Overcoming Glutathione *S*-Transferase P1–Related Cisplatin Resistance in Osteosarcoma

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Abstract

Cisplatin (*cis*-diamminedichloroplatinum, CDDP) is one of the most used drugs for osteosarcoma chemotherapy. By using a series of CDDP-resistant variants, which were established from the U-2OS and Saos-2 human osteosarcoma cell lines, we found that CDDP resistance was mainly associated with the increase of both the intracellular level and enzymatic activity of glutathione *S*-transferase P1 (GSTP1). On the basis of these findings, we evaluated the clinical effect of GSTP1 in a series of 34 high-grade osteosarcoma patients and we found that the increased expression of *GSTP1* gene was associated with a significantly higher relapse rate and a worse clinical outcome. These indications prompted us to assess the *in vitro* effectiveness of 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), a promising new anticancer agent that is a highly efficient inhibitor of GSTP1. NBDHEX was tested on a panel of 10 human osteosarcoma cell lines and 20 variants of the U-2OS or Saos-2 cell lines that were resistant to CDDP, doxorubicin, or methotrexate. NBDHEX proved to be very active on the vast majority of these cell lines, including those with higher GSTP1 levels and enzymatic activity. Drug combination studies showed that NBDHEX can be used in association with CDDP and provided useful information about the best modality of their combined administration. In conclusion, our findings show that GSTP1 has a relevant effect for both CDDP resistance and clinical outcome of high-grade osteosarcoma and that targeting GSTP1 with NBDHEX may be considered a promising new therapeutic possibility for osteosarcoma patients who fail to respond to conventional chemotherapy. [Cancer Res 2008;68(16):6661–8]

Introduction

Cisplatin (*cis*-diamminedichloroplatinum, CDDP) is a very active antitumor agent that shows a relevant clinical activity against a wide variety of human solid tumors. CDDP is widely used for high-grade osteosarcoma treatment, but despite this, only a small amount of information about the mechanisms that are responsible for resistance to this drug in human osteosarcoma cells has yet been reported (1–4).

CDDP produces DNA damages by inducing DNA interstrand or intrastrand cross-links, which finally drive cells to apoptosis (5). Several mechanisms can be responsible for CDDP resistance, including decreased drug accumulation, increased drug efflux, increased detoxification by glutathione or metallothionein systems, decreased DNA platination, or increased DNA repair (5, 6).

A key factor that severely limits the CDDP cytotoxic efficacy in several human tumors is the activity of cellular glutathione (GSH)-related detoxification system. In fact, once inside the cell, CDDP has to be activated through a series of spontaneous aquation reactions before being able to interact with DNA (6). The formation of aquated CDDP is rate limited by its interaction with many endogenous detoxification molecules, among which GSH is the most important and effective (5). When CDDP enters cells and is aquated, it becomes vulnerable to cytoplasmic inactivation by GSH. The reaction with GSH is catalyzed by the glutathione *S*-transferases (GST), a family of enzymes involved in xenobiotic detoxification, the dominant member of which is the GSTP1-1 isoenzyme (7, 8).

Differently from other human tumors, the relative effect of these mechanisms for the resistance to CDDP in human osteosarcoma cells still remains to be determined, mostly because of the rarity of adequate experimental models. In this study, we have investigated the bases of CDDP resistance in a series of CDDP-resistant human osteosarcoma cell lines. Moreover, because we have found that the acquisition of CDDP resistance in our experimental models mostly involved GSTP1-1 alterations, we evaluated the clinical effect of GSTP1-1 expression in a series of high-grade osteosarcoma patients, and we also assessed the *in vitro* effectiveness of 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX), a promising new anticancer agent that is a highly efficient inhibitor of GSTP1-1 and can accumulate in tumor cells by avoiding the extrusion mediated by multidrug resistance protein pumps (9–11).

Materials and Methods

Drugs. CDDP, doxorubicin (DX), and methotrexate (MTX) were purchased from Sigma-Aldrich. NBDHEX was synthetized as reported by Ricci and colleagues (9). Stock solutions of CDDP (500 μg/mL), DX (2 mg/mL), and MTX (25 mg/mL) were stored at 4°C. NBDHEX was dissolved in DMSO at a 50 mmol/L concentration and stock solution aliquots were stored in darkness at room temperature. Immediately before use, the integrity of NBDHEX molecule was verified by spectrophotometry. After dilution of NBDHEX stock solutions to the appropriate concentration required for the *in vitro* experiments, the final DMSO concentration never exceeded 0.01%, a dosage at which DMSO had no cytotoxic effect on our cell lines. For all drugs, working concentrations were prepared by diluting stock solutions in culture medium immediately before use.

Cell lines. CDDP-resistant variants were established by exposing the CDDP-sensitive U-2OS and Saos-2 human osteosarcoma cell lines [both purchased from the American Type Culture Collection (ATCC)] to step-by-step increases in CDDP concentrations. The *in vitro* continuous drug exposure resulted in the establishment of variants that were resistant to

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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300 ng/mL CDDP (U-2OS/CDDP300 and Saos-2/CDDP300), 1 μg/mL (U-2OS/CDDP1 μg and Saos-2/CDDP1 μg), 4 μg/mL (U-2OS/CDDP4 μg), or 6 μg/mL CDDP (Saos-2/CDDP6 μg). These drug concentrations corresponded to 1.0 μmol/L (300 ng/mL), 3.3 μmol/L (1 μg/mL), 13.3 μmol/L (4 μg/mL), and 20.0 μmol/L CDDP (6 μg/mL), respectively.

