EFFICACY OF PRO-APOPTOTIC RECEPTOR AGONISTS IN THE TREATMENT OF PRIMARY BREAST CANCER AND BONE METASTASIS

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A Thesis submitted in total fulfilment of the requirements

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in

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DECLARATION

NAME: Irene Zinonos **PROGRAM:** PhD

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ACKNOWLEDGMENTS

“Ithaca”, a very famous poem by Konstantinos Kavafis (one of the greatest Greek poets) describes Odysseus’ long journey to return home after the Trojan War. Kavafis explains how important his journey was, even though it was beset by perils and misfortunes. Odysseus learnt valuable lessons and gained experience, he never gave up and he eventually arrived home to his beautiful wife Penelope and son Telemachus.

.....Η Ιθάκη σ' έδωσε το ωραίο ταξίδι

Χωρίς αυτήν δεν θά ‘βγαινες στον δρόμο

Αλλο δεν έχει να σε δώσει πια

Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε

Ετσι σοφός που έγινες, με τόση πείρα,

ήδη θα το κατάλαβες οι Ιθάκες τι σημαίνουν

Ithaca has given you the beautiful voyage

Without her you would never have taken the road

But she has nothing more to give you

And if you find her poor, Ithaca has not defrauded you

With the wisdom you have gained, with so much experience,

you must surely have understood by then what Ithacas mean.

The poem suggests that, had it not been for the desire of Odysseus to return to Ithaca, all the experiences along the way would have been missed. So, the journey AND the destination share the same importance in life, which brings me to my Ithaca, my PhD! I have finally arrived to my destination! The desire to complete my PhD, made the journey so much more enjoyable and the journey, with all the great things it taught me along the way, made the destination so much more important. However, experiencing this journey and eventually arriving to my destination would not have been possible if it wasn't for the support, guidance and love of many great people.

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ABSTRACT

Breast cancer is the most common malignancy among women which frequently metastasises to the bone. Despite the significant improvements in detecting and treating primary breast cancer, metastatic breast cancer remains a challenging condition to treat. The studies presented in this thesis were aimed to exploit the therapeutic potential of Pro-Apoptotic Receptor Agonists (PARAs) including the recombinant TNF-related apoptosis-inducing ligand, Apo2L/TRAIL, and its agonistic monoclonal antibody, drozitumab, for the treatment of primary breast cancer and bone metastases *in vitro* and *in vivo*.

Drozitumab is a fully human agonistic monoclonal antibody which binds to Apo2L/TRAIL death receptor DR5 and triggers apoptosis. The anticancer efficacy of drozitumab was evaluated using murine models of breast cancer xenografted at the orthotopic site and in bone. *In vitro*, drozitumab induced apoptosis in various human breast cancer cell lines, without being toxic to normal cells. *In vivo*, drozitumab exerted remarkable tumour suppressive activity as a single agent and co-operated with chemotherapeutic drugs, for increased efficacy against mammary tumours. In addition, drozitumab treatment completely inhibited tumour growth in bone, even in animal having well-advanced tibial tumours, leading to complete resolution of osteolytic lesions.

Osteoprotegerin (OPG) is a soluble member of the TNF receptor superfamily, which binds the receptor activator of NF-kB (RANKL) and inhibits bone resorption. OPG can also bind and inhibit the activity of Apo2L/TRAIL, raising the possibility that the anticancer efficacy of Apo2L/TRAIL may be abrogated in the bone microenvironment, where OPG expression is high. *In vitro,* breast cancer cells engineered to overexpress OPG were protected from Apo2L/TRAIL-induced apoptosis. However, when mice were injected intratibially with cells overexpressing OPG, Apo2L/TRAIL treatment resulted in strong growth inhibition of

OPG overexpressing intratibial tumours indicating that OPG levels in bone, even in the face of supra-physiological concentrations, are unlikely to play a significant role in modulatingApo2L/TRAIL therapeutic potential.

Previous preclinical studies have shown that systemic administration of recombinant OPG inhibited tumour growth in bone and prevented cancer-induced osteolysis. However, the data presented in this thesis have demonstrated that while overexpression of OPG by breast cancer cells protected the bone from cancer-induced osteolysis, it was without effect on overall tumour burden. Despite the OPG-mediated bone protection, OPG overexpression led to a significant increase in the incidence of pulmonary metastasis. These results suggest that OPG-mediated inhibition of bone resorption modulates the bone microenvironment and it may affect the likelihood of cancer cells spreading elsewhere in the body. This also suggests that other anti-resorptive therapeutic agents including bisphosphonates (BPs), which have been the standard care for patients with skeletal malignancies, have the potential to harm by promoting cancer metastasis to other non-skeletal sites.

In conclusion, the data presented in this thesis demonstrate that drozitumab and Apo2L/TRAIL represent potent immunotherapeutic agents with strong activity as single agents and in combination with conventional chemotherapy against the development and progression of breast cancer. In addition, these studies provide important preclinical evidence that modulating the bone microenvironment by inhibiting osteoclastic bone resorption may not always be beneficial, a phenomenon which needs further investigation.

CONFERENCE PRESENTATIONS

- **Irene Zinonos,** Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vasilios Panagopoulos, Mark DeNichilo, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino, David M Findlay, and Andreas Evdokiou. Doxorubicin overcomes resistance to drozitumab based-immunotherapy in a mouse model of breast cancer**.** Research Day 12 October 2012, Basil Hetzel Institute, The Queen Elizabeth Hospital. Oral Presentation.

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  Doxorubicin overcomes resistance to drozitumab based-immunotherapy in a mouse model of breast cancer**.** Faculty of Health Sciences 2012 Postgraduate Research Expo, University of Adelaide, Adelaide-SA. Poster presentation.

- **Irene Zinonos,** Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino, David M Findlay, and Andreas Evdokiou.

  Local production of Osteoprotegerin by breast cancer cells inhibits cancer-induced osteolysis but promotes pulmonary metastasis**.** Research Day 14 October 2011, Basil Hetzel Institute, The Queen Elizabeth Hospital. Oral Presentation.

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Local production of Osteoprotegerin by breast cancer cells inhibits cancer-induced osteolysis but promotes pulmonary metastasis. Faculty of Health Sciences 2010 Postgraduate Research Expo, University of Adelaide, Adelaide-SA. Poster presentation.

- **Irene Zinonos**, Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino, David M Findlay, and Andreas Evdokiou.

  Local production of Osteoprotegerin by breast cancer cells inhibits cancer-induced osteolysis and intra-osseous tumour burden but fails to restrain extra-medullary tumour growth. ECTS/IBMS 3rd Joint Meeting of the European Calcified Tissue Society and the International Bone and Mineral Society, 7-11 May 2001, Athens, Greece. Poster presentation.

- **Irene Zinonos**, Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino, David M Findlay, and Andreas Evdokiou.

  Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin *in vivo*. Faculty of Health Sciences 2010 Postgraduate Research Expo, University of Adelaide, Adelaide-SA. Poster presentation.

- **Irene Zinonos**, Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino, David M Findlay, and Andreas Evdokiou.

  Apomab, a fully human agonistic DR5 monoclonal antibody, inhibits tumour growth and osteolysis in murine models of breast cancer development and progression. 20th

Annual Scientific Meeting of the ANZBMS 5-8 September 2010, Adelaide-SA. Oral Presentation.

- **Irene Zinonos**, Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino David M Findlay, and Andreas Evdokiou.

  Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin *in vivo*. 20th Annual Scientific Meeting of the ANZBMS 5-8 September 2010, Adelaide-SA. Poster Presentation.

- **Irene Zinonos**, Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino David M Findlay, and Andreas Evdokiou.

  Our Battle with Bone Cancer.3 minutes Thesis Competition. Faculty of Health Sciences, University of Adelaide. Oral Presentation.

- **Irene Zinonos**, Agatha Labrinidis, Michelle Lee, Vasilios Liapis, Shelley Hay, Vladimir Ponomarev, Peter Diamond, Andrew CW Zannettino David M Findlay, and Andreas Evdokiou.

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  Apomab, a fully human agonistic DR5 monoclonal antibody, inhibits tumour growth and osteolysis in murine models of breast cancer development and progression. 12th International TNF Conference, 26-29 April 2009, Madrid, Spain. Poster Presentation.

PRIZES AWARDED

- Best Oral Presentation in the category for Senior PhD Students (Laboratory), Research Day 12 October 2012, Basil Hetzel Institute, The Queen Elizabeth Hospital, Adelaide, SA.
- Best Poster Presentation for the School of Medicine. 2012 Postgraduate Research Expo, 31 August 2011, University of Adelaide, Adelaide-SA.
- Best Oral Presentation in the category for Senior PhD Students (Laboratory), Research Day 14 October 2011, Basil Hetzel Institute, The Queen Elizabeth Hospital, Adelaide, SA.
- Best Poster Presentation for the School of Medicine. 2011 Postgraduate Research Expo, 25 August 2011, University of Adelaide, Adelaide-SA.
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CHAPTER 1

INTRODUCTION

Breast Cancer

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer death among women worldwide. Every day, thousands of women around the world are diagnosed with this disease. Breast Cancer occurs in 1 in 9 women and accounts for 23% of all invasive new cancer cases and 14% of the total cancer deaths in 2008 (Ferlay, Shin et al. 2010; Jemal, Bray et al. 2011).

Classification

Breast cancers, based on their pathology at the time of diagnosis, are categorised in three different groups: a) benign, b) *in situ* or c) invasive. Benign breast cancers are not cancerous, do not grow uncontrollably, are easily treated and they are not life threatening. *In situ* breast cancer masses are confined within the lobules (milk production glands) or ducts (tubular structures connecting the lobules to the nipple) of the breast and are called lobular carcinoma *in situ* (LCIS) or ductal carcinoma *in situ* (DCIS) respectively. The majority of *in situ* breast cancers are DCIS accounting for about 80% of all *in situ* cases diagnosed during 2004-2008. Invasive or infiltrating breast cancers have escaped the duct or glandular walls at the time of diagnosis and have invaded the surrounding tissues. These cancers are called invasive ductal or lobular carcinomas depending on their origin. Microscopic analysis of the breast at the time of diagnosis will determine whether the cancer is *in situ* or invasive and lobular or ductal (ACS. 2011).

There are two main staging systems for cancer. The TNM classification of tumours describes tumour size and how far it has spread within the breast and nearby organs (T), lymph node involvement (N) and the presence or absence of distant metastases (M). Once the T,N and M are determined a stage of 0, I, II, III or IV is assigned with stage 0 being *in situ*, stage I being early stage invasive cancer and stage IV being the most advanced (ACS. 2011).

Incidence and Mortality

The global burden of breast cancer in women measured by incidence, mortality and economic costs is substantial and increases dramatically. Forouznafar MH and colleagues (Forouzanfar, Foreman et al. 2011) collected global breast cancer registry data on mortality and incidence for the last 30 years and demonstrated that the number of breast cancer incidence has increased steadily with an annual increase of 3.1 % between 1980 and 2010. Interestingly, more than two thirds of breast cancer cases in 2010 were accounted in women over 50 years, most of which originated from developed countries. However, for women aged 19-49, twice as many were diagnosed in developing countries than in the developed countries. Fortunately, the mortality to incidence ratio (MI ratio) has decreased substantially in both developing and developed regions. This decrease is a result of early detection through wider screening (mammography), increase in breast cancer awareness and improved treatment (Jemal, Siegel et al. 2010).

Risk Factors and epidemiology

Besides being a female, age is the most important risk factor for breast cancer. The incidence of breast cancer increases rapidly with age during the reproductive years and then increases at a slower rate after about 50 years, being the average age at menopause (Collaborative Group on Hormonal Factors in Breast Cancer 1997). In 2008, 95% of new breast cancer cases and 97% of breast cancer deaths occurred in women over 40 years of age and older.

The aetiology of breast cancer is not fully understood; however epidemiological studies demonstrate that hormonal factors play a significant role in the development and the aggressiveness of the disease. The female reproductive hormones, oestradiol and progesterone, stimulate the mitosis of breast epithelial cells, which influence breast cancer by increasing cell proliferation as well as promoting cancer growth. Early menarche and late

menopause are associated with increased risk of breast cancer by extending the relative lifetime exposure to high concentration of reproductive hormones (Kelsey, Gammon et al. 1993; Hulka and Moorman 2008; 2012; Britt 2012).

Pregnancy, especially at a younger age (< 30 years) is associated with decreased risk of breast cancer. Compared to nulliparous women, women who have had at least one full-term pregnancy have approximately a 25% reduction in breast cancer risk and this protection is increased with a greater number of pregnancies (Kelsey 1993; Lambe, Hsieh et al. 1994). Breastfeeding has also been shown to decrease a woman's risk to breast cancer, with greater benefit associated with longer duration (Collaborative Group on Hormonal Factors in Breast Cancer 2002). Oral contraceptives may slightly increase the risk of breast cancer; however women who stopped using oral contraceptives for 10 years or more have the same risk as women who never used them (Collaborative Group on Hormonal Factors in Breast Cancer 1996). In addition, hormonal therapy, with combined oestrogen and progesterone, for women undergoing menopause is associated with an increased risk of breast cancer and the risk increases with increasing duration of use. However, this increased risk diminishes within 5 years of discontinuation of hormone use.

Women with family history of breast cacner, especially in a first-degree relative (mother, sister, daughter) are at increased risk of developing breast cancer. A woman's risk of breast cancer is two or three times greater if she has a first degree relative that was diagnosed with breast cancer before the age of 50 and the younger the relative when she developed the disease the greater the risk. An estimated 10% of breast cancer cases are due to genetic predisposition and inherited mutations. So far, at least 10 mutations that predispose to breast cancer have been identified (The Cancer Genome Atlas Network 2012). These include mutations in the genes for BRCA1 and BRCA2 which account for 15-20% of familial breast cancers (Turnbull and Rahman 2008). These mutations are present in far less than 1% of the

general population but occur more often in certain ethnic groups such as those of Ashkenazi Jewish descent (Schwartz, Hughes et al. 2008).

Numerous studies have examined the effect of lifestyle factors on breast cacner risk. The observation that breast cancer rates are much higher in developed countries with high fat diets, led to the hypothesis that high fat diets increase the risk of breast cancer. However, no study to date has confirmed this association and the true relation between fat intake and breast cancer does not appear to be consistent (Hunter, Spiegelman et al. 1996). Obesity though is associated with a twofold increase of breast cancer in postmenopausal women but appears to protect against breast cancer before menopause. This is due to the fact that in postmenopausal women, circulating oestrogen is primarily produced by fat tissue. Thus having more fat tissue increases oestrogen levels and the likelihood of developing cancer. Physical activity on the other hand, appears to have a protective effect on breast cacner risk with stronger evidence in post menopausal than in premenopausal women (Neilson, Friedenreich et al. 2009). Numerous studies have confirmed the strong association between alcohol consumption and increased risk of breast cancer and the most likely mechanisms by which alcohol increases breast cancer risk is by increasing oestrogen and androgen levels (Singletary and Gapstur 2001). Lastly, smoking doesn't appear to be important in the aetiology of breast cancer, although avoiding exposure to tobacco has multiple health benefits (Miller, Marty et al. 2007; Luo, Margolis et al. 2011; Xue, Willett et al. 2011).

Breast Cancer Treatments

Treatment protocols for patients diagnosed with early breast cancer generally include local and systemic therapies which aim to improve survival and decrease the risk of recurrence. The mainstay of breast cancer treatment for localised tumours is surgery (partial or total mastectomy), which is often followed by radiation therapy. Systemic therapies consist of chemotherapy and hormone therapy which follow a certain pattern depending on different

clinical criteria of the patients (age, size of cancer, etc.) (Moreno-Aspitia and Perez 2009). These current therapies are proved to be very effective in controlling the symptoms of the disease, preventing serious complications, prolonging survival and improving the quality of life in women diagnosed with early breast cancer. Early diagnosis leads to patients having a very good prognosis with a 5-year disease free survival (DFS) of 89% and an overall survival (OS) rate of up to 98% (Brewster, Hortobagyi et al. 2008).

Breast Cancer and Metastasis

Despite the significant improvements in preventing, detecting and treating primary breast cancer over the last 20 years, 30% of women diagnosed with early breast cancer will eventually progress or relapse with locally advanced or metastatic breast cancer. Patients diagnosed with advanced disease experience poor prognosis and as a result the mortality rate increases dramatically to 70% (Beaumont and Leadbeater 2011). The high mortality rate is not a result of the primary cancer but is due to the subsequent metastasis to distant sites in the body, most common being the skeleton. Treatment protocols against metastatic breast cancer are limited to hormonal therapy and some chemotherapeutic agents including taxanes and anthracyclines (Lin, Zhang et al. 2003). However, due to intolerance toxicity and or/ development of drug resistance, such anticancer treatments are often discontinued and in patients with advanced disease, the treatment becomes only palliative.

Breast Cancer Metastasis to the Bone: a multistep process

An estimated 75-80% of patients with advanced breast cancer develop skeletal metastasis. Despite the significant progress in understanding other areas of cancer development the complexities of the metastatic process remain poorly understood (Coleman 2006; Siegel, Ward et al. 2011).

The process of metastasis involves multiple stages and at each stage the tumour cells must adapt to ensure their survival. Bone metastasis begins when breast cancer cells detach from the primary site of the tumour by forming new blood vessels and invade the blood and/or lymphatic system via a process called intravasation. For a successful metastatic process, the cancer cells must survive against the host immunoresponse while they are travelling in the circulation. To do so, they form aggregates with other cells such as platelets and lymphocytes and eventually adhere to the vascular endothelial cells of distant capillaries of the bone. Eventually, the tumour cells escape the circulation (extravasation), invade the marrow stroma and adhere to the endosteal surface of the bone. Tumour cells able to initiate and maintain colony proliferation form micrometastases which, following successful angiogenesis, grow into detectable metastases (Guise and Mundy 1998; Chambers, Groom et al. 2002; Hoon, Kitago et al. 2006; Pantel, Alix-Panabieres et al. 2009; Hoon, Ferris et al. 2011).

Epithelial to Mesenchymal Transition (EMT)

At the primary site, breast cancer cells have a basal epithelial phenotype. Epithelial cells are organised tightly to form a continuous layer above a basement membrane whereas mesenchymal cells are loosely anchored and have the capability of becoming mobile (Foroni, Broggini et al. 2012). For a successful metastatic process, breast cancer cells need to undergo diverse changes at the genetic and molecular level that drive cell transformation. One of these cell changes is EMT or Epithelial to Mesenchymal Transition (Hay DE 1968). EMT is characterised by the loss of epithelial polarity and the subsequent development of fibroblast-like phenotype (Patel, Ndabahaliye et al. 2010). EMT involves the loss of epithelial cell markers including E-cadherin, γ-catenin, zonula occludens (Zo-l) and the acquisition of mesenchymal markers such as vimentin, fibronectin and N-cadherin (Hollier, Evans et al. 2009; Moen, Oyan et al. 2009). In addition, the tumour microenvironment can trigger EMT

through induction via upregulation of specific cytokines and growth factors, including TGF-β (Romagnoli, Belguise et al. 2012). After losing their attachments from the basement membrane, the cancer cells enter the vasculature and become mobile and can take up residence at secondary sites, where they revert to an epithelial morphology and establish metastases.

Metastatic Theories

Different cancer types exhibit preference to specific organ sites for secondary metastases that are dependent from the primary site of the tumour, a phenomenon called organ tropism. For example, breast cancer preferentially metastasizes to the lymph nodes, lung, liver, brain and bone. The two main theories proposed to explain this organ tropism of cancer metastasis include the "seed and soil" hypothesis first proposed by Stephen Paget and Ewing's mechanical arrest theory.

In 1889, Stephen Paget proposed the "seed and soil" hypothesis to explain how the non-random metastatic process occurs: "When a plant goes to seed, the seeds are carried in all different directions; but they can only grow if they fall in fertile soil" (Paget 1989). Certain tumour cells (the 'seed') have specific affinity for certain tissues ('the soil') (Langley and Fidler 2011). In the case of breast cancer, the bone 'soil' is one of the most hospitable place for the breast cancer 'seed' to survive and grow because of the favourable microenvironment of its matrix and its ample blood supply (Mundy 2002). This theory was challenged by Ewing who suggested that organ tropism can be accounted by circulatory patterns within the body and that cells are mechanically arrested in the first capillary bed they encounter (Ewing 1928). Ewing suggested that breast cancer cells that leave the primary site, will be carried by the blood flow first through the heart and then to the capillary beds of the lungs. Some of them may arrest there and form micrometastases and some might pass through the lungs, enter the arterial system and get transported to other organs, such as bone. Scientists now believe that it

is more likely that the above two theories work in concert for a successful metastatic process to occur: the initial delivery and arrest of cancer cells seems to be mechanical due to circulatory and chemical signals. Once the cancer cells have been seeded to an organ, however, they require a suitable microenvironment to initiate proliferation and establish metastasis and that depends on the compatibility of the "seed" with the "soil". Recent findings suggest that the soil is primed and ready prior to the arrival of cancer cells, thereby creating a "landing dock" for future metastatic growth, a concept referred to as the "pre-metastatic niche"(Peinado, Lavotshkin et al. 2011).

Pre-metastatic niche

The phenomenon of organ specificity of different cancer types during the metastatic process remains the most intriguing aspect of carcinogenesis. The genetic and phenotypic make-up of the primary tumour is a major determinant for a successful metastatic process but a receptive microenvironment is necessary for establishing macrometastases and tumour growth. As the primary tumour progress, it modifies its own microenvironment but it can also "prime" distant sites for the arrival of disseminated tumour cells (DTCs) from the primary site, known as the pre-metastatic niche (Chu and Allan 2012; Wilson, Holen et al. 2012).

Breast cancer is one of the most common carcinoma to develop bone metastases due to the unique characteristics of the bone niche which not only provides homing signals to primary tumour cells but also the biochemical (cytokines, growth factors) and physical (acidic pH, high extracellular calcium concentration) properties of the bone provide an advantageous microenvironment for tumour growth (Guise 2010; Patel, Camacho et al. 2011).

Extensive studies in the last decade have demonstrated that bone-derived chemokines such as osteopontin, osteonectin and stromal-derived factor-1 (SDF1:CXCL12) play a significant role in trafficking of breast cancer cells to the bone (Jacob, Webber et al. 1999;

Ibrahim, Leong et al. 2000; Taichman, Cooper et al. 2002). In addition, tumour cells express patterns of chemokine receptors that match chemokines that are specifically expressed in organs to which these cancer cells commonly metastasise. Breast cancer cells in particular, express the chemokines receptor CXCR4 and home to organs with high levels of its ligand, SDF-1, such as the bone (Muller, Homey et al. 2001; Kang, Siegel et al. 2003; Luker and Luker 2006). Activation of CXCR4 stimulates a number of cellular processes involved in metastasis, including pseudopodia formation, invasion and migration (Luker and Luker 2006). CXRC4 also signals for integrin activation, which increases the affinity of tumour cells for microvascular endothelial cells and blocking of CXRC4 was found to inhibit metastasis of breast cancer cells in experimental animal models (Muller, Homey et al. 2001; Cardones, Murakami et al. 2003). In conclusion, the primary tumour and its production of a unique array of chemokines appear to orchestrate formation of the pre-metastatic niche which then prepares the bone "soil" for micrometastases and establishment of metastatic lesions (Chambers, Groom et al. 2002).

Establishment of the metastatic spread and vascularisation

Successful breast cancer expansion in the bone requires the differentiation of a new vasculature network to support tumour growth (Folkman and Kalluri 2004). This process is being orchestrated by a number of chemokines secreted by the tumour cells that promote vascularisation. Pro-angiogenic cytokines, expressed by the tumour cells, such as vascular endothelia growth factor (VEGF) induce homing endothelial progenitor cells (EPCs) and hematopoietic progenitor cells (HPCs), derived from the bone marrow, to express VEGF receptors 1 and 2 which then initiates the proliferation and differentiation of the neovascular network (Lyden, Hattori et al. 2001; Rafii, Lyden et al. 2002; De Palma, Venneri et al. 2003; Rafii and Lyden 2003; Okamoto, Ueno et al. 2005). Recent examination of the early stages of the metastatic process has revealed the recruitment of clusters of hematopoietic progenitor

cells expressing VEGFR-1 to pre-metastatic sites, including the bone, forming the pre-metastatic niche prior to the arrival of tumour cells which promotes the generation of a suitable microenvironment for the metastatic breast cancer cells (Kaplan, Riba et al. 2005). This was further substantiated by inhibiting the expression of VEGF receptors on HPCs and EPCs which then eliminated the pre-metastatic niche and subsequently establishment of micrometastases was blocked (Psaila, Kaplan et al. 2006).

Overall, the ability of cancer cells to metastasize and grow at a specific site depends on the cancer cells features themselves but also the characteristic features of the distant organ and the active interactions between primary and metastatic sites. Once the metastatic niche is successfully formed in the bone, micrometastases can occur which then proceed to form established metastatic lesions.

Normal Bone Remodelling - RANKL/RANK/OPG System

The pathologic complications of bone metastasis can have devastating effects and patients experience debilitating skeletal-related events (SREs) including, pathological skeletal fractures, hypercalcaemia of malignancy and spinal cord compression. SREs are accompanied by severe bone pain and loss of mobility which eventually leads to reduction of quality of life and survival (Coleman, Lipton et al. 2010; Guise, Brufsky et al. 2010; Coleman, Costa et al. 2011). These complications arise because the metastatic breast cancer cells disrupt the very important process of normal bone remodeling and cause extensive bone destruction.

The bone matrix is constantly being remodelled throughout life to maintain skeletal integrity (Sommerfeldt and Rubin 2001; Tanaka 2007). The dynamic and complex structure of the skeleton is the result of a balance between bone resorption and bone formation. These two procedures are controlled by the coordinated activity of the two main bone cells: the osteoblasts, the bone forming cells and the osteoclasts, the bone resorbing cells. Osteoclasts remove both mineral and organic components of damaged bone from the surfaces of trabecular and cortical bone, leaving behind eroded resorption lacuna. Osteoblasts are then recruited to the bone surface and subsequently fill in the gaps with new, healthy bone matrix (Guise and Mundy 1998; Karsenty and Wagner 2002; Boyce, Xing et al. 2003; Clines and Guise 2005).

Normal bone remodelling is affected by a number of processes that are still not completely understood. However, it is now becoming evident that these processes include damage on bone in response to normal wear and tear, changes in mechanical forces due to alterations in body shape or weight, and local production and release of cytokines or growth factors. Bone formation follows resorption and there are more than 1 million microscopic foci

of remodelling at any time in the adult skeleton (Boyce and Xing 2008; Kohli and Kohli 2011).

The discovery of the key factors involved in bone remodelling was of great importance for bone research. One major system that appears to play a fundamental role in both physiological and pathological bone remodelling, particularly in bone resorption is the RANKL/RANK/OPG axis (Hofbauer, Neubauer et al. 2001; Wittrant, Theoleyre et al. 2004). RANKL ('receptor activator of NF-κB ligand') is a member of the TNF family and is expressed by stromal cells and osteoblasts in the bone marrow microenvironment (Lacey, Timms et al. 1998). RANKL can bind to its signalling receptor RANK, on the surface of osteoclast precursors and mature osteoclasts, to induce osteoclast recruitment, differentiation, and bone resorption (Fig 1A) (Anderson, Maraskovsky et al. 1997; Ikeda, Kasai et al. 2001). Osteoprotegerin (OPG), which is the only soluble member of the TNF receptor family and produced by osteoblasts and other cell types, binds to and neutralises the effects of RANKL, preventing the association between RANKL and RANK, thereby inhibiting osteoclast activation and function (Fig 1B) (Simonet, Lacey et al. 1997; Tsuda, Goto et al. 1997; Yasuda 1998; Yasuda, Shima et al. 1998). According to recent findings, the RANKL/RANK/OPG system is the key regulator not only for normal but also for pathological bone metabolism.

