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Mechanisms of therapy resistance in osteosarcoma: a review

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Abstract

Therapy resistance remains a challenge in the treatment for osteosarcoma (OS). By studying therapy resistance and gaining biological understanding of resistance in OS, novel treatment targets to sensitise OS could potentially be discovered. The aim of this review is to give an overview of the mechanisms of resistance employed by OS cells after cytotoxic treatment, the key molecules involved in therapy resistance combined with the (pre)clinical research performed on this subject in a search to discover means to overcome therapy resistance and thus improve treatment efficacy for OS.

Keywords: Osteosarcoma, drug resistance, therapy, novel treatment

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour in children and adolescents. The gold standard for therapy consists of a combination of multi-agent neoadjuvant chemotherapy, followed by radical surgery and adjuvant chemotherapy. With this aggressive regimen, 5-year survival rates of approximately 65% are obtained in patients with localised disease. However, in the case of metastatic or recurrent disease, 5-year survival rates are reduced to only 20% [1-5]. In patients with axial OS and/or inoperable OS, local control is difficult to achieve and these patients run a high risk of local relapse and the development of metastatic disease. Therefore the prognosis for these patients is worse compared to patients with OS of the extremity and 5-year survival is approximately 25% [3,4,6,7]. Attempts to improve therapy efficacy by dose escalation, alterations in combinations of chemotherapy and irradiation therapy (particularly for patients with inoperable OS), either combined with chemotherapy or not, have not improved survival outcomes [5,8-15]. Resistance to therapy, both intrinsic and acquired, can be accountable for essential treatment failure as well as for recurrence after a disease-free interval. Methods to subvert therapy resistance and re-establish sensitivity of OS for existing treatments potentially increases therapy efficacy and thus survival outcome. This review summarises the literature published in the past decade on various mechanisms of therapy resistance encountered in OS and how they could be meaningful in the design of targeted therapies to improve the sensitivity of OS to existing treatments.

Resistance

Basic principles of anticancer therapy are proliferation, inhibition and (re)activation of apoptosis. Cancer cells can utilise various mechanisms to circumvent or counteract the cytotoxic stimuli induced by anticancer therapy, including: (1) non-proliferative state (i.e., maintaining a stem-cell like phenotype and/or dormancy), (2) cell cycle alterations, (3) enhanced drug efflux, (4) increased detoxification, (5) increased DNA repair (combined with cell cycle alterations) and (6) apoptosis resistance [14,16-19]. (**Table 1**) provides an overview of these mechanisms, including key molecules involved in the particular resistance mechanism. Resistance of cancer cells to therapy can be either intrinsic to the cancer cell, or acquired as the result of genomic instability. Acquired resistance would lead to the selection of resistant clones after a treatment that could, in a later stage of the disease, lead to relapse or metastatic disease progression. Intrinsic therapy resistance would lead to an essential poor response to therapy and account for the so-called 'poor responders' [14,20-25]. Both types of therapy resistance are, in general, the result of specific molecules or molecular signalling in the OS cells. In the following section, each of the abovementioned mechanisms of therapy resistance will be elucidated and the contributing molecules in OS cells will be discussed, followed by a summary of previous (pre)clinical efforts to target the specific resistance mechanisms in attempts to improve treatment sensitivity in OS.

Non-proliferative state

Most conventional cytotoxic treatments modalities induce DNA damage and target rapidly proliferating (cancer) cells [26-28]. Contrarily, non-proliferating cells are not (so much) susceptible to DNA damaging therapy and therefore the non-proliferative state can serve tumour cells a survival benefit after the administration of treatment. Non-proliferative states are found in quiescent cells, cells with stem-cell like phenotypes and dormant cells [29-31].

Stem-like cells

There is growing evidence that tumours may develop from

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Table 1. Mechanisms of resistance in OS and key molecules involved per mechanism.

Mechanisms of Resistance	Molecular involvement	Drug Target	Drug	References
I Non-proliferative state				
(a) Stem-like cells	P-glycoprotein			[32]
	TGF-β1			[30]
	STAT3			[33]
(b) Dormancy	PI3K/Akt			[26,42-44]
	Integrin-α5β1/NF-κB/ERK/p38-MAPK			[26,31]
	miR-190 (EPHA5, Angiomotin)	miR-190	miR-190 overexpression	[38]
II Cell cycle alterations				
G1 checkpoint	p53			[46-48,50,55,56]
		Cdk2		[16,69]
S-phase checkpoint	ATR/Chk1/Cdc25A		Zoledronate	[57,58]
		Chk1		[48,68]
		Chk1	Caffeine	[55]
G2/M checkpoint	ATM/Chk2/Cdc25C/Cdc2-CyclinB			[47,48,55,56,61]
	Cdc2 (Cdk1)			[59-61,67,68]
	STAT3			[62,63]
	NF-κB/Survivin			[43,64]
	GADD45α (MAPK3K-JNK/p39-MAPK)			[19,66,67]
		Cdc2 (Cdk1)	Flavopiridol	[16,48,68]
		Cdc2-CyclinB	WEE1 inhibitor	[60]
		Chk2	Caffeine	[55]
III Reduced drug accumulation	P-glycoprotein			[16,18,43,71-73]
		P-glycoprotein	RNAi: siABCB1	[78]
		Ezrin	stable Ezrin mutant (shRNA)	[73]
	Reduced Folate Carrier			[14,74,77]
IV Increased detoxification	Glutathione S-transferase P1 (GSTP1)			[17,65]
	GSTP1/JNK- c-Jun/p38-MAPK/ERK1/2			[79,80]
		GSTP1-1 -TRAF1	NBDHEX	[80]
		GST	NBDHEX	[79,80]
V Increased DNA repair	(ATM/DNA-PK) γH2AX			[81]
	APE-1			[82]
	ERCC2 or XPD gene			[83]
		γH2AX	si-γH2AX	[81]
		γH2AX	miR-138 overexpression	[81]
		APE-1	si-APE1	[82]
VI **Apoptosis resistance**	p53/BAX/NOXA/PUMA/p53AIP1			[43,55]
		p53/MDM2	Nutlin(-3a)	[105,106]
	Fas			[84,90-93]
		Fas	IL-12	[84,91,93]
		Fas	Gemcitabine	[21,90]
		Fas	Liposomal MTP-PE	[97,107-113]
		FasL	Ifosfamide	[84,90]
	NF-κB/Survivin			[64]
	JNK/c-Jun/AP-1			[65,94]

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Continuation of Table 1.