For the *in vitro* evaluation of NBDHEX effectiveness, in addition to U-2OS, Saos-2, and their CDDP-resistant variants, a group of other 8 human osteosarcoma cell lines and 14 drug-resistant variants were considered. Among these, the human osteosarcoma cell lines MG-63 and HOS were purchased from ATCC, whereas the IOR/OS9, IOR/OS10, IOR/OS14, IOR/OS15, IOR/OS18, and SARG cell lines were established from clinical specimens obtained from osteosarcoma patients at the Laboratorio di Ricerca Oncologica of the Istituti Ortopedici Rizzoli (12). The six DX-resistant and eight MTX-resistant variants included in this panel were selected from the U-2OS and Saos-2 cell lines, as previously described (13, 14). All cell lines were cultured in Iscove's modified Dulbecco's medium, supplemented with penicillin (100 units/mL)/streptomycin (100 μg/mL; Invitrogen Ltd.) and 10% heat-inactivated fetal bovine serum (BioWhittaker Europe). Each drug-resistant variant was continuously cultured in the presence of the drug concentration used for its selection. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

***In vitro* growth characteristics and drug sensitivity analyses.** The *in vitro* growth characteristics (doubling time and saturation density) of each cell line were determined as previously described (14, 15). Drug sensitivities of each cell line were calculated from the drug dose-response curves obtained by using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche Diagnostics GmbH) and expressed as IC_{50} (drug concentration resulting in 50% inhibition of cell growth after 96 hours of *in vitro* treatment).

Evaluation of drug-drug interactions. To evaluate the *in vitro* interactions between NBDHEX and CDDP, human osteosarcoma cell lines were incubated with different regimens of two-drug combinations. In the experiments of simultaneous exposure, cell lines were treated with combinations in which the two drugs were used at equitoxic concentrations, corresponding to dosages resulting in 10%, 20%, 30%, 50%, and 60% growth inhibitions (IC_{10}, IC_{20}, IC_{30}, IC_{50}, and IC_{60}) after 96 hours of single-drug treatment. In the drug sequence experiments, cell lines were sequentially exposed for 72 hours to equitoxic concentrations (IC_{10}, IC_{20}, IC_{30}, IC_{50}, and IC_{60} after 72 hours of single-drug treatment) of NBDHEX and then, after its removal, to the same equitoxic concentrations of CDDP for additional 72 hours. The same schedule was repeated by exposing cells to CDDP followed by NBDHEX. To define the type of interaction between NBDHEX and CDDP (in terms of synergism, additivity, or antagonism), the combination index of each two-drug treatment was calculated with the isobologram equation (16) by using the CalcuSyn software (Biosoft). By following the range of combination index values reported in the CalcuSyn software manual and recommeded by Chou and colleagues (16), we classified the drug-drug interaction as synergistic when combination index < 0.90, as additive when $0.90 \leq$ combination index ≤ 1.10, or as antagonistic when combination index > 1.10.

Assessment of drug-induced apoptosis and DNA fragmentation. Drug-induced apoptosis was estimated on the basis of morphologic assessment of apoptotic nuclei after nuclear staining with Hoechst 33258 (Sigma-Aldrich), as previously described (15). For DNA fragmentation analysis, DNA of CDDP-untreated and treated cells was isolated by using the Apoptotic DNA Ladder Kit (Roche) by following the manufacturer's procedure, and DNA samples were electrophoresed through 1.0% agarose gel in the presence of ethidium bromide to reveal the DNA smears under UV light.

Intracellular uptake of CDDP. The intracellular uptake of CDDP was evaluated and quantified on both parental cell lines and CDDP-resistant variants with the method reported by Molenaar and colleagues (17), which is based on the use of a carboxyfluorescein diacetate–conjugated CDDP (CFDA-Pt). The quantification of CFDA-Pt fluorescence intensity was done by image cytometry with a fluorescence microscope equipped with a Photometrics Sensys charge-coupled device camera (QUIPS XL Genetic Workstations, Abbott-Vysis, Inc.).

GSH and GSTP1-1 assays. Intracellular GSH concentration was estimated with the Bioxytech GSH-400 kit (OxisResearch) by following the manufacturer's procedure. The total protein concentration of each sample was determined with the bicinchoninic acid protein assay reagent (Sigma-Aldrich) and GSH content was referred to as picomoles per milligram of total proteins.

The GSTP1-specific activity was measured according to the method of Habig and colleagues (18) by using ethacrynic acid, a GSTP1 class–specific substrate (19). The reaction mixture (final volume of 0.2 mL) used for these assays contained 100 mmol/L potassium phosphate buffer (pH 6.5), 1 mmol/L EDTA (pH 6.5), 0.25 mmol/L glutathione, 0.2 mmol/L ethacrynic acid, and 200 μg of cellular proteins. The amount of ethacrynate-GSH conjugates was measured by spectrophotometry with the Nanodrop ND-1000 (Nanodrop Technologies, Inc.) at 270 nm. GSTP1 activity was defined as the amount of enzyme that was able to catalyze the conjugation of 1 nmol of ethacrynic acid with GSH per minute at 25°C, and it was referred to as the protein content of each sample. As for GSH assays, the protein concentration of each sample was determined with the bicinchoninic acid protein assay reagent (Sigma-Aldrich).

π-class GST, μ-class GST, and α-class GST protein levels were estimated by Western blot with the following primary antibodies: NCL-GSTpi (specific for π-class GST; Novocastra Lab), anti-GSTM1 (specific for μ-class GST), or anti-GSTA1 (specific for α-class GST; both from Novus Biologicals). All primary antibodies were diluted at 1:500 in TTBS/1% (w/v) nonfat milk. To verify the protein loading of each sample, after stripping of the primary antibody, the same membranes were immunostained with an anti–β-actin monoclonal antibody (Chemicon International). For each band, the amount of GST proteins was determined by densitometric analysis and normalized to that of β-actin.

The expression level of the *GSTP1* gene was analyzed by real-time PCR in parental cell lines and CDDP-resistant variants and in 34 tumor biopsies obtained from primary, nonmetastatic, high-grade osteosarcoma patients. All these patients were treated with neoadjuvant chemotherapy protocols based on the administration of DX, MTX, CDDP, and ifosfamide. Before RNA extraction, all clinical samples were histologically examined for tissue quality and representativity. Total RNA was extracted from both cell lines and snap-frozen tumor tissue samples by using the standard method with the TRIzol reagent (Invitrogen). For each sample, 500 ng of total RNA were reverse transcribed to cDNA in a 50 μL-reaction mixture using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR for *GSTP1* gene was done by using the TaqMan Low Density Arrays (Applied Biosystems) with predesigned TaqMan probe and primer sets. Samples were analyzed with the ABI Prism 7900HT system with a TaqMan LDA Upgrade (Applied Biosystems) according to the manufacturer's instructions. The expression level of the *GSTP1* gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the relative quantification analysis was based on the $\Delta\Delta C_t$ method. A pooled cDNA from human normal osteoblasts was used as calibrator for the comparative analysis. Clinical cases were stratified into high-expressing and low-expressing *GSTP1* by using the median *GSTP1* expression value as cutoff.

