0% after 24 hours of continuous drug exposure (Fig. 1A). Similar hypoxia selectivity in toxicity was also observed in three additional human osteosarcoma cell lines (S2SA-1, MG-63, and SAO52) tested (Appendix: Supplementary Fig. S1). An important attribute of TH-302 is the apparent selectivity in toxicity for tumor cells over normal cells. We observed that primary normal human osteoblasts, cultured from patients undergoing hip replacement surgery, were refractory to TH-302 treatment even under extreme hypoxia of 0.5% O2 (Fig. 1B), an effect that is likely related to the proliferative capacity of tumor cells when compared to normal cells.

TH-302 induces apoptosis of OS cells

TH-302-mediated cytotoxicity in both OS cell lines under hypoxic conditions was associated with morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation as assessed by DAPI staining of nuclei (Fig. 2A) and a concomitant activation of the effector caspase 3 assessed by the fluorometric caspase 3 assay (Fig. 2B). We next used the K-HOS cell line as a model system to dissect further the apoptotic signaling mediated by TH-302. We assessed some of the molecular determinants involved in both the extrinsic and intrinsic apoptotic signaling pathways and compared the results with those seen in normal primary human osteoblast for which no cytotoxicity was observed even under hypoxic conditions. In addition to the observed activation of caspase-3, TH-302 treatment resulted in the robust activation of caspase-9, which was clearly evident as early as 24 hours post treatment of K-HOS cells under hypoxic conditions (Fig. 2C). Activation of the initiator caspase-8 was not observed under these conditions, whereas the mitochondrial anti-apoptotic Bcl-2 family protein Bcl was cleaved and this was concomitant with processing of poly ADP-ribose polymerase (PARP) protein. The levels of Bcl-2, cIAP1, cIAP2 and XIAP under hypoxic conditions were significantly reduced with TH-302 treatment but this occurred only at the later time point of 48 hours indicating that these changes were an effect rather than a cause of TH-302-induced apoptosis. The levels of BAX remained unchanged (Fig. 2C). TH-302 mediated downregulation of cIAP1 and cIAP2 was not restricted to K-HOS cells but was also observed when BTX-143 cells were treated with TH-302 under the same conditions (Appendix: Supplementary Fig. S2). In contrast, TH-302 treatment of normal human bone cells was without effect on the caspase cascade. On the contrary, the levels of cIAP1, cIAP2 and XIAP proteins were significantly up-regulated in normal human bone cells with TH-302 treatment (Fig. 2C), suggesting a compensatory and protective response of normal cells to TH-302 death-inducing signals.

TH-302 inhibits tumor growth and protects the bone from osteosarcoma-induced bone destruction

To evaluate the efficacy of TH-302 against OS growth in bone, its effects on cancer-induced bone destruction and on the subsequent lung metastases, luciferase tagged BTX-143 and K-HOS OS cells were injected directly into the tibial marrow cavity of athymic nude mice. These cells were chosen because when transplanted intratibially in mice they replicate the bone cancer lesions commonly seen in OS patients [21]. BTX-143 cells give rise to predominantly osteolytic lesions and when compared to the contralateral non-tumor injected tibia, exhibit a net loss of bone volume (BV). In contrast, K-HOS OS cells when transplanted intratibially give rise to mixed osteolytic/osteosclerotic lesions and
while they are destructive in nature because of osteoclastic bone resorption, they also exhibit a characteristic but extensive spicular new bone formation extending from the periosteum resulting in an overall net gain of BV when compared to the contralateral non-tumor bearing tibia [21]. Importantly, as in human disease, both cell lines develop lung metastases three to four weeks after cancer cell transplantation, allowing assessment of the anti-metastatic activity of TH-302.

In the first animal model, the highly osteolytic BTK-143 cells expressing luciferase were injected directly into the tibial marrow cavity. Treatment with TH-302 was initiated 7 days post cancer cell transplantation after confirming that they established growth in bone. All vehicle treated animals showed an exponential increase of mean photon emission associated with an increase in tumor burden, which was clearly evident from day 7 onwards. By day 21, all vehicle treated mice were humanely killed because they developed large intratibial tumors that penetrated the cortical bone and the tumor mass extended into the surrounding soft tissue (Fig. 3A). In contrast, when mice were treated with TH-302 as a single agent, tumor growth was significantly delayed and tumor burden measured as a function of bioluminescence signal in bone was significantly reduced. Treatment with Dox as a single agent also resulted in a significant inhibition in tumor growth. Combination therapy with TH-302 and Dox was far superior in inhibiting tumor growth when compared to that seen with each agent alone. In fact, combination therapy completely prevented BTK-143 tumor progression in the bone marrow (Fig. 3A).

At the end of the experiment, the tibiae of all mice were dissected and assessed, using high resolution μCT for both quantitative and qualitative analysis (Fig. 3B). Extensive osteolysis was clearly evident in the vehicle treated animals such that the net loss in bone volume (BV) was 71% in the left tumor-bearing tibia when compared to the contralateral non-tumor-bearing tibia of the same animals (Fig. 3B). The extent of osteolysis was significantly reduced following treatment with TH-302 as a single agent, such that the net loss of BV decreased from 71% to 56%. Treatment with Dox alone was also effective, decreasing bone loss to 52%. Combination therapy with TH-302 and Dox demonstrated additional protection of the bone architecture such that the net loss of BV was only 20%. The inhibition of tumor growth observed in the bone marrow of animals treated with TH-302 alone or Dox alone translated to a 31% (4 of 9) and 25% (4 of 8) reduction in the number of mice that subsequently developed lung metastases respectively, as assessed by ex vivo bioluminescence imaging. However, the number of animals that developed lung metastases in the combination treatment group was not reduced further. While there was a reduction in the number of
Fig. 2. TH-302 induced apoptosis of O2 cells. A. DAPI nuclear fluorescence stain of untreated K-HOS cells showing nuclei homogeneously stained. Cells treated with 50 μM TH-302 under hypoxic conditions for 24 hours exhibit changes in the nuclei consistent with the induction of apoptosis. B. BT-143 and K-HOS cells were treated with 50 μM TH-302 for 24 hours under normoxic (21% O2) and hypoxic (1% O2) conditions and lysates were obtained to determine caspase activity using the caspase-3-specific Fluorogenic substrate, dFAMVD-AFC. The increase in caspase 3 activity is correlated with an increase in cell death by TH-302 under hypoxia. C. K-HOS and NHB cells were seeded at 2 x 10⁶ per 725 flask and were treated with TH-302 at 50 μM under normoxic and hypoxic (1% O2) conditions. Protein lysates were collected at 0, 6, 12, 24 and 48 hours after treatment and immunoblotted with various Abs, as shown.

Please cite this article in press as: Vouklos Liapis, et al., Hypoxia-activated pro-drug TH-302 exhibits potent tumor suppressive activity and cooperates with chemotherapy against osteosarcoma, Cancer Letters (2014), doi: 10.1016/j.canlet.2014.11.020
Fig. 3. TH-302 co-operates with Dox for increased anticancer efficacy against the highly metastatic BTX-145 tumors. A. BTX-143-TCL cells were injected directly into the tibial marrow cavity of 4 week female athymic mice (1 × 10^7/100 μl injection), as described in the methods, and mice were imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. Representative whole body bioluminescent images (BLI) of a single mouse from each group during the course of the experiment are shown. The line graph represents average tumor signal over time, measured as photons counts per second. All vehicle-treated animals were humanely killed on day 21 due to high tumor load. TH-302 and Dox as single agents reduced tumor growth and in addition, the combination of TH-302 and Dox resulted in an even greater inhibition of tumor growth. B. Quantitative assessment of total bone loss (%) comparing the tumor bearing tibiae of each group to the contralateral tibiae. The qualitative 3-D micro CT images showing the osteolytic nature of the BTX-143-TCL cell line, which was greatly reduced by TH-302 and Dox as single agents and by the combination of both agents. C. Average lung tumor growth was assessed via bioluminescence showing Dox and the combination of both agents caused a reduction in lung tumor growth of the BTX-143-TCL cell line. TH-302 did not significantly affect tumor burden in the lungs when compared to the vehicle group.

Please cite this article in press as: Voulkos Lasisi, et al., Hypertia-activated pro-drug TH-302 exhibits potent tumor suppressive activity and cooperates with chemotherapy against osteosarcoma, Cancer Letters (2014), doi: 10.1016/j.canlet.2014.11.020
animals with lung metastases in the TH-302 alone treated group the tumor burden measured as a function of bioluminescence signal in the lungs of the remaining mice with metastases was not significantly different from that of the vehicle treated animals, indicating that cancer cells that escaped from the bone marrow and lodged in the lungs were refractory to TH-302 treatment in this highly oxygenated environment. In contrast, Dox maintained cytotoxicity in the lungs, leading to a significant reduction in tumor burden in the Dox-alone group and also in the combination treatment group (Fig. 3C).

In the second OS model where K-HOS cells were injected intratibially, treatment with TH-302 as a single agent resulted in a significant delay in tumor growth within the first 7–14 days (Fig. 4A). However tumor growth persisted exponentially thereafter so that tumor burden was not statistically different from vehicle treated tumors by day 21. Treatment with Dox alone followed the same pattern seen with TH-302 alone. In contrast, combination therapy with TH-302 and Dox resulted in a longer delay in tumor growth, which translated to a significant reduction in the overall tumor burden within the bone marrow at each time, when compared to that seen with each agent alone (Fig. 4A). High resolution micro CT analysis of tibiae from the vehicle treated animals demonstrated extensive bone remodeling associated with cortical and trabecular bone destruction, representing the K-HOS lesions. In addition, extensive areas of spicular new bone formation extending from the cortical surface representing the osteosclerotic component of these lesions were also present in the tumor bearing tibiae (Fig. 4B).

The bone forming activity in the K-HOS cells was greater than the osteolytic activity since overall there was a net gain in BV of 41% in the left tumor-bearing tibiae when compared to the contralateral non-tumor bearing tibiae of the same animals (Fig. 4B). Comparison of the relative BV of tumor bearing tibiae and contralateral tibiae after TH-302 treatment or Dox treatment showed that the OS induced increase in BV was reduced from 41% to 3.5% and 13.9% respectively (Fig. 4B). In contrast, combination therapy with TH-302 and Dox showed significant preservation of the tibiae, with no difference in BV measurements between the tumor and non-tumor bearing tibiae. In this K-HOS OS model, 100% of the animals in the vehicle treated group developed lung metastases four weeks post-cancer cell transplantation. In contrast, the number of animals with lung metastases in the TH-302 alone, Dox alone and in the combination group decreased by 50% (4 of 8), 25% (2 of 8) and 50% (4 of 8) respectively. Similarly, what had been observed with BTK-143, tumors, metastatic tumor burden in the lungs of the remaining 50% of mice treated with TH-302 alone was no different when compared to the vehicle treated animals. In contrast lung tumor burden in the Dox alone treated group and in the combination group was significantly reduced (Fig. 4C).

Effect of TH-302 on normal bone metabolism

While prior clinical studies in patients with solid tumors have shown hematological toxicity with TH-302 therapy to be minimal [22,23], there is no data on the effects of TH-302, or indeed any other HAP, on normal bone metabolism. The intracranial injection model described in this study provided an opportunity to evaluate the consequence of TH-302 treatment on normal bone parameters. After three weeks of therapy, comparisons of the contralateral non-tumor bearing tibiae of TH-302-treated and untreated animals using high resolution micro-CT analysis demonstrated no significant changes in any of the micro architectural bone morphometric parameters, including total bone volume (BV), bone surface (BS), trabecular number (Tb.N), thickness (Tb.Th) or spacing (Tb.Sp) (Table 1).

Discussion

Osteosarcoma (OS) is the most common primary malignant tumor of bone in children and adolescent and accounts for 20% of all primary osteosarcoma metastases. It is a highly aggressive neoplasm, typically composed of spindle cells producing osteoid. The outcome for patients with OS is poor without the use of effective chemotherapy, with 2-year overall survival rates of 15%–20% following surgical resection and/or radiotherapy. Although OS can occur in any bone, it is most common in the metaphysis of long bones. The most common primary sites are the proximal humerus, distal femur, and proximal tibia, with approximately 50% of cases originating around the knee area. In addition to surgical intervention, the choice of treatment for OS is chemotherapy and multiple anticancer drugs such as Dox, cisplatin, etoposide and cyclophosphamide, are commonly used, either alone, or in combination. However, despite significant improvements in the treatment of the primary tumor and consequent patient survival, a significant proportion of these patients eventually develop pulmonary metastases and succumb to their disease even after conventional multi-stage chemotherapy and surgical excision. Therefore, there is a need to develop new and safe approaches to the treatment of OS [24–26].

In considering the use of HAPs in OS therapy, it is important to note that the bone marrow, and particularly the hematopoietic niche proximal to the endosteal surface, is hypoxic and this is a prerequisite for normal hemopoiesis. In humans, the average oxygen tension (pO2) in the bone marrow is between 1 and 7% (cf atmospheric oxygen = 21%) [27]. Cancer cells normally home to hypoxic endosteal niches where the oxygen tension is estimated to be <1.3%. Unlike soft tissue tumors, cancer cells in bone are adapted to survive and grow in a microenvironment, which is already hypoxic [28]. Therefore tumor hypoxia is a major contributor to the incurability of bone cancer. The molecule of HAPs has recently been tested in two separate mouse models of multiple myeloma [13,29]. In these studies, TH-302 treatment as monotherapy, and in combination with bortezomib, demonstrated efficacy against multiple myeloma (MM) both in vitro and in vivo and provided supporting evidence that targeting the hypoxic bone microenvironment niche is a useful and novel strategy for the treatment of cancer in bone.

Consistent with previously published data for other cancer cell types, TH-302 resulted in a dose-dependent increase in cytotoxicity of OS cells under hypoxic conditions, whereas primary normal human osteoblasts under the same conditions were relatively resistant to treatment, highlighting not only the hypoxic selectivity but also the tumor selectivity of the drug, which is likely related to the proliferative capacity of tumor cells when compared to normal cells. Mechanistically, we found that TH-302 mediated cytotoxicity was associated with caspase activation involving mitochondrial events since apoptosis induction by TH-302 was associated with caspase-9 activation and cleavage of the Bcl-2 family protein Bid. In addition, we found that the protein levels of anti-apoptotic Bcl-2, and inhibitor of apoptosis proteins, cIAP, cIAP2 and XIAP, were significantly decreased by TH-302 treatment under hypoxic conditions. However, these changes occurred 48 hours post TH-302 treatment, well after apoptosis induction and therefore likely constitute events of the cell death process rather than being causatively involved in TH-302-induced apoptosis.