Mechanisms of Resistance	Molecular involvement	Drug Target	Drug	References
		JNK	JIP1 inhibitor	[94]
	Wnt/β-catenin			[64,95,96]
	Wnt- β-catenin/ Syndecan-2			[24]
		(Wnt/β-catenin)	TCF	[24]
	Syndecan-2/endothelin-1/ ERK1/3/PIK3/ Akt/Calpain-6			[25]
		Calpain-6	RNAi si-calpain-6	[25]
	Syndecan-2/endothelin-1/NF-κB			[25]
	mTOR/PI3K/Akt			[86]
	mTORC1/AMPK			[86]
	CXCR-3/-4/MMP-2/-9			[14,24,25,30,35-37,64,86]
	CXCR-4/CXCL12/NF-kB			[36,37,98,99]
			Everolimus	[86]
EMT				
	TGF-β			
	E-cadherin/β-catenin	(Wnt/ β-catenin)		
Autophagy	HMGB1			[104]
		HMGB1		[104]

cancer stem cells that have characteristics similar to normal/ healthy stem cells but give rise to cell populations that do not mature into fully differentiated cells but rather cells that hold the ability to divide infinitely, thus producing cancer cells. Many solid tumours are known to contain small populations of stem-like cells, or cancer stem cells [32,33]. In OS, both in cell lines and in biopsies from human OS specimens, populations of stem-like cells have been detected. When cultured, the stem-like cells can form so-called sarcopheres that express mesenchymal stem cell markers such as CD133, CD117, CD105 and CD44 [30,33,34]. These cancer stem cells, alike normal stem cells, have self-renewal capacity, can be quiescent (that is, resting in G0 for prolonged periods of time) and can repair their DNA [32]. *Zhang* et al., [30] show that cancer stem cells in spheres exhibit insensitivity for cisplatinum and doxorubicin treatment. The quiescent state, by definition, renders the stem-like cells less vulnerable for DNA damaging treatments. In addition, the ability to repair DNA is proposed to allow for new mutations in cells exposed to cytotoxic agents, and/or for the selection of therapy resistant cells that, after re-activation, possess a high clonogenic capacity and can develop into overt tumour recurrence in a later stage of the disease. Also, the expression of ABC transporters, particularly P-glycoprotein encoded by the multidrug-resistance (MDR) gene, is reported to provide a resistant phenotype in stem-like cancer cells [32]. The importance of P-glycoprotein is more elaborately discussed in the section on drug efflux.

In OS, little is known about the molecules that steer the survival and activation of OS cancer stem cells. It was reported that Transforming Growth Factor β1 (TGF-β1) is a controlling molecule that can promote self renewal capacity, proliferation and chemoresistance in OS cancer stem cells. Inhibition of TGF-β1 reduced tumorigenicity and increased chemosensitivity in cancer stem cell spheres [30]. Furthermore, it was reported that in stem-like cells, STAT3 is activated to maintain self renewal capacity and luripotency. This transcription factor is also implicated in apoptosis resistance, as will be discussed further below in the section on cell cycle [33]. Pathways involving IGF, MAPK/ERK, Wnt, Notch and JNK signalling have also been implicated in the maintenance of cancer stem cells. In addition, the tumour microenvironment, and low oxygen tension in particular, is proposed to influence the stem-like state of cancer cells. In rapidly growing tumours, areas of (relative) hypoxia arise and in these areas tumour cells display reduced cell metabolism and cell division rates, leading to a stem-like phenotype and causing diminished sensitivity of these cells to DNA damaging treatments. In addition, hypoxia was shown to be instrumental in the maintenance of pluripotency in stem cells and could thus also be important in the maintenance of cancer stem cells within an osteosarcoma [30]. Hypoxia is also reported to instigate inflammation-like conditions in the tumour microenvironment which are generally favourable to survival of (stem-like) cancer cells [35-37]. The interactions between tumour cells and the microenvironment will be discussed in the section on apoptotic signalling.

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(Pre)Clinical studies: stem-like cells

While it is noted that most conventional cytotoxic therapies do not kill stem-like cancer cells, studies exploring these cells as therapeutic targets are extremely rare. Perhaps the fact that cancer stem cells only comprise a few percent of all cells within the tumour precludes the efficacy of targeting these cells on beforehand. Nonetheless, if indeed these cells are responsible for the development and growth of malignancies, it might be paramount to identify crucial survival molecules in cancer stem cells and gain new insights in how to combat this very small, though very essential cell population within OS.

Dormancy

Dormancy can also be considered a non-proliferative state and refers to a prolonged period of survival of single cancer cells or micro-metastases and is typically encountered during the process of metastasis. Dormant tumour cells can go undetected for years or even decades prior to overt outgrowth. This phenomenon can explain why patients can relapse or show progressive metastatic disease after a (sometimes considerable) disease-free interval [27,38-40]. The molecules regulating dormancy in OS remain largely unknown, however, it is likely that the molecules that ensure survival in single cells (i.e., in the case of anchorage independent survival) provide a survival benefit that also increases resistance to cytotoxic stimuli. The tumour microenvironment is also thought to play a role in both dormancy and re-activation of dormant tumour cells via cell-cell interactions or chemokines present in the microenvironment [15]. The influence of receptors and chemokines on survival shall be further discussed in the section on apoptosis resistance. In order to survive in the absence of cell-cell and cell-matrix signalling, the single tumour cells must rely on altered intracellular signalling to prevent apoptosis, or more particularly anoikis to occur [41]. It has been shown that ainoikis resistant OS cells can intrinsically activate PI3K and/or Akt survival pathways; both pathways that can possibly also contribute to therapy resistance [26,42-44]. The anti-apoptotic gene Bcl-XL, that is reported to provide prolonged survival of dormant tumour cells, can possibly also convey insensitivity to chemotherapy in these cells [20,45]. Another molecule considered important in the survival of dormant cells is Integrin-α5β1 that acts via activation of the NF-κB pathway and via modulation of the ratio between ERK and p38-MAPK kinases, tilting the intracellular balance toward survival [26,31].

