Chapter 7

Summary and discussion
SUMMARY
Despite aggressive treatment regimens, survival outcomes for patients suffering from osteosarcoma, especially for those with metastatic disease, remain dismal. The survival outcome for osteosarcoma has currently stagnated at approximately 65% 5-year survival for patients with localised disease, whereas patients with metastatic or relapsed disease have even lower survival rates of approximately 20% 5-year survival. [1,2] As posed in the introductory section, it seems that the current treatments for osteosarcoma lack efficacy, particularly in treating metastatic disease, and this inefficacy could, at least in part, be attributed to therapy resistance encountered in osteosarcoma cells. The observation that osteosarcoma metastases generally show a poorer response to chemotherapy than do primary tumours suggests that the metastatic cell has characteristics that not only allows it to metastasise, but also to (better) evade cell death after cytotoxic treatment. This is thoroughly discussed in chapter 2. Targeting these features in the metastatic cells might enhance the efficacy of current osteosarcoma treatments, however, OS metastasis is not yet well understood. Further unravelling the biology of OS metastasis should provide new molecular leads that could be used as a rational basis for innovative OS treatments. In chapter 3 we further evaluate the current knowledge that exists on the biological processes that contribute to therapy resistance in osteosarcoma cells and summarise what research efforts have thus far been undertaken to design novel treatment strategies to reverse or circumvent therapy resistance in osteosarcoma. From these first two chapters we conclude that, despite the multitude of pre-clinical and clinical research both in the treatment of osteosarcoma lung metastases and in overcoming treatment resistance in osteosarcoma, very few new agents or strategies have reached clinical application. A profound understanding of mechanisms that underlie therapy resistance in osteosarcoma should provide opportunities to design targeted treatments for osteosarcoma on a rational basis. In the research described in this thesis, we sought for new targeted treatment opportunities to subvert or circumvent the relative resistance to therapy observed in osteosarcoma. This targeted therapy could be achieved either by selectively targeting intracellular proteins essential for tumours to survive, or by a targeted delivery of therapeutics to the tumour by directing therapy selectively to extracellular surface proteins or receptors on tumour cells. Ideally, such new therapeutic strategies should target osteosarcoma-specific molecules, thereby selectively enhancing tumour cell kill while sparing healthy cells.

In search of intracellular treatment targets, we studied the sensitisation of osteosarcoma cells to radiotherapy and chemotherapy. In chapter 4 we describe the investigation of a candidate therapeutic target that was previously reported to be a radiosensitiser in other malignancies, [125,158,162,163] and studied its potential use for the radiosensitisation of osteosarcoma. We show that osteosarcoma can be sensitised to radiation therapy using a small molecule WEE1-inhibitor. Radiotherapy is not commonly applied in the treatment
of osteosarcoma because it is considered a radio-resistant tumour. However, a subgroup of patients, namely those with unresectable tumours (mainly located in the axial skeleton or head-and-neck region), painful skeletal metastases or patients who have undergone an intralesional resection of the primary tumour, could benefit from radiotherapy. Nonetheless, radiotherapy is only moderately effective and therefore we studied a possibility to improve the efficacy of this treatment modality. We observed that after irradiation-induced DNA damage, osteosarcoma cells sustain a prolonged G2 cell cycle checkpoint arrest, granting the cells time to perform DNA repair prior to entry into mitosis and thus evade cell death. WEE1 kinase can indirectly prevent cells from entering mitosis through inhibitory phosphorylation of CDC2, which is then hampered to bind to Cyclin-B, resulting in a G2 phase arrest. After WEE1 inhibition, osteosarcoma cells with damaged DNA are unable to sustain a prolonged G2 phase and are consequently forced into mitosis, leading to a type of apoptosis referred to as mitotic catastrophe. WEE1 expression analysis revealed that WEE1 gene- and protein expression is increased in osteosarcoma compared to healthy bone tissue, indicating that osteosarcoma patients treated with radiotherapy could benefit from concomitant WEE1 inhibition. Further in vivo work needs to be performed before translating these pre-clinical results to clinical testing of WEE1 inhibition combined with radiotherapy in osteosarcoma patients. The further development of WEE1 inhibition as a novel treatment strategy for the radiosensitisation of osteosarcoma is helped by the fact that WEE1 inhibitors have already been tested in clinical trials for other indications in oncology and therefore safety and dosage data are available. Nonetheless, we expect it to be very challenging to gather sufficient patients in which radiotherapy is indicated, to study the effectiveness of this new combination therapy.

