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Prevention of multidrug resistance (MDR) in osteosarcoma by NSC23925

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Background: The major limitation to the success of chemotherapy in osteosarcoma is the development of multidrug resistance (MDR). Preventing the emergence of MDR during chemotherapy treatment has been a high priority of clinical and investigational oncology, but it remains an elusive goal. The NSC23925 has recently been identified as a novel and potent MDR reversal agent. However, whether NSC23925 can prevent the development of MDR in cancer is unknown. Therefore, this study aims to evaluate the effects of NSC23925 on prevention of the development of MDR in osteosarcoma.

Methods: Human osteosarcoma cell lines U-2OS and Saos were exposed to increasing concentrations of paclitaxel alone or in combination with NSC23925 for 6 months. Cell sublines selected at different time points were evaluated for their drug sensitivity, drug transporter P-glycoprotein (Pgp) expression and activity.

Results: We observed that tumour cells selected with increasing concentrations of paclitaxel alone developed MDR with resistance to paclitaxel and other Pgp substrates, whereas cells cultured with paclitaxel–NSC23925 did not develop MDR and cells remained sensitive to chemotherapeutic agents. Paclitaxel-resistant cells showed high expression and activity of the Pgp, whereas paclitaxel–NSC23925-treated cells did not express Pgp. No changes in IC_{50} and Pgp expression and activity were observed in cells grown with the NSC23925 alone.

Conclusions: Our findings suggest that NSC23925 may prevent the development of MDR by specifically preventing the overexpression of Pgp. Given the significant incidence of MDR in osteosarcoma and the lack of effective agents for prevention of MDR, NSC23925 and derivatives hold the potential to improve the outcome of cancer patients with poor prognosis due to drug resistance.

Osteosarcoma is one of the most common and aggressive primary malignant bone tumours in childhood and adolescence. Current standard treatment of osteosarcoma involves surgical resection and combination chemotherapy (Marina *et al*, 2004; Chou *et al*, 2008). Although this therapy helps tumour cytoreduction and remission rate, the long-term survival rate remains at ∼60–70% (Marina *et al*, 2004; Sakamoto and Iwamoto, 2008). Osteosarcoma is still characterised by frequent relapse and metastatic disease that is associated with the development of multidrug resistance (MDR) to chemotherapy in late stages (Chou and Gorlick, 2006; Dieudonne *et al*, 2010). Tumour cells that are initially sensitive to

chemotherapeutic drugs often develop resistance to those drugs as well as to other functionally and structurally unrelated anticancer agents later, thus limiting the overall utility of chemotherapeutic drugs (Agarwal and Kaye, 2003; Chou and Gorlick, 2006). The development of MDR is a major obstacle to cancer chemotherapy and is a significant challenge to successful cancer treatment (Gillet and Gottesman, 2010). Several mechanisms have been shown to potentiate osteosarcoma to become resistant to chemotherapeutic agents (Chou and Gorlick, 2006). One of the best characterised MDR mechanisms is overexpression of ATP-binding cassette (ABC) drug transporters P-glycoprotein protein (Pgp) that causes

decreased drug uptake and increased drug efflux in MDR cells (Gottesman *et al*, 2002; Ozben, 2006; Higgins, 2007). P-glycoprotein protein is the protein product of multidrug resistance gene 1 (MDR1), and acts as an energy-dependent drug efflux pump requiring two ATPs as the energy source to pump out many structurally unrelated chemotherapeutic drugs (Gottesman *et al*, 2002). Overexpression of Pgp has been found in multiple MDR osteosarcoma cell lines and osteosarcoma drug resistance tissues (Bodey *et al*, 1995; Suto *et al*, 1998; Chano *et al*, 2004; Okada *et al*, 2006; Susa *et al*, 2010). Strategies of reversing and preventing MDR by targeting Pgp have been studied extensively in different MDR model systems including osteosarcoma but with limited success (Decaudin *et al*, 2002; Ryu *et al*, 2010; Kobayashi *et al*, 2012).

Most of the previous investigations have been focussed on the reversal rather than prevention of Pgp-mediated MDR. Four generations of Pgp inhibitors have been developed (Shukla *et al*, 2011), including verapamil, PSC833 (valspodar), VX-710 (biricodar), XR9576 (tariquidar), R101933 (laniquidar) and a natural product, curcumin (Lin *et al*, 2007; Gupta *et al*, 2012). However, preventing the development of MDR during the course of chemotherapy and exploring the underlying molecular machinery rather than reversing MDR once it has arisen may fundamentally aid in the design of strategies for overcoming MDR in cancer. In recent years, several studies have investigated the prevention of initiation of MDR in cancer after chemotherapy drug treatment (Batrakova *et al*, 2006; Tooker *et al*, 2007; Sharma *et al*, 2008). A number of drugs, such as verapamil, cyclosporin A (CsA), VX710, XR9576, P85 (Pluronic 85) and LGD1069 (bexarotene) have been tested as the preventers that avert the development of MDR (Cocker *et al*, 2001; Yen *et al*, 2004; Yen and Lamph, 2006). Several clinical trials have been performed using these preventers in different types of cancer patients (Baer *et al*, 2002; Minderman *et al*, 2004; Kelly *et al*, 2011). However, significant effects on prevention of MDR for most of these preventers have not been established, and notable survival benefits have not been shown in clinical trials (Gandhi *et al*, 2007; Lhomme *et al*, 2008; Kolitz *et al*, 2010; O'Brien *et al*, 2010). Therefore, development of more potent and selective MDR preventers is needed.

