Reversal to cisplatin sensitivity in recurrent human ovarian cancer cells by NCX-4016, a nitro derivative of aspirin


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Ovarian cancer is a gynecological malignancy that is commonly treated by cytoreductive surgery followed by cisplatin treatment. However, the cisplatin treatment, although successful initially, is not effective in the treatment of the recurrent disease that invariably surfaces within a few months of the initial treatment. The refractory behavior is attributed to the increased levels of cellular thiols apparently caused by the cisplatin treatment. This observation prompted us to choose a cytotoxic drug whose activity is potentiated by cellular thiols with enhanced specificity toward the thiol-rich cisplatin-resistant cells. We used NCX-4016 [2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester], a derivative of aspirin containing a nitro group that releases nitric oxide in a sustained fashion for several hours in cells and in vivo, and we studied its cytotoxic efficacy against human ovarian cancer cells (HOCCs). Cisplatin-sensitive and cisplatin-resistant (CR) HOCCs were treated with 100 μM NCX-4016 for 6 h, and/or 0.5 μg/ml cisplatin for 1 h and assayed for clonogenecity. NCX-4016 significantly reduced the surviving fractions of cisplatin-sensitive (63 ± 6%) and CR (70 ± 10%) HOCCs. Treatment of cells with NCX-4016 followed by cisplatin showed a significantly greater extent of toxicity when compared with treatment of cells with NCX-4016 or cisplatin alone. In conclusion, this study showed that NCX-4016 is a potential inhibitor of the proliferation of CR HOCCs and thus might specifically kill cisplatin-refractory cancer cells in patients with recurrent ovarian cancer.

cisplatin resistance | glutathione | nitric oxide | cytotoxicity | nonsteroidal antiinflammatory drug

Ovarian cancer is the second most commonly diagnosed gynecological malignancy and the fourth leading cause of mortality among women in the United States (1). The high mortality rate is attributed to the lack of early diagnosis of the malignancy and difficulties associated with treatment. The standard treatment includes cytoreductive surgery followed by the administration of chemotherapeutic agents. Platinum-based compounds (e.g., cisplatin and carboplatin) in combination with taxanes (e.g., taxol or paclitaxel), anthracyclines (e.g., doxorubicin), or alkylating agents (e.g., melphalan) are administered to treat the advanced-stage disease (2). However, the overall clinical response with such treatments is only 40–60%. The primary factor that limits the success of chemotherapy in ovarian cancer is the acquired drug resistance in the tumor cell population. Administration of cisplatin results in the development of drug resistance in the cancer cells. Postulated mechanisms of drug resistance include decreased intracellular accumulation of the drug, increased levels of thiols, which inactivate the platinum compound, and the enhancement of DNA repair (3). Even a slight increase in the cisplatin resistance of the tumor may pose a clinically important problem requiring administration of large doses of the drug, which may lead to severe multiorgan toxicities (4). Therefore, a new adjuvant treatment is necessary to specifically overcome the thiol-mediated drug resistance in the treatment of recurrent ovarian cancer.

Recently, Ignarro and coworkers (5, 6) showed that nitric oxide (NO) is capable of inhibiting the proliferation of rat aortic smooth muscle cells in a cGMP-independent pathway. The cytostatic action of NO was attributed to its inhibitory effect on ornithine decarboxylase, an enzyme that generates spermine, spermidine, and putrescine, which are required for cell proliferation. Earlier, Wink et al. (7) showed enhanced cytotoxicity of cisplatin in Chinese hamster V79 lung fibroblast cells pretreated with NO donors in a time-dependent manner. The enhancement of cisplatin cytotoxicity by pretreatment with diethylamine/NO (DEA/NO) and (propylamino)propylamine/NO (PAPA/NO) persisted up to 2 and 3 h, respectively, and thereafter the cytotoxicity of the drug returned to a level that was exhibited by the cisplatin treatment alone. In a more recent study by Riganti et al. (8), it was shown that NO reverted resistance to doxorubicin in a multidrug-resistant human epithelial colon cancer cell population by increasing the drug accumulation inside the cells in a cGMP-independent pathway. Thus, it appeared that NO-releasing drugs may have potential cytostatic effects on proliferating cells. However, it was not known whether the NO donors had a selective effect on cancer cells and what factors influenced the targeting of a particular cancer cell.