In the following chapter, (chapter 5) we focussed on chemosensitisation. We hypothesised that targeting essential survival pathways in combination with administering conventional therapy may lead to enhanced cell death. The identification of essential regulators of drug response in osteosarcoma is crucial in the design of such a new treatment strategy. To analyse the possibilities to sensitisie osteosarcoma cells to chemotherapy, we chose an unbiased functional genomics approach in which we performed siRNA library screening of kinase and kinase-associated genes on SaOS-2 osteosarcoma cells to detect critical survival kinases after cytotoxic treatment. Gene silencing of JNK-interacting protein 1 (JIP1) elicited the most potent sensitisation to doxorubicin. Using a small molecule JIP1-inhibitor we could confirm our findings in a panel of osteosarcoma cell lines. The observed sensitisation to doxorubicin treatment seemed to be partly dependent on p53 status, where osteosarcoma cells harbouring functional p53 showed a less pronounced sensitisation than osteosarcoma cells with mutated or absent p53. JIP1 is known to be a scaffold protein in the JNK-signalling pathway. It plays an important role in the activation of JNK signalling by assembling specific MAP kinases indispensable for JNK activation or for maintaining JNK phosphorylation.
Therefore, downregulation of JIP1 leads to defective JNK signalling in response to cytotoxic damage. Protein expression analysis of JIP1 revealed that approximately two-thirds of human osteosarcoma samples express JIP1 and that patients with JIP1 positive tumours show a trend toward inferior overall survival. Thus, in this chapter we have successfully applied a functional genomics approach to identify a candidate drug target to exploit as a chemosensitiser to doxorubicin in osteosarcoma. Through downregulation of JIP1, the cellular balance is tilted further toward cell death leading to increased cell kill after doxorubicin treatment. Also here, the results provide a promising lead to pursue in the development of an innovative sensitising treatment for osteosarcoma. Many steps remain to be taken prior to realising clinical trials or clinical implementation of a JIP1-inhibitor for osteosarcoma patients. For example, it might be interesting to test whether JIP1 inhibition can induce sensitisation to other compounds commonly used in osteosarcoma treatment, such as cisplatinum or methotrexate, as well. At present, no such data is available. Also, in vivo validation of the observed increased anti-tumour effect is needed, as well as information on dose and timing of administration of the JIP1-inhibitor in relation to doxorubicin. Previous in vivo work on diabetes mellitus showed that the JIP1-inhibitor we used in our investigations can be safely administered to mice. [176] The in vivo administration of JIP1-inhibitor is currently also under investigation by a group that focuses on Parkinson’s disease. (www.michaeljfox.org/foundation) If the observed chemosensitisation can be verified in an in vivo model of osteosarcoma, phase I clinical trials might be designed to progress to toxicity and dose testing in osteosarcoma patients.

