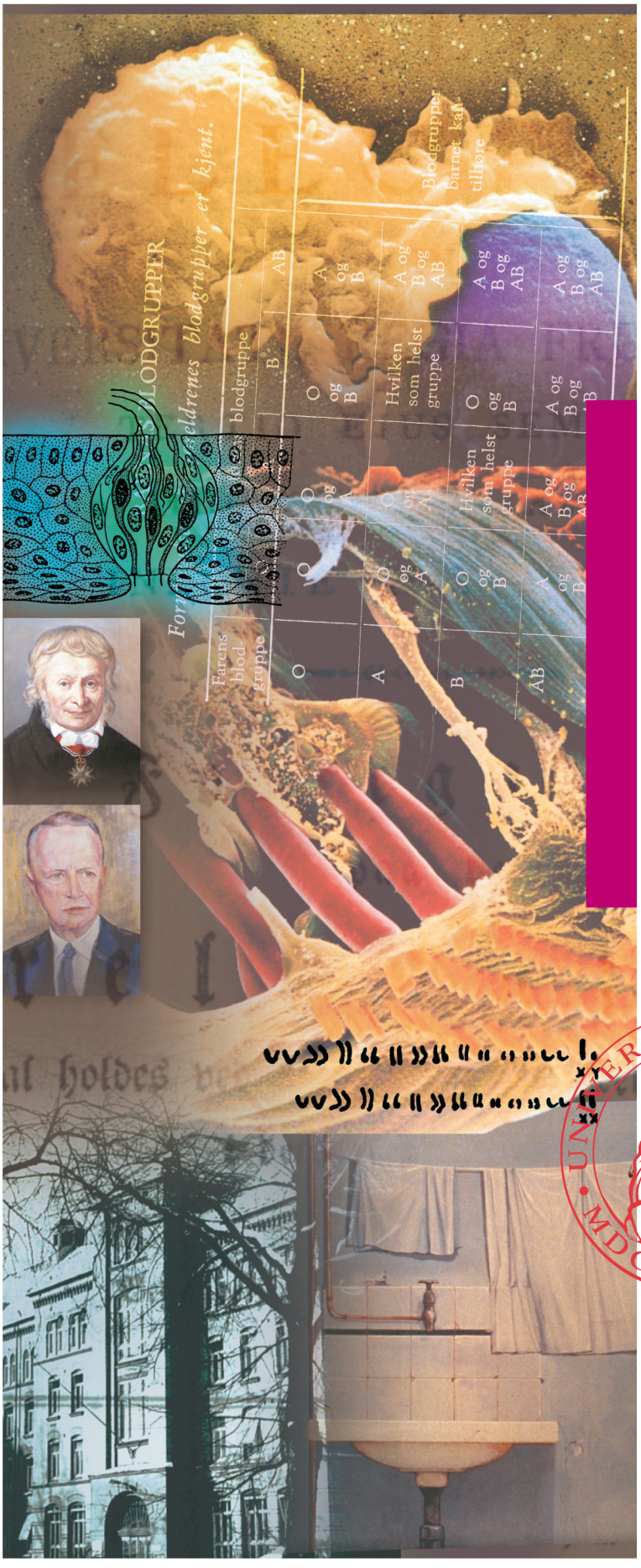
Christoph Rainer Müller

Novel approaches to the treatment of high-grade sarcoma



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&

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Contents

Preface

The work presented in this thesis originates from the Oncology Clinic and the Institute for Cancer Research at the Norwegian Radiumhospital (now part of Oslo University Hospital). Clinicians and scientists form the multidisciplinary sarcoma group at the Norwegian Radiumhospital, which is closely integrated with the Scandinavian Sarcoma Group (SSG[1]). Founded in 1979, SSG established a network of sarcoma specialists in research, diagnostics and clinical care facilitating translational research in the Nordic countries. Wider collaborations have been established through intergroup-projects with the Italian sarcoma group (ISG[2]) and in an extensive European-American study group on osteosarcoma (EURAMOS[3]). When working with rare tumours needing highly specialized care, centralization to expert centres and international collaboration is mandatory for improving the prospects for our patients.

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Und am Ende, ganz am Ende, wird das Meer in der Erinnerung blau sein[4].

Abbreviations

BAX	Bcl-2-associated X protein
BCD	combination chemotherapy of bleomycin, cyclophosphamide and dactinomycin
BCL2	B-cell CLL/lymphoma 2
BS	bone sarcoma
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2A
CML	chronic myelogenous leukaemia
COSS	Cooperative Osteosarcoma Study Group
DDLS	dedifferentiated liposarcoma
EBV	Ebstein Barr virus
EFT	Ewing family of tumours
EGFR	endothelial growth factor receptor
EOI	European Osteosarcoma Intergroup
EORTC	European Organisation for Research and Treatment of Cancer
EURAMOS	European American Osteosarcoma Study Group
FNCLCC	Fédération Nationale des Centres de Lutte Contre le Cancer
GIST	gastro intestinal stromal tumour
IFI16	interferon, gamma-inducible protein 16
IFITM1	interferon induced transmembrane protein 1 (9-27)
IFN	interferon
IL-15	interleukin 15
IOR-OS2	Istituto ortopedico Rizzoli – osteosarcoma study 2
IRF9	interferon-regulatory factor 9
ISG	Italian Sarcoma Group
ISGSSG1	Italian-Scandinavian Osteosarcoma Study 1
ISGSSG2	Italian-Scandinavian Osteosarcoma Study 2
JAK	Janus kinase
KI	Karolinska Institute
KS	Karolinska Hospital
LS	liposarcoma

MAP	combination chemotherapy of methotrexate, doxorubicin and cisplatinum
MDM2	Mdm2 p53 binding protein homolog (mouse)
MDM4	Mdm4 p53 binding protein homolog (mouse)
MPNET	malign peripheral neuroectodermal tumour
MTX	Methotrexate
NCI	National Cancer Institute
NF1	neurofibromin 1
OAS	oligo adenylate synthetase
OS	osteosarcoma
p53	tumour protein p53
PARP	poly (ADP ribose) polymerase
PEG	polyethylene glycol
RB1	retinoblastoma 1
SS18	synovial sarcoma translocation, chromosome 18
SSG	Scandinavian Sarcoma Group
SSGII	SSG study II (1. Scandinavian osteosarcoma study)
SSGVIII	SSG study VIII (2. Scandinavian osteosarcoma study)
SSX	synovial sarcoma, X breakpoint 1
STAT	signal transducer and activator of transcription
STS	soft tissue sarcoma
TP53	tumour protein p53
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
USP7	ubiquitin specific peptidase 7 (herpes virus-associated) (HAUSP)
VIG	combination chemotherapy of etoposide, ifosfamide and granulocyte growth factor
WDLS	well differentiated liposarcoma
XAF1	X-linked inhibitor of apoptosis (XIAP) - associated factor 1

List of papers

Paper I: Smeland S, **Müller C**, Alvegard TA, Wiklund T, Wiebe T, Bjork O, Stenwig AE, Willen H, Holmström T, Follerås G, Brosjö O, Kivioja A, Jonsson K, Monge O, Sæter G;
Scandinavian Sarcoma Group Osteosarcoma Study SSG VIII: prognostic factors for outcome and the role of replacement salvage chemotherapy for poor histological responders.
Eur J Cancer 2003, **39;** 488-494.

Paper II: **Müller CR**, Smeland S, Bauer H, Saeter G, Strander H:
Interferon-α as the only adjuvant treatment in high grade osteosarcoma: Long term results of the Karolinska Hospital series.
Acta Oncol 2005, **44**; 475-480.

Paper III: **Müller CR**, Namløs HM, Bjerner J, Østensen IHG, Sæter G, Smeland S, Bruland Ø, Myklebost O:
Characterization of Treatment Response to Interferon-α in Osteosarcoma Xenografts.
Manuscript submitted march 2010

Paper IV: **Müller CR**[*], Paulsen EB[*], Nordhuis P, Pedeutour F, Sæter G, Myklebost O:
Potential for treatment of liposarcomas with the MDM2 antagonist Nutlin-3A.
Int J Oncology, 2007, **121**; 199-205.

* contributed equally

Introduction

Sarcomas

The mesoderm gives rise to bone, cartilage, muscle, endothelium and blood cells. Non-haematological malignant tumours resembling these tissues are called sarcomas. The majority of sarcomas are thought to arise in pluripotent mesenchymal stem cells with the exception of a few neoplasms of probable neuroectodermal origin (Ewing family of tumours). Sarcomas are rare tumours with a stable incidence of 1% of all malignancies[5, 6], but are relatively more common in childhood and adolescence where they constitute 6-7%[7]. Sarcomas are traditionally subdivided into two main groups, soft tissue sarcomas (STS) originating from soft tissues and bone sarcomas (BS) originating from bone or cartilage.

Among over 50 subtypes of STS[8], the four most common adult subtypes are pleomorphic undifferentiated sarcoma constituting 20-70% of STS, liposarcoma 10-16%, fibrosarcoma <10-65% and leiomyosarcoma 5-10%. The ranges stated illustrate significant changes in the perception and definition of sarcoma histotypes over time and between pathologists[9]. Median age for STS as a group is 65 years[8]. The most common STS' of childhood are rhabdomyosarcoma and fibrosarcoma[7].

Bone sarcomas are divided into osteosarcoma (OS), chondrosarcoma and Ewing's sarcoma, with a peak incidence in late childhood/adolescence for both OS and Ewing's sarcoma, as opposed to chondrosarcoma, which is essentially a tumour of adulthood[10].

The *aetiology* of sarcomas is poorly understood. Risk factors include chemicals (phenoxyacetic acids and chlorophenols used as herbicides[11]; dioxin[12, 13] vinyl chloride[14, 15], previous radiotherapy [16, 17], viral infections (human herpes virus 8 is the causative agent of Kaposi's sarcoma)[18], EBV in the development of smooth muscle tumours[19] and acquired immunological defects. Some germ line mutations affecting tumour suppressor genes dispose for specific sarcomas (e.g. p53 in the Li Fraumeni syndrome[20], Rb in retinoblastoma[21], NF1 in neurofibromatosis related MPNET[22] and c-kit in GIST[23]).

Sarcomas may originate at any anatomic site and the distribution varies greatly by histological subtype and within subtypes. Roughly half of STS are localized in the extremities[24], and a similar distribution is found for bone sarcomas as a group. For OS approximately 90% are localized to the extremities[25].

Genetic alterations

An estimated 15-20% of sarcomas harbour specific chromosomal translocations[26] (table 1). The resulting chimeric proteins are important for the biology of these tumours, commonly acting as abnormal transcription factors deregulating downstream genes in critical signalling pathways. The strong relationship between specific translocations and distinct sarcoma types indicate that they represent early and tumour-driving events in tumour genesis[27], perhaps at the level of the suggested sarcoma stem cells. Fusions genes have become an important part of modern sarcoma diagnostics and variants of fusion genes may be associated with differences in outcome (e.g. in Ewing's sarcoma and synovial sarcoma[28]). The fusion gene products or their downstream signalling pathways may represent potential targets for treatment[29].

Table 1: characteristic fusion genes in sarcomas[32]

tumour	chromosomal aberration	fusion gene
alveolar rhabdomyosarcoma	t(1;13)(p36;q14)	PAX7–FOXO1
	t(2;13)(q36;q14)	PAX3–FOXO1
alveolar soft part sarcoma	t(X;17)(p11;q25)	ASPSCR1–TFE3
angiomatoid fibrous histiocytoma	t(2;22)(q33;q12)	EWSR1–CREB1
clear cell sarcoma	t(12;22)(q13;q12)	EWSR1–ATF1
dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	COL1A1–PDGFB
desmoplastic small round-cell tumour	t(11;22)(p13;q12)	EWSR1–WT1
endometrial stromal sarcoma	t(7;17)(p15;q11)	JAZF1–SUZ12
Ewing family of tumours	t(11;22)(q24;q12)	EWSR1–FLI1
	ins(21;22)(q22;q12q12)	EWSR1-ERG
	t(16;21)(p11;q22)	FUS-ERG
fibromyxoid sarcoma	t(7;16)(q34;p11)	FUS–CREB3L2
liposarcoma, myxoid	t(12;16)(q13;p11)	FUS–DDIT3
	t(12;22)(q13;p12)	EWSR1–DDIT3
synovial sarcoma	t(X;18)(p11;q11)	SS18–SSX

However, the majority of sarcomas have complex and variable genetic changes without balanced translocations. These sarcomas frequently have dysfunctional regulation of the cell cycle[30, 31], either by inactivating mutational events in central regulators (e.g. *TP53*, *CDKN2A* or *RB1*), or increased copy numbers of antagonists (e.g. *MDM2* or

CDK4). These and other yet unknown factors contribute to the genetic instability in these tumours.

Prognostic factors for sarcoma survival

At present, the treatment approach to sarcomas is based on the malignancy grade, tumour type, resectablitity and the presence or absence of overt metastases. Other patient, tumour or treatment specific factors have been proven to be of prognostic importance, and may guide treatment decisions to some extent, and several of these factors are common to most sarcoma entities (table 2).

Table 2: major factors with established beneficial impact on sarcoma outcome

factor		STS	OS	EFT
tumour	low malignancy grade	yes	yes	n.a.**
	non-metastatic	yes	yes	yes
	Extremity localization	yes	yes	yes
	small tumour volume	yes	yes	yes
treatment	complete surgical resection	yes	yes	yes
	chemotherapy	no*	yes	yes
	radiotherapy	yes	no	yes
	initial treatment at expert centre	yes	yes***	yes***

yes: of proven importance; no: not of proven importance

*exception for STS in children

** not applicable, Ewing's family of tumours are always regarded of high malignancy grade

*** low patient volumes make statistics uncertain, very few patients are treated outside paediatric oncology centres

Malignancy grade: Malignancy grading is applied for the majority of STS and BS to improve the prediction of local aggressiveness and metastatic potential. The malignancy grade is usually based on the pathologist's evaluation of intratumoural necrosis, mitotic rate and degree of cellular and architectural differentiation. The two most widely used systems are the FNCLCC[33] and the NCI system[34]. However, the risk of metastasis varies within the group of tumours with high malignancy grade, and more recently prognostication systems have been developed by combining several tumour features. Engellau et al. proposed a stepwise model of

predicting risk of metastatic relapse for non-metastatic high grade STS defining two risk groups[35]. High risk tumours showed vascular invasion or at least two of three other features: tumour size >8cm, tumour necrosis and peripheral infiltrative growth pattern. The high risk group had a cumulative incidence of metastasis at five years of 51% compared to 5% for the low risk group.

Histotype: Some sarcoma histotypes have a uniform behaviour and are thus independent of malignancy grading and prognostication systems. These include lipoma-like liposarcomas (relatively benign behaviour), myxoid round-cell liposarcomas (behaviour determined by the amount of round cells), dedifferentiated liposarcomas (highly malignant), synovial sarcoma (highly malignant), classical central OS (highly malignant), superficial (parosteal) OS (low malignancy) and EFT (highly malignant).

Metastatic status: The presence of overt metastatic disease is the most important adverse predictor of outcome for soft tissue and bone sarcomas (table2). The site and number of metastases has further importance as isolated pulmonary metastases carry significantly better prognosis in both bone sarcoma[36, 37] and STS[38]. This is due to the resectability of macroscopic disease, and underscores that chemotherapy is an ineffective substitute for complete surgical resection.

Site and size: Extremity localization is a positive prognosticator due to the resectability of the tumour[25]. Bone sarcomas localized to the axial skeleton carry a grave prognosis[25]. Retroperitoneal liposarcomas present with larger tumour volumes and are more often resected with marginal or intralesional margins than appendicular liposarcomas[39].
Small tumour volume is independently and positively related to outcome in the both STS and bone sarcomas[25, 35, 40].

Demographic factors: Young age is a positive predictor of survival in STS[38, 41], OS[25] and EFT[42]. Gender has been an inconsistent marker weakly favouring female sex and occasionally reaching statistical significance[25, 42].

Treatment related factors: Treatment at a sarcoma centre improves the outcome in STS and supposedly in bone sarcomas [43-46]. Achieving a complete resection is of

central importance for the risk of relapse and survival for STS and bone sarcomas (table2)[25, 41]. The likelihood of chemotherapy response is not sufficiently predicted by the factors mentioned above. High dose regimes can improve tumour sensitivity to methotrexate in OS[47], but a pre-treatment prediction of response to chemotherapeutic agents is not possible today. Recently described polymorphisms in folate metabolism modulating the response to methotrexate might prove useful[48]. Predictive impact of P-glycoprotein expression for chemotherapy resistance and survival has been shown in a prospective study but has so far not gained general acceptance or therapeutic impact[49 50, 51]. Preoperative chemotherapy in OS and Ewing sarcoma allows evaluation of the degree of residual viable tumour tissue at the time of the surgery, and this factor has been shown to be strongly predictive of long term outcome[25, 42, 52]; The hypothesis that it may be possible to improve the prospects for patients with initially poor tumour response is a central issue in the study forming the basis for paper 1.

Treatment and outcome

Treatment of the primary tumour: In both BS and STS, surgery is required to achieve local control of the primary tumour, and only in EFT may radiotherapy alone be successful in gaining local control[53, 54]. The importance of a complete resection of all identifiable tumour tissue has been demonstrated also for metastatic sarcoma[55-57]
With high quality surgery in extremity localized OS a local relapse rate of only ~5% is reported in recent series[25, 58], despite high rates of limb-salvage surgery. In unselected series of STS of the extremity and the trunk wall, a local relapse rate of 15-27% is observed[41, 59]. The commonly contaminated resection margins in retroperitoneal sarcomas are the main cause for a local relapse rate of 59-68%[60].
Pre-operative radiotherapy may render some STS operable and post-operative radiotherapy can improve local control for STS subgroups and EFT with inadequate surgery[61, 62].

Systemic treatment for patients at high risk of developing metastatic disease: Most sarcomas of childhood and adolescence are viewed as systemic diseases at the time of

diagnosis, and may be cured by adjuvant chemotherapy. In OS, four drugs are most active and have become the basis of modern combination treatment: methotrexate[63] , doxorubicin[64], cisplatinum[65] and Ifosfamide[66, 67]. Pre-operative chemotherapy for bone sarcomas was introduced in the late 1970ies[68, 69] to allow for the construction of custom made prostheses, and to attack probable micrometastatic disease as early as possible. However, the addition of pre-operative systemic treatment has not given any proven survival benefit[70]. Introduction of aggressive chemotherapy three decades ago has resulted in a considerable increase of 5-year overall survival for localized osteosarcoma, Ewing family of tumours and rhabdomyosarcoma from 10-25% to 60-80%[71-73].

Although high-grade adult STS carry a significant risk of distant metastases, the use of adjuvant chemotherapy remains controversial. According to a recent meta-analysis adjuvant doxorubicin improves local and distant control rates by 6% at ten years, and an update of this meta analysis in 2007 showed a significant survival benefit of 6%[74, 75]. However, the EORTC 62931 study randomizing adjuvant doxorubicin and ifosfamide vs. observation alone has reported no improved progression free survival or overall survival[76]. Thus, there is no definitive proven benefit of adjuvant chemotherapy in the broad group of adult STS. However, studies have commonly lumped together tumours with varying risk profiles and chemosensitivity, possibly masking effects in more chemosensitive subgroups like synovial sarcoma and myxoid liposarcoma[77]. The challenge lies in the small patient subgroups limiting the possibility for targeted subgroup studies.

For a mixed series of 1646 adult, resectable and nonmetastatic STS registered by the SSG, 5-year tumour related survival was over 70%[43]. Survival rates of retroperitoneal sarcoma are reported from 37-60%[78] and vary by the histological subtype. One group found a 92% 5-year survival rate for well differentiated liposarcoma compared to 36% for dedifferentiated liposarcoma[79].

Survival rates

Treatment of patients with overt metastases: A limited number of resectable metastases may be a curable situation in both OS and adult STS[55, 57]. Survival depends on whether a complete surgical remission can be achieved. 5-year overall survival for metastatic OS was reported with 29%; when a complete remission could be achieved, survival exceeded the 40% mark, whereas unresectable metastatic disease carried a very poor

prognosis with no survivors after 5 years[57]. Similarly for STS, patients selected for resectable pulmonary metastases achieved a 5-year overall survival of 38%[55] whereas survival remains poor for most patients with a primary metastatic STS. A Chochrane meta analysis of chemotherapy in metastatic STS[80] reports response rates to single agent doxorubicin ranging from 16-27% and median survival from 7.3 - 12.7 month with no additional benefit combining doxorubicin with additional agents. A combined analysis of 7 prospective EORTC studies including 2187 patients reported survival rates of 8% at 5 years and 5.6% at 8 years[38, 54]. The most significant predictor for long-term survival was response to chemotherapy and survivors were observed even in the patients with unfavourable prognosis as liver metastasis and high grade tumours. Dose escalation trials in adult STS have not been successful[81, 82], and single agent doxorubicin remains the standard treatment for metastatic adult STS[83, 84] with the notable exception of imatinib in GIST[85].

Current challenges in the treatment of sarcomas

Control of the primary tumour is not the main challenge, as the constant development of advanced surgical techniques and the combination with radiotherapy for selected patients in general gives low rates of local recurrence when performed by expert centres. Some tumour localizations remain problematic due to limited surgical access, e.g. in the head and neck, spine, thorax, retroperitoneum and pelvis.
The main challenge is to develop more efficient systemic treatment for patients with overt metastatic disease or for patients at high risk for developing such metastases.
In OS and EFT, where chemotherapy has improved outcome significantly, the challenge remains to increase event-free survival beyond 60-70% for patients with localized disease and beyond 10-30% for patients with initial metastases. To increase survival for poor histological responders after neo-adjuvant treatment is also imperative, as is the reduction of the current formidable toxicity.
For the heterogeneous group of STS the main challenges are to more accurately identify the patients at high risk of developing metastatic disease, to develop more effective systemic treatment and to adapt treatment to the tumour biology of the individual subgroups. In the majority of STS's the efficacy of existing systemic treatment

is poor; the role of adjuvant chemotherapy remains disputed and combination regimes have not clearly surpassed single agent doxorubicin as the standard of care.

Much progress has been made to elucidate the biological basis for some subtypes of sarcomas, and for a few of these, treatment success has been achieved by targeting key signalling pathways. This approach appears to be the most promising for all sarcomas. However, the low incidence, tumour heterogeneity and need for an expert multidisciplinary approach increase the challenges related to improving treatment results, and both centralization of patients to expert centres and large multicenter collaborations are important for further improvement. In fact it may be argued that a significant potential in improving particularly adult STS outcome remains unrealized by treating many patients at local hospitals without the necessary expertise. The effect of securing correct referral to expert centres without prior surgery for all patients may outweigh any novel research impact, at least in the short term.

New drug candidates investigated at The Norwegian Radium Hospital.

Our group has developed a specific interest in the possible role of interferon and mdm2 inhibitors. Interferon has the advantage of being a developed and well known drug for other conditions (table 3), and some data have suggested that interferon has activity in OS (paper 2). As for mdm2, our group's long standing interest in the 12q13-14 amplicon[86] was the background for our collaborative work with L. Vassilev[87] on preclinical studies of mdm2 amplified sarcoma cell lines.

Before discussing the actual work performed in this study a short general background will be given for these two new candidate approaches in sarcoma treatment.

Interferon

Five decades after their discovery, interferons (IFNs) have clearly defined indications in virology, neurology and oncology (table 3), but many important questions remain unanswered. The only sarcoma evidently sensitive to IFN is Kaposi's sarcoma[88], whereas a possible role in the treatment of Osteosarcomas is under investigation.

Table 3: clinical applications of type I interferons[89]

indication	
non-malignant	chronic viral hepatitis
	papillomatosis and condylomata acuminta
	haemangioma of infancy
	multiple sclerosis
malignant	AIDS-related Kaposi's sarcoma
	chronic myeloid leukaemia
	myelomatosis
	hairy cell leukaemia
	essential thrombocytopenia
	carcinoid tumours
	malignant melanoma
	renal cell carcinoma

Originally grouped according to the secreting cell type, IFNs are now classified into type I, II and III according to receptor specificity and sequence homology. The nine distinct type I IFNs bind to a common heterodimeric receptor. IFN-α (previously termed leukocyte IFN) consist of13 different subtypes whereas IFN-β, (previously fibroblast IFN) and other type I IFNs exist only in one form[90].

Antitumour activity of type I (α,β) IFNs was demonstrated in the late 1960ies. These effects can be separated into host dependent mechanisms (innate and adaptive immunity, angiostatic effect) and intrinsic tumour suppressor activity (mediated by expression of interferon stimulated genes)[91].

Immune-mediated effects: IFNs exert a broad range of immunoregulatory effects and promote immune functions. For example, type 1 IFNs activate dendritic cells, increase the cytolytic activity of macrophages and NK cells, induce the production of IL-15, prime T-cells and increase survival of T-cells leading to tumour cell kill, although the relative physiological relevance of these functions remains unclear[92]. Endogenous IFNs play a constitutive role in restricting emergence and development of tumours[91, 93]. Recently, both type 1 and 2 IFNs have been shown to be involved in the interactions between tumour and the immune system (immunoediting[92]).

Angiostatic effects: IFN was the first recognized angiostatic agent with clinical efficacy in angioproliferative tumours like Kaposi's sarcoma and life threatening haemangioma of infancy[88, 94]. This angiostatic effect is not related to the antiproliferative effects of interferons[95] and dependent on frequent administration of an optimal biological dose and not the maximal tolerated dose in a bladder cancer model[96].