Statistics. Differences among means were analyzed with the Student's *t* test. Two-tailed Fisher's exact test was used to evaluate the statistical association between two variables. Kaplan-Meier and log-rank methods were used to draw and evaluate the significance of survival curves.

Results

Selection and characterization of CDDP-resistant variants. CDDP-resistant variants of U-2OS and Saos-2 human osteosarcoma cell lines were obtained by initially exposing parental cell lines to 300 ng/mL (1.0 μmol/L) CDDP until they achieved a growth rate similar to that of untreated cells. Selection was then continued by step-by-step increases in CDDP concentrations up to 4 μg/mL (13.3 μmol/L) for U-2OS variants or up to 6 μg/mL (20.0 μmol/L) for Saos-2 variants. Establishment of adequate *in vitro* growth at

each new CDDP concentration required ~10 to 12 weeks (corresponding to 8–10 *in vitro* passages). All the experiments were done on CDDP-resistant variants that were maintained in culture for at least 6 months (30–35 *in vitro* passages) after establishment.

The CDDP IC_{50} values and the fold increase of resistance in the CDDP-resistant variants are listed in Table 1. In particular, the increase in CDDP resistance compared with the parental cell line ranged from 4.0- to 62.5-fold for U-2OS variants and from 7.4- to 112.1-fold for Saos-2 variants. Overall, CDDP-resistant variants did not show cross-resistance to DX or MTX, with the sole exception being U-2OS/CDDP1 μg, which was less sensitive to MTX, and U-2OS/CDDP4 μg, which showed a reduced sensitivity to DX and MTX (Table 1).

The doubling time of all CDDP-resistant variants did not significantly differ from those of their corresponding parental cell lines, whereas saturation density significantly decreased in U-2OS/CDDP1 μg, U-2OS/CDDP4 μg, and in Saos-2/CDDP6 μg variants (Supplementary Table S1).

To evaluate the sensitivity to CDDP-induced apoptosis, parental cell lines and CDDP-resistant variants were treated with equitoxic dosages of CDDP, corresponding to the IC_{50}, 5-fold IC_{50}, or 10-fold IC_{50} concentration of each cell line. No significant difference was found between CDDP-resistant variants and parental cell lines, although variants seemed to be slightly more sensitive to CDDP-induced apoptosis (Supplementary Fig. S1). In agreement with the apoptosis data, DNA fragmentation induced by treatment of cells with the same CDDP concentrations did not show any significant difference between parental cell lines and CDDP-resistant variants, all of these showing a similar extent of DNA fragmentation in CDDP-treated cells (data not shown).

Analysis of intracellular CDDP uptake showed that both parental cell lines and CDDP-resistant variants incorporated CDDP in almost all the cells. No difference was found about the intracellular CDDP distribution because all cell lines presented both cytoplasmic and nuclear uptake of CDDP and similar intracellular amounts of drug. Efflux cytometric analyses showed that both parental cell lines and CDDP-resistant variants were able to completely extrude CDDP from inside the cell with the same efficiency within 24 hours after drug removal (data not shown). Taken together, these data indicated that, in our experimental model, CDDP intracellular accumulation and distribution changes were not involved in development of CDDP resistance.

GSH and GSTP1-1 status in cell lines. Evaluation of the intracellular GSH concentration did not reveal any remarkable difference between CDDP-resistant variants and parental cell lines, although the two variants with the highest levels of CDDP resistance showed a trend toward an increase in GSH levels (Supplementary Table S2). The intracellular GSH levels of CDDP-resistant variants and parental cell lines were also inside the range of GSH content found in the other human drug-sensitive and drug-resistant osteosarcoma cell lines, which were included in the cell line panel used for this study (Supplementary Table S2).

Analyses of *GSTP1* gene expression and GST protein levels and enzymatic activity were done in all drug-sensitive and drug-resistant cell lines (data are summarized in Table 2). To compare the GST isoenzyme status in the group of drug-sensitive, human osteosarcoma cell lines, the U-2OS cell line was used as reference. GSTP1 status varied among these cell lines, but in all of them there was quite a good correlation between gene expression level, protein expression, and enzymatic activity. In fact, the level of GSTP1-specific enzymatic activity (assessed by using the ethacrynic acid as substrate) proceeded parallel to the extent of the *GSTP1* gene expression, the GSTP1-1 protein level, or both of them in the vast majority of cell lines (Table 2). In the group of drug-resistant variants, the following additional considerations must be done. Taking into account the parental cell line, the GSTP1-specific enzymatic activity progressively increased in all CDDP-resistant variants. However, whereas in U-2OS/CDDP-resistant variants the higher GSTP1-1 enzymatic activity was associated with an increase in both GSTP1-1 gene expression and protein level, in Saos-2/CDDP-resistant variants the increased GSTP1-1 enzymatic activity was not related to changes in gene expression and protein level (Table 2). An increased GSTP1-specific enzymatic activity of at least 2-fold compared with parental cells was also found in the four variants with the highest level of resistance to DX, in association with higher GSTP1-1 gene and protein expression (Table 2). Similar evidence was found for U-2OS/MTX30, U-2OS/MTX100, and U-2OS/MTX300, in which the increased GSTP1-specific enzymatic activity was associated with higher protein levels and enhanced

Table 1. CDDP-resistant variants of the human osteosarcoma U-2OS and Saos-2 cell lines and their corresponding drug sensitivities