The NSC23925 (2-(4-methoxyphenyl)-4-quinolinyl) (2-piperidinyl) methanol) is a small molecular compound that was identified as an effective and selective inhibitor of Pgp in previous studies. It was demonstrated that NSC23925 has the ability to reverse Pgp-mediated MDR in ovarian cancer by stimulating Pgp ATPase activity *in vitro*. The NSC23925 was 20-fold more potent than verapamil and 50-fold more potent than CsA at the same concentrations to reverse drug resistance (Duan *et al*, 2009a). Importantly, the combination of paclitaxel and NSC23925 produced a significant inhibitory effect on the growth of xenograft ovarian cancer MDR tumour models as compared with animals treated with paclitaxel alone (Duan *et al*, 2012). However, whether NSC23925 is able to prevent the development of MDR in cancer is unknown. Therefore, in this study, we evaluated the effects of NSC23925 on the prevention of the development of MDR in the osteosarcoma cell lines U-2OS and Saos.

MATERIALS AND METHODS

Cell lines, drugs and antibodies. Human osteosarcoma cell lines U-2OS and Saos were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Both of the cell lines were routinely cultured in RPMI-1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies) in 5% CO_2–95% air atmosphere at $37\,^\circ C$.

Prior analyses have demonstrated that there are two chiral centres in the structure of NSC23925 that lead to four different isomers. Isomer 11 of NSC23925 is the most potent one for the reversal of MDR (Duan *et al*, 2012). Therefore, isomer 11 was used in this study. Isomer 11 of NSC23925 (abbreviated as NSC23925) was synthesised by ChemPartner Co. (Chengdu, China) and has been validated to reverse MDR (Duan *et al*, 2012). Paclitaxel, docetaxel, doxorubicin, vincristine, gemcitabine, cisplatin and methotrexate were obtained from the pharmacy at the Massachusetts General Hospital. PM-00104 was supplied by PharmaMar (Madrid, Spain). The mouse monoclonal antibodies to human Pgp and MRP1 were purchased from Sigma-Aldrich (St Louis, MO, USA). The mouse monoclonal antibody to human BCRP was acquired from EMD Millipore (Bedford, MA, USA).

Development of resistant osteosarcoma cell lines. To evaluate the effect of NSC23925 on preventing the development of paclitaxel resistance, 1×10^5 U-2OS and Saos cells were seeded in $75\,cm^2$ tissue culture flasks containing RPMI-1640 with 10% FBS and 1% penicillin/streptomycin. Establishment of the resistant osteosarcoma cells lines followed similar previously described protocols (Cocker *et al*, 2001; Sharma *et al*, 2008). The culture medium was supplemented with either $0.0001\,\mu M$ paclitaxel alone, the combination of $0.0001\,\mu M$ paclitaxel and $1\,\mu M$ NSC23925 or $1\,\mu M$ NSC23925 alone. When growth of the cells reached 90% confluence, the cells were harvested and reseeded at the density of 1×10^5 cells/flask, and the concentration of paclitaxel was increased. At different selection points, cell sublines were stored in liquid nitrogen for further studies. The MDR cell line U-2OSR2, which was previously established from U-2OS, was used as a control (Duan *et al*, 2009b).

Cytotoxicity assay. Cytotoxicity of anticancer drugs in selected cell sublines was assessed by MTT assay as previously described (Duan *et al*, 2012). Briefly, cytotoxicity of anticancer drugs in selected cell sublines was assessed by MTT assay. Cells were seeded in 96-well plates at the density of 3.5×10^3 cells per well and exposed to different concentrations of anticancer drugs for 5 days. Subsequently, $20\,\mu l$ MTT (Sigma-Aldrich) was added to each well. After incubation for 4 h, the resulting formazan products were dissolved with acid-isopropanol. The absorbance at a wavelength of 490 nm was measured on a SpectraMax Microplate Spectrophotometer (Molecular Devices LLC, Sunnyvale, CA, USA). Experiments were performed in triplicate. Absorbance values were normalised assigning the value of the cells in medium without drug to 1.0 and the value of the no-cell control to 0.

Western blot assay. To evaluate the protein expression of Pgp and other ABC transporters including MRP1 and BCRP in different selected cell sublines, total proteins were extracted with $1 \times$ RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA, USA). Protein concentrations were evaluated by the DC Protein Assay (Bio-Rad, Hercules, CA, USA), and western blot analysis was then performed as previously described (Duan *et al*, 2009b). Briefly, $20\,\mu g$ of total protein was resolved on NuPAGE 4–12% Bis-Tris Gel (Life Technologies) and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was then blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% nonfat milk for 2 h and subsequently probed with primary antibodies (dilution: 1 : 1000) at $4\,^\circ C$ overnight. After washing three times with TBST, they were further incubated with respective secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) in a dilution of 1 : 20 000 for 1 h at room temperature. The membranes were washed again with TBST and rinsed with PBS. Finally, membranes were scanned using Odyssey CLx equipment (LI-COR Biosciences).

Immunofluorescence assay. Different U-2OS cell sublines were grown in 24-well plate for 48 h and fixed in 3.7% buffered

paraformaldehyde. After permeating with methanol for 20 min, the cells were blocked with 1% BSA in PBS for 1 h. Subsequently, the cells were incubated with Pgp antibody (dilution: 1 : 50) at 4 °C overnight. After washing three times with PBS, the cells were then incubated with Alexa Fluor 594 (Red)-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) in a dilution of 1 : 1000 for half an hour. The nuclei were stained with $1 \mu g \, ml^{-1}$ Hoechst 33342 (Life Technologies). Images were obtained by Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with a SPOT RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Drug uptake assay determined by fluorescence microscopy. For visualisation of the intracellular accumulation level of Pgp substrate Calcein AM and Rhodamine 123 (R123) in different U-2OS cell sublines, 2×10^4 cells were seeded in 12-well plate in duplicate the day before the assay. Cells were then incubated with either $0.25 \mu M$ Calcein AM or $1 \mu M$ R123 in RPMI-1640 medium for 2 h at 37 °C. After washing the cells with PBS, $1 \mu g \, ml^{-1}$ Hoechst 33342 was used to stain nuclei. Images were acquired by Nikon Eclipse Ti-U fluorescence microscope equipped with a SPOT RT digital camera.