Apart from shifting molecular balances within osteosarcoma cells to favour cell death after radiation and chemotherapy, another strategy to improve treatment efficacy could be to enhance the intracellular delivery of currently applied drugs to the tumour cells. In the case of cancer-specific treatment by targeted delivery of drugs, therapy should ideally be targeted to a cell surface molecule that is specific for the tumour, i.e. that is highly expressed on the surface of tumour cells, but not on healthy tissues. The identification of suitable receptors for targeted delivery of therapeutics to osteosarcoma is essential in the design and development of novel targeted treatment strategies and is described in chapter 6. In order to identify such a suitable receptor we performed a comparative surface proteomic analysis of five osteosarcoma cell lines compared to three healthy human bone cell cultures. As surface proteins are difficult to solubilise and are of relatively low abundance compared to intracellular proteins, we first enriched our study samples for surface proteins by biotinylation and selective retrieval of the surface proteins prior to high resolution mass spectrometric analysis. From our proteomic analysis we obtained a comprehensive dataset containing many surface proteins of interest. We found the Ephrin type-A receptor 2 (EPHA2) to be the most abundant surface protein on osteosarcoma cells, with a 12-fold upregulation compared to the healthy human primary osteoblasts. We validated EPHA2 surface expression
and significant EPHA2 upregulation on osteosarcoma cell lines compared to human primary osteoblasts using FACS analysis and EPHA2 expression in human tumour tissue samples. Analysis of human osteosarcoma tissue micro arrays revealed that around 80% of the tumour samples present on the array showed EPHA2 expression. Patients with EPHA2-expressing tumours showed a trend toward inferior overall survival. As a proof-of-concept we performed binding and internalisation studies of EPHA2 using a specifically designed GFP-expressing adenovirus (AdYSA) that is targeted to EPHA2 via the YSA peptide in its fibre knob. In these studies we showed that EPHA2 can specifically mediate the uptake of AdYSA into osteosarcoma cells, whereas the human primary osteoblasts remained unaffected due to their low EPHA2 expression. The results presented in this chapter show that EPHA2 is a highly upregulated surface receptor on osteosarcoma cells that can be used for the specific intracellular uptake of vectors directed toward EPHA2. The use of EPHA2 as receptor for targeted delivery does not need to be restricted to adenoviral vectors alone. Other groups have shown the application of nanoparticles coated with YSA peptide to direct them to EPHA2-expressing cells. One group showed the application of magnetic nanoparticles that can capture EPHA2-expressing cells and then clear them from the circulation of tumour bearing mice. [237] Another group demonstrated the use of YSA coated nanoparticles to specifically deliver siRNA to EPHA2-expressing tumour cells to increase chemosensitivity in vivo. [236] In the case of osteosarcoma, the targeted delivery of treatment modalities using the EPHA2 needs to be tested in a pre-clinical animal model as well. Eventually, the treatment and the vehicle used to deliver therapy to osteosarcoma cells might be various. For example, perhaps in future research we can combine the knowledge obtained in this and the previous chapter and deliver a JIP1 inhibitor to osteosarcoma cells specifically through internalisation via EPHA2.

**DISCUSSION**

In the recent past, so-called targeted therapy has gained tremendous interest in anti-cancer treatment, exploiting tumour-specific molecules for therapeutic goals. In this era of targeted treatments, the use of so-called ‘-omics’ approaches such as genomics, proteomics, and sequencing techniques allows the acquisition of vast amounts of information on tumour biology. It has allowed for the identification of crucial growth and survival genes and proteins in cancer cells; i.e. molecules that can be considered potential targets for targeted therapy. Thus, -omics approaches could be considered the cornerstone of the design of targeted anticancer therapy. Additionally, the advent of small molecule drugs that specifically target proteins or classes of proteins have further allowed the development of a very specific, or tailored, treatment of certain tumours based on the information provided by -omics techniques. Ultimately, tailored treatments should provide high anti-tumour efficacy...
combined with minimal toxicity for cancer patients. The use of -omics approaches can also be applied to identify cancer specific genetic profiles to predict therapy response or predictive or prognostic markers for clinical outcome. This can aid in treatment and dosing decisions and possible future stratification of patients to specific treatments that target a certain characteristic of their tumour and thus hopefully achieve improved treatment and survival outcomes.

However, both the investigational techniques for the identification of candidate drug targets and the development and implementation of novel drugs in the clinical setting is costly and cumbersome. The affordability of health care in general and cancer care in particular is a contemporary topic of political debate and even though it is clear that the development of novel treatment strategies for osteosarcoma is warranted, there are political and economical considerations to be made. Currently, it is estimated that the costs for anti-cancer medicine are approximately 1% of all health care spending. [239] As research techniques have become more sophisticated and high-tech, and the research questions surrounding cancer have become increasingly complex, the costs associated with the development of novel treatments are likely to increase as well. Furthermore, the value of the research and development of innovative treatments for small numbers of individuals is being questioned. For example, even though osteosarcoma is the most common primary bone tumour in children and adolescence, its incidence is extremely low (approximately 4/million/year). [1,2] In the Netherlands this would amount to only approximately 50-60 new patients each year. The combination of high expenditure in the development of novel treatments and only very few patients to be treated tends to lead to discussions about funding, pricing and costs of such anticancer treatments. [239] Multiple challenges exist surrounding the research and development of new treatment strategies for extremely rare diseases. Owing to its rarity, osteosarcoma is considered an orphan drug indication in oncology. One might state that there is no financial incentive for pharmaceutical companies to invest in the discovery and development of drugs for orphan indications in oncology because the expected returns are negligible. Therefore, legislation exists to encourage companies, universities and other research institutions to develop and manufacture treatments for orphan drug indications by for example granting the manufacturer market exclusivity for a specified period of time, granting a prolonged duration of patent, providing fiscal incentives for investors, as well as providing other forms of funding and support. Also, regulatory requirements for clinical trials might be adjusted for drug testing in orphan indications to reduce the complexity of executing a clinical trial, consequently reducing procedural costs as well, thus making drug development for these indications more attainable. [239,240]

