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NCX-4040, a nitric oxide-releasing aspirin, sensitizes drug-resistant human ovarian xenograft tumors to cisplatin by depletion of cellular thiols

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Abstract

Background: Ovarian carcinoma is the leading cause of mortality among gynecological cancers in the world. The high mortality rate is associated with lack of early diagnosis and development of drug resistance. The antitumor efficacy and mechanism of NCX-4040, a nitric oxide-releasing aspirin derivative, against ovarian cancer is studied.

Methods: NCX-4040, alone or in combination with cisplatin (*cis*-diamminedichloroplatinum, cDDP), was studied in cisplatin-sensitive (A2780 WT) and cisplatin-resistant (A2780 cDDP) cell lines as well as xenograft tumors grown in nude mice. Electron paramagnetic resonance (EPR) was used for measurements of nitric oxide and redox state. Immunoblotting analysis of A2780 cDDP tumor xenografts from mice was used for mechanistic studies.

Results: Cells treated with NCX-4040 (25 μ M) showed a significant reduction of cell viability (A2780 WT, $34.9 \pm 8.7\%$; A2780 cDDP, $41.7 \pm 7.6\%$; $p < 0.05$). Further, NCX-4040 significantly enhanced the sensitivity of A2780 cDDP cells (cisplatin alone, $80.6 \pm 11.8\%$ versus NCX-4040+cisplatin, $26.4 \pm 7.6\%$; $p < 0.01$) and xenograft tumors (cisplatin alone, $74.0 \pm 4.4\%$ versus NCX-4040+cisplatin, $56.4 \pm 7.8\%$; $p < 0.05$), to cisplatin treatment. EPR imaging of tissue redox and thiol measurements showed a 5.5-fold reduction ($p < 0.01$) of glutathione in NCX-4040-treated A2780 cDDP tumors when compared to untreated controls. Immunoblotting analysis of A2780 cDDP tumor xenografts from mice treated with NCX-4040 and cisplatin revealed significant downregulation of pEGFR (Tyr845 and Tyr992) and pSTAT3 (Tyr705 and Ser727) expression.

Conclusion: The results suggested that NCX-4040 could resensitize drug-resistant ovarian cancer cells to cisplatin possibly by depletion of cellular thiols. Thus NCX-4040 appears to be a potential therapeutic agent for the treatment of human ovarian carcinoma and cisplatin-resistant malignancies.

Background

Ovarian carcinoma is the leading cause of mortality among gynecological cancers in the world. The high mortality rate is attributed to the lack of early diagnosis of the malignancy and difficulties associated with treatment. Ovarian cancer is treated using cisplatin drugs; however, relapse of the disease involving a substantial population of cisplatin-resistant cells is commonly observed [1]. The development of drug resistance is a major impediment toward successful treatment of the recurrent cancer [2]. Substantially higher doses of cisplatin and paclitaxel are required to treat the recurrent disease. However, at high doses, these drugs have been known to cause undesirable side effects. For example, cisplatin causes significant nephro- [3], neuro- [4], and ototoxicity [5] while paclitaxel is associated with neurotoxicity and neutropenia [6]. Thus, there have been a number of studies aimed at understanding the causes of drug resistance, so as to develop strategies to overcome or avoid this complication [7-9]. To date, several mechanisms of cisplatin resistance in ovarian cancer cells have been proposed, including decreased cellular uptake and increased cellular efflux of cisplatin [8], inactivation of intracellular cisplatin by glutathione [10-12], and increased levels of DNA repair and DNA tolerance [12,13].

In the past, there have been enormous efforts to develop alternative drugs to treat the recurrent disease. Derivatives of cisplatin, such as carboplatin, oxaliplatin, paclitaxel, doxorubicin, and a variety of alkylating agents have been studied as potential agents to eliminate the resistant cells [1,2]. Also, several strategies were explored to resensitize the refractory cells to established modes of treatment without undesirable side effects. Depletion of cellular thiol levels in the drug-resistant cancer cells is one of the more promising strategies that has been shown to be significantly effective in a number of cases [14-17].

Recently, we showed that NCX-4016, a nitro derivative of aspirin, inhibited the proliferation of cisplatin-sensitive as well as cisplatin-resistant human ovarian cancer cell lines *in vitro* [18]. We also showed that NCX-4016, on incubation with the cisplatin-resistant cells, generated sustained levels of nitric oxide (NO) and substantially depleted cellular thiols. In a subsequent report, we further showed that NCX-4016, by itself, was capable of inhibiting the growth of cisplatin-resistant human xenograft tumors in mice [19]. We determined that NCX-4016 induced G₁ cell-cycle arrest and apoptosis by inhibiting the EGFR/PI3K and STAT3 signaling pathways. Subsequent to the initial reports on NCX-4016, there have been a few reports on NCX-4040 (Figure 1A), a positional isomer of NCX-4016 [20], that demonstrated significant cytotoxic effect on pancreatic [21], bladder [22], and colon cancer [23-25]. It was reported that NCX-4040 was 100 times more

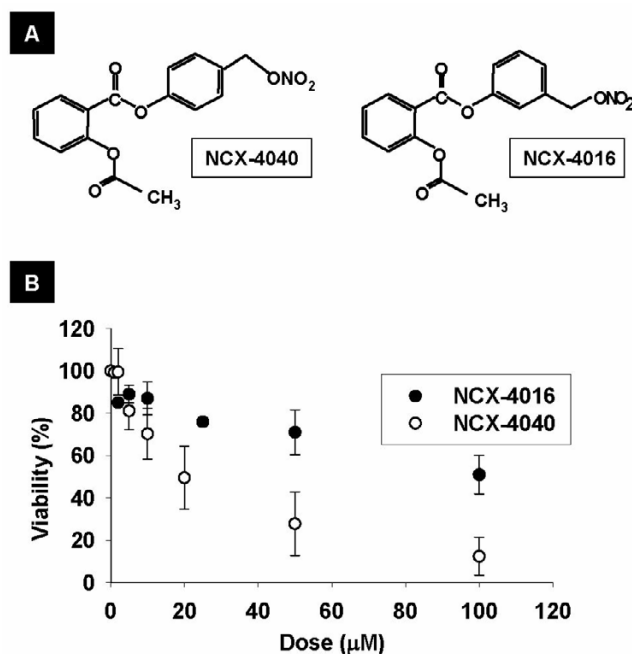


Figure 1
Comparison of the structure and effect of NCX-4040 and NCX-4016 on cell viability. (A) Molecular structure of NCX-4040 (acetyloxy)benzoic acid 4-(nitrooxymethyl)phenyl ester) and NCX-4016 (2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester). The nitroaspirins consist of acetylsalicylate (aspirin) linked to a spacer moiety by an ester bond. An NO-releasing moiety (-ONO₂) is attached to the spacer. (B) Effect of NCX-4040 and NCX-4016 (25 μM) on the viability of cisplatin-resistant human ovarian cancer cells (A2780 cDDP). The results show a dose-dependent cytotoxic effect, with NCX-4040 demonstrating a substantially higher effect compared to NCX-4016.

potent than NCX-4016 in HT-29 human colon cancer cells [26]. However, its cytotoxic effect on human ovarian cancer has not been investigated. Therefore, the goal of the present work was to study the antitumor efficacy and potential of NCX-4040 to sensitize cisplatin-resistant ovarian cancer cells to cisplatin treatment. The experiments were performed using cisplatin-sensitive (A2780 WT) and cisplatin-resistant (A2780 cDDP) human ovarian cancer cell lines and xenograft tumors. The results showed that administration of NCX-4040 decreased cellular thiol levels, thereby sensitizing the drug-resistant cells to cisplatin. NCX-4040, in combination with cisplatin, inhibited tumor growth by downregulation of EGFR and STAT3 signaling.