The "Vicious Cycle" of Bone Metastasis

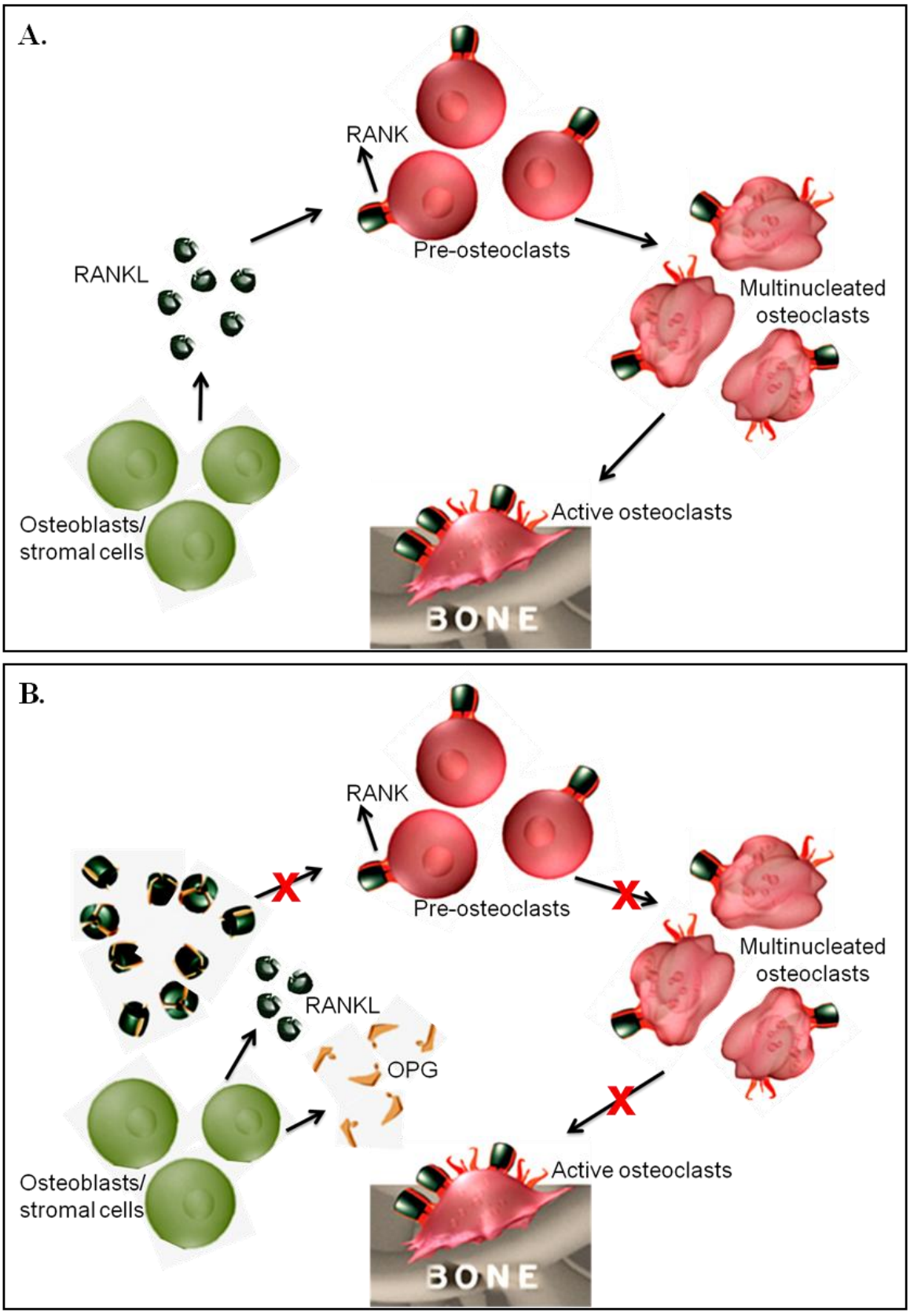
Bone metastasis begins when breast cancer cells detach from the primary site of the tumour by forming new blood vessels and invade the circulation (Kingsley, Fournier et al. 2007; Buijs and van der Pluijm 2009). For the metastatic process to be successful, cancer cells must survive against the host immune response while they are travelling in the circulation. To do so, they form aggregates with other cells such as platelets and lymphocytes and eventually

adhere to the vascular endothelial cells of distant capillaries of the bone. Eventually, the tumour cells escape the circulation, invade the marrow stroma and finally adhere to the endosteal surface of the bone (Guise and Mundy 1998; Guise, Kozlow et al. 2005). In order to invade and reach the endosteal surface, breast cancer cells need to degrade their surrounding hard mineralised matrix of the bone. Therefore, in the presence of breast cancer within the bone microenvironment, normal bone remodelling is altered and no longer in balance giving tumour cells the opportunity to home and proliferate in the bone ‘soil’ (Guise and Mundy 1998; Demers, Costa et al. 2000).

Figure 1. ***Normal Bone Remodelling - RANKL/RANK/OPG System***.

A. Osteoblasts and stromal cells produce RANKL which binds to its receptor RANK on pre-osteoclasts and stimulates their differentiation and maturation into functional osteoclasts.

B. OPG binds to RANKL, inhibiting the association between RANKL and RANK, thereby inhibiting osteoclast differentiation and function.


A.
RANK
Pre-osteoclasts
RANKL
Multinucleated
osteoclasts
Active osteoclasts
Osteoblasts/
stromal cells
BONE
B.
RANK
Pre-osteoclasts
RANKL
OPG
Multinucleated
osteoclasts
Active osteoclasts
Osteoblasts/
stromal cells
BONE

Bone metastases are classified as osteolytic or osteoblastic based on their radiographic appearance (Mundy 2002; Kozlow and Guise 2005; Kingsley, Fournier et al. 2007). Both types of lesions result from an alteration in the balance of normal bone remodelling. Osteolytic lesions are characterised by an increase in osteoclast activity with a concomitant impairment of osteoblastic activity. In turn, this leads to an abnormally high rate of bone resorption. In osteoblastic lesions there is an increase in bone formation around tumour cell deposits, followed by imbalance in osteolytic activity and increase in bone turnover. Both types of bone lesions result in significant local bone loss, with the eventual risk of fracture or, in the spine, potential vertebral collapse (Demers, Costa et al. 2000; Mundy 2002; Kozlow and Guise 2005; Kingsley, Fournier et al. 2007). Although approximately 15% of breast cancers are osteoblastic or mixed, most breast cancer lesions tend to be osteolytic. Osteolytic bone metastases are present in 80% of patients with stage IV disease (Demers, Costa et al. 2000; Kakonen and Mundy 2003).

Once breast cancer cells have invaded the bone marrow, they secrete a plethora of humoral factors that stimulate the recruitment of osteoclast progenitor cells which then undergo differentiation to mature osteoclasts and begin degradation of the bone. Breast cancer cells can also release growth factors that induce increased osteoblastic activity. Osteoblastic activation in turn releases growth factors that also stimulate tumour-cell growth, and further activation and differentiation of osteoclasts. The activation of osteoclasts, and in turn bone resorption, is thought to cause further release of bone-derived growth factors that enhance the survival and proliferation of tumour cells, thus establishing a "vicious cycle" of continuous bone destruction and local tumour growth (Fig 2) (Kakonen and Mundy 2003; Lipton 2004).

The vicious cycle, as described above, is propagated by four contributors: breast cancer cells, osteoblasts, osteoclasts and the bone matrix (Kozlow and Guise 2005). Metastatic cells in the bone microenvironment secrete parathyroid hormone-related peptide,

PTHrP, which stimulates osteoclastic bone resorption. This is accomplished by an increase in expression of RANKL by osteoblasts and stromal cells. After binding to RANK on osteoclast precursor cells, RANKL signals the differentiation and fusion of active osteoclasts (Kakonen and Mundy 2003). In addition, breast cancer cells produce other factors that increase the formation of osteoclasts, including interleukin-6 (IL-6), tumour necrosis factor- α (TNF-α) and many others. These factors increase the expression of RANKL, which directly induces osteoclast formation and eventually bone resorption (Guise 2000; Roodman 2004). As a consequence of osteoclastic bone resorption, several factors are released from the bone matrix, including transforming growth factor-β (TGF-β), insulin-like growth factors (IGFs) and others which stimulate further production of PTHrP by tumour cells, and tumour proliferation (Guise 2000; Roodman 2004; Guise, Kozlow et al. 2005). Additionally, bone destruction increases local calcium levels, which also promotes tumour growth and the production of PTHrP, leading to further amplification of the effects of the continuous vicious cycle.

Current treatment strategies for patients with breast cancer bone metastasis include the disruption of this vicious cycle by targeting cancer cells directly and/or by modulating the host bone microenvironment that ultimately inhibits proliferation of breast cancer growth in bone. RANKL plays a fundamental role in promoting this "vicious cycle" of breast cancer bone metastasis leading to continuous bone destruction and local tumour growth in the bone (Roodman 2004). Thus, in the context of modulation of the bone microenvironment, the RANKL/RANK/OPG system becomes an attractive therapeutic target. Directly targeting cancer cells is the major focus of metastatic cancer research and one key mechanism which is an important target for developing novel therapeutics for the treatment of metastatic breast cancer within the bone is apoptosis.

Apoptosis

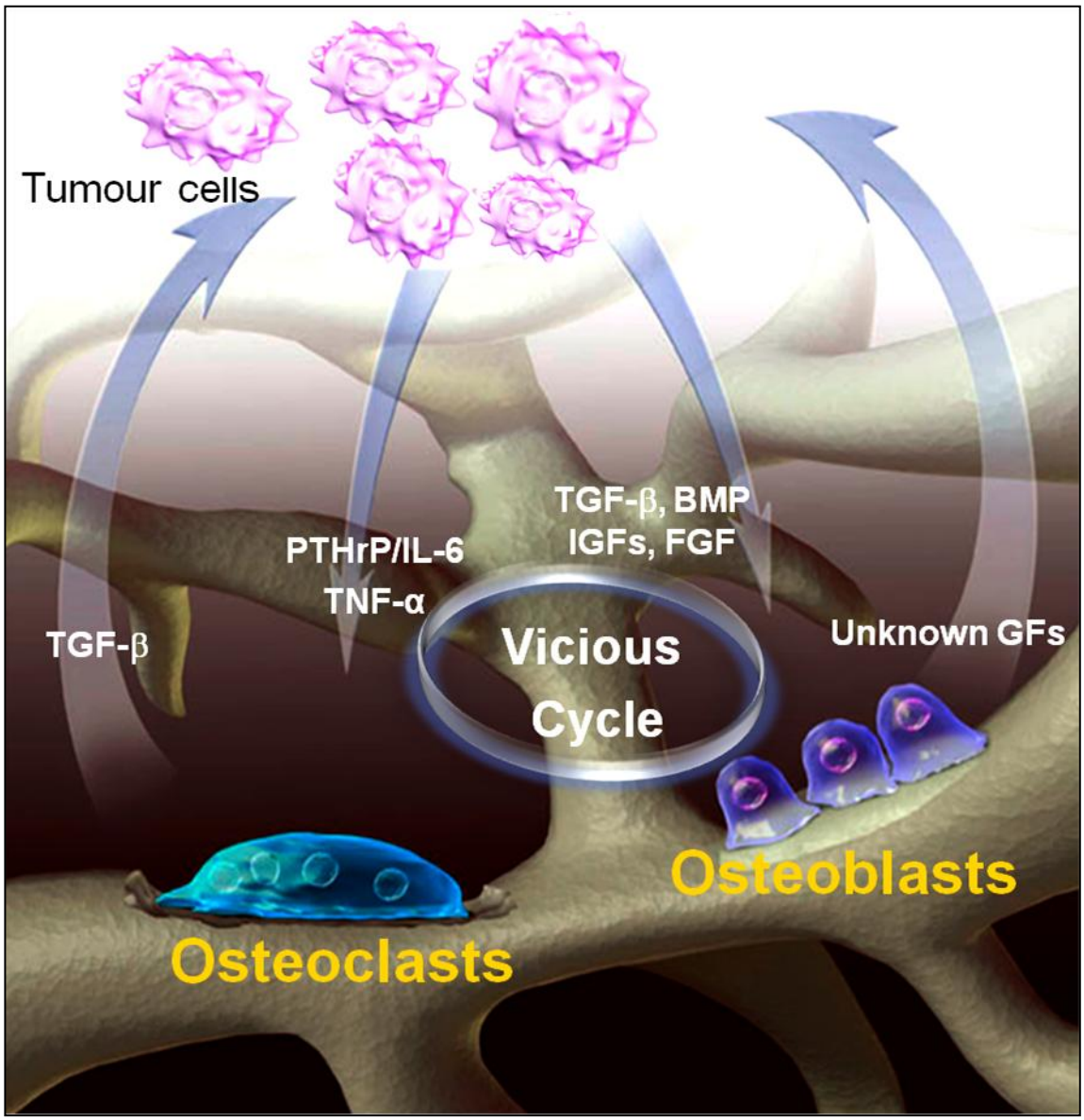
Apoptosis is a form of programmed cell death that plays an important role in normal development and tissue homeostasis in multicellular organisms (Prindull 1995). It is a major control mechanism, whereby cells will die if DNA damage is not repaired (Lowe and Lin 2000). Apoptosis is characterised by a number of specific features including cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, ending with the engulfment by macrophages or neighbouring cells, resulting in the cell disappearing without an inflammatory response (Savill and Fadok 2000). Induction of apoptosis is therefore considered to be the most suitable method of anticancer therapy because it aims to specifically induce tumour cell death while limiting possible cytotoxic effects in healthy tissues (Russo, Terrasi et al. 2006).

The Extrinsic and Intrinsic Apoptotic Pathways

In mammals, apoptosis occurs through two main pathways: the "extrinsic" or death receptor pathway and the "intrinsic" or the mitochondria pathway (Jin and El-Deiry 2005). The extrinsic pathway is mediated by transmembrane receptor-mediated interactions (Bouralexis, Findlay et al. 2005; Elmore 2007). These death receptors are members of the tumour necrosis factor (TNF) protein superfamily that contain a cytoplasmic domain of about 80 amino acids called the "death domain" (Ashkenazi and Dixit 1998; Locksley, Killeen et al. 2001). The TNF receptors include Fas, (Apo1,CD95), TNF-R, DR3, DR4 (tumour necrosis factor-related apoptosis-inducing ligand receptor 1, TRAIL R1) and DR5 (TRAIL R2) (Zapata, Pawlowski et al. 2001).

Figure 2. ***The Vicious Cycle of Bone Metastasis.***

When breast cancer cells metastasise to the bone, they start producing growth factors that can stimulate increased osteoclastic and osteoblastic activity, both eventually leading to bone degradation. As a result, the bone releases growth factors that can stimulate further proliferation of the tumours cells that establishes a vicious cycle of continuous bone destruction and local tumour growth. *(Diagram adapted from Medscape.)*


Tumour cells
TGF-β, BMP
IGFs, FGF
PTHrP/IL-6
TNF-α
TGF-β
Vicious
Cycle
Unknown GFs
Osteoblasts
Osteoclasts

Upon death ligand binding, oligomerisation of the receptors occurs, which leads to the recruitment of the cytoplasmic adaptor protein Fas-associated death domain (FADD) that binds to the receptors by its corresponding death domain. Consequently, the apoptosis-initiating proteases, caspase-8 and/or caspase-10 bind to FADD and form a death-inducing signalling complex (DISC), which leads to the auto-activation of these initiator caspases. The activation of caspase-8 at the DISC is followed by the activation of the effector caspases, including caspase-3, caspase-6 and caspase-7. Eventually, the initiation of this intracellular caspase cascade leads to programmed cell death (Ashkenazi and Dixit 1998).

The intrinsic pathway is triggered by apoptotic stimuli within the cell, such as radiation, hypoxia, hyperthermia and many others (Elmore 2007). These stimuli induce modifications (dephosphorylation and cleavage) of pro-apoptotic members of the Bcl-2 protein family including Bid, Bax, Bak and others, leading to their activation and translocation to the mitochondria (Cory and Adams 2002; Scorrano and Korsmeyer 2003). As a result, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome-c from the mitochondria to the cytosol (Saelens, Festjens et al. 2004). Once cytochrome-c is released in the cytosol, it binds to the apoptosis protease-activating factor 1 (Apaf-1) and pro-caspase-9, resulting to the formation of an intracellular DISC-like complex known as the "apoptosome" (Shi 2002; Hill, Adrain et al. 2004). Within the apoptosome, caspase-9 is cleaved and activated. This leads to activation of caspase-3, which subsequently activates a caspase cascade and eventually apoptosis (Shi 2002). In some cells, the death receptor pathway (extrinsic) interacts with the intrinsic apoptotic pathway *via* caspase-8 mediated cleavage of the pro-apoptotic Bcl-2 family member Bid, which leads to the subsequent release of cytochrome-c. Eventually this amplifies apoptosis through the cell-extrinsic pathway (Fig 3) (Li, Zhu et al. 1998; Esposti 2002).

Both the extrinsic and the intrinsic apoptotic pathways end at the point of the execution phase, which is the final step of apoptosis. The execution phase begins with the activation of the effector caspases (caspase-3, -6, and -7), which activate cytoplasmic endonucleases to degrade the nuclear material and proteases that in turn degrade the nuclear and cytoskeletal proteins (including PARP, NuMA). As a result, cell cycle regulation and signalling pathways are affected, leading to the morphologic manifestations of apoptosis such as DNA condensation, fragmentation, membrane blebbing and eventually to cell death (Thornberry and Lazebnik 1998; Slee, Adrain et al. 2001).

Targeting the Extrinsic Apoptotic Pathway

The idea of targeting the extrinsic apoptotic pathway in tumours for therapeutic purposes is very attractive. This is mainly because death receptors directly activate the caspase machinery and also initiate apoptosis independently of the p53 tumour suppressor gene, which is mutated and inactivated in most human cancers (Chicheportiche, Bourdon et al. 1997; Vogelstein, Lane et al. 2000; Strano, Dell'Orso et al. 2007). The best characterised death ligands and their receptors are the Fas ligand (also known as Apo1L or CD95L) that binds to Fas (Apo1, CD95), the TNF ligand that binds to TNFR1 (Chicheportiche, Bourdon et al. 1997; Marsters, Sheridan et al. 1998), the Apo3 ligand which binds to DR3 (Marsters, Sheridan et al. 1998) and Apo2 ligand, also called TRAIL (Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand), that binds to DR4 and DR5 (Wiley, Schooley et al. 1995; Pitti, Marsters et al. 1996; Pan, Ni et al. 1997).

Even though initially TNF and CD95L carried great promise as anticancer agents, their severe toxicity towards normal tissues precluded their clinical use. Systemic administration of certain TNF doses caused an inflammatory response syndrome and CD95L

induced apoptosis in normal hepatocytes, which express abundant CD95 (Sheridan, Marsters et al. 1997). In contrast, Apo2L/TRAIL is selectively toxic to cancer cells, while sparing normal cells (Ashkenazi and Dixit 1999; Ashkenazi 2002). Recent clinical trials demonstrated Apo2L/TRAIL to be safe and well-tolerated in patients with advanced cancer (Duiker, Mom et al. 2006; Pan 2007). Therefore, Apo2L/TRAIL is considered to be an exciting new anticancer agent for the treatment of solid and heamatological malignancies.

Apo2L/TRAIL and its Death and Decoy Receptors

Apo2L/TRAIL is a type II transmembrane protein but it also exists in a soluble form (Wiley, Schooley et al. 1995; Ashkenazi 2002; LeBlanc and Ashkenazi 2003; Wang and El-Deiry 2003; Huang and Sheikh 2007). Apo2L/TRAIL can engage apoptosis *via* the extrinsic apoptotic pathway by specifically binding to its death domain-containing receptors DR4 and DR5 as a homotrimer (Li, Wang et al. 2006; Huang and Sheikh 2007). When Apo2L/TRAIL binds to either DR4 and/or DR5, it activates the caspase cascade, which leads to programmed cell death. Apo2L/TRAIL can also activate the intrinsic pathway, involving the mitochondria system, by caspase-8 mediated cleavage and activation of Bid which eventually amplifies apoptosis as well (Fig 3) (Wiley, Schooley et al. 1995; Ashkenazi and Dixit 1999; LeBlanc and Ashkenazi 2003; Wang and El-Deiry 2003; Bouralexis, Findlay et al. 2005; Li, Wang et al. 2006; Falschlehner, Emmerich et al. 2007; Huang and Sheikh 2007).

In addition to the death receptors, Apo2L/TRAIL can also bind to three other "decoy" receptors, DcR1, DcR2 and osteoprotegerin (OPG) (Degli-Esposti, Dougall et al. 1997; Degli-Esposti, Smolak et al. 1997; Marsters, Sheridan et al. 1997; Pan, Ni et al. 1997; Emery, McDonnell et al. 1998). DcR1 and DcR2 resemble DR4 and DR5 but DcR1 lacks a death domain, and DcR2 has a truncated and non-functional death domain. Therefore they are

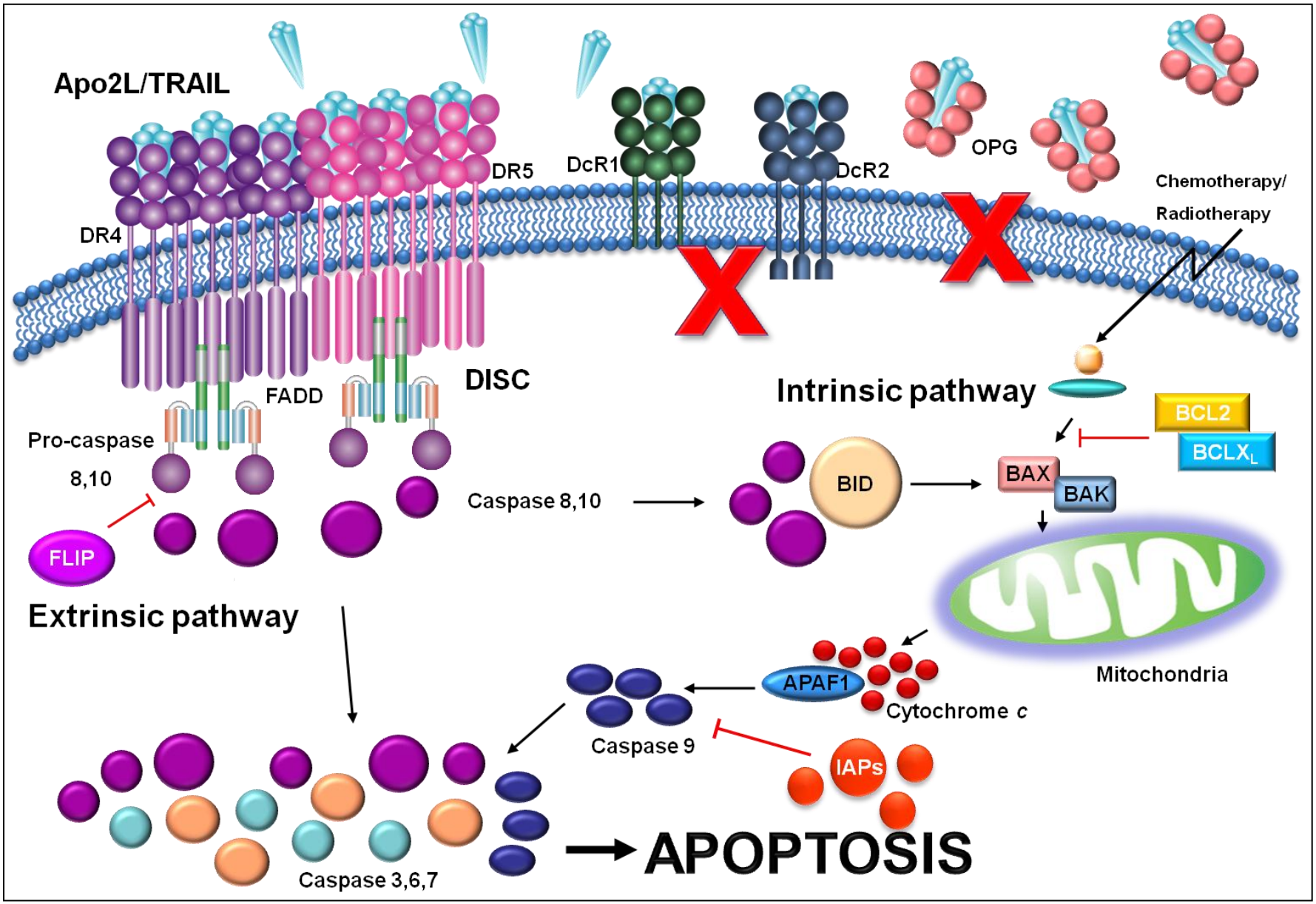
unable to signal for apoptosis induction upon ligand binding. These two receptors were found in abundance in many types of human normal tissues but not in most cancer cell lines, suggesting that they compete with the death receptors for Apo2L/TRAIL and are thought to protect normal cells from its apoptotic activity (Yagita, Takeda et al. 2004). OPG can also bind to RANKL, and prevent osteoclast bone resorption. OPG is a widely expressed soluble member of TNF receptor that is capable of binding to Apo2L/TRAIL and although it has lower affinity for Apo2L/TRAIL at normal physiological temperatures, it can block Apo2L/TRAIL-induced apoptosis, at least *in vitro* (Emery, McDonnell et al. 1998; Park, Min et al. 2003; Vitovski, Phillips et al. 2007).

Apo2L/TRAIL in Cancer Therapy

The potential of soluble Apo2L/TRAIL as an anticancer therapeutic agent has been well demonstrated in several mouse xenograft models of human soft tissue cancers including colorectal (Kelley, Harris et al. 2001), breast (Walczak, Miller et al. 1999; Thai le, Labrinidis et al. 2006), lung (Jin, Yang et al. 2004), multiple myeloma (Mitsiades, Treon et al. 2001; Labrinidis, Diamond et al. 2009) and glioma (Roth, Isenmann et al. 1999). Apo2L/TRAIL was active alone and in combination with certain chemotherapeutic agents or radiotherapy and caused marked regression or complete remission of tumours, with no evidence of toxicity to normal tissues (Gliniak and Le 1999; Chinnaiyan, Prasad et al. 2000). In addition, recent clinical trials demonstrated recombinant soluble Apo2L/TRAIL to be safe and well-tolerated in patients with advanced tumours (Pan 2007). However, not much is known about the actions of Apo2L/TRAIL in the bone microenvironment, where also OPG is found at high levels.

Figure 3. ***Apo2L/TRAIL Apoptotic Signalling Pathway.***

Apo2L/TRAIL engages apoptosis via the extrinsic apoptotic pathway by binding to its death domain-containing receptors DR4 and DR5. Upon ligand binding oligomerization of the receptors occurs and an enzymatic caspase cascade is activated which then mediates the apoptotic cell death program. Apo2L/TRAIL can also activate the intrinsic apoptotic pathway, involving the mitochondria system, which eventually amplifies apoptosis as well.


Apo2L/TRAIL
DR4
DR5
DcR1
DcR2
OPG
Chemotherapy/
Radiotherapy
DISC
FADD
Pro-caspase
8,10
FLIP
Extrinsic pathway
Caspase 8,10
Intrinsic pathway
BID
BCL2
BCLX$_L$
BAX
BAK
Mitochondria
APAF1
Cytochrome c
Caspase 9
IAPs
Caspase 3,6,7
APOPTOSIS

A recent study by this laboratory investigated the efficacy of Apo2L/TRAIL in skeletal malignancies and showed that recombinant soluble Apo2L/TRAIL decreased tumour burden within the bone and reduced cancer-induced bone destruction in a murine model of metastatic breast cancer (Thai le, Labrinidis et al. 2006). Unfortunately, treatment with Apo2L/TRAIL, as shown in this study, failed to completely eliminate tumours from the bone. This outcome suggests that a) the dose and schedule of Apo2L/TRAIL treatment was not 100% efficient, requiring further dose optimisation or b) prolonged treatment with Apo2L/TRAIL *in vivo* leads to acquired resistance to Apo2L/TRAIL. Recent research also showed that the decoy receptor OPG which binds to Apo2L/TRAIL may act as a functional inhibitor of its apoptotic activity (Vitovski, Phillips et al. 2007). These data raise the possibility that the activity of Apo2L/TRAIL is suppressed in the bone microenvironment where OPG is normally expressed in very high levels (Emery, McDonnell et al. 1998; Vitovski, Phillips et al. 2007). Another limitation is that the half-life of soluble Apo2L/TRAIL in the body is short, requiring frequent administration for Apo2L/TRAIL to maintain efficacy (Kelley, Harris et al. 2001; Pan 2007). Therefore, alternative therapeutic agents that specifically activate DR4/DR5 death receptors to induce apoptosis, and especially long-acting agents that do not bind to OPG, are clearly needed.

Monoclonal Antibodies in Cancer Therapy

In the past decade, a number of agonistic monoclonal antibodies (mAb) have been developed as alternative therapeutic agents for cancer therapy (Takeda, Okumura et al. 2007). They are effective molecule-specific targeting drugs which have a longer half-life *in vivo* than recombinant proteins and their binding affinity is usually higher than that of the ligands. Furthermore, mAb have been reported to induce death receptor-dependent apoptosis in human

cancer cells and showed potent antitumour activity against tumour xenografts in preclinical models, which is enhanced by combination with chemotherapy (Chuntharapai, Dodge et al. 2001; Ichikawa, Liu et al. 2001; Belyanskaya, Marti et al. 2007). As an alternative to soluble Apo2L/TRAIL, long-acting agonistic monoclonal antibodies (mAbs) specific for binding to Apo2L/TRAIL receptors DR4 and DR5 and inducing cell death have been developed (mapatumumab, lexatumumab, TRA-8, contanumumab, CS-1008) (Buchsbaum, Zhou et al. 2003; Georgakis, Li et al. 2005; Zeng, Wu et al. 2006; Herbst, Kurzrock et al. 2010; Wiezorek, Holland et al. 2010). These antibodies have several therapeutic advances over Apo2L/TRAIL including their no binding affinity for Apo2L/TRAIL decoy receptors, their longer half-life (6-21 days in the circulation compared to 30-60 minutes for Apo2L/TRAIL) and their potential to initiate antibody-dependent cellular cytotoxicity. For example, an anti-DR5 mAb, called TRA-8, when used alone or in combination with chemotherapy and/or radiotherapy exhibited significant antitumour activity in breast cancer xenograft models (Buchsbaum, Zhou et al. 2003). Furthermore, mapatumumab (HGS-ETR1), a DR4 agonistic mAb, and lexatumumab (HGS-ETR2), a DR5 agonistic mAb, exhibited strong cytotoxic activity against a variety of primary and cultured lymphoma cells (Georgakis, Li et al. 2005). Mapatumumab also induced apoptosis in human renal cell carcinoma cells (RCC) and suppressed tumour growth in a mouse xenograft model of RCC (Zeng, Wu et al. 2006). Both mapatumumab and lexatumumab have been evaluated as single agents and in combination with chemotherapy in early clinical trials in patients with advanced malignancies (Plummer, Attard et al. 2007; Tolcher, Mita et al. 2007; Mom, Verweij et al. 2009; Wakelee, Patnaik et al. 2010).