Another issue could be that, after innovative (perhaps unregistered) treatments for orphan indications in oncology have reached clinical implementation, it is not unlikely that they will not be reimbursed, or only partially reimbursed by health insurance companies.
High pricing of the novel drugs could be the result of high development costs and the need to produce and supply only low volumes of the drug. Furthermore, if the efficacy of a novel treatment is not fully proven yet, insurers are likely to be reluctant to reimburse the costs for the treatment. Consequently these novel treatments may become (or remain) unavailable to those patients that cannot afford to pay for their own treatment. Policymakers, regulatory organs or governments in the EU can hopefully achieve a way to balance development costs, pricing, drug regulation and availability for novel treatments for orphan indications in oncology such as osteosarcoma, to ensure fair access for patients to the best possible therapy for their disease. [239,240]

A different difficulty in the development of new treatments for osteosarcoma is the accrual of sufficient patients to perform adequately powered clinical trials. It is appreciated worldwide that appropriately performed clinical trials provide the best evidence on safety and efficacy of new treatments. To collect data and material from large enough numbers of patients is both cumbersome, extremely time consuming and also potentially expensive. In the case of osteosarcoma, international collaborations have been formed through which a more extensive patient population becomes available for centralised research. The European and American Osteosarcoma Study Group (EURAMOS) is an important example. EURAMOS was founded in 2001 and is a collaboration of four other international study groups, namely the COSS (Collaborative German-Austrian-Swiss Osteosarcoma Study Group), the COG (Children’s Oncology Group, USA), the EOI (European Osteosarcoma Group) and the SSG (Scandinavian Sarcoma Group). Their ultimate aim is to improve the survival from osteosarcoma by conducting large randomised clinical trials, to undertake extensive parallel biological studies on tumour material obtained from patients enrolled in their trials and to eventually develop a common understanding and language about osteosarcoma worldwide. (www.ctu.mrc.ac.uk/euramos) Their first clinical trial (EURAMOS-1) has now been completed and first results have been presented. These results are beyond the scope of the material discussed here. Part of the secondary objectives of EURAMOS-1 was to assess the feasibility of such an international operation in performing clinical trials for osteosarcoma. The presentation of first results suggests that this in fact, is the case.

Apart from testing the safety and efficacy of novel treatments or drugs, the collection of adequate amounts of human tumour tissue samples is also a very important achievement that can be realised by international collaborations, especially when follow-up data on disease progression and survival are present and complete. Commonly, in osteosarcoma research, the absence of sufficient primary tumour material or primary cell cultures leads to the use of OS cell lines to perform in vitro experiments to study osteosarcoma biology. We ourselves have also done so. Recently, Mohseny et al. [241] published a report in which they studied the appropriateness of OS cell lines to study the human disease. They identified at least eight cell lines that are representative for the human disease that could be used as
a model for osteosarcoma. Apart from this, various murine models exist (of both primary and metastatic osteosarcoma) to extrapolate in vitro data to an in vivo situation. [175, 242]

Although such pre-clinical models for osteosarcoma are suitable to study various aspects of osteosarcoma biology and behaviour, the study of human primary tumour tissue is absolutely indispensable. For example, protein expression levels of newly identified candidate drug targets need to be investigated in human tumour samples to validate their possible clinical relevance for patients suffering from osteosarcoma; this comprises an essential step from laboratory investigations to implementation of new strategies in the clinic. Apart from validation purposes, the collected human tissue samples and follow-up data could also be investigated to identify osteosarcoma specific predictive markers for therapy response or prognostic markers for survival outcome, neither of which currently exist for osteosarcoma. [7] Ultimately, prediction of treatment and/or survival outcomes may in the future allow for the stratification of patients to certain treatment schedules, thereby offering these patients a tailored therapy when it could be anticipated that they will benefit from specific targeted treatments.