Cell line	Cisplatin		Doxorubicin		Methotrexate	
	IC_{50} (μmol/L)	IC_{50} ratio to parental cell line	IC_{50} (μmol/L)	IC_{50} ratio to parental cell line	IC_{50} (μmol/L)	IC_{50} ratio to parental cell line
U-2OS	0.47 ± 0.11		0.03 ± 0.01		0.03 ± 0.01	
U-2OS/CDDP300	1.89 ± 0.17	4.0	0.02 ± 0.01	0.9	0.10 ± 0.02	3.3
U-2OS/CDDP1 μg	8.50 ± 0.19	18.1	0.04 ± 0.02	1.7	0.16 ± 0.05	5.7
U-2OS/CDDP4 μg	29.37 ± 3.23	62.5	0.24 ± 0.05	8.0	0.45 ± 0.10	15.0
Saos-2	0.42 ± 0.06		0.09 ± 0.01		0.08 ± 0.01	
Saos-2/CDDP300	3.09 ± 0.71	7.4	0.16 ± 0.04	1.8	0.14 ± 0.05	1.8
Saos-2/CDDP1 μg	6.42 ± 0.41	15.3	0.17 ± 0.09	2.0	0.15 ± 0.01	1.9
Saos-2/CDDP6 μg	47.10 ± 4.41	112.1	0.26 ± 0.10	2.9	0.17 ± 0.05	2.2

NOTE: IC_{50} values were determined by MTT assay and data refer to the mean values of three independent experiments (± SD).

Table 2. GST status in the panel of 10 human osteosarcoma cell lines and 20 drug-resistant variants of the U-2OS and Saos-2 cell lines considered in this study

	GSTP1 gene expression	GSTπ protein level	GSTμ protein level	GSTP1 enzymatic activity assessed with ethacrynic acid
Human osteosarcoma cell lines (fold change compared with U-2OS)				
U-2OS	1.0	1.0	1.0	1.0
Saos-2	0.2	1.8	2.7	1.8
MG-63	0.2	2.4	4.4	2.0
HOS	1.7	3.3	2.3	2.0
IOR/OS9	1.8	2.3	1.7	2.0
IOR/OS10	3.1	1.8	2.2	2.0
IOR/OS14	0.2	0.2	1.1	0.3
IOR/OS15	0.8	1.8	5.0	2.3
IOR/OS18	1.8	1.4	3.8	1.8
SARG	2.0	2.8	1.2	3.1
Drug-resistant variants (fold change compared with parental cell line)				
U-2OS/CDDP300	2.1	1.9	1.1	2.0
U-2OS/CDDP1 μg	2.2	2.2	1.2	3.3
U-2OS/CDDP4 μg	2.5	2.7	3.9	5.8
Saos-2/CDDP300	0.8	0.7	0.9	1.3
Saos-2/CDDP1 μg	0.9	0.8	0.9	1.4
Saos-2/CDDP6 μg	1.0	0.9	0.9	3.4
U-2OS/DX30	1.3	3.1	0.7	1.3
U-2OS/DX100	1.5	3.2	1.9	3.0
U-2OS/DX580	2.3	3.4	1.9	3.0
Saos-2/DX30	1.6	0.7	0.5	1.9
Saos-2/DX100	1.8	0.9	0.7	2.0
Saos-2/DX580	2.1	1.6	1.2	2.1
U-2OS/MTX3	0.4	2.0	1.2	1.8
U-2OS/MTX30	0.8	2.4	0.9	2.0
U-2OS/MTX100	1.8	2.5	1.9	2.0
U-2OS/MTX300	2.6	2.7	1.7	2.0
Saos-2/MTX30	1.3	0.7	0.9	1.1
Saos-2/MTX100	1.5	0.7	1.1	1.1
Saos-2/MTX300	2.4	1.3	1.3	1.6
Saos-2/MTX1 μg	2.6	1.6	1.4	1.7

gene expression, with the only exception being U-2OS/MTX30 (Table 2). As a whole, these findings indicated that, in the osteosarcoma cell lines included in this panel, enhanced GSTP1-specific enzymatic activity was very often associated with enhanced *GSTP1* gene expression, increased GSTP1-1 protein level, or both of them, as reported for many other human tumor cell lines (7).

Clinical effect of GSTP1 expression level. The clinical effect of *GSTP1* expression was analyzed in a series of 34 clinical samples taken from patients with primary, nonmetastatic osteosarcoma, whose clinicopathologic characteristics reflected those of conventional high-grade osteosarcoma (Supplementary Table S3). *GSTP1* gene expression level was assessed and quantified by real-time PCR and patients were classified into two subgroups by using as cutoff value the median *GSTP1* gene expression level of the whole series. The range of *GSTP1* expression level in clinical samples overlapped that of our drug-sensitive, human osteosarcoma cell lines (data not shown). A high expression of *GSTP1* at diagnosis was found in 17 of 34 (50%) patients. No relationship was found between *GSTP1* gene expression level (high or low) and clinicopathologic variables (gender, age, histologic subtype, type of surgery, surgical margins, and tumor necrosis; Supplementary Table S3).

With regard to clinical outcome, 16 (47%) patients relapsed and 18 (53%) patients did not show any evidence of disease. Relapse was significantly associated with *GSTP1* gene status, occurring in 12 of 17 (71%) patients with high *GSTP1* gene expression and in 4 of 17 (24%) patients with low *GSTP1* gene expression (P = 0.015, two-tailed Fisher's exact test). According to this evidence, event-free survival analysis showed a statistically significant worse outcome for patients with high *GSTP1* gene expression (Fig. 1).

NBDHEX *in vitro* activity and interactions with conventional drugs. Both parental cell lines and CDDP-resistant variants proved to be highly sensitive to NBDHEX, with a slight decrease of drug sensitivity in variants with the highest levels of CDDP resistance (Table 3). Parental cell lines and CDDP-resistant variants were also sensitive to the apoptotic effects of NBDHEX. In fact, at equitoxic concentrations, NBDHEX was able to induce apoptosis to a similar extent in both parental cell lines and CDDP-resistant variants (Supplementary Fig. S2).

The analysis of the *in vitro* activity of NBDHEX was also extended to the whole panel of our drug-sensitive and drug-resistant human osteosarcoma cell lines. All cell lines turned out to be highly sensitive to NBDHEX, showing low and rather homogeneous IC_{50} values, with no evidence of significant cross-resistance with conventional drugs (Table 3). Only the U-2OS/DX580 variant showed a 4.7-fold increased NBDHEX IC_{50} value compared with

parental cells, whose value was similar to those of U-2OS/CDDP1 μg or U-2OS/CDDP4 μg cell lines.