(Pre)Clinical studies: dormancy

The insensitivity of dormant tumour cells to cytotoxic therapy might not be an issue per se so long as the cells remain dormant, however, once the cells switch into the proliferative state, we are confronted with highly proliferative, therapy resistant cells. It could be argued that the cells should be kept dormant in order to address this issue optimally. In this light, some state that tumour dormancy could offer opportunities in anticancer treatment, however, the lack op appropriate models impairs the design of new strategies. Recently, *Almog* et al., [38] presented work on regulatory micro RNAs (miRs) that steer the switch between dormant cells and fast-growing cells of the 'angiogenic phenotype' in various cell types including OS. They show that, among others, overexpression of miR-190 could suppress the angiogenic phenotype and tumour growth in an *in vivo* model of OS and impose a dormant state of the tumour cells. Possible genes that play a role here are EphA5 and Angiomotin. Further insights in this switch from dormant to proliferative and efforts to interfere with this switch can offer promising opportunities for future treatments.

Cell cycle alterations

Alterations in cell cycle regulation are encountered in many types of cancer, including OS. Cell cycle alterations are excessively implicated in tumorigenesis, genomic instability and drug resistance [46-48]. Arrest in the cell cycle grants the cells extra time to repair their damaged DNA. Normally, re-entry in the cell cycle will only occur after DNA repair has been realised, but in selected cases cells are allowed to progress through the cell cycle without repair being fully completed. This attributes to genetically more instable clones which often have a more aggressive and invasive phenotype [21,47,49,50]. The cell cycle is tightly controlled and has three important checkpoints to maintain the genome. In general, these checkpoints are meant to prevent cells from replicating with damaged DNA, and in the case of DNA damage, to obstruct the cells from entering mitosis. (**Figure 1**) gives an overview of the cell cycle regulation, with key players involved in drug response.

Many malignant cells, including OS cells lack a functional p53 pathway. The rate of p53 alterations in patient tumour samples ranges between 22-45% [51-54]. In the absence of functional p53, the G1 cell cycle checkpoint is often surpassed and the other cell cycle checkpoints become of more importance to the tumour cell for DNA damage management and survival [14,46-48,50,55,56]. Both the S-phase and the G2/M-phase checkpoint have been described in drug response in OS and arrest of the cell cycle in G2/M-phase is commonly observed upon DNA damage in malignant cells of various types, including OS [47,48,57-60]. In the case of damage, DNA double strand breaks are detected by ATM, which relays a signal (mostly) to Chk2, which in turn phosphorylates Cdc25C. Cdc25C can phosphorylate the universal controller regarding the onset of mitosis, namely Cdc2. Inhibitory phosphorylation of Cdc2 inactivates the Cdc2/Cyclin-B complex, prevents the G2/M transition and consequently prevents the cell from entering mitosis, thus allowing time for DNA repair [47,48,55,56,61].

Many other molecules are involved in regulating the cell cycle at the various checkpoints. STAT3 is a transcription factor associated with resistance to chemotherapy-induced apoptosis in OS. While the exact mechanism of action remains unknown to day, there is an implication in cell cycle regulation at the

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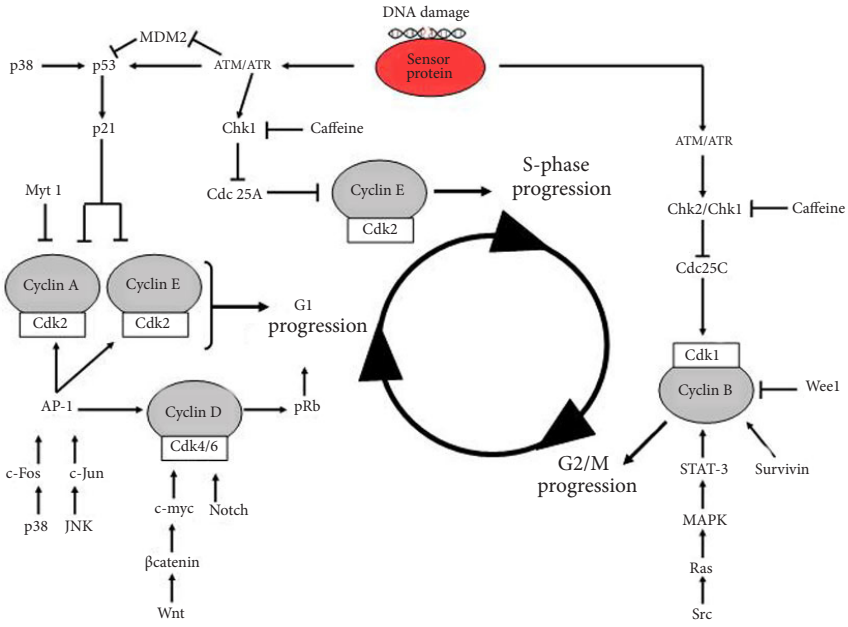

Figure 1. Representation of the cell cycle with key molecules involved in OS therapy resistance.

G2/M checkpoint and possibly in the activation of survival stimulatory genes [62,63].

Survivin is a protein known to have anti-apoptotic properties in OS, but it also has an effect on the cell cycle. It lies downstream in the NF-κB pathway and can regulate mitotic entry at the G2/M checkpoint [43,64]. Overexpression of Survivin allows for aberrant cell cycle progression of cells with minor DNA damage through the G2/M checkpoint to enter mitosis, resulting in cells with increased genomic instability and a more aggressive phenotype.

GADD45α is another, nuclear, protein known to interact with various cell cycle related proteins. Repression or inactivation of this protein by NF-κB can make tumour cells escape programmed cell death. Defects in the expression of GADD45α is associated with drug resistance. Furthermore, this molecule is also thought to interact with MAP3-kinase which can activate JNK and p38-MAPK signalling pathways, further stimulating cell survival [19,65,66].

(Pre)Clinical studies: Cell cycle alterations

Since cell cycle alterations can greatly benefit OS tumour cells in surviving cytotoxic treatments, molecules involved in cell cycle regulation represent promising therapeutic targets. As a result, a magnitude of research has been performed concerning this topic, ultimately to abolish these cell cycle alterations and either induce cell death directly or to sensitise OS cells to existing treatments.

Zoledronate has been shown to induce growth retardation and apoptosis by induction of arrest in the S- and G2/M-checkpoint. Upon treatment with Zoledronate, double strand DNA breaks occur, which activate the ATR/Chk1/Cdc25A cascade, leading to S-phase arrest, growth inhibition and possibly better tumour control [57,58].