Drozitumab

Drozitumab (PRO95780), formally known as apomab, is a fully human and long-acting agonistic mAb developed by Genentech Inc., which has been designed to directly and specifically bind to the Apo2L/TRAIL pro-apoptotic receptor DR5 on the surface of cancer cells and initiate apoptosis (Adams, Totpal et al. 2008).

Drozitumab has already been evaluated both *in vitro* and *in vivo*. *In vitro,* drozitumab induces apoptosis in various human cancer cell lines while sparing normal hepatocytes. Addition of an anti-Fc cross-linking antibody to drozitumab *in vitro* further augments DISC assembly and caspase-8 activation, enhancing pro-apoptotic signalling (Adams, Totpal et al. 2008). This enhanced apoptotic activity of drozitumab is due to the further aggregation of the death receptors, as a result of the cross-linking with the anti-Fc antibody. *In vivo,* drozitumab demonstrated tumour suppressive activity in various xenograft models of cancer, including colorectal cancer, NSCLC, pancreatic cancer and lung (Adams, Totpal et al. 2008; Jin, Yang et al. 2008). Cross-linking with anti-Fc antibody is not necessary *in vivo* due to the abundance of circulating Fc antibodies and the cell-to-cell interactions which enables for death receptors oligomerisation and formation of aggregates which eventually leads to the activation of the extrinsic apoptotic pathway.

Data from phase I studies evaluating the safety, pharmacokinetic profile and early evidence of antitumour efficacy of drozitumab in a cohort of patients with solid and haematological malignancies demonstrated drozitumab to be well-tolerated, and capable of producing prolonged stable disease in patients with advanced cancer (Camidge 2008). Phase II clinical trials are ongoing to evaluate drozitumab as a single agent, and in combination with chemotherapy, in a variety of malignancies. Early studies showed that drozitumab suppresses tumour growth in the mammary tissue, even in animals with well-advanced tumours. These

findings suggest that drozitumab may represent a novel therapeutic agent that is safe and non-toxic, for the treatment of breast cancer patients. However, to date, no studies have been reported using drozitumab for the treatment of skeletal malignancies.

Aims and Significance of the Project

Breast cancer is the most common cancer in women that frequently metastasises to bone and patients suffer from debilitating complications that erode the quality of their life. Current treatments for patients with breast cancer bone metastases remain only palliative. Therefore, there is a great need to identify novel approaches for the treatment of patients with advanced metastatic breast cancer.

Inhibiting the vicious cycle of bone metastasis appears to be an attractive approach for developing novel treatment strategies for patients with breast cancer bone metastasis. The disruption of this vicious cycle could be accomplished by targeting cancer cells directly using the mechanism of apoptosis and/or by modulating the host bone microenvironment that ultimately inhibits proliferation of breast cancer growth in bone.

Apo2L/TRAIL is a member of the TNF superfamily and it induces apoptosis in cancer cell lines. Potency and lack of toxicity to normal tissues makes activation of Apo2L/TRAIL signalling an ideal target for cancer therapy. However, recombinant soluble Apo2L/TRAIL failed to completely eliminate tumours from the bone giving rise to late recurrence. This outcome suggests a) the dose and schedule of Apo2L/TRAIL treatment was not 100% efficient, requiring further dose optimisation or b) prolonged treatment with Apo2L/TRAIL *in vivo* leads to acquired resistance to Apo2L/TRAIL. In addition, the decoy receptor OPG binds to Apo2L/TRAIL and acts as a functional inhibitor of its apoptotic activity, raising the possibility that the activity of Apo2L/TRAIL is suppressed in the bone microenvironment where OPG is normally expressed in very high levels. Furthermore, there is a move away from ligand therapy for reasons such as cost, immuno-reaction and poor pharmacokinetics. Drozitumab activates Apo2L/TRAIL receptor-mediated apoptotic pathways in a manner similar to soluble Apo2L/TRAIL and may have enhanced therapeutic potential compared to

Apo2L/TRAIL ligand, owing to its prolonged half life *in vivo*. This, together with the non-OPG binding properties, potentially makes drozitumab an effective therapeutic agent for the treatment of skeletal malignancies. Testing this possibility is an exciting project at the forefront of work in this area. In addition, an appropriate understanding of the mechanisms controlling cancer cells evasion from Apo2L/TRAIL /drozitumab-induced cell death will be required to optimally use these drugs in the clinic. These studies will provide a real opportunity to influence the treatment of patients with bone metastases.

The specific aims of these studies are:

1. To evaluate the efficacy of drozitumab as a single agent both *in vitro* and *in vivo* in animal models of osteolytic breast cancer.

2. To evaluate the efficacy of drozitumab in combination with chemotherapy against resistant breast cancer cells in animal models of orthotopic breast cancer.

3. To assess whether tumour-derived OPG can limit the anticancer efficacy of recombinant soluble Apo2L/TRAIL in the bone microenvironment, using animal models of osteolytic breast cancer.

4. To examine the effects of local production of OPG by breast cancer cells, as a way to disrupt the vicious cycle, in a mouse model of osteolytic breast cancer.

CHAPTER 2

APOMAB, A FULLY HUMAN AGONISTIC ANTIBODY TO DR5 EXHIBITS POTENT ANTITUMOUR ACTIVITY AGAINST PRIMARY AND METASTATIC BREAST CANCER

*Part of the work presented in this manuscript was conducted during my Honours Project, 2008-2009.

Apomab, a fully human agonistic antibody to DR5 exhibit potent antitumour activity against primary and metastatic breast cancer.

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Short running title: Apomab inhibits breast cancer development and progression

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Mol Cancer Ther. 2009 Oct;8(10):2969-80

STATEMENT OF AUTHORSHIP

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Mol Cancer Ther. 2009 Oct;8(10):2969-80

Irene Zinonos (Candidate)

Performed *in vitro* experiments and animal studies, BLI imaging and mCT imaging, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author

I hereby certify that the statement of contribution is accurate

Signed:…….....…….*Date:…01/06/2012*……

Agatha Labrinidis

Supervised development of work, transfection of breast cancer cells with triple reporter gene contract (SFG-NES-TGL), assistance with mCT analysis, data interpretation and manuscript evaluation

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

Signed:.....…….*Date:...23/05/2012*.....

STATEMENT OF AUTHORSHIP

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Mol Cancer Ther. 2009 Oct;8(10):2969-80

Michelle Lee

Assistance with animal handling

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Signed **…….....….***Date:…16/05/2012……*

Vasilios Liapis

Assistance with handling of animals and provided expert advice on western blotting and immunodetection

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

Signed:…….....…….Date:……01/06/2012..……

STATEMENT OF AUTHORSHIP

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Shelley Hay

Assistance with handling of animals, tissue excision and provided advice and support on histology

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Vladimir Ponomarev

Provided triple reporter gene construct (SFG-NES-TGL)

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STATEMENT OF AUTHORSHIP

Apomab, a fully human agonistic antibody to DR5 exhibit potent antitumour activity against primary and metastatic breast cancer.

Mol Cancer Ther. 2009 Oct;8(10):2969-80

Peter Diamond

Assistance with transfection of breast cancer cells with triple reporter gene contract (SFG-NES-TGL)

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Andrew C.W. Zannettino

Data interpretation and manuscript evaluation

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David M. Findlay

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Andreas Evdokiou

Principal supervisor, data interpretation and manuscript editing

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Faculty of 1000 Medicine Commentary

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Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer.
Zinonos I, Labrinidis A, Lee M, Liapis V, Hay S, Ponomarev V, Diamond P, Zannettino AC, Findlay DM, Evdokiou A
Mol Cancer Ther 2009 Oct **8**(10):2969-80 [abstract on PubMed]
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Evaluated 3 Nov 2009
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Faculty Comments & Author Responses

Faculty Member	Comments
Larry Suva University of Arkansas Medical Sciences, United States of America Rheumatology & Clinical Immunology New Finding Controversial	**Zinonos and co-workers describe the efficacy of a potent immunotherapeutic molecule in the experimental treatment of breast cancer and progression.** In developed countries, the diagnosis of a metastatic breast tumor mandates systemic treatment, along with local intervention targeting the primary tumor. Despite the progress resulting from early detection and improved adjuvant therapy, the prognosis of breast cancer patients is still limited by the occurrence of distant metastases, with bone metastasis especially common. The development of distant metastases is associated with eventual mortality from breast cancer, so therapies that inhibit the metastatic potential of advanced cancer would be a valuable addition to the oncologist's armamentarium. Apo2 ligand (Apo2L)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis selectively in a wide variety of cancer cells. Apo2L/TRAIL-induced apoptosis is mediated by interactions with specific death domain-containing cell surface receptors, death receptor 4 (DR4) and DR5, via caspase activation. Zinonos et al. tested the efficacy of a novel long-acting fully human agonist DR5 monoclonal antibody (Apomab) to inhibit breast cancer growing in the orthotopic site (i.e. the mammary gland) or in a distant metastatic site, namely bone. Apomab was capable of inhibiting not only breast cancer growth at the primary site but also growth in bone along with the associated bone destruction. It is perhaps not surprising that Apomab completely inhibited tumor growth in the early treatment protocol in the mammary gland, since subcutaneous efficacy has been demonstrated, but it is of particular significance that Apomab induced a striking and rapid inhibition in well-advanced mammary tumors in a treatment model. Perhaps most surprising was the apparent selectivity of Apomab for the inhibition of tumor cells, while enhancing healing of osteolytic lesions, even in mice bearing advanced and highly osteolytic tumors. This remarkable observation suggests that the therapeutic antibody may be able to treat cancers resident in bone and without any evidence of the detrimental side

effects of most treatments on normal bone remodeling. Whatever the efficacy of Apomab, it should be noted that these studies were performed in xenograft models using the well characterized MDA-MB-231-TXSA breast cancer cell line that was previously demonstrated to be highly sensitive to Apomab treatment. All xenograft studies using immunocompromised mice should be regarded as preliminary, and additional studies using syngeneic tumor models and genetic models of tumor progression and metastasis are clearly indicated and likely ongoing. In any case, the studies have identified a potent single-agent therapy with strong anti-tumor activity and positive bone effects that warrants further evaluation. The clinical utility of the antibody has the potential to impact the treatment of advanced breast cancer and should be evaluated in humans.

Competing Interests: No potential interests relevant to this article were reported.
Evaluated 3 Nov 2009

How to cite this evaluation

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Larry Suva: Faculty of 1000 Medicine, 3 Nov 2009 http://www.f1000medicine.com/article/id/1166534/evaluation

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Molecular Cancer Therapeutics, v. 8(10), pp. 2969-2980

CHAPTER 3

DOXORUBICIN OVERCOMES RESISTANCE TO DROZITUMAB BASED-IMMUNOTHERAPY IN A MOUSE MODEL OF BREAST CANCER

Doxorubicin overcomes resistance to Drozitumab based-immunotherapy in a mouse model of breast cancer.

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Short running title: Doxorubicin reverses resistance to drozitumab in breast cancer growth *in vivo*.

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Irene Zinonos (Candidate)

Performed *in vitro* experiments and animal studies, BLI imaging, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author

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Agatha Labrinidis

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Shelley Hay

Assistance with handling of animals, tissue excision and provided advice and support on histology

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Vasilios Panagopoulos

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Gerald Atkins

Data interpretation and manuscript evaluation

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David M. Findlay

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Abstract

Drozitumab is a fully human agonistic monoclonal antibody which binds selectively to Apo2L/TRAIL death receptor DR5 and triggers apoptosis through activation of the extrinsic apoptotic signalling pathway. We have shown previously that drozitumab induces apoptosis of breast cancer cells *in vitro* and reduces tumour growth *in vivo*. However, drozitumab resistance, like Apo2L/TRAIL resistance appears to be a major obstacle limiting anticancer efficacy. In this study, we examined the potential for the chemotherapeutic drug doxorubicin (DOX) to overcome resistance to drozitumab-induced apoptosis *in vitro* and to enhance anticancer efficacy in a xenograft model of breast cancer. We have developed MDA-MB-231-TXSA breast cancer cells which were completely resistant to apoptosis induction by drozitumab after prolonged exposure of parental sensitive cells to drozitumab. Notably, when compared to the parental sensitive cells the resistant cells, had reduced cell surface expression of DR5 and increased levels of cIAP. Treatment with DOX increased cell surface expression of DR5, reduced intracellular levels of cIAP and resensitised cells to drozitumab-induced apoptosis. Similarly, treatment with the cIAP antagonist, BV6 resensitised resistant cells to drozitumab-induced apoptosis identifying cIAP as a major determinant of drozitumab resistance in these cells. *In vivo*, all animals left untreated or treated with DOX or drozitumab alone developed large mammary tumours and were humanely killed at 4 weeks due to their high tumour load. In contrast, mice treated with a combination of drozitumab and DOX showed significant inhibition of tumour growth and increased survival. These results suggest that combination treatments of drozitumab and chemotherapy could provide a promising therapeutic approach for patients with breast cancer.

Introduction

Breast cancer is the most frequently diagnosed non-skin related type of malignancy and the second leading cause of cancer death among women, with a worldwide incidence of around 1 in 9 women (Jemal, Bray et al. 2011). Despite the improved diagnosis and treatment of primary breast cancer over the last 20 years, advanced disease remains a challenging condition to treat (Beaumont and Leadbeater 2011). Therefore, there is an urgent need to identify more effective treatments for breast cancer.

Apoptosis is thought to be one of the most suitable methods for anticancer therapy because it aims to specifically induce tumour cell death while limiting possible cytotoxic effects in healthy tissues. Drozitumab is a fully human agonistic monoclonal antibody that is designed to specifically bind to Apo2L/TRAIL death receptor DR5 and can engage apoptosis via the extrinsic apoptotic pathway (Adams, Totpal et al. 2008). Upon drozitumab binding, oligomerisation of the receptors occurs, which leads to the recruitment of the cytoplasmic adaptor protein Fas-associated death domain (FADD) that binds to the receptors by its corresponding death domain. Consequently, the apoptosis-initiating proteases, caspase-8 and/or caspase-10 bind to FADD and form a death-inducing signalling complex (DISC), which leads to the auto-activation of these initiator caspases. The activation of caspase-8 at the DISC is followed by the activation of the effector caspases, including caspase-3, caspase-6 and caspase-7. Eventually, the initiation of this intracellular caspase cascade leads to programmed cell death (Ashkenazi and Dixit 1998; Bouralexis, Findlay et al. 2005). Drozitumab can also activate the intrinsic apoptosis pathway *via* caspase-8 mediated cleavage of the pro-apoptotic Bcl-2 family member Bid, which eventually amplifies apoptosis as well (Li, Zhu et al. 1998; Esposti 2002).

Previous preclinical studies have demonstrated that drozitumab can induce apoptosis in various human cancer cell lines while sparing normal hepatocytes (Adams, Totpal et al. 2008; Jin, Yang et al. 2008; Kang, Lee et al. 2009). *In vivo*, drozitumab demonstrated tumour suppressive activity in various xenograft models of cancer, including colorectal cancer, NSCLC, pancreatic cancer and lung (Adams, Totpal et al. 2008; Jin, Yang et al. 2008). Data from phase I studies examining the safety, pharmacokinetic profile and antitumour efficacy in a cohort of patients with solid and haematological malignancies has shown that drozitumab is well-tolerated, and capable of inducing prolonged stable disease (Camidge, Herbst et al. 2010). Phase II clinical trials evaluating the efficacy of drozitumab as a single agent, and in combination with chemotherapy, in a variety of malignancies are ongoing. We have previously assessed the cytotoxic effect of drozitumab treatment against breast cancer cells *in vitro* and evaluated its antitumour activity in murine models of breast cancer development and progression in both the orthotopic mammary tissue and in bone (Zinonos, Labrinidis et al. 2009). *In vitro*, drozitumab induced apoptosis in a panel of breast cancer cell lines but was without effect on normal human primary osteoblasts, fibroblasts or mammary epithelial cells. *In vivo*, drozitumab exerted remarkable tumour suppressive activity as a single agent, leading to complete regression of well-advanced tumours within the mammary tissue, with the animals showing no evidence of recurrence. Importantly, drozitumab reduced tumour burden within the bone marrow cavity and protected the bone from breast cancer-induced osteolysis (Zinonos, Labrinidis et al. 2009).

Despite its potent apoptotic activity, some cancer cells remain resistant to drozitumab-induced apoptosis. The mechanisms behind this resistance are poorly understood but may involve reduced cell surface expression of DR5, high expression of FLIP which inhibits DISC assembly upon antibody binding, increased levels of IAP proteins that antagonise caspase activity and other determinants involved in apoptotic signalling. In addition to resistance

being an innate multi-factorial process, it could also be acquired which occurs due to adaptive changes in response to therapy (Li, Wang et al. 2006).

Previous studies have demonstrated that Apo2L/TRAIL or drozitumab acquired resistance can be reversed by combination with chemotherapeutic agents (Munshi, McDonnell et al. 2002; Lin, Zhang et al. 2003; Singh, Shankar et al. 2003; Jin, Yang et al. 2004; Jin, Yang et al. 2008; Lagadec, Adriaenssens et al. 2008; Moreno-Aspitia and Perez 2009; Wang, Ren et al. 2010; Morizot, Merino et al. 2011) Anthracyclines such as doxorubicin (DOX) are commonly used chemotherapeutics for treating breast cancer, especially in the adjuvant setting. In this study we developed a drozitumab-resistant subline of MDA-MB-231-TXSA cells, which are normally extremely sensitive to drozitumab-induced apoptosis, by prolonged exposure of these cells to drozitumab. We investigated the potential of DOX to overcome resistance to drozitumab-induced apoptosis *in vitro* and for co-operative anticancer efficacy against mammary tumours in mice. Our *in vitro* results demonstrated that DOX resensitised resistant cells to drozitumab-induced apoptosis and identified cIAP as a major determinant of drozitumab's resistance. *In vivo*, DOX cooperated with drozitumab to inhibit mammary tumour growth and to increase survival suggesting that this combination could provide a promising therapeutic approach for breast cancer patients.

Materials and Methods

Cell lines and tissue culture

The MDA-MB-231 derivative cell line, MDA-MB-231-TXSA was kindly provided by Dr Toshiyuki Yoneda (University of Texas Health Sciences Centre, San Antonio, Texas). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2 mM glutamine, 100 IU/ml penicillin, 160 μg/ml gentamicin, Hepes (20mM) and 10% fetal bovine serum (Biosciences, Sydney, Australia) in a 5% CO_2-containing humidified atmosphere.

Reagents

Drozitumab and BV6-pan-IAP inhibitor were a kind gift from Genentech, Inc (South San Francisco, CA). Affinity Pure Goat Anti-Human IgG Fc_γ Fragment was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). The Caspase Inhibitor-1, zVAD-fmk, was obtained from Calbiochem (Inc. La Jolla, CA, USA). Doxorubicin (DOX) was purchased from Ebewe Pharma (A-4866 Unterach, Austria).

Generation of drozitumab-resistant MDA-MB-231-TXSA-TGL cells *in vitro*

MDA-MB-231-TXSA-TGL cells were seeded in a T75 flask and grown until 75% confluency was reached. Cells were exposed to drozitumab at 100 ng/ml + anti human–Fc IgG for 8-10 weeks. During the selection period, cell debris was removed every three days and cells were incubated with fresh media containing drozitumab. By week 8 in the continual presence of drozitumab, clones that tolerated drozitumab treatment emerged. Thereafter, the

newly generated cell line, denoted MDA-MB-231-TXSA-droz-R, was cultured in drozitumab-free media until used for further experiments.

Cell viability assays

To determine the cytotoxic effects of drozitumab on cell growth, 1 x 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 drozitumab alone (0-1000 ng/ml) or with increasing concentrations of DOX (0-6.25 μM) or BV6 (0-0.5 μM) in combination with drozitumab at 100 ng/ml for 24 hours. Prior to treatment, drozitumab was cross-linked with an anti-human IgG Fc_γ for 30 minutes at 4°C (for all the *in vitro* experiments, drozitumab was cross-linked with anti-human IgG Fc_γ before use). Cell viability was assessed using the Cell Titer Blue Cell Viability Assay (Promega, Madison, WI, USA) as well as 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.

Flow cytometry analysis (FACs) for cell surface expression of DR4 and DR5

MDA-MB-231-TXSA and MDA-MB-231-TXSA-droz-R cells were seeded in T75 flasks and either left untreated or treated with DOX at 5μM for 24 hours. Cells were then washed with phosphate buffered saline (PBS) and detached using 2mM EDTA in PBS at 37°C for 5 min. For flow cytometric analysis, all subsequent incubation steps were performed on ice and centrifugation steps performed at 4°C (to achieve ultimate receptor binding). For analysis of Apo2L/TRAIL receptors on the cell surface, cells were washed twice in protein-free clear PBS. Cells were resuspended in wash buffer (1%BSA + 0.1% sodium azide in PBS)

at 1 x 10^6 cells/ml and washed by centrifugation. Cells were then resuspended in 50 µl blocking buffer (5% normal goat serum, 1% BSA in PBS + 0.1% sodium azide). Monoclonal antibodies against TRAIL-R1 (DR4) were purchased by R&D Systems whereas monoclonal antibodies against TRAIL-R2 (DR5) were purchased by Immunex. Monoclonal antibodies were diluted in blocking buffer at 20 µg/ml and added (150µl) to 50 µl aliquots of cell suspension and incubated for 45 minutes. Cells were then washed three times with 2 ml of wash buffer and collected by centrifugation. A PE-conjugated goat anti-mouse secondary antibody (50 µl) was added (aIgG-PE; Southern Biotech) to the resuspended cell pellets, diluted 1:50 in wash buffer and the cells were incubated for a further 45 minutes in the dark. After the last incubation step, cells were washed three times as above, then resuspended and fixed in fluorescent-activated cell sorting fix (FACs fix) solution (10 mL of 37% formalin stock, 20 g glucose, 2 mL 10% sodium azide stock, 988 µL PBS). Tubes were placed in 4°C until analysis**.**

Western blot analysis

MDA-MB-231-TXSA-droz-R cells were seeded in T25 flasks (2 x 10^6 cells/flask), treated as indicated (drozitumab 100 ng/ml, DOX 5 µM) and incubated at 5% CO_2–containing humidified atmosphere at 37ºC overnight. Cells were then lysed in buffer containing 10 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 2 mM sodium vanadate and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and stored at -70 ^oC until ready to use. Anti-caspase-8 and polyclonal antibodies (pAb) anti-caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA), mAb anti-caspase-10 from MBL (Naka-ku Nagoya, Japan) mAb anti-caspase-3 from Transduction Laboratories (Lexington, KY), pAb anti-Bid from Chemicon International (Temecula, CA, USA), mAb anti-Bcl-2 and anti-Bax from Santa Cruz (Santa Cruz,

California, USA), anti-cIAP1, anti-cIAP2 and anti-XIAP from R&D systems and pAb anti-PARP from Roche Diagnostics (Mannheim, Germany). Anti-actin mAb (SIGMA, Saint Louis, Missouri, USA) was used as a loading control. Membranes were then rinsed several times with PBS containing 0.1% Tween-20 and incubated with 1:5,000 dilution of anti-mouse, anti-goat or anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Pierce, Rockford, Illinois, USA) for 1 hour. Visualization and quantification of protein bands was performed using the ECF substrate reagent kit (GE Healthcare, Buckinghamshire, UK) on a FluorImager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Apoptosis analysis

4,6-Diamidine-2-phenylindole staining of nuclei (DAPI stain). Cells were seeded on plastic chamber slides at 1 x 10^4/well and either left untreated or treated with drozitumab alone at a 100 ng/ml, DOX alone at 5 μM or with the combination of drozitumab and DOX. Cells were fixed in ethanol: acetic acid (6:1) for 10 minutes, washed twice with PBS, and incubated with 0.8 mg/mL 4,6-diamidine-2-phenylindole (Roche Diagnostics GmbH, 68298 Mannheim, Germany) in methanol for 5 minutes at room temperature. After several washes in PBS, the coverslips were mounted with Prolong Gold Anti-fade reagent with DAPI (Life Technologies, Grand Island, NY, USA). 4,6-Diamidine-2-phenylindole 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 x 10^4/well) grown in 24-well plates were treated as indicated, washed once with PBS, and resuspended in 30 μl gepal lysis buffer containing 5 mmol/L Tris-HCl, 5 mmol/L EDTA, and 10% gepal (pH 7.5). Cell lysate (15-20

μg of protein) was added to each assay tube containing 8 μmol/L substrate in 1 mL flurometric protease buffer [50 mmol/L HEPES, 10% sucrose, 10 mmol/L DTT, 0.1% CHAPS (pH 7.4)]. After 4-5 hours at room temperature, fluorescence was quantified (Ex 400 and Em 505) in a Perkin-Elmer LS50 fluorescence spectrometer.

Animals

Female athymic nude mice at 8 weeks old (Institute of Medical and Veterinary Services Division, Gilles Plains, SA, Australia) were acclimatised 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 throughout 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-droz-R human breast cancer cells were cultured as described above until they reached 70-80% confluency. Adherent cells were removed from flasks with 2mM EDTA and resuspended in 1 x PBS at 0.5 x 10^6 cells/10μl and kept on ice in an eppendorf tube. An equal volume of Matrigel™-HC (BD Biosciences, Bedford, MA, USA) was added to the cells and resuspended. Mice were anaesthetised 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, a 25G needle was

inserted and 20 µl of cells were injected into the mammary fat pad. Mice were allowed to recover under the heat lamp before being transferred into cages.

In vivo bioluminescent imaging (BLI)

Non-invasive, whole body imaging to monitor luciferase-expressing MDA-MB-231-TXSA-droz-R cells in mice was performed weekly using the IVIS 100 Imaging system (Xenogen, Alameda, CA). Mice were injected intraperitoneally (i.p.) with 100 µl of the D-Luciferin solution at final dose of 3 mg/20 g mouse body weight (Xenogen Alameda, CA) and then gas-anaesthetized with Isoflurane (Faulding Pharmaceuticals, Salisbury, SA, Australia). Images were acquired for 0.5-30 seconds (images are shown at 1 second) from the front angle 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.

Data analysis and statistics

Experiments were performed in triplicate, and data presented as mean ± SE. All statistical analysis was performed using GraphPad Prism (San Diego, CA, USA) using the unpaired students't-test. Comparisons between groups were assessed using a one-way ANOVA test. In all cases, $p < 0.05$ was considered statistically significant.

Results

Generation of drozitumab-resistant MDA-MB-231-TXSA breast cancer cells

We have previously shown that the MDA-MB-231-TXSA breast cancer cells are extremely sensitive to drozitumab treatment (Zinonos, Labrinidis et al. 2009). The purpose of this study was to investigate the possibility of selecting for drozitumab-resistant clones from this highly sensitive parental cell line with continuous and prolonged *in vitro* exposure to drozitumab. The highly sensitive MDA-MB-231-TXSA breast cancer line was cultured in the presence of drozitumab at 100 ng/ml for at least 8 weeks, as described in the materials and methods. Fc crosslinked drozitumab induced a dose dependent increase in apoptosis in the parental sensitive MDA-MB-231-TXSA cell line after 24 hours treatment, reaching a maximum of 99% cell death at a dose of 250 ng/ml of drozitumab (Fig 1A). In contrast, the newly generated cell line, denoted MDA-MB-231-TXSA-droz-R, was completely refractory to the apoptotic effects of the antibody and showed no detectable drozitumab-induced apoptosis, even at the highest dose of 1 mg/ml (data not shown). In addition, drozitumab treatment induced a dose-dependent activation of caspase-3 in the sensitive MDA-MB-231-TXSA cells; whereas the resistant cell line demonstrated no evidence of caspase-3 activation when compared to the parental cell line (Fig 1B). Morphological changes characteristic of apoptosis, including chromatin condensation and DNA fragmentation were clearly evident following treatment with drozitumab only in the parental cells but not in the resistant subline (Fig 1C).