Methods

Reagents

NCX-4040 (2-(acetyloxy)benzoic acid 4-(nitrooxymethyl) phenyl ester) was obtained from NicOx (Sophia Antipolis, France). GSH (L-glutamyl-L-cysteinylglycine), aspirin (ASA), dimethylsulfoxide (DMSO), buthionine sulfoximine (BSO), S-Nitroso-N-acetylpenicillamine (SNAP), MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma. 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA) was obtained from Molecular Probes. Cisplatin (*cis*-diamminedichloroplatinum), ammonium iron(II) sulfate hexahydrate, and acetonitrile were purchased from Aldrich. Cell culture medium (RPMI medium 1640), fetal bovine serum (FBS), antibiotics, sodium pyruvate, trypsin, and phosphate-buffered saline (PBS) were purchased from GIBCO/BRL. The imidazoline biradical probes R₁S-SR₁ (bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)disulfide) and R₂S-SR₂ (bis((2,2,3,5,5-pentamethyl-1-oxyl-imidazolidinyl-4)-methyl)-disulfide) were synthesized as previously reported [27]. N-methyl-D-glucaminedithiocarbamate (MGD) was synthesized and purified in our laboratory [28]. Polyvinylidene fluoride (PVDF) membrane and molecular weight markers were obtained from Bio-Rad (Hercules, CA). Antibodies for pEGFR (Tyr845, Tyr992), EGFR, pSTAT3 (Tyr705 and Ser727), poly-adenosine diphosphate ribose polymerase (PARP), cleaved caspases-3, Bcl-x_L, and Bax were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for p53, Survivin, CycD1, and STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Ovarian cancer cell lines

Cisplatin-sensitive (A2780 WT) and cisplatin-resistant (A2780 cDDP) human ovarian cancer cell lines were used. The cisplatin-resistant cell line was originally developed from an *in vivo* tumor model by treating with cisplatin [29]. Cells were grown in RPMI medium 1640 supplemented with 10% FBS, 2% sodium pyruvate, and 1% penicillin/streptomycin. Culture and drug-treatment of cells were carried out at 37°C in an atmosphere of 95% air/5% CO₂. Cells were routinely trypsinized (0.05% trypsin/EDTA) and counted using an automated counter (NucleoCounter, New Brunswick Scientific Co., Edison, NJ).

Cell viability assay

Cytotoxicity of the compounds was determined using the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan via mitochondrial oxidation. Cells were grown in 75-cm² flasks to 70% confluence, trypsinized, counted, and plated at a density of 20,000 cells per well in 96 well plates. Cells

were incubated overnight and then treated with NCX-4040, ASA and/or cisplatin at variable concentrations for designated lengths of time. All experiments were repeated at least three times. NCX-4040 was dissolved in dimethyl sulfoxide (DMSO) and added into the culture. The final DMSO concentration never exceeded 1%, and this condition was used as the control in each experiment. Cisplatin was dissolved in DMSO (16 mg/ml) and stored at -20°C. The stock solution was diluted to the final concentration prior to each treatment.

The synergism of NCX-4040 and cisplatin was calculated as described [30]. An expected value of cell survival, S_{exp}, was defined as the product of the survival observed for NCX-4040 and the survival observed for cisplatin (cDDP): S_{exp} = S_{NCX-4040} * S_{cDDP}. The actual survival observed for the combination treatment was defined as S_{obs}. The synergistic ratio, R, was calculated as: R = S_{exp}/S_{obs}. Synergy was defined for R > 1.

Measurement of nitric oxide generation using EPR spectroscopy

Generation of NO from NCX-4040 by cells incubated with the compound was measured by EPR spectroscopy using Fe(MGD)₂ spin trap. The spin trap reacts with NO and forms a stable complex Fe(MGD)₂-NO which can be detected using EPR. A2780 WT and A2780 cDDP cells were cultured in 75-cm² flasks. When cells reached 70% confluence, they were trypsinized and resuspended to a final concentration of 20 million cells per milliliter. The cell suspension was added with NCX-4040 (100 μM) and Fe(II)(MGD)₂, an NO spin-trap prepared in deoxygenated PBS by mixing 1 mM Fe²⁺ with 5 mM MGD. The NO measurements were performed using a Bruker ER300 X-band EPR spectrometer. The EPR spectra were collected at 20-mW microwave power using the following settings: modulation amplitude, 3 G; modulation frequency, 100 kHz; time constant, 8 ms; scan time, 21 s. SNAP was used as a positive control for NO detection.

Confocal fluorescence microscopic imaging

Nitric oxide production in A2780 cDDP cells was analyzed using confocal fluorescence microscopic imaging. DAF-FM DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate), a green fluorescence probe specific for NO, was added (10 μM) to cells treated with NCX-4040 (100 μM; 0, 1, 2 and 4 h). The probe was diluted in serum and phenol red-free culture medium. Cells were incubated with DAF-FM DA for 30 min at 37°C and then for 15 min without probe to allow complete de-esterification of the intracellular diacetate. Fluorescence measurements were made using a Zeiss LSM 510 multiphoton confocal microscope (excitation, 488 nm; emission, 515 nm). Quantitative analysis of fluorescence intensity was performed using MetaMorph Software.

Animal housing and preparation

Female 6-week-old BALB/c nude mice were obtained from the National Cancer Institute. The animals were housed five per cage in a climate- and light-controlled room. Food and water were allowed *ad libitum*. All animals were used according to the Public Health Services Policy, the Federal Welfare Act, and ILACUC procedures and guidelines. The mice were anesthetized with ketamine (200 mg/kg b.w.) and xylazine (4 mg/kg b.w.) by intraperitoneal (i.p.) injection. The animals inhaled room air (21% O₂) during the *in vivo* spectroscopy, as well as for imaging. The body temperature of the animal was maintained at 37 ± 1 °C by an infrared lamp placed just above the animal during the measurements.

Ovarian cancer tumor xenografts in mice

Cisplatin-sensitive (A2780 WT) and cisplatin-resistant (A2780 cDDP) human ovarian cancer cells (5 × 10⁶ cells in 60 µl of PBS) were injected subcutaneously (s.c.) into the upper portion of the right hind limb of BALB/c nude mice. Both types of cells grew, *in vivo*, as a solid tumor. The size of the tumor was measured three times per week using a Vernier caliper. The tumor volume was determined from the orthogonal dimensions (d₁, d₂, d₃) using the formula (d₁*d₂*d₃)* π/6.

In vivo study of NCX-4040 efficiency and redox study

On the 7th day after inoculation, when the tumor size reached approximately 3–5 mm, the mice were subjected to the treatment regime. The mice received i.p. injections of (1) 5 mg/kg b.w. of NCX-4040 alone, daily; (2) 5 mg/kg b.w. of ASA alone, daily; (3) 8 mg/kg b.w. of cisplatin on the 11th day alone; or (4) 5 mg/kg b.w. of NCX-4040 daily, followed by cisplatin treatment on the 11th day (8 mg/kg b.w.). The control group was administered with the vehicle alone (5). At the end of experiment (25th day) the mice were sacrificed. The tumor tissues were then resected and stored in liquid nitrogen up to the time of thiol level determination and immunoblotting analysis.

Thiol (GSH) assay

Tissue levels of thiols were determined by X-band EPR spectroscopy, using a thiol-specific imidazoline biradical probe [31]. The probe reacts with reduced thiol and shows a characteristic EPR spectrum, which can be quantified by using known amounts of glutathione (GSH). One hundred mg of thawed tissue were homogenized, in 2 ml of PBS, using a tissue homogenizer (PCR Tissue Homogenizing Kit, Fisher Scientific) to obtain a uniform tissue suspension. The tissue homogenate was treated with the thiol-specific label R₁S-SR₁ (0.5 mM) at pH 7.0 for 7 min, and the EPR spectra were measured. The concentration of GSH was determined from a standard curve prepared with known concentrations of GSH under similar conditions.

In vivo redox and imaging measurements

Tumor-bearing mice were intratumorally (i.t.) injected with 15 µL of thiol-sensitive probe, R₂S-SR₂ [27]. Then EPR measurements were taken immediately and every 1.5 min thereafter for a total of 30 min, during which the signal-decay was observed. The measurements were performed using a home-built L-band (1.2 GHz) EPR spectrometer with a bridged loop-gap resonator [32]. *In vivo* EPR redox images were obtained on the 25th day of tumor growth. Mice were intratumorally injected with 15 µL of R₂S-SR₂ [27]. The probe upon reaction with thiols is converted to nitroxide. The total redox information can be obtained from the decay of the nitroxide EPR signal, which depends primarily upon reduction factors in the tissue. Mapping of the EPR probe location and its decay was obtained by spatial EPR imaging (EPRI) methods that are well established in our laboratory [32,33]. The EPRI measurements were performed by using a home-built L-band (1.2 GHz) EPR spectrometer with a bridged loop-gap resonator [32].