Because NBDHEX targets GSTP1-1, we have analyzed the relationship between NBDHEX activity and GSTP1-1 status inside each cell line. Globally, our data showed a trend toward a reduced *in vitro* activity of NBDHEX in cell lines with higher GSTP1-specific enzymatic activity, as assessed by using ethacrynic acid as a substrate (Fig. 2). In particular, CDDP-resistant variants showed progressively increasing NBDHEX IC_{50} values according to their level of GSTP1-specific enzymatic activity. The same tendency was also evident, even if at a much lower extent, in U-2OS/DX– and U-2OS/MTX–resistant variants. In drug-sensitive cell lines, the situation was more heterogeneous, with all these cell lines being highly sensitive to NBDHEX, with the sole exception of SARG. Most of them, however, had GSTP1-1 activities similar to that of the U-2OS/CDDP300 variant.

The *in vitro* efficacy of NBDHEX in combination with CDDP was evaluated after either simultaneous or sequential two-drug exposure in both parental cell lines and CDDP-resistant variants. As shown in Table 4, simultaneous exposure of all cell lines to NBDHEX and CDDP produced mostly additive interactions, with a synergistic effect in U-2OS/CDDP4 μg variant. Analyses of sequential drug exposure revealed mostly additive effects (Table 4). However, when cells were treated with NBDHEX as the first drug followed immediately by CDDP, synergistic effects, instead of additive ones, were observed on the variants with higher levels of CDDP resistance.

Discussion

CDDP is one of the most effective antitumor agents currently used for the chemotherapeutic treatment of several human neoplasms and it is almost invariably included in chemotherapy regimens for high-grade osteosarcoma, together with DX, high-dose MTX, and ifosfamide (20, 21). Although high-grade osteosarcoma can be considered as a CDDP-responsive tumor, it may present an inherent or acquired resistance to this drug, which severely limits its clinical efficacy (20).

CDDP resistance is multifactorial, with several different mechanisms that can simultaneously be involved. Understanding these mechanisms is critical for elucidating the bases of the CDDP-resistant phenotype inside each tumor type. To do this, however, adequate experimental models are essential. Studies done on these models may also have great relevance for better clarifying the bases of clinical unresposiveness to CDDP because there is evidence for a general agreement between experimental findings and CDDP resistance mechanisms that are clinically encountered (22).

Despite its high importance for high-grade osteosarcoma treatment, only scarce and partially discordant information about the CDDP resistance mechanisms that are specifically developed by human osteosarcoma cells has been reported thus far. In the study of Asada and colleagues (1), CDDP resistance acquired by one human osteosarcoma cell line seemed to be related to both reduced drug accumulation and increased drug efflux. Komiya and colleagues (2) reported an inverse correlation between the intrinsic GSH content and CDDP sensitivity of eight human osteosarcoma cell lines. In the studies of Perego and colleagues (3) and Martelli and colleagues (4), which were both done on the U-2OS cell line and its 6-fold resistant variant U2-OS/Pt, resistance to CDDP

Table 3. *In vitro* effectiveness of NBDHEX assessed by MTT assay on the panel of human osteosarcoma cell lines considered in this study

Cell line	NBDHEX IC_{50} (μmol/L)	IC_{50} ratio compared with parental cell line
U-2OS	0.67 ± 0.04	
Saos-2	1.00 ± 0.10	
MG-63	0.11 ± 0.05	
HOS	0.08 ± 0.02	
IOR/OS9	0.21 ± 0.02	
IOR/OS10	0.08 ± 0.01	
IOR/OS14	0.27 ± 0.01	
IOR/OS15	0.28 ± 0.08	
IOR/OS18	0.18 ± 0.04	
SARG	1.98 ± 0.37	
CDDP-resistant variants		
U-2OS/CDDP300	1.39 ± 0.12	2.1
U-2OS/CDDP1 μg	2.76 ± 0.30	4.1
U-2OS/CDDP4 μg	3.88 ± 1.31	5.8
Saos-2/CDDP300	1.02 ± 0.01	1.0
Saos-2/CDDP1 μg	1.56 ± 0.63	1.6
Saos-2/CDDP6 μg	2.58 ± 1.23	2.6
DX-resistant variants		
U-2OS/DX30	0.35 ± 0.15	0.5
U-2OS/DX100	0.60 ± 0.14	0.9
U-2OS/DX580	3.16 ± 0.46	4.7
Saos-2/DX30	0.57 ± 0.17	0.6
Saos-2/DX100	0.99 ± 0.32	1.5
Saos-2/DX580	1.13 ± 0.29	1.7
MTX-resistant variants		
U-2OS/MTX3	0.35 ± 0.05	0.5
U-2OS/MTX30	0.57 ± 0.13	0.9
U-2OS/MTX100	0.62 ± 0.21	0.9
U-2OS/MTX300	1.05 ± 0.24	1.6
Saos-2/MTX30	0.27 ± 0.08	0.3
Saos-2/MTX100	0.30 ± 0.08	0.3
Saos-2/MTX300	0.35 ± 0.10	0.4
Saos-2/MTX1 μg	0.85 ± 0.27	0.9

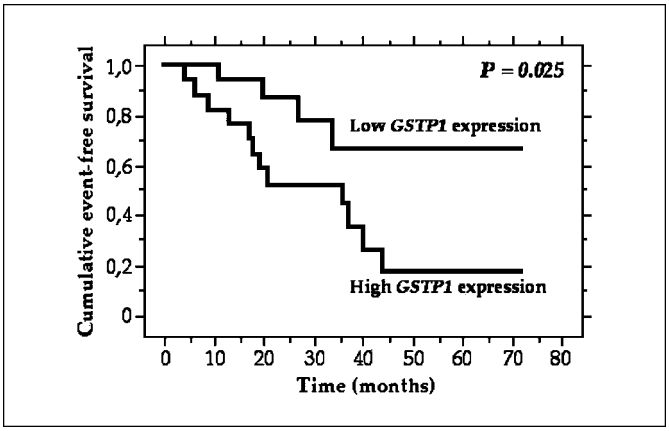

Figure 1. Event-free survival curves in 34 high-grade osteosarcoma patients stratified according to the *GSTP1* gene expression level at diagnosis. Comparison of survival curves was done with the log-rank test.

proved to be associated with a reduced susceptibility to drug-induced apoptosis, decreased drug accumulation and DNA damage, defects in mismatch repair, and a marginal increase in GSH content. Therefore, by looking to all these results, it is obvious that no conclusive evidence is available about the bases of CDDP resistance in human osteosarcoma cells. Further studies are still needed to clarify this phenomenon.