Intrinsic tumour suppressor activity: IFNs exert direct tumour suppressor activity although the detailed mechanism is not yet known. Interaction with the p53 pathway has been demonstrated[97]. IFNs can halt cell cycle progression and this process is paralleled by OAS increase, inhibition of proto-oncogene expression and it may be related to cell differentiation[98]. IFN-α-mediated apoptosis in malignant cells is largely dependent on the activation of different caspases, and is also associated with the disruption of mitochondrial integrity and release of cytochrome c[99]. Recent genome wide profiling efforts have established several hundreds of IFN stimulated genes with largely undefined importance for the treatment response[100-102].

MDM2 antagonists

The tumour suppressor protein p53, kept at very low levels in unstressed cells, is rapidly stabilized and activated in response to environmental and intracellular stress. p53 exerts its antitumour effects primarily by transcriptional activation leading to induction of cell cycle arrest, DNA repair, apoptosis or senescence[103] . In addition to transcriptional activation and probably less important, p53 exerts transcription independent pro-apoptotic function by BAX mediated stabilization of the mitochondrial membrane[104].

p53 and MDM2 form an auto-regulatory feedback loop by which the two proteins mutually control each other's function. Targeting the *MDM2* gene promoter.p53 increases cellular MDM2 levels. MDM2 inactivates p53 by blocking its transactivation domain[105], promotes nuclear export of p53[106] and p53 degradation[107]. Cellular stress signals release p53 from MDM2, a process thought to be mediated by specific

phosphorylation of both proteins[108]. More recent data indicate that MDM2 levels are critical for p53 control and that destabilisation of MDM2 is an important factor for initiating a p53 response[109].

Approximately half of all malignancies including sarcomas carry a deletion or inactivating point mutation of *TP53,* and a significant proportion of malignancies with wt *TP53* have defects in the signalling network making the p53 response dysfunctional[110]. In OS, 22-39% have been found to have p53 mutations[111, 112]; a further 16% of OS and 29% of liposarcomas deactivate p53 by an *MDM2* amplification[113]. MDM2 protein overexpression in the absence of gene amplification has been observed[113]. Investigating the *MDM2* gene variant SNP309, even a modest 2-4 fold overexpression of MDM2 could be shown to promote cancer development[114].

Treatment rationale: as tumours with *MDM2* gene amplification almost exclusively retain wild-type *TP53* but loose p53 tumour suppressive function[116, 117], inhibiting MDM2 might reactivate p53 in cancer cells. Recently, the first potent and selective small-molecule MDM2 antagonists, the nutlins, were identified from a class of cis-imidazoline compounds. Nutlins bind to the p53 pocket of MDM2 and inhibit the p53–MDM2 interaction with a high degree of specificity. In vivo treatment with Nutlin 3a was well tolerated in several xenografts models. Proliferating cancer cells retaining wild type p53 were effectively blocked in G1 and G2 phases, and underwent apoptosis when exposed to low micromolar concentrations of nutlins[87]. No anti tumour effect was observed in cells carrying a mutant or deleted p53 indicating that an intact p53 pathway is necessary for this effect.

Aims of the present study

During the last decades multidisciplinary efforts have changed the prospects for many sarcoma patients considerably. Further improvements in the treatment of these rare tumours will depend on conquering the challenge of inherent and acquired chemotherapy resistance, in particular in patients with inoperable or overt metastatic disease.

The effect of systemic treatment may be improved by at least three general strategies:

1. Optimization of the administration of established agents and combination regimens
2. Further research into identified promising agents which appear to have effect in sarcoma, but where current evidence is insufficient. Aims should be both to demonstrate clear clinical efficacy and to increase the knowledge of mechanisms of action.
3. Development of entirely new drugs which may be efficacious alone or in combination with known agents. Currently the most promising approach is targeting the signalling networks of sarcoma cells and their microenvironment.

This thesis is based on projects illustrating each of these three strategic approaches, with the following aims:

1. To examine the effect of increased chemotherapy aggressiveness in osteosarcoma, and to use replacement salvage chemotherapy to increase survival in patients with poor histological tumour response to pre-operative treatment
2. To further assess interferon as an agent in osteosarcoma by
   - Analysing long-term effects after adjuvant treatment with single agent interferon in a clinical series and
   - Investigating its antitumour effect and effects on signalling in a xenograft model
3. To study the effects of a new compound, the small molecular MDM2 antagonist Nutlin, in soft tissue sarcoma cell lines selected for amplification of the *MDM2* gene.

Summary of publications

Paper I:

Scandinavian Sarcoma Group Osteosarcoma Study SSG VIII: prognostic factors for outcome and the role of replacement salvage chemotherapy for poor histological responders.

SSG VIII applied a combination of the current most effective drugs for the treatment of osteosarcoma. The results with relapse-free and overall survival of 63% and 74% at 5 years represent an apparent improvement over the less intense preceding SSG II regimen, and the results are comparable to the best published series. The salvage chemotherapy approach with etoposide and ifosfamide failed to improve results for poor histological responders, as continued exposure to the conventional agents doxorubicin, cisplatinum and methotrexate appeared at least as effective as switching to a dose-intense regimen with the new drugs.
Unexpectedly, the data showed an independent gender specific survival advantage for female patients which remains unexplained.

Paper II:

Interferon-α as the only adjuvant treatment in high grade osteosarcoma: Long term results of the Karolinska Hospital series.

The Karolinska series represents a unique treatment approach for nonmetastatic high-grade osteosarcoma with primary resection followed by single agent (semi-purified, leukocyte) interferon-α. This consecutive series was started before the introduction of aggressive combination chemotherapy in 1971 and continued through 1990, enrolling 89 patients. The toxicity was limited and comparable to other series using long-term adjuvant IFN. The observed 10-year metastases-free and sarcoma specific survival rates were 39% and 43%, respectively. The apparently improved outcome as compared to historical controls could not be explained by second line chemotherapy at relapse, as only one of seven survivors after relapse received

chemotherapy. These observations suggest activity of interferon-α as adjuvant treatment in high-grade osteosarcoma.

Paper III:

Characterization of Treatment Response to Interferon –α in Osteosarcoma Xenografts

We screened five osteosarcoma xenografts for specific growth delay to IFN-α and explored molecular mechanisms involved in response and resistance by analyzing the transcriptional response. Only one of five xenografts displayed growth inhibition and this was compared to two resistant xenografts. A common set of 79 genes was identified in response to IFN-treatment independent of the growth inhibiting effect, and the majority represented well characterized interferon stimulated genes. The expression of 121 unique genes changed only in the IFN-sensitive xenograft, and subsets of these genes are involved in cell adhesion and osteogenic tissue development. Combination treatment with interferon and doxorubicin showed improved growth control rates.

Paper IV:

Potential for treatment of liposarcomas with the MDM2 antagonist Nutlin-3A

We examined the response to Nutlin-3a in a panel of five osteosarcoma and four liposarcoma cell lines. Wild type p53 cell lines displayed a dose dependent inhibition of cell proliferation when treated with increasing concentrations of Nutlin 3A. In cells with MDM2 amplification, Nutlin efficiently stabilized p53 and induced downstream p53 dependent transcription and apoptosis. An antiproliferative effect of Nutlin was also observed in cell lines with wt TP53 but without amplified MDM2, but apoptosis was not induced. Inhibiting the MDM2-p53 interaction in these cell lines reactivates an otherwise intact p53 pathway.

Results and Discussion:

Can outcome in OS be improved by further refinement of current treatment approaches?

To improve on the results from SSG II[52] two assumptions were made in the planning of SSG VIII. The first was that more aggressive pre-operative treatment would improve both histological response and survival. Thus pre-operative treatment was intensified by increasing the methotrexate dose and by adding doxorubicin and cisplatinum. In the postoperative phase supposedly less effective drugs (dactinomycin, bleomycin, and vincristine) were omitted to allow for continuation of the pre-operative agents at adequate dose intensity (in good responders). The second assumption was that in poor histological responders, postoperative replacement chemotherapy with other agents (etoposide and ifosfamide) would improve outcome. The rationale for this was that the persistence of viable primary tumour tissue indicated chemoresistance also at the micrometastatic level, and that the switch to an ifosfamide/etoposide combination could circumvent this resistance. Ifosfamide had shown considerable activity in second line OS treatment[66, 67], and there was already a positive Italian experience with ifosfamide/etoposide in poor histological responders[72].

SSGVIII was successful in increasing both histological response and survival as compared to the preceding SSG II, and the results were comparable to those of the best contemporary studies[72, 115, 116]. However, the gap in outcome between good and poor histological responders remained.

Histological response to pre-operative chemotherapy is recognized to be one of the most important predictors of outcome in high-grade OS, and several trials apart from SSGVIII have tried to adjust the post-operative treatment in poor responders to improve results. Poor response to preoperative methotrexate/BCD could not be compensated by postoperative addition of doxorubicin and cisplatinum[117, 52, 118, 119]. To our knowledge only one study[72] has been able to close the survival gap between

poor and good responders, and that was by continuing the same drugs postoperatively and add ifosfamide and etoposide in the poor responders. Our later SSG VIII study, where the same drugs were introduced but replaced the pre-operative drugs did not succeed. It should be underlined that even in poor responders survival is clearly increased over patients treated by surgery alone (from ~20% to ~55%). Poor histological response does thus not indicate total chemoresistance at the micrometastatic level, only somewhat decreased sensitivity. This is supported by the observation in the relatively large group of grade II responders, who where treated differently in two separate phases of the SSG VIII study. Patients who were switched to etoposide/ifosfamide (VIG) actually had a trend towards poorer metastasis-free survival than those continuing methotrexate, doxorubicin and cisplatinum (MAP). Therefore, the collective data from SSG VIII and IOR-OS2 indicate that adding etoposide/ifosfamide to MAP is superior to replacing MAP with the same agents.
It could be argued that the intermediate dose of ifosfamide applied in SSGVIII (4,5g/m2) was insufficient to overcome chemoresistance in the salvage arm. In retrospect a higher ifosfamide dose would appear feasible when considering the relatively low rate of grade 4 haematological toxicity after VIG.
SSGVIII supports that modern chemotherapy for OS should start with a three drug combination of methotrexate, doxorubicin and cisplatinum (MAP), and that these drugs should be continued postoperatively regardless of histological response. Our data support that adding ifosfamide and etoposide to postoperative MAP in poor responders is a better strategy than replacing MAP, and the effect of this strategy is currently being tested in the EURAMOS1 study.

The Cox regressional analysis identified low tumour volume, high serum methotrexate at 24h and female gender as independent predictors of improved metastasis-free survival. Histological response was a significant factor at the univariate level (p=0,03), but fell short of significance in the multivariate analysis. This may be due to the relatively small size of this study (113 patients). However, the increase in the fraction of good responders compared to the T10-based SSGII was not reflected by a comparable survival benefit. In their last randomized study, the EOI found that increased dose intensity correlated to the degree of necrosis but not survival indicating that necrosis is likely to be a protocol dependant variable,

and that its relative importance as predictor of survival decreases with increasing treatment intensity[120].
The strong predictive power of female gender in the Scandinavian OS population is unique in the literature, and so far remains unexplained. This effect of gender was not seen in historical controls treated by surgery alone[121], and indicates that one explanation could be differences in the effect of chemotherapy, e.g. through differences in drug sensitivity, pharmacokinetics and/or drug metabolism. However, there was no indication of gender-specific differences in serum methotrexate levels in SSG VIII or SSG II. Interestingly, a positive prognostic impact of female gender was also found in the KS IFN experience (paper 2), and related to a difference in drug efficacy, the combined data indicate that this would be true for both IFN and combination chemotherapy.

Earlier reports suggest that increased dose and dose intensity, in particular of doxorubicin and methotrexate, are associated with a better outcome[47]. Several groups including SSG have found correlations between high serum levels of methotrexate and improved histological response and survival[52]**.** SSG VIII supports these findings and found a serum methotrexate level >4,5 µM at 24h to be an independent predictor of improved metastasis-free survival, and in the subsequent ISGSSG1 study methotrexate doses were modified per patient and per course to achieve a pre-set target for serum methotrexate.
However, the data regarding the importance of chemotherapy intensity are not consistent, and a large retrospective analysis from the German cooperative osteosarcoma group could not prove that higher then average dose intensity of conventional chemotherapy correlated with better outcome[122]. Prospective studies have examined different strategies of further dose intensification. The ISGSSG1 trial attempted to "maximize" chemotherapy by combining ifosfamide 15 g/m2 with cisplatinum, doxorubicin, methotrexate and mandatory granulocyte colony stimulating factor support. Furthermore methotrexate treatment was optimized by modifying dose according to serum methotrexate measurements. Toxicity was increased but survival was similar to SSGVIII[123].
The concept of increasing the dose intensity by compressing the treatment interval (dose-dense chemotherapy) was tested by the last EOI randomized study. A dose dense schedule of the two drug regime of doxorubicin and cisplatinum was

compared to conventional intervals and did not show a survival benefit[120]. Dose escalation with peripheral blood stem cell support in primary metastatic or inoperable OS was addressed by the ISGSSG2 study. The toxicity was manageable but survival rates were discouraging[124]. Another trial following a similar strategy in bone sarcomas with unfavourable prognosis failed to show survival benefits and reported intolerable toxicity[125]. Similarly, high dose chemotherapy with stem cell support has not been shown to be superior to conventional relapse therapy in relapsed patients[126, 127].

Collectively the data show that osteosarcoma survival has improved during the last three decades through gradual identification of the most active agents, and to some degree by intensification of their combined use. However, further intensification beyond the current standards for methotrexate, cisplatinum, doxorubicin and ifosfamide is probably not the way for further improvement. Current short and long term toxicity is significant and needs to be reduced[128] . Future strategies should focus on better individualized and risk-adapted treatment with the development of robust pre-treatment markers for the individual patient. These may include individual pharmacokinetic factors. Whereas a general increase in dose intensity was not successful, an individualized dose adjustment based on drug serum levels may be pertinent for more drugs than methotrexate. However, the need for the development of new effective drugs with low toxicity is apparent.

Finally, SSG VIII represents a relatively small phase II study with all consequent limitations that are attached to the interpretation of its data. In order to properly address the future challenges for all rare tumours including OS, intergroup collaborations allowing proper randomized trial designs are essential.

Single agent IFN-α as adjuvant treatment in OS

During a twenty year period, patients with resectable non-metastatic osteosarcoma were offered adjuvant IFN at the Karolinska Hospital (KS) in Stockholm[129, 130] . The

series was initiated in 1971 before the international introduction of effective chemotherapy, in part based on the assumption that OS might be induced by an oncogenic virus. An early indication of an effect on relapse-free survival led to the continuation of this project[129]. Furthermore, evolving preclinical data from the Karolinska Institute (KI)[131, 132] and other institutions[133-135] confirming the antitumour effect of IFN-α in vitro and in vivo combined with limited toxicity[136, 137] was thought to strengthen the rationale for IFN use despite the internationally evolving combination chemotherapy. When a positive effect of IFN was indicated, the diagnosis of high grade OS in the first 28 patients was subjected to a confirmatory independent review in 1976[129]. Additional support for diagnostic accuracy in the IFN series is provided by the close cooperation between sarcoma pathologists within SSG, with regular slide reviews and a low rate of misclassification in later studies where KS has participated (SSGVIII, ISGSSG 1).

The 89 patients given adjuvant IFN represent a consecutive series from 1971 to 1990. The distribution of primary tumour sites, age and gender is comparable to other reports[25, 138], but only 9/102 patients admitted to KS were diagnosed as having detectable metastases at diagnosis. This fraction was lower than in other reports (12-20%)[25, 139], and may represent an under-diagnosis of pulmonary metastases in the KS series. A small decline in the number of IFN-treated patients in the last years is probably explained by a remapping of the health care regions leaving the Karolinska Hospital a smaller geographical catchment area.

The analysis of potential prognostic factors showed that in this limited patient material large tumour diameter, male sex and intralesional surgical margins were predictors of inferior survival. The first two factors were also reported for Scandinavian patients treated with chemotherapy (see discussion of paper 1). Inadequate margins are closely associated with the risk of local recurrence in OS[25], and the relatively high rate of intralesional surgery in this series (11%) resulted in a high rate of local recurrence (22%) and a 2,4-fold increased risk of sarcoma specific death. This may be related to the absence of pre-operative chemotherapy in parallel with a relatively high level of ambition as regards limb salvage surgery (42%).

A small and retrospectively collected group of patients treated in the early seventies by surgery alone at other Swedish centres had a slightly better survival than a historical

control group at the KI[140]. These groups were both of small size and it is important to note that survival in the KI control group (17% after 2.5 years) is comparable to both Scandinavian[121] and international reports[141, 142]. Thus there is no evidence of a change in the natural history of OS which can explain the improved survival in the INF-treated patients[143].

This long term follow-up study of the IFN-treated OS patients from KS shows that the percentage of survivors at 10 years (43%) is considerably higher than for historical controls treated by surgery alone (11-18%)[121, 140-142], but still appears lower then for modern combination chemotherapy (55-75%)[25, 72, 120]. The data allow no conclusion as to whether the IFN dose increase in the late series was more efficacious. The survival increase found can not be assigned to second line chemotherapy as the majority of survivors remain in their first remission, and only one of the long-term survivors has received chemotherapy at relapse.

An effect from adjuvant IFN is thus clearly suggested by this experience from KS. However, it should be emphasised that the data in the KS series are derived from a consecutive patient series rather than a formal clinical study, and that the results therefore must be interpreted with considerable caution. It should also be kept in mind that there are, to our knowledge, no reports indicating that IFN has effect in metastatic disease, which has traditionally been a pre-requisite for adjuvant treatment. Unpublished experiences from KS and the Rizzoli Institute (Hans Strander and Stefano Ferrari, personal communications) indicate that there is no effect of IFN on established metastatic disease.

Thus the effect of IFN in OS remains unclear and should to be put to the test in a well designed randomized controlled trial in combination with chemotherapy. This was attempted in the COSS 80 study where patients were randomized to receive IFN-β or not as maintenance treatment after completing chemotherapy[118] – no effect was seen. However, this trial used a low IFN dose for a short period of time. For the subgroup of good responders to preoperative chemotherapy the addition of maintenance IFN is currently being tested in EURAMOS-1. The question whether poor responders would benefit from IFN maintenance treatment is not addressed in that trial.

Antitumour effects of interferon in experimental models

In OS and other malignancies, resistance to IFN-α is common but poorly understood. A better understanding of the molecular mechanisms of response and resistance is required to effectively utilize this agent. Furthermore, biomarkers are required to select treatment options and may help to define new cellular targets.

Human OS xenografts in immuno-incompetent mice have been widely used as disease models[144]. Subcutaneous xenografts offer certain advantages in the study of OS. They are relatively easy to establish, make it possible to transplant human tumour samples without the prior selection process in cell culture, and have been shown to be relatively stable genetically over long passage times[145, 146], giving abundant access to fresh tumour tissue. Importantly, they give the opportunity to perform repetitive therapeutic experiments. These models have been shown to be important for the preclinical assessment of treatment response[144]. In our research group, OS xenografts have been used to profile the response to doxorubicin, cisplatinum, ifosfamide and methotrexate[147, 148] and to experimental agents. Disadvantages of this model include a necessary selection of aggressive tumours for growth to occur, and a low inherent tendency for metastasis, probably due to the rapid growth of the primary implant not leaving time for detectable metastases to develop. Furthermore, the immune deficiency of the host and the species specific action of IFN limit this model to the study of direct antitumour effects of human relevance. However, as regards the analysis of signalling and molecular effects secondary to IFN within the cancer cells, the model may be highly relevant.

Pegylation prolongs IFN half life and is expected to augment and prolong IFN signalling[149]. The unexpected low response rate in the reported xenograft panel (paper 3) when compared to an older series[131] and the short-lived effect on expression profiles, prompted us to examine response to unpegylated IFN, but our xenografts appeared equally resistant. Interestingly, a high daily dose of unpegylated IFN was able to induce complete remission in one IFN-sensitive xenograft. Examination of the elimination rate of PEG-IFN by radioiodine-labelling indicated a shorter half life than expected, and

shortening the dose interval improved the response to PEG-IFN in the sensitive xenograft. Others have shown that recombinant IFN-α was less effective than natural IFN-α in an OS-xenograft model[150] and that IFN-α subtypes have different efficacy in several other model systems[151]. The diversity of type I IFNs is further highlighted by superior efficacy of IFN-β over IFN–α in some cell lines[152]. Our findings may indicate that recombinant IFN α2b is not the optimal IFN for the treatment of OS, although there may be indirect effects through host stroma and immune system not detected in the xenogeneic models[92, 153].

To explain the low response rate it may be relevant that three of our tumours (including the sensitive one) were exposed to chemotherapy before xenografting, whereas all the Karolinska tumours were chemotherapy naïve. The sensitive xenograft being the only one of metastatic origin is interesting taking into account the disappointing clinical experience with IFN in overt metastatic osteosarcoma. Furthermore, our xenograft lines were maintained over a longer time before the experiments. Although passage number did not impact on response to chemotherapy in a previous study on these xenografts[148], we can not exclude that primary IFN resistance may be caused genetic or epigenetic alterations over time. However, interferon sensitivity was documented in all together three passages of the same xenograft. Finally, there were no apparent differences in the xenografting method between our and the Swedish xenograft series. Based on only one sensitive xenograft, no firm conclusions can be drawn and findings have to be viewed as preliminary.

For the sensitive xenograft, global transcription profiling did not indicate selective impact on cell cycle regulators or cell death genes at the mRNA level. Genes reported by others, e.g. IFITM1 (9-27)[101, 154] and IFI16[101] were expressed in all three cell lines without apparent relation to response. We describe IFN-induced changes in cellular adhesion genes and genes involved in differentiation and suggest that these pathways may be involved in the IFN response in this particular xenograft. Again, these findings have to be viewed with caution as they are based on one xenograft only, and because the fold changes are relatively low. However, IFN-a was shown to induce differentiation in an OS model[155] and increased

differentiation capacity in CML cells[156], and this mechanism may correlate or perhaps contribute to a reduced proliferation rate.

The global transcriptional response demonstrated activation of central IFN stimulated genes in all three xenografts independent of a growth inhibitory effect. The activation of genes related to the innate antiviral immune response and of the adaptive cellular immune response was not correlated with the growth inhibitory effect of IFN in our model system.

Resistance to IFN has been linked to defects in signal transduction. Others have described resistance due to circulating IFN receptors[157], suppressed expression of JAK[158] or STAT2[159], or methylation of the proapoptotic genes (XAF1)[152]. In our model, transactivation of IFN-stimulated genes with ISRE in promoters indicate an intact signal transduction. The observation is supported by the expression of two genes involved in the classical JAK STAT signal transduction cascade, STAT1 and IRF9. The activation of the proapoptotic genes Trial and XAF1 makes at least a broad demethylation of their promoter regions of IFN stimulated genes unlikely. Selective demethylation, post-transcriptional silencing by miRNA or selective destruction by targeting for ubiquitinilation remain untested in our model.

An interesting observation was done in pilot experiments combining IFN and doxorubicin. The combination appeared to have at least additive effects. Doxorubicin has been shown to induce cell death genes in the treatment of hepatocellular carcinoma[160], but we could not show a clear induction of these genes by doxorubicin after 24h in one xenograft. A number of potential predictors of responsiveness to doxorubicin have been described in our OS xenograft system[147] but were not confirmed in our study.

Once confirmed, these finding supports a strategy to combine IFN with other agents. IFN has been shown to kill multidrug resistant OS cell lines[161] and synergy with other agents has been shown in the treatment of non-Hodgkin lymphoma[162] and recently in the treatment of renal cell carcinoma[163]. The sensitisation of p53 by IFN[97] would be expected to make tumours with intact TP53 more prone to many types of chemotherapy. The notion is also supported by induction of a broad IFN response on

the molecular level even in our resistant xenografts. Finally, IFN was shown to mobilise stem cells in leukaemia rendering this tumour responsive to cytotoxic chemotherapy[164]. It remains to be shown whether this effect can be exploited in solid tumours.

Effect of mdm2 inhibition in OS and LS

The *in silico* development of a class of small protein antagonists of MDM2, the Nutlins, represented a major breakthrough in molecular biochemistry[87]. Targeting the MDM2-p53 regulatory circuit is expected to reactivate the p53 pathway in a large range of tumours. At the start of this work it had already been shown that Nutlin was effective in a single osteosarcoma *in vitro* cell line with *MDM2* amplification both when grown in vitro and in vivo. We wanted to explore the therapeutic potential of this drug in both osteosarcoma and liposarcomas depending on their status of *TP53* mutation and *MDM2* amplification. An initial collaboration[165] showed for the first time efficacy in a xenograft established directly from a patient's osteosarcoma.

In paper 4, we showed that Nutlin-3a had a dose dependent anti-proliferative effect in wild type *TP53* osteo- and liposarcoma cells. The p53 protein was stabilized and its level thus increased. In MDM2 amplified cell lines, we observed a Nutlin-induced transactivation of p53 targets *MDM2*, *CDKN1* (encoding p21) and *BAX,* and down regulation of *BCL2*. Consistent with activation of p53 function, analysis of the cell cycle profile confirmed the activation of both G1 and G2 checkpoints. TUNEL labelling, Caspase 3 activation and PARP cleavage confirmed p53 dependent induction of apoptosis as the mechanism of cell death. Nutlin at the maximal tested dose did not arrest the growth of control cell lines harbouring a *TP53* mutation. Wild type *TP53* cell-lines without MDM2 amplification responded only with cell cycle arrest but not apoptosis.