Calcein AM retention assay. At 1 day before the assay, 2×10^4 cells of different U-2OS cell sublines were cultured in a 96-well plate in triplicate. Cells were then incubated with $0.25 \mu M$ calcein AM for 15 min. After rinsing with cold culture medium, the cell fluorescence was measured at absorption wavelength 494 nm and emission wavelength 517 nm on a SPECTRAmax GeminXS fluorescent spectrophotometer (Molecular Devices), and normalised for cellular protein levels as determined by DC Protein Assay. The absorbance values were normalised against the value of the parental U-2OS and Saos cells.

R123 accumulation and efflux assay. Different U-2OS cell sublines were seeded at 4×10^4/well in two separate 96-well plates in triplicate 1 day before the assay. Both plates were washed three times with serum-free RPMI-1640 on day 2, and the cells were then incubated with $3.2 \mu M$ R123 in serum-free RPMI-1640 culture medium for 1 h at 37 °C. One plate was gently washed three times with ice-cold PBS to remove extracellular R123 and lysed in Triton X-100 (1.0%). Intracellular R123 content was determined using a SPECTRAmax GeminXS fluorescent spectrophotometer ($\lambda ex = 492 \, nm$, $\lambda em = 535 \, nm$) and normalised for cellular protein levels as determined by DC Protein Assay. This value was labeled as R_a. The second plate was washed three times with ice-cold serum-free RPMI-1640 culture medium, and incubated with serum-free RPMI 1640 for another 1 h. Subsequently, cells were washed three times with ice-cold PBS and lysed in Triton X-100 (1.0%). R123 content was determined as described above and the values were labeled as R_e. The relative R123 cellular efflux level was calculated by $(R_a - R_e)/R_a$. The absorbance values were normalised against the value of the parental U-2OS cells.

Statistical analysis. Statistical analysis was done using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the mean ± s.d. For comparison between two groups, a two-sided Student's t-test was used. $P < 0.05$ value was considered as statistically significant.

RESULTS

NSC23925 prevents the emergence of MDR during paclitaxel treatment. To establish MDR cell sublines, osteosarcoma cell lines U-2OS and Saos were cultured either with increasing concentrations of paclitaxel alone or in combination with $1 \mu M$ NSC23925 (paclitaxel–NSC23925). U-2OS cells selected with paclitaxel alone (U-2OS/paclitaxel) grew steadily in the presence of $0.2 \mu M$ paclitaxel in culture medium after 6 months of drug exposure. In contrast, cells cultured with paclitaxel–NSC23925 combination (U-2OS/paclitaxel–NSC23925) were unable to grow in the medium with more than $0.006 \mu M$ paclitaxel (Figure 1A). Similar to U-2OS, Saos cells selected with paclitaxel alone (Saos/paclitaxel) showed stable growth in the presence of $0.06 \mu M$ paclitaxel after half-year, whereas cells cultured with paclitaxel–NSC23925 combination (Saos/paclitaxel–NSC23925) could not tolerate more than $0.001 \mu M$
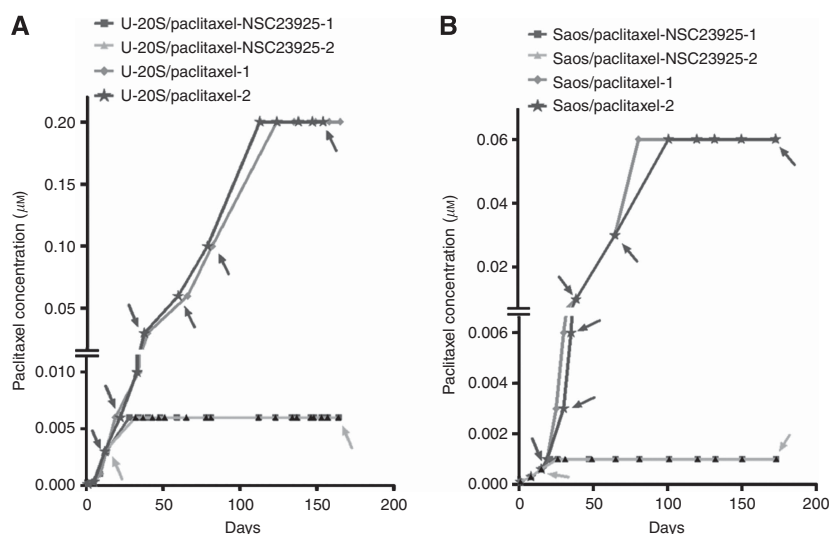

Figure 1. Development of drug resistance in U-2OS and Saos cell lines during paclitaxel treatment. (**A**) The time course of development of paclitaxel resistance in U-2OS cell line in the absence or in the presence of $1 \mu M$ NSC23925. (**B**) The time course of development of paclitaxel resistance in Saos cell line. Two independent experiments were established simultaneously in cells selected with paclitaxel alone, and resistance U-2OS and Saos cell lines developed on both occasions (paclitaxel-1 and paclitaxel-2). The experiments of cells cultured with the paclitaxel–NSC23925 combination were also repeated independently two times, in which the use of NSC23925 prevented the development of resistance to paclitaxel on both occasions (paclitaxel–NSC23925-1 and paclitaxel–NSC23925-2). Different selection points for cell sublines indicated by arrows in the time course of paclitaxel-1 and paclitaxel–NSC23925-1 were used in the further experiments.

paclitaxel in culture medium (Figure 1B). Two independent experiments were established simultaneously in osteosarcoma cells selected with paclitaxel alone, and resistant U-2OS and Saos cell lines were developed in all of these experiments. The experiments of cells cultured with paclitaxel–NSC23925 combination were also repeated independently two times, and NSC23925 was shown to prevent the introduction and development of resistance to paclitaxel in both of these events. Cell sublines used for further experiments were harvested and frozen at different selection points, as shown by arrows in Figure 1A and B. In addition, U-2OS and Saos cells cultured with 1 μM NSC23925 (U-2OS/NSC23925, Saos/NSC23925) alone were grown stably for 2 months (data were not shown).