Immunoblot analysis

The tumor tissues were homogenized in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM sodium orthovanadate, 0.5% NP40, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. The homogenate was clarified by centrifugation. The protein concentration in the lysates was determined using a Pierce detergent-compatible protein assay kit (Rockford, IL). For immunoblotting, 50 to 100 µg of tissue protein per sample was denatured in 2× SDS-PAGE sample buffer (0.5 mM Tris HCl, 60% glycerol, 10% SDS, and β-mercapto-ethanol, bromophenol blue) and subjected to SDS-PAGE on a 10% or 12% tris-glycine gel. The separated proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk powder (w/v) in Tris-buffered saline Tween-20 (TBST; 10 mM Tris, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature or overnight at 4 °C. The membranes were incubated overnight with the respective primary antibodies. The bound antibodies were detected with horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG or HRP-labeled donkey anti-rabbit IgG (Amersham Pharmacia Biotech) using an ECL detection system. The ECL image was digitized using a scanner (ScanJet 7400c, Hewlett-Packard, Palo Alto, CA) and quantified using densitometry software (Scion Image v0.4.0.2, Scion, Frederick, MD).

Statistical analysis

Data were expressed as mean ± SD (*in vitro* study) or mean ± SEM (*in vivo* study). Comparisons among groups were performed by using a Student's *t* test. The significance level was set at *p* < 0.05.

Results

NCX-4040 enhances cisplatin cytotoxicity in ovarian cancer cell lines

NCX-4040 is a positional isomer of NCX-4016 (Figure 1A), which we have previously reported to be cytotoxic to human ovarian cancer cell lines [18]. In order to evaluate the anticancer efficacy of NCX-4040 and to compare with that of NCX-4016, we performed a dose-response study using cisplatin-resistant cell line (A2780 cDDP). As seen in Figure 1B, a dose-dependent decrease of cell viability was observed in both cases. However, NCX-4040 showed a substantial decrease in cell viability when compared to NCX-4016. The cytotoxic effects of NCX-4040, either alone or in combination with cisplatin, on A2780 WT and A2780 cDDP cell lines were determined. Cells were treated with NCX-4040 (25 μ M) for 6 h followed by cisplatin (IC_{67} dose for A2780 WT cells) for 1 h with a 24-h follow up time. Cytotoxicity was assessed using an MTT viability assay. The results (Figure 2) showed that NCX-4040 alone significantly decreased the viability of both

A2780 WT ($34.9 \pm 8.7\%$) and A2780 cDDP ($41.7 \pm 7.6\%$) cell lines. Cisplatin (cDDP) alone significantly decreased the viability of A2780 WT ($31.5 \pm 3.4\%$), but not A2780 cDDP ($80.6 \pm 11.8\%$) cell lines. On the other hand, combination of cisplatin and NCX-4040 was more effective than cisplatin or NCX-4040 alone in decreasing the viability of both A2780 WT ($9.4 \pm 6.0\%$) and A2780 cDDP ($26.4 \pm 7.6\%$) cell lines. A weak synergism was observed ($R = 1.27$, see Methods for calculation) between NCX-4040 and cisplatin in the killing of A2780 cDDP cells. The results clearly demonstrated that NCX-4040 was not only cytotoxic, but also capable of sensitizing cisplatin-resistant ovarian cancer cells to cisplatin.

NCX-4040 generates NO in cells

To determine whether NCX-4040 can generate NO in cells, we performed EPR spectroscopic measurements on A2780 WT and A2780 cDDP cells incubated with 100 μ M of NCX-4040. Fe(MGD)₂ was used as a spin-trap for real-time detection of NO. Fe(MGD)₂ reacts with NO and forms a stable paramagnetic adduct, Fe(MGD)₂-NO, which can be detected by EPR spectroscopy [34]. NCX-4040 or cells alone in media did not generate any signal (Figure 3A). However, a prominent triplet signal with a hyperfine coupling constant of 12.5 G, characteristic of Fe(MGD)₂-NO, was observed upon incubation of NCX-4040 with the cells. The authenticity of the spectra was verified using S-nitroso-N-acetylpenicillamine (SNAP, 100 μ M), a known NO-releasing compound. Both cell lines demonstrated a time-dependent increase in the intensity of Fe(MGD)₂-NO signal (Figure 3B) starting at 40–50 min of co-incubation time. While the rate of NO generation was steady in the case of A2780 WT cells, a rapid increase followed by slow attenuation was observed in the case of A2780 cDDP cells. In order to determine whether or not the increased rate of NO generation in A2780 cDDP cells was due to thiols, we pre-incubated A2780 cDDP cells with 1 mM buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, for 24 h and then treated with NCX-4040. As seen in Figure 3B, the NO generation was significantly decreased upon BSO treatment, suggesting that the NO generation was mediated by cellular thiols. In contrast, NO generation by SNAP was instant and lasted only for a few minutes (data not shown). We further imaged the intracellular NO generation using DAF-FM DA (Figure 3C). These results shown in Figure 3D clearly demonstrated a sustained generation of NO by NCX-4040 in the cancer cells. Thus, the NO generation by NCX-4040 in cancer cells was low, but persisted for longer periods.

NCX-4040 inhibits tumor growth in mice

The anti-tumor efficacy of NCX-4040 was studied using A2780 cDDP tumor xenografts in mice. Tumor-bearing animals, on day 7 post-inoculation, were treated with

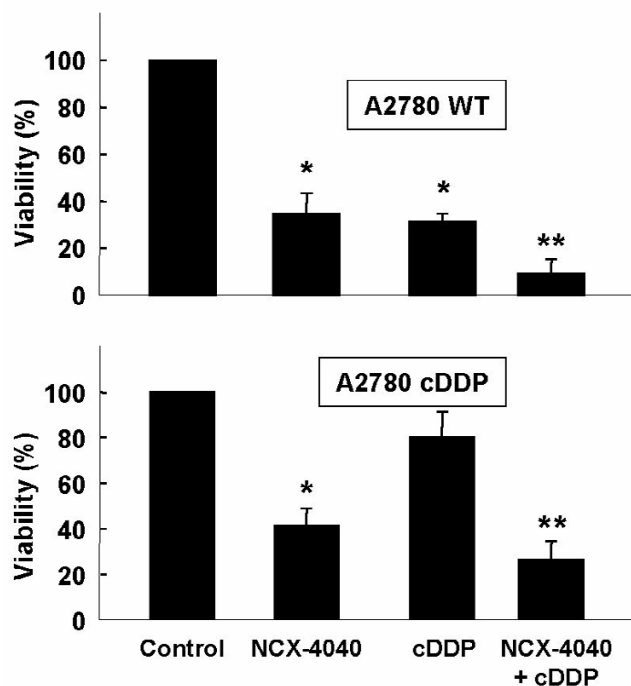
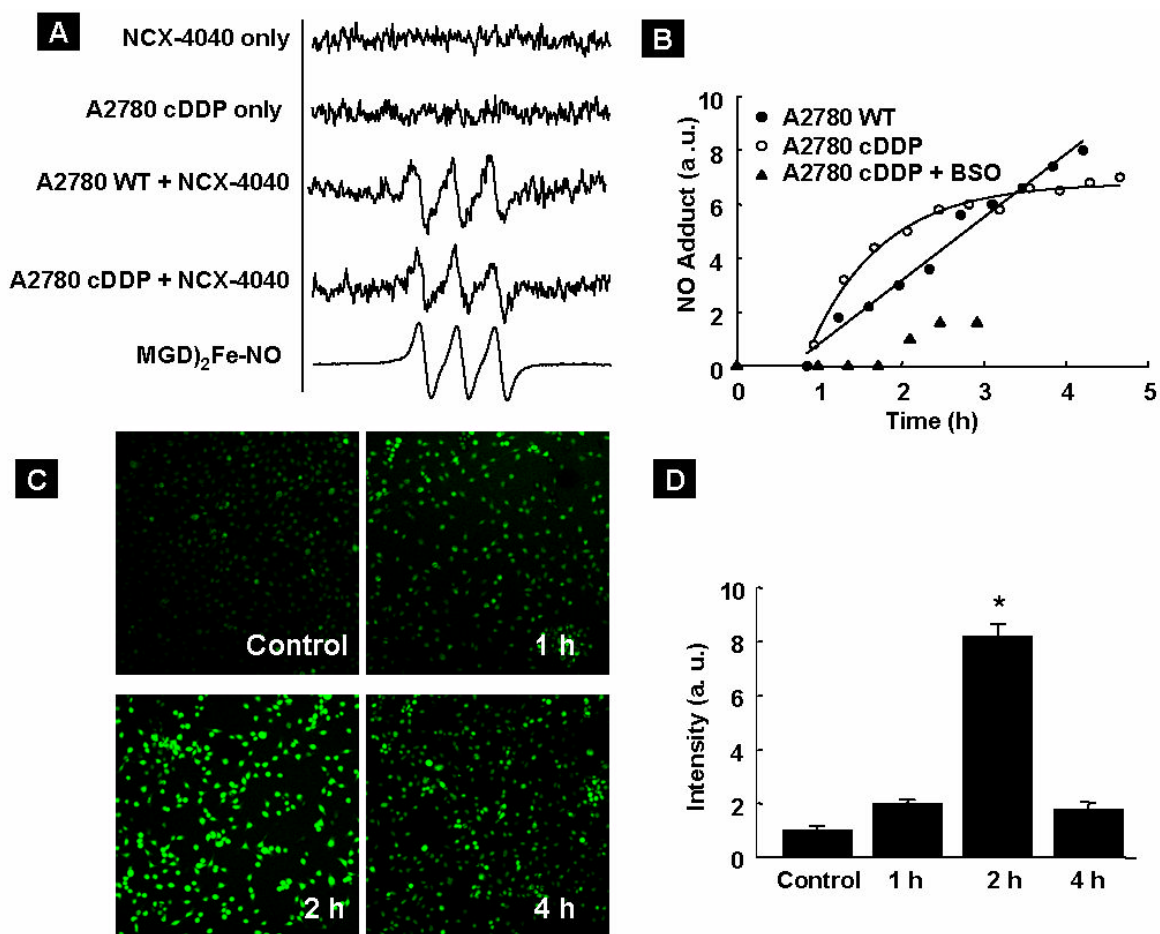