CDKs are key regulators of the cell cycle, therefore they represent a potential target for therapy. Cdc2 (also known as Cdk1) is a Cyclin-Dependent Kinase that plays a pivotal role in the onset of mitosis or the induction of a G2/M arrest. Abrogation of its inhibitory phosphorylation can abrogate the G2 arrest, initiate an unwanted acceleration of the cell cycle and force DNA damaged cells into mitosis, ultimately leading to enhanced apoptotic cell death [48,56,59-61,67,68]. Another way to intervene in cell cycle regulation is to manipulate CDK-regulatory pathways, rather than targeting the CDKs directly. For example, inhibition of Chk1 with a small molecule inhibitor has been reported to sensitise cells to DNA damage by abrogation of the G2-checkpoint leading to premature mitotic entry and consequently to apoptosis [48,68]. Similarly, inhibition of Wee1 with a small molecule inhibitor can sensitise OS cells to irradiation-induced DNA damage by abrogation of the G2/M arrest and consequent cell death via mitotic catastrophe [60].

Flavopiridol, a pan-CDK-inhibitor, was shown to exert effects on cell cycle regulation and enhance chemotherapy-induced cell death *in vitro,* also in chemoresistant cell lines. As Flavopiridol inhibits multiple CDKs, it can influence both the G1-checkpoint (via Cdk2) and the G2-checkpoint (via Cdk1). It can induce sustained checkpoint activation, followed by apoptosis via the mitochondrial pathway. Additionally, it would reduce the transcription of NF-κB via inhibition Cdk7 and Cdk9 and thus initiate apoptosis [16,69].

Caffeine is a naturally occurring substance which influences the cell cycle at both the G1- and G2/M-checkpoint. Although

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contradictory reports exist about this compound, the general consensus is that additional treatment with caffeine can override a G2/M arrest after DNA damaging treatment, induce apoptosis and therefore enhance the cytotoxic effects of chemotherapy. The reported effect seemed to be most potent in cells lacking a functional p53. The mechanism behind this abrogation of the G2/M arrest is contributed to an inhibitory effect of caffeine on Chk1, resulting in Cdc25C activation, subsequent Cdc2/Cyclin-B activation and ultimately G2/M progression [55]. Preclinical studies with caffeine have shown a sensitisation to cisplatin in OS cell lines. Moreover, in a retrospective study, addition of caffeine to chemotherapy regimens in patients resulted in an increased efficacy of doxorubicin and cisplatin treatment. Patients with metastatic OS receiving the so called 'caffeine potentiated chemotherapy' had prolonged survival compared to those treated with chemotherapy alone. This finding supports the notion that cell cycle manipulation could enhance conventional chemotherapy. In the case of caffeine however, concern exists regarding the dose that needs to be administered, which is high and adverse effects may eventually limit the feasibility of this compound as an additive to chemotherapy [55,70].

Reduced drug accumulation

P-glycoprotein is a transmembrane surface protein encoded by the MultiDrug Resistance 1 (MDR1) gene, its name already highly suggestive of a role in drug response in OS [16,18,43,71]. P-glycoprotein is indeed involved in resistance to various drugs as it functions as an efflux pump of both hydrophilic and hydrophobic compounds. In the case of OS, P-glycoprotein is especially active as an efflux pump for doxorubicin, but also vincristine and vinblastine, granting lower levels of intracellular (cytotoxic) drug accumulation and thus resulting is less cellular damage. The level of P-glycoprotein expression is reported to be correlated to the degree of drug resistance in tumours and to poor survival outcomes in OS [14,18,71-74].

Caronia et al., [72], conducted a so-called pharmaco-genetic study evaluating drug response and survival outcomes for 102 OS patients in relation to a set of 24 candidate genes known to be involved in drug efflux. They showed that two members of the MDR family, namely ABCC3 and ABCB1 (encoding P-glycoprotein) were associated with a higher risk of death and inferior survival outcomes, further indicating the importance of P-glycoprotein in drug resistance in OS.

It has been proposed that the resistant phenotype of OS cells is not so much defined by the level of P-glycoprotein overexpression per se, but by its functional localisation on the cell membrane. Recently it was shown that Ezrin, a membrane-cytoskeleton linker protein that was previously reported to be instrumental to OS metastasis and survival of OS cells, [75,76] plays an essential role in P-glycoprotein function by maintenance of P-glycoprotein-actin connections, thereby securing its cell membrane localisation and functionality [73].

Contrarily to increased efflux, reduced drug accumulation can also be the result of reduced drug influx into tumour cells. In OS, the intracellular uptake of Methotrexate (MTX) is mediated by the Reduced Folate Carrier (RFC). It was shown that in resection samples from patients with a poor response to induction chemotherapy, RFC levels were significantly lower compared with patients with a good chemotherapy response, suggesting that low RFC expression may confer resistance to (high-dose) MTX, most probably by impaired drug influx into the cancer cells [14,74,77].

(Pre)Clinical studies: Reduced drug accumulation

It is highly likely that inhibition of P-glycoprotein would lead to sensitisation of OS cells to chemotherapy and therefore this could potentially represent a new strategy to overcome drug resistance in OS. It was shown that direct downregulation of P-glycoprotein expression using siRNA directed against ABCB1 leads to an increased sensitivity to doxorubicin and methotrexate in an OS cell line [78]. Apart from targeting of P-glycoprotein expression directly, interference with P-glycoprotein function also holds promise for reduction or reversion of the resistant phenotype. For example, *Brambilla* et al., [73] showed that stable expression of an Ezrin mutant in OS cells abrogated the co-localisation of Ezrin and P-glycoprotein at the plasma membrane, thereby hampering P-glycoprotein function and reinstating sensitivity to cytotoxic therapy. Thus, p-glycoprotein represents a putative target for sensitisation of OS to chemotherapy.

Increased detoxification

Some cytotoxic treatments result in the formation of free oxygen radicals, the presence of which is potentially lethal to cells. Glutathione S-transferase P1 (GSTP1) is an intracellular enzyme that catalyses Glutathione (anti-oxidant) detoxification. Increased expression of the GSTP1 gene as well as an increase in enzymatic activity of this protein is beneficial for tumour cells to survive chemotherapy, as the detoxification following oxidative stress occurs more rapidly. Both mechanisms have been shown relevant in therapy response in OS. GSTP1 is also assumed to regulate apoptotic signalling, namely to have an anti-apoptotic effect through interaction with c-Jun/JNK and regulation of p38-MAPK and Erk1/2. Activation of each of these signalling pathways leads to enhanced survival, which will be further discussed in the section on apoptosis resistance [17,65,79,80].