These findings confirm that *MDM2* amplified liposarcomas and osteosarcomas contain an otherwise intact p53 pathway. A similar observation was recently made in synovial sarcoma cell lines where the fusion-protein SS18-SSX stabilizes MDM2[166]. Resistance to genotoxic stress by the topoisomerase II inhibitors

doxorubicin and etoposide is common in both liposarcoma and synovial sarcoma and it was shown that topoisomerase inhibitors did not disrupt the MDM2-p53 interaction and that p53 remained transcriptionally inactive. In these sarcomas, MDM2 antagonism targets the driving defect in the cancer genome and, in a parallel to inhibiting constitutively active tyrosine kinases in CML[167] and GIST[85], carries the promise of significant clinical efficacy.

Tumours with wild type p53 and normal or low level amplification of *MDM2*, as exemplified by the cell line U2OS, may also respond to MDM2 antagonists, although only with cell cycle arrest and not with apoptosis. This differential response may be caused by different affinity of p53 to the promoters of cell cycle regulators compared to proapoptotic genes, or may be explained by the complementary roles of the homologues MDM2 and MDM4 in regulating p53. It has been shown that loss of MDM2 mainly stabilized p53 levels whereas loss of MDM4 function increased p53 transactivational activity[168]. Whereas the MDM4 protein could be detected in U2OS, it is undetectable in cells with high level amplifications of MDM2, possibly because of MDM2 mediated degradation of MDM4[169]. Nutlin selectively blocks MDM2 but not MDM4[170]. The blocked apoptotic p53 response could be rescued by cellular stress signals that phosphorylated MDM4 dissociating the MDM4 complex with the deubiquitinylating enzyme USP7 (HAUSP) leading to reduced cellular MDM4 levels[171]. This mechanism could explain the synergistic effect of the selective MDM2 antagonist Nutlin and genotoxic agents in some cases[172] (Ohnstad et al., unpublished). Alternatively, another as yet undetected aberration in the p53 pathway could explain the differential block of p53 apoptotic functions.

The adverse effects of unspecific genotoxic agents, particularly induction of genetic instability and secondary malignancies are irreversible and often difficult to manage. With an increasing awareness of the quality of life of sarcoma survivors[128], nongenotoxic activation of the p53 pathway would therefore be an attractive therapeutic strategy for cancers with intact p53-dependent signalling[173]. Trials with small molecular antagonists of MDM2 have now been initiated [174].

Conclusions and perspectives

The present work has attempted to address different approaches to improve the systemic treatment in high-grade sarcoma, based on the three general strategies outlined on p. 21:

1a. Improve chemotherapy with established agents (combine more agents and increase dose levels).

This approach has been beneficial for childhood STS, but has been less effective in adult STS were single agent doxorubicin remains the standard of care[84]**.** In OS some intensification has been beneficial, as illustrated by the apparently improved results in SSG VIII when compared to SSG II. However, subsequent studies as exemplified by ISGSSG1 showed that further intensification added considerable toxicity without further improvement in tumour-related outcome[123]**.** Similar data have been reported for the Ewing family of tumours[175]. Thus further dose intensification is probably not the right strategy for further improvement in survival.

1b. Switch to replacement salvage chemotherapy in poor responders to pre-operative chemotherapy in osteosarcoma.

Poor response to chemotherapy remains an important treatment related prognostic factor for survival. With one possible exception[72], salvage strategies have not been able to compensate for an initial poor response. In SSG VIII a total replacement strategy with new agents postoperatively was unsuccessful. The collective current data indicate that when faced with a poor histological response to pre-operative chemotherapy, the *addition* of agents is better than altogether *replacing* the previous ones. It is important to realise that limited chemosensitivity as evaluated by primary tumour necrosis does not imply chemotherapy resistance at the micrometastatic level. SSGVIII and other studies show that even in poor responders, survival is elevated well above historical controls treated without chemotherapy.

2. Further studies on promising agents in clinical use having inadequate documentation of efficacy.

By doing a long term follow-up study of the Karolinska series we have confirmed that adjuvant IFN appears to give benefit in OS, but proof of efficacy is still lacking. Our attempts to further explore mechanisms of IFN sensitivity and resistance were limited by the low sample size and lack of IFN sensitivity in the xenografts.

In rare tumours with significant mortality there is a natural tendency for implementing new and promising drugs too early and outside well-designed clinical trials. This highlights the necessity for large cooperative efforts in the sarcoma field, as illustrated by the EURAMOS 1 trial, which to some degree addresses the IFN question in a randomized fashion.

3. The development of novel (targeted) agents.

Molecular profiling of sarcoma subtypes will probably have considerable impact in the future. Examples to date include the identification of GIST as a separate entity with a characteristic genetic alteration which has been targeted with successful treatment. Following the human cancer genome project a few critical signalling pathways have been identified[176]. Blocking a malfunctioning signalling network at single or multiple points in the cascade appears to be the most promising way forward. Among many emerging new agents we have selected Nutlin-3a and strengthened the case for further exploration of this substance in *MDM2* amplified sarcomas.

Further improvement in sarcoma survival is dependent on this type of strategy, where targeted drug development follows identification of critical signals driving the malignant phenotype. However, the increasing complexity of small-volume sarcoma subtypes and the aim to develop specific treatments for single tumour entities requires cooperation in large networks of expert centres, as established in EURAMOS, EUROEWING, CONTICANET and EUROBONET.

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Scandinavian Sarcoma Group Osteosarcoma Study SSG VIII: prognostic factors for outcome and the role of replacement salvage chemotherapy for poor histological responders

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Abstract

From 1990 to 1997, 113 eligible patients with classical osteosarcoma received neo-adjuvant chemotherapy consisting of high-dose methotrexate, cisplatin and doxorubicin. Good histological responders continued to receive the same therapy postoperatively, while poor responders received salvage therapy with an etoposide/ifosfamide combination. With a median follow-up of 83 months, the projected metastasis-free and overall survival rates at 5 years are 63 and 74%, respectively. Independent favourable prognostic factors for outcome were tumour volume <190 ml, 24-h serum methotrexate >4.5 μM and female gender. The etoposide/ifosfamide replacement combination did not improve outcome in the poor histological responders. In conclusion, this intensive multi-agent chemotherapy results in $>70\%$ of patients with classical osteosarcoma surviving for 5 years. The data obtained from this non-randomised study do not support discontinuation and exchange of all drugs used preoperatively in histological poor responders. As observed in previous Scandinavian osteosarcoma studies, female gender appears to be a strong predictor of a favourable outcome.

Keywords: Osteosarcoma; Clinical protocols; Chemotherapy; Neo-adjuvant therapy; Limb salvage; Prognosis; Scandinavia; Sex

1. Introduction

Adjuvant chemotherapy has significantly improved the outcome of patients with high-grade osteosarcoma [1–3]. Although never proven in controlled trials, neo-adjuvant therapy is generally agreed upon as the optimal treatment schedule. In addition to facilitating limb salvage surgery, it offers an opportunity to tailor postoperative chemotherapy after evaluation of histological response in the surgical specimen [4]. With this strategy, long-time overall survival rates of 70% are reported, and more than 80% of the patients can currently be expected to be operated upon with limb salvage surgery in major centres [5–9].

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Histological response as assessed by chemotherapy-induced tumour necrosis is regarded as a strong prognostic factor in osteosarcoma [4,7,10,11]. Consequently, a more intensive preoperative chemotherapy with more patients obtaining a favourable histological response has been associated with an improved outcome [6,9,12,13]. In the preceding Scandinavian SSG II study (1983–1990), single high-dose methotrexate was given preoperatively with only 17% of the patients achieving a good histological response [5]. In an attempt to increase the number of good responders, the subsequent SSG VIII study utilised an intensified regimen with high-dose methotrexate, cisplatin and doxorubicin given preoperatively to all patients.

The effect of salvage therapy to improve outcome for poor histological responders is not well documented. Ifosfamide has been viewed as an attractive candidate due to its reported efficacy in relapsed patients [14,15]. Etoposide has less single-agent activity than ifosfamide, but has demonstrated synergy with ifosfamide in the treatment of sarcomas [16]. In addition, adding ifosfamide and etoposide to salvage therapy for poor responders appeared successful in the Rizzoli IOR-II study [6]. Based upon these data, poor histological responders in the current trial were treated by an ifosfamide/etoposide combination that replaced the three drugs given up-front.

2. Patients and methods

2.1. Patients

From May 1990 to December 1997, 132 patients with high-grade extremity osteosarcoma from 14 centres in Sweden, Norway and Finland, were entered into the SSG VIII study. Further eligibility criteria were age <40 years and no evident metastases as assessed by a mandatory chest computed tomography (CT) and whole body bone scan. 19 patients were excluded due to metastatic disease ($n=12$), non-extremity tumour ($n=3$), age >40 years ($n=1$), revised diagnosis of Ewing's sarcoma ($n=1$) or malignant fibrous histocytoma ($n=1$) and definitive intralesional surgery before referral ($n=1$), leaving 113 eligible patients. Patient characteristics are summarised in Table 1. The diagnosis of osteosarcoma was confirmed by open biopsy in all cases. One reference pathologist from each participating country reviewed all of the slides and agreed upon the diagnosis, subtype and malignancy grade. Plain X-ray, technetium 99-MDP bone scan, CT scan and for most patients magnetic resonance imaging (MRI) of the entire bone involved, was used to assess the primary tumour. The tumour volume at diagnosis was reviewed by an expert panel of radiologists. Measurements were based upon MRI scans when available or otherwise by CT scan. Either the cylindrical formula ($V=\pi abc$) or the elliptic formula ($V=4/3\pi abc$) were chosen; usually the cylindrical formula was the model of choice [17].

Table 1
Patient characteristics

	Number (all)	5-year metastasis-free survival (%)	P value (logrank) in univariate analysis
Gender			
Female	44 (113)	81	0.001
Male	69	52	
Age (years)			
<15 years	34 (113)	73	0.11
$\geqslant 15$ years	79	59	
Country			
Sweden	68 (113)	67	0.31
Norway	28	64	
Finland	17	47	
Site			
Humerus	15 (113)	59	0.61
Femur	60	63	
Tibia	31	61	
Others	7	86	
Histology			
Osteoblastic	60 (74)	62	0.76
Chondroblastic	4	64	
Fibroblastic	2		
Telangiectatic	0		
Others	8		
Tumour volume (ml)			
<190	49 (98)	75	0.01
>190	49	51	
LDH			
Normal	68 (105)	64	0.88
elevated	37	64	
ALP			
Normal	17 (104)	76	0.16
elevated	87	61	

LD, lactate dehydrogenase; ALP, alkaline phosphatase.

2.2. Chemotherapy

The chemotherapy regimen is outlined in Fig. 1. All patients were intended to receive two cycles of paired high-dose methotrexate (HD-MTX) together with a cisplatin/doxorubicin (CDP/ADM) combination preoperatively. Methotrexate was given as a 4-h infusion followed by leucovorin rescue starting 24 h after the start of HD-MTX. Up to February 1993, the methotrexate dose for patients aged >12 years was 8 g/m^2 and for younger patients 12 g/m^2. As a result of a lack of age-related differences in serum methotrexate levels in the preceding SSG II study, all subsequent patients received methotrexate at 12 g/m^2. Cisplatin (90 mg/m^2) was given intravenously (i.v.) as a 4-h infusion. Doxorubicin was given as a daily 4-h infusion at a dose of 25

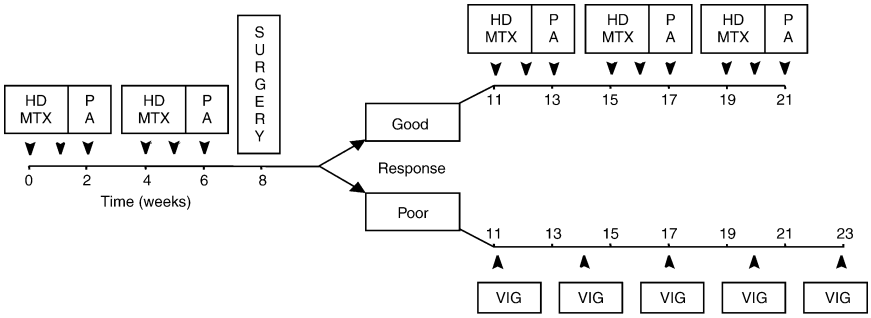

Fig. 1. Treatment schedule. HD-MTX = methotrexate 12 g/m^2, PA = cisplatin 90 mg/m^2, doxorubicin 75 mg/m^2. VIG = etoposide 600 mg/m^2, ifosfamide 4.5 g/m^2.

mg/m^2 for 3 consecutive days. Good responders were given three further cycles of methotrexate, cisplatin and doxorubicin postoperatively. The salvage regimen for poor responders consisted of 2-h infusions of 1.5 g/m^2 ifosfamide for 3 consecutive days in combination with a continuous 72-h infusion of 600 mg/m^2 etoposide (VIG regimen). Growth factor support with filgrastim was given after each VIG course and after CDP/ADM courses if a previous episode of febrile neutropenia had occurred. Good histological response was initially defined as Huvos grade II-IV. Based upon analyses of the SSG II study that revealed no difference in the outcome between grade I and II responders the current protocol was amended from February 1993, with grade II responders being treated as poor responders. Thus, due to the change of criteria for histological response, patients with Huvos grade II response were either given unchanged therapy or the VIG regimen. Relapse therapy was not included in protocol and left to the discretion of the responsible physician.

2.3. Pathological evaluation

Surgical margins were determined by the surgeon and the pathologist according to the classification of Enneking and colleagues [19]. The histological response to the preoperative chemotherapy was evaluated according to Huvos [20]. The initial pathological evaluation at each institution determined the postoperative chemotherapy. When possible (96%, $n = 108$), sections were reviewed for histological response by the three SSG reference bone tumour pathologists and this final evaluation was used in the prognostic factor analyses.

2.4. Response criteria and statistical analyses

Projected metastasis-free survival was calculated from the date of diagnosis until the date of distant metastasis or last follow-up. Event-free survival was calculated from the date of diagnosis until the date of distant metastasis, local recurrence, treatment-related death or last follow-up. Sarcoma-related survival was calculated from the date of diagnosis until death from osteosarcoma, treatment-related causes or last follow-up. For statistical analyses the Statistical Package SPSS for Windows (Release 10.1, SPSS Inc., Chicago, IL, USA) was used. The Kaplan–Meier method was used for the survival analysis and the curves were compared by the log-rank test. Missing values were not replaced by a default setting. A missing variable led to exclusion of the case in the analysis concerned. Continuous variables were categorised as below or above the median with the exception of age and alkaline phosphatase (ALP). Age groups were younger than 15 years or 15 years and older, and ALP was classified as normal or elevated after adjustment for age and gender according to Bacci [18]. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Compliance and toxicity

2 study patients were in need of prompt surgical treatment and did not receive neo-adjuvant chemotherapy: one because of an acute bacterial infection following tumour biopsy, the other due to pathological fracture after registration. The median delay from the start of chemotherapy to surgery was 20 days and the total treatment duration were 178 days in poor responders and 195 days in good responders. This represents a median delay of 66 days for good responders and 37 days for poor responders. 30% of the CDP/ADM courses and 8% of the VIG courses were followed by grade IV haematological toxicity. For 13 patients, chemotherapy was terminated early (less than 75% of intended dose was given) or modified in a major way because of toxicity. The individual drugs causing such major toxicity were methotrexate in 9 cases, cisplatin in 3 cases and unknown for 1 patient. We recorded three

treatment-related deaths. One patient (a good responder) developed acute myelogenous leukaemia within a year of diagnosis and died 16 months later. The second patient died of acute cardiac arrest aged 19 years, 26 months after the termination of chemotherapy. She had received 370 mg/m^2 of doxorubicin, and the diagnosis of anthracycline-induced cardiomyopathy was confirmed at autopsy. The third patient died postoperatively of acute respiratory distress syndrome after metastasectomy for pulmonary relapse. 7 patients who progressed radiologically and/or clinically during therapy were switched to an ifosfamide-based therapy, either the VIG regimen or high-dose ifosfamide (continuos infusion of 15 g/m^2 in 5 days). All these patients later developed distant metastases.

3.2. Surgery and local control

66 patients (58%) were treated with limb salvage surgery or rotation-plasty. In the period of 1990–1993, 42% were operated upon with a limb salvage approach compared with 77% in the period of 1994–1997. Information on the surgical margins was available for 106 patients. 87% of the patients operated upon with a limb salvage technique and 96% of the amputated patients obtained wide or radical margins according to Enneking [19]. 8 patients (7%) developed local recurrence at an average time of 20 months (range 5–36 months) from diagnosis. 6 of these patients were operated upon with wide margins. Of the 8 patients with local recurrence, only 1 did not develop distant metastases and is alive and in second complete remission, 42 months after local recurrence. 5-year projected risk of local recurrence is 7.5% (95% Confidence Interval (CI), 5–10%).

3.3. Histological response and postoperative chemotherapy

At the primary assessment, 63% of the patients were classified as having a good response. After review by the reference pathologist, the response grading was altered in 33 out of 108 tumours (31%). In most cases, the revised response remained within the same major response category (good/poor), and following revision 58% remained good histological responders. In the group of 41 patients with a revised grade II histological response (true histological grade II responders), 16 patients received an unchanged therapy (HD-MTX and CDP/ADM) postoperatively, 20 patients the ifosfamide/etoposide combination and 5 patients received a major modification of up-front chemotherapy other than the ifosfamide/etoposide combination due to toxicity or progression.

3.3.1. Survival and postrelapse outcome

With a median follow-up of 83 months for survivors (range 42–124 months), 80 patients are currently alive and 78 patients are in complete remission. The projected sarcoma-related survival at 5 years is 74% (70–78%) (Fig. 2). 68 patients (60%) are alive in first complete remission. Of the 43 (38%) patients who have relapsed, 12 are in second complete remission. The projected metastasis-free survival at 5 years is 63% (58.5–67.5%). The average time to distant metastasis was 18 months (range 1–62 months). The projected event-free survival at 5 years is 61% (56.5–65.5%), including one local recurrence and two treatment-related deaths in addition to the metastatic relapses. In a separate survival analysis of true histological grade II responders, there was a non-significant difference in metastasis-free survival in favour of the unchanged therapy compared with the salvage therapy (Fig. 3). The 5 patients that received a major modification of up-front chemotherapy other than the etoposide/ifosfamide combination were excluded from this analysis. Of these 5 patients, 3 are alive in first complete remission and 2 are dead of their disease.

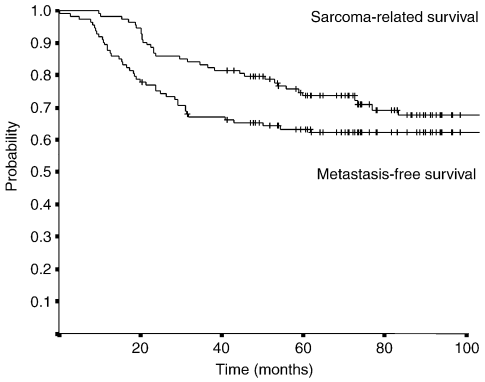

Fig. 2. Sarcoma-related and metastasis-free survival ($n = 113$).

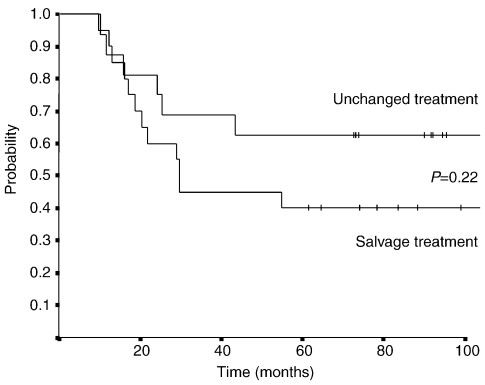

Fig. 3. True Huvos grade II responders—metastasis-free survival by postoperative chemotherapy. Unchanged treatment ($n = 16$): high-dose methotrexate, cisplatin and doxorubicin; salvage treatment ($n = 20$): etoposide and ifosfamide.

Of the 42 patients who developed distant metastases, 35 (83%) had lung metastases, 3 had skeletal metastases only and 4 had metastases at other locations. Relapse treatment was not defined by protocol and varied between centres; 73% received second-line chemotherapy and 76% underwent surgery. The overall survival at 5 years from relapse was 21%. For patients that were rendered macroscopically disease-free by metastasectomy, the corresponding survival rate was 38%.

3.4. Prognostic factor analyses

The following factors were taken into the univariate analyses of metastasis-free survival: age, gender, tumour volume, lactate dehydrogenase (LDH), ALP, site, type of surgery, serum-methotrexate (peak, 24 and 48 h values), histological response and type of postoperative chemotherapy (Tables 1 and 2). Independent favourable prognostic factors for outcome were female sex, tumour volume below median (190 ml, range 15–936 ml), and 24-h serum methotrexate serum-level above median (4.5 μM, range 1.0–7842 μM). Histological response to preoperative chemotherapy was not an independent prognostic factor in this analysis (Table 3).

Table 2
Treatment characteristics

	Number (all)	5-year metastasis-free survival (%)	*P* value (logrank) in univariate analysis
Surgery			
Amputation	46 (112)	56	0.28
Resection	66	68	
Margins			
Adequate	96 (106)	63	0.53
Inadequate	10	50	
Methotrexate serum peak (μmol/l)			
<1000	28 (86)	64	0.9
>1000	58	58	
Methotrexate serum level at 24 h (μM)			
<4.5	53 (106)	58	0.26
>4.5	53	67	
Response grade			
I	6 (111)	50	0.03
II	41	51	Good versus poor
III	49	69	
IV	15	87	
VIG postoperatively			
No	77 (113)	68	0.14
Yes	36	53	
Treatment duration			
<Median	45 (90)	55	0.31
>Median	45	64	

Table 3
Cox regression analysis of prognostic factors

Factor	Grouping	*n*	*P* value	HR	95% CI
Gender	Male	68	0.002	3.7	1.59–8.66
Age (years)	>15	79	NS		
Tumour volume (ml)	>190	50	0.017	2.4	1.18–5.05
Histological response	Good	64	NS		
Mean methotrexate at 24 h (μM)	>4500	53	0.017	0.4	0.21–0.88
VIG postoperative	Yes	36	NS		

HR, hazard ratio; CI, Confidence Interval; NS, non-significant.

4. Discussion

The survival data in the present study are comparable to the best published results, which are all obtained with combinations of the three or four most effective chemotherapeutic agents [6,9,21]. The SSG VIII survival data show a 9% increase in 5-year sarcoma-related survival compared with the SSG II study utilising the T-10 protocol [5].

During the study period, there was a considerable development towards more limb saving surgery with 3 out of 4 patients being operated upon with limb preservation from 1994 to 1997. This major achievement was obtained without an increase in the local recurrence rate compared with the SSG II study (7% versus 5%). The study confirms the association between local recurrence and metastatic disease as only 1 out of 8 relapsed patients is still alive.

Histological response is generally regarded as an important prognostic factor in osteosarcoma. The rate of good responders increased from 17% in our previous study [5] to 58% in the present study. This improvement in histological response did not translate into a comparative improvement in survival. This finding is in agreement with recent results from the Rizzoli Institute [22]. Thus, the fraction of patients achieving a favourable response cannot be regarded as a surrogate endpoint for survival. In a recent large study by the COSS group, histological response emerged as a key prognostic factor [11]. The survival difference between poor and good responders was comparable to that in our study (22–25%), and the fact that the histological response did not reach independent significance in the present study may merely reflect the relatively limited number of patients included.

Our results show that in poor responders replacement salvage chemotherapy with ifosfamide and etoposide failed to improve outcome. One reason may be that our ifosfamide dose was too low. Recently, Patel and colleagues documented a dose–response relationship for ifosfamide in osteosarcoma and recommended a dose of >10 g/m^2 [23]. In addition, the choice of etoposide may be questioned, as doxorubicin and etoposide have similar

mechanisms of action and share mechanisms of drug resistance [24]. Furthermore, the basis for withdrawal of the preoperative regimen in poor responders appears questionable in retrospect. The sub-group analysis of the true grade II histological responders indicates that a poor histological response does not equal chemoresistance. In the Rizzoli IOR-II study that showed a similar prognosis for good and poor histological responders, etoposide and ifosfamide were added to the up-front regimen in poor responders [6]. Our data support this strategy.

Modern osteosarcoma chemotherapy is very intense and several authors report reductions in dose-intensity which in turn may affect outcome [3,13,25]. We report a considerable toxicity-related prolongation in treatment. The use of growth factor support to overcome neutropenia may explain the shorter delay for the VIG regimen. However, this was not translated into a survival benefit. Methotrexate gives an opportunity to resume treatment despite incomplete bone marrow recovery and may have improved the overall treatment intensity and the results for patients treated with HD-MTX in the postoperative phase.

As other studies have shown, tumour volume was an important tumour-related factor predicting relapse [11,22,26]. Tumour volume is a candidate factor for stratification for therapy in a risk-adapted approach to osteosarcoma treatment. However, 2 out of 13 patients with a very small tumour volume (below 70 ml) died of their disease, demonstrating that small tumours can also be aggressive. As in SSG II, the methotrexate serum level at 24 h retained an independent prognostic value in the present study, whereas peak levels were not significant. Our study, with most patients receiving a dose of 12 g/m^2 confirms the importance of an adequate serum methotrexate level for outcome [27], and that doses of 12 g/m^2 methotrexate in patients up to 40 years of age is feasible. We would argue for tailoring of the methotrexate doses according to serum levels to ensure an adequate serum-level is obtained in most patients. Gender, in agreement with results from the SSG II study, is a prognostic factor for outcome, with a better prognosis for girls and women [28]. This gender difference is not observed in historical controls treated at the Norwegian Radium Hospital with surgery only or patients given sub-optimal chemotherapy [29,30]. We suggest that this may reflect some unknown gender-dependent genetic factor that is important for treatment efficacy.