IAP proteins represent one line of defense against apoptosis induction of cancer cells by inhibiting caspase activity, which is essential for tumor survival and the maintenance of therapeutic resistance. IAP proteins are expressed at elevated levels in many tumor types, including osteosarcoma, and as such makes IAP proteins attractive targets for anticancer therapy [30–32]. In contrast, the levels of IAPs were strongly upregulated in NRH donors treated with TH-302 under the same hypoxic condition, indicating the activation of
Fig. 4. TH-302 co-operates with Dox for increased anticancer efficacy against the mixed osteolytic/osteosclerotic K-HOS tumors. A. Tibial tumors were allowed to establish for 7 days and mice were randomized into 4 groups, which received PBS (vehicle), TH-302, Dox or a combination of both TH-302 and Dox as described in the Methods. Shown are representative whole body bioluminescent images, with the graph representing the average tumor signal over time, measured as mean photon counts per second. B. Representative CT images of the tumor affected the left tibia showing the osteosclerotic bone remodeling caused by K-HOS-TGL and the protection of the tibia with TH-302 and Dox as single agents and in combination. In addition, quantitative assessment of total bone volume (%) comparing the left untreated tibia to the cancer affected right tibia exhibited a reduction in osteosclerotic bone destruction in the TH-302, Dox and combination groups. C. The intensity of the lung metastases was quantified by BLI after lungs were removed. TH-302 had no significant effect on tumor burden when compared to the vehicle group. However, Dox and the combination of both TH-302 and Dox significantly reduced metastatic lung tumor burden. Data shown in each case are the average BLI from all animals in that group; points are mean ± SEM.
a compensatory mechanism where normal cells may be protected from TH-302 death-inducing signals.

OS has a variable bone-forming ability, but is destructive by virtue of its ability to expand in bone by inducing osteosteatostated-mediated bone resorption. The K-HOS and BTK-143 cell lines used in these studies were chosen on the basis that when injected subcutaneously in mice they replicate well the bone cancer lesions commonly seen in OS patients. K-HOS OS cells give rise to mixed osteolytic/osseoclastocytic but are destructive by virtue of their ability to induce osteoclast-mediated bone resorption, as we have shown previously [3]. In contrast, BTK-143 cells give rise to predominantly osteoclastocytic lesions. Importantly, as in human disease, both cell lines develop lung metastases three to four weeks post cancer cell transplantation. A limitation in measuring tumor burden in bone is that it is not possible to accurately assess the progression of tumor growth by palpation, as in soft tissue tumors, before they break through the cortical bone. However, non-invasive bioluminescence imaging used in these studies provided sensitive real-time in vivo qualitative and quantitative tracking of OS growth in bone and the subsequent metastasis to the lung. OS induced bone destruction and the consequence of drug treatment on bone architecture was assessed using high resolution micro-CT analysis.

Our results show that TH-302 monotherapy delayed tumor growth in bone and cooperated with Dox for increased antitumor efficacy. This effect was concomitant with significant protection from OS induced bone destruction and was observed in two distinct orthotopic mouse models of human OS giving rise to different bone lesions. TH-302 monotherapy reduced the incidence of lung metastases by 40%–50% in both models. However, the tumor burden within the lungs of the remaining animals with metastases was no different from those seen in the vehicle treated groups, suggesting that OS cells that escaped from the bone marrow and lodged into the lungs were refractory to TH-302 treatment in this highly oxygenated environment, highlighting the hypoxia selectivity of this drug. In contrast, Dox maintained its tumor suppressive activity in the lung.

Overall, the results presented in this study, together with the clinical activity observed in early phase I and II clinical trials of TH-302, and the lack of toxicity to normal bone metabolism, suggest that patients diagnosed with primary OS may benefit from TH-302 therapy when used either alone or in combination with conventional chemotherapy.

Acknowledgments

This work was supported by grants from the National Health and Medical Research Council (NH&MRC) of Australia @#D 627015, the Hospital Research Foundation, Adelaide, South Australia, (N/A), the National Breast Cancer Foundation and the Australian Breast Cancer Research.

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. We wish to thank Adelaide Microscopy at the University of Adelaide for technical assistance in bioluminescence imaging and Micro-CT.

Conflict of interest

None.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2014.11.020.

References

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Supplementary Figure 1:
Activity of TH-302 against OS cells in vitro: A. OS Cell lines MG-63, SAOS and SJSA-1 were seeded in 96 well plates at $1 \times 10^4$ cells per well and treated with TH-302 in normoxia (21% O$_2$) and of hypoxia (1% O$_2$) for 24 hours. TH-302 exhibited dose dependent cytotoxicity in hypoxic conditions to all 3 OS cell lines. Data points represent means of quadruplicate results from a representative experiment, repeated at least twice. Data are presented as the mean ±SD of quadruplicate wells, and are expressed as a percentage of the number of control cells.
Supplementary Figure 2: TH-302 downregulates c-IAP1 and c-IAP2 in the OS BTK-143
BTK-143 cells were seeded and treated in a similar manner to the K-HOS and NHB cells.
Protein lysates were then collected and immunoblotted with Abs against c-IAP1, c-IAP2, XIAP and Actin, as shown.
CHAPTER 3

ANTICANCER EFFICACY OF THE HYPOXIA-ACTIVATED PRO-DRUG EVOFOSFAMIDE (TH-302) IN OSTEOLYTIC BREAST CANCER MURINE MODELS
ANTICANCER EFFICACY OF THE HYPOXIA-ACTIVATED PRO-DRUG
EVOFOSFAMIDE (TH-302) IN OSTEOLYTIC BREAST CANCER MURINE
MODELS

Vasilios Liapis¹, Irene Zinonos¹, Agatha Labrinidis¹,², Shelley Hay¹, Vladimir Ponomarev²,
Vasilios Panagopoulos¹, Aneta Zysk¹, Mark DeNichilo¹, Wendy Ingman³, Gerald J Atkins⁴,
David M Findlay⁴, Andrew CW Zannettino⁵ and Andreas Evdokiou¹

¹ Discipline of Surgery, Breast Cancer Research Unit, Basil Hetzel Institute and Centre for
Personalised Cancer Medicine, University of Adelaide Woodville, South Australia, Australia
² Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, USA
³ Discipline of Surgery, School of Medicine at The Queen Elizabeth Hospital, University of
Adelaide, Woodville, Australia and Robinson Research Institute, University of Adelaide,
Australia
⁴ Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, South Australia,
Australia
⁵ School of Medical Sciences, Myeloma Research Laboratory Cancer Theme, South Australian
Health and Medical Research Institute (SAHMRI), Faculty of Health Science, University of
Adelaide, Australia

Address correspondence to: Professor Andreas Evdokiou
Breast Cancer Research Unit
Level 1, Basil Hetzel Institute, Queen Elizabeth Hospital
28 Woodville Road, Woodville
South Australia, AUSTRALIA
Fax: 618 8222 7451
E-mail: andreas.evdokiou@adelaide.edu.au

This work was supported by grants from the National Health and Medical Research Council
(NHMRC) of Australia ID# 627015, the Hospital Research Foundation, Adelaide, South
Australia, the National Breast Cancer Foundation and the Australian Breast Cancer Research.

There are no financial disclosures from any of the authors in this manuscript.

Short running title: Evofosfamide inhibits breast cancer growth

Keywords: tumor growth, hypoxia, TH-302, evofosfamide, breast cancer, chemotherapy

Total text pages: 25 Total figure pages: 5


² Agatha Labrinidis Present Address: Adelaide Microscopy, the Centre for Electron Microscopy and
Microstructure Analysis, University of Adelaide, Adelaide, South Australia, Australia
Statement of Authorship

Title of Paper
Anticancer efficacy of the hypoxia-activated prodrug evofosfamide (TH-302) in osteolytic breast cancer murine models

Publication Status
☑ Published
☐ Accepted for Publication
☐ Submitted for Publication
☐ Unpublished and Unsubmitted work written in manuscript style

Publication Details

Principal Author

<table>
<thead>
<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Vasilios Liapis</th>
</tr>
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<tbody>
<tr>
<td>Contribution to the Paper</td>
<td>Performed invitro experiments and animal studies, analysed all samples, wrote the manuscript and acted as corresponding author.</td>
</tr>
<tr>
<td>Overall percentage (%)</td>
<td>80%</td>
</tr>
<tr>
<td>Certification:</td>
<td>This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.</td>
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Signature

Date 7th November 2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate’s stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate to include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.
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<tr>
<th>Name of Co-Author</th>
<th>Contribution to the Paper</th>
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<tr>
<td>Irene Zinonos</td>
<td>Assisted with animal Ethics, manuscript evaluation and submission</td>
</tr>
<tr>
<td>Date</td>
<td>10th November 2016</td>
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<tr>
<td>Agatha Labrinidis</td>
<td>Supervised and assisted in the animal studies, assisted with the micro CT analysis, data interpretation and evaluating the manuscript.</td>
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<td>Date</td>
<td>7/11/16</td>
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<tr>
<td>Shelley Hay</td>
<td>Assisted with the invitro work and evaluated the manuscript.</td>
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<tr>
<td>Date</td>
<td>8/11/16</td>
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<td>Vladimir Ponomarev</td>
<td>Provided the triple reported construct (SFG-NES-TGL).</td>
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<td>Vasilios Panagopoulos</td>
<td>Assisted with the invitro work.</td>
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<tr>
<td>Aneta Zysk</td>
<td>Assisted with the invitro work.</td>
</tr>
<tr>
<td>Date</td>
<td>7th November 2016</td>
</tr>
<tr>
<td>Name of Co-Author</td>
<td>Contribution to the Paper</td>
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<tr>
<td>Mark DeNichilo</td>
<td>Data interpretation and manuscript evaluation.</td>
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<td>Wendy Ingman</td>
<td>Data interpretation and manuscript evaluation.</td>
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<tr>
<td>Gerald J. Atkins</td>
<td>Data interpretation and manuscript evaluation.</td>
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<tr>
<td>David M. Findlay</td>
<td>Data interpretation and manuscript evaluation.</td>
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<tr>
<td>Andrew C. W. Zannettino</td>
<td>Data interpretation and manuscript evaluation.</td>
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<tr>
<td>Andreas Evdokiou</td>
<td>Data interpretation and manuscript evaluation.</td>
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Cancer Medicine

ORIGINAL RESEARCH

Anticancer efficacy of the hypoxia-activated prodrug evosofamid (TH-302) in osteolytic breast cancer murine models

Vasilios Liapis¹, Irene Zinonos¹, Agatha Labrinidis¹,*, Shelley Hay¹, Vladimir Ponomarev², Vasilios Panagopoulos¹, Aneta Zysk¹, Mark DeNichilo¹, Wendy Ingman³,⁴, Gerald J. Atkins⁵, David M. Findlay⁵, Andrew C. W. Zannettino⁶ & Andreas Evdokiou¹

¹Discipline of Surgery, Breast Cancer Research Unit, Basil Hetzel Institute and Centre for Personalised Cancer Medicine, University of Adelaide Woodville, Woodville, South Australia, Australia
²Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, New York
³Discipline of Surgery, School of Medicine at The Queen Elizabeth Hospital, University of Adelaide, Woodville, South Australia, Australia
⁴Robinson Research Institute, University of Adelaide, Adelaide, South Australia, Australia
⁵Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, South Australia, Australia
⁶School of Medical Sciences, Myeloma Research Laboratory Cancer Theme, South Australian Health and Medical Research Institute (SAHMRI), Faculty of Health Science, University of Adelaide, Adelaide, South Australia, Australia

Keywords
Breast cancer, chemotherapy, evosofamid, hypoxia, TH-302, tumor growth

Abstract
Tumor hypoxia is a major cause of treatment failure for a variety of malignancies. However, hypoxia offers treatment opportunities, exemplified by the development of compounds that target hypoxic regions within tumors. Evosofamid (TH-302) is a prodrug created by the conjugation of 2-nitroimidazole to bromo-isophosphoramide mustard (Br-IPM). When evosofamid is delivered to hypoxic regions, the DNA cross-linking effector, Br-IPM, is released. This study assessed the cytotoxic activity of evosofamid in vitro and its antitumor activity against osteolytic breast cancer either alone or in combination with paclitaxel in vivo. A panel of human breast cancer cell lines were treated with evosofamid under hypoxia and assessed for cell viability. Osteolytic MDA-MB-231-TXSA cells were transplanted into the mammary fat pad, or into tibiae of mice, allowed to establish and treated with evosofamid, paclitaxel, or both. Tumor burden was monitored using bioluminescence, and cancer-induced bone destruction was measured using micro-CT. In vitro, evosofamid was selectively cytotoxic under hypoxic conditions. In vivo evosofamid was tumor suppressive as a single agent and cooperated with paclitaxel to reduce mammary tumor growth. Breast cancer cells transplanted into the tibiae of mice developed osteolytic lesions. In contrast, treatment with evosofamid or paclitaxel resulted in a significant delay in tumor growth and an overall reduction in tumor burden in bone, whereas combined treatment resulted in a significantly greater reduction in tumor burden in the tibia of mice. Evosofamid cooperates with paclitaxel and exhibits potent tumor suppressive activity against breast cancer growth in the mammary gland and in bone.

Keywords
Breast cancer, chemotherapy, evosofamid, hypoxia, TH-302, tumor growth

Correspondence
Andreas Evdokiou, Breast Cancer Research Unit, Level 1, Basil Hetzel Institute, Queen Elizabeth Hospital, 28 Woodville Road, Woodville, South Australia, Australia. Tel: 618 8222 7451; Fax: 618 8222 7872; E-mail: andreas.evdokiou@adelaide.edu.au

Present address
*A Agatha Labrinidis, Adelaide Microscopy, the Centre for Electron Microscopy and Microstructure Analysis, University of Adelaide, Adelaide, South Australia, Australia

Funding Information
This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia ID 627015, the Hospital Research Foundation, Adelaide, South Australia, the National Breast Cancer Foundation, and the Australian Breast Cancer Research.