(Pre)Clinical studies: Increased detoxification

Targeting GSTP1 (and other glutathione-S transferase (GST) enzymes) could be a promising strategy to obtain sensitisation of OS to cytotoxic therapies. It was shown that resistant cell lines show a increase in the expression of one or more GST isoenzymes as the cell lines acquire resistance [79]. NBDHEX is a GST targeting compound that disrupts complex formation between GSTP1-1 and TRAF2, resulting in prolonged JNK activation and ultimately to the induction

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of apoptosis [80]. Administration of NBDHEX to OS cells primarily led to impaired proliferation via G2/M arrest and after 48h of treatment apoptosis could be observed [79,80]. In a murine *in vivo* model of OS lung metastasis, a daily dose of NBDHEX proved to reduce both the formation and size of lung nodules compared to control [79]. The effect of combination treatment of NBDHEX and conventional cytotoxic drugs such as doxorubicin, cisplatinum and methotrexate was also investigated. NBDHEX combined with doxorubicin or cisplatinum was shown to have an additive to synergistic effect on cell death, whereas in the case of methotrexate the opposite was observed and thus NBDHEX must be considered to have an antagonistic interaction with methotrexate. One possible explanation for this phenomenon would be that the NBDHEX-induced G2/M arrest slows down the cellular proliferation rate and thus renders the OS cells less sensitive to methotrexate induced DNA damage [79]. It is also speculated that the different response to NBDHEX to different cytotoxic drugs and across different cell lines could be attributed to the release of TRAF2 in the cytoplasm, leading to the activation of various MAPKs which in turn could mediate opposite effects on cell survival in a cell- and context-dependent fashion [80]. Taken together, targeting GST enzymes in OS may be a potential strategy to achieve increase therapy efficacy, either through direct effects of GST inhibition on cell viability, or via alterations in apoptotic signalling.

Increased DNA repair

Most conventional cancer treatments are designed to damage the DNA of cells [48,56]. Enhanced repair of damaged DNA is a very efficient manner in which tumour cells evade chemotherapy-induced apoptosis. To achieve successful DNA repair, the first step is to signal that DNA damage has occurred, upon which repair proteins and pathways are recruited. At the same time, it is necessary for the cell to create time for repair, which is mainly realised by an arrest of the cell cycle as discussed previously [81]. Histone γH2AX plays an important role in the detection and repair of DNA damage. After DNA damage is signalled, activated ATM and DNA-PK kinases phosphorylates histone γH2AX which in turn recruits DNA repair proteins [81].

After DNA damage has been detected, repair can occur through four basic mechanisms, namely direct reversal, base-excision-repair, nucleotide-excision-repair and mismatch repair. All of these mechanisms are likely to be present in OS.

Base-excision-repair (BER) as a means to repair DNA has been studied by *Wang* et al., [82]. In BER, damaged DNA fragments are excised from the DNA, followed by polymerase B repair DNA synthesis. Enhanced repair of DNA damage after cytotoxic treatment in OS has been reported via this mechanism by upregulation of the APE-1 enzyme, which is a key enzyme in the BER-pathway. The expression of the APE-1 enzyme in OS is speculated to be of predictive value in treatment response.

Nucleotide-excision-repair (NER) typically repairs bulky DNA damage and is believed to be of influence on the response to cisplatinum. Recently, the effect of single nucleotide polymorphisms (SNPs) in NER genes in patients with OS was studied. It was shown that excision SNPs in the repair cross-complementing group 2 (ERCC2/XPD) gene correlated significantly with improved event-free survival, and this effect was directly proportional to the number of variant alleles in the gene, i.e., patients with a homozygously mutated genotype for this particular gene had better event-free survival compared to patients with a heterozygous mutation. Contrarily, no correlations were observed between mutations in NER genes and overall survival or response to chemotherapy. Nonetheless it is speculated that patients with XPD mutations may have an increased therapeutic benefit from platinum-based chemotherapy [83].

(Pre)Clinical studies: Increased DNA repair

Studies investigating DNA repair mechanism as therapeutic targets are scarce. However, a few reports have been published. For example, it was shown that microRNA-138 targets γH2AX leading to reduced γH2AX expression levels, γH2AX foci formation and increased sensitivity to DNA damaging agents and irradiation. Furthermore, γH2AX downregulation using siRNA leads to deficiencies in homologous recombination. Combination treatment studies showed that both siRNA-mediated downregulation of γH2AX and γH2AX downregulation via overexpression of miR-138 lead to enhanced sensitivity to cisplatinum treatment. It is therefore speculated that miR-138 could represent a therapeutic target in OS [81].

Preclinical studies on an OS cell line showed that downregulation of APE-1 using siRNA lead to an increased sensitivity of OS cells to treatment with thiotepa, etoposide and ionising radiation, yielding higher levels of apoptosis compared to cells with functional APE-1 levels [82].

Apoptosis resistance

Apoptosis resistance is a major issue in anti-cancer treatment failure. It is believed that the efficacy of cytotoxic therapies is supported by the activation of apoptotic pathways. The failure to induce apoptosis upon treatment is thought to be the result of a misbalance between pro- and anti-apoptotic signalling, in which there is a preference for the expression of anti-apoptotic genes. Restoration of this balance, creating an environment favourable of pro-apoptotic signalling should enhance treatment with cytotoxic agents [19,20,43,45,64,84,85].

Apart from changes in gene-expression, apoptotic signalling can be influenced by several different mechanisms. For example, constitutively activated receptor tyrosine kinase pathways (i.e., IGF, Wnt, ERK, PI3K, mTOR) that play a role in the development of OS can induce enhanced survival after cytotoxic treatments through aberrant survival signalling. Also, there is increasing evidence that interaction between tumour cells and their micro-environment via specific cell surface receptors and

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chemokine signalling can lead to increased downstream survival signalling and thus confer resistance to therapy [14,24,25,30,64,86]. A different phenomenon observed in tumour cells (although more often in cells from an epithelial lineage) is the Epithelial-Mesenchymal-Transition (EMT), which is a type of de-differentiation of tumour cells associated with an increase in malignant behaviour, metastasis and apoptosis resistance [20,45,87-89]. Therefore, these pathways and interactions shall also be discussed in this section on pro- and anti-apoptotic signalling.

p53 is probably the best known regulator in DNA-damage management. Apart from regulation of the G1 cell cycle checkpoint, it can induce the expression of the pro-apoptotic proteins BAX, NOXA, PUMA and p53AIP1 and thus create a situation in favour of apoptosis [43,55]. Unfortunately, as stated in the above section on cell cycle, many OS lack a functional p53, which could impair apoptosis after cytotoxic therapy and confer therapy resistance.