Figure 2
Effect of NCX-4040 and/or cisplatin on the viability of A2780 WT and A2780 cDDP cell lines. Cells were incubated with 25 μ M of NCX-4040 for 6 h, then with cisplatin for 1 h (IC_{67} dose for A2780 WT) with a 24 h follow up time. Cell viability was evaluated using MTT assay. Data represent mean \pm SD obtained from 4 independent experiments and expressed as a percentage of the control cells treated with vehicle (1% DMSO). * $p < 0.01$ versus Control group. ** $p < 0.05$ versus cDDP or NCX-4040 group.

**Figure 3**

Generation of nitric oxide (NO) by NCX-4040 in A2780 WT and A2780 cDDP cells. (A) Representative EPR spectra obtained from control (NCX-4040, A2780 cDDP cells) and NCX-4040 treatment (100 μ M for both A2780 WT and A2780 cDDP) groups. The cells were incubated with NCX-4040 in the presence of spin trap (MGD)₂Fe. Also shown is an EPR spectrum of an authentic complex of NO with Fe(MGD)₂ obtained by adding SNAP (an NO donor) to spin trap. (B) Time-course of nitric oxide release by NCX-4040 in A2780 WT, A2780 cDDP, and A2780 cDDP cells treated with 1 mM buthionine sulfoximine (BSO) for 24 h. (C) Representative DAF-FM DA fluorescence images of untreated (control) and treated (1, 2 or 4 hours) with NCX-4040 (100 μ M). (D) Quantitative results on the DAF-FM DA fluorescence data. **p* < 0.01 versus Control group.

NCX-4040 and/or cisplatin, as described in the Methods section. The tumor volumes were measured every 2 days for 19 days post-inoculation. As shown in Figure 4, NCX-4040 treatment alone did not show any significant reduction in tumor volume ($85.8 \pm 9.8\%$ versus Control on day 19; Figure 4B). Cisplatin (cDDP) treatment alone significantly reduced the tumor growth volume ($74.0 \pm 4.4\%$ versus Control). However, animals treated with a combination of cisplatin and NCX-4040 showed a significant reduction in tumor volume visible from second day after cisplatin treatment and reached $56.4 \pm 7.8\%$ versus Control on day 19. Aspirin (ASA), a metabolic product of NCX-4040, did not show any significant effect on tumor growth volume (Figure 4B). The results clearly demon-

strated that NCX-4040, in combination with cisplatin, was more effective than cisplatin alone in inhibiting the growth of cisplatin-resistant ovarian cancer xenografts in mice.

NCX-4040 depletes thiol levels in the tumor xenografts

Since cisplatin-resistance in ovarian cancer is associated with excessive thiol levels, we next wanted to check whether NCX-4040 treatment could modify cellular thiol levels in the solid tumor, *in vivo*. We performed *in vivo* redox imaging of tumor xenografts in mice treated with NCX-4040. As we have reported previously, the *in vivo* redox mapping using EPR imaging enables noninvasive and *real-time* visualization of tumor redox status, which is

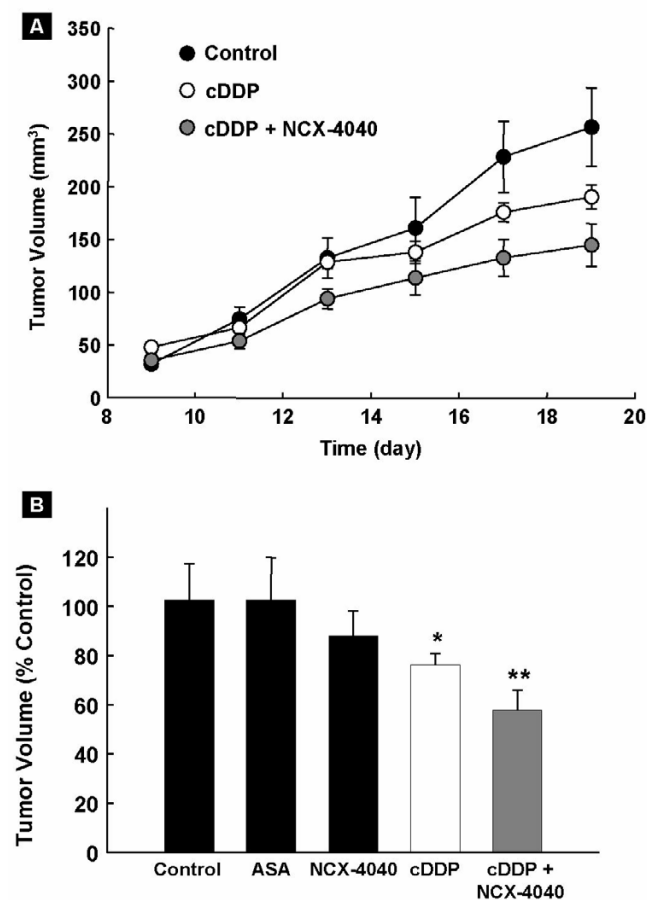


Figure 4
Effect of NCX-4040 and/or cisplatin (cDDP) on the growth volume of human ovarian cancer xenograft tumor in mice. Groups of mice (4–6 per group) were inoculated with the A2780 cDDP human ovarian cancer cells in the upper portion of hind leg. Seven days after inoculation, two groups were injected daily (i.p.) with NCX-4040 (5 mg/kg), of which one group received a single i.p. injection of cisplatin on day 11 (8 mg/kg). The third group received single dose of cisplatin on day 11 (8 mg/kg). One group received aspirin (5 mg/kg, daily). Control group received vehicle. **(A)** Tumor growth curve for control, cisplatin, and combination of treatment (NCX-4040 and cisplatin). **(B)** Tumor growth volume data (mean \pm SE, expressed as percent of control group) on the 19th day after injection of cancer cells. * $p < 0.05$ compared to control group. ** $p < 0.05$ versus cDDP group. NCX-4040 and aspirin (ASA), a metabolic product of NCX-4040, showed no significant effect. The results show that pretreatment with NCX-4040 was effective in enhancing the efficacy of cisplatin in inhibiting the growth of cisplatin-resistant ovarian cancer xenografts in mice.

a measure of total reducing equivalents, including reduced thiols, in the tissue [35]. We used a thiol-specific di-nitroxyl redox probe R_2S-SR_2 , which on reaction with SH groups breaks down to a mono-nitroxyl with a charac-

teristic triplet EPR signal. The rate of conversion of di- to mono-nitroxyl is a measure of thiol concentration in the tumor. However, in solid tumors, the conversion is very rapid due to excess concentration of thiols compared to the probe. However, the subsequent slower reduction of the EPR-active mono-nitroxyl to EPR-silent hydroxylamine by the cellular reductants including thiols can be monitored and used as a measure of tissue "redox" state. Figure 5A shows typical time-course "redox" images obtained after intra-tumoral injection of R_2S-SR_2 (100 mM, 15 μ l) in the cisplatin-sensitive (A2780 WT) and cisplatin-resistant (A2780 cDDP) ovarian xenograft tumors treated with NCX-4040. The time-course images showed