In the present study, we have described the establishment and characterization of a panel of CDDP-resistant variants derived from the U-2OS and Saos-2 human osteosarcoma cell lines, which were used to define the effect in osteosarcoma cells of the most relevant mechanisms reported to be responsible for CDDP resistance in other human solid tumors. Our experimental model differs from those mentioned above because it includes a panel of six

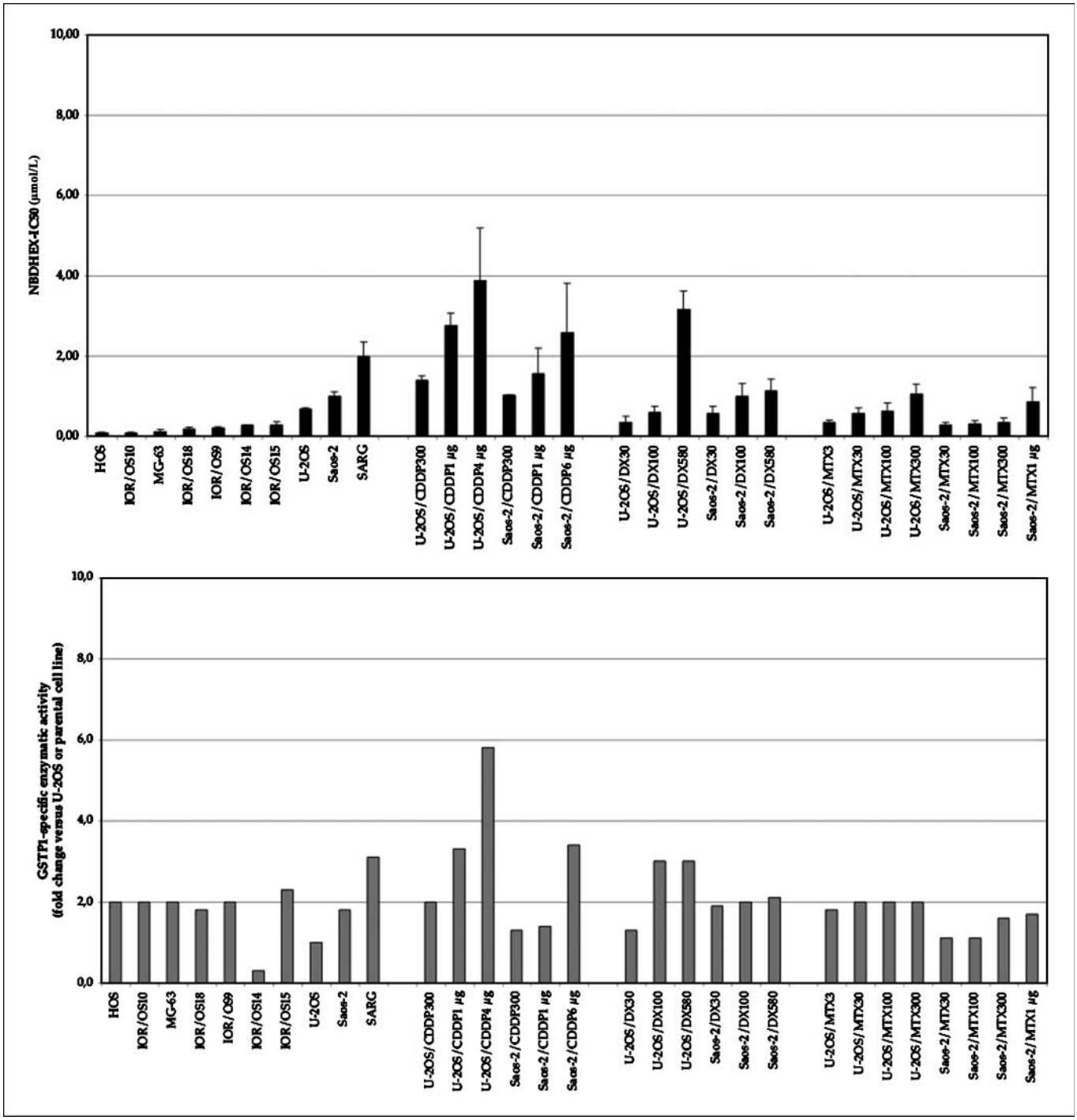

Figure 2. Comparison between NBDHEX *in vitro* efficacy estimated on the basis of IC_{50} values (*top*) and GSTP1-specific enzymatic activity (*bottom*) in the 10 drug-sensitive and 20 drug-resistant human osteosarcoma cell lines included in this study. Inside each group, cell lines were listed according to an increasing order of NBDHEX IC_{50} value.

Table 4. Effects of the *in vitro* simultaneous or sequential administration of NBDHEX and cisplatin (CDDP)

Cell line	Simultaneous drug administration	Sequential drug administration	
	NBDHEX + CDDP (CI)	NBDHEX→CDDP (CI)	CDDP→NBDHEX (CI)
U-2OS	ADD (0.97)	ADD (0.91)	ADD (0.97)
Saos-2	ADD (1.09)	ADD (0.90)	ADD (0.92)
U-2OS/CDDP300	ADD (1.04)	ADD (0.90)	ADD (0.90)
U-2OS/CDDP1 μg	ADD (1.09)	SYN (0.82)	ADD (0.90)
U-2OS/CDDP4 μg	SYN (0.51)	SYN (0.86)	ADD (0.91)
Saos-2/CDDP300	ADD (0.92)	ADD (0.90)	ADD (0.91)
Saos-2/CDDP1 μg	ADD (1.09)	SYN (0.81)	ADD (0.90)
Saos-2/CDDP6 μg	ADD (0.93)	SYN (0.86)	ADD (0.90)

Abbreviations: CI, combination index; ADD, additive ($0.90 \leq CI \leq 1.10$); SYN, synergistic ($CI < 0.90$).