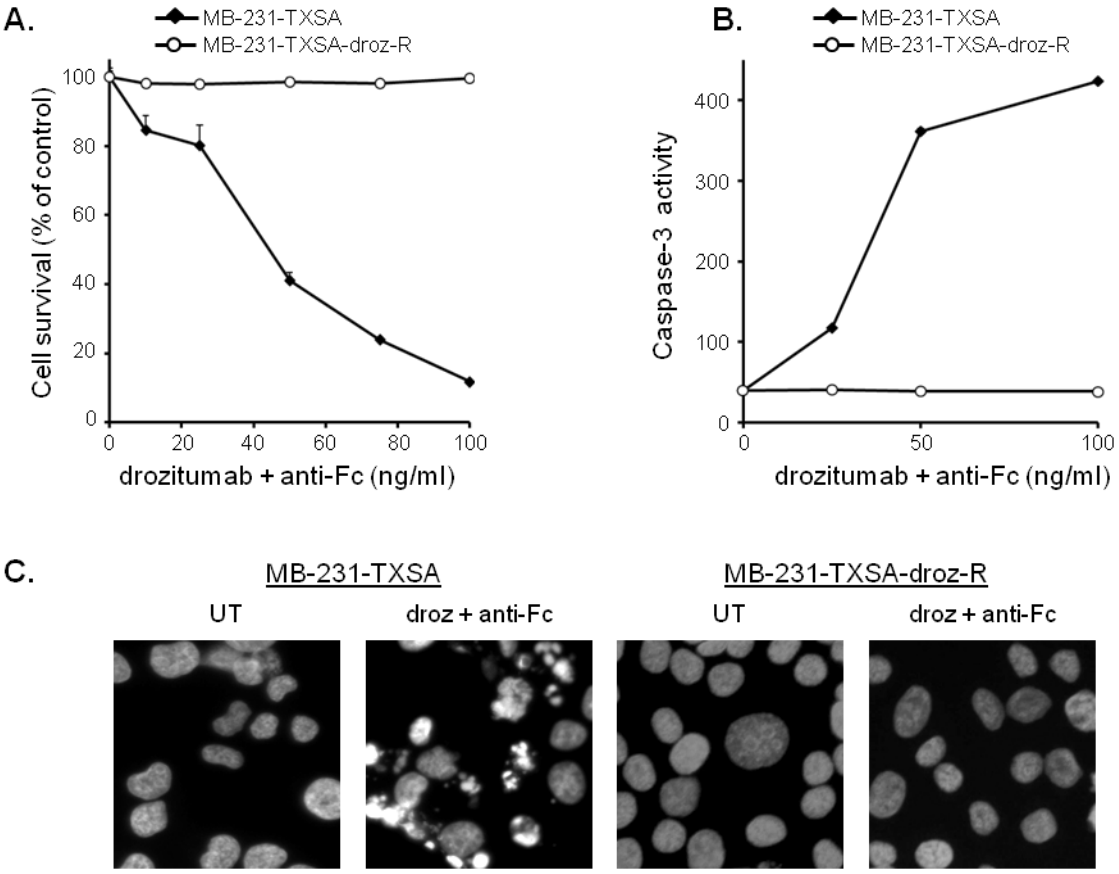
To investigate the reason for the development of drozitumab resistance *in vitro*, we compared the cell surface expression profile of Apo2L/TRAIL death receptors between sensitive and resistant cells using flow cytometry. The results demonstrated that the parental

Figure 1. ***Development of drozitumab-resistant MDA-MB-231-TXSA breast cancer cells with prolonged treatment in vitro.***

A. Drozitumab-resistant cells were generated *in vitro* by culturing the parental MDA-MB-231-TXSA breast cancer cells continuously in medium containing drozitumab for 8 weeks as described in the "Materials and Methods" section. The resistant cells (denoted MDA-MB-231-TXSA-droz-R) and the parental cells (MDA-MB-231-TXSA) were seeded in 96-well plates at 1 x 10^4 cells / well and treated with increasing doses of drozitumab plus anti-human IgG Fc, as indicated. Cell viability was assessed by Titer Blue assay or crystal violet staining, 24 hours after treatment. MDA-MB-231-TXSA-droz-R were refractory to drozitumab-induced apoptosis showing 90% viability even at the highest dose of 1 μg/mL when compared to the parental sensitive cell line. Data are presented as the mean ±SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as a percentage of the number of control cells.

B. MDA-MB-231-TXSA-droz-R and MDA-MB-231-TXSA cells were treated with increasing doses of drozitumab, as indicated. Cell lysates were collected at 24 hours after treatment and used to determine caspase-3-like activity, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC, as described in the Methods. MDA-MB-231-TXSA-droz-R showed lack of drozitumab-induced caspase-3 activity compared to the parental cells.

C. MDA-MB-231-TXSA-droz-R and MDA-MB-231-TXSA cells were seeded on plastic chamber slides at 1 x 10^4 cells per chamber and were treated for 24 hours with drozitumab at 100 ng/mL plus anti Fc. Cells were fixed with ethanol: acetic acid and incubated with DAPI, before washing in PBS and mounting on Prolong Gold Anti-fade reagent with DAPI. DAPI staining was visualised by fluorescence microscopy.


A.
MB-231-TXSA
MB-231-TXSA-droz-R
Cell survival (% of control)
0
20
40
60
80
100
0
20
40
60
80
100
drozitumab + anti-Fc (ng/ml)
B.
MB-231-TXSA
MB-231-TXSA-droz-R
Caspase-3 activity
0
100
200
300
400
0
50
100
drozitumab + anti-Fc (ng/ml)
C.
MB-231-TXSA
MB-231-TXSA-droz-R
UT
droz + anti-Fc
UT
droz + anti-Fc

(sensitive) MDA-MB-231-TXSA cells express high levels of cell surface DR4 and DR5. While cell surface expression of DR5 appears to have decreased somewhat in MDA-MB-231-TXSA-droz-R cells, when compared to the parental cell line, nonetheless these levels remain high and therefore unlikely to have contributed to drozitumab resistance (Fig 2A). Similarly, western blot analysis demonstrated no significant difference in the basal levels of several pro- and anti-apoptotic proteins involved in the extrinsic and the intrinsic apoptotic pathways between the resistant and sensitive cells (Fig 2B). Notably, however, there was a pronounced increase in the levels of cIAP2 protein in the drozitumab resistant cells when compared to the sensitive parental cells, suggesting that IAP proteins may be involved in determining resistance of these cells to drozitumab.

Combination of drozitumab and DOX against MDA-MB-231-TXSA-droz-R cells *in vitro*

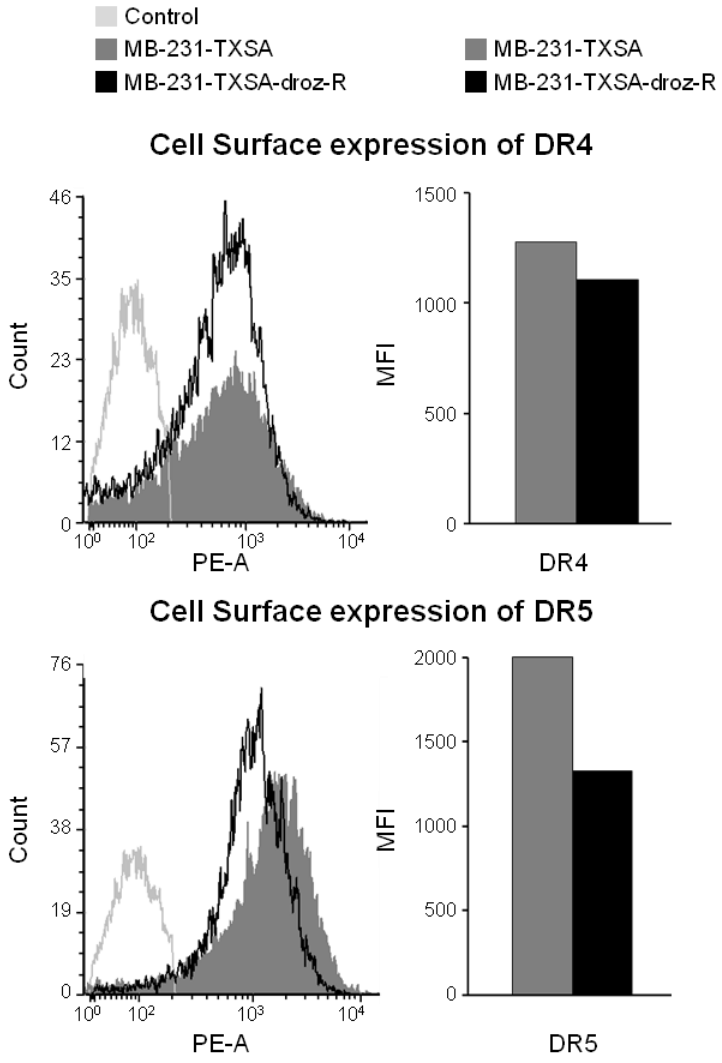
Our laboratory and others have previously shown that certain conventional chemotherapeutic agents in combination with pro-apoptotic receptor agonists, including Apo2L/TRAIL and agonistic antibodies to DR4 and DR5, cooperate synergistically for apoptosis induction *in vitro* and potentiate anticancer efficacy in various xenograft cancer models (Nimmanapalli, Perkins et al. 2001; Singh, Shankar et al. 2003; Jin, Yang et al. 2004; Jin, Yang et al. 2008; Wang, Ren et al. 2010). To determine whether the MDA-MB-231-TXSA-droz-R cells could be resensitised to drozitumab induced apoptosis, we used the chemotherapeutic agent doxorubicin (DOX) which is commonly used for the treatment of patients with breast cancer. Dose escalation treatment of MDA-MB-231-TXSA-droz-R cells with DOX showed a maximum 30% cell death only at the highest dose of 6.25 μM, demonstrating that these cells are relatively resistant to DOX alone. However, combination of

Figure 2. ***Comparison of cell surface expression of death receptors levels do not explain the resistance of MB-231-TXSA-droz-R cells to drozitumab.***

A. Flow cytometry analysis of cell surface expression of Apo2L/TRAIL death receptors in drozitumab-sensitive MDA-MB-231-TXSA cells versus resistant MDA-MB-231-droz-R cells. Graphs were obtained after staining with anti-human DR4 and DR5 mAbs as described in "Materials and Methods" section.

B. MDA-MB-231-TXSA cells and MDA-MB-231-TXSA-droz-R cells were seeded in T25 flasks (2 x 10^6 cells/flask) and left to attached overnight. Cells were then lysed and total cell lysates were analysed by PAGE and transferred to PVDF membranes for immunodetection. There was no detectable difference in the basal levels of anti- and pro-apoptotic proteins between the two cell lines, whereas cIAP2 levels were extremely high in the drozitumab resistant cells compared to the sensitive parental cell line.

A.


Control
MB-231-TXSA
MB-231-TXSA-droz-R
MB-231-TXSA
MB-231-TXSA-droz-R
Cell Surface expression of DR4
Count
PE-A
MFI
DR4
Cell Surface expression of DR5
Count
PE-A
MFI
DR5

B.


MB-231-TXSA
MB-231-TXSA-droz-R
Caspase 8
Caspase 10
Bid
Caspase 9
Caspase 3
Bcl-2
Bax
cIAP1
cIAP2
XIAP
Actin

DOX with drozitumab at 100 ng/ml induced a dose dependent increase in apoptosis showing a 30% cell death only at a dose of 2 μM and reaching 70% death at the maximum dose of 6.25 μM (Fig 3A). Furthermore, the loss in cell viability with the combination treatment of DOX and drozitumab was well-correlated with a significant increase in caspase-3 activity whereas DOX treatment alone had no effect on caspase-3 activation even at the highest dose (Fig 3B). In addition, DAPI staining of drozitumab-resistant cells, treated with DOX and drozitumab in combination, demonstrated all the characteristics of apoptosis (cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation), confirming that the combination of DOX and drozitumab has a more pronounced synergistic effect on these resistant cells, when compared to the effect of each agent alone (Fig 3C).

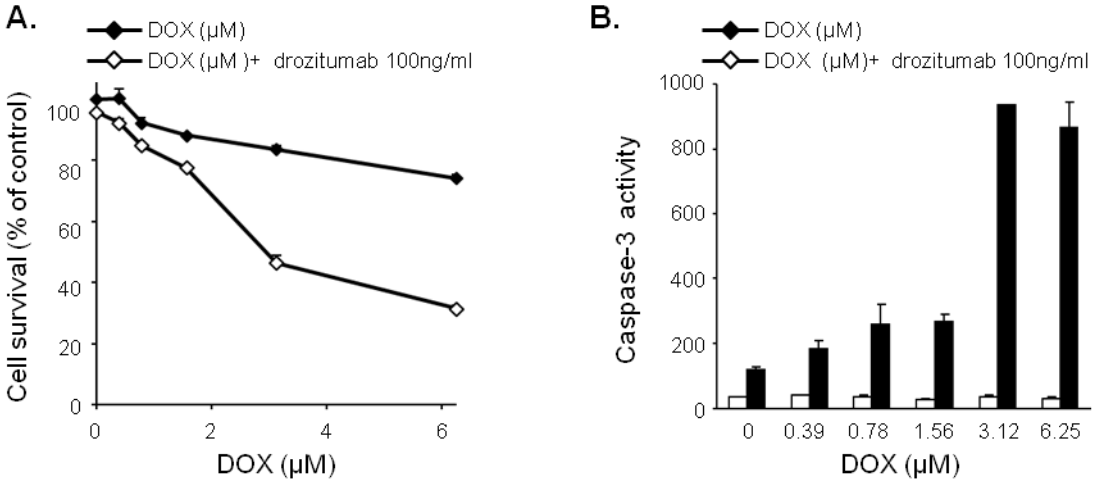
While the basal cell surface expression of DR4 and DR5 was intrinsically high in the resistant cells, treatment with DOX led to a substantial increase in cell surface expression of both DR4 and DR5 likely accounting for the increased sensitivity of the resistance cells to drozitumab when combined with DOX treatment. Western blot analysis performed on cell lysates treated with DOX and drozitumab alone or in combination, has shown that reversal of resistance by combination treatment was associated with processing and activation of the initiator caspase-8, leading to cleavage pro-caspase-3, which was concomitant with cleavage of the apoptosis target protein poly(ADP-ribose) polymerase (Fig 4B). Engagement of the intrinsic apoptotic signalling pathway was also demonstrated since apoptosis induction by the combination of DOX and drozitumab was associated with cleavage of the Bcl-2 protein family member Bid and enhanced processing and activation of caspase-9. Interestingly, cIAP1 and cIAP2 but not XIAP levels were dramatically decreased by DOX treatment alone and by the combination with drozitumab, suggesting that IAPs may play a significant role in inhibiting drozitumab-induced apoptosis in these resistant cells, an effect which could be reversed following DOX treatment.

Figure 3. ***Interaction of drozitumab and DOX in apoptosis induction of resistant cells in-vitro.***

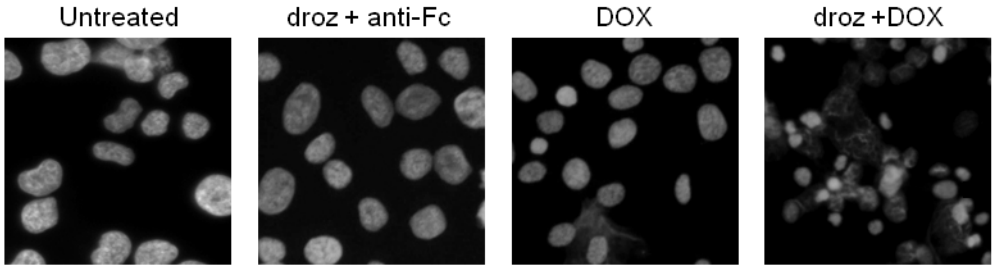
A. MB-231-TXSA-droz-R cells were in 96 well plates at 1 x 10^4 cells/well and treated with increasing concentrations of DOX (0–6.25 μM) in combination with drozitumab plus anti-human IgG Fc at 100 ng/ml. Cell viability was assessed by Titer Blue assay or crystal violet staining, 24 hours after treatment. DOX alone resulted in a 30% cell death only at the highest dose of 6.25 μM whereas the combined treatment with drozitumab led to a significant loss in cell viability.

B. MDA-MB-231-TXSA-droz-R cells were treated as above and cell lysates were used to determine caspase-3-like activity using the caspase-3 specific fluorogenic substrate, zDEVD-AFC, as described in Materials and Methods. The combined treatment resulted in a dramatic increase in caspase-3 activation. Data are presented as the mean ± SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as percentage of the number of control cells.

C. MDA-MB-231-TXSA-droz-R cells were seeded on plastic chamber slides at 1 x 10^4 cells per chamber and were treated for 24 hours either with DOX at 10 μM or drozitumab plus anti Fc at 100 ng/ml alone or in combination. Cells were fixed with ethanol: acetic acid and incubated with DAPI, before washing in PBS and mounting on Prolong Gold Anti-fade reagent with DAPI. DAPI staining was visualised by fluorescence microscopy.


A.
DOX (μM)
DOX (μM)+ drozitumab 100ng/ml
Cell survival (% of control)
100
80
60
40
20
0
0
2
4
6
DOX (μM)
B.
DOX (μM)
DOX (μM)+ drozitumab 100ng/ml
Caspase-3 activity
1000
800
600
400
200
0
0
0.39
0.78
1.56
3.12
6.25
DOX (μM)

C.


Untreated
droz + anti-Fc
DOX
droz +DOX

To assess the role of IAP proteins in inhibiting drozitumab-induced apoptosis in the MDA-MB-231-TXSA-droz-R cells, we investigated the potential of a small-molecule IAP antagonist, BV6, to overcome resistance to drozitumab-induced apoptosis (Varfolomeev, Alicke et al. 2009). Cells were treated with increasing concentrations of BV6 alone or in combination with drozitumab at 100 ng/ml and cell viability was assessed 48 hrs after treatment. Drozitumab alone as expected had no effect on cell viability and BV6 alone had minor effect on MDA-MB-231-TXSA-droz-R cells, showing only a 9% cell death at the higher dose after 48 hours (Fig 4C). In contrast, co-administration of drozitumab and BV6 resulted in a synergistic increase in cell death, reaching 48% at the higher dose, when compared to when drugs were used alone. These data provide supporting evidence of the potential significant role of IAP proteins in the apoptotic signalling pathway, particularly in drozitumab-induced apoptosis.

Anticancer efficacy of drozitumab and DOX on the growth of mammary xenografts

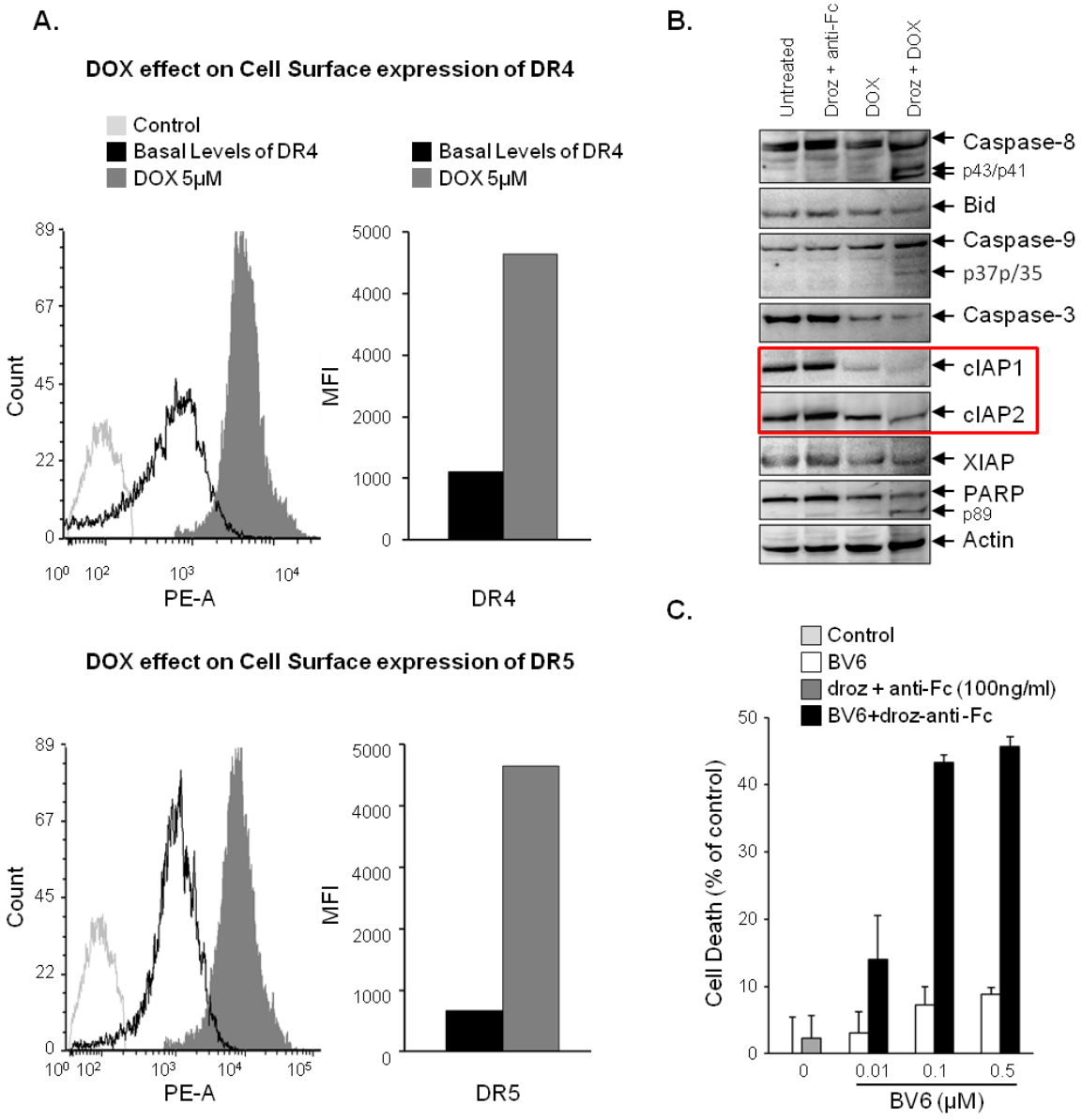
To examine if the synergistic activity of drozitumab and DOX seen *in vitro* could be translated into *in vivo* efficacy, we assessed the anticancer efficacy of each drug when administered alone and in combination against mammary tumours developing after direct transplantation of MDA-MB-231-TXSA-TGL-droz-R cells into the mammary fat pad of athymic female nude mice. For non-invasive bioluminescence imaging (BLI) of tumour growth, the breast cancer cells were retrovirally infected with a triple-fusion protein reporter construct encoding herpes simplex virus thymidine kinase (TK), green fluorescent protein (GFP) and firefly luciferase (Luc) (Ponomarev, Doubrovin et al. 2004; Zinonos, Labrinidis et al. 2009). After infection, MDA-MB-231-TXSA-droz-R cells were enriched for high level expression of GFP by two rounds of fluorescence-activated cell sorting.

Figure 4. ***DOX treatment upregulates DR4 and DR5 and decreases IAP levels on MB-231-TXSA-droz-R cells.***

A. Flow cytometry analysis of cell surface expression of Apo2L/TRAIL death receptors in MDA-MB-231-droz-R cells 24 hours after DOX treatment at 5 μM. Graphs were obtained after staining with anti human TRAIL-R1 (DR4) and TRAIL-R2 (DR5) mAbs as described in "Materials and Methods". DOX treatment dramatically increased the expression of DR4 and DR5 on the surface of the MDA-MB-231-droz-R cells.

B. MB231-TXSA-droz-R cells were seeded at 2 x 10^6 per T25 flask and were either left untreated or treated with drozitumab alone at a concentration of 100 ng/ml, or DOX alone at 5 μM or with the combination of drozitumab and DOX. Cells were then lysed and protein was isolated at 24 hours after treatment. Cell lysates were analysed by polyacrylamide gel electrophoresis and transferred to PVDF membranes for immunodetection as described in the "Materials and Methods". The caspase-8, caspase-9 and poly (ADP-ribose) polymerase antibodies detect both full-length and processed forms of the antigen, whereas caspase-3 and Bid antibodies detect only the full-length antigens. The combination treatment led to activation of caspases -8, -3, -9 and also the apoptotic proteins BID and PARP. In addition there was dramatic processing of the IAP proteins, cIAP1, and cIAP2 following combination treatment of drozitumab and DOX.

C. MB-231-TXSA-droz-R cells were seeded in 96 well plates at 1 x 10^4 cells/well and treated with increasing doses of BV6 (0-0.5 μM) alone or in combination with drozitumab plus anti-human IgG Fc, at 100 ng/ml. Cell viability was assessed by Titer Blue assay or crystal violet staining, 48 hours after treatment. BV6 alone had no effect on drozitumab-resistant MB-231-TXSA cells, whereas combination treatment of BV6 and drozitumab, led to a significant increase of cell death in these cells. Data are presented as the mean ± SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as percentage of the number of control cells.


A.
DOX effect on Cell Surface expression of DR4
Control
Basal Levels of DR4
DOX 5μM
Basal Levels of DR4
DOX 5μM
Count
PE-A
MFI
DR4
DOX effect on Cell Surface expression of DR5
Count
PE-A
MFI
DR5
B.
Untreated
Droz + anti-Fc
DOX
Droz + DOX
Caspase-8
p43/p41
Bid
Caspase-9
p37p/35
Caspase-3
cIAP1
cIAP2
XIAP
PARP
p89
Actin
C.
Control
BV6
droz + anti-Fc (100ng/ml)
BV6+droz-anti-Fc
Cell Death (% of control)
BV6 (μM)

The sub-line designated MDA-MB-231-TXSA-droz-R-TGL exhibited a 1000-fold induction of luciferase activity when analysed *in vitro*. When compared to non-infected cells, MDA-MB-231-TXSA-droz-R-TGL cells were equally resistant to drozitumab, with both lines showing low sensitivity to the apoptotic effects of the antibody and with similar kinetics of caspase-3 activity (data not shown). Following cancer cell transplantation tumours were allowed to establish in the mammary gland of the mice for two weeks before treatment was initiated. At day 14 mice were randomized into 4 groups, and treated with vehicle, drozitumab, DOX or the combination of drozitumab and DOX.

Tumours in the vehicle-treated group showed an exponential increase of mean photon emission associated with a rapid increase in tumour burden, which was clearly evident from day 14 onwards and continue to increase rapidly until day 28 at which point mice were humanely killed due to the high tumour load (Fig 5A). Animals treated with drozitumab alone or DOX alone showed a small delay in tumour progression until day 21 with tumours rapidly expanding thereafter until animals were humanely killed on day 35. In contrast combined treatment with drozitumab and DOX caused a slight regression of tumour growth and a substantial delay in tumour progression which translated to a significant increase in survival. Taken together these data suggest that DOX overcomes drozitumab-mediated apoptosis and toxicity against resistant human breast tumours and resensitise these cells to drozitumab both *in vitro* and *in vivo*.

Figure 5. ***Antitumour activity of Drozitumab in combination with DOX against resistant orthotopic mammary tumours in vivo.***

Eight-week-old female nude mice were injected with 0.5×10^6 MDA-MB-231-TXSA-drozR-TGL cells into the mammary fat pad, as described in the "Materials and Methods". Two weeks after cancer cell transplantation, mice were randomised into 6 groups of 5 mice per group and received the following treatments:

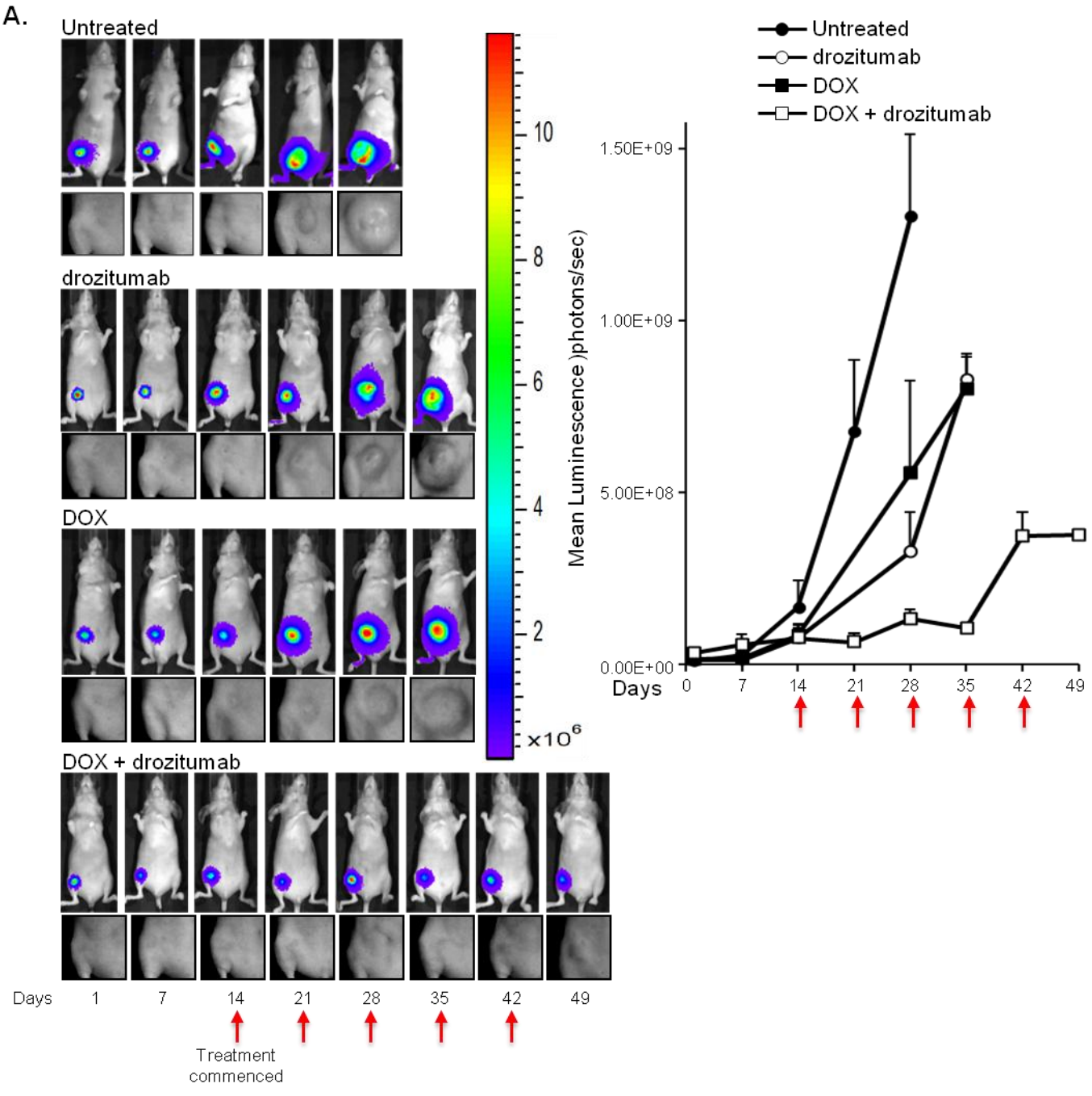
a) Group 1: Untreated

b) Group 2: Single i.p. injection of drozitumab at 3mg/kg (in clear PBS) once weekly

c) Group 3: Single intravenously (i.v.) injection of DOX at 4mg/kg (in saline) once weekly

d) Group 4: DOX and drozitumab treatments as described above, with a four hour gap between the two injections.