In conclusion, the current study shows that intensive combination chemotherapy combined with centralised surgery resulted in a 5-year survival rate of 74%. Most patients were operated upon using a limb salvage approach. Prognostic factor analyses revealed female gender, small tumour volume and high serum-methotrexate as favourable factors for outcome. In this non-randomised study, adjusting chemotherapy by a complete change of the drugs used in the poor responders failed to improve outcome. Our findings, supported by previous results [6], indicate that if a salvage strategy is chosen, it should be given in addition to, and not as a replacement for, first-line therapy.

Acknowledgements

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ORIGINAL ARTICLE

Interferon-α as the only adjuvant treatment in high-grade osteosarcoma: Long term results of the Karolinska Hospital series

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Abstract

This experience of single agent interferon-α treatment in high-grade osteosarcoma was based on observed anti-osteosarcoma activity in laboratory models and was started before introduction of aggressive combination chemotherapy. From 1971 to 1990, 89 consecutive patients with non-metastatic high-grade osteosarcoma received semi-purified, leukocyte interferon-α as adjuvant treatment. From 1971 to 1984, 70 patients were given a dose of 3 MIU daily for one month followed by 3 times weekly for an additional 17 months. For 19 patients treated from 1985 to 1990 the dose was increased to 3 MIU daily and the treatment duration extended to 3–5 years. All patients underwent surgery prior to interferon treatment. The toxicity was mainly constitutional and long-term toxicity was virtually absent. With a median follow-up of 12 years the observed 10-year metastases-free and sarcoma specific survival rates were 39% and 43%, respectively. Only one of seven survivors after relapse received chemotherapy.This work suggests activity of interferon-α as adjuvant treatment in high-grade osteosarcoma. The efficacy of interferon in combination with standard therapy should be explored in randomized trials.

Interferons are a group of cytokines with pleiotropic effects including immunostimulation, antiangiogenic activity and direct antitumour activity [1–3]. They have shown high activity against osteosarcoma in vitro and in xenograft models [4–6]. Early preclinical data raised hopes to improve the dismal prognosis of osteosarcoma patients treated with amputation alone [7–9] and the availability of semi-purified interferon-α produced by human leukocytes lead to the initiation of interferon treatment for osteosarcoma at the Karolinska Hospital in 1971. Due to an apparent improvement in outcome the series was extended until 1990 [10–12]. At this time, neoadjuvant chemotherapy was introduced in order to improve the attack on micrometastatic disease and to facilitate limb-salvage surgery [13]. However, subsequent prospective multicentre trials have shown that even the most aggressive combination chemotherapy regimes appear unable to lift the survival above 70–75%, and new treatment options are mandatory for further progress [14].

In this context interferon-α may be a candidate agent in combination with chemotherapy, and the present work represents the final report from this single institution experience on interferon-α treatment of high-grade osteosarcoma, combining two patient series and extending the follow-up from previous preliminary reports [10–12].

Patients and methods

Patients

The Karolinska Hospital is the largest Swedish sarcoma centre serving approximately 30% of the national population. One hundred and two consecutive patients with primary osteosarcoma were admitted to Karolinska Hospital between 1971 and 1990. Nine patients had metastases at diagnosis and four patients had low-grade tumours leaving 89 consecutive patients to be presented in this report. Median age was 17 years (range 5–74), the male: female ratio was 1.6:1 and 92% of the tumours were

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localized to the extremities with 71% originating in the tibia or femur. Six patients (7%) had pelvic tumours and one tumour originated in a rib. Median maximal tumour extension as determined by the soft tissue mass was 8 cm (range 2–20). Informed consent for participation in the study was obtained from all patients or their parents according to institutional guidelines. The diagnosis was established by open biopsy. Due to the apparent improvement of results all patients entered up to May 1976 (n = 28) were subjected to a detailed review by National Cancer Institute scientists who confirmed the diagnosis of high-grade osteosarcoma [10]. Prior to treatment, all patients had conventional radiographs of the affected bone and a chest x-ray. CT scans of the chest were only done on the suspicion of lung metastases. Diagnostic procedures and follow-up schedules have been reported earlier [10–12].

Treatment

All patients underwent surgery. Before 1976, radiotherapy was given preoperatively to the primary tumour area in 10 patients at doses ranging from 10 to 64 Gy [10]. Thereafter, preoperative radiotherapy was omitted and postoperative radiotherapy reserved for a few patients with intra-lesional surgical margins [12]. No patients received prophylactic lung irradiation.

Limb-salvage procedures were performed in 37% of the patients treated before 1985 and in 50% thereafter. The surgical margins were classified according to Enneking [15] and ten patients (11%) achieved an intralesional margin only.

The interferon used was a semi purified preparation of human leukocyte interferon containing different subtypes of interferon-α, provided by Kari Cantell at the Central Public Health Laboratory and the Finish Red Cross Blood Transfusion Service. The purity and the concentration of the preparation improved by various procedures during the study period [16,17].

The series is divided into two patient cohorts (Table I). Between 1971 and 1984, 70 patients received three million units daily as a single i.m. injection for one month, followed by three doses weekly for another 17 months. Based on laboratory data suggesting a dose-dependent effect of interferon [5,6] and clinical observations of tumour regrowth after discontinuation of the drug, both dose and treatment duration were increased from 1985 onwards. Thus 19 patients were treated with three million units daily s.c. for three to five years.

Upon relapse treatment was individualized. Local relapse was resected when possible. Thoracotomies for pulmonary metastases were performed increasingly during the trial period and combined with pulmonary radiotherapy (20 Gy) in some patients. In the 1970's, interferon was discontinued at relapse, and salvage chemotherapy containing either methotrexate or doxorubicin was commenced. In the 1980's, interferon treatment was continued or resumed following resection of the recurrent tumour. Chemotherapy was considered for all patients with unresectable relapses, but was not given in combination with interferon. Unfortunately, the details of treatment at relapse are only available for long-term survivors.

Statistical analysis

Metastases-free survival was calculated from the date of biopsy until the date of distant metastases. Sarcoma-specific survival was calculated from the date of biopsy until death from osteosarcoma or treatment-related causes. Survival was estimated with the Kaplan Meier method and the log-rank test was used for comparisons. Prognostic factor analyses were based on sarcoma specific survival.

Table I. Characteristics of patients receiving adjuvant interferon-α in Stockholm between 1971 and 1990.

		all	1971–1984	1985–1990
patients		89	70	19
age	median (years)	17 (5–74)	16 (5–74)	23 (11–50)
sex	male	55	44	11
	female	34	26	8
localization	extremity	82	67	15
	non-extremity	7	3	4
largest tumor extension	median (cm)	8 (2–20)	9 (2–20)	7 (5–11)
histology	osteoblastic	49	45	4
	non-osteoblastic	29	25	4
surgery	resection	37	28	9
	amputation	52	42	10
margins	intralesional	10	8	2
	marginal or better	79	62	17

Variables with p-values of 0.2 or less in univariate analyses were entered into a Cox proportional hazard model. $P < 0.05$ was considered as statistically significant. Computed statistical analyses applied SPSS for Windows (Release 11.5, SPSS Inc., Chicago, IL, USA).

Results

Toxicity

In the first cohort, fever was reported in 62%, pain at the injection site in 38%, hair-loss in 27%, itching erythema in 17%, coryza-like symptoms in 14% and headache, body stiffness, fatigue and excess perspiration in 10% [18]. Local pain, erythema and body-stiffness were markedly reduced for patients treated with the more purified interferon preparation and in general symptoms tended to decline with time. Interferon did not delay wound healing or new bone formation [12]. Body development was not disturbed by the treatment [19]. Within the first five years of this series two patients committed suicide four and six month into treatment (female, 62 years, male 29 years, both femural amputees). In the second cohort, of eleven patients surviving longer then five years, only two decided to discontinue interferon treatment after three years (due to constitutional symptoms of toxicity), whereas nine continued for five years or more. Detailed data on toxicity for the period after 1979 are not available, but the good treatment compliance indicates no major additional toxicity.

Long term outcome

Median follow-up for survivors was 12 years (range 2–16), and all but two surviving patients were followed for at least ten years (two patients were lost to follow up after 24 and 51 months). Two patients died from unrelated causes (cardiac failure and lung cancer, 13 and 16 years after sarcoma diagnosis) and one died 16 years after diagnosis from an unspecified cause.

For all 89 patients, metastases-free survival (MFS) and sarcoma specific survival (SSS) at 10 years was 39% (95% confidence interval 29–49%) and 43% (33–54%), respectively (Figure 1). By treatment period, SSS was 38% in the first cohort and 63% in the second cohort but this difference is not significant (Figure 2). Median time to metastasis was 8 months (range 1–60); information of the site of metastasis is incomplete but pulmonary metastases were most common.

Local relapse was observed in 22% of the patients, and projected local recurrence-free survival at ten years was 75%. The median time to local recurrence was 5.5 months (range 1–60). Patients operated with intralesional margins had a high rate of local recurrence (80%) and a particularly poor outcome with a 10 year SSS of only 10%.

Twenty-eight of the 35 survivors have remained in first remission after systemic treatment with interferon only. Seven of 49 relapsed patients are free of disease after a median of 11.3 years (range 2.7–12.3) from relapse. Two of these had local relapses alone, three had pulmonary metastases and two patients had both local and pulmonary recurrences. All had complete resections of their local relapses and/or metastases, three continued interferon for 5 years and only one survivor from relapse received chemotherapy.

Analysis of prognostic factors

The following variables were included in univariate analyses: age (below versus above median), sex, tumour site (extremity versus other), largest tumour extension (below versus above median), histology (osteoblastic versus non-osteoblastic), margins (intra lesional versus marginal or better), surgery (amputation versus resection) and interferon dose (Table II). Sex, tumour extension, surgical margins and interferon dose were investigated further in multivariate analyses, where female sex and marginal or better surgical margins had independent positive prognostic impact (Table III).

Discussion

In the present series we report a 10-year sarcoma specific survival rate of 43%. With little evidence for

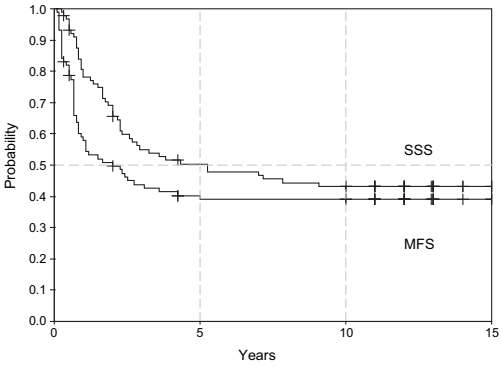

Figure 1. Sarcoma specific survival (SSS) and metastasis free survival (MFS) in 89 interferon-treated patients.

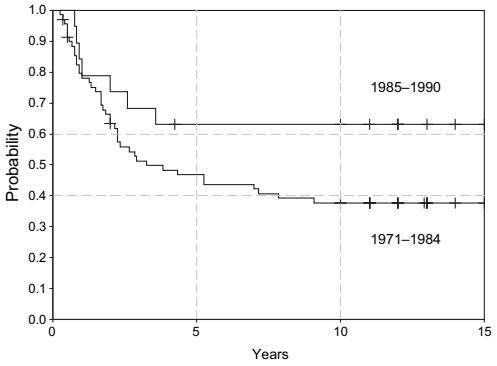

Figure 2. Sarcoma specific survival for two treatment periods, 1971–1984 (n = 70) and 1985–1990 (n = 19) (log rank p = 0.1).

a change in the natural history of osteosarcoma yielding a survival of 15–20% with surgery alone [20,21], and assuming the absence of serious bias, this suggests an effect of interferon-α in primary high-grade osteosarcoma. Although we cannot rule out selective referral of patients with favourable prognosis throughout the series, the number of patients treated matches the expected referral in the Stockholm region, and the age and sex distribution is close to what would be expected. Salvage

Table II. Univariate analysis of prognostic factors.

	number	SSS (%)*	95%CI	p **
Age				
<16 y	43	47	31–62	0,64
>16 y	46	41	26–56	
Sex				
male	55	35	22–48	0,03
female	34	58	41–74	
Localisation				
extremity	82	41	30–52	0,23
nonextremity	7	71	38–100	
Largest tumour diameter				
<8 cm	32	50	33–68	0,16
>8 cm	56	39	26–52	
Histology				
osteoblastic	49	41	26–55	0,66
non-osteoblastic	29	45	27–64	
Surgery				
resection	37	50	33–66	0,41
amputation	52	39	26–53	
Margins				
intralesional	10	10	0–29	0,1
marginal or better	79	48	37–60	
Cohort				
1971–1984	70	38	27–50	0,10
1985–1990	19	63	41–85	

(* Sarcoma specific survival at 10 years; **log rank).

Table III. Cox proportional hazards model based on sarcoma specific survival at 10 years of 88 patients (one patient with incomplete data is excluded).

	Number	p	HR	95% CI*
Male sex	54	0,04	1,93	1,02–3,64
Tumour diameter >8 cm	56	0,10	1,60	0,90–3,13
Intralesional margins	10	0,01	2,41	1,24–5,49
Interferon-dose 1971–1984	70	0,26	1,67	0,71–3,58

*Confidence Interval.

treatment, and in particular second line chemotherapy in combination with metastasectomy, may save some relapsing patients [22]. However, most survivors in this study are in their first remission after surgery and interferon, and only one long-time survivor received chemotherapy for relapse.

Only one large, randomized trial has explored the effect of interferon in primary osteosarcoma (COSS 80) [23]. For this trial interferon-β was used based on in vitro evidence of superior efficacy compared to interferon-α [4,23], and was given as maintenance therapy for 22 weeks after completed post-operative chemotherapy. There was no survival difference between the interferon group and the control group. Interferon was however given at a relatively low dose (100 000 IU/kg twice weekly) and for a comparably short time (22 weeks), which may explain the lack of demonstrable effect. Furthermore, interferon was given to patients who already achieved a 60% survival rate, making an effect more difficult to demonstrate.

In our series compliance to treatment was good and all patients continued on interferon according to protocol or until relapse. Two suicides early in the series may be treatment related, and previously published data indicate that depression is associated with interferon therapy [24]. In this context we cannot rule out that patients who have undergone amputation may be particularly vulnerable.

The number of resections with intralesional margins in this experience explains the high rate of local relapse and surgical margins were of independent prognostic impact for survival.

Although a further improvement in survival is suggested in the last cohort of patients, this cannot be attributed to the increase in interferon dose and treatment duration. Not only were the two treatment cohorts separated in time, but tumour size was smaller in the second cohort (Table I). However, interferon trials in other malignancies have shown longer treatment duration and higher weekly dose to result in improved response and survival [25].

Female sex was an independent prognostic factor for improved survival in this series. It is of interest that a subsequent Scandinavian chemotherapy trial

also reported gender as an independent risk factor for metastases [13], whereas there is no evidence of a sex difference outside Scandinavia or in Scandinavian patients treated with surgery alone [8,9,14,26]. The underlying mechanism for the sex difference in survival is unclear, but could be due to genetic features in the Scandinavian population affecting response to medical treatment.

During the last two decades, several prospective multicentre studies combining three or four of the most active drugs have been shown to cure at best 70–75% of patients with primary non-metastatic osteosarcoma [13,26], and further improvement depends on new drugs with novel mechanisms of action. Interferon-α may be a candidate in combination with chemotherapy. Experimental data suggest that multidrug resistant osteosarcoma cell-lines are sensitive to interferon-α [27]. Moreover, interferon has been shown to increase the chemotherapy sensitivity of several drug resistant cell-lines including osteosarcoma lines [27,28], and has been shown to modulate cytotoxicity by induction of p53 [29]. Interferon's role as maintenance treatment has been extensively studied with favourable results for some cancers [30,31], and pegylation of interferon-α has reduced toxicity in the treatment of hepatitis and chronic myelogenous leukaemia [32].

In our opinion, interferon-α is an attractive candidate for future studies. An important issue will be how to optimally incorporate interferon with current standard chemotherapy for this disease.

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III

Characterization of Treatment Response to Recombinant Interferon-α2b in Osteosarcoma Xenografts

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Abstract

Interferons (IFNs) may target cancer cells both through their regulation of the immune response, effect on angiogenesis and through direct effect on cancer cells. Treatment response has been demonstrated in osteosarcoma patients, but tumour resistance to IFN-α is common. Hence, understanding the molecular mechanisms involved in response and resistance is essential for improving therapeutic efficacy. Of five xenografts screened for specific growth delay in response to treatment with unconjugated and PEGylated IFN-α2b, one displayed growth inhibition and tumour shrinkage. Growth inhibition increased on a dosing schedule of PEGylated IFN every third day. Xenografts resistant to PEGylated IFN were similarly resistant to unconjugated IFN. Combination treatment with IFN-α2b and doxorubicin resulted in improved growth control rates. Transcriptional profiling analysis of the one sensitive and two resistant xenografts identified a common set of 79 genes significantly affected by IFN-α2b treatment independent of tumour growth inhibition. All but four of the 79 genes were up-regulated. The majority of these genes were well characterized IFN-stimulated genes and core members of the IFN-α signalling pathway. The expression of a set of 128 unique genes changed only in the sensitive xenograft; 52/128 genes were up-regulated. The specific gene-expression pattern seen in the responsive xenograft identified possible pathways important for the antitumor effect of IFN-α in osteosarcoma, including ssubsets of genes involved in cell adhesion and osteogenic tissue development. The observed improved control rates of combined treatment with IFN and doxorubicin are encouraging and should be further explored.

Keywords:

Osteosarcoma, xenograft, interferon, gene expression profiling

Abbreviations

IFN – Interferon

ISG – interferon stimulated gene

ISRE- IFN-stimulated response elements

OS - osteosarcoma

PEGylation – conjugated to poly-ethylene-glycol

Background

Osteosarcoma (OS) is the most frequent primary malignant tumour of the skeletal system in children and young adolescents. Although a multidisciplinary approach with specialized surgery and combination chemotherapy has led to significant survival improvement during the last three decades, a relapse rate of 30-40% within five years remains (Souhami et al. 1997; Bacci et al. 2000; Bielack et al. 2002; Smeland et al. 2003; Ferrari et al. 2005). Further improved survival appears to depend on the optimal integration of novel drugs into the existing treatment protocols.

Interferons (IFN) are biological response modifiers that may inhibit cancer cell growth by regulation of the immune response, inhibition of angiogenesis and direct antitumour activity (Balkwill 1985; Gresser 1989). By interactions with their specific cell surface receptors IFNs activate signal transducer and activator of transcription (STAT) complexes and initiate the classical Janus kinase-STAT (JAK-STAT) signalling pathway (Fu et al. 1992; Schindler et al. 1992). Activated STATs form complexes that bind to specific IFN-stimulated response elements (ISRE) or IFN-γ-activated site (GAS) elements within promoters of interferon stimulated genes (ISGs) to initiate transcription. In addition, non-classical signalling (involving MAPK, PI3K and NF-κB) is necessary to explain the full extent of the transcriptional gene response (Platanias 2005; Du et al. 2007).

IFN-α subtypes differ in their binding affinities to the IFN receptor and also have variable efficacy on tumour growth (Balkwill 1985; Foster and Finter 1998; Yamaoka et al. 1999). Whereas the early studies were performed with mixtures of IFNs extracted from white blood cells, most clinical trials have been performed with single-species recombinant IFN-α, and

more recently with variants conjugated to poly-ethylene-glycol (PEGylation). PEGylation delays the elimination of IFN, allowing for once weekly treatment in humans (Glue et al. 2000), and carries a promise of higher efficacy and reduced toxicity (Eggermont et al. 2008).

Direct antitumour activity in osteosarcoma has been demonstrated *in vitro* (Strander and Einhorn 1977; Dannecker et al. 1985) and *in vivo* (Masuda et al. 1983; Hofmann et al. 1985; Brosjo 1989). Pioneering work at the Karolinska Hospital in Stockholm strongly indicated single agent activity of adjuvant human IFN-α in patients with non metastatic high-grade osteosarcoma (Muller et al. 2005; Strander 2007). No clear benefit of IFN-β was observed in the only completed randomized trial testing the additional effect of IFN following conventional chemotherapy (Winkler et al. 1984). EURAMOS1, a major randomized OS trial (EURAMOS) explores sequential maintenance treatment with PEGylated IFN-α2b for the favourable prognostic subgroup with good histologic response to neoadjuvant chemotherapy with cisplatinum, doxorubicin and high-dose methotrexate. Although IFN is in some clinical use in advanced osteosarcoma, the relative contributions of direct antitumour effects, inhibition of angiogenesis and indirect immune-mediated effects are not known. Resistance to IFN is frequently seen, and an understanding of the molecular mechanisms involved in response and resistance is essential for improving the therapeutic efficacy. Furthermore, IFN-α has been effective in the treatment of multidrug resistant osteosarcoma cell lines (Manara et al. 2004) and may be a way to overcome chemotherapy resistance. We therefore investigated the growth response of PEGylated IFN-α2b and unconjugated IFN-α2b treatment and the effect of PEGylated IFN-α2b on gene expression in osteosarcoma tissue using human xenograft models. Finally, aiming at integrated bio-chemotherapy, we extended our

experimental series by combining IFN treatment with doxorubicin, which is one of the four active drugs in OS treatment (Blaney et al. 1993).

Material and methods

Animals

Female athymic mice (Balb/c: nu/nu) were bred in our animal facility, weaned after 21 days and maintained in a pathogen-free environment at controlled temperature (21 +/- 0.5˚C) and humidity (55-65%) on a 12 hour light cycle. Sentinels were tested according to FELASA's health monitoring recommendations. Groups of up to eight mice were kept in transparent polycarbonate cages (Tecniplast Eurostandard type III, Scanbur BK, Nittedal, Norway) on aspen chip bedding (B&K Universal, Hull, UK) with pellet feed (RM3, Special Diets Services, Witham, UK) and acidified water supplied *ad libitum*. Morbidity was controlled for by daily inspections focusing on behaviour, posture or weight loss. Animals were sacrificed for ethical reasons in case of weight loss >10% or tumour diameter >20 mm. Before transplantation or sacrifice by cervical dislocation mice were anaesthetised with intraperitoneal injection of 0.1mg/kg fentanyl, 5 mg/kg fluanison (Janssen Pharmaceutica, Beerse, Belgium), and 2.5 mg/kg midazolam (Roche, Basel, Switzerland).

All procedures involving animals were performed according to protocols approved by the National Research Authority in compliance with the European Convention for the Protection of Vertebrates Used for Scientific Purposes.

Xenografts

Tumour fragments were sampled from adolescent high grade osteosarcomas, four from primary tumours (TSx, KPBx, OHSx, MPAx) and one from a pulmonary metachronous metastasis (HPBx). Tumour tissue was implanted in the flanks of nude mice and propagated by serial transplantation (Bruheim et al. 2004).

The mice were allocated to treatment groups when tumours reached 50-70 µl. Tumours were measured twice weekly and tumour volumes calculated as 0.5 x length x width^2. Relative tumour volume (RTV) was calculated for each individual tumour for a specific number of days after start of treatment: RTV = $Volume_{day\ X}$ *100 / $Volume_{day\ 0}$. Tumour doubling time (TD) was defined as the time from the start of treatment to the first doubling of the median RTV. The drug effect was expressed as specific growth delay SGD = ($TD_{treated}$ – $TD_{control}$)/ $TD_{control}$ and treated to control rate T/C (%) = $RTV_{treated}$ x 100 / $RTV_{control}$. Based on earlier experience SGD of > 1.0 and T/C of < 50% were defined as antitumour activity (Bruheim et al. 2004)

Radio-labelling of PEGylated IFN

To estimate elimination half life in mouse serum, PEGylated IFN was radio-labelled with ^{125}I. For this analysis, PEGylated IFN was radio-labelled with ^{125}I at an equimolar ratio. An Iodogen tube (Pierce) was prewashed with 1 ml of a pH 7.5 iodination buffer (25 mM Tris, 0.4 M NaCl, all Sigma). 30 µl iodination buffer and 20 MBq ^{125}I dissolved in 7 µl water was added to the iodination tube and incubated for 5 min under continuous shaking, transferred to a tube containing 50 µg interferon-α in 50 µl water and incubated for another 5 min.

Iodination was terminated by adding 50 µl of iodination buffer with 10 mg/ml of tyrosine (Sigma). Finally free and bound iodine were separated on a column (Paus et al. 1982).

Eight mice were randomly assigned to two groups**.** Mice were pre-treated with potassium iodide 10 µg/day in the drinking water for 5 days before subcutaneous injection with ^{125}I-labeled PEGylated interferon-α2b at 10 and 100 µg/kg (0.3 and 3 MBq). Each mouse was sampled daily for 20-40 µl blood with a heparinised microcapillary (capillary tubes for microhematokrit, art. Nr. 110690, Kebo-Lab Stockholm) (Hem et al. 1998). At the indicated time points (24 h, 7 days) mice were anesthetized, cardially aspirated and killed before sampling tumour-tissue and hind leg muscle. Serum was spun for 7 minutes at 12 800 rpm in a Hematokrit 24 centrifuge (Hettich Zentrifugen), diluted in PBS, and tissue samples were minced in PBS before filtration on a NAP-5 column (Pharmacia Biotech) equilibrated with PBS, and the radioactivity was counted in a Wallac automatic gamma counter (Perkin Elmer Life Science) together with samples of known activity. We found excellent correlation between the capillary plasma and cardial serum measurements ($r = 0.99$).

