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## **Doxorubicin-resistant osteosarcoma: novel therapeutic approaches in sight?**

High-grade osteosarcoma (HGOS) is the most common malignant tumour of bone. One of the most active drugs for HGOS treatment is doxorubicin, which is invariably included in HGOS chemotherapy protocols together with methotrexate and cisplatin, with the possible addition of ifosfamide. Several studies have shown that HGOS patients may be inherently resistant to doxorubicin or may become unresponsive to this drug during chemotherapeutic treatment [1, 2]. The most relevant mechanism of doxorubicin resistance in HGOS has been demonstrated to be the ATP-binding cassette (ABC) transporters-mediated drug efflux, with ABCB1 (P-glycoprotein, MDR1) playing a predominant role [2, 3]. Furthermore, ABCB1 overexpression has been reported independently by several authors to be associated with an adverse clinical outcome [1, 2]. Based on this evidence, in 2011, the evaluation of ABCB1 protein expression level at diagnosis has been introduced to tailor the treatment of HGOS patients according to their ABCB1 expression status (ClinicalTrials.gov Identifier: NCT01459484). However, in this trial, which is presently ongoing and recruiting patients, ABCB1 expression level is used to stratify HGOS patients but not as therapeutic target.

A possible strategy to overcome the clinical resistance against doxorubicin may be based on the use of ABC transporter inhibitors, with the aim to revert doxorubicin-resistant tumour cells toward a drug-sensitive phenotype. Although several ABC transporter inhibitors have entered phase I-II-III trials for different human tumours, their clinical use has invariably been limited by the severe toxicity exerted at the concentrations required to efficiently inhibit ABC transporter activity [4]. In the past ten years, in order to overcome this limitation, a new generation of ABC transporter inhibitors has been developed and characterised [4], showing promising preclinical results also in HGOS cells [3].

In the next years, this new generation of ABCB1 inhibitors might therefore provide novel agents that may be considered for doxorubicin-unresponsive HGOS patients.

An alternative strategy that has been suggested to overcome the ABCB1-mediated drug resistance may be based on the use of curcumin, a phenolic compound used in the traditional Indian medicine, which was shown to down-modulate the function of ABCB1 and to partially revert doxorubicin resistance in human HGOS cell lines [3]. Some years ago, curcumin was included in a phase I-II clinical trial for relapsed or metastatic HGOS patients (ClinicalTrials.gov Identifier: NCT00689195). The recruitment status of this trial is unfortunately unknown and no

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3 results have been posted yet. It is therefore impossible to understand whether this approach might  
4 be of help to overcome the ABCB1-mediated drug resistance in HGOS patients.

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6 Another additional possibility to interfere with ABCB1 activity in human tumour cells has  
7 been highlighted by recent studies on protein kinase inhibitor drugs. Some of them proved to  
8 downregulate ABCB1 activity and, therefore, act as chemoresistance revertants [5]. We have  
9 recently provided evidence that this situation also occurs in HGOS cells, and that the combined  
10 treatment of specific kinase inhibitor drugs with doxorubicin can overcome the ABCB1-mediated  
11 drug resistance in human HGOS cell lines [6, 7]. This may represent a real perspective of  
12 intervention to improve the treatment response and outcome of doxorubicin-resistant HGOS  
13 patients.

14  
15 Doxorubicin-unresponsive HGOS patients may be also treated with alternative,  
16 conventional chemotherapeutic drugs. There are, in fact, clinical studies demonstrating that HGOS  
17 patients may be efficiently treated with etoposide, methotrexate, and ifosfamide [8, 9].  
18 In a French randomized trial that enrolled 234 children/adolescents with localized HGOS, the  
19 efficacy of preoperative chemotherapy with high-dose methotrexate plus doxorubicin was  
20 compared to that of high-dose methotrexate plus etoposide and ifosfamide [8]. A good histological  
21 response (tumour necrosis greater than 95%) was achieved in 56% of the etoposide-ifosfamide  
22 arm patients versus 39% of the doxorubicin arm ( $P = 0.009$ ). Although no significant difference  
23 was found regarding five-year event-free and overall survival between the two protocol arms, this  
24 study showed that treatment with methotrexate, etoposide and ifosfamide is effective and can lead  
25 to survival rates similar to those achievable with standard regimens based on methotrexate and  
26 doxorubicin.

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28 The same group also performed the multicenter OS2006 phase III study, which enrolled 318  
29 HGOS patients and was primarily aimed to assess the efficacy of zoledronic acid [9]. Preoperative  
30 chemotherapy addressed to pediatric patients consisted in methotrexate, ifosfamide and etoposide,  
31 whereas adult patients received ifosfamide, doxorubicin and cisplatin. Patients with a good  
32 histological response received a postoperative treatment with the same drugs as in the preoperative  
33 phase. Poor responder pediatric patients were treated with a regimen in which ifosfamide and  
34 etoposide were replaced by cisplatin and doxorubicin, whereas poor responder adult patients  
35 received etoposide plus ifosfamide. Patients were randomly assigned to receive the  
36 aforementioned chemotherapy with or without ten zoledronate intravenous infusions.