As noted above, molecular profiling of tumours using -omics techniques allows the generation of extensive amounts of data on tumour biology. Apart from the identification of oncogenes, crucial survival genes, predictive or prognostic biomarkers, etc. these techniques have also unveiled the extent of tumour subtypes and/or tumour heterogeneity within tumour types. [243] Osteosarcoma is known to be a highly heterogeneous tumour that harbours complex chromosomal aberrancies and regularly displays chromosomal instability. [5, 7, 243] This heterogeneity, both between tumours in different individuals but particularly within one tumour and between a primary tumour and its metastases, has implications for biomarker discovery, response to (chemo)therapy and for the design of targeted therapy. In this sense, the tumour heterogeneity may have negative implications for the newly identified drug targets described in this thesis. Although we have found relatively high expression levels of JIP1 (67%) and EPHA2 (84%) in our tested osteosarcoma samples, verification of this expression in independent, preferably large datasets is very much needed to ascertain that indeed, these therapeutic targets are broadly expressed in osteosarcoma patients. It has been observed, in osteosarcoma as well as other types of cancer, that genomic or chromosomal instability can give rise to a genetic diversity of cells within one tumour that exhibit a different behaviour than the general cell population within that tumour. Apart from chromosomal instability, (miR-induced) post-translational modifications and epigenetic changes can lead to the development of different clonal populations within one tumour. After a cycle of treatment, resistant clones remain to give rise to tumour recurrence in a later stage of the disease. Thus, tumour heterogeneity can allow a tumour to adapt to extra-tumoural circumstances and give rise to, for example, therapy resistance but also other phenomena such as metastasis. [47, 51, 243] It is essential
to understand this heterogeneity and try to ascertain that newly defined drug targets are very broadly and preferably stably expressed among osteosarcomas and osteosarcoma metastases to be sure that most patients could potentially benefit from the newly designed therapy. If the tumour might ‘adapt’ to treatment, than perhaps so should the treatment schedule be adapted to the tumour behaviour. Among others, this is one reason why single agent therapy is very unlikely to be successful in heterogeneous tumours. It may eventually be necessary to switch from one compound to the other along the course of osteosarcoma treatment.

Contrary to this perhaps disturbing notion that -omics approaches revealed a heterogeneity within tumours which could potentially impede the design of one single targeted therapy for a certain type of cancer, whole genome sequencing, for example, can also provide valuable information on common gene mutations across different types of cancer. Recently, in the Netherlands, a Center for Personalised Cancer Treatment (CPCT) has been founded where patients suffering metastatic disease are included, biopsies from metastases are obtained and then subjected to ‘Next Generation Sequencing’ (NGS). Two thousand genes per tumour biopsy are analysed and thus the biopsies are screened for known predictive or prognostic markers and for novel biomarkers for therapy response and survival. The ultimate goal of this project is to provide each patient suffering from metastatic disease with a personalised treatment schedule, based on the molecular profile of their metastases and not the primary tumour. Also, patients can be enrolled in phase 1 clinical trials based on the genetic profile of their tumour rather than on the type of cancer that they suffer. Patients suffering from metastatic osteosarcoma are also enrolled in phase 1 clinical testing of novel compounds targeting their specific molecular profile. (www.cpct.nl) This formulation of pharmacogenetic profiles of tumours may eventually allow a better use of already existing anti-cancer drugs, where drugs initially developed and registered for other indications might be rationally administered to (and tested for efficacy in) patients suffering from osteosarcoma with a specific molecular profile. Although at present, the sequencing techniques are still costly and relatively time-consuming, in the future it may prove to be cost-effective to selectively stratify osteosarcoma patients to treatment with readily available anti-cancer compounds for other indications.