The IC_{50} of paclitaxel was then evaluated to further determine paclitaxel resistance. As expected, the IC_{50} of paclitaxel in cell sublines revealed the same trend as their tolerance to paclitaxel during selection (Figure 2A and B). The IC_{50} of paclitaxel in cells selected with paclitaxel alone increased as the time and concentration of drug exposure increased. U-2OS cells selected with $\geqslant 0.06 \mu$M of paclitaxel alone showed significantly higher IC_{50} of paclitaxel as compared with the parental cells. An increase of 72.1-fold in IC_{50} of paclitaxel was observed in U-2OS cells selected with 0.2 μM paclitaxel alone (U-2OS/paclitaxel$_{0.2}$) as compared with parental cells. However, the IC_{50} of paclitaxel in cells cultured with 0.006 μM paclitaxel–NSC23925 combination (U-2OS/paclitaxel$_{0.006}$–NSC23925) increased by 0.8-fold only, further suggesting that paclitaxel–NSC23925 combination prevented the development of paclitaxel resistance. Specifically, the IC_{50} of paclitaxel in U-2OS/paclitaxel$_{0.2}$ cells was 40.1-fold as high as that of U-2OS/paclitaxel$_{0.006}$–NSC23925 cells (Figure 2A and Supplementary Table 1). Similar results were shown in Saos cell sublines (Figure 2B and Supplementary Table 2). There was no change in IC_{50} of paclitaxel in cells cultured with 1 μM NSC23925 alone (Figure 2).

Because paclitaxel–NSC23925 prevented the emergence of paclitaxel resistance, we further examined whether this combination could prevent acquired drug resistance to other cytotoxic agents. The IC_{50} of docetaxel, doxorubicin, vincristine, PM-00104, gemcitabine, cisplatin and methotrexate in U-2OS cell sublines were used to examine the cross-resistance profiles. U-2OS/paclitaxel$_{0.2}$ cells revealed 28.9-fold increased cross-resistance

to doxorubicin, whereas U-2OS/paclitaxel$_{0.006}$-NSC23925 cells remained sensitive to doxorubicin. Sensitivity to other well-known Pgp substrate drugs, such as docetaxel, vincristine and PM-00104, was also notably reduced in U-2OS/paclitaxel$_{0.2}$ cells. Nevertheless, U-2OS/paclitaxel$_{0.006}$–NSC23925 cells remained sensitive to docetaxel, vincristine and PM-00104 (Figure 3 and Supplementary Table 3). Therefore, cells selected with paclitaxel alone naturally acquired MDR, whereas MDR did not develop in cells cultured with paclitaxel–NSC23925 combination. These results, taken together, indicated that NSC23925 has the ability to prevent the emergence of MDR in cancer cells treated with paclitaxel. In addition, the cytotoxicity of cisplatin and methotrexate that are known to be unaffected by Pgp mechanisms were not altered in U-2OS/paclitaxel$_{0.006}$–NSC23925 and U-2OS/paclitaxel$_{0.2}$ cells (Figure 3 and Supplementary Table 3).

NSC23925 specifically prevents Pgp overexpression during chemotherapeutic drug treatment. The expression levels of Pgp in different cell sublines were evaluated to further identify the underlying mechanisms of preventing MDR development by NSC23925. Western blot analysis demonstrated that U-2OS/paclitaxel and Saos/paclitaxel cells selected with $\geqslant 0.06 \mu$M paclitaxel alone expressed significantly higher levels of Pgp; however, no significant induction of Pgp expression was observed in U-2OS/paclitaxel–NSC23925 and Saos/paclitaxel–NSC23925 cells as compared with parental cells (Figure 4A and B). The relative Pgp expression level was analysed by densitometry and the results are presented in Figure 4C. The increase in Pgp level detected in the different cell sublines showed a strong association with the increase in the concentration of paclitaxel tolerated by cells. These results suggested that exposure of cells to paclitaxel led to overexpression of Pgp, and that increased Pgp expression level was responsible for the emergence of MDR. NSC23925 can prevent the development of MDR in cancer cells through the inhibition of Pgp expression. These findings were further confirmed by immunofluorescence. The U-2OS/paclitaxel$_{0.2}$ cells displayed significantly higher Pgp staining than U-2OS/paclitaxel$_{0.006}$–NSC23925 cells (Figure 4D). Cells selected with 1 μM NSC23925 alone did not affect Pgp expression.
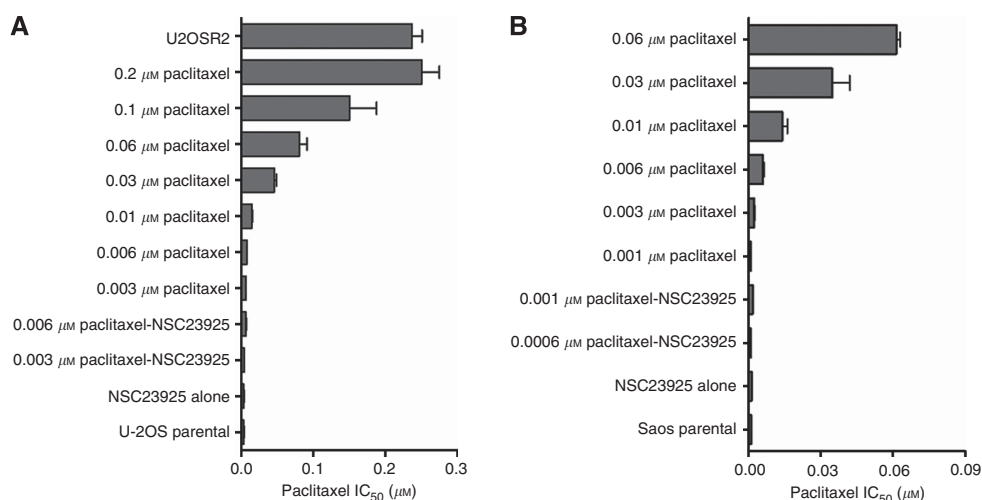