A. Mice were imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. Representative whole body BLI and photographs of the mammary tumours of a single animal from each group of mice during the course of the experiments are shown. All vehicle-treated animals were humanely killed on day 28 due to high tumour load. Animals received treatment with a single agent also showed an increase in tumour growth and were humanely killed at day 35. In contrast, all mice receiving a combination treatment with drozitumab and DOX showed delayed or even inhibition of tumour growth which was also concomitant with prolonged survival of the mice until the end of the experiment.


A.
Untreated
drozitumab
DOX
DOX + drozitumab
10
8
6
4
2
×10⁶
Days 1 7 14 21 28 35 42 49
Treatment commenced
Untreated
drozitumab
DOX
DOX + drozitumab
Mean Luminescence)photons/sec)
1.50E+09
1.00E+09
5.00E+08
0.00E+00
Days
0 7 14 21 28 35 42 49

Discussion

Previous preclinical studies have demonstrated that drozitumab is a promising anticancer treatment for solid and haematological malignancies. *In vitro,* drozitumab can induce apoptosis in various human cancer cell lines while sparing normal cells (Adams, Totpal et al. 2008; Zinonos, Labrinidis et al. 2009). *In vivo,* drozitumab has shown potent antitumour activity as a single agent in various xenograft animal models of human cancer, including colorectal (Adams, Totpal et al. 2008), lung (Jin, Yang et al. 2008) and breast cancer (Zinonos, Labrinidis et al. 2009). In addition, the anticancer efficacy of drozitumab was evaluated in combination with chemotherapy (irinotecan and gemcitabine) in murine models of colorectal and pancreatic cancer and showed greater tumour suppressive activity than each agent alone (Adams, Totpal et al. 2008). Data from phase I studies evaluating the safety, pharmacokinetic profile and antitumour efficacy of drozitumab in a cohort of patients with solid and hematological malignancies demonstrated drozitumab to be well-tolerated, and capable of producing prolonged stable disease in patients with advanced cancer (Camidge, Herbst et al. 2010). Phase II clinical trials are ongoing to evaluate drozitumab as a single agent, and in combination with chemotherapy, in a variety of malignancies. We have previously evaluated the antitumour activity of drozitumab in murine models of breast cancer development and progression in both the orthotopic mammary tissue and in bone. Drozitumab exerted remarkable tumour suppressive activity as a single agent leading to complete regression of well-advanced tumours within the mammary tissue, with the animals showing no evidence of recurrence. Similarly, drozitumab reduced tumour burden within the bone marrow cavity and protected the bone from breast cancer-induced osteolysis thus highlighting the need to clinically evaluate drozitumab in patients with primary and metastatic breast cancer.

Unfortunately, drozitumab resistance either intrinsic or acquired, like Apo2L/TRAIL resistance, is a major obstacle limiting anticancer efficacy. We have previously shown that

not all breast cancer cell lines are sensitive to drozitumab-induced apoptosis and have now shown that prolonged exposure of sensitive breast cancer cells with drozitumab selects for a population of cells which is refractory to apoptosis induction by drozitumab. Such resistance resembles drug resistance that develop in cancer patients following prolonged treatments with certain anticancer agents (Djeu and Wei 2009; Marquette and Nabell 2012). Our aim was to evaluate the potential for the chemotherapeutic drug DOX to overcome resistance to drozitumab-based immunotherapy *in vitro* and to evaluate the anticancer efficacy of this combination approach *in vivo* using a xenograft model of breast cancer.

The breast cancer cell line MDA-MB-231-TXSA is extremely sensitive to drozitumab-induced apoptosis. However, we were able to generate a drozitumab-resistant subline with prolonged exposure of these cells with drozitumab for 8-10 weeks. These cells, denoted MB-231-TXSA-droz-R, were completely refractory to drozitumab-induced apoptosis and maintained resistance, even after a long period of time from the removal of drozitumab from their growth media. However, development of resistance to drozitumab in the MB-231-TXSA cells could not be explained by major differences in receptor expression between sensitive and resistant cells, indicating that other factors in the apoptotic pathways may be involved. Indeed, more detailed examination of the drozitumab-resistant cells, by western blot analysis, demonstrated that these cells express elevated levels of IAP proteins, particularly cIAP2, compared to the sensitive cells. IAP proteins are inhibitors of apoptosis that block cell death in response to diverse stimuli through interactions with pro- and anti-apoptotic factors (LaCasse, Mahoney et al. 2008). Our results are in line with various preclinical and clinical studies that have demonstrated IAP proteins to be expressed at very high levels in human malignancies (Ambrosini, Adida et al. 1997; LaCasse, Baird et al. 1998; Tamm, Kornblau et al. 2000; Vucic, Stennicke et al. 2000; LaCasse, Mahoney et al. 2008; Fulda and Vucic 2012). This raises the hypothesis that IAP proteins may act as important modulators of the apoptotic

signalling pathways and therefore may play a significant role in causing resistance to apoptosis in cancer.

In our attempts to reverse the resistance of the MB-231-TXSA-droz-R cells to drozitumab, we tested these cells for their sensitivity to the chemotherapeutic agent DOX. DOX, when used alone, showed a slight cytotoxic effect on the MB-231-TXSA-droz-R cells. However when combined with drozitumab, DOX cooperated synergistically for apoptosis induction when compared to the effect of each agent alone. This effect was associated with a significant degradation of cIAP1 and cIAP2 proteins, an effect that was more profound when combined with drozitumab treatment. To assess if IAP proteins play a role in determining the resistance of MB-231-TXSA-droz-R cells to drozitumab, we used a pan-IAP-antagonist, BV6, to inhibit their action in these cells. MB-231-TXSA-droz-R cells treated with drozitumab alone showed a maximum of 2 % cell death whereas in combination with BV6 cell death increased to 50 %. These data suggest that IAP proteins play a significant role in inhibiting drozitumab-induced apoptosis.

To examine if the synergistic activity of drozitumab and DOX seen *in vitro* could be translated into *in vivo* efficacy, MB-231-TXSA-droz-R cells were transplanted into the mammary gland of female athymic mice and allowed to establish for 2 weeks. Animals treated with vehicle developed aggressive, rapidly growing mammary tumours. When animals were administered drozitumab or DOX as single agents tumour growth was somewhat delayed by about one week. In contrast, drozitumab cooperated positively with DOX showing an initial reduction in tumour growth and a significant delay in tumour progression which was associated with increased survival benefit.

This study provides evidence that drozitumab resistance in breast cancer cells can be reversed by combination with DOX and identified IAPs as major determinants involved in drozitumab's resistance. *In vivo*, DOX cooperated with drozitumab to inhibit mammary

tumour growth and to increase survival suggesting that this combination could provide a promising therapeutic approach for patients with breast cancer. The identification of specific biomarkers for detecting acquired or innate resistance to these agents is of great importance and when this is achieved, new combination treatments can be developed to improve the survival of cancer patients. Taken together, these results suggest that combination of drozitumab with agents that modulate IAP levels including IAP antagonists could potentially be a very useful strategy to target tumour cell resistance in cancer patients and improve survival.

CHAPTER 4

ANTICANCER EFFICACY OF APO2L/TRAIL IS RETAINED IN THE PRESENCE OF HIGH AND BIOLOGICALLY ACTIVE CONCENTRATIONS OF OSTEOPROTEGERIN *IN VIVO*.

Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin *in vivo*.

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Running title: Efficacy of Apo2L/TRAIL in the presence of OPG

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JBMR, 2011, March;26(3):630-43

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JBMR, 2011, March;26(3):630-43

Irene Zinonos (Candidate)

Performed *in vitro* experiments and animal studies, BLI imaging and mCT imaging, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author

I hereby certify that the statement of contribution is accurate

Signed:…….....…….*Date:*…*01/06/2012*……

Agatha Labrinidis

Supervised development of work, transfection of breast cancer cells with triple reporter gene contract (SFG-NES-TGL), assistance with mCT analysis, data interpretation and manuscript evaluation

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

Signed:.....……..*Date:*...*23/05/2012*.....

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Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin *in vivo*.

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Michelle Lee

Assistance with animal handling

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Signed **…….....….***Date:***…***16/05/2012***……**

Vasilios Liapis

Assistance with handling of animals and provided expert advice on western blotting and immunodetection

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JBMR, 2011, March;26(3):630-43

Shelley Hay

Assistance with handling of animals, tissue excision and provided advice and support on histology

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

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Vladimir Ponomarev

Provided triple reporter gene construct (SFG-NES-TGL)

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

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Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin *in vivo*.

JBMR, 2011, March;26(3):630-43

Peter Diamond

Assistance with generating breast cancer cells overexpressing native human OPG and with transfection of breast cancer cells with triple reporter gene contract (SFG-NES-TGL)

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

Signed:……..……..*Date:…24/05/2012*……

David M. Findlay

Data interpretation and manuscript evaluation

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Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin *in vivo*.

JBMR, 2011, March;26(3):630-43

Andrew C.W. Zannettino

Data interpretation and manuscript evaluation

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

Signed:…….....*Date:…23/05/2012*……

Andreas Evdokiou

Principal supervisor, data interpretation and manuscript editing

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

Signed:……..*Date:…01/06/2012*……

Zinonos, I., Labrinidis, A., Lee, M., Liapis, V., Hay, S., Ponomarev, V., Diamond, P., Findlay, D.M., Zannettino, A.C.W. & Evdokiou, A. (2011) Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin in vivo.
Journal of Bone and Mineral Research, v. 26(3), pp. 630-643

CHAPTER 5

LOCAL PRODUCTION OF OSTEOPROTEGERIN BY BREAST CANCER CELLS INHIBITS CANCER-INDUCED OSTEOLYSIS AND INTRA-OSSEOUS TUMOUR BURDEN BUT PROMOTES PULMONARY METASTASIS

Local production of Osteoprotegerin by breast cancer cells inhibits cancer-induced osteolysis and intra-osseous tumour burden but promotes pulmonary metastasis.

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Short running title: Local OPG production by breast cancer cells promotes pulmonary metastasis.

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Local production of Osteoprotegerin by breast cancer cells inhibits cancer-induced osteolysis and intra-osseous tumour burden but promotes pulmonary metastasis.

Text in manuscript

Irene Zinonos (Candidate)

Performed *in vitro* experiments and animal studies, BLI imaging and mCT imaging, analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author

I hereby certify that the statement of contribution is accurate

*Signed***:**…….....…….*Date:…01/06/2012*……

Agatha Labrinidis

Supervised development of work, transfection of breast cancer cells with triple reporter gene contract (SFG-NES-TGL), data interpretation and manuscript evaluation

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

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STATEMENT OF AUTHORSHIP

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Vasilios Liapis

Assistance with handling of animals and provided expert advice on western blotting and immunodetection

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Shelley Hay

Assistance with handling of animals, tissue excision and provided advice and support on histology

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Peter Diamond

Assistance with generating breast cancer cells overexpressing native human OPG and with transfection of breast cancer cells with triple reporter gene contract (SFG-NES-TGL)

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Vladimir Ponomarev

Provided triple reporter gene construct (SFG-NES-TGL)

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Text in manuscript

Gerald Atkins

Data interpretation and manuscript evaluation

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David M. Findlay

Data interpretation and manuscript evaluation

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Andrew C.W. Zannettino

Data interpretation and manuscript evaluation

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Andreas Evdokiou

Principal supervisor, data interpretation and manuscript editing

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Abstract

Osteoprotegerin (OPG) is a secreted member of the TNF receptor superfamily which binds to receptor activator of nuclear factor kB ligand (RANKL) and inhibits osteoclast activity and bone resorption. Systemic administration of recombinant OPG was previously shown to inhibit tumour growth in bone and prevent cancer-induced osteolysis. In this study we examined the effect of OPG when produced locally by breast cancer cells in bone using a mouse model of osteolytic breast cancer. MDA-MB-231-TXSA breast cancer cells, tagged with a luciferase reporter gene construct and engineered to overexpress full-length human OPG were transplanted directly into the tibial marrow cavity of nude mice. Tumour progression was monitored in live animals using bioluminescence imaging, whereas the development of breast cancer-induced osteolysis was measured using high resolution micro-computer tomography and histology. Animals transplanted with empty vector-transfected cells developed large osteolytic lesions with tumour growth extending into the surrounding soft tissues. In contrast, over-expression of OPG by breast cancer cells protected the bone from breast cancer-induced osteolysis, diminished intra-osseous tumour growth but had no effect on extra-skeletal tumour growth. This was associated with a significant reduction in the number of osteoclast lining the bone surface. Despite the bone protection, OPG over-expression resulted in a significant increase in the incidence of pulmonary metastasis. Our results demonstrate for the first time that OPG secreted by tumours in bone may affect the behaviour of cancer cells within the bone microenvironment and their likelihood of spreading and establishing metastases elsewhere in the body.

Introduction

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer death among women worldwide accounting for 23% of the total cancer cases and 14% of the cancer deaths in 2008 (Jemal, Bray et al. 2011). Despite the significant improvements in detecting and treating early breast cancer, an estimated 75-80% of patients with advanced disease develop bone metastasis (Siegel, Ward et al. 2011). The pathologic complications of bone metastasis can have devastating effects and patients experience debilitating skeletal-related events (SREs) including, pathological skeletal fractures, hypercalcaemia of malignancy and spinal cord compression. SREs are accompanied by severe bone pain and loss of mobility which eventually leads to reduction of quality of life and survival (Coleman, Lipton et al. 2010; Guise, Brufsky et al. 2010; Coleman, Marshall et al. 2011).

Osteoprotegerin (OPG) is the only secreted member of the TNF receptor superfamily and plays an important role in bone remodelling and especially in osteoclastogenesis (Simonet, Lacey et al. 1997; Tsuda, Goto et al. 1997). Osteoblasts and stromal cells express receptor activator of NF-kB ligand (RANKL) which binds to its receptor RANK on pre-osteoclasts and stimulates their differentiation and maturation into functional osteoclasts. OPG, produced by osteoblasts and other cell types, binds to RANKL and prevents the association between RANKL and RANK, thereby inhibiting osteoclast activation and function. It was shown previously that systemic administration of recombinant OPG inhibited tumour growth in bone by inhibiting cancer-induced osteolysis and prevented bone loss in animal models of experimental bone metastasis (Croucher, Shipman et al. 2001; Buijs, Que et al. 2009). In this study, we examined the effects of local tumour production of OPG by breast cancer cells in a mouse model of osteolytic breast cancer. Our data showed that over-expression of OPG by breast cancer cells protected the bone from cancer-induced osteolysis but without effect on the overall tumour burden. More importantly, and despite the bone

protection, OPG over-expression led to a significant increase in the incidence of pulmonary metastasis. These results demonstrate, for the first time, that OPG secreted by tumours in bone may affect the behaviour of cancer cells within the bone microenvironment and their likelihood of spreading and establishing metastases elsewhere in the body.

Materials and Methods

Cell lines and tissue culture

The MDA-MB-231 derivative cell line, MDA-MB-231-TXSA was kindly provided by Dr Toshiyuki Yoneda (University of Texas Health Sciences Centre, San Antonio, Texas). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2 mM glutamine, 100 IU/ml penicillin, 160 μg/ml gentamicin, Hepes (20mM) and 10% fetal bovine serum (Biosciences, Sydney, Australia) in a 5% CO2-containing humidified atmosphere. The generation of luciferase-tagged MB-231-TXSA-TGL-p-RUF and p-OPG over-expressing human breast cancer cells were previously described (Zinonos, Labrinidis et al. 2011).

Animals

Female athymic nude mice at 5 weeks old (Institute of Medical and Veterinary Services Division, Gilles Plains, SA, Australia) were acclimatised 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 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.

Intratibial injection model

MB-231-TXSA-TGL-p-RUF and p-OPG over-expressing human breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2 mM glutamine, 100 IU/ml penicillin, 160 μg/ml gentamicin, Hepes (20mM) and 10% fetal bovine serum (Biosciences, Sydney, Australia) in a 5% CO_2-containing humidified atmosphere, until they reached 70-80% confluency. Adherent cells were removed from flasks with 2 mM EDTA and resuspended in 1 x PBS at 0.5 x 10^5 cells/10 μl and kept on ice in an eppendorf tube. Mice (n=10 per cell line) were anaesthetised by Isoflurane (Faulding Pharmaceuticals, SA, Australia), the left tibia was wiped with 70% ethanol and a 27 G needle coupled to a Hamilton syringe, was inserted through the tibial plateau with the knee flexed and 0.5 x 10^5 cells resuspended in 10 μl of PBS were injected into the marrow space. Tumour volume was monitored regularly and mice were imaged on a weekly basis using the bioluminescence imaging (BLI) system (see below). Mice were humanely killed 4 weeks after cancer cell transplantation, due to high tumour load.

Preparation of blood serum and detection of OPG by ELISA

Blood was collected from all the mice at day 18 (tail bleeds) and at the time of termination of the experiment to determine the OPG concentration in the blood serum of the mice. Blood was collected in MiniCollect tubes (0.8 ml LH Lithium Hep Sep) and stored in the fridge until the procedure was completed for all the mice. Blood was then spinned for 5 mins and the separated clean serum was transferred into eppendorf tubes and stored at -80ºC until ready for use. The concentration of OPG in blood serum collected from all animals was determined using a commercial ELISA kit as per the manufacturer's instructions (KB 1011, Immunodiagnostics AG,Wien, Austria).

Bioluminescence imaging (BLI) of tumour growth

Non-invasive, whole body imaging for assessment of tumour growth was performed once weekly using the IVIS 100 Imaging system (Xenogen, Alameda, CA). Mice were injected i.p. with 100 µl of the D-Luciferin solution at final dose of 3 mg/20 g mouse body weight (Xenogen Alameda, CA) and then gas-anaesthetised with Isoflurane (Faulding Pharmaceuticals, Salisbury, SA, Australia). Images were acquired for 0.5-30 seconds (images are shown at 1 second) from the side angle 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.

Micro-computed (µCT) tomography analysis

Limbs for µCT analysis were surgically resected and scanned using the SkyScan-1174 high-resolution µCT Scanner (Skyscan, Belgium). During µCT scanning the tibiae were placed vertically in tightly fitting plastic tubes. The µCT Scanner was operated at 50kV, 800 µA, rotation step 0.4, 0.25 mm Al filter and scan resolution of 7.78 µm/pixel. The cross sections were reconstructed using a cone-beam algorithm (software NRecon, Skyscan). Files were then imported into CTAn software (Skyscan) for 3D analysis and 3D image generation. 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. All images were viewed and edited using CTvol visualisation software. Histograms are representing bone volume (mm^3), total and trabecular, from tumour bearing tibiae and were generated and compared to the contralateral non-tumour bearing tibiae. Tumour burdens, measured in mm^3, were determined using the Skyscan software.

Histology

Tibiae were fixed in 10% (v/v) buffered formalin (24 hours at 4°C), followed by 2-4 weeks of decalcification in 0.5M EDTA/0.5% paraformaldehyde in PBS, pH 8.0 at 4°C. Complete decalcification of the tibiae was confirmed by radiography and tibiae were then paraffin embedded. Five micron (5μM) longitudinal sections were prepared and stained with Hematoxylin and Eosin (H&E). Additional sections were used for osteoclast-specific tartrate-resistant acid phosphatase 5 (ACP5/TRAP) (Sigma-Aldrich, St Louis, MO) staining, following the manufacture's protocol. Analysis was performed on an Olympus CX41 microscope and images were taken using the NanoZoomer Digital Pathology (NDP-Hamamatsu). Tumour area measured in mm^2 was assessed using the Nanozoomer software. Lungs were also fixed in 10% (v/v) buffered formalin and were then paraffin embedded and sectioned at 5 μM at three different levels, followed by H&E staining. Total lung area and metastatic foci area was measured in mm^2 using the Nanozoomer 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. Comparisons between groups were assessed using a one way ANOVA test. In all cases, $p < 0.05$ was considered statistically significant.

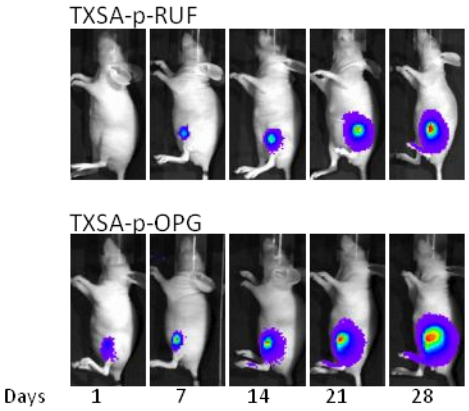
Figure 1. ***Effect of OPG over-expression by breast cancer cells on tumour growth.***

A. Five week old female nude mice were injected with 0.5 x 10^5 MB-231-TXSA-TGL-pRUF or p-OPG cells directly into the marrow cavity of their right tibia. Mice were imaged weekly using the Xenogen IVIS 100 bioluminescence imaging system. Representative whole body BLI images of a single animal from each group (n=10 per cell line) during the course of the experiments are shown. All animals, in both groups, were humanely killed on day 28 for ethical reasons, due to high tumour load.

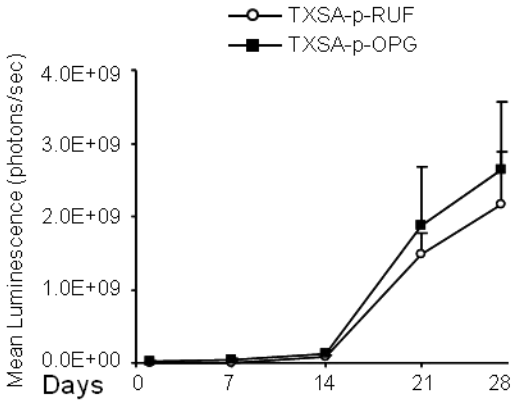
B. The line graph represents the average tumour signal over time measured as mean photon counts per second and it demonstrates that OPG over-expression has no effect on tumour growth when compared to p-RUF tumours.

C. OPG concentration in the blood serum of mice (n=10 per cell line) collected at two different time points during the experiment as measured by ELISA, bars +/- SEM.

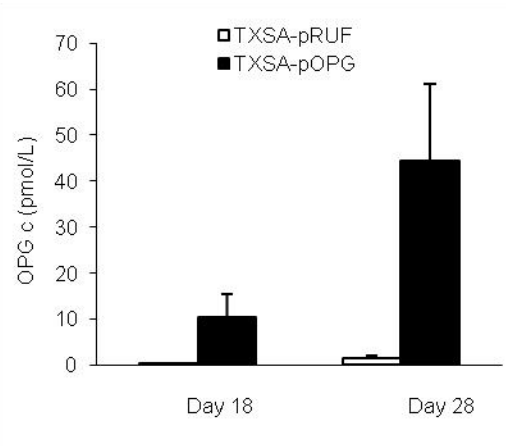
A.


TXSA-p-RUF
TXSA-p-OPG
Days
1
7
14
21
28

B.


TXSA-p-RUF
TXSA-p-OPG
Mean Luminescence (photons/sec)
4.0E+09
3.0E+09
2.0E+09
1.0E+09
0.0E+00
Days
0
7
14
21
28

C.


TXSA-pRUF
TXSA-pOPG
OPG c (pmol/L)
70
60
50
40
30
20
10
0
Day 18
Day 28

Results

OPG over-expressed by breast cancer cells does not affect total tumour burden *in vivo*

To evaluate the effect of OPG over-expression by breast cancer cells on tumour growth, we used a xenograft animal tumour model, in which the empty vector or OPG-over-expressing MB-231-TXSA breast cancer cells were transplanted directly into the tibial marrow cavity of female athymic nude mice. We established non-invasive bioluminescence imaging approaches, which provided sensitive real time *in vivo* assessment of breast cancer growth in bone. Development of breast cancer-induced bone destruction was qualitatively and quantitatively assessed using high resolution micro-CT analysis. For non-invasive bioluminescence imaging (BLI) of tumour growth, the empty vector and OPG transfected cells were infected with a triple-fusion protein reporter construct encoding herpes simplex virus thymidine kinase (TK), green fluorescent protein (GFP) and firefly luciferase (Luc) as described previously (Labrinidis, Diamond et al. 2009; Zinonos, Labrinidis et al. 2011). After infection, cells were enriched for high level expression of GFP by two rounds of fluorescence-activated cell sorting, thus generating the sub-lines MB-231-TXSA-pRUF-TGL and MB-231-TXSA-pOPG-TGL which were then transplanted into the mice. In this study, tumour growth was monitored regularly and mice were imaged once weekly. All animals inoculated with the empty vector transfected cells showed an increase of mean photon emission associated with an increase in tumour burden, which was clearly evident from day 14 onwards (Fig 1 A and B). Similar results were found with mice bearing OPG over-expressing tumours with BLI showing no significant differences between the two groups. Bloods were collected from all animals at different time points during the experiment and the levels of circulating OPG measured by ELISA (Fig 1C). The data show that the basal levels of circulating OPG in animals bearing empty vectors transfected cells were approximately 0.2 pmol/L, when measured on day 18 post-cancer cell transplantation. These levels were

increased 50 fold to approximately 10 pmol/L in mice bearing OPG over-expressing tumours. Mice were humanely killed on day 28 for ethical reasons, due to the high tumour load in the tibiae. OPG levels in mice bearing OPG-over-expressing tumours increased further to 45 pmol/L, clearly indicating that p-OPG cells maintained expression of OPG over the entire period of the study.

OPG over-expression inhibits cancer-induced osteolysis

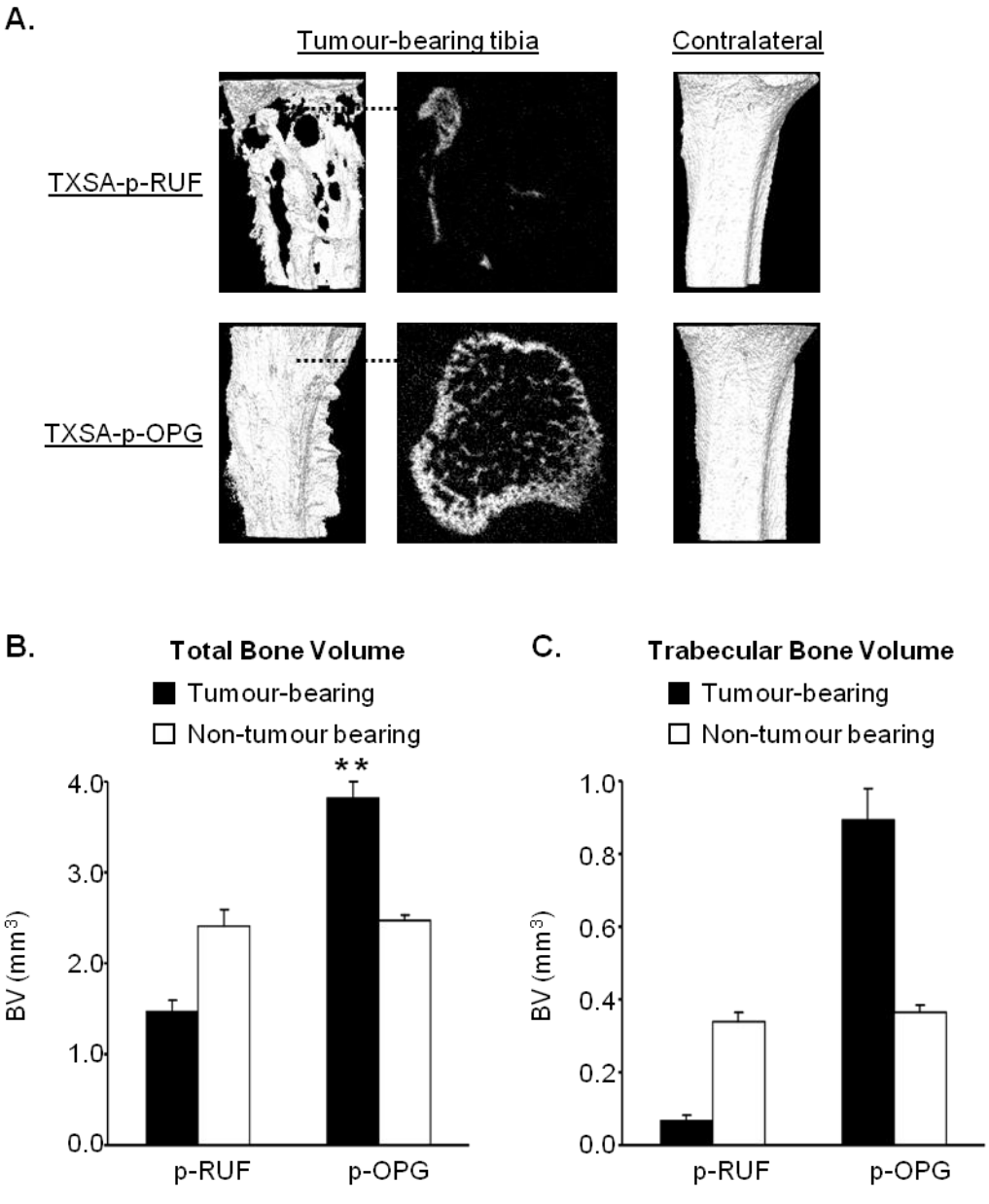
To assess the effects of OPG over-expression on bone structure, the lower hind limbs of all mice were surgically resected at the end of the study and μCT analysis was performed as described in the "Material and Methods" section. Reconstructed 3D micro-CT images of representative empty vector transfected tumour-bearing tibiae demonstrated extensive osteolysis when compared to the contralateral non-tumour bearing tibiae (Fig 2A). In contrast, and consistent with the role of OPG in inhibiting osteoclastic bone resorption, all animals inoculated with the p-OPG transfected cells showed preservation of the integrity of bone around the tumours and protection from tumour-induced osteolysis, as shown in Fig 2A. To quantify the total bone volume (BV), we compared the tumour-bearing with the contralateral non-tumour bearing tibiae of all the animals in each group at a selected region beginning at the growth plate and extending downwards 400 x 7.8 μm slices, which encompassed all of the cancer lesions. As seen in Fig 2B, the amount of bone lost in the tibiae of mice inoculated with empty vector transfected cells exceeded 40% in the tumour-bearing tibiae when compared to the contralateral non-tumour bearing tibiae. In contrast, OPG released by cancer cells demonstrated remarkable protection from breast cancer-induced osteolysis translating to a significant increase (55%) in BV in the OPG-bearing tibiae when compared to the contralateral tibia.