**FUTURE PERSPECTIVE**

The research presented in this dissertation was mainly aimed at the identification of novel treatment targets in osteosarcoma to formulate strategies to improve the current treatment regimens. We speculate that therapy resistance in osteosarcoma cells is accountable for therapeutic failure and poor outcome and that research efforts for the design of novel treatment strategies should be aimed at overcoming or subverting this resistance. This
can be achieved either by the selective targeting of intracellular proteins that grant the osteosarcoma cells a survival benefit after cytotoxic treatment, or by selectively targeting treatment modalities (be it conventional, small molecules or gene therapy) to osteosarcoma cells in order to obtain a higher effective dose at the site of the tumour whilst sparing normal cells and tissues.

By performing an siRNA library screening and mass spectrometry based proteomics we obtained two extensive datasets on intracellular regulators of doxorubicin response and osteosarcoma specific surface molecules, respectively. We have selected one candidate from each dataset for follow-up studies and present these as molecules to be exploited for the design of a new, targeted, treatment for osteosarcoma. In the case of EPHA2, the specific delivery of treatment modalities via this receptor can be realised by conjugating the particular modality with the YSA peptide which covalently binds to EPHA2, leading to receptor internalisation and thus the intracellular delivery of the YSA-coupled modality. The moieties delivered to osteosarcoma cells could encompass various entities. YSA could be conjugated to multiple modalities, for example liposome encapsuled chemotherapy or small molecules, but also gene therapy such as siRNAs, short hairpin RNAs or microRNAs, also encapsuled in a delivery vehicle of some sort.

siRNA library screening identified JIP1 (among others) as chemosensitiser to doxorubicin treatment in osteosarcoma. Validation studies with separate siRNA clones targeting the JIP1 gene verified that siRNA-mediated gene silencing led to a depletion of intracellular JIP1 with consequent increased cell death after cytotoxic treatment with doxorubicin. We then proceeded to use a small molecule JIP1-inhibitor for further combination treatment studies. The systemic administration of siRNA to treat tumours remains a challenge today and therefore the use of small molecule drugs that can selectively inhibit proteins or protein classes is an attractive method to use for in vivo and clinical studies following target identification. Whereas RNA interference is a powerful and efficient method for the systematic discovery of potential drug targets, the applicability of siRNAs in the clinical setting is still limited, mainly due to delivery issues. For example, the intracellular uptake of naked siRNA is hampered by its low membrane permeability. Also, proteases in the blood stream lead to enzymatic degradation, combined with rapid renal clearance results in a very short circulation half-life of siRNAs and therefore a limited biodistribution. Furthermore, siRNAs can elicit an interferon response and thus an undesirable induction of the immune system when systemically administered. [244,245] Notwithstanding delivery issues, the use of siRNA for targeted treatment has advantages over the use of small molecules. For example, siRNAs have extremely high target selectivity and therefore the chance of off-target effects are smaller compared to small molecules. Additionally, the design of siRNA sequences is relatively uncomplicated, rapid and potentially less expensive. [244] Coupling of siRNA to a targeting ligand can improve cell specific delivery of siRNAs, however, due to
their charge siRNAs cannot cross the cell membrane by diffusion and so a packaging modality remains necessary for the siRNA to reach the intracellular compartment. [244] Thus, prior to the successful application of siRNAs in targeted treatment in humans, the development of a delivery system that protects the siRNA against degradation and elimination from the bloodstream is needed.

Viral delivery of gene therapy to osteosarcoma cells has been successfully shown by our group in the past. [145,246,247] Viral vectors can also be adjusted to specifically infect tumour cells, an approach that we used for proof-of-principle studies in chapter 5. Viral vectors are reported to have a high transduction efficiency and have a profound capacity to deliver the gene therapy intracellularly, potentially granting high exogenous siRNA and thus ‘gene-silencing’ levels. Furthermore, they can be quickly produced in high titres. The use of (replication deficient) viruses in patients has been subject of discussion due to concerns surrounding safety, such as the possibility of virus induced insertional mutagenesis and immune and/or toxic reactions in the patient that have been observed in the past. Currently, the use of oncolytic viruses in patients has been proven safe, however, therapeutic efficacy remains relatively low due to delivery and tumoural uptake issues.