Figure 2. The NSC23925 prevented the emergence of paclitaxel resistance during chemotherapeutic agents. (A) The IC_{50} of paclitaxel in U-2OS parental cell and selected cell sublines. The labels correspond to U-2OS parental cells (U-2OS parental); cells cultured with 1 μM NSC23925 alone (NSC23925 alone); cells cultured with 0.003 μM paclitaxel in the presence of 1 μM NSC23925 (0.003 μM paclitaxel–NSC23925); cells selected with 0.006 μM paclitaxel in the presence of 1 μM NSC23925 (0.006 μM paclitaxel–NSC23925); and cells selected with paclitaxel alone at different concentrations: 0.003 μM (0.003 μM paclitaxel), 0.006 μM (0.006 μM paclitaxel), 0.01 μM (0.01 μM paclitaxel), 0.03 μM (0.03 μM paclitaxel), 0.06 μM (0.06 μM paclitaxel), 0.1 μM (0.1 μM paclitaxel) and 0.2 μM paclitaxel (0.2 μM paclitaxel); U-2OSR2. **(B)** The IC_{50} of paclitaxel in Saos parental cell and selected cell sublines. Data are presented as mean ± s.d.
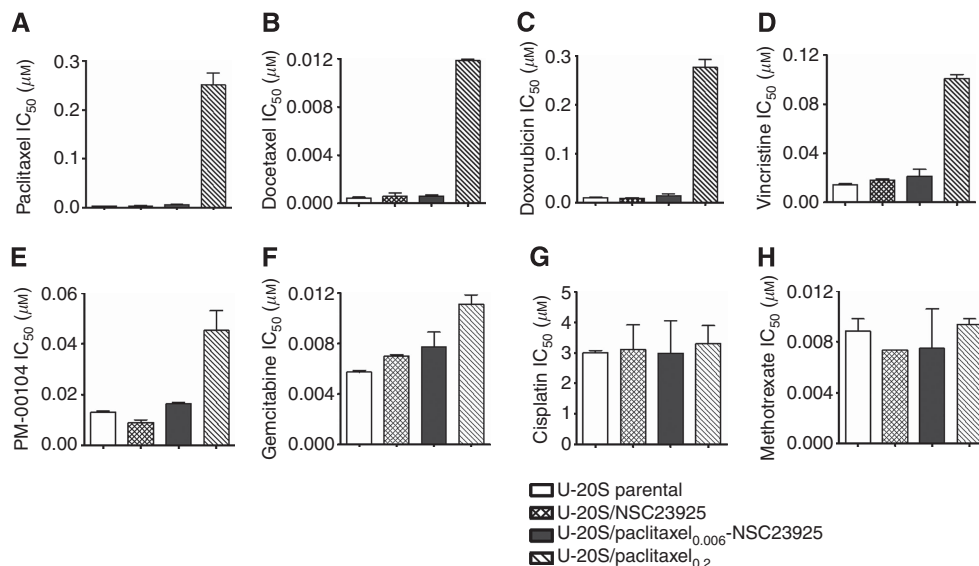
Figure 3. The NSC23925 inhibited the introduction of cross-resistance to chemotherapeutic agents. The U-2OS/paclitaxel$_{0.006}$–NSC23925 cells showed sensitivity to paclitaxel (**A**), docetaxel (**B**), doxorubicin (**C**), vincrisine (**D**), PM-00104 (**E**) and gemcitabine (**F**), whereas U-2OS/paclitaxel$_{0.2}$ cells displayed resistance to these drugs. The cytotoxicity of cisplatin (**G**) and methotrexate (**H**) did not change in both U-2OS/paclitaxel$_{0.006}$–NSC23925 and U-2OS/paclitaxel$_{0.2}$ cells. No changes in cytotoxicity of chemotherapeutic agents were seen in U-2OS/NSC23925 cells. Data are presented as mean ± s.d.

It is well known that Pgp is not the sole mechanism of MDR. Therefore, the expression level of other important drug transporter proteins including BCRP and MRP1 was also assessed in different cell sublines. All of the U-2OS and Saos cell sublines exhibited low expression of BCRP, and protein expression of MRP1 was undetectable (Figure 4A and B). No difference of MRP1 and BCRP levels among the cell sublines revealed that MRP1 and BCRP were not associated with the introduction of drug resistance selected by paclitaxel treatment. Thus, these observations suggested that NSC23925 specifically suppressed the expression of Pgp to prevent the emergence of MDR during chemotherapeutic drug treatment.