Figure 2. ***Effects of OPG over-expression by breast cancer cells on bone.***

A. Qualitative 3D m-CT images of representative animals from each group. Tibiae of mice inoculated with the empty vector transfected cells (MB-231-TXSA-p-RUF) had developed large intratibial tumours and demonstrated extensive osteolysis when compared to the contralateral non-tumour bearing tibiae. In contrast, all animals inoculated with the p-OPG transfected cells showed preservation of the integrity of bone around the tumours and protection from breast cancer-induced osteolysis.

B. Quantitative assessments of total bone volume (BV) in mm^3 in the tumour-bearing tibiae when compared to the contralateral non-tumour bearing tibiae, bars +/-SEM * $p < 0.01$.

C. Quantitative assessments of trabecular bone volume (BV) in mm^3 when compared to the contralateral non-tumour bearing tibiae, bars +/-SEM * $p < 0.01$.


A.
Tumour-bearing tibia
Contralateral
TXSA-p-RUF
TXSA-p-OPG
B.
Total Bone Volume
Tumour-bearing
Non-tumour bearing
**
BV (mm3)
4.0
3.0
2.0
1.0
0.0
p-RUF
p-OPG
C.
Trabecular Bone Volume
Tumour-bearing
Non-tumour bearing
BV (mm3)
1.0
0.8
0.6
0.4
0.2
0.0
p-RUF
p-OPG

The effect of OPG on trabecular bone volume (TbBV) was more pronounced. Mice inoculated with the empty vector transfected cells showed a dramatic loss of their trabecular bone (> 80%) when compared to the contralateral leg and to the tibiae of tumours containing OPG over-expressing cells. More interestingly, mice bearing OPG over-expressing tumours demonstrated 141% gain in their TbBV when compared to the contralateral right tibiae (bars +/- SEM, * $p < 0.01$). The observed inhibition of osteolysis by OPG was due to the suppression of osteoclastic bone resorption since p-OPG released by tumour cells significantly decreased the number of osteoclasts lining the bone surface. Fig 3C shows that TRAP+ osteoclasts were abundantly present and attached to the bone surfaces in tumour lesions from the vector only transfected cells. In contrast, there was complete absence of TRAP+ osteoclasts in tibiae preparations of animals inoculated with p-OPG transfected cells, which also confirms the biological activity of OPG *in vivo*.

OPG secreted by breast cancer cells maintains skeletal integrity but alters the intra- and extra-medullary tumour distribution

Tumour burden as a function of bioluminescence showed no significant differences in the average tumour signal between the mice bearing p-OPG tumours when compared to the mice with p-RUF tumours, suggesting that there are no differences in the total tumour burden between the two groups. However, a detailed histological examination of the tibiae, using high resolution imaging using the Nanozoomer software, showed that the distribution of the tumour in the bone was different in animals with OPG-overexpressing tumours compared to animals bearing empty vector transfected tumours. In mice bearing empty vector transfected tumours there was persistent growth of cancer cells within the bone marrow cavity representing 76.4% of the overall tumour burden, which then penetrated the cortical bone and

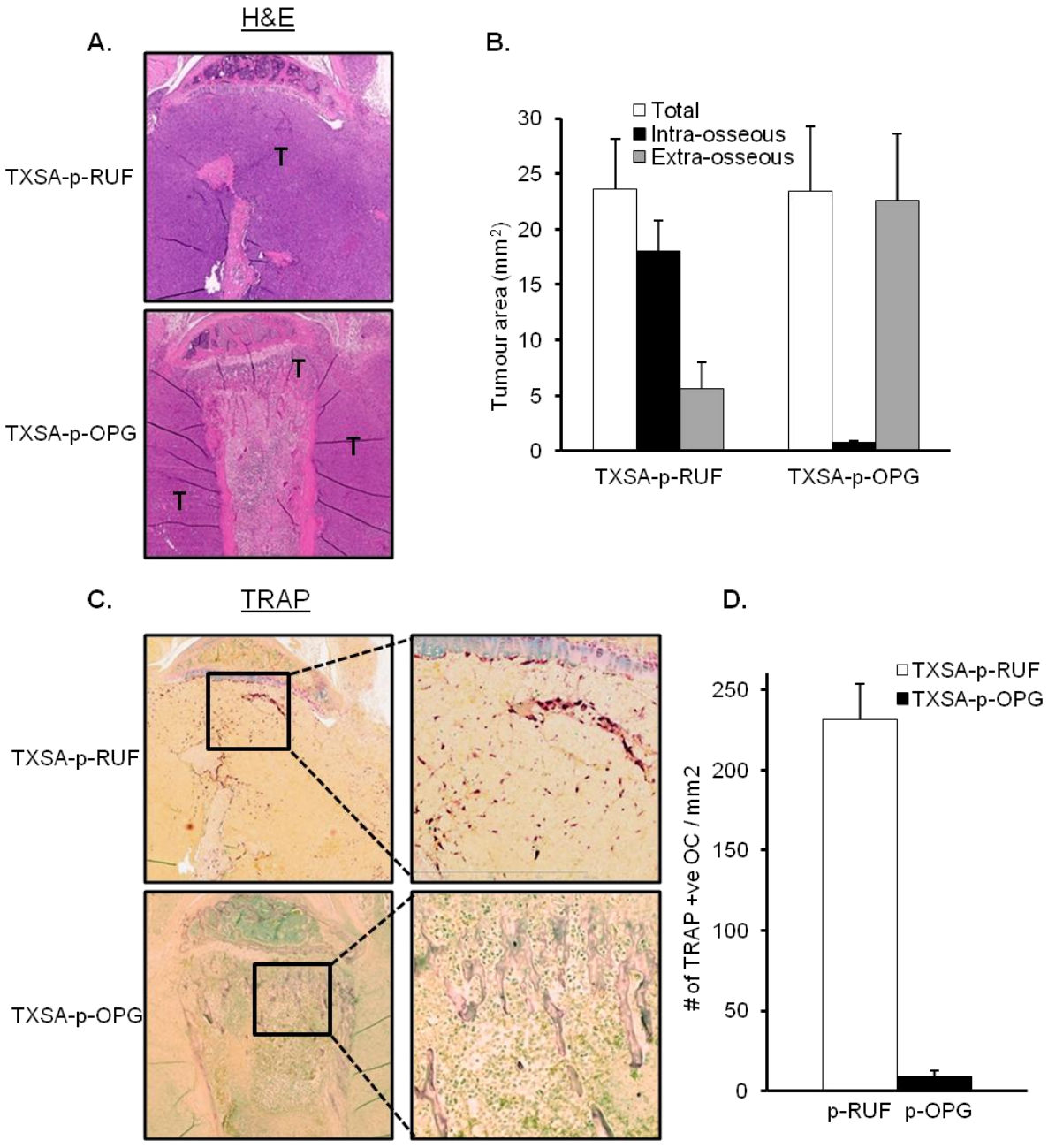
Figure 3. ***OPG over-expression maintains bone integrity but alters the intra- and extra-medullary tumour distribution.***

A. Representative H&E stained tibial sections from mice inoculated with MB-231-TXSA-TGL-pRUF or p-OPG cells showing the differential distribution of intra-and extra medullary tumour growth.

B. Quantitative assessment of intra- and extra- medullary tumour area measured in mm^2 using the histological images and the area is expressed as an average per group.

C. TRAP staining of histological sections showing absence of TRAP positive osteoclasts in tibiae preparations of animals inoculated with OPG transfected cells when compared to vector transfected cells in which osteoclasts were abundantly present and attached to the bone surfaces.

D. Quantitative assessment of the number of TRAP + osteoclasts.


A.
H&E
TXSA-p-RUF
TXSA-p-OPG
T
T
T
T
B.
Total
Intra-osseous
Extra-osseous
Tumour area (mm2)
30
25
20
15
10
5
0
TXSA-p-RUF
TXSA-p-OPG
C.
TRAP
TXSA-p-RUF
TXSA-p-OPG
D.
TXSA-p-RUF
TXSA-p-OPG
# of TRAP +ve OC / mm2
250
200
150
100
50
0
p-RUF
p-OPG

invaded the surrounding soft tissue. In contrast, histological sections of tibiae with OPG over-expressing tumours showed that the intra-osseous tumour burden was significantly decreased, accounting only for 3% of the overall tumour burden. In these tibiae, cancer cells were almost undetectable within the bone marrow space and this was also associated with a dramatic increase in trabecular bone density and a concomitant decrease in bone marrow volume (Fig 3A). Importantly, OPG over-expressing cancer cells escaped the marrow cavity and continued to grow in the extra-medullary space, accounting for 96.8% of the total tumour burden. Fig 3B shows the intra- and extra-osseous tumour burden in the tibiae, expressed as an average tumour area per group. TRAP stain was also performed on histological sections of tibiae of mice from both groups and it was found that osteoclasts were abundantly present and attached to the bone surfaces in tumour lesions from the vector only transfected cells (Fig 3C). In contrast, there was almost complete absence of TRAP+ osteoclasts in tibiae preparations of animals inoculated with p-OPG transfected cells. It was found that there was 24.5 fold decrease in the number of TRAP+ in the tibiae of mice bearing OPG over-expressing tumours compared to the mice inoculated with vector only transfected cells, accounting for the protective effect of OPG over-expression on bone destruction and also confirming the biological activity of OPG *in vivo* (Fig 3D).

OPG secreted by breast cancer cells promotes pulmonary metastasis by failing to restrain extra-medullary tumour growth

While OPG over-expression protected the bone from cancer-induced bone destruction, and the intra-osseous tumour mass was almost completely diminished, there was persistent cancer cell growth in the extra-medullary space. At the end of the experiment, the lungs from each group of animals were excised and tumour burden as a function of photon counts per

second was quantified *ex-vivo* using BLI. The mean luciferase activity of lungs from mice bearing OPG-over-expressing tumours was significantly higher than that of the animals bearing empty vector transfected tumours (Fig 4A). The graph in Fig 4B represents the average metastatic tumour signal in the lungs over time, measured as mean photon counts per second, confirming the qualitative images. Histological examination of lungs confirmed metastatic tumour growth within the lungs of mice bearing OPG-over-expressing tumours (6/10) compared to the mice inoculated with empty vector transfected cells (1/10) (Figure 4C). The increase incidence of pulmonary metastasis in the mice with p-OPG tumours was also confirmed by quantification of the metastatic nodules using the Nanozoomer software. It was found that the number of metastatic foci was increased by 23 fold in the mice with p-OPG tumours when compared to the animals bearing empty vector transfected cancer cells (Fig 4D). In addition, the percentage of tumour burden of the lung area was approximately 105 fold higher in the mice with OPG over-expressing cancer cells compared to the p-RUF mice (Fig 4E). These results indicate that in addition to OPG having no effect on tumour burden within the bone, it actually increased the propensity of breast cancer cells to metastasise to the lungs.

Figure 4. ***Effects of OPG over-expression on pulmonary metastasis.***

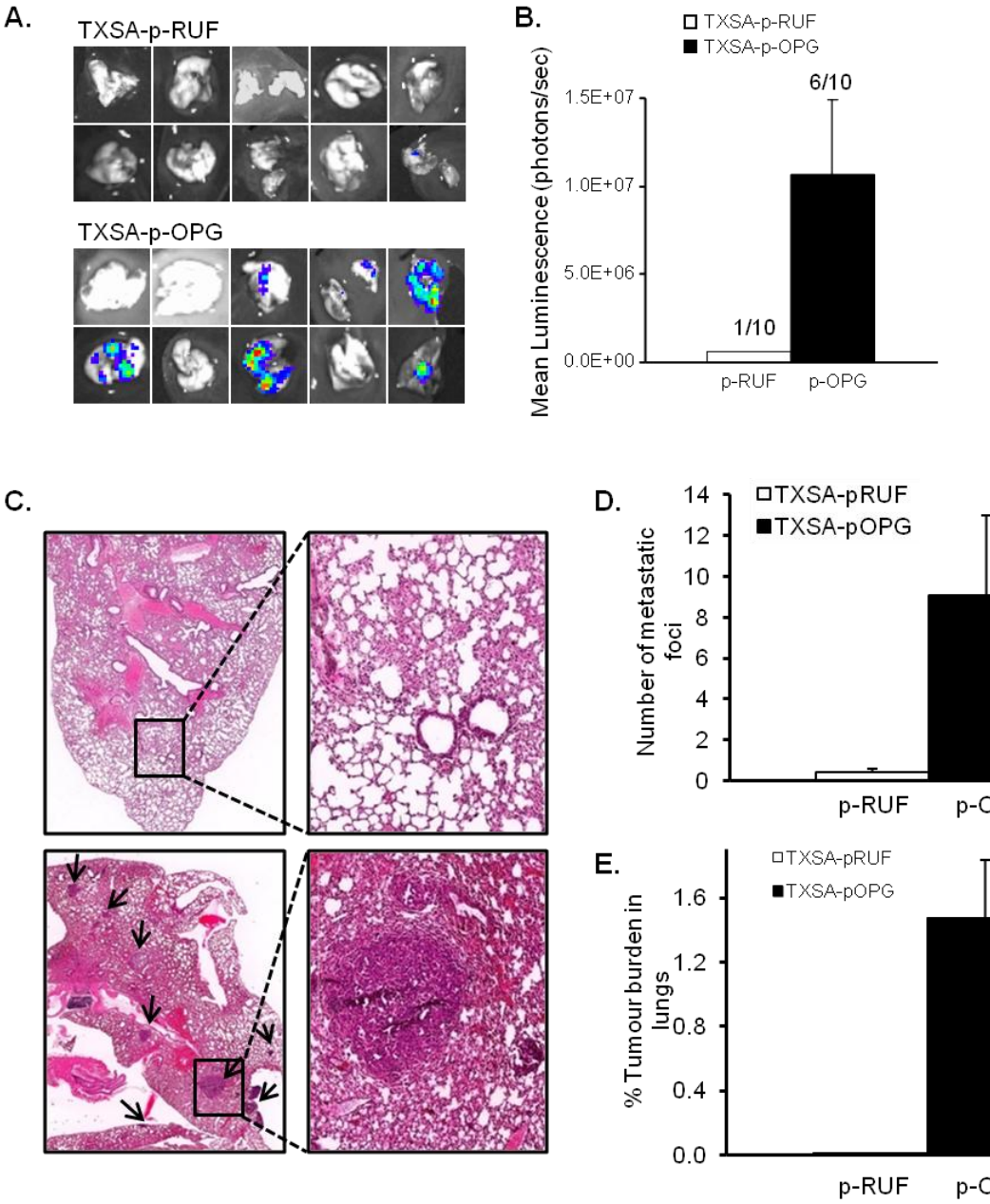
A. BLI images of the lungs of all the mice inoculated with MB-231-TXSA-TGL-pRUF or p-OPG cells. From all the mice bearing tumours with empty vector transfected cells only one showed a very small BL signal whereas 6 out of 10 mice from the MB-231-TXSA-p-OPG group showed significant BL signal of pulmonary metastasis.

B. The line graph represents the average metastatic BL tumour signal in the lungs over time measured as mean photon counts per second.

C. Representative histological sections of the lungs of mice from each group stained with H&E, confirming tumour within the lungs in the mice bearing OPG-over-expressing tumours compared to the lungs of mice bearing empty-vector transfected tumours.

D. Graph represents the tumour area as percentage of total and is expressed as an average per group.

E. Graph represents the average number of metastatic foci per group. Data shown in each case are an average from a representative section of each animal. bars +/- SEM, * $p < 0.01$.


A.
TXSA-p-RUF
TXSA-p-OPG
B.
TXSA-p-RUF
TXSA-p-OPG
Mean Luminescence (photons/sec)
1.5E+07
1.0E+07
5.0E+06
0.0E+00
6/10
1/10
p-RUF
p-OPG
C.
D.
TXSA-pRUF
TXSA-pOPG
Number of metastatic foci
14
12
10
8
6
4
2
0
p-RUF
p-OPG
E.
TXSA-pRUF
TXSA-pOPG
% Tumour burden in lungs
1.6
1.2
0.8
0.4
0.0
p-RUF
p-OPG

Discussion

In this study, we assessed the effects of native full-length human OPG when produced and secreted locally by human breast cancer cells in the bone microenvironment *in vivo*. We have engineered the MB-231-TXSA cells to over-express full length human OPG and we have previously shown that the transfected cells produce and secrete in their growth media very high levels of biologically functional OPG (Zinonos, Labrinidis et al. 2011). In this study we used a well-established murine model of osteolytic breast cancer in which MB-231-TXSA cells over-expressing full length human OPG are transplanted directly into the tibial marrow cavity of nude mice. This *in vivo* model mimics the late stages of bone metastasis and is ideal for monitoring the effects of OPG over-expression on breast cancer growth in the bone and also on cancer-induced bone destruction.

OPG plays a key role in bone remodelling by inhibiting osteoclastic bone resorption and bone remodelling. This study demonstrates that over-expression of biologically active OPG inhibits osteoclast formation and bone resorption, protecting cortical and trabecular bone integrity and preventing further development of osteolytic lesions in animals bearing OPG over-expressing tumours. However, despite this bone protection, OPG over-expression failed to reduce the overall tumour burden as measured by BLI. Interestingly though, histological examination of tibiae of mice injected with OPG over-expressing breast cancer cells demonstrated that their intra-osseous tumour burden diminished significantly whereas cancer cells persisted to grow in the extra-medullary space. In addition, there was a significant increase in the trabecular bone density in the p-OPG bearing tibiae, which resulted in a significant reduction in bone marrow volume. The fact that intra-osseous tumour growth was prevented could be explained by this decrease in bone marrow space which forced the cancer cells to escape and invade the surrounding soft tissue. These effects were associated with a

significant decrease in the number of TRAP+ osteoclasts lining the bone surface of tibiae bearing OPG over-expressing tumours.

These results substantiate our recently published data showing that osteoclast ablation with zoledronic acid (ZOL), while it protected against osteosarcoma (OS) induced bone destruction, had no significant effect on the overall tumour burden due to the persistent growth of osteosarcoma cells in the extra-medullary space (Labrinidis, Hay et al. 2009). More importantly, the mean luciferase activity of lungs from mice treated with both a weekly and a single dosing regimen of ZOL was significantly higher than that of the vehicle-treated animals. This increase in pulmonary metastasis associated with zoledronic treatment indicates that in addition to ZOL having no effect on OS burden within the bone, it actually increased the propensity of osteosarcoma cells to metastasise to the lungs. Furthermore, in another study, Endo-Munoz et al in collaboration with our lab, conducted a transcriptomic screen of osteosarcoma biopsies and found that expression of ACP5/TRAP is significantly downregulated in primary OS compared with non-malignant bone (Endo-Munoz, Cumming et al. 2010). Moreover, within the OS patient cohort, the patients who developed pulmonary metastases had significantly lower ACP5/TRAP expression and osteoclast numbers than those patients who did not develop metastases. Therefore, they examined whether metastasis-competent (MC) OS cells could induce loss of TRAP+ osteoclasts and contribute to metastasis. The results demonstrated that MC OS cell lines can inhibit osteoclastogenesis *in vitro* and *in vivo* and also osteoclasts can inhibit the migration of MC OC cells *in vitro*. Finally they reported that ablation of osteoclasts with ZOL increases the number of metastatic lung lesions in an orthotopic OS model. In conclusion, Endo-Munoz et al demonstrated that the metastatic potential of OS is determined in tumour development and that loss of osteoclasts in the primary lesion enhances OS metastasis. Interestingly, the present study demonstrates the exact same results, since mice bearing OPG over-expressing tumours

demonstrated a significant increase in the pulmonary metastasis incidence, compared to animals bearing empty vector-transfected cells. These results suggest that OPG secreted by tumours in bone inhibits osteoclast activation and function therefore modulates the bone microenvironment and affects the likelihood of cancer cells to spread elsewhere in the body.

It is worth mentioning that Fisher et al have demonstrated that over-expression of full length OPG in breast cancer cells was associated with enhanced tumour growth *in vivo*, with an accompanied increase in osteolysis, whereas Fc-OPG treatment of mice inhibited osteolysis and tumour advance in the bone (Fisher, Thomas-Mudge et al. 2006). In this particular paper MCF-7 cells were transfected with PTHrP (parathyroid hormone related protein) and full length human OPG and then transplanted directly into the tibial marrow cavity of mice. Similar to our results, Fisher et al, have demonstrated that there was minimal osteolytic damage in mice inoculated with MCF-7 cells overexpressing OPG alone whereas, as expected, MCF-7 over-expressing PTHrP promoted bone loss and osteolysis. However, co-overexpression of PTHrP and OPG resulted in enhanced osteolysis and increased tumour growth. On the contrary, our results have demonstrated that OPG over-expression had no effect in the overall tumour burden and most importantly it protected the bone from cancer-induced osteolysis. The different observation between the two studies are probably due to the fact that the OPG over-expressing cells in the Fisher study were also transfected with PTHrP which influence osteolysis by inducing the expression of RANKL by osteoblasts. This PTHrP over-expression most possibly counteracted the effects of OPG in the bone, resulting in increased osteolysis.

There are at least two possible explanations for the increased lung metastasis observed in this study; either the cells themselves are more aggressively metastatic or the bone microenvironment is altered such that more cancer cells escape into the circulation from the tumour mass and they are then mechanically arrest in the lungs. Although the MDA-MB-231-

TXSA cell form more rapidly growing and aggressive tumours than the MCF-7 breast cancer cells, the most possible explanation for this increased incidence in lung metastasis observed in the present study is that OPG by inhibiting osteoclastic bone resorption increases trabecular bone density and this translates to a decrease in bone marrow volume. These changes within the bone microenvironment which may also include changes in vasculature likely promote cancer cells to escape into the extramedullry space which then increases their likelihood of spreading to other organs with the circulation.

Taken together these findings indicate that OPG plays a significant but a controversial role in bone metastases in which accumulating experimental evidence on one hand support its anti-osteoclastogenic and tumour inhibiting actions while in certain other situations it appears to stimulate osteolysis and tumour growth. These contradictory observations require additional experimental designs to delineate the role of OPG in bone metastases.

This inhibition of osteoclast activation and function by OPG raises the very serious question whether other therapeutic agents that inhibit cancer-induced bone breakdown by osteoclast inhibition, such as bisphosphonates, have the same potential to harm by promoting cancer spread to other non-skeletal sites.

Several clinical trials have shown the ability of oral and intravenous bisphosphonates to reduce the incidence and frequency of SREs and skeletal morbidity and prevent bone loss in patients with breast cancer bone metastasis when administrated alone or in combination with adjuvant therapy (Paterson, Powles et al. 1993; Rosen, Gordon et al. 2001; Rosen, Gordon et al. 2003; Body, Diel et al. 2004; Bundred, Campbell et al. 2008; Brufsky, Bosserman et al. 2009; Guise, Brufsky et al. 2010). However, there were some conflicting results in a recent clinical trial with Zolendronic acid and adjuvant therapy. In the AZURE study (Coleman, Marshall et al. 2011) over 3000 patients with stage II/III breast cancer were randomized to receive standard therapy (chemotherapy, endocrine therapy, radiation) or

standard therapy plus 4 mg of zolendronic acid. Unfortunately, there were no significant differences between the patients receiving ZOL in addition to adjuvant therapy compared to patients receive only standard therapy in terms of disease-free survival (DFS) events. However, in a subgroup analysis that evaluated the patients receiving ZOL plus adjuvant therapy by their menopausal status, a 25% reduction in invasive DFS events was found in postmenopausal women compared to patients who were premenopausal or their menopausal status was unknown. Moreover, there was a 30% reduction in extraskeletal recurrences in postmenopausal patients whereas pre- or peri- menopausal women showed an increase risk of extraskeletal metastasis.

These clinical findings suggest that osteoclast inhibition by zolendronic acid treatment leads to an increase in pulmonary metastasis, confirming the results from our preclinical studies. These data also suggest that the reproductive hormones play a key role in the bone remodeling process and on the behavior of cancer cells in the bone microenvironment, increasing their likelihood of spreading elsewhere in the body. This implies that the hormonal environment influences the zolendronic effect on the metastatic potential of the cancer cells.

Taken together, all these data suggest that therapeutic agents which inhibit osteoclastic bone resorption may cause more harm than good by promoting extraskeletal metastasis in patients with osteolytic breast cancer, especially in an environment with high reproductive hormones. It is clearly evident that this phenomenon needs to be further investigated and more studies need to be conducted for a better understanding of the role of osteoclast inhibition and bisphosphonates as antitumour agents for patients with bone metastasis.

CHAPTER 6

DISCUSSION

Discussion

Currently, there is a plethora of treatment modalities for women with early stage breast cancer and early diagnosis leads to patients having a very good prognosis with prolonged term survival (Brewster, Hortobagyi et al. 2008). Unfortunately, 30% of women diagnosed with early stage breast cancer, will eventually progress or relapse with locally advanced or metastatic disease. Metastatic breast cancer consists of subsequent metastasis to distant sites in the body, with skeletal metastases occurring in more than a third of patients with advanced disease where it is devastating and often incurable (Coleman 2001). Breast cancer metastasis to bone is associated with bone destruction resulting in bone loss and a considerable increase in morbidity and erosion of the patient's quality of life (Lipton 2004). Skeletal complications of bone metastases arise because of progressive bone destruction leading to bone pain, spinal cord compression, hypercalcaemia, bone fractures and the need for extensive orthopaedic surgical intervention (Lipton, Uzzo et al. 2009; Smith 2011). The cost of dealing with these complications is enormous. The clinical management of bone metastases in breast cancer patients is thus a significant challenge and, despite recent advances, treatment options remain essentially palliative (Smith 2011). Therefore there is a great need to identify novel, safe and effective therapies for the treatment of metastatic breast cancer.

Apoptosis induction is a key element of the tumour suppressive activity of many conventional cancer therapeutics including chemotherapy and radiation (Prindull 1995; Lowe and Lin 2000; Adams, Totpal et al. 2008). Conventional chemotherapy mediates apoptosis primarily *via* activation of the intrinsic apoptotic signalling pathway involving the mitochondria and modulation of the Bcl-2 family of proteins (Elmore 2007). However, these events are often dependent on functional p53, and mutations leading to p53 inactivation found in more than half of cancers provide a key resistance mechanism by which cancer cells escape

apoptosis induction in response to conventional therapies (Lowe and Lin 2000; Sigal and Rotter 2000). In addition, the development of systemic toxicities associated with conventional anticancer therapies limit their effectiveness in cancer patients. In contrast, activation of the extrinsic apoptotic signalling pathway triggers apoptosis independently of p53 and target cancer cells more selectively with limited or no toxicities to normal tissues and organs (Chinnaiyan, Prasad et al. 2000). Therefore, therapies designed to directly activate this pathway have the potential to induce apoptosis in a wide variety of cancer types irrespective of p53 status, thus evading treatment resistance.

One promising anticancer approach that is gaining momentum is directed towards activation of the extrinsic apoptotic signalling pathway using pro-apoptotic receptor agonists (PARAs) including recombinant soluble Apo2L/TRAIL and agonistic antibodies to its death receptors, currently in early phase clinical trials (Ashkenazi 2008). The potential of soluble Apo2L/TRAIL as an anticancer agent has been well demonstrated in several mouse xenograft models of human soft tissue cancers (Roth, Isenmann et al. 1999; Walczak, Miller et al. 1999; Kelley, Harris et al. 2001; Mitsiades, Treon et al. 2001; Jin, Yang et al. 2004). Previous work from this laboratory has shown that recombinant soluble Apo2L/TRAIL has the ability to induce apoptosis, in a broad range of human cancer cell lines *in vitro* and demonstrated tumour suppressive activity in various xenograft models of cancer including breast cancer and multiple myeloma but most importantly, with no evidence of toxicity to normal cells, tissues and organs (Thai le, Labrinidis et al. 2006; Labrinidis, Diamond et al. 2009). Results from recent clinical trials showed that Apo2L/TRAIL was safe and well-tolerated in patients with advanced tumours indicating Apo2L/TRAIL to be an emerging and exciting anticancer agent for the treatment of solid and haematological malignancies (Pan 2007).