Cellular thiols have been shown to be involved in the mechanism of action of certain NO donor molecules. For example, it was reported that NO donors such as glyceryl trinitrate (GTN) and sodium nitroprusside induced cytotoxicity and mutations in Mutatet cancer cell lines and that the extent of mutagenicity was dependent on the cellular glutathione (GSH) levels (9). Chen et al. (10) identified the enzymatic bioactivation of GTN in mouse macrophage RAW cells mediated by mitochondrial aldehyde dehydrogenase that generates nitrite and, subsequently, NO. It has been suggested that thiols (2-mercaptoethanol or DTT) were required for this conversion. These observations prompted us to hypothesize that the thiol-rich environment in cisplatin-resistant (CR) ovarian cancer cells may potentiate NO production from a similar nitrate-based drug and may exert...
preferential cytostatic effect. The elevated levels of thiols in the CR cells presumably may increase the production of NO from a donor drug and potentiate the tumoricidal efficacy of cisplatin.

The aim of the present work was to test the potency of a novel NO-releasing compound, NCX-4016 [2-(acetyloxy)benzoic acid 3-nitrooxymethyl] phenyl ester (Fig. 1), in inducing cytotoxicity in human ovarian cancer cells (HOCCs) with a particular emphasis on CR cells. NCX-4016, a nitro derivative of aspirin (nitosalicylate), had been shown to generate NO in human umbilical vein endothelial cells (11). Our hypothesis was that the sustained release of NO by nitroaspirin in the thiol-rich ovarian cancer cells would decrease cell survival and proliferation. We further hypothesized that the cytotoxicity of NCX-4016 might augment the effect of cisplatin by decreasing the thiol content in the cells. We tested our hypothesis by performing cell-survival (clonogenic) assays on cisplatin-sensitive (CS) and CR HOCCs treated with NCX-4016 and cisplatin. The results showed that NCX-4016 was a potent inhibitor of proliferation of ovarian cancer cells and further suggested that this drug can be used in combination with cisplatin for treatment of advanced human ovarian cancer.

Results

Effect of Cisplatin on the Clonogenicity of CS and CR Cells. The integrity of the CS and CR ovarian cancer cell lines, as well as their IC50 dose of cisplatin, were established by determining the effect of cisplatin on the colony-forming ability of the cells. Cells were treated with varying doses of cisplatin (0–30 μg/ml) for 1 h, and clonogenic assays were performed as described in Materials and Methods. Fig. 2 shows the surviving fractions of CS and CR cells as a function of cisplatin dose. The surviving fraction of CS cells was markedly reduced by cisplatin even at a low dose of the drug (for example, 8% survival at 1 μg/ml). The proliferation of the CS cells was almost completely inhibited by cisplatin at a dose of 5 μg/ml. The IC50 dose of cisplatin for the CR cells was 0.6 μg/ml. On the other hand, cisplatin exerted lower cytotoxicity on CR cells. The observed IC50 dose of cisplatin for the CR cells, 16 μg/ml, was ~25 times higher than that for the CS cells. The results clearly established that the CR cells were resistant to cisplatin treatment and that the CS cells were sensitive to cisplatin treatment.