CDDP-resistant variants that were derived from two different parental cell lines and with increasing levels of resistance. From our point of view, this fact can lead to a better delineation of the mechanisms that are most relevant for both the acquisition and the degree of CDDP resistance in osteosarcoma cells.

In our experimental models, the degree of CDDP resistance seemed to be mainly associated with the increase of both intracellular levels and enzymatic activity of GSTP1-1. A possible, less relevant contribution to CDDP resistance of these cells may also derive from the increase of μ-class GST, which, however, did not seem to be as relevant as that of GSTP1-1. In fact, higher levels of μ-class GST proteins were detected only in the U-2OS/CDDP4 μg variant.

Other common mechanisms of CDDP resistance, including reduced susceptibility to drug-induced apoptosis, decreased drug accumulation or increased drug efflux, and decreased sensibility to drug-induced DNA fragmentation, did not turn out to play a significant role in the acquisition of CDDP resistance of our resistant variants. The same evidence was found for GSH intracellular content, although the two variants with the highest levels of CDDP resistance showed 1.4- to 1.8-fold increase in GSH levels. This may suggest a possible involvement of this mechanism in the acquisition of CDDP resistance of our cell lines, something which will be more specifically analyzed in further studies.

Our findings are in line with a large part of evidence showing that GSTP1-1 plays a central role in the degree of CDDP unresponsiveness in several human tumor cell lines (5, 7, 23). In particular, in osteosarcoma, it has recently been reported that induction of GSTP1-1 overexpression in Saos-2 cells is associated with the acquisition of resistance to CDDP and DX, whereas GSTP1-1 silencing in HOS cells is responsible for an increased chemosensitivity to these two drugs (24). In agreement with this evidence, we have observed a low-level cross-resistance to DX and MTX in the cisplatin-resistant cell lines with the highest levels of GSTP1-1–specific activity (i.e., U-2OS/CDDP1 μg and U-2OS/CDDP4 μg). These findings may be explained by the fact that the activity of drugs that interact with DNA (e.g., DX) or interfere with DNA synthesis and cell cycle (e.g., MTX) may be partly mediated by c-jun NH_2-terminal kinase (JNK) activation (25). Therefore, the collateral low-level resistance against DX and MTX exhibited by these two cell lines may be explained by taking into account the antiapoptotic effect of GSTP1-1 through its interaction with JNK; this assumption, however, needs further experimental confirmation. The cross-resistance to DX may also be explained by taking into account the reported evidence of a possible role of GSTP1 in the formation of DX-GSH adducts, which can be recognized by ABC transporters and extruded from the cells (26).

The GSTP1-1 increase has also been reported to significantly contribute to clinical CDDP resistance in different human tumors (5, 23). Among these, in head and neck squamos cell carcinoma cell lines and primary tumors, amplification and overexpression of *GSTP1* have been shown to be related to CDDP resistance and poor clinical outcome of patients treated with CDDP-based therapies (27). In gliomas, the high expression of *GSTP1* has been shown to be associated with a more aggressive clinical course and to have a strong adverse prognostic value for patient survival (28).

The few data reported thus far for osteosarcoma have suggested that overexpression of *GSTP1* after preoperative chemotherapy seems to be related to drug treatment unresponsiveness of high-grade osteosarcoma patients (29). In agreement with these observations, we also found that, in high-grade osteosarcoma patients, high expression of the *GSTP1* gene at diagnosis was significantly associated with a higher relapse rate and a worse event-free survival. Unfortunately, due to the low amount of tumor material, we could not quantify either the GSTP1-1 protein level or the GSTP1-1 enzymatic activity in our clinical samples. It is worth noting that the range of *GSTP1* expression level of our drug-sensitive osteosarcoma cell lines overlapped the one found in clinical samples, further underlining the adequacy of our experimental models. Therefore, on the basis of the evidence derived from our cell lines, in which the *GSTP1* gene expression and protein levels were frequently related to those of GSTP1-specific enzymatic activity, it might be speculated that osteosarcoma patients with high *GSTP1* gene expression at diagnosis may have a worse treatment response due to increased GSTP1 activity–related mechanisms. Considering all these findings together, GSTP1-1 emerged as a potential drug target, which may have potential for consideration to overcome CDDP resistance and improve the clinical outcome of high-grade osteosarcoma patients.

To circumvent CDDP resistance, the modification of platinum-based compounds has been proposed as a promising approach for the development of non–cross-resistant CDDP analogs for clinical use (5, 22). Unfortunately, several studies have shown that the

cellular and molecular mechanisms responsible for the resistance to these CDDP analogs are either very similar or even identical to those to CDDP, a fact that severely limits their clinical usefulness (5). This situation has been slightly improved with the development of triplatinum complexes, some of which have been reported to be able to overcome CDDP resistance in experimental models (3). However, there is still a need for new anticancer agents that are able to circumvent CDDP resistance.

In our search for drugs targeting GSTs, we focused on the new anticancer agent NBDHEX, which has been proved to predominantly target GSTP1-1, to be very active in several human tumor cell lines, and to not be extruded from tumor cells by multidrug resistance protein pumps (9–11). Moreover, the low concentrations that are necessary to exert cytotoxic effects on human tumor cells, together with the low toxicity exhibited in mice, have identified NBDHEX as a promising new drug of potential clinical use (10). For these reasons, NBDHEX seems to be a very interesting agent for testing in our experimental models.

The results obtained in this study show that NBDHEX is very active on human osteosarcoma cell lines and that it also has a relevant efficacy on drug-resistant variants with increased GSTP1-1 protein levels and enzymatic activities. Drug combination studies further suggest that NBDHEX is a promising new drug for osteosarcoma treatment, showing that it can be used together with CDDP. The modality of administration, however, must be carefully defined on the basis of the evidence provided by this study.