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38 Unfortunately, the addition of zoledronic acid did not improve treatment efficacy and prognosis,  
39 but the observation that the rate of good histological response was higher in patients treated with  
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3 methotrexate, ifosfamide and etoposide compared to those who received ifosfamide, doxorubicin  
4 and cisplatin, confirmed the previous findings reported by the same group [8].

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6 Approaches based on the use of doxorubicin derivatives aiming to improve the local  
7 efficacy of the native drug, have also been considered to treat unresponsive, relapsed HGOS  
8 patients.

9  
10 Doxil (liposomal doxorubicin hydrochloride) is a liposomal encapsulated doxorubicin with a  
11 longer half-life in blood and an improved drug incorporation into tumour cells.

12  
13 Doxil was first used in a group of 47 sarcoma patients (including six HGOS) who were  
14 unresponsive to doxorubicin and standard chemotherapy [10]. Clinical benefit was observed in  
15 three of the six HGOS patients. All of them had previously received doxorubicin and ifosfamide,  
16 indicating that doxil may be considered for treating patients refractory to conventional doxorubicin  
17 [10].

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19 After this study, doxil has been considered for other clinical trials for patients with HGOS  
20 (ClinicalTrials.gov Identifiers: NCT00949325; NCT00019630; NCT02557854).

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22 The NCT00949325 phase I/II trial has been performed to test the ability of temsirolimus (CCI-  
23 779; an mTOR inhibitor) to potentiate the cytotoxic effect of liposomal doxorubicin [11]. This  
24 trial, which enrolled 15 bone and soft-tissue sarcoma patients (including one patient with HGOS),  
25 showed that the combination of liposomal doxorubicin with temsirolimus can be safely  
26 administered to heavily pretreated patients with recurrent or refractory bone and soft tissue  
27 sarcomas and that the toxicity of this combination was manageable and reversible [11]. The  
28 NCT00019630 phase I trial has been completed, but no study results have been posted yet.

29  
30 The NCT02557854 phase I trial is not open yet for recruitment. Its estimated primary completion  
31 date is December 2018.

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33 Although there is some evidence that doxil could be a possible alternative drug to native  
34 doxorubicin, it is still too early to define its actual value for treating doxorubicin-resistant HGOS  
35 patients.

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37 More recently, aldoxorubicin (formerly DOXO-EMCH, then renamed as INNO-206), an albumin-  
38 binding prodrug of doxorubicin with acid-sensitive properties, has emerged as an agent superior to  
39 native doxorubicin in several preclinical tumour models [12]. Phase I and II studies showed that  
40 aldoxorubicin was able to induce tumour regression in breast cancer, small cell lung cancer, and  
41 sarcomas [13]. Aldoxorubicin also appeared to be more effective than doxorubicin in advanced  
42 soft-tissue sarcoma patients [14], despite producing a higher incidence of grade 3-4 neutropenia  
43 [15]. In the past years, trials based on the use of aldoxorubicin alone or in combination with other  
44 chemotherapeutic drugs have been launched for treating patients with advanced solid tumours and  
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3 sarcomas. However, most of them have been either completed without providing results  
4 (ClinicalTrials.gov Identifiers: NCT01337505, NCT01673438) or are active but not recruiting  
5 patients (ClinicalTrials.gov Identifiers: NCT02049905, NCT02235688). At present, there is one  
6 phase I/II trial (ClinicalTrials.gov Identifier NCT02235701) that is recruiting participants. Its  
7 completion date is scheduled for December 2018. This trial is aimed to investigate the safety and  
8 activity of aldoxorubicin plus ifosfamide/mesna in patients with metastatic, locally advanced, or  
9 unresectable soft-tissue sarcomas and it may provide information which can be of interest also for  
10 HGOS. However, similarly to doxil, the actual clinical value of aldoxorubicin in HGOS still needs  
11 to be established.  
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18 Other chemically modified doxorubicins have recently been developed and proved to be  
19 effective against drug-resistant tumour cells overexpressing ABCB1 [16, 17]. A mitochondria-  
20 targeting doxorubicin recently proved to be more effective and less cardiotoxic than doxorubicin  
21 in murine osteosarcoma preclinical models and to overcome the ABCB1-mediated doxorubicin  
22 resistance in HGOS cell lines [16].

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28 Other compounds obtained by combining doxorubicin with H<sub>2</sub>S donors through an ester linkage  
29 at C-14 showed to be less cardiotoxic to in vitro cultured cardiomyocytes and more active than the  
30 native drug also in ABCB1-overexpressing, doxorubicin-resistant human HGOS cell lines [17].  
31 These findings indicate these doxorubicin derivatives as promising new chemotherapeutic drugs  
32 for a possible future clinical application in doxorubicin-unresponsive HGOS patients.  
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Very recently, the progress of nanomedicine has offered new hope for improving  
chemotherapeutic drug efficacy, as well as for overcoming chemotherapy resistance and reducing  
collateral toxicities. Nanomedicine techniques have also been applied to doxorubicin, in order to  
increase its in vivo stability and to control its intracellular drug release.