NSC23925 prevents decreased drug intracellular accumulation by preventing the overexpression of Pgp during paclitaxel treatment. The intracellular accumulation and efflux levels of Pgp substrates were examined to confirm the functional activity of the Pgp in different U-2OS cell sublines. U-2OS/paclitaxel$_{0.006}$–NSC23925 cells showed a considerable higher intracellular accumulation of Calcein AM and R123 as compared with U-2OS/paclitaxel$_{0.2}$ cells. Notably, U-2OS/paclitaxel$_{0.006}$–NSC23925 cells showed similar Pgp substrate intracellular accumulation levels when compared with parental cells (Figure 5A and B). Furthermore, a statistical lower relative efflux level of R123 was detected in U-2OS/paclitaxel$_{0.006}$–NSC23925 cells than in U-2OS/paclitaxel$_{0.2}$ cells (Figure 5B). The accumulation and efflux activity of Pgp substrates were consistent with the degree of Pgp expression. In addition, cells with longer exposure to NSC23925 alone had no obvious effect on Pgp activity. These results strongly indicated that the increased expression of Pgp resulted in the decreased intracellular accumulation and increased efflux of Pgp substrates.

DISCUSSION

NSC23925 is a novel methoxyphenyl piperidinyl compound (http://pubchem.ncbi.nlm.nih.gov/) with a molecular weight of 348 g mol^{-1}. The information about the pharmacological action of

NSC23925 is lacking. Reflective of our findings that NSC23925 can reverse MDR by stimulating Pgp ATPase activity and inhibiting the function of Pgp in a variety of MDR cancer cells (Duan *et al*, 2009a, 2012), the present study further demonstrated that NSC23925 has the ability to prevent the development of MDR in human osteosarcoma U-2OS and Saos cells. The U-2OS and Saos cells selected with paclitaxel alone developed a MDR phenotype with resistance to paclitaxel and cross-resistance to Pgp substrates doxorubicin, docetaxel, vincristine and PM-00104. However, cells selected with paclitaxel–NSC23925 simultaneously remained sensitive to chemotherapeutic agents. Interestingly, cells selected with paclitaxel alone showed significant higher Pgp expression than cells selected with paclitaxel–NSC23925 combination and parental cells. Specifically, cells selected with paclitaxel–NSC23925 combination displayed similar Pgp expression level to their parental cells. The extent of MDR in selected cells correlated with the Pgp expression level. Furthermore, cells selected with paclitaxel or paclitaxel–NSC23925 combination remained sensitive to non-Pgp substrates cisplatin and methotrexate, and no changes in BCRP and MRP1 expression were seen in different cell sublines. Our findings indicate that NSC23925 can prevent the emergence of MDR in osteosarcoma by specifically preventing the expression of Pgp during chemotherapeutic drug treatment.

We observed that exposure of cells to paclitaxel led to overexpression of Pgp, and increased Pgp expression level was responsible for the emergence of MDR, consistent with previous research results suggesting that overexpression of Pgp plays a major role in the molecular mechanisms of MDR (Suto *et al*, 1998; Gottesman *et al*, 2002; Ozben, 2006; Sharma *et al*, 2008). The Pgp rapidly pumps out functionally and structurally distinct chemotherapeutic drugs from cells, acting as an energy-dependent membrane transporter. Therefore, overexpression of Pgp results in reduced intracellular accumulation of drugs and prevents the drugs from exerting their cytotoxic effects (Szakacs *et al*, 2006). Our data showed that paclitaxel-selected drug-resistant cells displayed decreased drug intracellular accumulation and enhanced drug efflux. In comparison, cancer cells selected with paclitaxel–NSC23925 combination showed similar drug uptake/efflux level as parental sensitive cells. NSC23925 prevents the increased
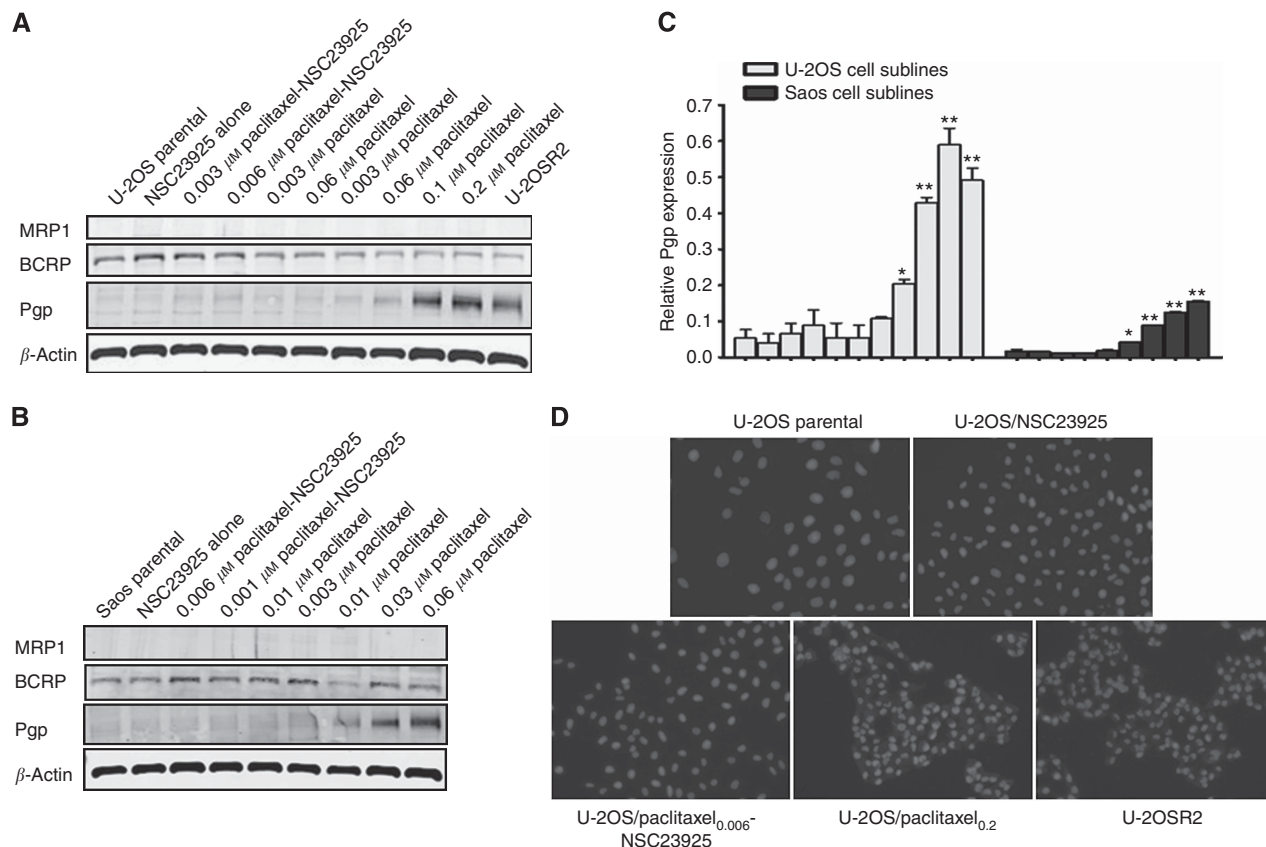
Figure 4. The NSC23925 specifically prevented Pgp overexpression during paclitaxel treatment. (**A** and **B**) Expression level of Pgp, BCRP and MRP1 in different U-2OS and Saos cell sublines detected by western blot, respectively. Western blot analysis demonstrated that U-2OS/paclitaxel and Saos/paclitaxel cells eventually expressed significantly higher Pgp. However, no significant change in Pgp expression level was observed in U-2OS/paclitaxel–NSC23925 and Saos/paclitaxel–NSC23925 cells compared with parental cells. Increases in the Pgp level detected in the different cell sublines showed a strong association with the increase in the concentration of paclitaxel tolerated by cells. No significant difference of MRP1 and BCRP levels were observed among the different cell sublines. (**C**) Relative Pgp expression of western blot from (**A** and **B**) were analysed by densitometry. The labels correspond to those given in (**A** and **B**). Data are presented as means ± s.d. and analysed using Student's t-test (two tailed, unequal variance). Statistical comparisons between selected and parental cells are presented: $*P < 0.05$, $**P < 0.005$, $***P < 0.001$. (**D**) Expression level of Pgp in U-2OS cell sublines analysed by immunofluorescence. The U-2OS/paclitaxel$_{0.2}$ cells displayed significantly higher Pgp staining than U-2OS/paclitaxel$_{0.006}$–NSC23925 cells.