The Fas receptor is a member of the Tumor Necrosis Factor (TNF) receptor superfamily and can induce receptor-mediated apoptosis. This extrinsic apoptotic pathway is activated by binding of Fas to its Fas-ligand (FasL), either on neighbouring cells, or by cross-linkage on one and the same cell. Fas/FasL interaction leads to activation of Fas receptor signalling which consecutively leads to cell death. The Fas death receptor pathway and aberrations in the cell surface expression of the Fas receptor on OS has been extensively studied, mainly in the context of OS metastasis. It has been observed that metastatic cell populations show a down-regulation of the Fas receptor on the cell surface. Consequently, Fas activation is impaired and Fas-induced apoptosis is reduced. It was shown that absence of Fas expression in lung metastases from OS patients correlated with disease progression and poor survivial outcome [90-93]. Furthermore, the Fas-pathway is also reported to be of influence on chemotherapy-induced apoptosis [84]. Given its role in apoptosis and the observed down-regulation in OS cells with increased malignant behaviour, the Fas receptor might represent a potential target for the sensitisation of OS to existing therapies.

The NF-κB pathway is also involved in regulation of apoptosis after chemotherapeutic treatment. Upon activation it activates multiple anti-apoptotic proteins such as Inhibitor of Apoptosis Proteins (IAPs) and Bcl-XL, thus putting more weight at the anti-apoptotic side of the balance and providing a survival benefit to the tumour cells. Survivin, which has been noted earlier, is a known anti-apoptotic protein that also belongs to the NF-κB pathway and influences drug response in OS [64,74].

JNK pathway activation is observed following cellular stress such as DNA damage, cytotoxic stress and γ-irradiation. However, the exact influence of JNK and it downstream molecules c-Jun and AP-1 on cell death is ambiguous and reported to be cell and context dependent. It seems that JNK exerts anti-apoptotic properties mainly in p53 deficient cells lines [65,94].

The Wnt/β-catenin pathway is implicated in the development and metastatic progression of OS [64,95,96]. Recently, it was reported that Wnt signalling is likely to influence doxorubicin response in OS cells by repression of syndecan-2 expression after the administration of doxorubicin. Syndecan-2 is a proteoglycan reported to influence both caspase-dependent and independent apoptosis and is upregulated by doxorubicin treatment to allow cell death. However, β-catenin activation was reported to impair syndecan-2 upregulation and therefore reduce sensitivity to doxorubicin in OS cells [24]. Syndecan-2 is reported to modulate ERK, PI3K/Akt and NF-κB pathways and thus influence cell survival after treatment with cytotoxic drugs. The proposed mechanism through which this occurs is that syndecan-2 inhibits endothelin-1-induced activation of ERK1/2 and PI3K/Akt which leads to reduced calpain-6 gene expression. Calpain-6 is implicated in positive stimulation of cell survival and proliferation in OS. Furthermore, the syndecan-2 mediated decrease in endothelin-1 signalling leads to reduced NF-κB activation and impaired survival signalling [25]. Thus, downregulation of syndecan-2 provides OS cells with a possible survival benefit and restoration or overexpression on syndecan-2 might be a method to sensitise OS cells to therapy.

Mammalian target of rapamycin (mTOR) signalling has been implicated in survival and proliferation of OS cells via the PI3K/Akt/mTOR pathway [15,18,85,86,97]. Very recently, a study was conducted to analyse the influence of mTOR on Sorafenib (a multi-kinase inhibitor) response in OS. It is observed that Sorafenib response is OS is commonly short-lived and that resistance to this treatment develops rapidly. mTOR can form two active protein complexes, mTORC1 and mTORC2, both of which can act as a component of PI3K/Akt signalling. It was shown that Sorafenib inhibits mTORC1, but contrarily activates mTORC2. It is noted that mTORC1 can activate AMP-activated protein kinase (AMPK) via ERK1/2 signalling and thus initiate apoptosis. Possibly, mTORC2 and/or the crosstalk between both complexes conveys resistance to Sorafenib and interfering with complex formation might overcome the resistance [86].

The tumour microenvironment is known to influence important features in OS development, metastasis and survival. It can stimulate tumour cell survival and therefore response to cytotoxic stress via cell-cell interactions, cell-extracellular matrix interactions and chemokine signalling. In OS, it is known that hypoxia can upregulate the expression of chemokine receptors CXCR-3 and -4. This can start a cascade in which the CXCR expression leads to increased expression of matrix-metalloproteinase-2 (MMP-2) and MMP-9 on the cell surface which can degrade the extracellular matrix, leading to the release of inflammatory cytokines such as interleukins, which, eventually can provide a survival benefit for the tumour cells [35-37]. Binding of CXCR-3 and -4 to their corresponding ligands (i.e., CXCL9/10/11 and CXCL12 respectively) has been shown to be instrumental in OS metastasis, providing not

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only adherence, but also survival and proliferation. Binding of CXCR-4 to its corresponding ligand, CXCL12 can activate the NF-κB pathway via ERK and further induce anti-apoptotic signalling in OS cells. Furthermore, it was found that CXCR-4 expression in patient samples inversely correlated with survival in these patients, suggesting that CXCR-4 is a relevant molecule for patients suffering OS [36,37,98,99]. Thus, CXCR/CXCL interactions in OS cells lead to apoptosis resistance and disruption of CXCR/CXCL interactions may be an opportunity to improve OS survival outcomes.