Received: 11 June 2015; Revised: 5 November 2015; Accepted: 6 November 2015

Cancer Medicine 2016; 5(3):534-545
doi: 10.1002/cam4.599

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Introduction

Breast cancer is the most common cancer and the leading cause of cancer mortality in women worldwide [1]. Bone metastasis occurs in over 75% of patients with breast cancer and is associated with extensive bone destruction, leading to pathological fractures, bone pain, spinal cord compressions, and hypercalcemia [2]. While much progress has been made in the diagnosis and treatment of primary disease, metastatic breast cancer remains a challenging condition to treat and patients with advanced disease continue to die due to metastatic burden. Therefore, there is a great need to identify more effective anticancer therapeutics to improve patients’ disease-free survival, especially in patients with advanced or metastatic disease.

Solid tumors, including breast cancer, are less well-oxygenated than the normal tissues from which they arise. This so-called tumor hypoxia is described by a significant portion of the tumor mass, usually the center of the tumor, becoming less oxygenated due to its long distance from blood vessels and the slower rate of proliferation of the cancer cells in that area [3, 4]. This makes cancer cells residing in the hypoxic regions most likely to be resistant to chemotherapy and radiotherapy which then allows the tumor to recur and metastasize. Hence, tumor hypoxia is a major cause of treatment failure, poor outcomes, and recurrence for a variety of malignancies.

These low oxygen levels found in tumor subregions are rarely observed in normal tissues. Tumor hypoxia can therefore serve as the basis for selective cancer therapy, and there are a variety of therapeutic strategies being pursued for the selective targeting of hypoxic tumor cells. Hypoxia-activated produgs (HAPs) are exciting new therapeutics that enable the selective delivery of cytotoxic or cytostatic agents to hypoxic tumor cells. Eovosfamide (formally known as TH-302) is a HAP composed of 2-nitromidazole conjugated to bromo-isophosphoramide mustard (Br-IPM) [5]. The 2-nitromidazole component of eovosfamide acts as an oxygen sensor, causing eovosfamide to fragment in a one electron reductase-dependent process selectively under hypoxic conditions, releasing the bisalkylating effector, bromo-isophosphoramide (Br-IPM) which is a potent alkylating agent creating DNA cross-links. The DNA damage is recognized by the DNA damage response (DDR). The variant histone H2AX is phosphorylated, producing γH2AX and activating Chk1 and cell cycle arrest [6]. In addition, recent studies have shown that under hypoxic conditions, eovosfamide causes the inactivation of thioredoxin reductase [7], which regulates several cellular processes including the protection from damage caused by reactive oxidant species (ROS) and apoptosis by the apoptosis signal-regulating kinase 1 enzyme (ASK-1) [8].

Eovosfamide is currently in various stages of clinical trials, due to its efficacy against numerous cancer types including pancreatic cancer, multiple myeloma, soft-tissue sarcoma, and melanoma [9, 10]. However, to date, there has been no investigation looking at the anticancer efficacy of eovosfamide for the treatment of breast cancer at the primary site and, in particular, its growth in bone. It must be noted that the bone marrow itself is hypoxic, when compared with other tissues and metastatic cancer cells such as breast cancer cells, preferentially seed in these hypoxic niches [11]. Unlike soft-tissue tumors, cancer cells in bone adapt to survive in this hypoxic bone microenvironment.

Therefore, the ability to target bone metastases in this hypoxic environment is an important advantage that eovosfamide has over other cancer treatments. Taking this into consideration and the fact that conventional chemotherapeutics are usually cytotoxic to normal cells in the bone marrow, combination of chemotherapeutic agents with eovosfamide should reduce toxicity to these bone cells, especially since we have recently shown eovosfamide to be nontoxic to normal bone cells [12]. This study assessed the cytotoxic activity of eovosfamide against a panel of human breast cancer cell lines in vitro and evaluated its antitumor efficacy alone and in combination with the chemotherapeutic agent paclitaxel in vivo, using animal models of osteolytic breast cancer.

Materials and Methods

Cells

The human breast cancer cell lines MDA-MB-468, MDA-MB-453, MCF-7, T47D, andZR-75 and the breast epithelial cell lines MCF-10A and MCF-12A were obtained from ATCC (Manassas, VA). The MDA-MB-231 derivative cell line, MDA-MB-231-TXS, was kindly provided by Dr. Toshiyuki Yoneda (formerly at University of Texas Health Sciences Centre, San Antonio, TX). The generation of luciferase-tagged MDA-MB-231-TXS-TGL was described previously [13]. Normal mammary fibroblast cells were provided by Wendy Ingman (University of Adelaide, Australia), and the dermal fibroblasts were a gift from John E. Greenwood (Adult Burn Centre, Royal Adelaide Hospital, Australia). The breast cancer lines and fibroblasts were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 2 mmol/L glutamine, 100 IU/mL penicillin, 160 μg/mL gentamicin, and 10% fetal bovine serum (Life Technologies, Carlsbad, CA). The epithelial cell lines were cultured in membrane epithelial basal media (Lonza, Basel, Switzerland) containing 10% fetal bovine serum. All cell lines were maintained in a 5% CO₂-containing humidified atmosphere.
Drugs
Evozosamid was provided by Threshold Pharmaceuticals (South San Francisco, CA) and dissolved in sterile saline at a concentration of 13.2 mmol/L. The ZVAD-fmk (Caspase Inhibitor-1) was purchased from Calbiochem (Inc. La Jolla, CA), and Paclitaxel was purchased from Ebeve Pharma (A-4866; Unterach, Austria).

Cell viability assay
To determine the cytotoxic effects of evosofamid on cell growth, 1 × 10^4 cells per well were seeded in 96-well microtiter plates and allowed to adhere overnight. Cells were then treated with increasing concentrations of evosofamid (1-50 μmol/L) for 48 h as shown in Figure 1A or 24 h (1-100 μmol/L) as shown in Figure 1B, under normoxic and various hypoxic conditions. Cell viability was determined by crystal violet staining, and optical density was measured at 570 nm wavelength (OD570). Experiments were performed in triplicate and repeated at least 3 times. Results of representative experiments are presented as the mean ± SD.

Apoptosis analysis
DAPI staining of nuclei
Cells were seeded on plastic chamber slides and treated at 1% O₂ with evosofamid at 50 μmol/L for 24 h. After two washes with PBS, cells were fixed in ethanol-acetic acid (6:1) for 10 min, washed again with PBS, and incubated with 0.8 mg/ml of 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI; Roche Diagnostics, Mannheim, Germany) in methanol for 15 min. After several washes in PBS, the coverslips were mounted on ProLong® Gold antifade (Life Technologies), DAPI staining was visualized by fluorescence microscopy.

Measurement of DEVD-caspase activity
DEVD-caspase activity was assayed by cleavage of zDEVD-AFC, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerase. Cells (1 × 10^5/well) grown in 96-well plates were treated as indicated, washed once with PBS, and resuspended in 30 μl lysis buffer containing 5 mmol/L Tris-HCl, 5 mmol/L EDTA, and 10% Igepal (pH 7.5). Cell lystate (15–20 μg of protein) was added to each assay tube containing 8 μmol/L substrate in 1 ml fluorometric protease buffer (50 mmol/L HEPES, 10% sucrose, 10 mmol/L DTT, 0.1% CHAPS [pH 7.4]). After 4-5 h at room temperature, fluorescence was quantified (Ex 400 and Em 505) in a Perkin-Elmer LS50 fluorescence spectrometer. Results were expressed relative to the protein concentration of the sample and determined using a commercial BCA protein assay reagent from Thermo Fisher Scientific (Waltham, MA).

Western blot analysis
Cells were treated with evosofamid at 50 μmol/L in a time-dependent manner (0, 6, 12, 24, 48 h) under normoxic (21% O₂) and hypoxic (1% O₂) conditions and lysed in buffer containing 10 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium deoxycholate (SDS), 2 mmol/L sodium vanadate, and a protease inhibitor cocktail (Roche Diagnostics). Protein lysates were heated at 70°C for 10 min and loaded into 4–12% polyacrylamide gels for electrophoresis under reducing conditions. Separated proteins were electrophoretically transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK) and blocked in PBS containing 5% blocking reagent (GE Healthcare) for 1 h at room temperature.

Immunodetection was performed overnight at 4°C in PBS/blocking reagent containing 0.1% Tween 20, using the following primary antibodies at the dilutions suggested by the manufacturer: mAb anticaspase-8, mAb anticaspase-3, pAb antibid, pAb anticaspase-9, pAb P13 kinase Class III, pAb phospho-p44/42 MAPK, pAb p44/42 MAPK, pAb phospho-Akt, and pAb Akt were purchased from Cell Signaling Technology (Beverly, MA), pAb anti-inhibitor of apoptosis 1 (cIAP1), pAb anti-inhibitor of apoptosis 2 (cIAP2), pAb anti-XIAP from R&D systems, pAb anti-BAX, pAb anti-p53, pAb anti-p21, and mAb anti-Bcl-2 were purchased from Santa Cruz Biotechnology (Dallas, Texas) and pAb anti-poly-(ADP-Ribose) polymerase (PARP) from Roche Diagnostics. Anti-actin mAb (Sigma, Saint Louis, MO) was used as a loading control. Membranes were then rinsed several times with PBS containing 0.1% Tween-20 and incubated with 1:5000 dilution of anti-mouse, anti-goat, or anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h. Visualization and quantification of protein bands were performed using the ECF substrate reagent kit (GE Healthcare) on a FluorImager (Molecular Dynamics Inc., Sunnyvale, CA).

Animals
Female athymic mice at 5 weeks old (Institute of Medical and Veterinary Services Division, Gilles Plains, SA, Australia) were acclimatized to the animal housing facility for a minimum period of 1 week prior to the commencement of experimentation. The general physical well-being and weight of animals were monitored continuously throughout
Figure 1. Activity of evosfosamide against breast cancer, epithelial cells, and fibroblasts in vitro. (A) Six breast cancer cell lines were seeded in 96-well plates at 1 × 10^4 cells per well and treated with increasing doses of evosfosamide in normoxic (21% O₂) and hypoxic (1% O₂) conditions for 48 h. (B) The breast cancer cell line MDA-MB-231-TXSA was treated with an increasing dose of evosfosamide under normoxic (21% O₂) and in various hypoxic conditions from 0% to 5% O₂ for 24 h. (C) Evosfosamide reduced cell viability in both epithelial breast cell lines MCF-10A and MCF-12A selectively under hypoxic conditions (1% O₂) 48 h after treatment. Dermal and mammary fibroblasts were relatively resistant to evosfosamide under the same conditions. Cell viability of all cell lines was assessed by crystal violet staining. (D) DAPI nuclear fluorescence stain of untreated MDA-MB-231-TXSA cells showing nuclei homogenously stained. Cells treated with 50 μmol/L evosfosamide under hypoxic conditions for 24 h exhibit changes in the nuclei consistent with the induction of apoptosis. Data points show means of quadruplicate results from a representative experiment, repeated at least twice and presented as the mean ± SD of quadruplicate wells and expressed as a percentage of the number of control cells.
the experiments. All mice were housed under pathogen-free conditions, and all experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research and were approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, SA, Australia.

Mammary fat pad injections of breast cancer cells

MDA-MB-231-TXSA-TGL human breast cancer cells were cultured as described above until they reached 70–80% confluency. Cells were removed from flasks with 2 mmol/L EDTA and resuspended in 1 × PBS at 1 × 10^5 cells/10 μL and kept on ice in an eppendorf tube. An equal volume of Matrigel™–HC (BD Biosciences, Bedford, MA) was added to the cells and resuspended. The mice were anesthetized by Isoflurane (Faulding Pharmaceuticals, SA, Australia), the mammary fat pad area of the mice was wiped with ethanol, and the skin was lifted over the left outermost nipple. Finally, 20 μL of cells was injected into the mammary fat pad area using a 25-gauge needle, and animals were allowed to recover under a heat lamp before being transferred into cages. Mice were then assigned randomly into groups of 10 animals each. Eovosamide was given via intraperitoneal injection at a dose of 50 mg/kg once a day for 5 consecutive days, followed by 2 days rest. Paclitaxel was given at a dose of 6.25 mg/kg subcutaneously once a week. The dosing started 7 days after cancer cell implantation and was given every week until the end of the experiment, at day 21.

Intratibial injections of breast cancer cells

MDA-MB-231-TXSA-TGL cells were cultured as described above. The left tibia was wiped with 70% ethanol, and a 27-gauge needle coupled to a Hamilton syringe was inserted through the tibial plateau with the knee flexed; 1 × 10^5 MDA-MB-231-TXSA-TGL cells resuspended in 10 μL of PBS were then injected in the marrow space. All animals were injected with PBS in the contralateral tibia as the control. Mice were then assigned randomly into groups of 10 animals per group and received the same treatments described in the mammary fat pad model.

In vivo bioluminescent imaging

Noninvasive, whole-body imaging to monitor luciferase-expressing MDA-MB-231-TXSA-TGL cells in mice was performed weekly using the IVIS 100 Imaging system (Xenogen, Alameda, CA). Mice were injected i.p. with 100 μL of the α-Luciferin solution at final dose of 3 mg/20 g mouse body weight (Xenogen) and then gas anesthetized with Isoflurane (Faulding Pharmaceuticals). Images were acquired for 0.5–30 sec (representative images are shown at 1 sec), and the photon emission transmitted from mice was captured and quantitated in photons/sec/cm^2/sr using Xenogen Living image (Igor Pro version 2.5) software.

Microcomputed tomography ex vivo analysis

Limbs for μCT analysis were surgically resected and scanned using the SkyScan-1072 high-resolution μCT Scanner (SkyScan, Kontich, Belgium). The scanner was operated at 80 kV, 120 μA, rotation step 0.675, 0.5 mm Al filter and scan resolution of 5.2 μm/pixel. The cross-sections were reconstructed using a cone-beam algorithm (software Cone rec, SkyScan). Using the 2D images obtained from the μCT scan, the growth plate was identified and 400 sections were selected starting from the growth plate/tibial interface and moving down the tibia. For quantification, 3D evaluation was performed on all data sets acquired by selecting total bone of the proximal tibia, to determine 3D bone morphometric parameters (software CTAn, SkyScan). The cross-sections were reconstructed using a cone-beam algorithm (software Cone_rec, SkyScan). Files were then imported into CTAn software (SkyScan) for 3D analysis and 3D image generation. All images are viewed and edited using ANT visualization software (SkyScan).