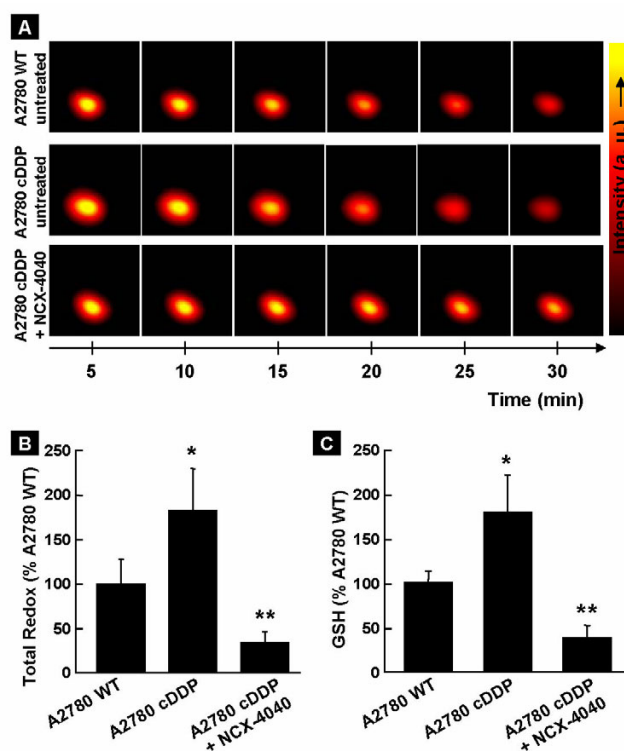


Figure 5
In vivo redox images of ovarian xenograft tumors in mice. A redox-sensitive probe (R_2SSR_2) was injected intra-tumorally (100 mM; 15 μ l), then sequential images were obtained for 30 min. The EPR images show the probe distribution in the tumor, while the tumor itself is not visible. **(A)** Representative images of time-course of redox probe distribution in untreated A2780 WT and A2780 cDDP tumors, as well as in A2780 cDDP tumor treated with NCX-4040 (5 mg/kg, daily). **(B)** Total *in vivo* redox levels expressed as a percent of A2780 WT level. **(C)** Glutathione (GSH) levels in the tumors excised on day 19 post-inoculation. Data represent mean \pm SD obtained from 4 mice/group. * $p < 0.05$ versus A2780 WT group. ** $p < 0.05$ versus A2780 cDDP group. The results demonstrate that NCX-4040 depleted thiol levels in the cisplatin-resistant tumor.

that the decay of the redox signal was faster in the cisplatin-resistant tumor as compared to that of the cisplatin-sensitive tumor, presumably due to higher cellular reductants in the former cell type. Further, it was observed that NCX-4040 treatment resulted in a substantial slow-down of the redox decay, suggesting that the treatment might have depleted the reductants in the cisplatin-resistant tumor. Quantitative redox data from multiple experiments (Figure 5B) showed that the total redox level was significantly elevated in A2780 cDDP tumor, $182.1 \pm 47.4\%$ of A2780 WT tumors' redox. NCX-4040 treatment significantly depressed the total redox level, to $33.3 \pm 12.8\%$ (5.5-fold decrease), in A2780 cDDP tumor. We next wanted to measure the modulation of thiol-only levels in the tumors. We performed GSH assay from excised tumor tissues (*in vitro*) using EPR spectroscopy with another thiol-specific probe R_1SSR_1 , as described in Methods. As seen in Figure 5C, the GSH level was $179.5 \pm$

42.3% in A2780 cDDP tumor and it was depleted to $38.5 \pm 14.1\%$ (a 4.7-fold decrease) after treatment with NCX-4040. The results clearly showed that NCX-4040 depleted thiol level in the cisplatin-resistant tumor.

NCX-4040 induces apoptosis by downregulating EGFR and STAT3 signaling

We recently observed that NCX-4016, an isomeric form of NCX-4040, inhibited tumor growth by modulating EGFR/PI3K and STAT3 signaling pathways [19]. Therefore, we analyzed the EGFR and STAT3 proteins in the A2780 cDDP tumor xenografts from mice treated with NCX-4040 and/or cisplatin. The immunoblots showed that the expressions of pEGFR (Tyr845 and Tyr992) and pSTAT3 (Tyr705, Ser727) were clearly downregulated in the tumors treated with NCX-4040 and cisplatin, when compared to NCX-4040 or aspirin alone groups (Figure 6A). The combination treatment also downregulated the cell-

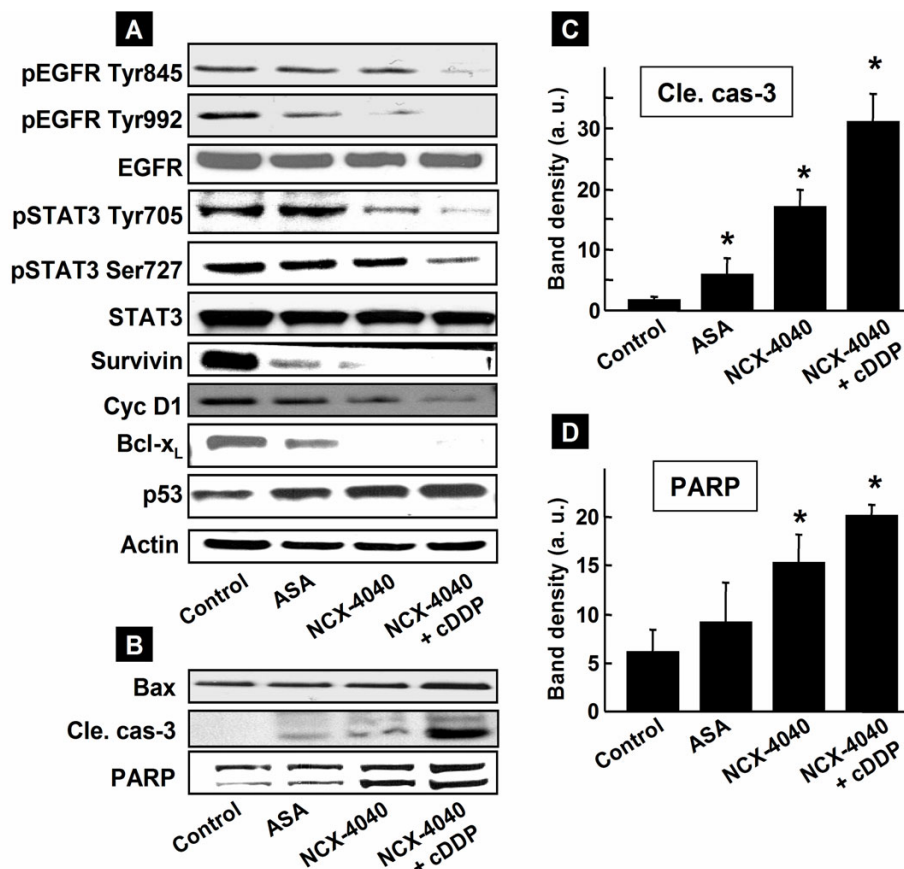


Figure 6

Immunoblot analysis of tumor tissue. (A) Representative images of EGFR/PI3K and STAT3 signaling pathways. EGFR, pEGFR, pSTAT3, STAT3, cyclin D1, Bcl-x_L, survivin, p53, actin; (B) images for Bax, cleaved caspase-3, and PARP proteins. (C) Analysis of cleaved caspase-3 protein band showing an increase of cleaved caspase-3 in cisplatin-resistant tumor treated with aspirin (ASA) or NCX-4040 (5 mg/kg, daily), and NCX-4040 (5 mg/kg, daily) with cisplatin (cDDP, 8 mg/kg, single dose). (D) Analysis of PARP protein band showing an increase of PARP in cisplatin-resistant tumor treated with aspirin (ASA), NCX-4040, and NCX-4040 with cisplatin (cDDP). **p* < 0.05 versus untreated (Control) group.

survival proteins including survivin, cyclin D1, Bcl-x_L, and upregulated the p53 protein. The expression of the proapoptotic protein Bax and apoptotic markers, including cleaved caspase-3 and PARP, were upregulated in the co-treatment group (Figure 6B–D). The results suggested that administration of NCX-4040, in combination with cisplatin, inhibited tumor growth by inducing apoptosis through downregulation of EGFR and STAT3 signaling in the cisplatin-resistant ovarian cancer xenograft in mice.