A different phenomenon that is speculated to confer an increased malignant phenotype and possibly resistance to therapy is the Epithelial-Mesenchymal-Transition (EMT). EMT is a type of de-differentiation that is accompanied by a change in expression of surface molecules, namely the cadherins, that define either an epithelial or mesenchymal phenotype. It has been broadly implicated in the process of metastasis. Loss of E-cadherin and/or switch to N-cadherin on the cell surface of cells characterises EMT, resulting in a more mesenchymal, primitive phenotype [20,45,87-89]. EMT can be induced by Transforming Growth Factor (TGF)-β signalling, but other pathways such as the canonical Wnt signalling pathway, Notch pathway and MAP signalling are also associated with this process. Via these pathways EMT not only provides motility to OS cells, but also apoptosis resistance [20,100,101]. At an intracellular level, cadherin switching can stimulate increased survival via β-catenin, a downstream molecule in the Wnt-signalling pathway. At the intracellular domain of E-cadherin, β-catenin is bound to E-cadherin via α-catenin. Consequently, loss of E-cadherin at the plasma membrane increases the cytoplasmic availability of β-catenin and thus induces survival [96,100,102]. Of note is that E-cadherin is typically expressed by epithelial cells rather than mesenchymal cells and EMT has been more extensively studied in epithelial than mesenchymal tumours. Its importance in mesenchymal tumours has not yet been fully elucidated. However, *Wheelock* et al., state that cadherin switching can also include situations in which E-cadherin expression levels do not alter significantly while there is a significant increase in N-cadherin expression [103]. This situation may be applicable to changes in cadherin expression in OS. The therapeutic opportunities to target or counter EMT in an attempt to sensitise OS cells to cytotoxic treatments most likely lie at the level of β-catenin and/or the Wnt pathway.

A less commonly described mechanism through which OS cells can exhibit chemotherapy resistance is autophagy. Autophagy is a cellular protection mechanism through which a cell can dispose of dysfunctional or damaged cellular components, and in doing this can prevent apoptotic cell death after cytotoxic stress. High Mobility Group Box 1 (HMGB1) protein is a bone active cytokine and, when overexpressed, associated with all hallmarks of cancer, including evasion of apoptosis. It is also reported to be a critical regulator of autophagy through its influence on the formation of Beclin1/PI3KC3 complexes and consequent vesicle formation. In OS cells, HMGB1 is reported to be overexpressed following treatment with doxorubicin, cisplatinum and methotrexate, leading to autophagy of damaged structures and thus creating a survival benefit for the treated cells [104].

(Pre)Clinical studies: Apoptosis resistance

(Re)activation of apoptotic signalling, or impairment of anti-apoptotic signalling could theoretically lead to an enhanced therapy efficacy in OS. This field is intensively studied and many publications describe research efforts to exploit apoptotic signalling pathways to gain sensitisation of OS to a variety of treatments.

As stated in previous sections, p53 is an important regulator of the G1 cell cycle checkpoint, but also an important modulator of pro-apoptotic signalling, for example via BAX, NOXA, PUMA and p53AIP1. In OS, inactivating p53 mutations are encountered, as well as increased expression of negative regulators of OS such as mouse double minute 2 (MDM2) protein [15,43,55,74,105,106]. MDM2 is a ligase that binds to p53 and subsequently promotes its degradation. Nutlin-3a (Nutlin in short) is a small molecule inhibitor of MDM2 by binding at the p53 binding-site thereby rendering MDM2 inactive through prevention of MDM2-p53 binding [105,106]. Previous work from our group demonstrated that treatment with Nutlin induced cell death in p53 wild-type OS cells. Combination treatment of Nutlin with an adenovirus introducing exogenous p53 further augmented tumour cell kill [105]. Thus, in principle, combining conventional therapies with Nutlin could potentially increase therapeutic efficacy. Recently, however, a report was published describing that treatment of cells with Nutlin could possibly lead to newly formed mutations in p53 which in turn may lead to selection for p53-mutated cells [106]. This unwanted side effect may eventually limit the applicability of Nutlin in clinical practice.

The Fas death receptor pathway is a promising pathway for therapeutic intervention. It was shown that OS cells with decreased Fas expression have a survival benefit. Restoration of the Fas death pathway has been tested in different preclinical models of OS and results are promising. Upregulation of Fas expression can be achieved by the cytokine Interleukin-12 (IL-12) and by Gemcitabine. Liposomal MTP-PE (muramyl tripeptide phosphatidyl ethanolamine) is a synthetic analogue of a component of bacterial cell walls that can induce endogenous IL-12 production and thus indirectly induce an upregulation of Fas. Additionally, MTP-PE was also reported to stimulate macrophages and monocytes to exert anti-tumour activity. Furthermore, IL-12 could stimulate cytotoxic T-cells and NK-cells to clear OS cells from the circulation of the host. Additionally, Ifosfamide, commonly used in the treatment of OS was reported to augment FasL expression on the surface of OS tumour cells [21,84,90,93]. In an *in vivo* model of OS, the intranasal delivery of IL-12 was shown to establish an overexpression of Fas death receptor in OS lung metastases,

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leading to a decrease in tumour burden as single agent treatment already. Combination therapy of intranasal IL-12 with Ifosfamide further increased the anti-tumour efficacy [84]. Despite these promising pre-clinical findings, it must be noted that a major drawback in the use of IL-12 is its potent immunostimulatory effect that could lead to serious side effects such as fever, chills, headache, myalgia, nephro- and hepatotoxicity, when delivered systemically [21,91].

The use of liposomal MTP-PE could provide a possible solution for this issue because it can stimulate endogenous IL-12 production without inducing the systemic toxicity encountered after administration of exogenous IL-12 [21]. Combination therapy of liposome encapsuled MTP-PE with conventional treatment agents has been proven to enhance therapy efficacy *in vitro*, *in vivo* and in trial setting [107-110]. The addition of liposomal MTP-PE to conventional treatment schedules has been shown to give improved overall survival in a phase III clinical trial in patients with high-grade conventional OS [97,111-113]. These results suggest that stimulating extrinsic apoptotic pathways can enhance the cytotoxic potential of chemotherapy and are beneficial for patients suffering from OS.

The role of and interfering with JNK-signalling after DNA damaging treatment in OS has also been studied [65,94]. Although JNK activation is reported to have both pro- and anti-apoptotic properties in a cell and context dependent manner, we recently showed that interference of JNK-signalling, either using RNAi techniques or by small-molecule inhibition renders OS cells more sensitive to doxorubicin therapy [94]. Literature on JNK modulation and chemotherapy response in OS remains scarce and further studies need to be conducted in order to appreciate the potential suitability of JNK and its pathway as a target for therapy in OS.