Histology

Tibiae were fixed in 10% (v/v) buffered formalin (24 h at 4°C), followed by 2 weeks of decalcification in 0.5 mol/L EDTA/0.5% paraformaldehyde in PBS, pH 8.0 at 4°C. Complete decalcification was confirmed by radiography and tibiae were then paraffin embedded. Five-micron longitudinal sections were prepared and stained with H&E. Analysis was performed using the Nanozoomer and the Hamamatsu software.

Data analysis and statistics

Experiments were performed in triplicate, and data presented as mean ± SE. All statistical analysis was performed using SigmaStat for Windows version 3.0 (Systat Software, Inc., Port Richmond, CA) using the unpaired Students’ t-test. Measures of association between two variables were assessed using the Spearman’s rank correlation coefficient. Comparisons between groups were assessed using a one-way analysis of variance test. In all cases, P < 0.05 was considered statistically significant.
Results

Evofosfamide exhibits hypoxia-selective cytotoxicity on human breast cancer cells

A panel of human breast cancer cell lines (MDA-MB-231-TXSA, MDA-MB-453, MDA-MB-468, ZR-75, MCF-7, and T47D) were assessed for their sensitivity to the cytotoxic effects of evofosfamide under normoxic (21% O₂) and hypoxic (1% O₂) conditions. Evofosfamide exhibited relatively minimal toxicity under normoxic conditions, whereas under hypoxic conditions (1% O₂), evofosfamide reduced cell viability in a dose-dependent manner after 48 h of treatment, with IC₅₀ values ranging between 1 and 25 μmol/L and a maximum of 50–90% loss of viability at the highest dose of 50 μmol/L (Fig. 1A). The hypoxia selectivity of evofosfamide, in the MDA-MB-231-TXSA cells measured as a ratio of IC₅₀ values under varying conditions of hypoxia (0%, 0.5%, 1.0%, and 5.0% oxygen), was greater than 200-fold during extreme hypoxia of <1.0%.

Figure 2. Evofosfamide-induced apoptosis in breast cancer cells in vitro. (A) Four breast cancer cell lines were seeded in 96-well plates at 1 × 10⁴ cells per well and treated with evofosfamide at 50 μmol/L or coincubated with the broad specificity caspase inhibitor z-VAD-fmk (50 μmol/L). To exclude possible toxic effects of the inhibitor, cells were also treated with the inhibitor alone under normoxic and hypoxic (1% O₂) conditions. Cell lysates were used to determine caspase-3-like activity, using the caspase-3-specific fluorogenic substrate, zDEVD-AFC, and cell viability was assessed via crystal violet staining. Data points show means of quadruplicate results from a representative experiment, repeated at least twice; bars ± SD.
after 24 h of treatment (Fig. 1B). Of interest, evosofamide was also cytotoxic to MCF-10A and MCF-12A cells lines, normally regarded as normal epithelial cell lines of breast origin, when subjected under the same hypoxic conditions as the breast cancer cell lines, with IC$_{50}$ values of 25 μmol/L and 2 μmol/L, respectively. In contrast normal human dermal fibroblasts and human primary mammary fibroblasts were relatively resistant to the cytotoxic activity of evosofamide, with IC$_{50}$ values of >50 μmol/L and 40 μmol/L, respectively (Fig. 1C). Evosofamide-mediated cytotoxicity in the breast cancer cell line MDA-MB-231-TXSA under hypoxic conditions was associated with morphological changes characteristic of apoptosis, including chromatid condensation and nuclear fragmentation, as assessed by DAPI staining of nuclei (Fig. 1D).

**Caspase activation is secondary in the apoptotic activity of evosofamide**

The observed decrease in cell viability with evosofamide treatment under hypoxic conditions was associated with morphological changes characteristic of apoptosis, including chromatid condensation and nuclear fragmentation, concomitant with an increase in caspase-3 activation. However, coadministration with the pan-caspase inhibitor ZVAD-fmk failed to prevent the cytotoxic activity of evosofamide under hypoxic conditions, despite irreversibly inhibiting caspase-3 activity (Fig. 2A) suggesting that the mechanisms of evosofamide-mediated cytotoxicity are in part caspase independent. This is also supported by the observation that evosofamide under hypoxic conditions was potently cytotoxic against MCF-7 cells which are caspase-3 deficient. [14] (Fig. 2B).

As a prelude to testing the anticancer efficacy of evosofamide in vivo, the MDA-MB-231-TXSA cell line was chosen to further characterize the molecular determinants involved in apoptotic signaling, mediated by evosofamide (Fig. 3A). Under hypoxic conditions (1% O$_2$), evosofamide treatment resulted in the prominent activation of the caspase cascade with robust cleavage of the initiator caspase-8 followed by caspase-9, and caspase-3 and the concomitant processing of the mitochondrial proapoptotic Bcl-2 family protein BID and the poly ADP-ribose polymerase (PARP) protein. While these changes were observed under hypoxic conditions, nonetheless this occurred only at the later time point of 48 h after the onset of cell death, indicating that

![Figure 3. Apoptotic signaling of evosofamide as a single agent against MDA-MB-231-TXSA cells.](image)

(A) Normoxia | Hypoxia  
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<td>Caspase-3</td>
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(B) Normoxia | Hypoxia  
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these changes were an effect rather than a cause of evofosamid-induced apoptosis. The levels of inhibitor of apoptosis proteins 1 and 2 (cIAP1/2) under hypoxic conditions were significantly reduced with evofosamide treatment, which occurred at the 24- and 48-h time points, whereas the levels of XIAP, BAX, and Bcl-2 remained unchanged. Due to the DNA alkylation caused by the BR-IPM component of evofosamide, upregulation of p53 was observed after 48 h of evofosamide exposure in both normoxic and hypoxic conditions, leading to the upregulation of the p53 target protein p21 after 48 h in normoxia and after 24 h in hypoxia. Additional signaling pathway analysis demonstrated a robust reduction in PI3 kinase and inhibition of phosphorylated AKT which was evident at 24 and 48 h after treatment with evofosamide. However, no significant changes in the levels of either phosphorylated or total MAPK were detected following treatment.

**Effect of evofosamide and paclitaxel on the growth of orthotopic breast cancer xenografts**

To investigate the anticancer efficacy of evofosamide, paclitaxel, and the combination of both against tumors growing in the orthotopic site, luciferase-tagged MDA-MB-231-TXSA cells were injected directly into the mammary fat pad of athymic female nude mice and treatment was initiated 7 days post cancer cell transplantation. These cells form aggressive, rapidly growing tumors when injected into the mammary fat pad, which can be accurately monitored and quantified using noninvasive bioluminescence imaging [13]. Animals treated with vehicle showed an exponential increase of mean photon emission, associated with an increase in tumor burden, which was evident from day 7 onwards, reaching a maximum signal at day 21, at which point animals had to be humanely killed. In contrast, all animals treated with evofosamide or paclitaxel as single agents showed a significant reduction in tumor burden over the same period, whereas the combination of both was highly effective which completely prevented tumor growth (Fig. 4A and B).

**Figure 4.** Paclitaxel cooperates with evofosamide to reduce MDA-MB-231-TXSA-TGL orthotopic tumors in vivo. Mice were treated as described in Materials and Methods section and imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. (A) Representative whole body bioluminescent images of the mammary tumors of a single animal from each group and (B) the line graph, showing average tumor signal over time, expressed as mean photon counts per second during the course of the experiments are shown. Animals receiving treatment with evofosamide and paclitaxel as single agents showed a significant delay in tumor growth. In addition, all mice receiving the combination of evofosamide and paclitaxel showed a further delay of tumor growth when compared with each agent individually. Data shown in each case are the average bioluminescent imaging from all animals in that group; points are means ± SEM.
destruction, a xenogeneic tumor model was used, in which the MDA-MB-231-TXSA-TGL cells were transplanted directly into the tibial marrow cavity of athymic mice. All vehicle-treated animals showed an exponential increase of mean photon emission associated with an increase in tumor burden, which was clearly evident from day 14 onwards. By day 21, all animals developed large intratibial tumors that penetrated the cortical bone with the tumor mass, invading the surrounding soft tissue. For ethical reasons, all vehicle-treated animals were humanely killed on day 21 due to high tumor load and extensive osteolysis. In contrast, animals treated with evosofamide or paclitaxel as single agents showed a significant delay in tumor growth and an overall reduction in tumor burden, whereas the combination of both agents demonstrated a far superior anticancer efficacy in bone (Fig. 5A and B).

At the end of the experiment, the tibiae of all mice were dissected and assessed, using high-resolution μCT.

**Figure 5.** Paclitaxel cooperates with evosofamide for increased anticancer efficacy against breast cancer line MDA-MB-231-TXSA-TGL in vivo. MDA-MB-231-TXSA-TGL cells were injected directly into the tibial marrow cavity of 4-week-old athymic mice, allowed to establish for 7 days, as described in Materials and Methods section, mice were imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. (A) Representative whole-body bioluminescent images of a single mouse from each group during the course of the experiment are shown. (B) The line graph represents average tumor signal over time, measured as photon counts per second. Evosofamide and paclitaxel as single agents reduced tumor growth, and in addition, the combination of both resulted in an even greater inhibition of tumor growth. (C) Quantitative assessment of total bone loss (%) comparing the tumor-bearing tibiae of each group to the contralateral tibia and the qualitative 3-D micro-CT images show the osteolytic nature of the MDA-MB-231-TXSA-TGL cell line, which was reduced by paclitaxel and the combination of evosofamide and paclitaxel. (D) Representative H&E-stained tibial sections from mice showing an inhibition of intra- and extramedullary growth with evosofamide, paclitaxel, and the combination of both.
for both quantitative and qualitative analysis of bone destruction (Fig. 5C). Extensive osteolysis was clearly evident in the vehicle-treated animals such that the net loss in bone volume (BV) was 94% in the left tumor-bearing tibiae when compared with the contralateral nontumor-bearing tibiae. While evofosfamide treatment reduced tumor burden, this did not translate to prevention of bone destruction such that the extent of osteolysis was not significantly different than the vehicle-treated group. In contrast, treatment with paclitaxel alone was significantly effective, decreasing bone loss to 21%. Combination therapy with evofosfamide and paclitaxel demonstrated additional protection of the bone architecture such that the net loss of BV was only 6%, although this improvement in bone protection by the combination therapy was not statistically significant when compared with paclitaxel alone. Histological examination of representative sections of the tibial sections show the MDA-MB-231-TXSA-TGL tumor growing within the marrow cavity and in the surrounding tissue (Fig. 5D). Trabecular and cortical bone destruction was also evident, confirming the micro-CT data. Treatment with evofosfamide, paclitaxel, and the combination of both significantly reduced tumor size in the bone marrow confirming the bioluminescence data but did not completely eliminate breast cancer growth in bone.

**Discussion**

Tumor hypoxia is a major cause of treatment failure and poor outcome for a wide variety of malignancies [15]. Within most solid tumors, there are significant areas of hypoxia, which contain cancer cells that resist conventional anticancer chemotherapy and radiotherapy, and this pre-disposes to tumor recurrence and metastasis [16, 17]. However, tumor hypoxia also offers treatment opportunities, exemplified by the development of highly active compounds that can specifically target tumor hypoxic zones. In this study, we assessed the hypoxia-selective cytotoxicity of evofosfamide against a panel of human breast cancer cell lines in vitro and investigated the anticancer efficacy of evofosfamide as monotherapy and in combination with paclitaxel against breast cancer growing in the orthotopic site of the mammary gland and in the bone marrow using the aggressive and highly osteolytic MDA-MB-231-TXSA breast cancer cells. Consistent with our previous published data [12], evofosfamide exhibited relatively minimal toxicity under normoxic conditions, whereas under increasing hypoxia evofosfamide treatment of a variety of human breast cancer cell lines dose dependently decreased cell viability. Normal breast epithelial cell lines MCF-10A and MCF-12A were also equally sensitive to evofosfamide under hypoxic conditions, which may be related to their proliferative capacity being similar to that of breast cancer cells. In contrast, primary dermal fibroblasts and normal mammary fibroblasts were relatively resistant to the cytotoxic activity of evofosfamide under the same conditions.

It is well established that the clinical management of breast cancer patients with different amplifications of markers such as ER+, PR+, and HER2 indicates different therapeutic strategies. Our results do not show any direct correlation between these markers and the cytotoxic activity of evofosfamide, suggesting perhaps a universally effective response. However, recent studies using a limited number of cell lines have shown a link between BRCA-1, triple negativity of breast cancer and its response to evofosfamide [18]. In this context, an extensive investigation using a large cohort of breast cancer cell lines and patient data will be required to delineate such differences in evofosfamide therapeutic response.

While evofosfamide treatment induced changes characteristic of apoptosis induction, coaddition with the broad-spectrum caspase inhibitor zVAD-fmk failed to protect breast cancer cells from the cytotoxic activity of evofosfamide under hypoxia, suggesting the involvement of caspase independent mechanism. This is also reflected by the activation of caspase-3, caspase-8, caspase-9, cleavage of the Bcl-2 family protein Bid, and the downregulation of c-IAP1/2, which occurred 48 h post evofosfamide treatment, well after apoptosis induction and therefore, likely to represent events of the cell death process rather than a cause of evofosfamide-induced apoptosis. When evofosfamide is activated under hypoxia, the cytotoxic compound Br-IPM induces 1’5’-cross-linkage of DNA, which results in S139 phosphorylation of the histone H2AX leading to cell death [19]. This DNA damage is often associated with caspase independent mechanisms as previously reported [20] and is reflected by PARP cleavage leading to the upregulation of p53 followed by upregulation of the p53 target protein and cell cycle regulating gene p21 as clearly observed here.