Drugs

Unconjugated IFN-α2b (Intron A, Schering Plough, Oslo, Norway, specific activity 2.6×10^8 IU/mg protein) was administered using a prefilled injection pen. MonoPEGylated IFN-α2b (PEG-INTRON®, Schering Plough, specific activity 6.4×10^7 IU/mg protein) was reconstituted in water. Stock solutions of diluted PEGylated IFN were stored for a maximum of 2 weeks at -80˚C. Immediately before use the stock was diluted with PBS + BSA (Sanceau et al. 2002) to the final concentration in a volume of 0.1 ml. IFNs were injected subcutaneously (s.c.) to

the lower back of the mice. Control mice were treated with s.c. PBS + BSA. Doxorubicin (Adriamycin, Pharmacia Upjohn, Stockholm, Sweden) was dissolved in physiological saline and administered intravenously (i.v.).

Unconjugated IFN was administered at daily doses of 30,000, 300,000 or 1 million IU/mouse (equivalent to weekly doses of 40, 400 or 1350 μg/kg). PEGylated IFN was tested at weekly doses of 10, 100 or 300 μg/kg or every third day at an equivalent dose of 4.3, 43 or 129μg/kg. The total treatment interval for IFN was 3 weeks. Combined treatment with IFN and doxorubicin was started with 10 or 100 μg/kg/week PEGylated IFN followed 24 hours later by 8mg/kg doxorubicin equalling the maximal tolerated dose (Bruheim et al. 2004); the doxorubicin injection was repeated once after a week.

For microarray experiments mice were treated with 100 μg/kg PEGylated IFN-α2b and the tissue were sampled both 24 and 48 hours later. In the combination group, mice were treated with 100 μg/kg PEGylated IFN-α2b, followed 24 hours later by 8 mg/kg doxorubicin. Tissue was sampled 24 hours after the doxorubicin treatment**.**

Gene expression microarray analysis

RNA isolation, labelling and microarray hybridizations. Tissue samples were snap frozen on liquid nitrogen and stored at -80°C until use. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and stored at -80°C. Samples were quantified on a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and

RNA integrity was determined on Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA from several animals was pooled. In the initial experiments hybridizations were done on cDNA arrays printed at the core facility of the Norwegian Microarray Consortium at the Norwegian Radium Hospital (Oslo, Norway) as described earlier (Prasmickaite et al. 2006). For the final experiments amplification and labelling of 500 ng RNA from treated and untreated TSx, MPAx and HPBx xenografts was performed using the Illumina TotalPrep RNA amplification kit (Illumina Inc., San Diego, CA, USA). The microarray experiments were done using the Whole-Genome Gene Expression Direct Hybridization Assay and Illumina Human-6 Expression BeadChips version 2 arrays (Illumina Inc.) consisting of >48,000 different probes represented with an average 30-fold redundancy across the array. Technical replicates were hybridized for all samples. All laboratory processing and hybridisations were performed according to manufacturer's protocols.

Microarray pre-processing and analysis. The slides were scanned with the BeadArray Reader (Illumina, Inc.) and data extraction and initial quality control of the bead summary raw data were performed using BeadStudio (version 3.1.3.0) from Illumina and the Gene Expression module 3.2.6. Additional quality control before and after quantile normalization and pre-processing was performed with the R package (Du et al. 2007; Du et al. 2008; Lin et al. 2008) which is a part of the Bioconductor project (R-Development-Core-Team 2009) and the data was annotated using the HumanWG-6_V2_R4_11223189_A annotation file from Illumina. Changes in gene expression were detected at the probe level with one gene represented by one or several probes. To find probes that were differentially expressed between IFN treated and untreated samples for each of the xenografts, we applied linear models for microarray data (Limma) significance analysis with a moderated t-statistic using a

simple Bayesian model (Smyth 2004). The cut-off value for log2-fold-change was set to 0.57 (fold- change <1.5) and the p-value was adjusted for multiple testing by Benjamini and Hochberg's method (Benjamini and Hochberg 1995) with restriction p <0.01. When identifying probes related to IFN resistance, the difference in expression levels between the sensitive and each of the resistant xenografts had to be at least 1.5 fold. The discriminatory gene lists were further analysed in the online Database for Annotation, Visualization and Integrated Discovery (DAVID, release 2008) (DAVID ; Dennis et al. 2003) to organize the genes and identify enrichment based on common functional features in KEGG biochemical pathways and Panther ontologies Biological Process and Molecular Function. To measure the gene-enrichment in annotation terms the number of genes on the differentially expressed lists were compared to all the genes on the Illumina array. The significance of enrichment was indicated by p-values calculated as EASE score (Hosack et al. 2003), a modified Fisher Exact P-Value for gene enrichment analysis, and the cut-off was set to p <0.01. The data was submitted to ArrayExpress with accession E-TABM-707.

Interferome, a recently published database of ISGs and putative ISRE in the promoters of these genes (Interferome ; Samarajiwa et al. 2008) was used to investigate IFN signatures in the gene lists of differentially expressed genes.

Results

Treatment response of the xenografts

Of a total of five osteosarcoma xenografts screened for growth inhibition in response to treatment with PEGylated IFN-α2b, only one (HPBx) was found to be sensitive (Figure 1). Xenografts resistant to PEGylated IFN were also resistant to treatment with unconjugated IFN (Figure 1; G, H). In the sensitive xenograft (HPBx), weekly administration of 100 μg/kg PEGylated IFN gave a weak response (specific growth delay of 0.25 and a treated to control rate of 69% (Figure 1C). Due to strong growth inhibition of unpegylated IFN in the same xenograft (Figure 1A), we examined the half life PEGylated IFN in our model. Iodination of PEGylated IFN indicated a half-life of 15-16 hours in nude mice, compared to 27-39 hours in humans (Glue et al. 2000) (Figure 2). An adapted treatment schedule of PEGylated IFN every third day at a dose equivalent of 100 and 300 μg/kg/week improved efficacy compared to weekly administrations and strongly suppressed tumour growth (specific growth delay of 1.25 and 7.5; treated to control rate of 15% and 5%, Figure 1B . Daily treatment with unconjugated IFN at a dose equivalent of 40μg/kg/week (30,000 IU/mouse/day) was less effective (specific growth delay of 0.75 and a treated to control rate of 30%) whereas a dose of 400μg/kg/week (300,000 IU/mouse/day) for 21 days prevented any regrowth of the sensitive xenograft for a period of at least 90 days after end of treatment. We observed a dose-response relationship for both agents (Figure 1B).

Combined treatment with doxorubicin and PEGylated IFN. Pilot experiments were performed examining the potential of concomitant treatment of the sensitive xenograft (HPBx) with IFN and doxorubicin. When the IFN-sensitive xenograft HPBx was treated with 8 mg/kg doxorubicin as monotherapy a moderate growth delay was obtained (specific growth delay 1.25, treated to control rate 44%). Treatment with PEGylated IFN followed by doxorubicin 24 hours later was considerably more effective than monotherapy with either doxorubicin or IFN (specific growth delay up to 2.5, treated to control rate of 26%; Figure 1D).

Response in gene expression

Initial analyses of time course and dose response. Initial experiments were performed to investigate the time course and dose-dependence of the response to PEGylated IFN (Figure 3). Known ISGs were among the strongest induced, and showed highest expression levels with the highest dose and peaked within 12-24 hours. Based on these initial results we selected a dose of 100 μg/kg PEGylated IFN for 24 hours for further experiments with the three xenografts HPBx, TSx and MPAx, representing the one sensitive and two resistant xenografts, respectively.

Global changes in gene expression. Treatment with PEGylated IFN caused a significant change in the expression detected by 300 probes in HPBx, 325 in TSx and 1,777 in MPAx when comparing the treated and untreated xenografts pair-wise. The probe lists were filtered to identify probes that were specific for the sensitive and resistant xenografts (see Supplementary Tables 1, 2, 3) and were further classified according to biological and functional characteristics (Supplementary Table 4).

Genes affected by IFN in all xenografts. Ninety-nine of the probes detecting significant expression changes upon IFN treatment were common for all three xenografts. Five of these probes showed opposite effects in some of the three xenografts and were removed. This resulted in a set of 94 probes, representing 79 genes; 75 genes were up-regulated and only four were down-regulated in all xenografts. Mean fold change for these genes were 2.4, 6.6 and 3.9 in HPBx, TSx and MPAx, respectively (Supplementary Table 1).

This group contains several core members of the IFNα signalling pathway (*STAT1, ISGF3G, IRF7*) and central well characterized ISGs (*OAS1-3, MX1, EIF2AK2*, members of the *HLA* and *PARP* families, and *ISG15*) (Supplementary Table 1). As expected, these genes were enriched in immunity related gene ontology groups and pathways (Supplementary Table 4A).

Sixty-six of the 79 common genes were identified as known ISGs in the Interferome database (Interferome ; Samarajiwa et al. 2008). Thirty-six of these 66 genes were found to contain putative ISRE and 16 had a putative STAT1 binding site in the promoter. The high number of ISRE-containing genes illustrates the induction of a specific IFN response in all three xenografts examined. For the majority of genes, this effect of IFN treatment was more pronounced in the resistant xenografts.

Genes differentially expressed only in the sensitive xenograft. One hundred and twenty-eight probes identified genes that were differentially expressed upon treatment with PEGylated IFN-α only in the sensitive xenograft HPBx . 72 of these probes, representing 70 unique genes, had a fold-change difference of ≥ 1.5 between the sensitive and each of the two resistant xenografts. 24 of these genes were induced and 48 were repressed. Only seven of

these genes were identified as known ISGs by the Interferome database and only one of the seven had a putative ISRE in the promoter (Supplementary Table 2).

The 70 genes were significantly enriched for gene ontology annotations related to signalling (MF00016) and receptors (BP00108), extracellular matrix (MF00179) and actin binding protein (MF00262), development (BP00193) or cell adhesion (BP00124) and KEGG pathway calcium signalling pathway (hsa04020) (Supplementary Table 4B).

IFN down-regulated a number of genes related to the extracellular matrix of connective tissue and its maturation, including the collagens *COL2A1*, *COL9A1* and *COL10A1*, osteomodulin (*OMD*), matrilin 4 (*MATN4*), and metallopeptidase 3 (*MMP3*). The adhesion receptor galectin-7 (*LGALS7*) and *LOC728910* similar to galectin-7 is down-regulated whereas galectin-9 (*LGALS9*) and the highly similar *LGALS9C* are up-regulated.

Up-regulated genes related to developmental processes included chordin-like 2 (*CHRDL2*) involved in osteo- and myoblast differentiation and *T1560* involved in thyroid development. Down-regulated genes included the Wnt pathway inhibitor dickkopf1 (*DKK1*), chordin (*CHRD*) which is an inhibitor of bone morphogenetic proteins, SRY (sex determining region Y)-box 8 (*SOX8*) involved in pro-osteoblast differentiation, secretoglobin (*SCGB3A2*) involved in lung development, and the tyrosine kinase receptor ephrin-A1 (*EFNA1*) involved in development, tumourigenesis and metastasis.

Genes with similar IFN response in the two resistant xenografts. Of the probes detecting significant expression changes, 58 were common only to the two resistant xenografts TSx and MPAx, but not significantly changed in the sensitive HPBx. Only 19 of these probes, representing 17 unique genes, were similarly regulated in both resistant xenografts and had a

fold-change above 1.5 when compared to the sensitive xenograft (Supplementary Table 3). Eight of these genes are known as ISGs in the Interferome database of which one had a putative ISRE and five a putative STAT1 promoter binding site. Functional annotation analysis yielded enrichment in the Panther ontology group MF00001: receptor.

Gene expression analysis of combination of doxorubicin and PEGylated IFN. Limma analyses revealed no significant differences in the gene expression patterns of the xenografts treated with IFN alone compared to the combination with doxorubicin at the time point investigated (data not shown).

Discussion

The effect of IFN on tumour growth

In an earlier series of 14 osteosarcoma xenografts in nude mice, natural buffy coat-derived IFN-α at a daily dose of 200,000 IU induced tumour regression or growth arrest in five, partial growth inhibition in eight whereas one tumour could only be arrested at a higher dose (Brosjo 1988). In contrast, in this report only one of five xenografts were sensitive to PEGylated IFN-α2b. This may be explained by differences in the tumour panels or the therapeutic agents. It may be relevant that three of our tumours (including the sensitive one) were exposed to chemotherapy before xenografting, whereas all the Karolinska tumours were chemotherapy naïve. The sensitive xenograft being the only one of metastatic origin is an observation of uncertain significance: small series with IFN in metastatic osteosarcoma did

not indicate clinically relevant efficacy. Furthermore, our xenograft lines were maintained over a longer time before the experiments. Although passage number did not seem to affect response to chemotherapy in a previous study on this panel (Bruheim et al. 2004), we can not exclude that primary IFN resistance may be caused by passage-related genetic or epigenetic alterations over time. As human IFNs are not thought to evoke a host response in mice (Balkwill 1985), it seems unlikely that a different immune status of our animals would explain the different treatment response.

The difference may also be due to the type of IFNs used. Natural IFN, as used in the Karolinska experiments, contains a mixture of α-interferon subtypes that have varying properties and anti-proliferative activity (Thomas and Balkwill 1991; Foster et al. 1996; Foster and Finter 1998; Yano et al. 2006). It is also possible that PEGylation may directly influence the antitumour effect. Natural α-IFNs have been shown to have higher anti-tumour activity in a small panel of osteosarcoma xenografts compared with recombinant IFN-α2c (Bauer et al. 1987), but have not been directly compared with PEGylated IFN-α. Unconjugated and PEGylated IFN-α2a induced similar growth inhibition and expression profiles in melanoma-xenografts (Certa et al. 2003; Krepler et al. 2004), whereas growth inhibition in hepatocellular carcinoma xenografts was stronger for PEGylated IFN-α2b (Yano et al. 2006). Significant variation in the IFN-related response depending on the targeted cell line and IFN–α subtype has been reported by others (Balkwill et al. 1985; Foster and Finter 1998; Yamaoka et al. 1999; Yanai et al. 2001). In our panel, xenografts resistant to PEGylated IFN were similarly resistant to unconjugated IFN. Complete growth arrest in the sensitive xenograft was only obtained by unconjugated IFN, but this could be due to the higher dose administered per week compared to the PEGylated moiety. Importantly, lack of

effect in our model system on tumour growth is only reflecting the absence of a direct antitumour effect of IFN and does not rule out clinically important effects mediated through immune- or angiogenesis-mediated mechanisms.

Changes in gene expression in response to IFN

With only one sensitive xenograft, we limited the microarray analysis to compare the one sensitive to two resistant xenografts. Obviously such a limited analysis cannot provide definite answers, but may indicate a list of candidate genes and pathways related to IFN treatment response. We restricted the investigation of the transcriptional response to stimulation with PEGylated IFN, as previous studies have shown that unconjugated and PEGylated IFN give indistinguishable transcriptional patterns and are equally potent activators of IFN gene expression (Certa et al. 2003; Krepler et al. 2004).

When comparing the sensitive and the resistant osteosarcoma xenografts we identified 79 genes with similarly altered expression after IFN treatment regardless of tumour response. A subset of 36 genes contained ISRE, indicating a functional JAK/STAT signalling pathway in all three xenografts. Genes exclusively induced in the sensitive xenograft rarely contained ISRE; a comparable group of genes was observed earlier in an IFN sensitive melanoma cell line and termed IFN secondary response genes (Certa et al. 2003). It was postulated that resistance to IFN is caused by abnormal secondary signalling rather than by primary defects in induction of JAK-STAT signalling pathway (Certa et al. 2003; Holko and Williams 2006).

LGALS9, and the similar *LGALS9C*, are among the ISGs induced by IFN in the sensitive xenograft. Galectins are evolutionary highly conserved β-galactoside-binding lectins involved

in basic cellular mechanisms (cell interaction, proliferation, migration, apoptosis, mRNA splicing) and modulation of immunity. LGALS9 expression is correlated with a better prognosis in malignant melanoma (Kageshita et al. 2002), and is possibly involved in IFN-induced apoptosis in the melanoma cell line WM9 (Leaman et al. 2003). Two other members of the galectin family, *LGALS7* and *LOC728910*, were found to be down-regulated, and these proteins have previously been described both as a positive and negative regulatory factors in tumour development, involved in proapoptosis, neoangiogenesis and metastatic tendency through metastatic proteins such as MMP9 (Saussez and Kiss 2006).

IFN-α has been shown to induce bone differentiation in a xenotransplanted human osteosarcoma (Forster et al. 1988). Osteosarcomas are known to express several bone morphogenetic proteins (BMPs) (Bauer and Urist 1981; Gobbi et al. 2002; Khan et al. 2008), the central proteins in the fine-tuning of bone development**.** Both CHRD, a regulator of dorsoventral patterning in early embryogenesis, and CHRDL2, a structurally related protein expressed preferentially in chondrocytes of developing cartilage, are BMP binding inhibitors of bone differentiation (Zhang et al. 2007)**.** In our sensitive xenograft, IFN induced up-regulation of *CHRDL2* whereas *CHRD* was down- regulated, the significance of which is unclear.

Further, the transcription factor *SOX8* was repressed as a response to IFN treatment. SOX8 is involved in early chondrogenesis and SOX8-deficient mice display an osteopenic phenotype. SOX8-deficient preosteoblasts proliferate slowly probably caused by RUNX2-induced exit from the cell cycle (Schmidt et al. 2005), and the reduced expression of *SOX8* may thus be related to the tumour response.

Finally, *DKK1* was down-regulated following IFN treatment in the sensitive xenograft only. DKK1 inhibits Wnt signalling and impairs osteoblast function. DKK-1 has been shown to be expressed maximally at the periphery of the tumour and it is thought that DKK1 can contribute to tumour expansion by inhibiting repair of the surrounding bone (Lee et al. 2007). Osteosarcoma patients have elevated DKK1 serum levels and the surviving fraction of circulating osteosarcoma cells is proportional to DKK1 levels in a xenograft model (Lee et al. 2007); it has been suggested that DKK1 levels could be used to monitor the effect of osteosarcoma treatment, and this might be applicable also to interferon. However, our findings contrast *in vitro* data of interferon induced overexpression of DKK1 in a hepatoblastoma cell line (Qu et al. 2007) .

In summary, several genes involved in cell adhesion and osteogenic tissue development were altered by IFN in the sensitive xenograft. However, the analysis of only one sensitive xenograft, and the fact that the magnitude of change in these genes appeared low, makes caution in the interpretation imperative.

Our observations that combined treatment with IFN and doxorubicin has an increased efficacy indicate that IFN may moderate response to other cytotoxic drugs. An exploratory analysis did not find transcriptional changes of combined IFN and doxorubicin as compared to IFN alone. Doxorubicin has been shown to induce both cell cycle regulators and death genes in hepatocellular cancer cell lines (Wang et al. 2009). The lack of specific mRNA changes in this report may point at a posttranscriptional regulation by the combination in the sensitive xenograft. Others have shown that one of the many genes induced by IFN is the tumour suppressor TP53 (Takaoka et al. 2003), and that IFN-related genes such as ISGF3G directly influence p53 expression (Munoz-Fontela et al. 2008). Doxorubicin can induce apoptosis

through p53 in osteosarcoma cells (Yuan et al. 2007; Yuan et al. 2008). In this study, no change in TP53 mRNA levels was observed between IFN treated and untreated osteosarcoma xenografts, but we cannot rule out effects at the protein level. Finally, it has been shown that IFN-α can mobilise quiescent leukemia stem cells, thus making them sensitive to chemotherapy (Essers et al. 2009). It remains to be seen if such mechanisms also can be exploited in non-haematological cancers.

Conclusions

We found a direct antitumour effect of IFN in one of five xenografts. At the doses tested, both types of IFN reduced the growth rate, but only unconjugated interferon induced growth arrest. Known ISGs were induced independent of tumour response. The antitumor response seen in one xenograft may be related to interference with genes involved in cell adhesion or osteogenic tissue development. Our preliminary data of combined treatment with IFN and doxorubicin are encouraging and should be further explored.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1

Growth response of osteosarcoma xenografts to treatment with PEGylated IFN (PEG-IFN), unpegylated IFN (IFN), doxorubicin (DOX) or a combination of DOX-IFN compared to a control group (CTR). Doses are given in equivalents to μg/kg/week. IFN was always given daily. PEG-IFN was given weekly in Figure A, C, D, E and F and every third day in Figure B, G, H. Doxorubicin was given at the maximal tolerated dose of 8 mg/kg 24 hours after start of treatment with PEG-IFN 100 (DOX-IFN). Volume is annotated as median relative tumour volume (RTV).

Figure 2

Elimination half-life of PEGylated IFN in mouse serum.

Serum concentration of radiolabeled PEGylated IFN-α2b in NCR mice following a single treatment with a dose of 10 and 100 μg/kg.

Figure 3

Hierarchical clustering of gene expression patterns in initial time and dose response experiments. **A**. 369 differentially expressed probes in xenograft TSx treated with 100 μg/kg/week PEGylated IFN-α2b for 12, 24 and 48 hours. **B.** Expanded view of lower

subcluster (red) from A, showing induction of numerous interferon-regulated genes **C**. 168 differentially expressed probes in xenograft TSx treated with 1, 10 or 100 µg/kg/week PEGylated IFN-α2b for 24 hours. An untreated control sample (TSx Ctrl) was also included. The signal of each probe is given as log2 of the ratio between the treated and untreated samples that was co-hybridized to the arrays, represented as mean value of two replicated assays. The probes presented in the clusters were at least 2 times up- or down-regulated in at.

Supplementary files

Supplementary table 1

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Differentially expressed probes common in three xenografts.

Relative intensity level of 94 probes (log2 ratios) that detected a common gene expression response in HPBx, TSx and MPAx following treatment with 100 µg/kg PEGylated IFN-α2b for 24 hours. The cut-off for the Limma analysis was set to $p<0.01$ and and fold-change <1.5 (log2 fold-change< 0.585). IFN-regulated genes (in bold) and putative ISRE and STAT1 binding sites in proximal promoter regions 1000 bp upstream of the transcription start site and the 5'UTR of the genes was identified in the Interferome database.

Supplementary table 2

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Differentially expressed probes in the sensitive xenograft only.

Relative intensity level of 72 probes (log2 ratios) that detected differentially expressed genes in the sensitive xenograft HPBx, but not in the resistant xenografts TSx and MPAx following treatment with 100 µg/kg PEGylated IFN-α2b for 24 hours. The cut-off for the Limma analysis was set to $p<0.01$ and and fold-change <1.5 (log2 fold-change< 0.585). In addition the difference in expression level between the sensitive and each of the resistant xenografts was at least 1.5 fold. IFN-regulated genes (in bold) and putative ISRE and STAT1 binding sites in proximal promoter regions 1000 bp upstream of the transcription start site and the 5'UTR of the genes was identified in the Interferome database.

Supplementary table 3

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Differentially expressed probes in two resistant xenografts

Relative intensity levels (log2 ratios) of 19 probes representing genes that were differentially expressed in both of the resistant xenografts TSx and MPAx, but not in the sensitive xenograft HPBx following treatment with 100 µg/kg PEGylated interferon-α2b for 24 hours. The cut-off for the Limma analysis was set to $p<0.01$ and fold-change <1.5 (log2 fold-change< 0.585). In addition the difference in expression level between the sensitive and each of the resistant xenografts was at least 1.5 fold. IFN regulated genes (in bold) and putative ISRE and STAT1

binding sites in proximal promoter regions 1000 bp upstream of the transcription start site and the 5'UTR of the genes was identified in the Interferome database.

Supplementary table 4

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Metabolic pathways representing genes overrepresented among those with altered expression. Pathways are shown that appeared enriched using ontologies from Panther Biological Process (BP), Molecular function (MF) and KEGG pathways[a]. Enriched categories from analysis of **A:** 94 probes with similar IFN response in HPBx, TSx and MPAx. **B:** 70 probes differentially expressed in the sensitive xenograft HPBx only. **C:** 19 probes with similar IFN response in the resistant xenografts TSx and MPAx.