Measurement of NO Generation in NCX-4016-Treated Cells. Although NCX-4016 has been shown to generate NO in human umbilical vein endothelial cells (11), it was not known whether it could be capable of generating NO in HOCCs. We used EPR spectroscopy to detect the generation of NO in HOCCs treated with NCX-4016. CR cells (2 × 106 cells per milliliter) were incubated with 500 μM NCX-4016, and EPR measurements were performed by adding a solution of iron-N-methyl-d-glucamine dithiocarbamate (Fe-MGD), a water-soluble NO trap that forms a stable EPR-detectable paramagnetic complex, Fe(MGD)2-NO. After 25 min of incubation of cells with NCX-4016, a characteristic triplet spectrum was observed, which continued to increase up to 90 min (Fig. 3). NCX-4016 alone or cells without NCX-4016 treatment did not show the characteristic NO spectrum upon treatment with Fe-MGD. The authenticity of the triplet EPR spectrum was established by measuring the EPR spectrum in a freshly mixed solution containing Fe-MGD and Δ-nitroso-N-acetylpenicillamine (SNAP), an NO-donor (Fig. 3). The triplet spectrum observed in the cells treated with NCX-4016 was identical to that observed in the SNAP control. Thus, the EPR data showed that NO was generated by NCX-4016 in HOCCs.
significant effect compared with the control (data not shown). We observed that NCX-4016 treatment for 1 h did not show any surviving fractions were evaluated after 7 days in culture. It was assayed for their clonogenecity after 7 days. Data represent mean /SEM obtained from 4–10 independent experiments and normalized to the value of the corresponding control cells. The data showed that NCX-4016 exhibited a dose-dependent cytotoxicity in both the CS and CR cells.

Effect of NCX-4016 on the Clonogeneity of HOCCs. To assess the effect of NCX-4016 on the proliferation of HOCCs, clonogenic cell-survival assays on CR and CS cells treated with NCX-4016 were performed. Fig. 4 shows the surviving fractions of the CS and CR cells as a function of NCX-4016 dose at 6 h of drug incubation followed by 7 days in culture. The surviving fractions of both the CS and CR cells were significantly (* P < 0.001) reduced by NCX-4016 in a dose-dependent fashion. Although the cytotoxic effect of NCX-4016 was consistently greater on CR cells compared with that on CS cells, the difference was not statistically significant.

We also studied the effect of exposure time of cells to NCX-4016 on the surviving fraction of cells. The cells were incubated for 1 or 6 h with 100 μM NCX-4016, and their surviving fractions were evaluated after 7 days in culture. It was observed that NCX-4016 treatment for 1 h did not show any significant effect compared with the control (data not shown). However, the same treatment for 6 h showed ~30% reduction in the number of surviving colonies of both CS and CR cells.

To check whether the cytotoxicity of NCX-4016 was caused by the aspirin that arises from the metabolic cleavage of NCX-4016, we performed the clonogenecity assay in the presence of 100 μM aspirin and found that aspirin alone did not cause significant cytotoxic effect (Fig. 5). This suggested that cytotoxicity depended on the nitrate moiety of the drug.

We also verified whether the cytotoxic effect of NCX-4016 was due to its slow NO-releasing ability in cells. We performed additional experiments with 3-morpholinosydnonimine (SIN-1), which released NO in a relatively short time (~10 min), in aqueous solutions, under physiological conditions (12). Treatment of the CR cells with 100 μM SIN-1 did not alter the surviving fraction (Fig. 5), suggesting that, at least in the case of CR cells, long-term exposure to NO was required for the observed cytotoxic effect.

Effect of NCX-4016 on the Cytotoxicity of Cisplatin. We further investigated the effect of NCX-4016 on cisplatin-induced cytotoxicity to HOCCs. CR and CS cells were treated with 100 μM NCX-4016 for 6 h, followed by 0.5 μg/ml cisplatin for 1 h, and then were assayed after 7 days of treatment. Fig. 6 shows the surviving fractions of cells treated with cisplatin and/or NCX-4016. The results showed that cisplatin was ineffective in CR cells (90 ± 9% surviving fraction) but caused a significant reduction in the number of colonies in both CS and CR cells. However, NCX-4016 significantly reduced the number of colonies in both CR (70 ± 10%) and CS (63 ± 6%) cells. On the other hand, treatment of cells with NCX-4016 followed by cisplatin treatment caused significantly higher cell killing (CR cells, 52 ± 7%; CS cells, 46 ± 3%) compared with cisplatin or NCX-4016 treatment alone.