The therapeutic advantage of evofosfamide is expected to be greatest in combination with adjuvant cytotoxic chemotherapy. In this context, we tested the activity of evofosfamide in combination with paclitaxel, in a preclinical model of breast cancer development and progression. We selected the breast cancer cell line MDA-MB-231-TXSA, which we have shown in vitro to be the least sensitive to evofosfamide, such that any additive or synergistic activity will be readily detected. The antitumor effect of evofosfamide as a single agent in the mammary gland was most prominent from day 14 onwards when tumor size was approaching 1.0 cm$^3$ which coincided with the presence of hypoxic tumor cores seen when tumors were excised from parallel untreated animals and assessed histologically at the same time (data not shown). While
Evofosamide Inhibits Breast Cancer Growth

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Paclitaxel as a single agent was highly effective in reducing tumor burden, the combination of both completely prevented growth of the tumor within the mammary gland. MDA-MB-231-TXSA cells are highly osteolytic and when transplanted in to the tibial marrow cavity of mice lead to extensive bone destruction [13, 21]. This in vivo model mimics the late stages of bone metastasis and is ideally suited for monitoring the effects of drug treatment on breast cancer growth in the bone and also on cancer-induced bone destruction [13]. To date, the activity of evofosamide against breast cancer growing in bone has not been reported. When breast cancer cells were injected intratibially, evofosamide treatment inhibited tumor growth in bone, leading to a significant reduction in the overall tumor burden. However, the reduction in tumor burden did not translate to a significant inhibition of osteolysis attesting to the aggressive osteolytic properties of these cells. In contrast, paclitaxel as a single agent was highly effective in reducing tumor load in bone while also protecting the bone from cancer-induced bone destruction. The combined treatment resulted in a reduction in tumor burden.

It is important to note that while hematological toxicity with evofosamide treatment has been minimal, to date no data exist on the effects of evofosamide or indeed any other HAP, on normal bone metabolism in the context of osteoclasts, osteoblasts, or osteocyte survival and function. We have compared the micro architectural bone morphometric parameters of the contralateral nontumor injected tibiae from untreated and evofosamide-treated animals and showed no obvious histological abnormalities on bone parameters including the number and viability of osteoclasts, or osteoblasts, per surface area (data not shown), whereas high-resolution micro-CT analysis demonstrated no changes in microarchitectural bone parameters, such as total or trabecular BV measurements with evofosamide treatment (data not shown).These results are consistent with our previously published data in this context using a model of osteosarcoma [12].

Evofosamide is currently being evaluated in numerous phase III and phase II clinical trials against a variety of cancer types either as monotherapy or in combination with conventional chemotherapy with promising results. Our data further support the clinical development of evofosamide as a novel approach in the treatment of patients with breast cancer, especially those with existing bone metastases.

Acknowledgments

This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia ID# 627015, the Hospital Research Foundation, Adelaide, South Australia, the National Breast Cancer Foundation, and the Australian Breast Cancer Research. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Adelaide Microscopy at the University of Adelaide for technical assistance in bioluminescence imaging and Micro-CT.

Conflict of Interest

There are no financial disclosures from any of the authors in this article.

References


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CHAPTER 4
ANTICANCER EFFICACY OF THE HYPOXIA ACTIVATED PRODRUG EVOFOSFAMIDE IS ENHANCED IN COMBINATION WITH PROAPOPTOTIC RECEPTOR AGONISTS AGAINST OSTEOSARCOMA
## Statement of Authorship

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<td>Publication Status</td>
<td>□ Published  □ Accepted for Publication  ✔ Submitted for Publication  □ Unpublished and Unsubmitted work written in manuscript style</td>
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### Principal Author

| Name of Principal Author (Candidate) | Vasilios Liapis |
| Contribution to the Paper | Performed invitro experiments and animal studies, analysed all samples, wrote the manuscript and acted as corresponding author. |
| Overall percentage (%) | 80% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date 7th November 2014 |

### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.
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<tr>
<td>Aneta Zysk</td>
<td>Assisted in the animal studies, assisted with the micro CT and bioluminescent analysis, data interpretation and evaluating the manuscript.</td>
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<td>7th November 2014</td>
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<tr>
<td>Mark DeNichilo</td>
<td>Data interpretation and manuscript evaluation.</td>
<td></td>
<td>7th November 2016</td>
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<tr>
<td>Irene Zinonos</td>
<td>Assisted with animal Ethics, manuscript evaluation and submission</td>
<td></td>
<td>10th November 2016</td>
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<tr>
<td>Shelley Hay</td>
<td>Assisted with the <em>in vitro</em> work and evaluated the manuscript.</td>
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<td>7/11/16</td>
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<tr>
<td>Vasilios Panagopoulos</td>
<td>Assisted with the <em>in vitro</em> work and evaluated the manuscript.</td>
<td></td>
<td>7/11/16</td>
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<tr>
<td>Alexandra Shoubridge</td>
<td>Assisted with the <em>in vitro</em> work and the micro CT results.</td>
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<td>07/11/2016</td>
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<tr>
<td>Christopher Difelice</td>
<td>Assisted with the <em>in vitro</em> work.</td>
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<td>8/11/16</td>
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<tr>
<td>Name of Co-Author</td>
<td>Vladimir Ponomarev</td>
<td>Contribution to the Paper</td>
<td>Provided the triple reported construct (SFG-NES-TGL)</td>
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Anticancer efficacy of the hypoxia activated prodrug evofosfamide is enhanced in combination with proapoptotic receptor agonists against osteosarcoma

Vasilios Liapis¹, Aneta Zysk¹, Mark DeNichilo², Irene Zinonos¹, Shelley Hay¹, Vasilios Panagopoulos¹, Alexandra Shoubridge¹, Christopher Difelice¹, Vladimir Ponomarev³, Wendy Ingman⁴, Gerald J Atkins⁵, David M Findlay⁵, Andrew CW Zannettino⁶ and Andreas Evdokiou¹

¹Discipline of Surgery, Breast Cancer Research Unit, Basil Hetzel Institute and Centre for Personalised Cancer Medicine, University of Adelaide, Woodville, South Australia, Australia

²Vascular Biology and Cell Trafficking Laboratory, Centre for Cancer Biology, University of South Australia, Adelaide, South Australia, Australia

³Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, USA

⁴Discipline of Surgery, School of Medicine at The Queen Elizabeth Hospital, University of Adelaide, Woodville, Australia and Robinson Research Institute, University of Adelaide, Australia

⁵Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, South Australia, Australia

⁶School of Medical Sciences, Myeloma Research Laboratory Cancer Theme, South Australian Health and Medical Research Institute (SAHMRI), Faculty of Health Science, University of Adelaide, Australia

Address correspondence to: Professor Andreas Evdokiou
Breast Cancer Research Unit
Level 1, Basil Hetzel Institute, Queen Elizabeth Hospital
28 Woodville Road, Woodville South Australia, AUSTRALIA
Phone: E-mail: andreas.evdokiou@adelaide.edu.au

This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia ID# 627015, the Hospital Research Foundation, Adelaide, South Australia, the National Breast Cancer Foundation and the Australian Breast Cancer Research. We wish to thank Adelaide microscopy Adelaide Microscopy at the University of Adelaide for technical assistance in Micro-CT.

There are no financial disclosures from any of the authors in this manuscript.

Keywords: Hypoxia, Evofosfamide, Osteosarcoma, Dulanermin, Drozitumab

Total text pages: 26 Total figure pages: 4

Cancer Medicine: Accepted 31st March 2017
Abstract:

Tumor hypoxia is a major cause of treatment failure for a variety of malignancies. However, hypoxia also leads to treatment opportunities as demonstrated by the development of compounds that target regions of hypoxia within tumors. Evofosfamide is a hypoxia activated prodrug that is created by linking the hypoxia seeking 2-nitroimidazole moiety to the cytotoxic bromo-isophosphoramide mustard (Br-IPM). When evofosfamide is delivered to hypoxic regions of tumors, the DNA cross linking toxin, Br-IPM, is released leading to cell death. This study assessed the anticancer efficacy of evofosfamide in combination with the Pro Apoptotic Receptor Agonists (PARAs) dulanermin and drozitumab against human osteosarcoma in vitro and in an intratibial murine model of osteosarcoma.

Under hypoxic conditions in vitro, evofosfamide cooperated with dulanermin and drozitumab, resulting in the potentiation of cytotoxicity to osteosarcoma cells. In contrast under the same conditions, primary human osteoblasts were resistant to treatment. Animals transplanted with osteosarcoma cells directly into their tibiae developed mixed osteosclerotic/osteolytic bone lesions and consequently developed lung metastases three weeks post cancer cell transplantation. Tumour burden in the bone was reduced by evofosfamide treatment alone and in combination with drozitumab and prevented osteosarcoma-induced bone destruction while also reducing the growth of pulmonary metastases.

These results suggest that evofosfamide may be an attractive therapeutic agent, with strong anticancer activity alone or in combination with either drozitumab or dulanermin against osteosarcoma.
**Introduction:**

Osteosarcoma (OS) is the most common primary type of cancer that develops in the bone and accounts for 20% of all primary osseous neoplasms (Campanacci, 1999; Pringle, 1999). Most OS occur in young adults and children and usually develops in areas where the bone is rapidly growing such as the proximal tibia, distal femur and proximal humerus (Tang, Song, Luo, Haydon, & He, 2008). Metastatic spread of OS preferentially occurs in the lungs which is correlated with poor survival and is seen in 20% of patients with OS (Link et al., 1991; Saeter et al., 1997). Over the past 20 years treatment of OS has advanced considerably due to the increased efficacy of conventional chemotherapeutic agents. The type, combination as well as the doses of chemotherapeutic agents given as well as the sensitivity of the tumour cells determine the patients’ response to treatment. Despite these advances in treatment, drug resistance still remains a problem (Chan, Grogan, Haddad, DeBoer, & Ling, 1997). In addition, conventional chemotherapeutic drugs have a significant impact on normal bone health, leading to a greater risk of developing osteoporosis and myelosuppression due to toxicities in the bone marrow (Gralow et al., 2009; Lustberg, Reinbolt, & Shapiro, 2012).

The characteristics of bone lesions caused by OS are based on their radiologic appearance which can be either osteoblastic (osteosclerotic), osteolytic, or a combination of both (Mundy, 2002b). Osteolysis is common with OS and is caused by the bone resorbing activity of osteoclasts (Goltzman, 2001; Taube, Elomaa, Blomqvist, Beneton, & Kanis, 1994). Tumour growth is stimulated by factors released from the bone and in turn tumour cells produce factors that stimulate osteoclastic bone resorption, resulting in a mutual relationship of bone destruction between the cell types known as “the vicious cycle” (Chirgwin & Guise, 2000). In contrast, osteoblastic lesions are associated with tumour cells that stimulate osteogenesis (Goltzman, 1997; Goltzman, Karaplis, Kremer, & Rabbani, 2000).
As with most solid tumours, early stage OS displays significant regions of hypoxia, where resistant tumour cells reside which results in tumour recurrence and metastasis, leading to treatment failure and poor outcomes. These hypoxic conditions found in tumor sub-regions are rarely observed in normal tissue, tumor hypoxia can therefore provide the basis for selective cancer therapy and there are a number of strategies currently being investigated to selectively target tumor cells in this hypoxic environment. Hypoxia-activated prodrugs (HAPs) selectively deliver cytostatic or cytotoxic agents to hypoxic sub regions.

Evofofosfamide (formerly TH-302) is a hypoxia-activated prodrug composed of 2-nitroimidazole linked to bromo-isophosphoramid mustard (Br-IPM) (Jian-Xin Duan, Monica Banica, & W. Steve Ammons, 2007). The 2-nitroimidazole component of evofofosfamide serves as an oxygen sensor, releasing the crosslinking DNA-alkylating Br-IPM into the hypoxic regions of tumors. To date, evofofosfamide has been investigated both as a stand-alone agent and in combination with chemotherapy and other targeted cancer drugs against numerous solid tumor types and blood cancers (Borad et al., 2014; Chawla et al., 2014; Wojtkowiak et al., 2015).

Pro-Apoptotic Receptor Agonists (PARAs), either as monotherapy or in combination with other agents, are generally well-tolerated by patients with very few side effects (Dine et al., 2016) and although phase 1/1b studies provided encouraging preliminary results, findings from randomized Phase 2 studies failed to demonstrate significant clinical benefit (Herbst et al., 2010; Kindler et al., 2012; Soria et al., 2010; Younes et al., 2010). Despite these clinical observations, there has been no investigation examining the anticancer efficacy of evofofosfamide alone or in combination with either the Pro-Apoptotic Receptor Agonists (PARAs) dulanermin (formerly known as Apo2L/TRAIL), or drozitumab for the treatment of osteosarcoma.
This study investigates the cytotoxic activity of evofosfamide alone and in combination with dulanermin and drozitumab against human OS cells in vitro and in vivo, using a clinically relevant orthotopic mouse model of OS and on their subsequent lung metastases.

**Materials and Methods:**

**Cells**

The human OS cell lines BTK-143 and K-HOS were obtained from ATCC (Manassas, VA, USA) and were authenticated by DNA (STR) profiling. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 2mM glutamine, 100 IU/ml penicillin, 160 µg/ml gentamicin and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) in a 5% CO₂-containing humidified atmosphere. The generation of luciferase-tagged BTK-143-TGL has been described previously (Irene Zinonos et al., 2009).

Normal human osteoblasts (NHB) were obtained from bone marrow aspirations from the iliac crest of normal healthy donors or from the trabecular bone of osteoarthritic patients at joint replacement surgery, grown in αMEM (SIGMA, Saint Louis, Missouri, USA) containing L-ascorbic acid 2-phosphate (Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum. Medium was then replaced at 4 day intervals cells were then consequently sub cultured by treatment with a (0.1%) (w/v) mixture of collagenase and dispase. In all experiments, cells from the first passage were used in all experiments.

**Drugs**

Threshold Pharmaceuticals (South San Francisco, CA, USA) provided the evofosfamide powder which was dissolved in a sterile saline solution at a concentration of 13.2mM. The Caspase Inhibitor-1 ZVAD-fmk, was purchased from Calbiochem Inc. (La Jolla, CA, USA).
Both drozitumab and dulanermin were a gift from Dr Avi Ashkenazi, Genentech, Inc. (South San Francisco, CA, USA). Affinity Pure Goat anti-human IgG Fcγ fragment was purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA).

**Cell Viability Assay**

To determine the cytotoxicity of evofosfamide on cell growth, $1 \times 10^4$ cells per well were seeded in 96-well microtitre plates and allowed to attach overnight. Cells were then treated with increasing concentrations of evofosfamide (1-100μM) alone and in combination with 100ng/ml of either dulanermin or drozitumab for 24 hours under both hypoxic (1% O$_2$) and normoxic conditions. Drozitumab was cross-linked with an anti-human IgG Fcγ for 30 minutes at 4°C prior to treatment before all in vitro experiments. Crystal Violet staining was used to determine cell viability and optical density was measured at 570 nm wavelength (OD570). Results of representative experiments are presented as the mean +/- SD which were performed in triplicate and repeated at least 3 times.