Figure 1 Growth response of osteosarcoma xenografts

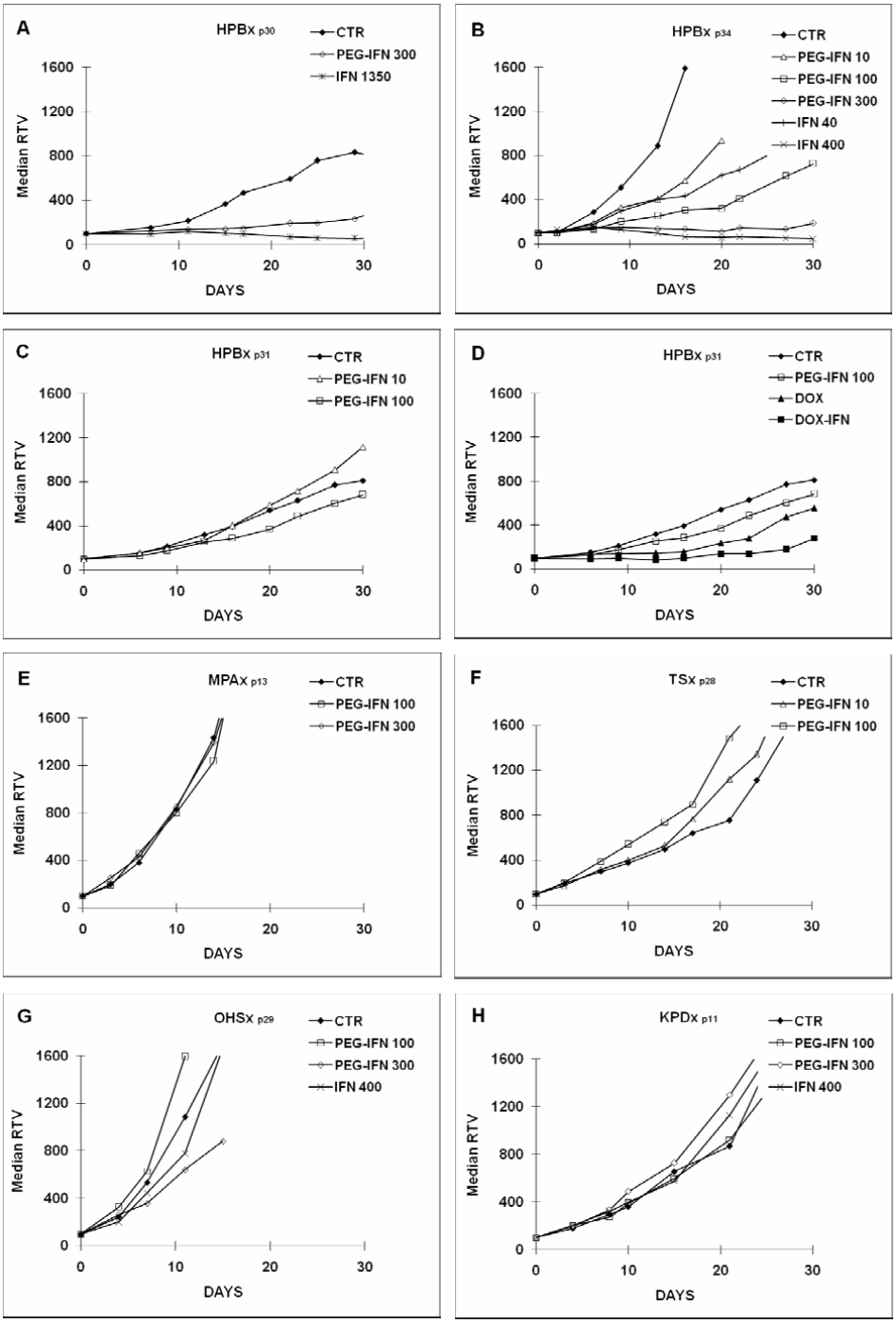
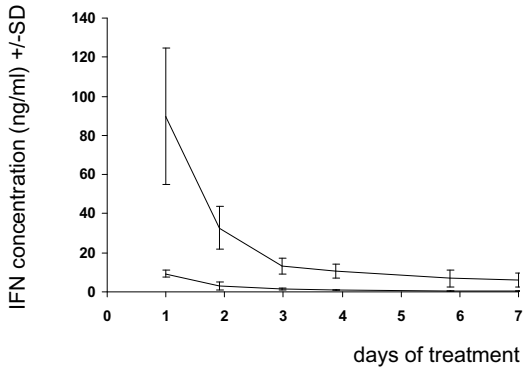
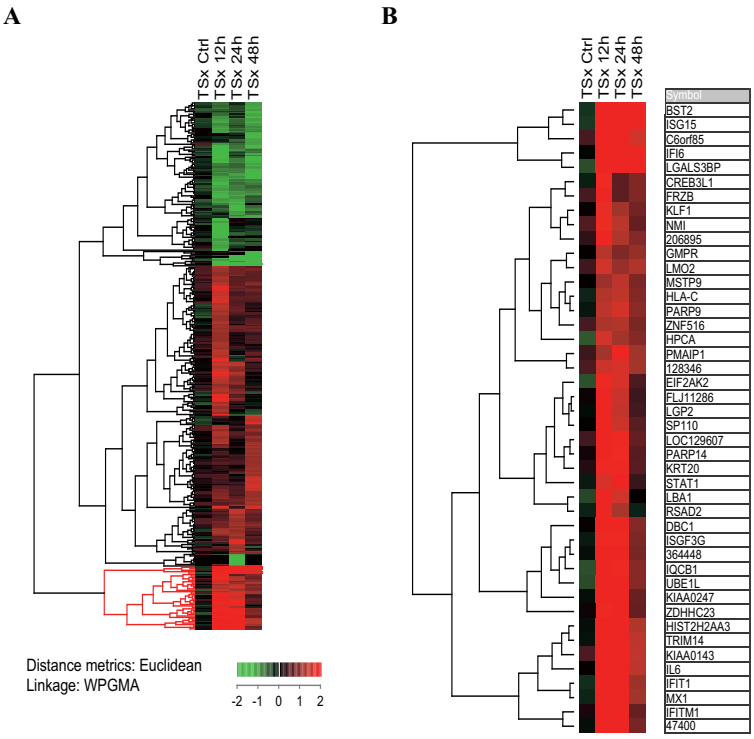


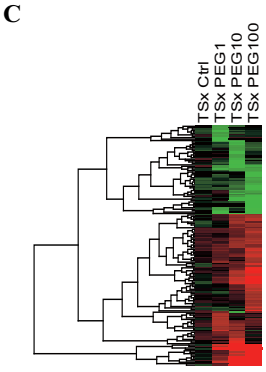
Figure 2 Elimination half-life of PEGylated IFN in mouse serum



dose/day	1	2	3	4	6	7
10	9.3 +/- 1.8	3.1 +/- 2	1.5 +/- 0.7	1.05 +/- 0.1	0.62 +/- 0.1	0.59 +/- 0.2
100	89.8 +/- 35	32.7 +/- 11	13.2 +/- 4	10.8 +/- 3.5	7.1 +/- 4.3	6.0 +/- 3.7

Figure 3 Hierarchical clustering of gene expression patterns in initial time and dose response experiments





Supplementary Table 1. Differentially expressed probes common in three xenografts

ILMN_Gene	Source_Reference_ID	Definition	log2FC HPBx/HPXCtrl	log2FC TSx/TSxCtrl	log2FC MPAx/MPAxCtrl	ISRE	STAT1
ACO1	NM_002197.1	aconitase 1, soluble	-0.665	-0.708	-0.948		
ADAR	NM_015840.2	adenosine deaminase, RNA-specific	0.681	1.212	0.809		
ASPN	NM_017680.3	asporin	-0.853	-1.039	-1.209		
B2M	NM_004048.2	beta-2-microglobulin	1.136	1.248	1.423	x	
BST2	NM_004335.2	bone marrow stromal cell antigen 2	1.619	4.573	1.506	x	x
CD68	NM_001251.1	CD68 antigen	1.016	1.673	1.134		
CFB	NM_001710.4	complement factor B	1.137	1.401	1.745	x	
DDX58	NM_014314.2	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	0.690	1.434	1.581	x	
DTX3L	NM_138287.2	deltex 3-like (Drosophila)	0.747	1.606	1.003	x	x
EIF2AK2	NM_002759.1	eukaryotic translation initiation factor 2-alpha kinase 2	0.715	1.551	1.022	x	x
EPSTI1	NM_033255.2	epithelial stromal interaction 1 (breast)	1.613	2.928	2.443	x	
FLJ11000	NM_018295.1	hypothetical protein FLJ11000	0.703	0.593	1.279	x	x
FLJ11286	NM_018381.1	hypothetical protein FLJ11286	0.801	1.757	1.607		
FLJ20035	NM_017631.3	hypothetical protein FLJ20035	1.074	2.291	1.078	x	
G1P3	NM_002038.2	interferon, alpha-inducible protein (clone IFI-6-16)	0.955	2.393	1.898	x	x
GBP1	NM_002053.1	guanylate binding protein 1, interferon-inducible, 67kDa	1.194	0.87	1.306	x	
HCP5	NM_006674.2	HLA complex P5	1.927	1.215	1.801		
HERC5	NM_016323.1	hect domain and RLD 5	0.841	2.766	0.939	x	
HERC6	NM_001013005.1	hect domain and RLD 6	1.935	4.498	3.123		
HES4	NM_021170.2	hairy and enhancer of split 4 (Drosophila)	0.603	1.654	1.543		
HLA-A	NM_002116.5	major histocompatibility complex, class I, A	1.075	0.737	1.405	x	
HLA-B	NM_005514.5	major histocompatibility complex, class I, B	2.425	1.72	2.424	x	
HLA-C	NM_002117.4	major histocompatibility complex, class I, C	1.657	1.345	1.652	x	
HLA-E	NM_005516.4	major histocompatibility complex, class I, E	1.652	0.819	1.927		
HLA-F	NM_018950.1	major histocompatibility complex, class I, F	2.072	1.544	1.258	x	
HLA-H	NR_001434.1	major histocompatibility complex, class I, H (pseudogene),non-coding RNA.	1.127	1.183	1.656		
HS.125087	Hs.125087	AGENCOURT_7914287 NIH_MGC_71 cDNA clone IMAGE:6156595 5, mRNA sequence	1.494	3.798	1.613		
HS.371609	Hs.371609	cDNA clone IMAGE:5261213	-0.626	-1.113	-1.278		
HS.489254	Hs.489254	cDNA clone IMAGE:5277162	0.836	0.902	0.697		
IFI27	NM_005532.3	interferon, alpha-inducible protein 27	1.824	3.852	3.869	x	x
IFI30	NM_006332.3	interferon, gamma-inducible protein 30	1.276	1.09	1.44		
IFI35	NM_005533.2	interferon-induced protein 35	1.442	2.494	2.65		x
IFI44	NM_006417.2	interferon-induced protein 44	1.261	1.99	0.716		
IFI44L	NM_006820.1	interferon-induced protein 44-like	1.911	5.484	1.686		
IFIT1	NM_001548.2	interferon-induced protein with tetratricopeptide repeats 1	1.758	3.687	2.352	x	
IFIT2	NM_001547.3	interferon-induced protein with tetratricopeptide repeats 2	1.047	2.809	0.84		
IFIT3	NM_001549.2	interferon-induced protein with tetratricopeptide repeats 3	1.740	3.425	1.102	x	
IFIT3	NM_001549.2	interferon-induced protein with tetratricopeptide repeats 3	1.910	3.849	2.996	x	
IFIT3	NM_001031683.1	interferon-induced protein with tetratricopeptide repeats 3	1.336	1.946	1.869	x	
IFITM1	NM_003641.2	interferon induced transmembrane protein 1 (9-27)	1.368	4.585	2.9	x	
IFITM2	NM_006435.1	interferon induced transmembrane protein 2 (1-8D)	1.554	3.716	1.387	x	x
IFITM3	NM_021034.1	interferon induced transmembrane protein 3 (1-8U)	2.541	3.92	1.768		
IRF7	NM_004030.1	interferon regulatory factor 7	1.083	2.117	2.135		
ISG15	NM_005101.1	ISG15 ubiquitin-like modifier	1.257	4.749	3.305	x	
ISG20	NM_002201.4	interferon stimulated exonuclease gene 20kDa	0.831	1.664	2.524	x	
ISGF3G	NM_006084.3	interferon-stimulated transcription factor 3, gamma 48kDa	0.663	1.689	1.47		
LAP3	NM_015907.2	leucine aminopeptidase 3	0.770	0.864	1.181	x	x
LBA1	XM_940627.1	PREDICTED: lupus brain antigen 1	0.683	1.894	0.679		
LGALS3BP	NM_005567.2	lectin, galactoside-binding, soluble, 3 binding protein	0.932	3.245	2.708		x
LGP2	NM_024119.1	likely ortholog of mouse D11lgp2	0.794	2.246	1.547	x	
LOC129607	NM_207315.1	hypothetical protein LOC129607	1.033	0.617	1.596		
LY6E	NM_002346.1	lymphocyte antigen 6 complex, locus E	0.859	3.629	1.446		
MX1	NM_002462.2	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	1.557	6.066	2.395	x	
OAS1	NM_001032409.1	2',5'-oligoadenylate synthetase 1, 40/46kDa	1.987	3.768	3.002		x
OAS1	NM_002534.2	2',5'-oligoadenylate synthetase 1, 40/46kDa	1.954	0.915	2.796		x
OAS1	NM_016816.2	2',5'-oligoadenylate synthetase 1, 40/46kDa	0.622	1.309	0.826		x
OAS2	NM_016817.2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1.007	1.24	1.062	x	
OAS2	NM_016817.2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1.725	4.345	3.617	x	
OAS2	NM_001032731.1	2'-5'-oligoadenylate synthetase 2, 69/71kDa	0.924	2.616	1.795	x	
OAS2	NM_002535.2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1.244	1.199	2.22	x	
OAS3	NM_006187.2	2'-5'-oligoadenylate synthetase 3, 100kDa	1.525	2.767	3.052	x	
OASL	NM_003733.2	2'-5'-oligoadenylate synthetase-like	1.292	3.153	2.497	x	
OASL	NM_003733.2	2'-5'-oligoadenylate synthetase-like	0.692	1.737	1.527	x	
PARP10	XM_937629.1	PREDICTED: poly (ADP-ribose) polymerase family, member 10	1.373	1.845	1.854		
PARP10	NM_032789.1	poly (ADP-ribose) polymerase family, member 10	1.170	1.907	1.948		
PARP10	NM_032789.1	poly (ADP-ribose) polymerase family, member 10	1.410	1.606	1.913		
PARP12	NM_022750.2	poly (ADP-ribose) polymerase family, member 12	1.087	2.48	2.251		
PARP14	NM_017554.1	poly (ADP-ribose) polymerase family, member 14	1.241	1.897	1.613		
PARP9	NM_031458.1	poly (ADP-ribose) polymerase family, member 9	1.382	2.838	1.883		
PLSCR1	NM_021105.1	phospholipid scramblase 1	1.134	2.187	2.046	x	x
PRIC285	NM_033405.2	peroxisomal proliferator-activated receptor A interacting complex 285	0.916	1.345	2.429	x	
PSMB8	NM_004159.4	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	1.372	0.97	1.524		x
PSMB8	NM_148919.3	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	1.201	1.067	1.378		x
PSME1	NM_006263.2	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	0.737	0.96	1.1		
PSME2	NM_002818.2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	0.796	0.663	0.817		
RARRES3	NM_004585.2	retinoic acid receptor responder (tazarotene induced) 3	1.850	2.351	1.862		
RSAD2	NM_080657.3	radical S-adenosyl methionine domain containing 2	1.827	3.056	2.7	x	
SAMD9L	NM_152703.2	sterile alpha motif domain containing 9-like	0.888	1.277	1.204	x	x
SLC15A3	NM_016582.1	solute carrier family 15, member 3	1.782	1.958	3.215	x	
SP110	NM_004510.2	SP110 nuclear body protein	0.695	1.089	1.064		x
SP110	NM_004510.2	SP110 nuclear body protein	1.037	1.613	1.39		x
STAT1	NM_139266.1	signal transducer and activator of transcription 1, 91kDa	1.364	2.584	1.979	x	
STAT1	NM_139266.1	signal transducer and activator of transcription 1, 91kDa	1.302	2.743	2.222	x	
STAT1	NM_007315.2	signal transducer and activator of transcription 1, 91kDa	1.136	2.28	1.854	x	
TAP1	NM_000593.5	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	2.018	1.5	2.491		
TNFSF10	NM_003810.2	tumor necrosis factor (ligand) superfamily, member 10	1.404	1.469	1.215		
TRIM22	NM_006074.2	tripartite motif-containing 22	1.784	1.149	2.064		
UBA7	NM_003335.2	ubiquitin-like modifier activating enzyme 7	1.155	2.554	1.624		
UBE2L6	NM_004223.3	ubiquitin-conjugating enzyme E2L 6	1.176	1.031	2.013		
UBE2L6	NM_004223.3	ubiquitin-conjugating enzyme E2L 6	1.067	1.153	2.514		
USP18	NM_017414.2	ubiquitin specific peptidase 18	0.641	1.175	0.977	x	x
USP41	XM_937988.1	PREDICTED: ubiquitin specific peptidase 41	0.737	1.237	1.142		
XAF1	NM_199139.1	XIAP associated factor 1	1.422	1.418	2.112	x	
ZDHHC14	NM_024630.2	zinc finger, DHHC-type containing 14	0.705	0.826	0.854		

Supplementary table 2. Differentially expressed probes in the sensitive xenograft only

ILMN_Gene	Source_Reference_ID	Definition	log2FC HPBx/HPXC	log2FC TSx/TSxCtrl	log2FC MPAx/MPAxCtrl	ISRE	STAT1
AIF1	NM_001623.3	allograft inflammatory factor 1	0.841	-0.044	-0.066		
ATOH8	NM_032827.3	atonal homolog 8 (Drosophila)	0.734	-0.227	0.039		
BEST4	NM_153274.1	vitelliform macular dystrophy 2-like 2	-0.829	-0.139	-0.043		
BNIP3	NM_004052.2	BCL2/adenovirus E1B 19kDa interacting protein 3, nuclear gene encoding mitochondrial protein	-0.824	-0.175	0.112		
CA9	NM_001216.1	carbonic anhydrase IX	-1.073	0.099	0.028		
CBLN4	NM_080617.4	cerebellin 4 precursor	-1.446	0.075	-0.015		
CCK	NM_000729.3	cholecystokinin	1.644	0.021	0.315		
CFH	NM_001014975.1	complement factor H	1.242	0.257	-0.334	X	
CHRD	NM_177978.1	chordin	-0.714	0.055	-0.105		
CHRDL2	NM_015424.3	chordin-like 2	1.201	-0.103	0.391		
COL10A1	NM_000493.2	collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)	-1.072	-0.084	0.2		
COL2A1	NM_001844.3	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	-1.097	-0.06	-0.27		
COL9A1	NM_001851.3	collagen, type IX, alpha 1	-1.423	0.072	-0.185		
CORT	NM_001302.3	cortistatin	0.719	0.005	-0.11		
CRYBA2	NM_057093.1	crystallin, beta A2	0.951	-0.028	0.158		
CRYGS	NM_017541.2	crystallin, gamma S	1.14	-0.057	-0.398		
CTSC	NM_001814.2	cathepsin C	0.806	0.116	0.192		
DIO2	NM_001007023.1	deiodinase, iodothyronine, type II	1.181	-0.19	0.007		
DKK1	NM_012242.2	dickkopf homolog 1 (Xenopus laevis)	-0.674	-0.095	0.262		
EDNRA	NM_001957.1	endothelin receptor type A	0.586	-0.127	-0.119		
EFNA1	NM_004428.2	ephrin-A1	-0.7	0.098	0.133		
ENO2	NM_001975.2	enolase 2 (gamma, neuronal)	-0.908	-0.053	0.53		
FGFBP2	NM_031950.2	fibroblast growth factor binding protein 2	-0.975	-0.105	-0.004		
FGFR1	NM_023110.1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	0.637	-0.131	-0.328		
GAS2L2	NM_139285.1	growth arrest-specific 2 like 2	-0.594	0.056	0.105		
GPC1	NM_002081.1	glypican 1	-0.882	-0.142	0.301		
GRIN2C	NM_000835.3	glutamate receptor, ionotropic, N-methyl D-aspartate 2C	-0.693	0.176	0.01		
HS.131773	Hs.131773	AGENCOURT_6563847 NIH_MGC_119 cDNA clone IMAGE:5744832 5, mRNA sequence	0.656	0.015	0.012		
HS.436379	Hs.436379	full-length cDNA clone CS0DM002YA18 of Fetal liver of (human)	-0.637	0.019	0.098		
HS.453381	Hs.453381	in27e08.x1 Human Fetal Pancreas 1B cDNA clone IMAGE: 3, mRNA sequence	0.762	-0.368	0.108		
HS.573729	Hs.573729	UI-E-EO1-ajc-m-13-0-UI.s1 UI-E-EO1 cDNA clone UI-E-EO1-ajc-m-13-0-UI 3, mRNA sequence	0.728	0.065	-0.406		
IBSP	NM_004967.2	integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)	-0.821	0.149	0.018		
IL1R2	NM_173343.1	interleukin 1 receptor, type II	0.691	-0.104	0.003		
ISYNA1	NM_016368.3	inositol-3-phosphate synthase 1	-0.722	-0.002	0.055		
ITLN2	NM_080878.2	intelectin 2	-0.855	-0.043	0.126		
KCTD5	NM_018992.1	potassium channel tetramerisation domain containing 5	-0.704	0.193	0.006		
KRT85	NM_002283.2	keratin, hair, basic, 5	1.928	-0.001	0.213		
LGALS7	NM_002307.1	lectin, galactoside-binding, soluble, 7, galectine 7	-0.785	-0.083	0.167		
LGALS9	NM_009587.1	lectin, galactoside-binding, soluble, 9 (galectin 9)	1.26	0.318	0.433		
LGALS9C	XM_934665.1	lectin, galactoside-binding, soluble, 9C	1.197	0.571	0.228		
LOC728910	XM_927748.1	PREDICTED: similar to Galectin-7 (Gal-7) (HKL-14) (PI7) (p53-induced protein 1)	-0.848	0.044	0.051		
MAGEB2	NM_002364.3	melanoma antigen family B, 2	0.758	-0.044	-0.005		
MATN4	NM_003833.2	matrilin 4	-0.933	-0.006	-0.033		
MATN4	NM_030590.1	matrilin 4	-1.121	0.083	0.066		
MFI2	NM_033316.2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	-1.644	0.026	0.116		
MMP3	NM_002422.3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	-1.264	-0.092	0.16		
MOXD1	NM_015529.1	monooxygenase, DBH-like 1	-0.787	-0.152	0.178		
NDUFA4L2	NM_020142.3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	-0.725	0.23	0.542		
NELF	NM_015537.3	nasal embryonic LHRH factor	-1.047	0.232	0.11		
OLFML2B	NM_015441.1	olfactomedin-like 2B	0.764	-0.032	-0.178		
OMD	NM_005014.1	osteomodulin	-1.155	-0.24	-0.124		
PLCD1	NM_006225.1	phospholipase C, delta 1	-0.607	0.068	0.114		
PPP1R3C	NM_005398.3	protein phosphatase 1, regulatory (inhibitor) subunit 3C	-0.955	-0.082	0.232		
PRSS35	NM_153362.1	protease, serine, 35	-1.045	0.071	-0.099		
ROPN1	NM_017578.2	ropporin, rhophilin associated protein 1B	-1.065	-0.042	-0.098		
ROPN1B	NM_001012337.1	ropporin, rhophilin associated protein 1B	-0.651	0.016	0.001		
ROPN1B	XM_940725.1	PREDICTED: ropporin, rhophilin associated protein 1B, transcript variant 2	-1.172	-0.104	-0.081		
S100P	NM_005980.2	S100 calcium binding protein P	-0.775	0.042	0.019		
SCGB3A2	NM_054023.2	secretoglobin, family 3A, member 2	-0.842	0.051	-0.044		
SERPINA3	NM_001085.4	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	-0.859	-0.054	-0.001		
SH3BP4	NM_014521.1	SH3-domain binding protein 4	0.831	-0.062	0.026		
SLAMF9	NM_033438.1	SLAM family member 9	-0.969	-0.101	0.063		
SLC36A2	NM_181776.1	solute carrier family 36 (proton/amino acid symporter), member 2	-0.606	0.143	0.002		
SLC8A3	NM_183002.1	solute carrier family 8 (sodium/calcium exchanger), member 3	-0.782	0.259	0.084		
SNCAIP	NM_005460.2	synuclein, alpha interacting protein	1.023	-0.118	0.034		
SORCS2	NM_020777.1	sortilin-related VPS10 domain containing receptor 2	-0.855	-0.075	-0.268		
SOX8	NM_014587.2	SRY (sex determining region Y)-box 8	-1.069	0.106	0.227		
SYK	NM_003177.3	spleen tyrosine kinase	-0.685	0.068	0.135		
T1560	NM_199048.1	T1560 protein	1.552	0.065	-0.037		
UGT2B7	NM_001074.1	UDP glucuronosyltransferase 2 family, polypeptide B7	-0.75	-0.121	-0.084		
UNQ1940	NM_205855.1	HWKM1940	-0.6	0.083	0.008		
UNQ830	NM_206895.1	ASCL830	-0.992	0.172	0.005		

Supplementary Table 3. Differentially expressed probes in two resistent xenografts