Discussion

NCX-4040 belongs to a class of nitric oxide-releasing, non-steroidal anti-inflammatory drugs (NO-NSAID) that were originally developed to overcome the limitation of classical NSAIDs [36]. The NCX-4040 molecule contains an aspirin backbone attached to a -ONO₂ moiety linked via an aromatic molecular spacer. The -ONO₂ group, which is responsible for the release of NO, has been shown previously to be required for its anticancer activity [20-22,26]. NCX-4040 is related to NCX-4016 by a positional variation of the -ONO₂ group; whereas the -ONO₂ group is at the *para* position in NCX-4040, it is at *meta* position in NCX-4016. The positional isomers, including the *ortho* version (NCX-4060), have been shown to be substantially different in their anticancer efficacies in HT-29 human colon cancer and pancreatic cancer cell lines [20,21,26]. The *ortho*- and *para*-isomers showed similar IC₅₀ values (1–5 μM) for cell-growth inhibition over 72 h, whereas the IC₅₀ of the *meta*-isomer was 200 to 500 μM. A structure-activity study of the three isomers also revealed that substituting an aliphatic for the aromatic spacer or removing the -ONO₂ group profoundly diminished their ability to inhibit cell growth, whereas removal of the acetyl group on the aspirin moiety did not affect cell growth inhibition [20,21,26]. In addition, the three positional isomers exhibited substantial differences in their metabolism and these differences correlated with their differential effects on cancer cell growth, underscoring the importance of positional isomerism in modulating drug effects [20,21,26].

Cisplatin is a widely used chemotherapeutic agent against a number of malignancies, including ovarian cancer [37]. However, development of drug-resistance following prolonged treatment or up on relapse of the disease is an undesirable effect [1]. There is a huge volume of published data that implicates intracellular glutathione as an important factor in cisplatin resistance. Overexpression of glutathione-S-transferase enzymes, which catalyze coupling of glutathione to multiple reactive substrates, has also been correlated with low response rate to cisplatin

therapy [38-40]. The glutathione-mediated cisplatin resistance has been shown to occur through a number of mechanisms including decreased cellular levels of cisplatin [8], inactivation of cisplatin by formation of glutathione-cisplatin adduct [10-12], increased level of DNA repair and DNA tolerance [12,13], and inhibition of some of the mitochondrial alterations associated with apoptotic commitment [41]. Our results demonstrated a 4.7-fold depression of thiol levels in A2780 cDDP tumor up on treatment with NCX-4040 (Figure 5). This, presumably, was responsible for the resensitization of the tumor to cisplatin, resulting in a significant reduction (56.4%) in tumor volume (Figure 4). The mechanism by which NCX-4040 depletes thiol, however, is not well understood. Gao et al. showed the involvement of GSH and glutathione-S-transferase in the biotransformation of NCX-4040 in the cytosolic fraction of rat liver and colon tissues and in intact HT-29 human colon cancer cells [20]. Other possible mechanisms of thiol depletion might include the thiol-dependent metabolic conversion of the nitrate group (-ONO₂) to NO in cells and/or formation of nitrosothiols. For example, formation of S-nitrosoglutathione (GSNO) from GSH and NO has been shown to result in a dramatic decrease in GSH levels in activated neutrophils [42]. GSNO itself has been shown to be involved in the modulation of the activity of various enzymes including glutathione-S-transferase [43] and glutathione reductase [44], which is responsible for maintaining high intracellular concentrations of GSH. There is also a recent report which shows that the reaction of other metabolites of nitroaspirin with GSH responsible for thiol depletion [45].

While the cytotoxic effect of NCX-4040 against cisplatin-sensitive (A2780 WT) cells was comparable to that of cisplatin under the conditions used, its effect was significantly higher in the resistant (A2780 cDDP) cells (Figure 2). This indicates that the anticancer potential of NCX-4040 is not inhibited, but in fact potentiated to some extent, by the elevated thiol concentrations in the resistant cells. The substantially higher cytotoxicity induced by the co-administration of NCX-4040 and cisplatin seems to suggest a possible synergistic effect, presumably due to NCX-mediated thiol depletion (Figure 5). Overall, the results demonstrated a dual role for NCX-4040 in the inhibition of drug-resistant ovarian tumor growth by inducing NO-mediated cytotoxicity and resensitizing the refractory cells to cisplatin. Further work is necessary to elucidate their synergistic behavior.

In a previous study, we showed that the cytotoxic effect of NCX-4016 was due to its slow metabolism leading to NO release in cells [18]. We also showed that treatment of cisplatin-resistant ovarian cancer cells with NCX-4016 depleted intracellular thiol levels [18]. In the present

work, we have shown thiol content-dependent generation (time-course) NO in cells (Figure 3) and substantial depletion of thiols, as well as total redox, in the tumor tissue (Figure 5). The results clearly demonstrated a cause/effect advantage of thiol utilization/depletion by NCX-4040-mediated NO generation in the cisplatin-resistant ovarian carcinoma. Thus, we have shown that the anti-tumoral and cisplatin-sensitization activities of NCX-4040 were closely linked to its capacity to release NO in the ovarian cancer cells. This is particularly important in the context of a recent report that formation of a quinone methide intermediate from *para*- (NCX-4040) and *ortho*- (NCX-4060) nitrospirin seems to be responsible for biological activity of these compounds [45-47]. According to this report, the ASA derivative generates the *p*-quinone and *o*-quinone methides, which rapidly react with GSH and activate the antioxidant responsive element (ARE) and NAD(P)H-dependent quinone oxidoreductase (NQO1), probably via reaction with thiol-rich sensor proteins [47]. In general, the metabolism of the meta-form of nitrospirin (NCX-4016) was slower. NCX-4016 could not form a quinone, but ARE activation and NQO1 induction were observed with slower reaction with thiol-rich proteins [47].

Epidermal growth factor receptor (EGFR) is over-expressed in a wide variety of epithelial malignancies including non-small cell lung, head and neck, colon, and breast cancers [48]. Over-expression and increased activity of EGFR are key characteristics of human tumors and are frequently linked to more aggressive tumor behaviors, including increased proliferation, metastasis, and therapeutic resistance [49]. As such, EGFR is considered to be a key therapeutic target for human cancers [50]. Recent studies have shown that activation of EGFR and STAT3 (signal transducers and activators of transcription 3) confers resistance to chemotherapy-induced apoptosis in epithelial malignancies [49,51]. STAT3 can be activated by various protein tyrosine kinases including Janus tyrosine kinases (JAK) and the proto-oncogene tyrosine kinase (Src), as well as membrane-bound growth factor receptor tyrosine kinases such as EGFR [52]. Many synthetic compounds that inhibit phosphorylated STAT3 and EGFR tyrosine kinase activity have been shown to be effective in preclinical studies and in clinical trials of advance stages of non-small cell lung cancer, breast, and ovarian cancer [53-55]. However, a direct and consistent link between the EGFR and iNOS/NO pathways has not been established. For example, while EGF was shown to induce iNOS expression in normal astrocytes and head and neck squamous cell carcinomas [56], such a regulation was not found in other cell systems. In ovarian carcinoma cells, both exogenously applied NO and endogenously synthesized NO have been shown to inhibit tumor growth [57]. Recently, we have shown that NCX-4016 suppressed the

growth of cisplatin-resistant human ovarian xenograft tumor (A2780 cDDP) in mice by inducing G₁ cell-cycle arrest and apoptosis through inhibition of EGFR/PI3K and STAT3 signaling pathways [19]. In the present study, we also observed that treatment with NCX-4040 led to down-regulation of EGFR and STAT3 levels. The blockage of EGFR and STAT3-signaling cascades seemingly increased the levels of p53, Bax and decreased levels of Bcl-x_L, survivin, and cyclin D1 proteins. This, in turn, inhibited cellular proliferation, and induced apoptosis in cisplatin-resistant tumors. STAT3 is constitutively activated in a variety of tumor cell types including A2780 cDDP ovarian cancer cells [58]. The constitutively active STAT3 has been implicated in the induction of resistance to apoptosis, possibly through the expression of survivin, Bcl-2 and Bcl-x_L [55]. Bcl-x_L, which is regulated by both STAT3 Tyr705 and Ser727, is known to be overexpressed in cells resistant to chemotherapy [51,58]. It has been shown that blockage of EGFR and STAT3 cascades resulted in the inhibition of cancer cell proliferation and growth of prostate and liver cancer via cell cycle arrest and apoptosis [50,53].