Increased potency and lack of toxicity to normal tissues and organs make Apo2L/TRAIL a new promising anticancer therapeutic. However, in the field of designing

new and safe anticancer agents, there is a shift away from protein-based therapy for reasons such as cost, immuno-reaction and poor pharmacokinetics. Furthermore, the half-life of soluble Apo2L/TRAIL in the body is short, requiring frequent administration for Apo2L/TRAIL to be efficient (Kelley, Harris et al. 2001). Therefore, alternative therapeutics which are long-acting but still activate the extrinsic apoptotic pathway in the same manner as Apo2L/TRAIL will have an added advantage. Drozitumab is an alternative to soluble Apo2L/TRAIL, a fully human and long-acting agonistic monoclonal antibody, designed by Genentech Inc. to specifically bind to the Apo2L/TRAIL pro-apoptotic receptor DR5 and initiate apoptosis by activating the Apo2L/TRAIL signalling pathway (Adams, Totpal et al. 2008).

The first aim of this project was to assess the efficacy of drozitumab *in vitro* and in animal models of orthotopic and osteolytic breast cancer. *In vitro,* drozitumab exhibited potent apoptotic activity against a panel of breast cancer cell lines but was without effect on normal cells. *In vivo,* drozitumab exerted remarkable tumour suppressive activity in an animal model of orthotopic breast cancer leading to complete regression of well-advanced mammary tumours. To assess the efficacy of drozitumab at a metastatic site, animals were transplanted with breast cancer cells directly into their tibiae with all developing large osteolytic lesions with tumour extending into the surrounding soft tissues three to four weeks after cancer cell transplantation that eroded the cortical bone. In contrast, treatment with drozitumab inhibited both intra- and extra-osseous tumour growth and prevented breast cancer induced osteolysis. In a delayed treatment protocol, drozitumab treatment resulted in the complete regression of advanced tibial tumours with progressive restoration of both trabecular and cortical bone leading to full resolution of osteolytic lesions. Therefore, drozitumab represents a potent immunotherapeutic agent with strong activity against the development and progression of

breast cancer and is currently being evaluated in patients with primary and metastatic disease (Suva 2009; Zinonos, Labrinidis et al. 2009).

Unfortunately not all breast cancer cell lines were sensitive to drozitumab-induced apoptosis. Four out of seven cell lines tested exhibited strong resistance to the apoptotic effects of the antibody and the same cell lines were previously shown to be resistant to Apo2L/TRAIL (Zinonos, Labrinidis et al. 2009). Although the mechanisms behind the resistance of these cells are not completely understood, they may involve multiple components of the Apo2L/TRAIL signalling pathway, including downregulation of DR5 or increased expression of the Apo2L/TRAIL decoy receptors DcR1 or DcR2 and the over-expression of intracellular inhibitory proteins such as FLIP or intracellular inhibitor of apoptosis molecules (IAPs). Overall, innate resistance to Apo2L/TRAIL or drozitumab is a process depended upon the balance between the levels of anti-apoptotic and pro-apoptotic factors in the cell. However, resistance could also be acquired which occurs due to adaptive changes in response to therapy (Li, Wang et al. 2006). Indeed, we were able to demonstrate that prolonged treatment of a sensitive breast cancer cell line with drozitumab resulted in the isolation of clones exhibiting extreme resistance to apoptosis induction by this antibody. Such resistance resembles drug resistance that develop in cancer patients following prolonged treatments with certain anticancer agents (Djeu and Wei 2009; Marquette and Nabell 2012). Hence, identifying factors that dictate resistance or sensitivity to anticancer agents, including Apo2L/TRAIL and drozitumab, is of great importance, since identification of such specific biomarkers will identify patients that are likely to benefit from this therapy and help avoid unnecessary treatment on cancer patients that are unlikely to respond.

An important goal of anticancer strategies is the selective induction of apoptosis in cancer cells but not normal cells. In the search for more efficient treatments for cancer, combinations of agents with synergistic or additive activity are attractive because they may

reduce the probability of intrinsic or acquired resistance to either therapy. Combination therapy will also likely enable the use of lower drug doses and reduced toxic side effects. Therefore, the second aim of this project was to investigate the possibility of overcoming drozitumab resistance using chemotherapy regimens that are currently in use for the treatment of breast cancer. DOX is a potent chemotherapeutic agent from the family of anthracyclines, currently in use, in combination with a variety of other types of chemotherapy, for treating patients with primary or metastatic breast cancer. *In vitro* experiments presented in this thesis, demonstrated that combination of drozitumab with DOX treatment resulted in cooperative activity leading to enhanced apoptosis of drozitumab resistant cells. These experiments also identified IAPs to be major determinants involved in drozitumab's resistance, since DOX treatment resulted in significant downregulation of IAP levels in the drozitumab-resistant cells. *In vivo*, DOX treatment in combination with drozitumab resulted in significant inhibition of mammary tumour growth when compared to animals treated with each agent alone and this translated into a survival benefit of mice bearing mammary tumours. Therefore, this study indicates a cooperative interaction between drozitumab and DOX in this orthotopic mammary cancer model and provides support for further clinical investigation of this combination approach in patients with advanced or metastatic breast cancer.

Resistant cancer cells have defects in their ability to die *via* apoptosis, and unfortunately, Apo2L/TRAIL or drozitumab therapy may not always be effective in cells that have certain defects in apoptotic pathways. Differential expression of Apo2L/TRAIL receptors may in some instances play a significant role in determining Apo2L/TRAIL resistance. However the studies presented here and many others have shown no consistent correlation between Apo2L/TRAIL receptor expression and sensitivity to Apo2L/TRAIL-induced apoptosis (Ashkenazi and Dixit 1999; Huang and Sheikh 2007). A previous study by this laboratory investigating the efficacy of Apo2L/TRAIL in skeletal malignancies showed

that recombinant soluble Apo2L/TRAIL decreased tumour burden within the bone marrow and reduced cancer-induced bone destruction in a murine model of osteolytic breast cancer (Thai le, Labrinidis et al. 2006). However, treatment with soluble Apo2L/TRAIL failed to completely eradicate tumours from the bone, giving rise to late recurrence and suggested that Apo2L/TRAIL therapy in bone may not be 100% efficient. To understand the possible reasons for the acquired Apo2L/TRAIL resistance leading to recurrence in this setting, subsequent studies presented in this thesis aimed to investigate the role of OPG in this context for the following reasons. OPG is a key regulator of normal bone metabolism and is expressed in high levels in the bone. In addition, OPG is a decoy receptor to Apo2L/TRAIL and acts as a functional inhibitor of its apoptotic activity, raising the possibility that the activity of Apo2L/TRAIL may be suppressed in the bone microenvironment where OPG expression is normally high.

Therefore, the third aim of this study was to address whether local production of OPG by breast cancer cells limits Apo2L/TRAIL antitumour efficacy. For these studies, human breast cancer cells were engineered to overexpress full length human OPG and were transplanted directly into the tibial marrow cavity of athymic nude mice where the efficacy of Apo2L/TRAIL was assessed. The results of this study demonstrated that while Apo2L/TRAIL-induced apoptosis was abrogated *in vitro* by OPG overexpression, the *in vivo* anticancer efficacy of recombinant soluble Apo2L/TRAIL was retained in the bone microenvironment even in the face of biologically active OPG when secreted at supra-physiological concentrations. Therefore, these results provide convincing evidence that OPG present within the bone microenvironment is unlikely to play a significant role in modulating the therapeutic potential of recombinant soluble Apo2L/TRAIL *in vivo*, unlike what has been suggested by others in the field (Emery, McDonnell et al. 1998; Truneh, Sharma et al. 2000;

Holen, Croucher et al. 2002; Shipman and Croucher 2003; Neville-Webbe, Cross et al. 2004; Holen and Shipman 2006; Sandra, Hendarmin et al. 2006).

OPG plays a significant role in bone remodelling by inhibiting osteoclastic bone resorption (Hofbauer, Neubauer et al. 2001; Ando, Mori et al. 2008). When breast cancer cells metastasise to the bone, they release growth factors that stimulate bone resorption. These growth factors, stimulate further proliferation of the tumour cells, and that establishes a vicious cycle of continuous tumour cell growth and stimulation of osteolysis. Disrupting the vicious cycle and targeting these interactions and pathways in the tumour-bone microenvironment by inhibiting bone resorption has been an encouraging strategy for bone metastasis therapy.

The previous study described in this thesis (Zinonos, Labrinidis et al. 2011) provided an opportunity to also investigate the potential of OPG in modulating the vicious cycle of cancer-induced bone destruction. Previous studies have shown that systemic administration of OPG-Fc decreased tumour burden in bone and protected the bone from osteolysis (Buijs, Que et al. 2009; Zinonos, Labrinidis et al. 2011). To investigate if inhibition of the vicious cycle could result in inhibition of tumour growth in the bone, a separate study was contacted in which the effects of native full-length human OPG when produced and secreted locally by human breast cancer cells in the bone microenvironment were assessed. The results demonstrated that local production of biologically active OPG inhibited osteoclast formation and bone resorption, and maintained cortical and trabecular bone integrity. Notably, despite the bone protection, OPG over-expression failed to reduce the overall tumour burden as assessed by bioluminescence imaging (BLI). However, a detailed histological analysis of the tibiae demonstrated that intra-osseous tumour burden was diminished significantly whereas cancer cells persisted to grow in the extra-medullary space of mice bearing tumours over-expressing OPG. Interestingly, this failure to eliminate tumour growth in the extra-medullary

space was associated with a significant increase in pulmonary metastasis. These data substantiate recently published results by this lab showing that osteoclast ablation with zoledronic acid (ZOL) had no effect on osteosarcoma burden within the bone but it actually increased the propensity of osteosarcoma cells to metastasise to the lungs (Labrinidis, Hay et al. 2009). Furthermore, Endo-Munoz et al, (Endo-Munoz, Cumming et al. 2010) in collaboration with this laboratory, provided clinical evidence that loss of osteoclasts due to bisphosphonate treatment contributed to increased incidence of lung metastasis in osteosarcoma patients. Collectively, these data suggest that disturbing the vicious cycle via osteoclast ablation, using anti-resorptive agents, such as bisphosphonates or recombinant OPG may modulate the bone microenvironment in a way that affects the likelihood of cancer cells to spread and establish metastases in other distant sites in the body. While the mechanisms for the observed increase in metastases following osteoclast ablation in this context are not fully understood, results of the recent AZURE clinical trial provide further insight into the factors involved.

In the AZURE clinical study (Coleman, Marshall et al. 2011), 3360 women with stage II or III breast cancer were randomized to receive adjuvant standard therapy (96% were treated with chemotherapy, including anthracyclines and taxanes, and only 152 women were treated with endocrine therapy only) or standard therapy plus 4 mg of zolendronic acid intravenously. Collectively, the results from the study showed no significant effect of zolendronic acid treatment either on disease free or overall survival. However, in a subset analysis that evaluated patients based on the menopausal status, zolendronic acid showed a significant effect on overall survival in a subgroup of women (n=1101) with a postmenopausal status for at least 5 years. These women demonstrated a 29% reduction in the risk of dying compared to pre- or peri-menopausal women receiving the same treatment.

Moreover, there was a 30% reduction in extraskeletal recurrences in postmenopausal patients whereas pre- or peri- menopausal women showed an increase risk of extraskeletal metastasis.

All these data raise the very important question of the role of reproductive hormones in the bone microenvironment and specifically in bone remodeling in postmenopausal women, which is regulated by different hormones than in young women (activins and BMPs instead of estradiol and inhibins). These hormonal changes obviously have a dramatic effect on tumour cells in the bone but more importantly it is clearly evident that the outcome of anti-resorptive treatments like ZOL are heavily depended on the hormonal status of each patient. The reason why ZOL works better in an estrogen deprived environment as opposed to an estrogen rich environment is clearly something that needs further investigation. Additional studies are urgently needed to clarify the role of the vicious cycle in metastatic breast cancer but more importantly future studies need to assess the role of reproductive hormones in the metastatic process, in both preclinical and clinical settings. While the majority of experts in the bone and metastasis field support the adjuvant bisphosphonate therapy in breast cancer, this approach should only be considered in an environment of low reproductive hormones. They also agree that bisphosphonates are effective anti-resorptive agents and might be quite helpful for women with a postmenopausal status.

Critical Evaluation

The studies presented in this thesis exploit the therapeutic potential of pro-apoptotic receptor agonist including, Apo2L/TRAIL and drozitumab, in animal models of primary and metastatic breast cancer. In this section a critical evaluation will be presented of the animal models used, methodology and data collected.

Animal Xenograft Models

Xenograft models of breast cancer growth are a useful tool in cancer research and are widely used for the evaluation of the efficacy of anticancer agents. Many human breast cancer–derived cell lines are tumorigenic in nude mice and selected cell variants, which have increased incidence of metastasis to bone, have been generated. We used the oestrogen-independent MDA-MB-231 cell line derivative MDA-MB231-TXSA, which was selected *in vivo* for its preferential bone metastasis properties and enhanced osteolytic activity.

MDA-MB231-TXSA cells injected in the mammary gland form aggressive tumours rapidly which then allows the evaluation of our anticancer agents at the orthotopic site. When transplanted directly in bone, these tumour cells grow and produce osteolytic lesions in the area of injection. We used the intratibial injection animal model for these studies for a number of reasons. Firstly, there is a need to establish whether the pharmacodistribution and pharmacokinetics of Apo2L/TRAIL and drozitumab ensures their efficient accumulation in the bone microenvironment and their ability to exert anticancer activity. Second, this model results in localized tumour growth at a single bony site that produces a consistent measurable outcome, in which the effect of Apo2L/TRAIL and drozitumab treatment could be assessed.

A limitation in measuring tumour burden in bone is that it is not possible to accurately assess the progression of tumour growth by palpation as in soft tissue tumours, because these tumours cannot be felt before they break through the cortical bone. To overcome this

limitation, we used non-invasive BLI, which enabled extremely sensitive tracking of breast cancer burden in bone in real-time before and after treatment in live animals. In addition, the micro-CT imaging not only allowed a precise qualitative description of Apo2L/TRAIL and drozitumab treatment effects on bone architecture but also provided accurate quantitative bone morphometry.

The implantation of established cell lines derived from human breast cancer in animals is relatively simple and allows the genetic or pharmacological manipulation of the implanted cells. However, there are clear limitations to xenograft models. First, immune responses, which have a key role during tumour development, are impaired in immunocompromised mice. Second, stromal components are not of tumour origin and therefore the stroma-tumour interactions which are essential for tumour progression are suppressed. Last, human cells are not fully adapted to grow in a murine environment. A specific limitation of the intratibial model is that breaching bone integrity, for example by drilling can result in ectopic bone formation even though this has not been observed in our animal models. In addition, when removing the needle from the tibia following the cancer cell transplantation, there is an increased risk of residual growth of cancer cells in the extramedullry space which could account for the increased metastatic potential of these cells.

Generation of cacner cell-resistance

The breast cancer cell line MDA-MB-23-TXSA was cultured in the presence of drozitumab for a prolonged period of time and as a result there was generation of a resistant subline, denoted as MDA-MB-23-TXSA-droz-R. Although this technique closely resembles the acquired resistance often seen in patients following prolonged period of treatment with certain anticancer agents, it is possible that it enabled selection of already resistant clones to this antibody rather that generation of resistance. Another limitation is that there is an increased possibility of the resistant clones to become resistant to the combination of

drozitumab and DOX, despite their current increased sensitivity to this combination of agents. This could happen both *in vitro* and *in vivo* in the same way acquired resistant was obtained in the first place, by generating clones that are already intrinsically resistant to both agents. This result will require identification of a different therapeutic combination of agents against breast cancer cells which are resistant to both DOX and drozitumab.

Future Directions

The basis for the sensitivity and resistance of cells to Apo2L/TRAIL or drozitumab-induced apoptosis is not well understood and is under intense investigation by many laboratories around the world. It is then of paramount importance to define novel pathways and identify the mechanisms that regulate susceptibility/resistance to Apo2L/TRAIL or drozitumab in cancer cells. Furthermore, future studies should focus on the identification of specific biomarkers that predict treatment efficacy of Apo2L/TRAIL or drozitumab with the aim to prevent chemotherapy-induced toxicity and the unnecessary burden of adverse events on cancer patients unlikely to respond.

A recent study trying to identify biomarkers that dictate this resistance to rhApo2L/TRAIL demonstrated that elevated expression of specific O-glycosyltransferases, which translates into death receptor modifications, significantly correlated with sensitivity to Apo2L/TRAIL (Stern, Padilla et al. 2010). According to Wagner et al, O-glycosylation of death receptors in cancer cells modulates sensitivity to Apo2L/TRAIL by promoting ligand-induced receptor clustering and consequent caspase-8 activation. These findings suggest the potential utility of specific O-glycosylation enzymes or their modified targets as predictive biomarkers for Apo2L/TRAIL-based cancer therapy.

Various preclinical studies, including studies from this laboratory, have revealed that while many cancers express both DR4 and DR5, activation of the death receptors with Apo2L/TRAIL or drozitumab is not sufficient enough to achieve significant efficacy, particularly in cancer cells displaying strong resistance to these agents (Bouralexis, Findlay et al. 2003; LeBlanc and Ashkenazi 2003). This suggests that other factors, downstream of the apoptotic signalling pathway play a significant role in limiting the anticancer efficacy of these agents. Data presented in this thesis showed that drozitumab-resistant cells express high levels

of inhibitors of apoptosis proteins (IAPs) and DOX treatment, dramatically reduced IAP levels in these cells, thus restoring their sensitivity to drozitumab. This suggests that IAP proteins may play a critical role in modulating Apo2L/TRAIL or drozitumab apoptosis activity, as also demonstrated recently by Varfolomeev and colleagues, and therefore could represent a potential biomarker to identify patients that are likely to respond to treatment with these agents (Varfolomeev, Alicke et al. 2009; Varfolomeev and Vucic 2011). Future studies assessing this phenomenon are required and will prove very useful in clinical outcomes.

Another family of molecules that may represent an attractive target in anticancer therapeutic protocols and its currently gaining momentum due to their implications in cancer is microRNAs. MicroRNAs (miRNAs) are a class of recently discovered non-coding RNA genes that regulate cell differentiation, cell proliferation and apoptosis through translational inhibition and mRNA degradation (Fabbri, Valeri et al. 2009; Ma, Bai et al. 2012; Pritchard, Cheng et al. 2012). It is now becoming clear that miRNAs play an important role in regulating gene expression. Recent evidence indicates that alterations in miRNAs expression may play a critical role in cancer initiation and progression (Calin and Croce 2006; Calin and Croce 2006; Stahlhut Espinosa and Slack 2006). MiRNA expression profiles may also be a more accurate and useful method for the classification, diagnosis, or prognosis of some human malignancies (Esquela-Kerscher and Slack 2006; Volinia, Calin et al. 2006). Our understanding of miRNA expression patterns in cancer patients is starting to emerge, with several miRNAs reported to be associated with clinical outcome of certain tumours (Yu, Chen et al. 2007). Previously in this laboratory, a genome-wide expression profiling of miRNAs was performed in Apo2L/TRAIL sensitive and resistant breast cancer cells. In Apo2L/TRAIL-sensitive MDA-MB-231-TXSA-**S** cells, the levels of several miRNAs were increased dramatically, and in particular, miR-146a**,** mirR-319, miR540, and miR1423 were

more than 10 fold higher when compared to the Apo2L/TRAIL resistant MDA-MB-231-TXSA-R cells.

The intention of this laboratory is to focus on miR-146, since this miRNA was recently implicated as a negative regulator of Toll-like receptor (TLR) signalling. In the context of this miRNA screening it was recently shown that miR-146 is upregulated in an NF-κB dependent manner in response to TLR activation by microbial components and pro-inflammatory mediators. In addition, IRAK was shown to be a direct target for miR-146, suggesting that miR-146 may function as a novel negative regulator that helps to fine-tune the immune response (Taganov, Boldin et al. 2006). Recent findings showed that IRAK physically associates with FADD (Zhande, Dauphinee et al. 2007), a pivotal adaptor protein required for the efficient assembly of the Apo2L/TRAIL DISC, leading to the recruitment and activation of caspase 8. To date there have been no data implicating miR-146 and/or IRAK in Apo2L/TRAIL-induced apoptosis. Previous studies in this lab have shown that indeed IRAK1 is a target of miR-146 and is dramatically down-regulated in the Apo2L/TRAIL sensitive breast cancer cells, in which expression of miR-146 is high. However, the consequence of modulating miR-146 expression in sensitive/resistant cells has not yet been assessed.

Based on this new information, it is hypothesised that Apo2L/TRAIL sensitive cells have *high* levels of miR-146 leading to degradation of IRAK1. Conversely, Apo2L/TRAIL resistant cells with *low* levels of miR-146 would accumulate high levels of IRAK1, capable of binding to FADD, thus sequestering FADD away from the Apo2L/TRAIL DISC and resulting in the abrogation of Apo2L/TRAIL-drozitumab signalling. Consistent with this idea, this laboratory has previously shown, using Apo2L/TRAIL DISC immunoprecipitation experiments that the level of FADD recruited to the Apo2L/TRAIL DISC in Apo2L/TRAIL resistant cells is impaired, resulting in the failure of caspase 8 to be recruited and activated, despite the presence of death receptors DR4 and DR5 within the DISC. An additional line of

evidence for this hypothesis is that an independent genome-scale miRNA and small interfering RNA screening has also identified IRAK as a modulator of the Apo2L/TRAIL-induced apoptosis pathway in breast cancer cells, further supporting a role of IRAK in this process (Ovcharenko, Kelnar et al. 2007). Taken together, these findings define a previously unrecognised pathway that may regulate susceptibility/resistance to Apo2L/TRAIL-drozitumab in cancer cells, and indicate that IRAK may be a therapeutic target in cancer therapy with diagnostic and prognostic value. Therefore, a better understanding of the molecular mechanisms, by which IRAK modulates Apo2L/TRAIL susceptibility, will have important clinical implications for Apo2L/TRAIL and drozitumab therapy. Future directions of this project consist of the investigation in detail the potential role of miR-146 and IRAK1 in regulating Apo2L/TRAIL sensitivity/resistance using well-established *in vitro* model systems.

In conclusion, the studies presented in this thesis have demonstrated that PARAs, including Apo2L/TRAIL and drozitumab, represent novel anticancer agents with exciting therapeutic potential. However, the efficacy of Apo2L/TRAIL and drozitumab, alone or in combination with conventional chemotherapeutics, needs to be further investigated in future preclinical and clinical settings, particularly in cancers exhibiting resistance to these agents. Further assessment of the molecular mechanisms that dictate this resistance and identification of specific biomarkers for these agents will provide valuable information to identify the most effective therapy for primary and metastatic breast cancer.

BIBLIOGRAPHY

Bibliography

*(2012). "Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies." Lancet Oncol **13**(11): 1141-1151.*

ACS. (2011). "Breast Cancer Facts and Figures 2011-2012." American Cancer Society Inc, NW, Atlanta, GA.

*Adams, C., K. Totpal, et al. (2008). "Structural and functional analysis of the interaction between the agonistic monoclonal antibody Apomab and the proapoptotic receptor DR5." Cell Death Differ **15**(4): 751-761.*

*Ambrosini, G., C. Adida, et al. (1997). "A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma." Nat Med **3**(8): 917-921.*

*Anderson, D. M., E. Maraskovsky, et al. (1997). "A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function." Nature **390**(6656): 175-179.*

*Ando, K., K. Mori, et al. (2008). "RANKL/RANK/OPG: key therapeutic target in bone oncology." Curr Drug Discov Technol **5**(3): 263-268.*

*Ashkenazi, A. (2002). "Targeting death and decoy receptors of the tumour-necrosis factor superfamily." Nat Rev Cancer **2**(6): 420-430.*

*Ashkenazi, A. (2008). "Directing cancer cells to self-destruct with pro-apoptotic receptor agonists." Nat Rev Drug Discov **7**(12): 1001-1012.*

*Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**(5381): 1305-1308.*

*Ashkenazi, A. and V. M. Dixit (1999). "Apoptosis control by death and decoy receptors." Curr Opin Cell Biol **11**(2): 255-260.*

*Beaumont, T. and M. Leadbeater (2011). "Treatment and care of patients with metastatic breast cancer." Nurs Stand **25**(40): 49-56.*

*Belyanskaya, L. L., T. M. Marti, et al. (2007). "Human agonistic TRAIL receptor antibodies Mapatumumab and Lexatumumab induce apoptosis in malignant mesothelioma and act synergistically with cisplatin." Mol Cancer **6**: 66.*

*Body, J. J., I. J. Diel, et al. (2004). "Oral ibandronate improves bone pain and preserves quality of life in patients with skeletal metastases due to breast cancer." Pain **111**(3): 306-312.*

*Bouralexis, S., D. M. Findlay, et al. (2003). "Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy." Br J Cancer **89**(1): 206-214.*

*Bouralexis, S., D. M. Findlay, et al. (2005). "Death to the bad guys: targeting cancer via Apo2L/TRAIL." Apoptosis **10**(1): 35-51.*

*Boyce, B. F. and L. Xing (2008). "Functions of RANKL/RANK/OPG in bone modeling and remodeling." Arch Biochem Biophys **473**(2): 139-146.*

Boyce, B. F., L. Xing, et al. (2003). "Regulation of bone remodeling and emerging breakthrough drugs for osteoporosis and osteolytic bone metastases." Kidney Int Suppl(85): S2-5.

*Brewster, A. M., G. N. Hortobagyi, et al. (2008). "Residual risk of breast cancer recurrence 5 years after adjuvant therapy." J Natl Cancer Inst **100**(16): 1179-1183.*

*Britt, K. (2012). "Menarche, menopause, and breast cancer risk." Lancet Oncol **13**(11): 1071-1072.*

*Brufsky, A. M., L. D. Bosserman, et al. (2009). "Zoledronic acid effectively prevents aromatase inhibitor-associated bone loss in postmenopausal women with early breast cancer receiving adjuvant letrozole: Z-FAST study 36-month follow-up results." Clin Breast Cancer **9**(2): 77-85.*

*Buchsbaum, D. J., T. Zhou, et al. (2003). "Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model." Clin Cancer Res **9**(10 Pt 1): 3731-3741.*

*Buijs, J. T., I. Que, et al. (2009). "Inhibition of bone resorption and growth of breast cancer in the bone microenvironment." Bone **44**(2): 380-386.*

*Buijs, J. T. and G. van der Pluijm (2009). "Osteotropic cancers: from primary tumor to bone." Cancer Lett **273**(2): 177-193.*

*Bundred, N. J., I. D. Campbell, et al. (2008). "Effective inhibition of aromatase inhibitor-associated bone loss by zoledronic acid in postmenopausal women with early breast cancer receiving adjuvant letrozole: ZO-FAST Study results." Cancer **112**(5): 1001-1010.*

*Calin, G. A. and C. M. Croce (2006). "MicroRNA-cancer connection: the beginning of a new tale." Cancer Res **66**(15): 7390-7394.*

*Calin, G. A. and C. M. Croce (2006). "MicroRNA signatures in human cancers." Nat Rev Cancer **6**(11): 857-866.*

*Camidge, D. R. (2008). "Apomab: an agonist monoclonal antibody directed against Death Receptor 5/TRAIL-Receptor 2 for use in the treatment of solid tumors." Expert Opin Biol Ther **8**(8): 1167-1176.*

*Camidge, D. R., R. S. Herbst, et al. (2010). "A phase I safety and pharmacokinetic study of the death receptor 5 agonistic antibody PRO95780 in patients with advanced malignancies." Clin Cancer Res **16**(4): 1256-1263.*

*Cardones, A. R., T. Murakami, et al. (2003). "CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via beta(1) integrin." Cancer Res **63**(20): 6751-6757.*

*Chambers, A. F., A. C. Groom, et al. (2002). "Dissemination and growth of cancer cells in metastatic sites." Nat Rev Cancer **2**(8): 563-572.*

*Chicheportiche, Y., P. R. Bourdon, et al. (1997). "TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis." J Biol Chem **272**(51): 32401-32410.*

*Chinnaiyan, A. M., U. Prasad, et al. (2000). "Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy." Proc Natl Acad Sci U S A **97**(4): 1754-1759.*

*Chu, J. E. and A. L. Allan (2012). "The Role of Cancer Stem Cells in the Organ Tropism of Breast Cancer Metastasis: A Mechanistic Balance between the "Seed" and the "Soil"?" Int J Breast Cancer **2012**: 209748.*

*Chuntharapai, A., K. Dodge, et al. (2001). "Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4." J Immunol **166**(8): 4891-4898.*

*Clines, G. A. and T. A. Guise (2005). "Hypercalcaemia of malignancy and basic research on mechanisms responsible for osteolytic and osteoblastic metastasis to bone." Endocr Relat Cancer **12**(3): 549-583.*

Coleman, R., L. Costa, et al. (2011). "Consensus on the utility of bone markers in the malignant bone disease setting." Crit Rev Oncol Hematol.