expression of Pgp and therefore prevents increased efflux and/or decreased influx of drugs during chemotherapy treatment. These findings provide an indication of the mechanism by which NSC23925 prevents the emergence of MDR in osteosarcoma (Figure 6). NSC23925 specifically prevented the overexpression of Pgp during the chemotherapeutic drug treatment that subsequently allowed for the retention of intercellular drug accumulation and decreased drug efflux. Consequently, NSC23925 enabled tumour cells to remain sensitive to chemotherapeutic drug and prevented the emergence of MDR during chemotherapy treatment.

The inclusion of drugs or agents that can prevent the development of MDR may enable patients who initially responded to therapy to continue responding without the development of MDR, and therefore provide significant clinical benefits. Preventing the overexpression of Pgp has been identified as an efficient approach to inhibit the development of MDR during chemotherapeutic drug treatment (Cocker *et al*, 2001; Yen and Lamph, 2006; Zatelli *et al*, 2007). There are several drugs that act on the same mechanism of preventing the development of MDR with NSC23925 by suppressing the overexpression of Pgp, such as PSC833, VX710 and P85 (Cocker *et al*, 2001; Batrakova *et al*, 2006; Batrakova and Kabanov, 2008; Sharma *et al*, 2008). Unfortunately, PSC833, a nonimmunosuppressive cyclosporin D derivative, was shown to have pharmacokinetic interactions with paclitaxel and