Conclusion

The present study demonstrated that NCX-4040 is not only cytotoxic to human ovarian cancer cells, but is also capable of resensitizing drug-resistant, recurrent tumors to cisplatin treatment. NCX-4040 is capable of releasing nitric oxide and depleting thiols in human ovarian cancer cells. NCX-4040 can resensitize and potentiate the anti-cancer effect of cisplatin through downregulation of EGFR and STAT3 signaling in cisplatin-resistant human ovarian tumor xenografts in mice. Thus, NCX-4040 appears to be a potential therapeutic agent for the treatment of human ovarian carcinoma.

Abbreviations

cDDP – *cis*-diamminedichloroplatinum; cisplatin; DAF-FM DA – 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate; EPR – electron paramagnetic resonance; EPRI – electron paramagnetic resonance imaging; GSH – L-glutamyl-L-cysteinylglycine; glutathione; MGD – N-methyl-D-glucamine dithiocarbamate; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCX-4040 – 2-(acetyloxy)benzoic acid 4-(nitrooxymethyl)phenyl ester; nitrospirin; NCX-4016 – 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester; nitrospirin; NSAID – non-steroidal anti-inflammatory drug; SNAP – S-Nitroso-N-acetylpenicillamine; R₁S-SR₁ – bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)disulfide; R₂S-SR₂ – bis((2,2,3,5,5-pentamethyl-1-oxyl-imidazolidinyl-4)-methyl)-disulfide.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AB performed the *in vitro* and *in vivo* experiments, data analysis, and writing of the manuscript. KS was responsible for immunoblotting experiments and data analysis. TW and AB analyzed the redox imaging data. VVK provided the thiol-specific probe. LJI participated in the study design. PK was involved in the study design and write up of the manuscript. All authors read and approved the final manuscript.

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References

- Harries M, Gore M: **Part I: chemotherapy for epithelial ovarian cancer-treatment at first diagnosis.** *Lancet Oncol* 2002, **3(9)**:529-536.
- Harries M, Gore M: **Part II: chemotherapy for epithelial ovarian cancer-treatment of recurrent disease.** *Lancet Oncol* 2002, **3(9)**:537-545.
- Yao X, Panichpisal K, Kurtzman N, Nugent K: **Cisplatin nephrotoxicity: a review.** *Am J Med Sci* 2007, **334(2)**:115-124.
- Screnci D, McKeage MJ: **Platinum neurotoxicity: clinical profiles, experimental models and neuroprotective approaches.** *J Inorg Biochem* 1999, **77(1-2)**:105-110.
- Rybak LP, Whitworth CA, Mukherjee D, Ramkumar V: **Mechanisms of cisplatin-induced ototoxicity and prevention.** *Hear Res* 2007, **226(1-2)**:157-167.
- Guastalla JP 3rd, Dieras V: **The taxanes: toxicity and quality of life considerations in advanced ovarian cancer.** *Br J Cancer* 2003, **89 Suppl 3**:S16-22.
- Johnson SW, Ozols RF, Hamilton TC: **Mechanisms of drug resistance in ovarian cancer.** *Cancer* 1993, **71(2 Suppl)**:644-649.
- Kartalou M, Essigmann JM: **Mechanisms of resistance to cisplatin.** *Mutat Res* 2001, **478(1-2)**:23-43.
- Agarwal R, Kaye SB: **Ovarian cancer: strategies for overcoming resistance to chemotherapy.** *Nat Rev Cancer* 2003, **3(7)**:502-516.
- Eastman A: **Cross-linking of glutathione to DNA by cancer chemotherapeutic platinum coordination complexes.** *Chem Biol Interact* 1987, **61(3)**:241-248.
- Ishikawa T: **The ATP-dependent glutathione S-conjugate export pump.** *Trends Biochem Sci* 1992, **17(11)**:463-468.
- Rabik CA, Dolan ME: **Molecular mechanisms of resistance and toxicity associated with platinating agents.** *Cancer Treat Rev* 2007, **33(1)**:9-23.
- Kelland L: **The resurgence of platinum-based cancer chemotherapy.** *Nat Rev Cancer* 2007, **7(8)**:573-584.
- Lewandowicz GM, Britt P, Elgie AW, Williamson CJ, Coley HM, Hall AG, Sargent JM: **Cellular glutathione content, in vitro chemoresponse, and the effect of BSO modulation in samples derived from patients with advanced ovarian cancer.** *Gynecologic oncology* 2002, **85(2)**:298-304.
- Liebmann JE, Hahn SM, Cook JA, Lipschultz C, Mitchell JB, Kaufman DC: **Glutathione depletion by L-buthionine sulfoximine antagonizes taxol cytotoxicity.** *Cancer Res* 1993, **53(9)**:2066-2070.
- Rudin CM, Yang Z, Schumaker LM, VanderWeele DJ, Newkirk K, Egorin MJ, Zuhowski EG, Cullen KJ: **Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance.** *Cancer Res* 2003, **63(2)**:312-318.
- Schnelldorfer T, Gansauge S, Gansauge F, Schlosser S, Beger HG, Nussler AK: **Glutathione depletion causes cell growth inhibition and enhanced apoptosis in pancreatic cancer cells.** *Cancer* 2000, **89(7)**:1440-1447.
- Bratasz A, Weir NM, Parinandi NL, Zweier JL, Sridhar R, Ignarro LJ, Kuppusamy P: **Reversal to cisplatin sensitivity in recurrent human ovarian cancer cells by NCX-4016, a nitro derivative of aspirin.** *Proc Natl Acad Sci U S A* 2006, **103(10)**:3914-3919.
- Selvendiran K, Bratasz A, Tong L, Ignarro LJ, Kuppusamy P: **NCX-4016, a nitro-derivative of aspirin, inhibits EGFR and STAT3 signaling and modulates Bcl-2 proteins in cisplatin-resistant human ovarian cancer cells and xenografts.** *Cell Cycle* 2008, **7(1)**:81-88.
- Gao J, Kashfi K, Rigas B: **In vitro metabolism of nitric oxide-donating aspirin: the effect of positional isomerism.** *J Pharmacol Exp Ther* 2005, **312(3)**:989-997.
- Rosetti M, Tesei A, Ulivi P, Fabbri F, Vannini I, Brigliadori G, Amadori D, Bolla M, Zoli W: **Molecular characterization of cytotoxic and resistance mechanisms induced by NCX 4040, a novel NO-NSAID, in pancreatic cancer cell lines.** *Apoptosis* 2006, **11(8)**:1321-1330.
- Fabbri F, Brigliadori G, Ulivi P, Tesei A, Vannini I, Rosetti M, Bravaccini S, Amadori D, Bolla M, Zoli W: **Pro-apoptotic effect of a nitric oxide-donating NSAID, NCX 4040, on bladder carcinoma cells.** *Apoptosis* 2005, **10(5)**:1095-1103.
- Leonetti C, Scarsella M, Zupi G, Zoli W, Amadori D, Medri L, Fabbri F, Rosetti M, Ulivi P, Ceconetto L, Bolla M, Tesei A: **Efficacy of a nitric oxide-releasing nonsteroidal anti-inflammatory drug and cytotoxic drugs in human colon cancer cell lines in vitro and xenografts.** *Mol Cancer Ther* 2006, **5(4)**:919-926.
- Tesei A, Ulivi P, Fabbri F, Rosetti M, Leonetti C, Scarsella M, Zupi G, Amadori D, Bolla M, Zoli W: **In vitro and in vivo evaluation of NCX 4040 cytotoxic activity in human colon cancer cell lines.** *J Transl Med* 2005, **3(1)**:7.
- Tesei A, Rosetti M, Ulivi P, Fabbri F, Medri L, Vannini I, Bolla M, Amadori D, Zoli W: **Study of molecular mechanisms of pro-apoptotic activity of NCX 4040, a novel nitric oxide-releasing aspirin, in colon cancer cell lines.** *J Transl Med* 2007, **5(1)**:52.
- Kashfi K, Borgo S, Williams JL, Chen J, Gao J, Glekas A, Benedini F, Del Soldato P, Rigas B: **Positional isomerism markedly affects the growth inhibition of colon cancer cells by nitric oxide-donating aspirin in vitro and in vivo.** *J Pharmacol Exp Ther* 2005, **312(3)**:978-988.
- Khrantsov VV, Yelinova VI, Glazachev Yu I, Reznikov VA, Zimmer G: **Quantitative determination and reversible modification of thiols using imidazolidine biradical disulfide label.** *J Biochem Biophys Methods* 1997, **35(2)**:115-128.
- Kuppusamy P, Wang P, Samouilov A, Zweier JL: **Spatial mapping of nitric oxide generation in the ischemic heart using electron paramagnetic resonance imaging.** *Magn Reson Med* 1996, **36(2)**:212-218.
- Andrews PA, Jones JA, Varki NM, Howell SB: **Rapid emergence of acquired cis-diamminedichloroplatinum(II) resistance in an in vivo model of human ovarian carcinoma.** *Cancer communications* 1990, **2(2)**:93-100.
- Kern DH, Morgan CR, Hildebrand-Zanki SU: **In vitro pharmacodynamics of I-beta-D-arabinofuranosylcytosine: synergy of antitumor activity with cis-diamminedichloroplatinum(II).** *Cancer Res* 1988, **48(1)**:117-121.
- Khrantsov VV, Yelinova VI, Weiner LM, Berezina TA, Martin VV, Volodarsky LB: **Quantitative determination of SH groups in low- and high-molecular-weight compounds by an electron spin resonance method.** *Anal Biochem* 1989, **182(1)**:58-63.
- Kuppusamy P, Afeworki M, Shankar RA, Coffin D, Krishna MC, Hahn SM, Mitchell JB, Zweier JL: **In vivo electron paramagnetic resonance imaging of tumor heterogeneity and oxygenation in a murine model.** *Cancer Res* 1998, **58(7)**:1562-1568.
- Ilangovan G, Bratasz A, Li H, Schmalbrock P, Zweier JL, Kuppusamy P: **In vivo measurement and imaging of tumor oxygenation using coembedded paramagnetic particulates.** *Magn Reson Med* 2004, **52(3)**:650-657.
- Komarov A, Mattson D, Jones MM, Singh PK, Lai CS: **In vivo spin trapping of nitric oxide in mice.** *Biochem Biophys Res Commun* 1993, **195(3)**:1191-1198.
- Kuppusamy P, Li H, Ilangovan G, Cardounel AJ, Zweier JL, Yamada K, Krishna MC, Mitchell JB: **Noninvasive imaging of tumor redox status and its modification by tissue glutathione levels.** *Cancer Res* 2002, **62(1)**:307-312.
- del Soldato P, Sorrentino R, Pinto A: **NO-aspirins: a class of new anti-inflammatory and antithrombotic agents.** *Trends Pharmacol Sci* 1999, **20(8)**:319-323.
- Cohen SM, Lippard SJ: **Cisplatin: from DNA damage to cancer chemotherapy.** *Prog Nucleic Acid Res Mol Biol* 2001, **67**:93-130.