Nanotechnology is a newly developing technique that has evolved dramatically over the past decade and that may provide an important method to deliver therapeutics to tumour cells in the future. Nanoparticles can be lipid-, polymer- or peptide/protein-based and can be designed to form various different special configurations, depending on the requested properties of the particle, the route of administration and the moiety that it should encapsulate. It is reported that nanoparticles can also carry combinations of, for example, doxorubicin and siRNA, as well as small molecule inhibitors, leading to a ‘dual action’ particle where both cytotoxic agent and sensitiser can be delivered concomitantly. Furthermore, they can be coated with targeting ligands to stimulate a more targeted delivery of the nanoparticles to the tumour cells. Importantly, nanoparticles can be designed to be both biocompatible and -degradable and thus induce relatively little toxicity. [244,248,249] As mentioned above in the summary section, the use of the EPHA2 receptor for the specific targeting of EPHA2-expressing tumour cells with YSA-coated magnetic nanoparticles has been described to clear tumour cells from the circulation and peritoneal fluid of tumour-bearing mice, thus indicating that EPHA2 could be suitable as a targeting molecule for nanoparticles. [237]

Very recently, an in vitro study presented the successful delivery of doxorubicin and siRNA using biocompatible and biodegradable polymeric nanoparticles to osteosarcoma cells. The particles were loaded either with doxorubicin or siRNA and treatment of cells with doxorubicin-loaded nanoparticles led to increased cytotoxicity compared to doxorubicin treatment alone; treatment of cells with siRNA-loaded nanoparticles led to gene-silencing of the target gene with accompanying depletion of the protein product. [245]
Thus, nanotechnology can be applied to osteosarcoma cells in laboratory investigations. Theoretically, the combined findings of our functional genomics and proteomics analyses could provide a recipe for the design of an EPHA2-directed, doxorubicin/siJIP1-carrying nanoparticle that could be used for the sensitisation of osteosarcoma cells to doxorubicin treatment. Alike, an EPHA2-directed nanoparticle carrying siWEE1 or small molecule WEE1-inhibitor might be used to achieve radiosensitisation in patients that need to undergo radiation therapy for their tumour.

At present, nanotechnology is still a developing field and therefore not (broadly) applied in clinical practice. The novelty and complexity is still high and with that the development costs also. It was noted that the entry of nanomedicines onto the market was delayed because there was little collaboration between research institutes and pharmaceutical companies to actually develop and produce the medicine. [248] As with any evolving technology it could be anticipated that as this discipline progresses further, the development and production costs of nanosystems for drug delivery will decrease and the use of this new technique will become a more realistic therapeutic entity in anticancer treatment somewhere in the future.

CONCLUSION
The research presented in this dissertation was mainly focussed at the identification of novel therapeutic targets in osteosarcoma in order to formulate new, targeted treatment strategies to improve the current treatment regimens for this disease. Resistance to therapy remains an issue in the treatment of cancer, particularly in disseminated disease today. The results from our investigations provide leads (molecules) through which the cytotoxicity of existing therapies may be enhanced in osteosarcoma, ultimately leading to higher treatment efficacy, reduced toxicity and improved clinical outcomes for osteosarcoma patients. The exact development of targeted treatment based on our candidate molecules has yet to take form. We live in an era where growing understanding of tumour biology and rapid technological advancements in research platforms seem to provide endless opportunities in designing novel, targeted treatments for cancer. At the same time, the economical climate, policymakers, financiers, etc., ask for a certain cost-effectiveness in the development and clinical use of newly designed treatments. Therefore, we believe that in future osteosarcoma treatment smart use of available techniques and data is required. For example, -omics approaches may reveal common mutations in osteosarcoma and other tumours, such that compounds readily available for other indications can be applied in the treatment of osteosarcoma. Furthermore, in the case of osteosarcoma, its rarity poses difficulties in (pre-) clinical research and the design, development and production of novel treatments. Therefore, orchestrated international collaborations are of absolute importance in order to enable osteosarcoma research to develop innovative treatment strategies.
Eventually, application of -omics approaches on tumour material, international collaboration between research institutes and, perhaps most importantly, the determination of researchers and clinicians employed in this field, will be conducive to a new era of targeted treatments for osteosarcoma.