**Apoptosis Analysis**

**Measurement of DEVD-caspase activity with and without Caspase Inhibitor 1, ZVAD-fmk**

DEVD-caspase activity was assayed by cleavage of the fluorogenic substrate zDEVD-AFC and based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerase. Cells were grown in 96 well plates at a density of $1 \times 10^4$/well and treated for 24 hours as indicated, washed once with PBS, and resuspended in 30μl lysis buffer containing 5mmol/L EDTA, 5mmol/L Tris-HCl and 10% Igepal (pH 7.5). Cell lysate containing 20μg of protein was added to each well containing 8μmol/L substrate in 1ml fluorometric protease buffer which contained 10% sucrose, 50mmol/L HEPES, 0.1% CHAPS (pH 7.4), 10mmol/L DTT.

Fluorescence was then quantified (Ex 400 and Em 505) after 4 hours at room temperature using a BMG FLUOstar OPTIMA microplate reader.
Results were expressed relative to the protein concentration of the sample, which was determined using a commercial BCA protein assay reagent from Thermo Fisher Scientific (Waltham, MA, USA). Caspase Inhibitor 1, ZVAD-fmk, was resuspended at a concentration of 50mM and added to the cells at 50μM alone, with evofosfamide at 50μM, drozitumab + anti-human IgG Fcγ or dulanermin at 100ng/ml.

**Western Blot analysis**

Cells were treated with 50μM of evofosfamide, alone or in combination with 100ng/ml of either dulanermin or drozitumab (cross-linked with an anti-human IgG Fcγ), under hypoxic (1% O₂) and normoxic (21%O₂) conditions for 24 hours and lysed in buffer containing 150mM NaCl, 10mM Tris HCl (pH 7.6), 0.1% sodium dodecyl sulphate, 1% Triton X-100, 2mM sodium vanadate and a protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany). Protein lysates were heated for 10 minutes at 70°C and loaded under reducing conditions into 4-12% polyacrylamide gels for electrophoresis. Separated proteins were transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK) electrophoretically and blocked in PBS containing 5% blocking reagent (GE Healthcare, Buckinghamshire, UK) and 0.1% Tween 20 for 1 hour at room temperature.

Immunodetection was performed at 4°C overnight in blocking reagent/PBS, using the following primary antibodies mAb anti-caspase-8, pAb anti-caspase-9, mAb anti-caspase-3 and pAb anti-bid which were purchased from Cell Signaling Technology (Beverly, MA, USA), pAb anti-Inhibitor of Apoptosis 2 (cIAP2), pAb anti-Inhibitor of Apoptosis 1 (cIAP1), pAb anti-XIAP, pAb death receptor 4 (DR4), pAb death receptor 5 (DR5), pAb decoy receptor 1 (dcR1) and pAb decoy receptor 2 (dcR2) purchased from R&D systems, pAb anti-Poly-(ADP-Ribose) Polymerase (PARP) from Roche Diagnostics (Mannheim, Germany). Anti-actin mAb was used as a loading control and was purchased from SIGMA, Saint Louis, Missouri, USA.
All primary antibodies were used at the dilutions suggested by their manufacturers. Membranes were then rinsed three times with PBS containing 0.1% Tween-20 and incubated for 1 hour with a 1:5,000 dilution of anti-goat, anti-mouse, or anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). The ECF substrate reagent kit purchased from (GE Healthcare, Buckinghamshire, UK) and the FluorImager (Molecular Dynamics Inc., Sunnyvale, CA, USA) were used to visually assess and quantify the protein bands.

**Animals**

For a minimum period of 1 week prior to the commencement of experimentation, 4 week old female athymic mice were acclimatized to the animal housing facility under pathogen free conditions (Institute of Medical and Veterinary Services Division, Gilles Plains, SA, Australia). Throughout the experiments the general physical well-being and weight of animals were monitored. All experimental procedures on animals were carried out with strict adherence to the guidelines and rules for the ethical use of animals in research and were approved by the Animal Ethics Committees of the Institute of Medical and Veterinary Science and the University of Adelaide, SA, Australia.

**Intra-tibial injections of osteosarcoma cells**

The BTK-143-TGL OS cell line was cultured as described previously until 70-80% confluency was reached. Cells were removed from flasks with 2mM EDTA and resuspended at 1x10^5 cells per 10μl PBS and kept on ice in an eppendorf tube. The left tibia was wiped with 70% ethanol and with the knee flexed, coupled to a Hamilton syringe a 27 gauge needle was inserted through the tibial plateau and 1x10^5 BTK-143-TGL cells resuspended in 10μl of PBS were injected in the marrow space. As the control all animals were injected with PBS into the contralateral tibia. Mice were randomly assigned into groups of 7 animals and 7 days after cancer cell transplantation drug dosing started.
Evofosfamide was administered via i.p injection once a day for 5 days followed by 2 days of rest at 50mg/kg body weight, whereas drozitumab was administered at 3mg/kg i.p once a week until the end of the experiment.

**In vivo bioluminescent imaging**

The IVIS 100 Imaging system (Xenogen, Alameda, CA) was used weekly to perform non-invasive, whole body imaging to monitor the luciferase-expressing OS cell line BTK-143-TGL in mice using 100 μl of the D-Luciferin (Xenogen Alameda, CA) solution at final dose of 3mg/20g mouse body weight was injected i.p. Mice were then gas-anaesthetized with Isoflurane (Faulding Pharmaceuticals, Salisbury, SA, Australia). Images were acquired from the side angle for 0.5-30 seconds (representative images are shown at 1 second) and the Xenogen Living image (Igor Pro version 2.5) software was used to capture and quantify photon emission from mice in photons/sec/cm².

**Micro-computed tomography ex vivo analysis**

The SkyScan-1072 high-resolution μCT Scanner (Kontich, Skyscan, Belgium) was operated at 80kV, 120 μA, rotation step 0.675, with a 0.5 mm Al filter and scan resolution of 5.2μm/pixel was used to scan surgically resected limbs. Cross sections of the samples were reconstructed using a cone-beam algorithm (software Cone rec, Skyscan). The growth plate was identified using the 2D images obtained from the μCT scan and starting from the growth plate/tibial interface and moving down the tibia 450 sections were selected for quantification.

To determine 3D bone morphometric parameters (software CTAn, Skyscan), 3D evaluation was performed on all data sets acquired by selecting total bone of the proximal tibia, the cross sections were reconstructed using a cone-beam algorithm (software Cone_rec, Skyscan). Files were then imported into CTAn software (Skyscan) for 3D analysis and 3D image generation. All images are viewed and edited using ANT visualisation software (Skyscan).
Data Statistics and Analysis

Experiments were performed in triplicate and data presented as mean ± SE. SigmaStat for Windows version 3.0 (Systat Software, Inc., Port Richmond, CA) was used for all statistical analysis using the unpaired Students’ T test. Spearman Rank correlation coefficient was used to assess the association between two variables and comparisons between groups were assessed using a one way ANOVA test. In all cases, p < 0.05 was considered statistically significant.
Results

_Evofosfamide cooperates with drozitumab and dulanermin, displaying increased hypoxia-selective cytotoxicity against OS cells._

Human OS cell lines K-HOS and BTK-143 were assessed for their sensitivity to the cytotoxic activity of evofosfamide alone and in combination with a maximum dose of 100ng/ml of drozitumab or dulanermin for 24 hours under normoxic (21% O\(_2\)) and hypoxic (1% O\(_2\)) conditions. In both OS cell lines as a single agent, evofosfamide had minimal toxicity under normoxic conditions. In contrast, under hypoxic conditions, evofosfamide dose dependently decreased cell viability in both OS cell lines, with 43% viability for the BTK-143 cells and 65% viability for the K-HOS cells at 25μM. Under normoxic conditions, both OS cell lines were resistant to the cytotoxic activity of drozitumab and dulanermin alone at 100ng/ml. However under hypoxic conditions, K-HOS cells were comparably more sensitive to the cytotoxic activity of both drozitumab and dulanermin alone (39 and 47% viability respectively), whereas BTK-143 cells were relatively resistant (94 and 77% viability).

Both OS cell lines showed a significant increase in cytotoxicity when either drozitumab or dulanermin were combined with evofosfamide in a dose dependent manner under hypoxic conditions, resulting in 95% loss of viability at 25μM for both OS cell lines (Fig. 1A). In contrast, primary normal human osteoblasts, cultured from patients undergoing hip replacement surgery, were resistant to the cytotoxic activity of evofosfamide at 100μM in combination with either drozitumab or dulanermin under similar conditions (Fig. 1B).
Figure 1: Activity of evofosfamide in combination with drozitumab and dulanermin against OS cells and primary normal human osteoblasts in vitro. (A) OS cell lines BTK-143 and K-HOS were seeded in 96 well plates at $1 \times 10^4$ cells per well and treated with increasing doses of evofosfamide alone and in combination with either drozitumab or dulanermin under normoxic (21% $O_2$) and hypoxic (1% $O_2$) conditions for 24 hours. (B) Primary normal human osteoblasts were resistant to evofosfamide and the combination with either drozitumab or dulanermin under the same conditions. Cell viability was assessed by crystal violet staining. Data points show means of quadruplicate results from a representative experiment, repeated at least twice and presented as the mean ±SD of quadruplicate wells and expressed as a percentage of the number of control cells.
Fig. 1

**A**

**BTK-143**

- 21% O$_2$
- 21% O$_2$ + Drozitumab IgG
- 1% O$_2$
- 1% O$_2$ + Drozitumab IgG

**K-HOS**

- 21% O$_2$
- 21% O$_2$ + Drozitumab IgG
- 1% O$_2$
- 1% O$_2$ + Drozitumab IgG

**B**

**NHB**

- 21% O$_2$
- 1% O$_2$
- 21% O$_2$ + Drozitumab IgG
- 1% O$_2$ + Drozitumab IgG
- 1% O$_2$ + Dulanermin
Evofosfamide mediated OS cytotoxicity is only partly caspase 3 dependant.

The increase in caspase-3 activation with 50μM of evofosfamide treatment under hypoxic conditions alone and in combination with drozitumab or dulanermin (100ng/ml) was associated with a decrease in cell viability. However co-administration with ZVAD-fmk, a pan-caspase inhibitor did not prevent the reduction in cell viability caused by evofosfamide in both OS cell lines under hypoxic conditions, despite irreversibly inhibiting the activity of caspase-3 (Fig. 2), suggesting that the mechanisms involved in evofosfamide-mediated cytotoxicity are not entirely caspase dependent.

However ZVAD-fmk completely reversed the cytotoxic activity of both drozitumab and dulanermin in both OS cell lines, indicating that the cytotoxicity of these PARAs against these OS cells is largely caspase dependant, this being in line with the well-established mechanism of action of these pro-apoptotic agents. When either drozitumab or dulanermin was combined with evofosfamide and the caspase inhibitor ZVAD-fmk was added, there was a significant reduction in cytotoxicity against both OS cell lines when compared to the combination of these drugs without ZVAD-fmk.

The molecular determinants involved in evofosfamide-mediated apoptotic signaling alone and in combination with drozitumab or dulanermin were characterised (Fig. 3). Evofosfamide alone treatment at 50μM under hypoxic conditions (1% O₂), for 24 hours activated the caspase cascade with robust cleavage of the initiator caspase-8, caspase-9, caspase-3 and cleavage of poly ADP-ribose polymerase (PARP). The mitochondrial pro-apoptotic Bcl-2 family protein BID, inhibitor of apoptosis proteins cIAP1, cIAP2 and XIAP however remained unchanged.
The combination of evofosfamide with dulanermin or drozitumab in both OS cell lines resulted in increased processing of caspases 8, 9, 3 and PARP. Importantly, combination treatment under hypoxia resulted in the robust cleavage of BID, likely resulting in the amplification of apoptotic signaling.

Interestingly we observed a significant decrease in the levels of inhibitor of apoptosis proteins cIAP1, cIAP2 and XIAP in the K-HOS cell line. The levels of cIAP2 in the BTK-143 remained unchanged.

Under hypoxic conditions evofosfamide alone and in combination with either dulanermin or drozitumab up-regulated the death receptor DR5 in both cell lines. In the K-HOS cell line which was more sensitive to dulanermin and drozitumab when compared to the BTK-143 cell line, dulanermin and drozitumab alone also upregulated DR5 as single agents under both normoxic and hypoxic conditions. There were no significant differences in the expression of the other agonistic receptors DR4 or decoy receptors DcR1 and DcR2 following treatment.
Figure 2: The cytotoxic activity of dulanermin and drozitumab is caspase dependant, whereas evofosfamide is not. OS cell lines were seeded in 96 well plates at 1x10^4 cells per well and treated with evofosfamide alone at 50μM and with drozitumab IgG 100ng/ml, dulanermin 100ng/ml or co-incubated with the broad specificity caspase inhibitor z-VAD-fmk (50μM). To exclude possible toxic effects of the inhibitor, cells were also treated with the inhibitor alone under normoxic and hypoxic (1% O_2) conditions. Cell lysates were used to determine caspase-3-like activity, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC and cell viability was assessed via crystal violet staining. Data points show means of quadruplicate results from a representative experiment, repeated at least twice; bars ± SD.
Fig. 2

**BTK-143**

**K-HOS**
**Figure 3:** Apoptotic signalling of evofosfamide, dulanermin and drozitumab against OS cells. OS cells were seeded at 2 x 106 per T25 flask and were treated with evofosfamide at 50μM, dulanermin and drozitumab at 100ng/ml under normoxic (21% O2) and hypoxic (1% O2) conditions. After 24 hours cells were lysed and protein was collected. Cell lysates were analysed by polyacrylamide gel electrophoresis and transferred to PVDF membranes for Immunodetection as described in the Materials and Methods and immunoblotted with various Ab, as shown.

Fig. 3

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<th>K-HOS</th>
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<td>1% O₂</td>
</tr>
<tr>
<td>Control</td>
<td>Dulanermin</td>
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<tr>
<td>Evofosfamide</td>
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<td>Evofosfamide + Drozitumab</td>
<td>Dulanermin</td>
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<tr>
<td>Caspase-8</td>
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<td>Caspase-9</td>
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<td>Caspase-3</td>
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<tr>
<td>PARP</td>
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<tr>
<td>BID</td>
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<tr>
<td>c-IAP-1</td>
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<td>c-IAP-2</td>
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<td>XIAP</td>
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<tr>
<td>DR4</td>
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<tr>
<td>DR5</td>
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<td>Actin</td>
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Cytotoxic activity of evofosfamide and drozitumab against osteosarcoma-induced bone destruction

Drozitumab was specifically chosen as opposed to dulanermin in this preclinical study due to its ability to specifically bind to DR5 and not the TRAIL decoy receptors. In addition, drozitumab has a longer half-life when compared to dulanermin (Ashkenazi, Holland, & Eckhardt, 2008).