38. Nishimura T, Newkirk K, Sessions RB, Andrews PA, Trock BJ, Rasmussen AA, Montgomery EA, Bischoff EK, Cullen KJ: **Immunohistochemical staining for glutathione S-transferase predicts response to platinum-based chemotherapy in head and neck cancer.** *Clin Cancer Res* 1996, **2(11)**:1859-1865.
39. Shiga H, Heath EI, Rasmussen AA, Trock B, Johnston PG, Forastiere AA, Langmacher M, Baylor A, Lee M, Cullen KJ: **Prognostic value of p53, glutathione S-transferase pi, and thymidylate synthase for neoadjuvant cisplatin-based chemotherapy in head and neck cancer.** *Clin Cancer Res* 1999, **5(12)**:4097-4104.
40. Nishimura T, Newkirk K, Sessions RB, Andrews PA, Trock BJ, Rasmussen AA, Montgomery EA, Bischoff EK, Hanigan MH, Cullen KJ: **Association between expression of glutathione-associated enzymes and response to platinum-based chemotherapy in head and neck cancer.** *Chem Biol Interact* 1998, **111-112**:187-198.
41. Hockenbery DM, Oltvai ZN, Yin XM, Millman CL, Korsmeyer SJ: **Bcl-2 functions in an antioxidant pathway to prevent apoptosis.** *Cell* 1993, **75(2)**:241-251.
42. Clancy RM, Levartovsky D, Leszczynska-Piziak J, Yegudin J, Abramson SB: **Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrophils: evidence for S-nitrosoglutathione as a bioactive intermediary.** *Proc Natl Acad Sci U S A* 1994, **91(9)**:3680-3684.
43. Clark AG, Debnam P: **Inhibition of glutathione S-transferases from rat liver by S-nitroso-L-glutathione.** *Biochem Pharmacol* 1988, **37(16)**:3199-3201.
44. Becker K, Gui M, Schirmer RH: **Inhibition of human glutathione reductase by S-nitrosoglutathione.** *Eur J Biochem* 1995, **234(2)**:472-478.
45. Hulsman N, Medema JP, Bos C, Jongejan A, Leurs R, Smit MJ, de Esch J, Richel D, Wijtman M: **Chemical insights in the concept of hybrid drugs: the antitumor effect of nitric oxide-donating aspirin involves a quinone methide but not nitric oxide nor aspirin.** *J Med Chem* 2007, **50(10)**:2424-2431.
46. Kashfi K, Rigas B: **The mechanism of action of nitric oxide-donating aspirin.** *Biochem Biophys Res Commun* 2007, **358(4)**:1096-1101.
47. Dunlap T, Chandrasena RE, Wang Z, Sinha V, Wang Z, Thatcher GR: **Quinone formation as a chemoprevention strategy for hybrid drugs: balancing cytotoxicity and cytoprotection.** *Chem Res Toxicol* 2007, **20(12)**:1903-1912.
48. Salomon DS, Brandt R, Ciardiello F, Normanno N: **Epidermal growth factor-related peptides and their receptors in human malignancies.** *Crit Rev Oncol Hematol* 1995, **19(3)**:183-232.
49. Coley HM, Shotton CF, AJose-Adeogun A, Modjtahedi H, Thomas H: **Receptor tyrosine kinase (RTK) inhibition is effective in chemosensitising EGFR-expressing drug resistant human ovarian cancer cell lines when used in combination with cytotoxic agents.** *Biochem Pharmacol* 2006, **72(8)**:941-948.
50. Woodworth CD, Michael E, Marker D, Allen S, Smith L, Nees M: **Inhibition of the epidermal growth factor receptor increases expression of genes that stimulate inflammation, apoptosis, and cell attachment.** *Mol Cancer Ther* 2005, **4(4)**:650-658.
51. Duan Z, Foster R, Bell DA, Mahoney J, Wolak K, Vaidya A, Hampel C, Lee H, Seiden MV: **Signal transducers and activators of transcription 3 pathway activation in drug-resistant ovarian cancer.** *Clin Cancer Res* 2006, **12(17)**:5055-5063.
52. Lo HW, Hsu SC, Ali-Seyed M, Gunduz M, Xia W, Wei Y, Bartholomew G, Shih JY, Hung MC: **Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway.** *Cancer Cell* 2005, **7(6)**:575-589.
53. Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur U, Nair AS, Shishodia S, Aggarwal BB: **Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappaB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells.** *Blood* 2007, **109(6)**:2293-2302.
54. Choudhari SR, Khan MA, Harris G, Picker D, Jacob GS, Block T, Shailubhai K: **Deactivation of Akt and STAT3 signaling promotes apoptosis, inhibits proliferation, and enhances the sensitivity of hepatocellular carcinoma cells to an anticancer agent, Atiprimod.** *Mol Cancer Ther* 2007, **6(1)**:112-121.
55. Selvendiran K, Koga H, Ueno T, Yoshida T, Maeyama M, Torimura T, Yano H, Kojiro M, Sata M: **Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids.** *Cancer Res* 2006, **66(9)**:4826-4834.
56. Torroglosa A, Murillo-Carretero M, Romero-Grimaldi C, Matarredona ER, Campos-Caro A, Estrada C: **Nitric oxide decreases subventricular zone stem cell proliferation by inhibition of epidermal growth factor receptor and phosphoinositide-3-kinase/Akt pathway.** *Stem cells (Dayton, Ohio)* 2007, **25(1)**:88-97.
57. Rieder J, Jahnke R, Schloesser M, Seibel M, Czechowski M, Marth C, Hoffmann G: **Nitric oxide-dependent apoptosis in ovarian carcinoma cell lines.** *Gynecologic oncology* 2001, **82(1)**:172-176.
58. Yu H, Jove R: **The STATs of cancer--new molecular targets come of age.** *Nat Rev Cancer* 2004, **4(2)**:97-105.

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