Although this approach has not specifically been studied in HGOS, findings provided in other  
human tumours have indicated that the nanodrug delivery system may be a promising selective  
modality to overcome the ABCB1-mediated doxorubicin resistance [18, 19], and to improve its  
treatment efficacy by simultaneously reducing the risk for cardiotoxicity [20].

As representative examples, we briefly mention here two doxorubicin nanoassemblies that have  
emerged as promising candidates for being transferred into clinical settings.

Using a so-called “squalenoylation” technology, doxorubicin can be chemically linked onto  
squalene, a natural lipid precursor of the cholesterol biosynthesis. The resulting squalenoyl  
doxorubicin proved to improve doxorubicin response of human tumour cells both in in vitro and in  
vivo experimental models, as well as to reduce the cardiotoxicity compared to the native  
doxorubicin [21].

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3 Of particular interest for a possible clinical application in the near future is the very recent  
4 development of an injectable nanoparticle generator (iNPG) which can be loaded with poly(l-  
5 glutamic acid)-conjugated doxorubicin (pDox) [22]. The resulting iNPG-pDox was able to  
6 overcome multiple biological barriers to cancer drug delivery and resistance. In fact, it has been  
7 shown that, after intravenous injection, iNPG-pDox accumulated at tumours and released pDox  
8 nanoparticles that were internalised by neoplastic cells. Intracellularly, pDox nanoparticles were  
9 transported to the perinuclear region and cleaved into doxorubicin, thereby avoiding excretion by  
10 drug efflux pumps like ABCB1 [22].

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16 Finally, novel indications for modulating HGOS patients' treatment may derive from  
17 pharmacogenomic studies. Several tumour (somatic) and normal cell (germline) markers have  
18 been suggested to modulate responsiveness to conventional chemotherapeutic drugs in HGOS.  
19 Although pharmacogenomic studies in HGOS are still at their beginning, some interesting  
20 evidence has already emerged (reviewed in: [23]).

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The role of somatic ABCB1 overexpression in HGOS cells for doxorubicin resistance is well  
understood, whereas there is less consensus regarding the impact of germline ABC transporter  
polymorphisms for patients with HGOS ([23] and references therein). Among the transporter  
genes involved in doxorubicin transport, ABCB1\_rs1128503 was reported to correlate variably  
with response to combined doxorubicin chemotherapy and survival in HGOS ([23] and references  
therein). ABCC2\_rs717620 was associated with poor histological response and a decreased risk  
for hematological or liver toxicity, and ABCC3\_rs939338 with worse outcome ([23] and  
references therein). Moreover, in a recent study, ABCC2\_rs2273697 was associated with poor  
survival and an increased risk for hematological toxicity [24]. However, it should be noted that in  
none of these studies toxicity was evaluated in relation to doxorubicin cycles alone. Therefore,  
these polymorphisms might be associated with the combined effect of all the drugs administered.

In one study, evaluating toxic events also in relation to doxorubicin alone, the variant  
GSTP1\_rs1695 was associated with increased risk of leukopenia and cardiotoxicity [25]. Other  
genes with reported clinical impact in HGOS patients in relation to doxorubicin belong mainly to  
several DNA repair pathways and drug metabolising enzymes ([23] and references therein).

Recent pharmacogenetic investigations have tried to identify genomic risk factors for  
anthracycline-induced cardiotoxicity in children ([26] and references therein). Although several  
germline variants have been reported in transporter, carbonyl reductase genes and the hyaluron  
synthase 3 gene, none of them can currently be used in clinical routine to tailor doxorubicin  
therapy. More studies are necessary to identify those genomic variants that are invariably



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3 associated with toxicity caused by doxorubicin treatment in HGOS patients, in order to suggest  
4 them for either modified doxorubicin or alternative drugs.

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6 Although almost all the described genomic variants need to be functionally characterised before  
7 their possible translation to the clinical setting, once validated, they will drive the transition to  
8 genetically guided decisions for personalised therapies. This approach, using the patients'  
9 pharmacogenomic background to optimise the treatment efficacy, is expected to overcome the  
10 present limitations of standard clinical procedures, which modulate drug dosage on the basis of  
11 patients' body surface area and age without considering the fact that patients inherently have  
12 different capabilities to metabolise and respond to chemotherapeutic agents.

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14 In conclusion, since new promising agents and therapeutic strategies, which may overcome  
15 resistance against doxorubicin in HGOS and other musculo-skeletal tumours, are currently under  
16 development and evaluation, it is possible to speculate that outcomes of these patients will  
17 reasonably improve in the near future.

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