To investigate the anticancer efficacy of drozitumab and evofosfamide against osteosarcoma progression and metastasis, an orthotopic model of OS was used in which luciferase tagged BTK-143-TGL cells were directly transplanted into the tibial marrow cavity of female athymic nude mice and accurately monitored and quantified using non-invasive bioluminescence imaging over a 28 day period (I. Zinonos et al., 2009). Treatment with drozitumab, evofosfamide or the combination of both agents commenced 7 days after the intratibial OS cell injections. All vehicle treated animals showed an increase in mean photon emission exponentially, which indicated an increase in tumor burden palpable from day 7 onwards, reaching a maximum signal at day 28, at which point animals were humanely killed. In contrast, treatment with evofosfamide or drozitumab showed a reduction in tumor burden over the same period in all animals. Importantly, the combination demonstrated a far greater anticancer efficacy in the bone (Fig 4A and B). The tibiae of all mice were dissected at the end of the experiment and the qualitative and quantitative assessment of bone destruction was analysed using high resolution µCT (Fig 4C). In the vehicle treated animals extensive osteolysis was clearly evident such that the net loss in bone volume (BV) was 69% in the left tumor-bearing tibiae when compared to the contralateral non-tumor bearing right tibiae.

Although tumor burden was reduced by evofosfamide treatment, this did not prevent bone destruction such that the extent of osteolysis was not significantly different when compared to the vehicle treated group.
Remarkably, treatment with drozitumab alone resulted in extensive bone remodeling, resulting in a gain of bone volume of 27% when compared to the untreated right tibia. Micro-CT analysis showed extensive bone remodeling that was noticeable under the growth plate and extended down the length of the tibia where the tumor resided. Tumour healing in the treatment of osteosarcoma in patients is often correlated with a significant increase in calcification, which would account for the increase in calcification in the tumour affected tibia of mice treated with drozitumab (S. Ferrari, Balladelli, Palmerini, & Vanel, 2013). In animals treated with the combination of drozitumab and evofosfamide, the tibia had a normal appearance due to the full mineralization of the cortical bone, demonstrating additional protection of the bone architecture, less calcification and more advanced bone remodeling, such that the net gain of BV was reduced to 6%.

Ex-vivo bioluminescence imaging showed no differences between the treated groups (3 out of 7 mice) in the number of mice that developed lung metastases. However the tumor burden in the lungs of the mice with metastases which was measured as a function of bioluminescence signal showed a reduction in tumour growth with evofosfamide treatment. In addition, drozitumab maintained its cytotoxicity against metastatic OS cells in the lungs, which led to the tumour burden being reduced in both the drozitumab and combination groups (Fig 4D).
Figure 4: Drozitumab co-operates with evofosfamide to reduce OS intratibial tumours in vivo. BTK-143-TGL cells were injected directly into the tibial marrow cavity of 4 week female athymic mice, allowed to establish for 7 days, as described in the methods, mice were imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. (A). Representative whole body bioluminescent images of a single mouse from each group during the course of the experiment are shown. (B). The line graph, showing average tumor signal over time, expressed as mean photon counts per second during the course of the experiments are shown. Animals receiving treatment with evofosfamide and drozitumab as single agents showed a significant delay in tumor growth. In addition, all mice receiving the combination of evofosfamide and drozitumab showed a further delay of tumor growth when compared with each agent individually. (C). Quantitative assessment of Total bone loss (%) comparing the tumor bearing tibiae of each group to the contralateral tibiae and the qualitative 3-D micro CT images show the osteolytic nature of the BTK-143-TGL cell line, which was reduced by drozitumab alone and the combination of evofosfamide and drozitumab. (D). Average lung tumor growth was assessed via bioluminescence showing evofosfamide, drozitumab and the combination of both agents caused a reduction in lung tumor growth of the BTK-143-TGL cell line when compared to the vehicle group. Data shown in each case are the average BLI from all animals in that group: points are means ±SEM.
**Effect of evofosfamide on bone metabolism**

Our experimental approach also provides an opportunity to assess the normal bone parameters after treating the mice with evofosfamide, drozitumab and the combination of both. After three weeks of treatment, the use of high resolution micro-CT analysis to compare the contralateral non tumor bearing tibiae of treated and untreated animals showed no differences in any of the micro architectural bone morphometric parameters, which included total bone volume, bone surface, trabecular number, trabecular thickness or trabecular spacing (Table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle control</th>
<th>Evofosfamide</th>
<th>Drozitumab</th>
<th>Evofosfamide + Drozitumab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Bone Volume (mm³)</td>
<td>2.34 ± 0.05</td>
<td>2.27 ± 0.07</td>
<td>2.31 ± 0.14</td>
<td>2.30 ± 0.11</td>
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<tr>
<td>Bone Surface (mm²)</td>
<td>191.68 ± 4.07</td>
<td>194.55 ± 4.94</td>
<td>188.71 ± 6.58</td>
<td>195.52 ± 10.91</td>
</tr>
<tr>
<td>Inter-section Surface (mm²)</td>
<td>0.36 ± 0.03</td>
<td>0.32 ± 0.04</td>
<td>0.38 ± 0.03</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>Trabecular Space (mm)</td>
<td>1.53 ± 0.02</td>
<td>1.49 ± 0.04</td>
<td>1.57 ± 0.04</td>
<td>1.45 ± 0.03</td>
</tr>
<tr>
<td>Trabecular Number (1/mm)</td>
<td>0.16 ± 0.07</td>
<td>0.18 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Trabecular Thickness (mm)</td>
<td>0.05 ± 0</td>
<td>0.05 ± 0</td>
<td>0.06 ± 0</td>
<td>0.05 ± 0</td>
</tr>
<tr>
<td>Trabecular Pattern factor (1/mm)</td>
<td>24.24 ± 1.37</td>
<td>21.17 ± 1.79</td>
<td>23.90 ± 0.77</td>
<td>22.05 ± 1.05</td>
</tr>
<tr>
<td>Structure Model Index</td>
<td>2.14 ± 0.04</td>
<td>2.04 ± 0.23</td>
<td>2.08 ± 0.03</td>
<td>1.92 ± 0.06</td>
</tr>
</tbody>
</table>

Bone volume, bone surface, inter-section surface, trabecular space, trabecular number, trabecular thickness, trabecular pattern factor and structure model index were measured by three dimensional analysis of μCT images of the contralateral tibial bone. Results are expressed as mean ± SE. Significance of results is with respect to untreated animals obtained using Student’s t test.
Discussion

In addition to surgical intervention, chemotherapeutic agents such as doxorubicin, etoposide, cisplatin and cyclophosphamide used alone, or in combination have significantly improved overall survival for patients with OS. Yet despite these improvements in treating the primary tumour, a large number of patients with OS eventually develop lung metastases, even after surgical excision and conventional chemotherapy. There is a need to therefore, develop safe and new approaches for OS treatment (Botter, Neri, & Fuchs, 2014; Jin-Peng He & Jin Guo, 2014; Yang & Zhang, 2013).

It must be noted that when compared to other tissues, the bone marrow and in particular the haemopoietic niche close to the endosteal surface is hypoxic, which is required for normal haemopoiesis to occur (Miharada et al., 2011). Unlike soft tissue tumors, OS can also adapt to this hypoxic bone microenvironment. The ability to target OS in this hypoxic bone environment is therefore an important feature that evofosfamide has over other cancer therapies. In addition conventional chemotherapeutics are usually cytotoxic to normal bone cells in the bone marrow, an important goal of anticancer treatment is to selectively target cancer cells but not normal bone cells.

A combinatorial approach using agents with additive or synergistic cytotoxic activities are appealing because they allow lower drug doses to be used, which reduce harmful side-effects, particularly in the bone. Consistent with our previous published data (V. Liapis et al., 2015; V. Liapis et al., 2016) under normoxic conditions evofosfamide alone resulted in minimal toxicity against OS, whereas under hypoxic conditions evofosfamide decreased OS cell viability. This cytotoxic activity was further increased when evofosfamide was combined with either drozitumab or dulanermin under hypoxic conditions.
The combination of the chemotherapeutic agents’ drozitumab and dulanermin with evofosfamide was not toxic to either normal human bone cells *in vitro* or normal bone metabolism *in vivo*, corroborating with previous studies which demonstrate that these agents individually are nontoxic to normal bone. (Agatha Labrinidis et al., 2009; V. Liapis et al., 2015; I. Zinonos et al., 2009). These results highlight not only the hypoxic selectivity of evofosfamide, but also the specific tumor selectivity of both evofosfamide and PARAs.

In the search for more effective treatments for OS, PARAs including recombinant dulanermin and the agonistic antibody drozitumab induce apoptosis through different but overlapping signaling pathways, whereas evofosfamide induces apoptosis mainly through caspase-independent mechanisms as described previously (Vasilios Liapis et al., 2016). As a result, the combination of PARAs and evofosfamide were considerably more cytotoxic to tumour cells that resist cytotoxic activity through a single pathway, where inhibiting caspase activity to prevent the activity of both drozitumab and dulanermin still resulted in both OS cell lines under hypoxic conditions being sensitive to the cytotoxic activity of evofosfamide. This is also reflected by the activation of caspase-8, caspase-9, caspase-3, PARP, cleavage of Bid, a member of the Bcl-2 family protein, and the downregulation of c-IAP1 when evofosfamide was combined with dulanermin or drozitumab as well as both PARAs activating the extrinsic pathway by the upregulation of DR5.

Based on our *in vitro* results, the therapeutic potential of evofosfamide was expected to be greatest in combination with adjuvant cytotoxic chemotherapy. When transplanted into the tibial marrow cavity of mice, BTK-143 cells are highly osteolytic and this results in extensive bone destruction and the development of metastases to the lung three to four weeks post cancer cell transplantation.
This *in vivo* model mimics OS activity in the bone as seen in patients with the disease and is ideal for determining the potential of drug treatment on cancer growth in the bone as well as cancer-induced bone destruction (Vasilios Liapis et al., 2015; I. Zinonos et al., 2009). The activity of evofosfamide in combination with drozitumab was tested in this context, in a preclinical model of OS progression and development for the following reasons. In contrast to dulanermin, which has a short bioavailability of 30 minutes, which requires daily treatment for patients and the inability to bind to death-inducing TRAIL receptors in various cancer types, preferring to bind with the decoy TRAIL receptors (Amarante-Mendes & Griffith, 2015; Ashkenazi et al., 1999), drozitumab is a fully agonistic human monoclonal antibody that specifically binds to and activates DR5 in the same manner as dulanermin (I. Zinonos et al., 2009). Drozitumab has a half-life ranging from several days to weeks and has been developed to specifically target DR5 (Ashkenazi, 2008a) and not the TRAIL decoy receptors. In addition, circulating Fragment Crystalline Gamma (Fcγ) receptors expressed on the surface of various immune cells (Holland, 2013; Robak, 2013), crosslink with drozitumab which leads to enhanced antibody-dependent, cell-mediated cytotoxicity (ADCC) (Wilson et al., 2011), resulting in immune cell activation leading to recruitment of other Fcγ receptor expressing cells to the tumor microenvironment (Amarante-Mendes & Griffith, 2015; Wilson et al., 2011). The apoptotic tumor cells are then phagocytosed by the activated Fcγ receptor expressing immune cells (Takeda et al., 2004), further enhancing the cytotoxic activity of drozitumab against cancer.

The activity of drozitumab against OS in bone has yet to be reported and in addition, this OS cell line is relatively resistant to drozitumab *in vitro*, allowing the detection of any synergistic or additive activity to be easily observed. As a single agent evofosfamide had limited impact in reducing tumour growth in the tibia or protecting the tibia from the cancer induced bone destruction caused by this highly aggressive osteolytic cell line.
The cytotoxic activity displayed by drozitumab in vivo contradicts the resistance of this human osteosarcoma shown in vitro. A possible explanation to account for the increase in cytotoxicity of drozitumab in vivo is the circulation of Fcγ receptors expressed by leukocytes in mice. The engagement of leukocyte Fcγ receptors by antibody-antigen complexes leads to an enhanced antibody-dependent, cell-mediated cytotoxicity (ADCC) (Wilson et al., 2011), which can interact more efficiently with the DR5 agonistic antibody drozitumab when compared to artificial Fc crosslinking in vitro, leading to improved cytotoxicity against the human osteosarcoma in the tibia and lungs of the mice.

The combination of both evofosfamide and drozitumab had a profound effect in preventing growth of the tumor within the tibia which also translated to increased bone protection and a reduction in tumour burden in the lung. This may be related to the ability of evofosfamide to upregulate DR5 expression under hypoxic conditions, resulting in increased sensitivity to Drozitumab as observed in previous studies (Alexiou, Tsamis, & Kyritsis, 2015). In addition, each drug specifically targets tumours regions of different oxygen tensions accordingly.

PARAs including drozitumab and dulanermin have been tested either alone or in combination with other agents in phase I and II clinical trials (Dine et al., 2016), with little clinical benefit observed to date which has led to the discontinuation of the development of PARAs in many cases (Holland, 2014). However, none of these clinical trials have examined the anticancer efficacy of PARAs against cancers in the bone such as OS.

Evofosfamide is currently being evaluated both as monotherapy and in combination with conventional chemotherapy and radiotherapy in numerous phase I and phase II clinical trials against a variety of cancer types.
To date, two phase 3 trials targeting unresectable or metastatic soft tissue sarcoma NCT01440088 and unresectable pancreatic adenocarcinoma NCT01746979 (Alama, Orengo, Ferrini, & Gangemi, 2012) failed to meet their primary endpoint of improving overall survival with statistical significance. Nonetheless, from the observations in phase I and II clinical trials of evofosfamide and PARAs, and from the results presented in this study, which indicate that these compounds are nontoxic to normal bone metabolism suggest that OS patients may benefit from evofosfamide when used in combination with PARAs.