inhibited non-MDR-related transporters (Kang *et al*, 2001; Bates *et al*, 2004). Although VX-710 has no pharmacokinetic interactions with paclitaxel and doxorubicin, myelosuppression and nonhaematologic toxicity have restricted this clinical application (Seiden *et al*, 2002). The addition of VX-710 to vincristine and doxorubicin therapy did not statistically enhance antitumour activity or survival in recurrent small cell lung cancer (Gandhi *et al*, 2007). In the present study, no significant changes on cell chemosensitivity, Pgp expression or Pgp activity were observed in osteosarcoma cells with longer exposure to NSC23925 alone *in vitro*. Moreover, both NSC23925 alone and paclitaxel–NSC23925 combination do not alter Pgp expression levels and activity in osteosarcoma cells. On the basis of animal weight and mortality, no significant toxicity was observed and the mice appeared to have tolerated it well when NSC23925 was administered alone in our previous study (Duan *et al*, 2012). Taken together, these observations may suggest that NSC23925 has no pharmacokinetic interactions with paclitaxel, and cannot increase drug toxicity *in vitro*. However, whether NSC23925 has pharmacokinetic interactions with anticancer drugs and other side effects *in vivo* deserves further investigation. P85 has also been demonstrated to prevent the introduction of MDR1 phenotype by preventing the overexpression of Pgp in leukemia cell line P388 as well as human breast carcinoma cell line MCF7 (Batrakova *et al*, 2006; Sharma *et al*, 2008). The IC$_{50}$ of cells
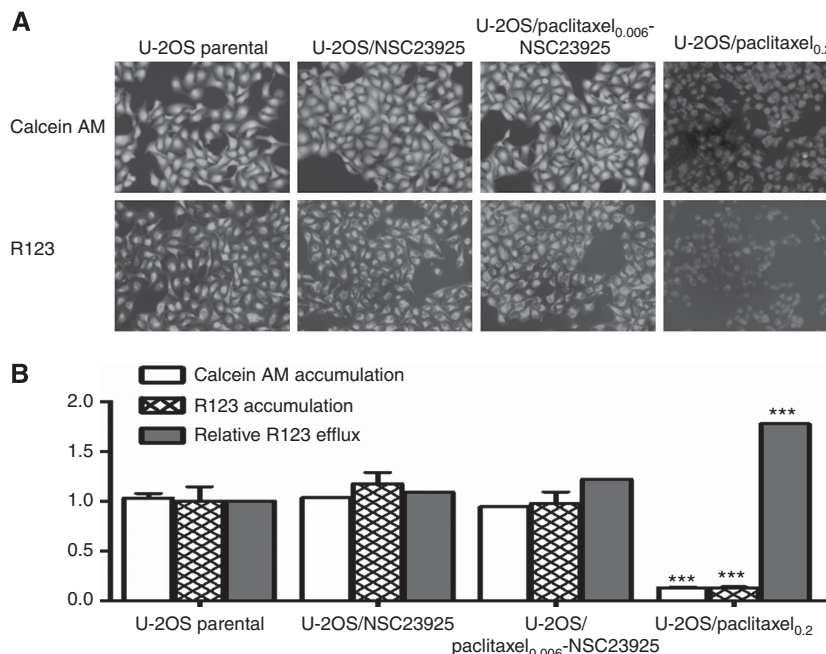
Figure 5. Pgp activity in different cell sublines. (A) Drug intracellular accumulation images of U-2OS cell sublines incubated with Calcein AM and R123. The U-2OS/paclitaxel$_{0.006}$–NSC23925 cells demonstrated a significantly higher accumulation of Calcein AM and R123 as compared with U-2OS/paclitaxel$_{0.2}$ cells. (B) Calcein AM accumulation and R123 accumulation and efflux level in U-2OS cell sublines analysed by Microplate Spectrofluorometer. The U-2OS/paclitaxel$_{0.006}$–NSC23925 cells showed a statistically higher accumulation of Calcein AM, R123 and lower relative efflux level of R123 than U-2OS/paclitaxel$_{0.2}$ cells. Cells cultured with NSC23925 alone had no obvious effect on Pgp activity. Data are presented as mean ± s.d. and analysed using Student's t-test (two tailed, unequal variance). Statistical comparisons between selected and parental cells are presented: *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$.
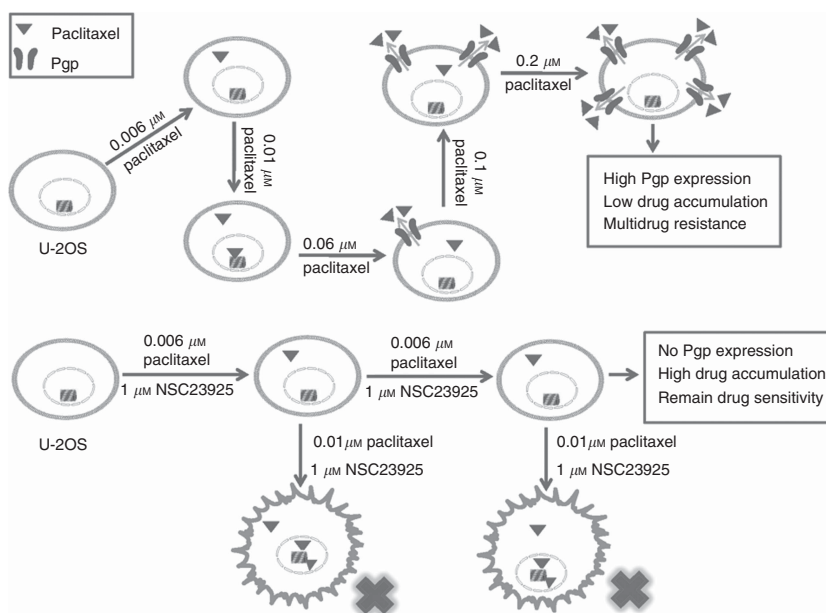

Figure 6. The mechanism by which NSC23925 acts to prevent the emergence of MDR in osteosarcoma. The U-2OS cells selected with paclitaxel alone developed a MDR phenotype with resistance to Pgp substrates, as well as decreased drug intracellular accumulation and enhanced drug efflux. However, NSC23925 specifically prevented overexpression of Pgp during chemotherapeutic drug treatment that subsequently allowed for the retention of intercellular drug accumulation and decreased drug efflux. Consequently, NSC23925 enabled tumour cells to remain sensitive to chemotherapeutic drugs and prevented the emergence of MDR during chemotherapy treatment.

selected with doxorubicin in the presence of P85 increased 6.13-fold *in vitro* and 5.58-fold *in vivo* as compared with parental P388 cells. In contrast, IC$_{50}$ of cells selected with paclitaxel in the presence of NSC23925 increased by 0.8- or 0.6-fold compared with IC$_{50}$ of parental cells. Hence, NSC23925 may have a greater effect on preventing the development of MDR than P85.

In summary, our findings indicate that NSC23925 can prevent the development of MDR in osteosarcoma by specifically preventing the overexpression of Pgp during chemotherapeutic drug treatment. These results suggest that NSC23925 will be useful in extending the long-term efficacy of chemotherapy by preventing the development of Pgp-mediated MDR in the clinical setting.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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