*Coleman, R. E. (2001). "Metastatic bone disease: clinical features, pathophysiology and treatment strategies." Cancer Treat Rev **27**(3): 165-176.*

*Coleman, R. E. (2006). "The role of bone markers in metastatic bone disease." Cancer Treat Rev **32 Suppl 1**: 1-2.*

*Coleman, R. E., A. Lipton, et al. (2010). "Metastasis and bone loss: advancing treatment and prevention." Cancer Treat Rev **36**(8): 615-620.*

*Coleman, R. E., H. Marshall, et al. (2011). "Breast-cancer adjuvant therapy with zoledronic acid." N Engl J Med **365**(15): 1396-1405.*

*Collaborative Group on Hormonal Factors in Breast Cancer (1996). "Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Collaborative Group on Hormonal Factors in Breast Cancer." Lancet **347**(9017): 1713-1727.*

*Collaborative Group on Hormonal Factors in Breast Cancer (1997). "Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer." Lancet **350**(9084): 1047-1059.*

*Collaborative Group on Hormonal Factors in Breast Cancer (2002). "Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease." Lancet **360**(9328): 187-195.*

*Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-656.*

*Croucher, P. I., C. M. Shipman, et al. (2001). "Osteoprotegerin inhibits the development of osteolytic bone disease in multiple myeloma." Blood **98**(13): 3534-3540.*

*De Palma, M., M. A. Venneri, et al. (2003). "Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells." Nat Med **9**(6): 789-795.*

*Degli-Esposti, M. A., W. C. Dougall, et al. (1997). "The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain." Immunity **7**(6): 813-820.*

*Degli-Esposti, M. A., P. J. Smolak, et al. (1997). "Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family." J Exp Med **186**(7): 1165-1170.*

*Demers, L. M., L. Costa, et al. (2000). "Biochemical markers and skeletal metastases." Cancer **88**(12 Suppl): 2919-2926.*

*Djeu, J. Y. and S. Wei (2009). "Clusterin and chemoresistance." Adv Cancer Res **105**: 77-92.*

*Duiker, E. W., C. H. Mom, et al. (2006). "The clinical trail of TRAIL." Eur J Cancer **42**(14): 2233-2240.*

*Elmore, S. (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.*

*Emery, J. G., P. McDonnell, et al. (1998). "Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL." J Biol Chem **273**(23): 14363-14367.*

*Endo-Munoz, L., A. Cumming, et al. (2010). "Loss of osteoclasts contributes to development of osteosarcoma pulmonary metastases." Cancer Res **70**(18): 7063-7072.*

*Esposti, M. D. (2002). "The roles of Bid." Apoptosis **7**(5): 433-440.*

*Esquela-Kerscher, A. and F. J. Slack (2006). "Oncomirs - microRNAs with a role in cancer." Nat Rev Cancer **6**(4): 259-269.*

Ewing, J. (1928). "A Treatise on Tumors." W.B. Saunders Company,Philadelphia, Pa, USA.

*Fabbri, M., N. Valeri, et al. (2009). "MicroRNAs and genomic variations: from Proteus tricks to Prometheus gift." Carcinogenesis **30**(6): 912-917.*

*Falschlehner, C., C. H. Emmerich, et al. (2007). "TRAIL signalling: decisions between life and death." Int J Biochem Cell Biol **39**(7-8): 1462-1475.*

*Ferlay, J., H. R. Shin, et al. (2010). "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008." Int J Cancer **127**(12): 2893-2917.*

*Fisher, J. L., R. J. Thomas-Mudge, et al. (2006). "Osteoprotegerin overexpression by breast cancer cells enhances orthotopic and osseous tumor growth and contrasts with that delivered therapeutically." Cancer Res **66**(7): 3620-3628.*

*Folkman, J. and R. Kalluri (2004). "Cancer without disease." Nature **427**(6977): 787.*

*Foroni, C., M. Broggini, et al. (2012). "Epithelial-mesenchymal transition and breast cancer: role, molecular mechanisms and clinical impact." Cancer Treat Rev **38**(6): 689-697.*

*Forouzanfar, M. H., K. J. Foreman, et al. (2011). "Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis." Lancet **378**(9801): 1461-1484.*

*Fulda, S. and D. Vucic (2012). "Targeting IAP proteins for therapeutic intervention in cancer." Nat Rev Drug Discov **11**(2): 109-124.*

*Georgakis, G. V., Y. Li, et al. (2005). "Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death." Br J Haematol **130**(4): 501-510.*

*Gliniak, B. and T. Le (1999). "Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity in vivo is enhanced by the chemotherapeutic agent CPT-11." Cancer Res **59**(24): 6153-6158.*

*Guise, T. (2010). "Examining the metastatic niche: targeting the microenvironment." Semin Oncol **37 Suppl 2**: S2-14.*

*Guise, T. A. (2000). "Molecular mechanisms of osteolytic bone metastases." Cancer **88**(12 Suppl): 2892-2898.*

*Guise, T. A., A. Brufsky, et al. (2010). "Understanding and optimizing bone health in breast cancer." Curr Med Res Opin **26 Suppl 3**: 3-20.*

*Guise, T. A., W. M. Kozlow, et al. (2005). "Molecular mechanisms of breast cancer metastases to bone." Clin Breast Cancer **5 Suppl**(2): S46-53.*

*Guise, T. A. and G. R. Mundy (1998). "Cancer and bone." Endocr Rev **19**(1): 18-54.*

*Herbst, R. S., R. Kurzrock, et al. (2010). "A first-in-human study of conatumumab in adult patients with advanced solid tumors." Clin Cancer Res **16**(23): 5883-5891.*

*Hill, M. M., C. Adrain, et al. (2004). "Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes." EMBO J **23**(10): 2134-2145.*

*Hofbauer, L. C., A. Neubauer, et al. (2001). "Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases." Cancer **92**(3): 460-470.*

*Holen, I., P. I. Croucher, et al. (2002). "Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells." Cancer Res **62**(6): 1619-1623.*

*Holen, I. and C. M. Shipman (2006). "Role of osteoprotegerin (OPG) in cancer." Clin Sci (Lond) **110**(3): 279-291.*

*Hollier, B. G., K. Evans, et al. (2009). "The epithelial-to-mesenchymal transition and cancer stem cells: a coalition against cancer therapies." J Mammary Gland Biol Neoplasia **14**(1): 29-43.*

*Hoon, D. S., R. Ferris, et al. (2011). "Molecular mechanisms of metastasis." J Surg Oncol **103**(6): 508-517.*

*Hoon, D. S., M. Kitago, et al. (2006). "Molecular mechanisms of metastasis." Cancer Metastasis Rev **25**(2): 203-220.*

*Huang, Y. and M. S. Sheikh (2007). "TRAIL death receptors and cancer therapeutics." Toxicol Appl Pharmacol **224**(3): 284-289.*

*Hulka, B. S. and P. G. Moorman (2008). "Breast cancer: hormones and other risk factors." Maturitas **61**(1-2): 203-213; discussion 213.*

*Hunter, D. J., D. Spiegelman, et al. (1996). "Cohort studies of fat intake and the risk of breast cancer--a pooled analysis." N Engl J Med **334**(6): 356-361.*

*Ibrahim, T., I. Leong, et al. (2000). "Expression of bone sialoprotein and osteopontin in breast cancer bone metastases." Clin Exp Metastasis **18**(3): 253-260.*

*Ichikawa, K., W. Liu, et al. (2001). "Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity." Nat Med **7**(8): 954-960.*

*Ikeda, T., M. Kasai, et al. (2001). "Determination of three isoforms of the receptor activator of nuclear factor-kappaB ligand and their differential expression in bone and thymus." Endocrinology **142**(4): 1419-1426.*

*Jacob, K., M. Webber, et al. (1999). "Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone." Cancer Res **59**(17): 4453-4457.*

*Jemal, A., F. Bray, et al. (2011). "Global cancer statistics." CA Cancer J Clin **61**(2): 69-90.*

*Jemal, A., R. Siegel, et al. (2010). "Cancer statistics, 2010." CA Cancer J Clin **60**(5): 277-300.*

*Jin, H., R. Yang, et al. (2004). "Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand cooperates with chemotherapy to inhibit orthotopic lung tumor growth and improve survival." Cancer Res **64**(14): 4900-4905.*

*Jin, H., R. Yang, et al. (2008). "Cooperation of the agonistic DR5 antibody apomab with chemotherapy to inhibit orthotopic lung tumor growth and improve survival." Clin Cancer Res **14**(23): 7733-7740.*

*Jin, Z. and W. S. El-Deiry (2005). "Overview of cell death signaling pathways." Cancer Biol Ther **4**(2): 139-163.*

*Kakonen, S. M. and G. R. Mundy (2003). "Mechanisms of osteolytic bone metastases in breast carcinoma." Cancer **97**(3 Suppl): 834-839.*

*Kang, H. J., S. H. Lee, et al. (2009). "Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB in breast cancer cells and potentiates the growth inhibitory effect of paclitaxel in a breast cancer nude mice model." Breast J **15**(3): 223-229.*

*Kang, Y., P. M. Siegel, et al. (2003). "A multigenic program mediating breast cancer metastasis to bone." Cancer Cell **3**(6): 537-549.*

*Kaplan, R. N., R. D. Riba, et al. (2005). "VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche." Nature **438**(7069): 820-827.*

*Karsenty, G. and E. F. Wagner (2002). "Reaching a genetic and molecular understanding of skeletal development." Dev Cell **2**(4): 389-406.*

*Kelley, S. K., L. A. Harris, et al. (2001). "Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety." J Pharmacol Exp Ther **299**(1): 31-38.*

*Kelsey, J. L. (1993). "Breast cancer epidemiology: summary and future directions." Epidemiol Rev **15**(1): 256-263.*

*Kelsey, J. L., M. D. Gammon, et al. (1993). "Reproductive factors and breast cancer." Epidemiol Rev **15**(1): 36-47.*

*Kingsley, L. A., P. G. Fournier, et al. (2007). "Molecular biology of bone metastasis." Mol Cancer Ther **6**(10): 2609-2617.*

*Kohli, S. S. and V. S. Kohli (2011). "Role of RANKL-RANK/osteoprotegerin molecular complex in bone remodeling and its immunopathologic implications." Indian J Endocrinol Metab **15**(3): 175-181.*

*Kozlow, W. and T. A. Guise (2005). "Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy." J Mammary Gland Biol Neoplasia **10**(2): 169-180.*

*Labrinidis, A., P. Diamond, et al. (2009). "Apo2L/TRAIL inhibits tumor growth and bone destruction in a murine model of multiple myeloma." Clin Cancer Res **15**(6): 1998-2009.*

*Labrinidis, A., S. Hay, et al. (2009). "Zoledronic acid inhibits both the osteolytic and osteoblastic components of osteosarcoma lesions in a mouse model." Clin Cancer Res **15**(10): 3451-3461.*

*LaCasse, E. C., S. Baird, et al. (1998). "The inhibitors of apoptosis (IAPs) and their emerging role in cancer." Oncogene **17**(25): 3247-3259.*

*LaCasse, E. C., D. J. Mahoney, et al. (2008). "IAP-targeted therapies for cancer." Oncogene **27**(48): 6252-6275.*

*Lacey, D. L., E. Timms, et al. (1998). "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation." Cell **93**(2): 165-176.*

*Lagadec, C., E. Adriaenssens, et al. (2008). "Tamoxifen and TRAIL synergistically induce apoptosis in breast cancer cells." Oncogene **27**(10): 1472-1477.*

*Lambe, M., C. Hsieh, et al. (1994). "Transient increase in the risk of breast cancer after giving birth." N Engl J Med **331**(1): 5-9.*

*Langley, R. R. and I. J. Fidler (2011). "The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs." Int J Cancer **128**(11): 2527-2535.*

*LeBlanc, H. N. and A. Ashkenazi (2003). "Apo2L/TRAIL and its death and decoy receptors." Cell Death Differ **10**(1): 66-75.*

*Li, H., H. Zhu, et al. (1998). "Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis." Cell **94**(4): 491-501.*

*Li, Y., H. Wang, et al. (2006). "Inducible resistance of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand receptor 2-mediated apoptosis by generation of a blockade at the death domain function." Cancer Res **66**(17): 8520-8528.*

*Lin, T., L. Zhang, et al. (2003). "Combination of TRAIL gene therapy and chemotherapy enhances antitumor and antimetastasis effects in chemosensitive and chemoresistant breast cancers." Mol Ther **8**(3): 441-448.*

*Lipton, A. (2004). "Pathophysiology of bone metastases: how this knowledge may lead to therapeutic intervention." J Support Oncol **2**(3): 205-213; discussion 213-204, 216-207, 219-220.*

*Lipton, A., R. Uzzo, et al. (2009). "The science and practice of bone health in oncology: managing bone loss and metastasis in patients with solid tumors." J Natl Compr Canc Netw **7 Suppl 7**: S1-29; quiz S30.*

*Locksley, R. M., N. Killeen, et al. (2001). "The TNF and TNF receptor superfamilies: integrating mammalian biology." Cell **104**(4): 487-501.*

*Lowe, S. W. and A. W. Lin (2000). "Apoptosis in cancer." Carcinogenesis **21**(3): 485-495.*

*Luker, K. E. and G. D. Luker (2006). "Functions of CXCL12 and CXCR4 in breast cancer." Cancer Lett **238**(1): 30-41.*

*Luo, J., K. L. Margolis, et al. (2011). "Association of active and passive smoking with risk of breast cancer among postmenopausal women: a prospective cohort study." BMJ **342**: d1016.*

*Lyden, D., K. Hattori, et al. (2001). "Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth." Nat Med **7**(11): 1194-1201.*

Ma, S. Y., Y. Bai, et al. (2012). "[Recent research progress of biogenesis and functions of miRNA]." Yi Chuan **34**(4): 383-388.*

*Marquette, C. and L. Nabell (2012). "Chemotherapy-resistant metastatic breast cancer." Curr Treat Options Oncol **13**(2): 263-275.*

*Marsters, S. A., J. P. Sheridan, et al. (1998). "Identification of a ligand for the death-domain-containing receptor Apo3." Curr Biol **8**(9): 525-528.*

*Marsters, S. A., J. P. Sheridan, et al. (1997). "A novel receptor for Apo2L/TRAIL contains a truncated death domain." Curr Biol **7**(12): 1003-1006.*

*Miller, M. D., M. A. Marty, et al. (2007). "The association between exposure to environmental tobacco smoke and breast cancer: a review by the California Environmental Protection Agency." Prev Med **44**(2): 93-106.*

*Mitsiades, C. S., S. P. Treon, et al. (2001). "TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications." Blood **98**(3): 795-804.*

*Moen, I., A. M. Oyan, et al. (2009). "Hyperoxic treatment induces mesenchymal-to-epithelial transition in a rat adenocarcinoma model." PLoS One **4**(7): e6381.*

*Mom, C. H., J. Verweij, et al. (2009). "Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study." Clin Cancer Res **15**(17): 5584-5590.*

*Moreno-Aspitia, A. and E. A. Perez (2009). "Anthracycline- and/or taxane-resistant breast cancer: results of a literature review to determine the clinical challenges and current treatment trends." Clin Ther **31**(8): 1619-1640.*

*Morizot, A., D. Merino, et al. (2011). "Chemotherapy overcomes TRAIL-R4-mediated TRAIL resistance at the DISC level." Cell Death Differ **18**(4): 700-711.*

*Muller, A., B. Homey, et al. (2001). "Involvement of chemokine receptors in breast cancer metastasis." Nature **410**(6824): 50-56.*

*Mundy, G. R. (2002). "Metastasis to bone: causes, consequences and therapeutic opportunities." Nat Rev Cancer **2**(8): 584-593.*

*Munshi, A., T. J. McDonnell, et al. (2002). "Chemotherapeutic agents enhance TRAIL-induced apoptosis in prostate cancer cells." Cancer Chemother Pharmacol **50**(1): 46-52.*

*Neilson, H. K., C. M. Friedenreich, et al. (2009). "Physical activity and postmenopausal breast cancer: proposed biologic mechanisms and areas for future research." Cancer Epidemiol Biomarkers Prev **18**(1): 11-27.*

*Neville-Webbe, H. L., N. A. Cross, et al. (2004). "Osteoprotegerin (OPG) produced by bone marrow stromal cells protects breast cancer cells from TRAIL-induced apoptosis." Breast Cancer Res Treat **86**(3): 269-279.*

*Nimmanapalli, R., C. L. Perkins, et al. (2001). "Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels." Cancer Res **61**(2): 759-763.*

*Okamoto, R., M. Ueno, et al. (2005). "Hematopoietic cells regulate the angiogenic switch during tumorigenesis." Blood **105**(7): 2757-2763.*

*Ovcharenko, D., K. Kelnar, et al. (2007). "Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway." Cancer Res **67**(22): 10782-10788.*

*Paget, S. (1989). "The distribution of secondary growths in cancer of the breast.1889." Cancer and Metastasis Reviews **8**(2): 98-101.*

*Pan, G., J. Ni, et al. (1997). "An antagonist decoy receptor and a death domain-containing receptor for TRAIL." Science **277**(5327): 815-818.*

*Pan, Y. (2007). "Application of pharmacodynamic assays in a phase Ia trial of Apo2L/TRAIL in advanced tumours." Journal of clinical oncology (ASCO) **Abstract 3535**.*

*Pantel, K., C. Alix-Panabieres, et al. (2009). "Cancer micrometastases." Nat Rev Clin Oncol **6**(6): 339-351.*

*Park, H. R., S. K. Min, et al. (2003). "Expression of osteoprotegerin and RANK ligand in breast cancer bone metastasis." J Korean Med Sci **18**(4): 541-546.*

*Patel, L. R., D. F. Camacho, et al. (2011). "Mechanisms of cancer cell metastasis to the bone: a multistep process." Future Oncol **7**(11): 1285-1297.*

*Patel, S. A., A. Ndabahaliye, et al. (2010). "Challenges in the development of future treatments for breast cancer stem cells." Breast Cancer (London) **2**: 1-11.*

*Paterson, A. H., T. J. Powles, et al. (1993). "Double-blind controlled trial of oral clodronate in patients with bone metastases from breast cancer." J Clin Oncol **11**(1): 59-65.*

*Peinado, H., S. Lavotshkin, et al. (2011). "The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts." Semin Cancer Biol **21**(2): 139-146.*

*Pitti, R. M., S. A. Marsters, et al. (1996). "Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family." J Biol Chem **271**(22): 12687-12690.*

*Plummer, R., G. Attard, et al. (2007). "Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers." Clin Cancer Res **13**(20): 6187-6194.*

*Ponomarev, V., M. Doubrovin, et al. (2004). "A novel triple-modality reporter gene for whole-body fluorescent, bioluminescent, and nuclear noninvasive imaging." Eur J Nucl Med Mol Imaging **31**(5): 740-751.*

*Prindull, G. (1995). "Apoptosis in the embryo and tumorigenesis." Eur J Cancer **31A**(1): 116-123.*

*Pritchard, C. C., H. H. Cheng, et al. (2012). "MicroRNA profiling: approaches and considerations." Nat Rev Genet **13**(5): 358-369.*

*Psaila, B., R. N. Kaplan, et al. (2006). "Priming the 'soil' for breast cancer metastasis: the pre-metastatic niche." Breast Dis **26**: 65-74.*

*Rafii, S. and D. Lyden (2003). "Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration." Nat Med **9**(6): 702-712.*

*Rafii, S., D. Lyden, et al. (2002). "Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy?" Nat Rev Cancer **2**(11): 826-835.*

Romagnoli, M., K. Belguise, et al. (2012). "Epithelial-to-Mesenchymal Transition Induced by TGF-beta1 Is Mediated by Blimp-1-Dependent Repression of BMP-5." Cancer Res.

*Roodman, G. D. (2004). "Mechanisms of bone metastasis." Discov Med **4**(22): 144-148.*

*Rosen, L. S., D. Gordon, et al. (2001). "Zoledronic acid versus pamidronate in the treatment of skeletal metastases in patients with breast cancer or osteolytic lesions of multiple myeloma: a phase III, double-blind, comparative trial." Cancer J **7**(5): 377-387.*

*Rosen, L. S., D. Gordon, et al. (2003). "Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial--the Zoledronic Acid Lung Cancer and Other Solid Tumors Study Group." J Clin Oncol **21**(16): 3150-3157.*

*Roth, W., S. Isenmann, et al. (1999). "Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts in athymic mice in the absence of neurotoxicity." Biochem Biophys Res Commun **265**(2): 479-483.*

*Russo, A., M. Terrasi, et al. (2006). "Apoptosis: a relevant tool for anticancer therapy." Ann Oncol **17 Suppl 7**: vii115-123.*

*Saelens, X., N. Festjens, et al. (2004). "Toxic proteins released from mitochondria in cell death." Oncogene **23**(16): 2861-2874.*

*Sandra, F., L. Hendarmin, et al. (2006). "Osteoprotegerin (OPG) binds with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): suppression of TRAIL-induced apoptosis in ameloblastomas." Oral Oncol **42**(4): 415-420.*

*Savill, J. and V. Fadok (2000). "Corpse clearance defines the meaning of cell death." Nature **407**(6805): 784-788.*

*Schwartz, G. F., K. S. Hughes, et al. (2008). "Proceedings of the international consensus conference on breast cancer risk, genetics, & risk management, April, 2007." Cancer **113**(10): 2627-2637.*

*Scorrano, L. and S. J. Korsmeyer (2003). "Mechanisms of cytochrome c release by proapoptotic BCL-2 family members." Biochem Biophys Res Commun **304**(3): 437-444.*

*Sheridan, J. P., S. A. Marsters, et al. (1997). "Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors." Science **277**(5327): 818-821.*

*Shi, Y. (2002). "Mechanisms of caspase activation and inhibition during apoptosis." Mol Cell **9**(3): 459-470.*

*Shipman, C. M. and P. I. Croucher (2003). "Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells." Cancer Res **63**(5): 912-916.*

*Siegel, R., E. Ward, et al. (2011). "Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths." CA Cancer J Clin **61**(4): 212-236.*

*Sigal, A. and V. Rotter (2000). "Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome." Cancer Res **60**(24): 6788-6793.*

*Simonet, W. S., D. L. Lacey, et al. (1997). "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density." Cell **89**(2): 309-319.*

*Singh, T. R., S. Shankar, et al. (2003). "Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma in vivo." Cancer Res **63**(17): 5390-5400.*

*Singletary, K. W. and S. M. Gapstur (2001). "Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms." JAMA **286**(17): 2143-2151.*

*Slee, E. A., C. Adrain, et al. (2001). "Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis." J Biol Chem **276**(10): 7320-7326.*

*Smith, H. S. (2011). "Painful osseous metastases." Pain Physician **14**(4): E373-405.*

*Sommerfeldt, D. W. and C. T. Rubin (2001). "Biology of bone and how it orchestrates the form and function of the skeleton." Eur Spine J **10 Suppl 2**: S86-95.*

*Stahlhut Espinosa, C. E. and F. J. Slack (2006). "The role of microRNAs in cancer." Yale J Biol Med **79**(3-4): 131-140.*

*Stern, H. M., M. Padilla, et al. (2010). "Development of immunohistochemistry assays to assess GALNT14 and FUT3/6 in clinical trials of dulanermin and drozitumab." Clin Cancer Res **16**(5): 1587-1596.*

*Strano, S., S. Dell'Orso, et al. (2007). "Mutant p53: an oncogenic transcription factor." Oncogene **26**(15): 2212-2219.*

Suva, L. (2009). Zinonos and co-workers describe the efficacy of a potent immunotherapeutic molecule in the experimental treatment of breast cancer and progression (http://www.f1000medicine.com), Faculty of 1000 Medicine.

*Taganov, K. D., M. P. Boldin, et al. (2006). "NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses." Proc Natl Acad Sci U S A **103**(33): 12481-12486.*

*Taichman, R. S., C. Cooper, et al. (2002). "Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone." Cancer Res **62**(6): 1832-1837.*

*Takeda, K., K. Okumura, et al. (2007). "Combination antibody-based cancer immunotherapy." Cancer Sci **98**(9): 1297-1302.*

*Tamm, I., S. M. Kornblau, et al. (2000). "Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias." Clin Cancer Res **6**(5): 1796-1803.*

*Tanaka, S. (2007). "Signaling axis in osteoclast biology and therapeutic targeting in the RANKL/RANK/OPG system." Am J Nephrol **27**(5): 466-478.*

*Thai le, M., A. Labrinidis, et al. (2006). "Apo2l/Tumor necrosis factor-related apoptosis-inducing ligand prevents breast cancer-induced bone destruction in a mouse model." Cancer Res **66**(10): 5363-5370.*

*The Cancer Genome Atlas Network (2012). "Comprehensive molecular portraits of human breast tumours." Nature **490**(7418): 61-70.*

*Thornberry, N. A. and Y. Lazebnik (1998). "Caspases: enemies within." Science **281**(5381): 1312-1316.*

*Tolcher, A. W., M. Mita, et al. (2007). "Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1." J Clin Oncol **25**(11): 1390-1395.*

*Truneh, A., S. Sharma, et al. (2000). "Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor." J Biol Chem **275**(30): 23319-23325.*

*Tsuda, E., M. Goto, et al. (1997). "Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis." Biochem Biophys Res Commun **234**(1): 137-142.*

*Turnbull, C. and N. Rahman (2008). "Genetic predisposition to breast cancer: past, present, and future." Annu Rev Genomics Hum Genet **9**: 321-345.*

*Varfolomeev, E., B. Alicke, et al. (2009). "X chromosome-linked inhibitor of apoptosis regulates cell death induction by proapoptotic receptor agonists." J Biol Chem **284**(50): 34553-34560.*

*Varfolomeev, E. and D. Vucic (2011). "Inhibitor of apoptosis proteins: fascinating biology leads to attractive tumor therapeutic targets." Future Oncol **7**(5): 633-648.*

*Vitovski, S., J. S. Phillips, et al. (2007). "Investigating the interaction between osteoprotegerin and receptor activator of NF-kappaB or tumor necrosis factor-related apoptosis-inducing ligand: evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways." J Biol Chem **282**(43): 31601-31609.*

*Vogelstein, B., D. Lane, et al. (2000). "Surfing the p53 network." Nature **408**(6810): 307-310.*

*Volinia, S., G. A. Calin, et al. (2006). "A microRNA expression signature of human solid tumors defines cancer gene targets." Proc Natl Acad Sci U S A **103**(7): 2257-2261.*

*Vucic, D., H. R. Stennicke, et al. (2000). "ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas." Curr Biol **10**(21): 1359-1366.*

*Wakelee, H. A., A. Patnaik, et al. (2010). "Phase I and pharmacokinetic study of lexatumumab (HGS-ETR2) given every 2 weeks in patients with advanced solid tumors." Ann Oncol **21**(2): 376-381.*

*Walczak, H., R. E. Miller, et al. (1999). "Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo." Nat Med **5**(2): 157-163.*

*Wang, S. and W. S. El-Deiry (2003). "TRAIL and apoptosis induction by TNF-family death receptors." Oncogene **22**(53): 8628-8633.*

*Wang, S., W. Ren, et al. (2010). "TRAIL and doxorubicin combination induces proapoptotic and antiangiogenic effects in soft tissue sarcoma in vivo." Clin Cancer Res **16**(9): 2591-2604.*

*Wiezorek, J., P. Holland, et al. (2010). "Death receptor agonists as a targeted therapy for cancer." Clin Cancer Res **16**(6): 1701-1708.*

*Wiley, S. R., K. Schooley, et al. (1995). "Identification and characterization of a new member of the TNF family that induces apoptosis." Immunity **3**(6): 673-682.*

Wilson, C., I. Holen, et al. (2012). "Seed, soil and secreted hormones: Potential interactions of breast cancer cells with their endocrine/paracrine microenvironment and implications for treatment with bisphosphonates." Cancer Treat Rev.

*Wittrant, Y., S. Theoleyre, et al. (2004). "RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis." Biochim Biophys Acta **1704**(2): 49-57.*

*Xue, F., W. C. Willett, et al. (2011). "Cigarette smoking and the incidence of breast cancer." Arch Intern Med **171**(2): 125-133.*

*Yagita, H., K. Takeda, et al. (2004). "TRAIL and its receptors as targets for cancer therapy." Cancer Sci **95**(10): 777-783.*

*Yasuda, H. (1998). "[Osteoclastogenesis inhibitory factor (OCIF)]." Seikagaku **70**(5): 385-390.*

*Yasuda, H., N. Shima, et al. (1998). "Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL." Proc Natl Acad Sci U S A **95**(7): 3597-3602.*

*Yu, S. L., H. Y. Chen, et al. (2007). "Unique MicroRNA signature and clinical outcome of cancers." DNA Cell Biol **26**(5): 283-292.*

*Zapata, J. M., K. Pawlowski, et al. (2001). "A diverse family of proteins containing tumor necrosis factor receptor-associated factor domains." J Biol Chem **276**(26): 24242-24252.*

*Zeng, Y., X. X. Wu, et al. (2006). "Monoclonal antibody to tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) induces apoptosis in primary renal cell carcinoma cells in vitro and inhibits tumor growth in vivo." Int J Oncol **28**(2): 421-430.*

*Zhande, R., S. M. Dauphinee, et al. (2007). "FADD negatively regulates lipopolysaccharide signaling by impairing interleukin-1 receptor-associated kinase 1-MyD88 interaction." Mol Cell Biol **27**(21): 7394-7404.*

*Zinonos, I., A. Labrinidis, et al. (2011). "Anticancer efficacy of Apo2L/TRAIL is retained in the presence of high and biologically active concentrations of osteoprotegerin in vivo." J Bone Miner Res **26**(3): 630-643.*

*Zinonos, I., A. Labrinidis, et al. (2009). "Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer." Mol Cancer Ther **8**(10): 2969-2980.*