Dieudonné et al., [24] describe how the Wnt/β-catenin/T-Cell Factor (TCF) pathway impairs the apoptotic response to doxorubicin and that modulation of this pathway could increase doxorubicin sensitivity. They show that downregulation of TCF transcriptional activity using a small molecule inhibitor increased syndecan-2 levels and consequently increased doxorubicin sensitivity in both sensitive and resistant OS cell lines. Others confirm that re-establishment of syndecan-2 expression sensitises OS cells to drug induced apoptosis in OS cell lines and in a murine model for OS. RNAi mediated knockdown of calpain-6 is also shown to re-sensitise cells with a resistant phenotype. It is shown that syndecan-2 is crucial in the control of calpain-6 expression and that (syndecan-2 mediated) downregulation of calpain-6 leads to enhanced apoptosis after treatment with doxorubicin. Furthermore, cells that stably overexpressed syndecan-2 were less tumorigenic and showed less invasive growth compared to controls [25]. Thus, modulation of syndecan-2 expression, either via inhibition of TCF of calpain-6 may be a strategy for the sensitisation of OS to doxorubicin treatment.

mTOR is a molecule that can form two active complexes, mTORC1 and mTORC2 and is was shown that mTORC2 mediates resistance of OS to Sorafenib treatment. Combination therapy regimens of Sorafenib and the mTOR inhibitor Everolimus however, reversed the resistant phenotype in OS cells and resulted in increased apoptotic response to Sorafenib. In OS cell lines, combination therapy of Sorafenib and Everolimus showed a synergistic effect on apoptotic cell death. Furthermore, combination treatment resulted in decreased cell motility and reduced tumorigenicity and angiogenesis in an *in vivo* model for OS. This reversal is attributed to disassembly of the mTORC2 complex, possibly resulting in increased mTORC1 complex formation, activation of AMPK and apoptosis [86]. Apart from providing insights in mTOR signalling and its role in resistance, this study also provides proof that new combination of existing and registered drugs can lead to improved treatment efficacy in OS.

As described above, HMBG1 protein is involved in autophagy in response to cytotoxic treatments in *OS*. *Huang* et al., described that RNAi mediated knockdown of HMGB1 resulted in increased sensitivity of OS cells and an increase in apoptosis after cytotoxic treatment. Moreover, in an *in vivo* model, it was shown that HMGB1 knockdown tumours had inferior growth potential compared to controls, and, more importantly, showed increased therapy response compared to control tumours [104]. These data would lead to believe that if cells are not permitted to execute autophagy after cytotoxic stress, apoptosis is increased and thus targeting this mechanism could have potential to overcome therapy resistance in OS.

Conclusion

In conclusion, therapy resistance remains a major issue in the treatment of patients with OS. Conventional treatments have reached their limits to improve survival outcomes in OS due to the encountered therapy resistance. This review summarises mechanisms and molecules that may contribute to therapy resistance in OS. A multitude of research in this field has already been conducted to discover new therapeutic options to sensitise OS to existing treatments. We hypothesise that successful treatment of therapy resistant OS should target molecules or pathways that are crucial in survival after exposure to cytotoxic treatment, and be given as a complement to conventional treatment. Thus far, re-establishment or augmentation of apoptotic signalling upon cytotoxic treatment seems to be most successful in the sensitisation of OS. Additionally, the advent of small molecule inhibitors provides the possibility to target certain molecules or classes of molecules very specifically. Ultimately, the goal is to establish treatments with a higher specificity and lower toxicity for patients suffering from OS. Despite all the preclinical research efforts endeavoured, very few new agents have reached clinical use for OS patients. It seems that on the one hand, gaining a profound understanding of mechanisms that underlie and induce therapy resistance in OS should give us the opportunity to design targeted treatments for OS on a rational basis. On the other hand, to

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pursue promising sensitising treatments into clinical use might be even more crucial for the improvement of survival of OS patients. Maybe, centralised discussions to identify the most promising sensitising modalities and to reach consensus on strategy development may push forward a new era of treating OS.

List of abbreviations

ABC: ATP binding cassette (transporter)
AMPK: 5' AMP-activated protein kinase
AP-1: Transcription factor AP-1
APE-1: Apurinic-apyrimidinic endonuclease 1
ATM: Ataxia telangiectasia mutated
ATR: ATM and Rad3-related
BAX: Bcl2-like protein 4
Bcl-XL: Bcl2-like 1
BER: Base excision repair
CD: Cluster of differentiation/designation
Cdc: Cyclin dependent kinase
CDK: Cyclin dependent kinase
Chk: Checkpoint kinase
CXCL: Chemokine (C-X-C-motif) ligand
CXCR: Chemokine (C-X-C-motif) receptor
EMT: Epithelial-mesenchymal-transition
EphA5: Ephrin type-A receptor 5
ERK: Extracellular signal regulated kinase
FasL: Fas ligand
GADD45α: Growth arrest and DNA damage-inducible protein GADD45 alpha
GST(P1): Glutathione S-transferase (pi 1)
HMGB1: High mobility group protein B1
IAP: Inhibitor of apoptosis
IGF: Insulin-like growth factor
IL: Interleukin
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
MDM2: (Mouse) Double minute 2 protein
MDR: Multidrug resistance
miR: Micro RNA
MMP: Matrix-metalloproteinase
mTOR(C): Mammalian target of Rapamycin (complex)
MTP-PE: Muramyl tripeptide phophatidyl ethanolamine
NBDHEX: 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol
NER: Nucleotide excision repair
NF-κB: Nuclear factor-kappa B
NOXA: Phorbol-12-myristate-13-acetate-induced protein 1
OS: Osteosarcoma
p53AIP1: p53-regulated apoptosis-inducing protein 1
PI3K: Phosphatidyl inositol 3-kinase
PUMA: Bcl2-binding component 3
RFC: Reduced folate carrier
RNAi: RNA interference
siRNA: Small interfering RNA
SNP: Single nucleotide polymorphism
STAT3: Signal transducer and activator of transcription 3
TCF: T-cell factor
TGF-β1: Transforming growth factor
TNF: Tumor necrosis factor
TRAF2: TNF receptor-associated factor 2

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	JPDB	BJVR	MNH
Research concept and design	✓	--	--
Collection and/or assembly of data	✓	--	--
Data analysis and interpretation	✓	--	--
Writing the article	✓	--	--
Critical revision of the article	--	--	✓
Final approval of article	✓	✓	✓
Statistical analysis	--	--	--

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