In conclusion, we have shown that GSTP1-1 is significantly involved in CDDP resistance of human osteosarcoma cells and that it has a relevant effect on the clinical outcome of high-grade osteosarcoma patients. Moreover, our findings about the *in vitro* efficacy of NBDHEX indicate that targeting GSTP1-1 with this drug opens up a promising new therapeutic possibility for high-grade osteosarcoma patients unresponsive to conventional chemotherapeutic regimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Asada N, Tsuchiya H, Ueda Y, Tomita K. Establishment and characterization of an acquired cisplatin-resistant subline in a human osteosarcoma cell line. Anticancer Res 1998;18:1765–8.
2. Komiya S, Gebhardt MC, Mangham DC, Inoue A. Role of glutathione in cisplatin resistance in osteosarcoma cell lines. J Orthop Res 1998;16:15–22.
3. Perego P, Caserini C, Gatti L, et al. A novel trinuclear platinum complex overcomes cisplatin resistance in an osteosarcoma cell system. Mol Pharmacol 1999;55:528–34.
4. Martelli L, Di Mario F, Botti P, Ragazzi E, Martelli M, Kelland L. Accumulation, platinum-DNA adduct formation and cytotoxicity of cisplatin, oxaliplatin and satraplatin in sensitive and resistant human osteosarcoma cell lines, characterized by p53 wild-type status. Biochem Pharmacol 2007;74:20–7.
5. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
6. Kelland LR. Preclinical perspectives on platinum resistance. Drugs 2000;59:1–8.
7. Tew KD, Monks A, Barone L, et al. Glutathione-associated enzymes in the human cell lines of the National Cancer Institute Drug Screening Program. Mol Pharmacol 1996;50:149–59.
8. Goto S, Iida T, Cho S, Oka M, Kohno S, Kondo T. Overexpression of glutathione *S*-transferase π enhances the adduct formation of cisplatin with glutathione in human cancer cells. Free Radic Res 1999;31:549–58.
9. Ricci G, De Maria F, Antonini G, et al. 7-Nitro-2,1,3-benzoxadiazole derivatives, a new class of suicide inhibitors for glutathione *S*-transferases. Mechanism of action of potential anticancer drugs. J Biol Chem 2005;280:26397–405.
10. Turella P, Cerella C, Filomeni G, et al. Proapoptotic activity of new glutathione *S*-transferase inhibitors. Cancer Res 2005;65:3751–61.
11. Turella P, Filomeni G, Dupuis ML, et al. A strong glutathione *S*-transferase inhibitor overcomes the P-glycoprotein-mediated resistance in tumor cells. 6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol (NBDHEX) triggers a caspase-dependent apoptosis in MDR1-expressing leukemia cells. J Biol Chem 2006;281:23725–32.
12. Benini S, Baldini N, Manara MC, et al. Redundancy of autocrine loops in human osteosarcoma cells. Int J Cancer 1999;80:581–8.
13. Serra M, Scotlandi K, Manara MC, et al. Establishment and characterization of multidrug-resistant human osteosarcoma cell lines. Anticancer Res 1993;13:323–9.
14. Serra M, Reverter-Branchat G, Maurici D, et al. Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells. Ann Oncol 2004;15:151–60.
15. Pasello M, Hattinger CM, Stoico G, et al. 4-Demethoxy-3′-deamino-3′-aziridinyl-4′-methylsulphonyl-daunorubicin (PNU-159548): a promising new candidate for chemotherapeutic treatment of osteosarcoma patients. Eur J Cancer 2005;41:2184–95.
16. Chou TC, Motzer RJ, Tong Y, Bosl GJ. Computerized quantitation of synergism and antagonism of Taxol, topotecan and cisplatin against teratocarcinoma cell growth: a rational approach to clinical protocol design. J Natl Cancer Inst 1994;86:1517–24.
17. Molenaar C, Teuben J-M, Heetebrij RJ, Tanke HJ, Reedijk J. New insights in the cellular processing of platinum antitumor compounds, using fluorophore-labeled platinum complexes and digital fluorescence microscopy. J Biol Inorg Chem 2000;5:655–65.
18. Habig WH, Pabst MJ, Jakoby WB. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–9.
19. Mannervik B, Alin P, Guthenberg C, et al. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc Natl Acad Sci U S A 1985;82:7202–6.
20. Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther 2006;6:1075–85.
21. Ferrari S, Palmerini E. Adjuvant and neoadjuvant combination chemotherapy for osteogenic osteosarcoma. Curr Opin Oncol 2007;19:341–6.
22. Giaccone G. Clinical perspectives on platinum resistance. Drugs 2000;59:9–17.
23. Townsend DM, Tew KD. The role of glutathione-*S*-transferase in anti-cancer drug resistance. Oncogene 2003;22:7369–75.
24. Huang G, Mills L, Worth LL. Expression of human glutathione *S*-transferase P1 mediates the chemosensitivity of osteosarcoma cells. Mol Cancer Ther 2007;6:1610–9.
25. MacCorkle RA, TanTH. Mitogen-activated protein kinases in cell cycle control. Cell Biochem Biophys 2005;43:451–61.
26. Goto S, Ihara Y, Urata Y, et al. Doxorubicin-induced DNA intercalation and scavenging by nuclear glutathione *S*-transferase π. FASEB J 2001;15:2702–14.
27. Cullen KJ, Newkirk KA, Schumaker LM, Aldosari N, Rone JD, Haddad BR. Glutathione *S*-transferase amplification is associated with cisplatin resistance in head and neck squamos cell carcinoma cell lines and primary tumors. Cancer Res 2003;63:8097–102.
28. Ali-Osman F, Brunner JM, Kutluk TM, Hess K. Prognostic significance of glutathione *S*-transferase π expression and subcellular localization in human gliomas. Clin Cancer Res 1997;3:2253–61.
29. Uozaki H, Horiuchi H, Ishida T, Iijima T, Imamura T, Machinami R. Overexpression of resistance-related proteins (metallothioneins, glutathione-*S*-transferase pi, heat shock protein 27, and lung resistance-related protein) in osteosarcoma. Relationship with poor prognosis. Cancer 1997;12:2336–44.