ILMN_Gene	Source_Reference_ID	Definition	log2FC HPBx/HPXCtrl	log2FC TSx/TSxCtrl	log2FC MPAx/MPAxCtrl	ISRE	STAT1
AGRN	NM_198576.2	agrin	0.31	1.30	0.881		
AIM2	NM_004833.1	absent in melanoma 2	0.17	1.61	1.738		X
ARHGDIB	NM_001175.4	Rho GDP dissociation inhibitor (GDI) beta	0.06	-0.91	-0.668		
CD97	NM_078481.2	CD97 molecule	0.02	0.70	1.003		X
ELLS1	NM_152793.1	hypothetical protein Ells1	-0.46	-1.16	-1.174		
HIST2H2AC	NM_003517.2	histone cluster 2, H2ac	0.02	0.73	0.608		
HS.145414	Hs.145414	aq28b01.y5 Barstead prostate BPH HPLRB4 1 cDNA clone IMAGE:2032201 5, mRNA sequence	0.33	2.02	2.211		
HS.540498	Hs.540498	UI-E-EJ0-ahs-h-11-0-UI.r1 UI-E-EJ0 cDNA clone UI-E-EJ0-ahs-h-11-0-UI 5, mRNA sequence	-0.12	3.18	0.707		
IL4I1	NM_152899.1	interleukin 4 induced 1	0.54	1.30	2.113		
LOXL3	NM_032603.2	lysyl oxidase-like 3	-0.06	0.66	0.78		
MLKL	NM_152649.1	mixed lineage kinase domain-like	0.17	0.74	0.823		
PABPC1	NM_002568.3	poly(A) binding protein, cytoplasmic 1	0.01	-0.75	-0.642		X
PDGFRA	NM_006206.3	platelet-derived growth factor receptor, alpha polypeptide	-0.02	-0.61	-1.102		X
PRRX2	NM_016307.3	paired related homeobox 2	-0.05	0.64	0.732		
REC8L1	NM_005132.1	REC8-like 1 (yeast)	0.34	1.49	1.31		
TNFRSF10D	NM_003840.3	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	-0.06	-0.85	-0.77		X
TNFSF7	NM_001252.2	tumor necrosis factor (ligand) superfamily, member 7	0.04	0.60	0.757		
TXNDC12	NM_015913.2	thioredoxin domain containing 12 (endoplasmic reticulum)	-0.42	-1.46	-1.615		
ZC3HAV1	NM_024625.3	zinc finger CCCH-type, antiviral 1	0.39	1.80	1.092	X	

Supplementary table 4 Metabolic pathways representing genes overrepresented among those with altered expression

Term	Gene Count[b]	%[c]	p-value
A. Genes with similar IFN response in HPBx, TSx and MPAx			
Panther BP			
BP00148:Immunity and defense	19	25.7	9.1E-08
BP00031:Nucleoside, nucleotide and nucleic acid metabolism	22	29.7	1.9E-03
BP00156:Interferon-mediated immunity	3	4.1	7.7E-03
BP00101:Sulfur metabolism	7	9.5	8.8E-03
BP00104:G-protein mediated signalling	21	28.4	1.4E-02
BP00049:mRNA polyadenylation	5	6.8	2.0E-02
BP00150:MHCI-mediated immunity	22	29.7	2.2E-02
BP00076:Electron transport	8	10.8	2.5E-02
BP00024:Acyl-CoA metabolism	4	5.4	2.8E-02
BP00071:Proteolysis	24	32.4	3.0E-02
BP00060:Protein metabolism and modification	16	21.6	3.7E-02
BP00065:Protein methylation	4	5.4	4.9E-02
Panther MF			
MF00197:Miscellaneous function	13	17.6	3.5E-05
MF00170:Ligase	10	13.5	6.9E-05
MF00042:Nucleic acid binding	41	55.4	1.0E-04
MF00007:Interferon receptor	4	5.4	1.2E-03
MF00006:Interleukin receptor	8	10.8	1.8 E-03
MF00141:Hydrolase	11	14.8	2.2 E-03
MF00262:Non-motor actin binding protein	21	28.4	3.0 E-03
MF00035:Other ion channel	7	9.5	4.0 E-03
MF00051:Helicase	8	10.8	5.0 E-03
MF00067:mRNA polyadenylation factor	6	8.1	6.2 E-03
MF00234:Other cytokine	7	9.5	7.4 E-03
MF00242:RNA helicase	12	16.2	8.8 E-03
MF00275:Transcription cofactor	7	9.5	1.0 E-02
MF00098:Large G-protein	8	10.8	1.3 E-02
MF00146:Deacetylase	5	6.8	1.3 E-02
MF00153:Protease	7	9.5	1.6 E-02
MF00044:Nuclease	7	9.5	2.8 E-02
MF00039:Other transcription factor	9	12.2	3.0 E-02
KEGG Pathway			
hsa04612:Antigen processing and presentation	9	12.1	2.3E-09
hsa04940:Type I diabetes mellitus	4	5.4	9.9E-04
hsa04514:Cell adhesion molecules (CAMs)	4	5.4	2.5E-02
hsa04650:Natural killer cell mediated cytotoxicity	4	5.4	2.6E-02
B. Genes differentially expressed in HPBx only			
Panther BP			
BP00124:Cell adhesion	9	13.6	4.9E-04
BP00193:Developmental processes	12	18.2	1.7E-02
BP00108:Receptor protein tyrosine kinase signaling pathway	5	7.6	3.9E-02
BP00289:Other metabolism	15	22.7	4.2E-02
Panther MF			
MF00016:Signaling molecule	9	13.6	1.8E-03
MF00174:Complement component	6	9.1	3.7E-03
MF00179:Extracellular matrix structural protein	5	7.6	1.9E-02
MF00262:Non-motor actin binding protein	17	25.8	2.1E-02
MF00137:Glycosyltransferase	9	13.6	2.3E-02
MF00015:Other receptor	6	9.1	2.9E-02
MF00250:Serine protease inhibitor	11	16.7	4.7E-02
KEGG pathway			
hsa04020:Calcium signaling pathway	4	6.1	4.9E-02
C. Genes with similar IFN response in TSx and MPAx			
Panther BP			
BP00196:Oogenesis	3	17.7	4.8E-02
Panther MF			
MF00001:Receptor	5	29.4	2.4E-02
KEGG pathway			
MF00001:Receptor	5	29.4	2.4E-02

Supplementary table 4 Metabolic pathways representing genes overrepresented among those with altered expression

[a]Based on lists of probes with significantly changed expression following IFN treatment identified by Limma analysis with restriction $p < 0.01$.

[b]To avoid over-counting duplicated genes, the probe lists are converted to corresponding DAVID gene IDs by which all redundancy in original IDs are removed

[c]The gene-enrichment of functional categories and pathways was measured by determining the number of genes belonging to the functional group in the list, weighted against the total number of analyzed genes on arrays using Fisher Exact statistical test with restriction $p < 0.05$.

IV

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SHORT REPORT

Potential for treatment of liposarcomas with the MDM2 antagonist Nutlin-3A

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The MDM2-antagonist Nutlin 3A can efficiently induce apoptosis in osteosarcoma cell lines with amplified *MDM2*. However, Nutlin-based therapy could be even more important in more common sarcoma types where this aberration is frequent. The well- and de-differentiated liposarcomas have complex marker chromosomes, consistently including multiple copies of the *MDM2* locus. Since amplification seems to be a primary aberration in these tumors, whereas amplification in osteosarcoma generally is a progression marker, the underlying biological mechanisms may be different. We have therefore investigated the molecular response to Nutlin treatment in several liposarcoma cell lines with such markers, as well as a panel of other sarcoma cell lines. We report that Nutlin efficiently stabilized p53 and induced downstream p53 dependent transcription and apoptosis in liposarcoma cells with amplified *MDM2 in vitro*. Some effect of Nutlin was also observed on cell lines without amplified *MDM2* but with wt *TP53*, but no apoptosis was induced. The MDM4 protein, reported to interfere with the reactivation of p53, was undetectable in cells with amplified *MDM2*. Thus, Nutlin represents a promising new therapeutic principle for the treatment of an increasing group of sarcomas.

Key words: therapy; amplification; TP53; apoptosis; MDM4; osteosarcoma

The activity and level of the tumor suppressor protein p53 is negatively regulated by the E3 ligase MDM2, which controls both its ability to trans-activate downstream genes and its proteasome-mediated degradation.[1] In tumors with wild type p53, its activity may be blocked through amplification and overexpression of the *MDM2* locus.[2,3] Due to the central role of p53 in oncogenesis and therapy response, the p53-MDM2 interaction is an interesting target for small-molecular therapy. The goal is to reactivate p53 and induce downstream effects leading to programmed cell death or increased response to DNA-damaging therapies, as has been pursued by several groups.[4–9] One of the most promising MDM2 antagonists is Nutlin 3A, which has been shown to activate wild type p53 in cancer cell lines, inducing cell cycle arrest and apoptosis in various cell lines, including osteosarcomas with amplified *MDM2*.[10,11] However, amplification of *MDM2* is much more frequent in the more common subtype of well-differentiated liposarcoma (WDLS) and dedifferentiated variants, where virtually all tumors contain complex marker chromosomes that always include multiple copies of *MDM2*.[12] Since the role of MDM2 in the etiology of these tumors seems different from that in osteosarcomas, we set out to investigate the potential for Nutlin treatment and its molecular effects. This particular type of liposarcoma can be hard to eradicate and may dedifferentiate to aggressive variants with high malignancy. Since inactivation of p53 may also be achieved by amplification and overexpression of MDM4,[13–15] we also investigated whether MDM4 could be important for the response in WDLS, although we have found only low levels of amplification of *MDM4* in our sarcoma panel (Ohnstad, unpublished data).

For these investigations we chose 5 cell lines with amplified *MDM2*, including 3 WDLS-derived lines, and, since Nutlin has been reported to induce apoptosis in haematological cancers even when *MDM2* is not amplified,[4,16,17] added 5 control cell lines with normal MDM2-level, of which 3 had either no or mutated *TP53* mRNA (Table I). The cell lines with high-level amplification consistently expressed very high levels of *MDM2* mRNA (Table I), and protein (Fig. 3), whereas the cell lines with normal gene number all had low levels in comparison.

We first examined the *in vitro* response of our cell panel to the active Nutlin 3A enantiomer and, as control, the virtually inactive 3B enantiomer (Table I). All cell lines with amplified *MDM2* responded well to Nutlin 3A, with half-maximal growth inhibitory concentration (IC_{50}) from 0.6–1.9 μM, although MHM did not reach total growth inhibition under the conditions used to determine IC_{50}. In two of the control cell lines with wt *TP53* (RMS13 and U2OS) partial growth inhibition was observed (IC_{50} from 3.3–3.5 μM), whereas the lines with mutated *TP53* (SW872, KPD, SAOS2) did not respond to Nutlin ($IC_{50} > 10$ μM). We documented a similar response pattern across the cell panel for drug exposure of 24 and 72 hr. Nutlin 3B did not inhibit growth in any of the cell lines tested (data not shown).

We further investigated the transcriptional response downstream of p53 with either of the Nutlin enantiomers. Transcript levels for *BAX*, *BCL2*, *p21*, *p53*, *MDM2* and $p14^{ARF}$ were assayed after 4, 24 and 48 hr of treatment (Figs. 1*a* and 1*b*). 48 hr data exist only for p21, p53 and MDM2 (supplementary Figs. II and III). As expected, cell lines with high *MDM2* copy number showed a strong and Nutlin 3A-dependent upregulation of p21 transcripts (Fig. 1*a*, unpaired *t*-test *vs.* control, $p < 0.005$) and protein (Fig. 3), indicating reactivation of p53. This was supported by increased p53 protein levels (Fig. 3) probably due to protein stabilisation.[5,10,23] However, upon treatment with 10 μM Nutlin 3A, p53 mRNA levels reproducibly decreased in all our sensitive liposarcoma cells (Fig. 1*a*, unpaired *t*-test *vs.* control $p < 0.01$), although less so with Nutlin 3B (Fig. 1*b*). This is contrary to what was observed in OSA cells (Fig. 1*b*, Refs. 10–11.). Although we cannot explain this behaviour at present, we note that in the MHM cells, originating from a parosteal osteosarcoma, also having marker chromosomes similar to those in WDLS, p53 showed the same decline, perhaps due to a similar etiology.

Consistent with p53 activation, levels of MDM2 mRNA and protein increased upon Nutlin 3A treatment (except in FU-DDLS-1; Figs. 1*a* and 3, unpaired *t*-test *vs.* control $p < 0.025$). Only moderate effects on $p14^{ARF}$ mRNA could be observed.

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TABLE I – DRUG SENSITIVITY, GENOTYPES AND CLINICAL AND BIOLOGICAL CHARACTERISTICS OF THE SARCOMA CELL LINE PANEL

Cell line	Histology	Patient sex/age	Origin	Site	TP53	MDM2 copy number	MDM2 mRNA level	IC50 (μM)	TGI (μM)
T449	WDLS	F/68	Primary	Retroperitoneum	wt	94.6 ± 6.0	77 ± 6	0.6 ± 0.1	1.9 ± 0.6
T778	WDLS	F/69	Relapse	Retroperitoneum	wt	59.8 ± 1.8	28.5 ± 1.6	1.0 ± 0.3	2.3 ± 0.9
FU-DDLS-1	DDLS	M/61	Relapse	Retroperitoneum	wt	70.6 ± 6.1	87 ± 10.9	0.6 ± 0.2	2.1 ± 1.0
OSA	OS	M/19	Primary	Femur	wt	49.1 ± 1.1	43.2 ± 9.4	0.9 ± 0.3	2.9 ± 0.4
MHM	OS	F/42	Relapse	Pelvis	wt	22 ± 1.6	59.5 ± 1.9	1.9 ± 0.3	>10
SW872	LS	M/36	Unknown	Unknown	mut	1.1 ± 0.1	0.7 ± 0	>10	>10
KPD	OS	M/8	Primary	Femur	mut	0.5 ± 0	1.1 ± 0	>10	>10
RMS13	RMS	M/17	Primary	Unknown	wt	1.9 ± 0.2	0.9 ± 0	3.5 ± 1.3	>10
SAOS2	OS	F/11	Unknown	Unknown	mut	1.4 ± 0.2	2.5 ± 0.2	>10	>10
U2OS	OS	F/15	Primary	Tibia	wt	0.7 ± 0.1	3.1 ± 0.2	3.3 ± 1.1	>10

WDLS, well differentiated liposarcoma; DDLS, dedifferentiated liposarcoma; OS, osteosarcoma; LS, undifferentiated liposarcoma, unspecified; RMS, rhabdomyosarcoma; wt, wild type; mut, mutated; TGI, total growth inhibition; $IC_{50,}$ concentration required for 50% growth inhibition. The drug concentration required for 50% growth inhibition (IC50), and total growth inhibition (TGI) was determined using the Sulphorhodamine B (SRB) assay[18] after 120 h exposure to Nutlin 3A. IC_{50} and TGI are presented as mean ±SD of at least 3 experiments. *MDM2* copy number was determined with SYBR-green RT-PCR on 5 nanograms of genomic DNA, initially purified using a phenol-based procedure adapted from.[19] The albumin gene was used as internal normalization reference. Values were expressed relative to human genomic female DNA (Promega, Southampton, UK). Primers: MDM2 forward 5′ AAGCCAAACTGGAAAACTCAACAC 3′. MDM2 reverse 5′ CAGGAACATCAAAGCCCTCTTC 3′. Albumin forward 5′ TTTATTCACATCATTCTCTC 3′. Albumin reverse 5′GAGTGAGATATGAGTTGAG 3′. Relative MDM2 expression was determined as described in the legend of Figure 1. TP53 genotypes were determined by the Roche TP53 Genechip, as described elsewhere (Ohnstad *et al*., manuscript in preparation). The cell lines MHM (OS45[20]) and KPD (OS06[21]) were established from primary patient samples at the Norwegian Radium Hospital. T449 (93449) and T778 (94778) were established from primary and relapsed tumor of the same patient at Hopital de l'Archet.[12] Dr. A. Thomas Look at St Jude's hospital, Memphis, USA, kindly donated the cell lines RMS13 (Rh30) and OSA (SJSA-1 or CRL2098; also available from the ATCC). The FU-DDLS-1 cell line was kindly provided by Dr. J. Nishio, Fukuoka University, Japan.[22] SAOS2 (HTB85), U2OS (HTB96) and SW872 (HTB92) cells were purchased from the American Type Culture Collection, Rockville, MD.

In some of the *MDM2*-amplified lines, in particular MHM and T778, but also, *e.g*., for MDM2 in OSA, some weaker effects could also be observed following treatment with the 200-fold less active Nutlin 3B enantiomer.

By expression analysis, a proapoptotic change of the BAX/BCL2 transcript ratio was observed (Figs. 1*a* and 1*b*), and Nutlin 3A induced cell cycle arrest and DNA fragmentation (positive TUNEL-labeling) consistent with apoptosis after 24 hr only in cell lines with amplified *MDM2* (Figs. 2*a* and 2*b*). In the wt *TP53*-cell lines U2OS and RMS13 where *MDM2* was not amplified, we observed cell cycle arrest (Fig. 2a), but no increase of apoptotic cell fraction upon Nutlin 3A treatment (Fig. 2b, supplementary Fig. I).

Apoptosis as the mechanism of cell death was confirmed by activation of Caspase 3 (CASP3) and cleavage of Poly(ADP-Ribose)-Polymerase (PARP) in *MDM2*-amplified cells (Fig. 3), although a specific PARP cleavage product of 62 kDa, perhaps involving calpain,[24] could only be detected in the MHM cells.

The structurally related MDM4 protein can also inhibit p53 activity, may form heterodimers with MDM2, and can apparently diminish the Nutlin response because it is insensitive to inhibition by Nutlin.[25–27] An exception has been reported for retinoblastoma, where MDM4 is amplified but Nutlin is efficiently killing retinoblastoma cells.[28] Although *MDM4* has been reported to be amplified in several cancer types,[14,29] this was generally not the case in our tumor panel (Ohnstad *et al*., unpublished). We nevertheless investigated the possible involvement of MDM4 in the cellular response to Nutlin in 4 of the cell lines. As can be seen in Figure 3, the 80 kDa MDM4 protein could be detected in the U2OS control line, but not in the *MDM2* amplified lines, regardless of Nutlin treatment. This is most likely caused by proteasomal degradation of MDM4 mediated by the very high MDM2 levels, as previously reported.[30] Furthermore, a smaller band of ~47 kDa appeared upon treatment with Nutlin in the amplified cell lines, which may be a degradation intermediate because of some inhibitory effect of Nutlin. MDM4 isoforms of this size have been reported previously in other cell lines.[29] But the degradation mechanisms are complex, and further investigations are evidently necessary to determine the exact nature of these bands.

In contrast to *e.g*. osteosarcomas, the well-differentiated subtype of liposarcomas and its more malignant derivatives have wild type p53 and amplified *MDM2*.[12] However, little is still known of the impact of $p14^{ARF}$ and MDM4 on p53 activity in this sarcoma subtype. In this work, we detected Nutlin-induced apoptosis only in cell lines with high MDM2 levels and wild type *TP53*, and with $p14^{ARF}$ seemingly intact. This is in line with the hypothesis that tumors with amplified and overexpressed MDM2 contain an (otherwise) intact p53 pathway,[10] although contradictory findings have been described in samples with low MDM2 expression and various genotypes of MDM4, $P14^{ARF}$ and TP53.[4,7,16,17,31,32] However, we cannot compare the absolute MDM2 levels with these studies, due to lack of data at gene, transcript and protein level. Therefore, at present, the key requirement for Nutlin sensitivity in sarcomas seems to be wild type *TP53*, in line with previous publications on Nutley inhibitors.[6–8,10,11,17,23] In U2OS and RMS13, representing the large fraction of sarcomas where both *MDM2* and *TP53* are wild type and show only low-level overexpression of the MDM2 locus, some other, as yet undetected, aberration most likely blocks the pathway downstream of p53, preventing apoptosis. Although several of such aberrations are known,[33,34] the limited selection of liposarcoma cell lines available here does not allow us to elucidate how this pathway may be deranged in such cells. More work on *e.g*. MDM4 must be done to clarify what separates regulation of MDM2-p53 in (lipo)sarcomas from the other classes of human cancers sensitive to MDM2 antagonists.

In conclusion, Nutlin 3A induces apoptosis efficiently in a major subset of liposarcomas, and from our data one would expect that *TP53* genotype together with *MDM2* amplification predicts sensitivity to this therapeutic agent. Both the mostly juvenile osteosarcomas, and adult liposarcomas, with their high mortality, are very much in need of new treatment options. Depending on the status of the p53 pathway, Nutlin could act as a sensitizer to cytyotoxic therapy or protect proliferating normal cells from chemotherapy- or radiation-associated DNA-damage,[35] As proposed lately by several groups,[15,26] the lack of response to Nutlin in some tumor types suggests the design of a novel MDM4 inhibitor that may be combined with Nutlin 3A to increase the group of cancers where p53 may be reactivated. The promise of an orally administered specific and efficient drug with low toxicity makes this MDM2 inhibitor an attractive new treatment option for an increasing range of tumors. Treatment of human sarcoma-xenografts in immunodeficient mice with Nutlin 3A has already been successful,[11] and clinical trials in sarcoma patients may now be contemplated.

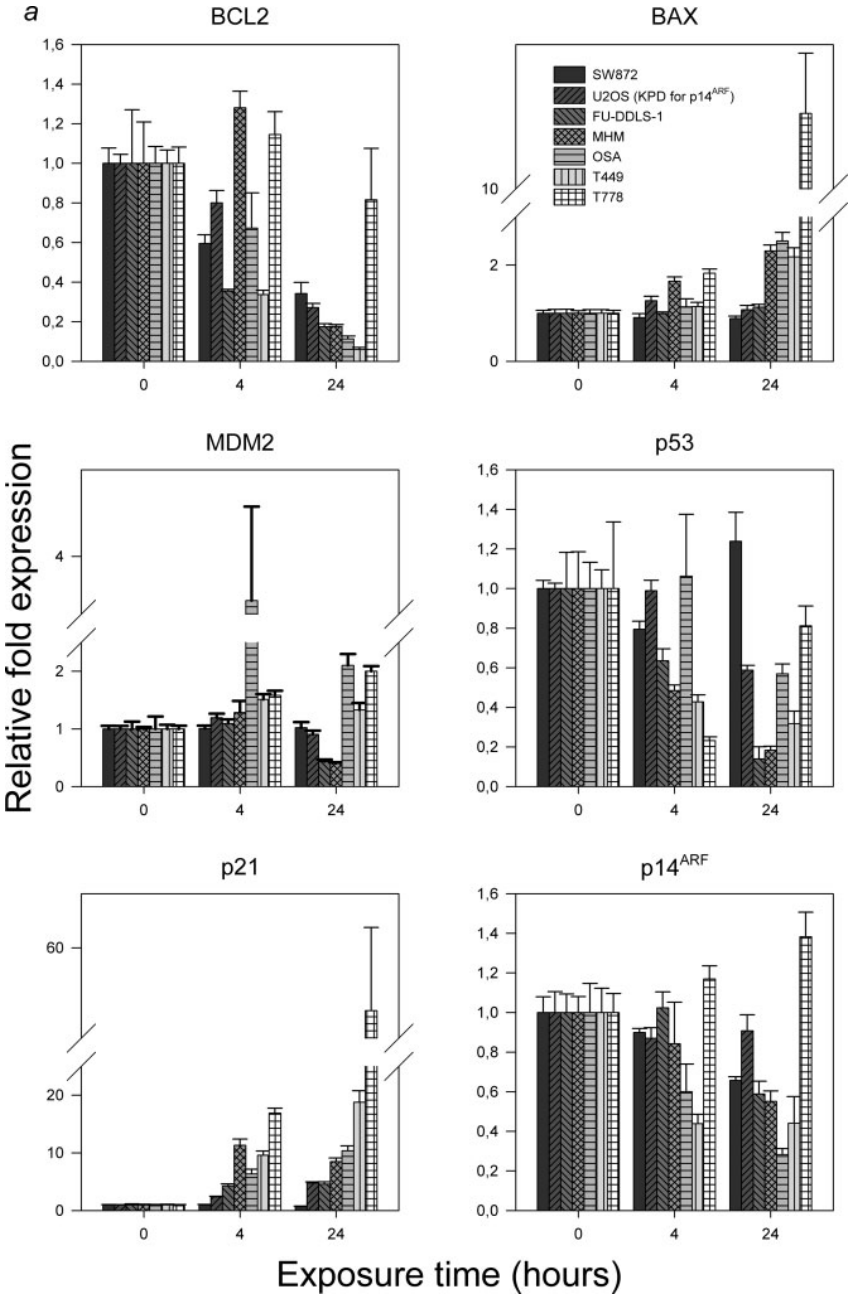


FIGURE 1 – Expression of selected genes in a cell line panel after treatment with (*a*) 10 μM Nutlin 3A or (*b*) 10 μM Nutlin 3B. Data are presented as fold-change expressed relative to untreated cell line control ($t = 0$) ±SD of at least 3 experiments. Three of the noninformative control cell lines were omitted to simplify the figure. All RT-PCR analyses were performed using Applied Biosystems 7500 thermal cycler and software. Briefly, total RNA was isolated using Trizol (Invitrogen, Paisley, UK) following the manufacturer's protocol. 2–5 ng cDNA was amplified, and standard curves generated using universal microarray reference RNA (UMR, Stratagene, La Jolla, CA). As internal normalization reference we used VIC-labeled beta-2-microglobulin, *B2M*. The NanoDrop apparatus (NanoDrop Technologies, Wilmington, DE) and the 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) were used to assay RNA/DNA quality and purity. (TaqMan assay IDs are listed in supplementary Table I). The legend is uniform for 5 of 6 panels except as indicated for $p14^{ARF}$, where KPD results were plotted instead of U2OS.