Acknowledgments

This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia ID# 627015, the Hospital Research Foundation, Adelaide, South Australia, the National Breast Cancer Foundation and the Australian Breast Cancer Research. We wish to thank Adelaide microscopy Adelaide Microscopy at the University of Adelaide for technical assistance in Micro-CT.
CHAPTER 5

DISCUSSION
Discussion

Tumor hypoxia is a major cause of treatment failure and poor outcome for a wide variety of malignancies. Within most solid tumours, there are significant areas of hypoxia, which contain cancer cells that resist conventional chemotherapy and radiotherapy, predisposing to tumor recurrence and metastasis. However, tumor hypoxia also provides treatment opportunities, exemplified by the development of highly active compounds known as HAPs that can specifically target tumor hypoxic zones.

In considering the use of the HAP evofosfamide specifically for cancers in the bone, it is important to note that the bone marrow, particularly the hematopoietic niche proximal to the endosteal surface, is hypoxic and this is a prerequisite for normal haemopoiesis. In humans, the average oxygen tension (pO2) in the bone marrow is between 1 and 7% (cf atmospheric oxygen = 21%) (Carreau, El Hafny-Rahbi, Matejuk, Grillon, & Kieda, 2011). Cancer cells normally home to hypoxic endosteal niches where the oxygen tension is estimated to be <1.3% (Spencer et al., 2014). Unlike soft tissue tumours, cancer cells in bone are adapted to survive and grow in a microenvironment, which is already hypoxic (Brahimi-Horn, Chiche, & Pouyssegur, 2007). Therefore tumor hypoxia is a major contributor to the incurability of bone cancer.

The results of the first aim which investigated the anticancer activity of evofosfamide against osteosarcoma showed that in vivo evofosfamide dose-dependently was cytotoxic to human OS cells selectively under hypoxic conditions, whereas primary normal human osteoblasts under the same conditions were relatively resistant to treatment, highlighting not only the hypoxic selectivity but also the tumor selectivity of the drug, which is likely related to the more rapid proliferation of tumor cells when compared to normal cells. In vivo evofosfamide as a single agent delayed tumor growth in bone and cooperated with doxorubicin for increased anticancer efficacy.
This effect was associated with significant protection from OS induced bone destruction and was observed in two distinct intratibial mouse models of human OS giving rise to different types of bone lesions. Evofosfamide alone reduced the incidence of lung metastases in both OS models. However, the tumor burden within the lungs of the remaining animals with metastases was no different from those seen in the vehicle treated groups, suggesting that osteosarcoma cells that escaped from the bone marrow and lodged into the lungs were refractory to evofosfamide treatment in this highly oxygenated environment, highlighting the hypoxia selectivity of this drug \textit{in vivo}. In contrast, doxorubicin maintained its tumor suppressive activity in the lung.

To date, the activity of evofosfamide against breast cancer both at the orthotopic site and bone metastases has not been reported. Therefore the second aim investigated the anticancer efficacy of evofosfamide in a preclinical model of breast cancer. \textit{In vitro} evofosfamide exhibited relatively minimal toxicity under normoxic conditions, against a panel of human breast cancer lines, whereas under increasing hypoxia evofosfamide treatment dose dependently decreased the cell viability of a variety of human breast cancer cell lines. Normal breast epithelial cell lines MCF-10A and MCF-12A were also equally sensitive to evofosfamide under hypoxic conditions, which may be related to their proliferative capacity being similar to that of breast cancer cells. In contrast, primary dermal and normal mammary fibroblasts were relatively resistant to the cytotoxic activity of evofosfamide under the same conditions.

The anticancer efficacy of evofosfamide alone and in combination with paclitaxel against breast cancer growing in the orthotopic site and in the bone marrow was assessed by using the aggressive and highly osteolytic human MDA-MB-231-TXSA breast cancer cell line \textit{in vivo}. 

When these human breast cancer cells were injected into the mammary fat pad of mice, treatment with evofosfamide or paclitaxel as single agents showed a significant reduction in tumor burden over the same period, whereas the combination of both was more effective in reducing tumor growth. In the intratibial model, evofosfamide treatment inhibited tumor growth in bone, leading to a significant reduction in the overall tumor burden. However, the reduction in tumor burden did not translate to a significant inhibition of osteolysis attesting to the aggressive osteolytic properties of these cells. In contrast, paclitaxel as a single agent was highly effective in reducing tumor load in bone while also protecting the bone from cancer induced bone destruction. The combined treatment resulted in a further reduction in tumor burden.

The third aim investigated evofosfamide in combination with the PARAs drozitumab and dulanermin. To date, there has been no assessment of PARAs for the treatment of osteosarcoma. PARAs have been investigated previously against breast cancer growth in bone and were shown to inhibit intra and extra osseous bone growth while being non-toxic towards normal bone metabolism (I. Zinonos et al., 2009). Consistent with the results of the first aim, evofosfamide alone exhibited relatively minimal toxicity under normoxic conditions, whereas under hypoxia evofosfamide decreased OS cell viability. This cytotoxic activity was further increased when evofosfamide was combined with either drozitumab or dulanermin under hypoxic conditions. In contrast, normal human osteoblasts were resistant to the cytotoxic activity of evofosfamide alone and in combination with either drozitumab or dulanermin under the same conditions.

As single agents in vivo, evofosfamide and in particular drozitumab were highly effective in reducing primary tumor burden and metastatic burden in the lungs. Evofosfamide however did not significantly reduce the cancer induced bone destruction caused by this highly aggressive osteolytic osteosarcoma cell line.
However the combination of both agents further prevented growth of the tumor within
the tibia which also translated to increased bone protection and a reduction in tumour burden
in the lung. PARAs including drozitumab and dulanermin, have been tested alone or in
combination with other agents in phase I and II clinical trials and little clinical benefit has
been observed to date which has led to the discontinuation of the development of PARAs in
many cases (Holland, 2014). However, none of these clinical trials have examined the
anticancer efficacy of PARAs against cancers in the bone such as OS.

To date no data exist on the effects of evofosfamide or any other HAP for that matter on
normal bone metabolism in the context of osteoclasts, osteoblasts, or osteocyte survival and
function. From these three studies, the micro architectural bone morphometric parameters of
the contralateral non tumor injected tibiae from untreated and evofosfamide treated animals
were compared using high resolution micro-CT which demonstrated no changes in micro
architectural bone parameters including, total or trabecular BV measurements with
evofosfamide treatment.

The results obtained in addressing the aims of this thesis as well as results obtained
from the early stages of clinical trials of evofosfamide for the treatment of other cancer types,
including the lack of toxicity to normal bone cells, suggest that patients diagnosed with
primary OS may benefit from evofosfamide therapy when used in combination with the
conventional chemotherapeutic drug doxorubicin. These studies also indicate that patients
may also benefit from evofosfamide when used in combination with PARAs as both these
compounds are nontoxic to normal bone metabolism when compared to chemotherapeutic
treatment of patients suffering from OS.
Data generated the second aim which investigated the anticancer efficacy of evofosfamide against breast cancer further supports the clinical development of evofosfamide as a novel approach in the treatment of patients with breast cancer, especially those with existing bone metastases.

The data presented in this thesis provides an important preclinical evaluation for the treatment of cancer that originates in the bone such as osteosarcoma or metastasizes to the bone such as breast cancer using the HAP evofosfamide with either conventional chemotherapeutic drugs such as doxorubicin and paclitaxel, or drugs such as the PARAs drozitumab and dulanermin which, although the PARAs drozitumab and dulanermin have failed to progress further than phase II clinical trials, they are continually investigated by researchers and clinicians because they are well tolerated by patients with only small side effects.

While outcomes for the treatment of patients suffering from bone cancers have improved over the past 30 years with the use of aggressive chemotherapeutics, these chemotherapeutic drugs cause many side effects and often give rise to second malignancies. In addition, the frequent acquisition of drug resistance as well as the toxic side effects which is often associated with the use of these drugs is a serious problem. Therefore evofosfamide and PARAs may have potential roles in the treatment of bone cancer, given that data generated in this thesis and form previous preclinical studies suggest that both evofosfamide and PARAs are non-toxic and safe.
CHAPTER 6

FUTURE DIRECTIONS
Future Directions

Tumour hypoxia has been pursued as a potential selective cancer drug treatment for over 30 years. Yet despite the link between hypoxia with treatment resistance and poor prognosis, a number of HAPs including evofosfamide have failed to demonstrate efficacy in phase III clinical trials and as yet no HAPs have been FDA approved.

Evofosfamide, which was the most advanced and the least toxic of all HAPs that have entered clinical trials, failed two phase III trials for the treatment of advanced pancreatic cancer (MAESTRO) and advanced soft tissue sarcoma (TH-CR-406/SARC021). The reason for the failure of the MAESTRO study was that evofosfamide did not improve overall survival in combination with gemcitabine when compared to gemcitabine plus placebo.

Pancreatic ductal adenocarcinoma is incurable and the most lethal common cancer because it is usually diagnosed at an advanced stage which is resistant to therapy (Ryan, Hong, & Bardeesy, 2014). Although the primary endpoint of overall survival in the MAESTRO study narrowly missed statistical significance (p=0.0589, where p=0.05 is statistically significant), secondary endpoints, overall response rates and progression free survival gave significant improvements for patients treated with gemcitabine plus evofosfamide compared to gemcitabine plus placebo (Russo & Saif, 2016). In addition a subgroup of 123 patients enrolled in the treatment arm at Japanese and South Korean sites had a risk of death reduction of 42% when compared to the control arm (Zeng, 2016). These results, which include the narrowly missed statistical significance of p=0.058, require further analysis to determine whether the phase III failure is due to the trial design or the drug evofosfamide itself, leading to the possibility that there may still be an opportunity to treat patients with pancreatic cancer with evofosfamide.
Importantly, in the context of cancer in the bone, updated data from the phase II study investigating evofosfamide in combination with bortezomib and low dose dexamethasone (NCT01522872) suggests that of the 18 patients in the trial with advanced multiple myeloma, 14 patients showed a clinical benefit rate of 29% (Zeng, 2016). This preliminary data suggest that the combination of evofosfamide with dexamethasone is active in patients who have failed conventional therapy for the treatment of multiple myeloma.

In addition to the phase II multiple myeloma trial, a phase I study investigating the clinical activity of evofosfamide for the treatment of patients with relapsed leukaemia (NCT01149915) demonstrated that evofosfamide as a single agent has activity in heavily pretreated leukaemia patients. To characterise the extent of the hypoxic bone environment in these leukaemia patients, this study also incorporated the hypoxia markers HIF-1a and CAIX, which were highly expressed in leukemic bone marrow and were significantly reduced after evofosfamide therapy (Badar et al., 2016).

Although both multiple myeloma and leukaemia are defined as blood cancers, the bone marrow microenvironment promotes the growth of these cancer types, which ultimately leads to bone destruction and resistance to conventional therapies (Hideshima, Mitsiades, Tonon, Richardson, & Anderson, 2007). Therefore based on these phase I and II trials and preclinical data that investigated evofosfamide for the treatment of various types of cancer that occurs in the bone, patients with these bone cancers may benefit from evofosfamide therapy. In addition as indicated in the preclinical studies conducted as part of this thesis, evofosfamide may also have a potential role in the treatment of osteosarcoma and metastatic breast cancer. This will require further preclinical studies with more osteosarcoma and breast cancer cell lines in order to generate enough evidence to warrant future clinical trials investigating evofosfamide against osteosarcoma and metastatic breast cancer.
In addition to investigating the role of evofosfamide on osteosarcoma and metastatic breast cancer as outlined in this thesis, other cancer types that metastasize to the bone should also be investigated such as prostate, lung kidney and thyroid cancers.

However there are limitations in the use of HAPs such as evofosfamide to target hypoxic regions in solid tumours and bone cancers, which may explain why evofosfamide failed to meet its primary endpoints in both phase III trials. Hypoxia between and within the tumour including in bone cancers is highly variable and is not always the cause of resistance to treatment.

The use of direct and indirect hypoxia measuring factors in order to determine the levels of hypoxia have shown large variations of hypoxia between cancers within patients. These levels of hypoxia also determine the possibility of treatment failure by radiotherapy and conventional chemotherapy which are most effective in the normoxic regions of the tumour (Dhani et al., 2015; Milosevic et al., 2012). There have been no phase II and III clinical trials that have incorporated biomarkers that accurately measure hypoxia in patients. This would explain why some patients in these clinical trials had little benefit for HAPs as their tumours would have had very few areas of hypoxia (F. W. Hunter, Wouters, & Wilson, 2016).

Secondly, not only is the efficacy of HAPs against cancer dependent on the levels of oxygen in hypoxic regions to activate the drug, but also the level of resistance of the cancer to the cytotoxic effector compound. Defining predictive biomarkers for HAPs in order to predict the efficacy of their effector drugs against tumours will determine their potential in the treatment of cancer. The dosing schedule of HAPs in combination with conventional chemotherapeutic agents will also be a contributing factor in determining tumour response while reducing side effects and allowing these drugs to complement each other (Lindsay, Garvey, Mumenthaler, & Foo, 2016).
Therefore biomarkers that can accurately detect and quantify the hypoxic tumour microenvironment as well as determining the tumour resistance against the effector compound are needed to determine the potential of HAPs in the treatment of cancer, including cancer in the bone.

Taken together, despite the lack of success of HAPs in phase III clinical trials, HAP research and development has produced a wealth of knowledge, understanding and expertise leading to novel approaches in targeting hypoxia in the tumour and bone microenvironment which will implement the principles and experience gained from over 30 years of developing HAPs and incorporate them into the design of future preclinical and clinical studies.
BIBLIOGRAPHY
References


Jian-Xin Duan, † Hailong Jiao, † Jacob Kaizerman, † Timothy Stanton, † James W. Evans, † Leslie Lan, † Gustavo Lorente, † Monica Banica, D. J., † Jinwei Wang, † Huaiyu Ma, † Xiaoming Li, † Zhijian Yang, † Robert M. Hoffman, †, & W. Steve Ammons, C. P. H., † and Mark Matteucci*, †. (2007). Potent and Highly Selective Hypoxia-Activated Achiral Phosphoramidate Mustards as Anticancer Drugs. J. Med. Chem., 51, 2412–2420.


Kaisa R Luoto1†, R. K., 2† and Robert G Bristow1. (2013). Tumor hypoxia as a driving force in genetic instability. GENOME INTEGRITY.


Manley, E., Jr., & Waxman, D. J. (2013). Impact of tumor blood flow modulation on tumor sensitivity to the bioreductive drug banoxantrone. J Pharmacol Exp Ther, 344(2), 368-377. doi:10.1124/jpet.112.200089