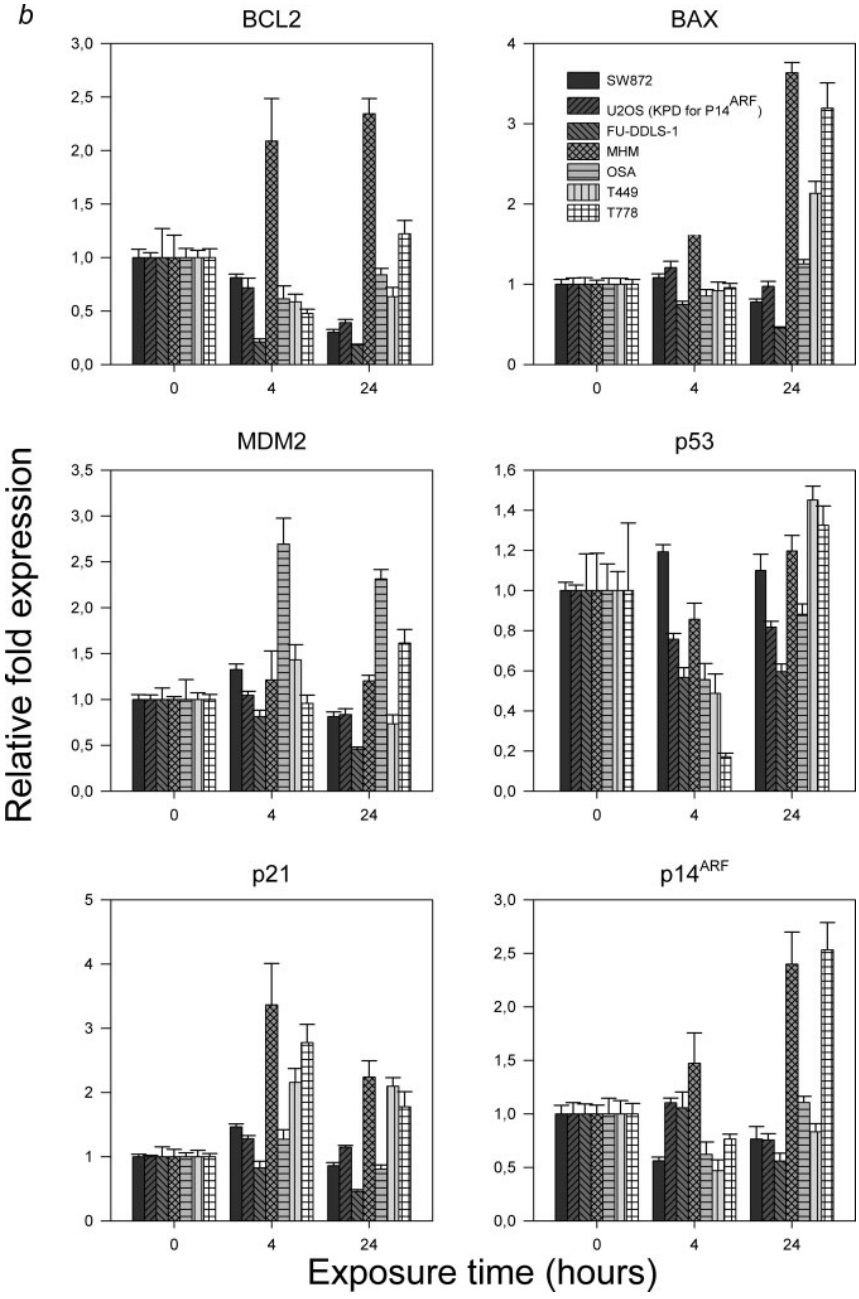
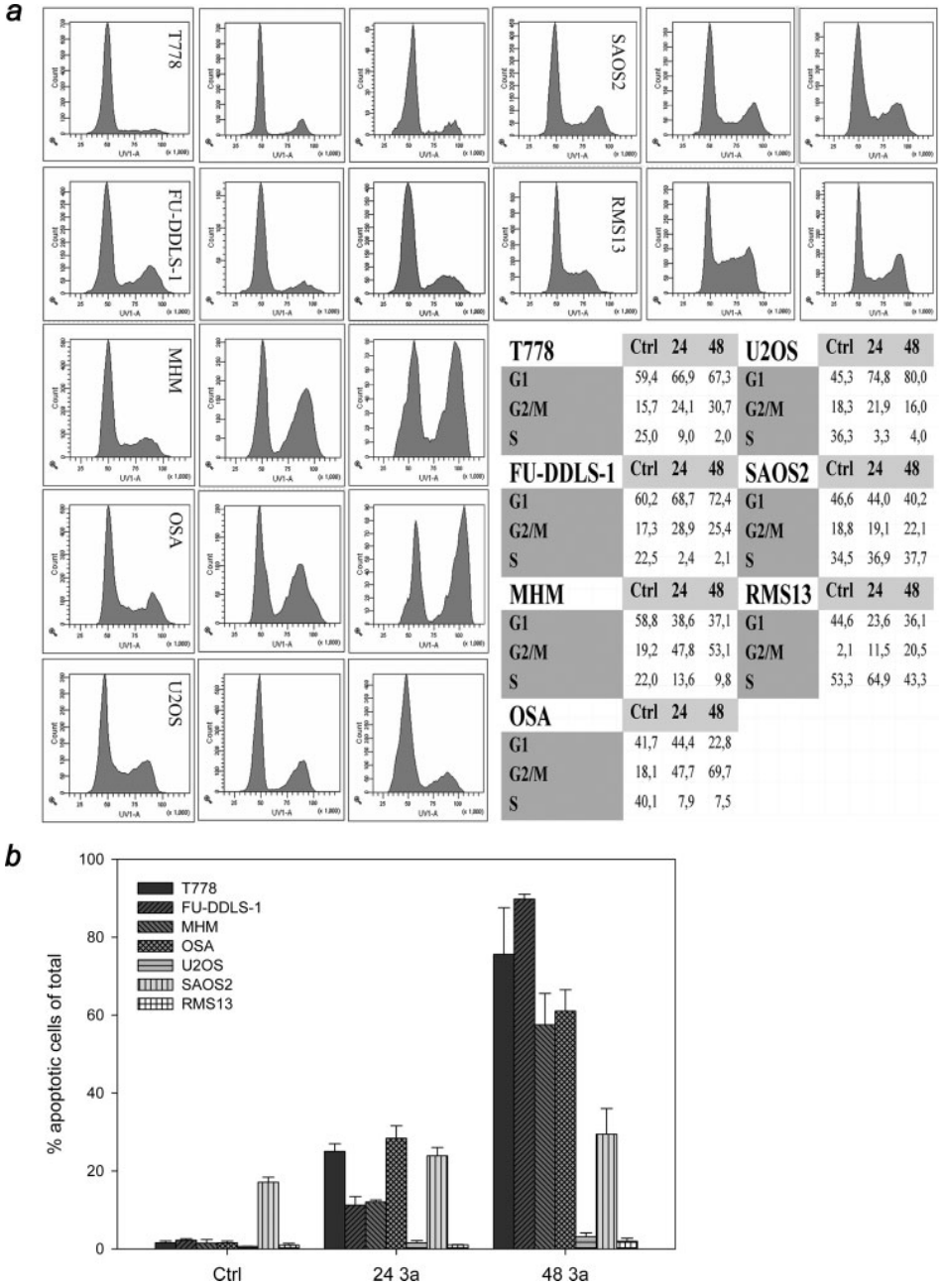


FIGURE 1 – (CONTINUED)



T778	Ctrl	24	48	U2OS	Ctrl	24	48
G1	59,4	66,9	67,3	G1	45,3	74,8	80,0
G2/M	15,7	24,1	30,7	G2/M	18,3	21,9	16,0
S	25,0	9,0	2,0	S	36,3	3,3	4,0
FU-DDLS-1	**Ctrl**	**24**	**48**	**SAOS2**	**Ctrl**	**24**	**48**
G1	60,2	68,7	72,4	G1	46,6	44,0	40,2
G2/M	17,3	28,9	25,4	G2/M	18,8	19,1	22,1
S	22,5	2,4	2,1	S	34,5	36,9	37,7
MHM	**Ctrl**	**24**	**48**	**RMS13**	**Ctrl**	**24**	**48**
G1	58,8	38,6	37,1	G1	44,6	23,6	36,1
G2/M	19,2	47,8	53,1	G2/M	2,1	11,5	20,5
S	22,0	13,6	9,8	S	53,3	64,9	43,3
OSA	**Ctrl**	**24**	**48**				
G1	41,7	44,4	22,8				
G2/M	18,1	47,7	69,7				
S	40,1	7,9	7,5				

FIGURE 2 – Apoptotic response of seven selected sarcoma cell lines to treatment with 10 μM Nutlin 3A for 24 and 48 hr compared to untreated controls. Data represent the mean of minimum 2 experiments. 10,000 cells were counted in each run. (*a*) Cytograms and cell cycle-distributions in viable cells, expressed as percentage of total population, rounded to the full percentage value. (*b*) Apoptotic percentage of total, presented with standard deviation. Apoptotic cells were quantified after methanol-fixation by TUNEL labeling (Gavrieli *et al*., 1992) using a kit (#333574) from Hoffmann-La Roche, NJ, USA. The biotin-labeled cleavage sites were labeled with FITC-conjugated streptavidin (RPN1232VI, Amersham Biosciences, Little Chalfont, England). Two μg/ml Hoechst 33258 (Sigma-Aldrich, St. Louis, MD) was used to stain genomic DNA. Cells were analyzed with the ModFit and Becton Dickinson FACS DiVa software and flow cytometer with Argon (488 nm) and Krypton UV lasers (356 nm).

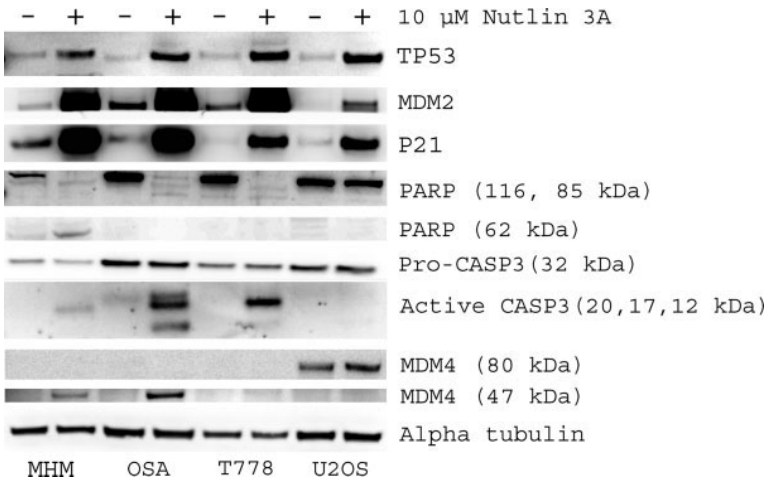


FIGURE 3 – Activation of the p53 pathway in selected cell lines after 10 µM Nutlin 3A-treatment for 26 hours. Complete cleavage of PARP and activation of CASP3 indicates apoptotic activity in cell lines with *MDM2* amplification. Data are representative of one of three separate experiments. Total protein was obtained with lysis buffer from a caspase assay kit (BioSource, Camarillo, CA), and concentration was measured using the BCA (bicinchoninic acid) method (kit #23225, Pierce, Rockford, IL). 25 µg of lysate was separated on 4–12% NuPage Bis-Tris gradient gels (Invitrogen, Paisley, UK) and transferred to PVDF membranes (Millipore, Bedford, MA) in blotting buffer with 20% (v/v) methanol, using wet blot equipment (BIO-RAD, Hercules, CA). Bands were visualized using SuperSignal West Dura ECL (#34076, Pierce, Rockford, UK) and the Kodak IR2000 Imager and software (Eastman Kodak, Rochester, NY). Antibodies (monoclonal except when noted): anti-PARP (1:2,000, Calbiochem 512739/Merck, Darmstadt, Germany), anti-CASP3 (1:2,000, R&D Systems AF-605-NA, Abingdon, UK), polyclonal anti-HDMX/4 (1:20,000, Bethyl Laboratories BL1258, Montgomery, TX), anti-p53 (1:3,000 Santa Cruz sc-6243, Biotechnology, Santa Cruz, CA), anti-MDM2 (1:200, Chemicon MAB1434, Temecula, CA), anti-tubulin (1:200, Calbiochem CP06/Merck, Darmstadt, Germany), polyclonal anti-p21 (1:250, Santa Cruz sc-397, Biotechnology, Santa Cruz, CA). All HRP-conjugated secondary antibodies were from DAKO, Glostrup, Denmark.

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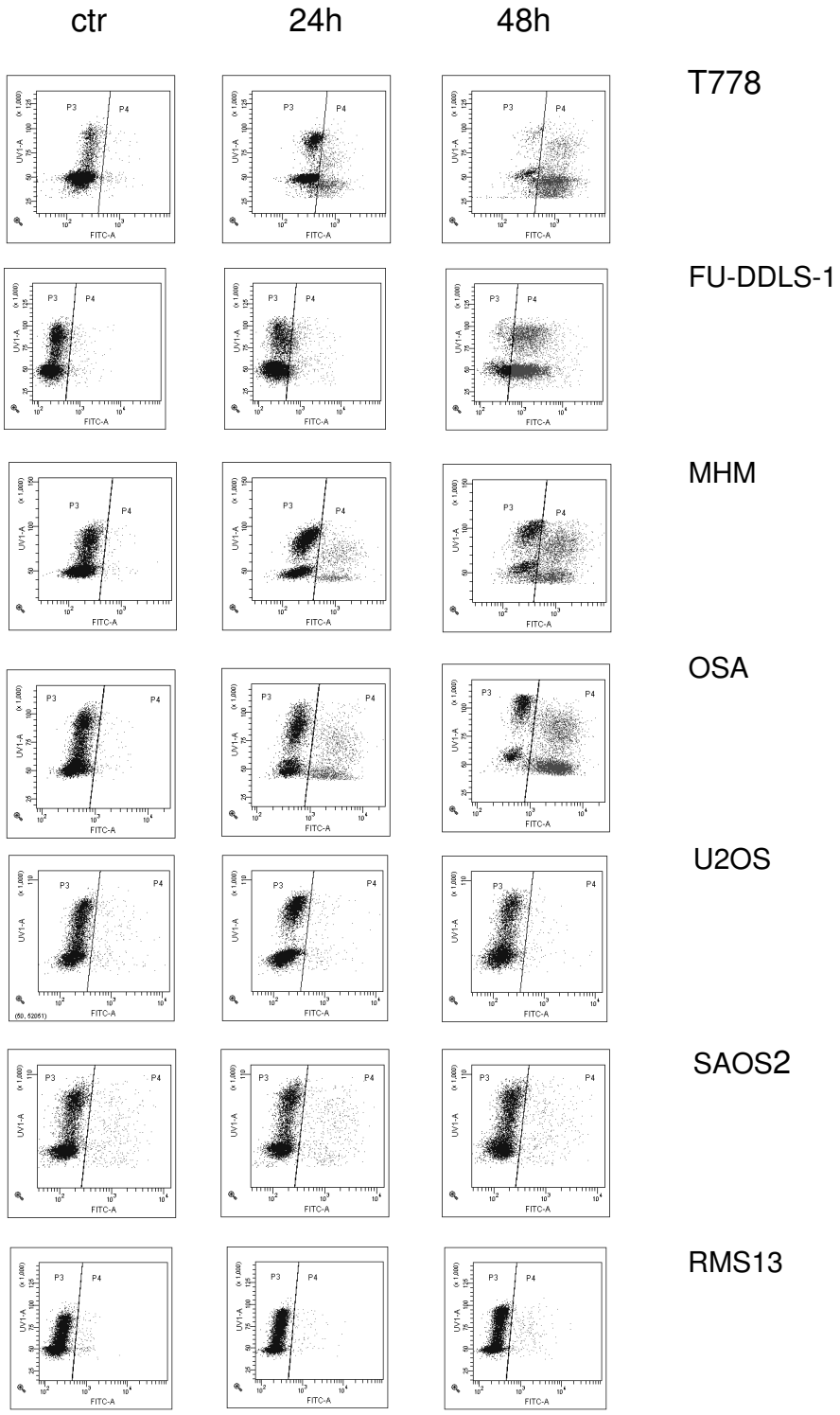
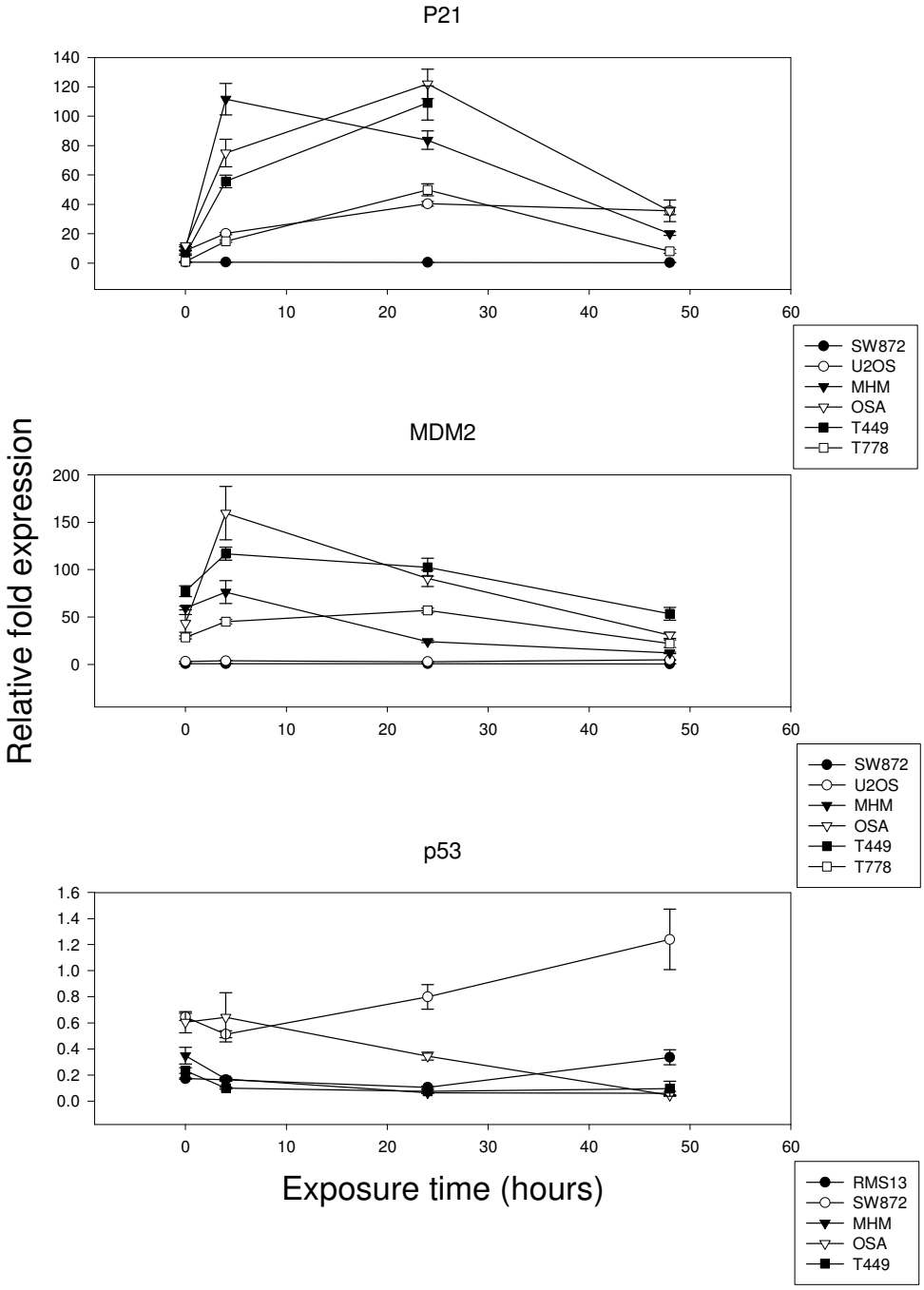


Supplementary figure I: Flow cytometry of sarcoma cell lines following treatment with 10µM Nutlin 3A for 24 and 48 hours. Biotin-streptavidin FITC TUNEL (abscissa) by Hoechst (ordinate).

BAX	Hs00180269_m1
BCL2	Hs00153350_m1
MDM2	Hs00234753_m1
TP53	Hs00153349_m1
P21	Hs00355782_m1
B2M	Hs99999907_m1
P14ARF	Hs00924091_m1

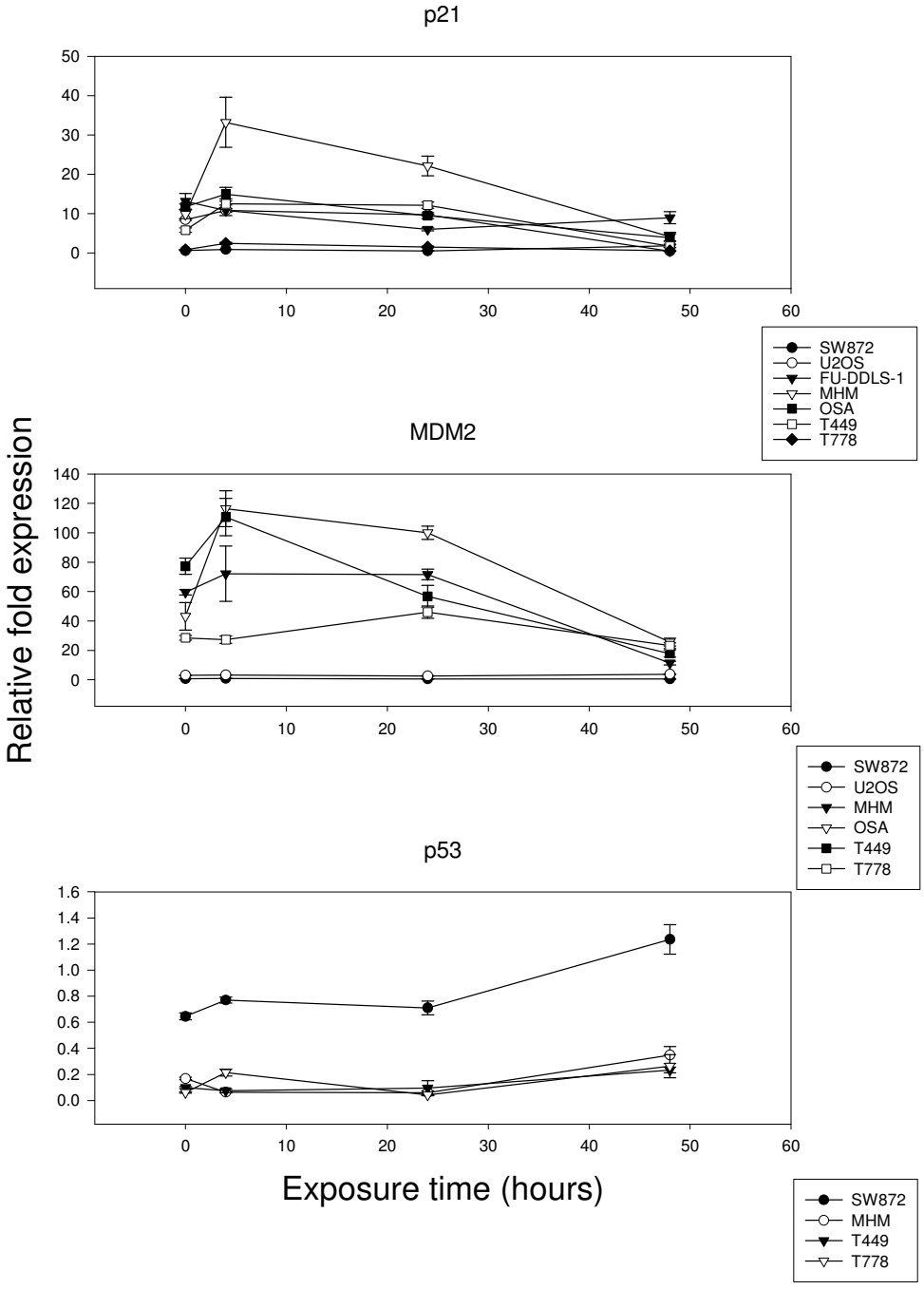
Supplementary Table 1: TaqMan Expression Assay ID's.


P21
MDM2
p53
Relative fold expression
Exposure time (hours)
SW872
U2OS
MHM
OSA
T449
T778
RMS13

Supplementary figure II: Relative expression of *p21*, *p53* and *MDM2* in selected cell lines after treatment with 10 µM Nutlin 3A. Y-axis: Gene/B2M ratio fold expression relative to UMR. X-axis: Time after Nutlin 3A-addition (hours). Each data point represents ≥3 independent experiments with respective standard deviations. B2M levels were not affected by Nutlin 3A-treatment (results not shown). UMR = universal microarray reference, B2M = beta-2 microglobulin.

Unpaired t-tests for sample towards untreated control in MDM2-amplified cell lines:

Gene	**24 hours**	**48 hours**
MDM2	P<0,025	P<0,05
P21	P<0,005	P<0,005
P53	P<0,01	P<0,025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40
30
20
10
0
0
10
20
30
40
50
60
SW872
U2OS
FU-DDLS-1
MHM
OSA
T449
T778
MDM2
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
SW872
U2OS
MHM
OSA
T449
T778
p53
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
SW872
MHM
T449
T778
Relative fold expression
Exposure time (hours)

Supplementary Figure III: Relative expression of *p21*, *p53* and *MDM2* in selected cell lines after treatment with 10 µM Nutlin 3B. Y-axis: Gene/B2M ratio fold expression relative to UMR. X-axis: Time after Nutlin 3B-addition (hours). Each data point represents ≥3 independent experiments with respective standard deviations. B2M levels were not affected by Nutlin 3B-treatment (results not shown). Unpaired t-tests for sample towards untreated control at 24 and 48 hours for MDM2-amplified cell lines: $p < 0{,}025$ for *MDM2*, *P21* and *P53*. UMR = universal microarray reference, B2M = beta-2 microglobulin.

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