We further evaluated the effect of cisplatin and NCX-4016 on the proliferation of a noncancer cell line, namely, Chinese hamster ovary (CHO) cells. The CHO cells were treated with NCX-4016 (100 μM) and/or cisplatin (0.5 μg/ml) as described above. The results showed significantly better cell-killing ability of the drugs in CHO cells compared with CR or CS cells. For example, the combination treatment in CHO cells showed 31 ± 2% survival compared with CS cells, which showed 46 ± 3% survival.

We further investigated whether the cellular concentration of NO during the pretreatment period was important in the potentiation of cisplatin-induced cytotoxicity and performed additional experiments with SIN-1. CR cells treated with SIN-1 (100 μM) for 1 h followed by 0.5 μg/ml cisplatin for 1 h showed survival of 55 ± 5%, which was significantly different from the survival of cells treated with SIN-1 alone (101 ± 3%) (Fig. 5). These results suggested that pretreatment of CR cells with NO potentiated the efficacy of cisplatin in overcoming the acquired drug resistance. The mode of NO generation, however, apparently was not critical for the regained cytotoxic effect of cisplatin.
whereas cells treated with cisplatin did not show any intracellular GSH level in CR cells treated with cisplatin and those of CS cells (data not shown). Fig. 7 shows changes in the thiol levels in CS and CR cells. The cell proliferation assays described above suggested that the NCX-4016 alone was equally cytotoxic to both CS and CR cells and that the cytotoxic efficacy apparently did not depend on the nature of cisplatin sensitivity among cells. This observation was in striking contrast to the results obtained with cisplatin, whose cytotoxic efficacy showed a strong dependence on the nature of cisplatin sensitivity of the cell. Because it was established that cisplatin resistance in HOCCs was primarily governed by elevated levels of GSH, we further determined the thiol content of these cells. The CR cells that we used had 75 ± 10% higher levels of GSH compared with those of CS cells (data not shown). Fig. 7 shows changes in the intracellular GSH level in CR cells treated with cisplatin and NCX-4016. Whereas cells treated with cisplatin did not show any significant change in their thiol content, cells treated with 100 μM NCX-4016 showed a substantially reduced level of GSH (50.3 ± 8.1%). Secondary incubation of the NCX-4016-treated cells with cisplatin did not show any significant change in the GSH level compared with the cells treated with NCX-4016 alone. The data suggested that GSH was involved in the antiproliferative action of NCX-4016 in CR cells.

**Thiol Levels in CS and CR Cells.** The cell proliferation assays described above suggested that the NCX-4016 alone was equally cytotoxic to both CS and CR cells and that the cytotoxic efficacy apparently did not depend on the nature of cisplatin sensitivity among cells. This observation was in striking contrast to the results obtained with cisplatin, whose cytotoxic efficacy showed a strong dependence on the nature of cisplatin sensitivity of the cell. Because it was established that cisplatin resistance in HOCCs was primarily governed by elevated levels of GSH, we further determined the thiol content of these cells. The CR cells that we used had 75 ± 10% higher levels of GSH compared with those of CS cells (data not shown). Fig. 7 shows changes in the intracellular GSH level in CR cells treated with cisplatin and NCX-4016. Whereas cells treated with cisplatin did not show any significant change in their thiol content, cells treated with 100 μM NCX-4016 showed a substantially reduced level of GSH (50.3 ± 8.1%). Secondary incubation of the NCX-4016-treated cells with cisplatin did not show any significant change in the GSH level compared with the cells treated with NCX-4016 alone. The data suggested that GSH was involved in the antiproliferative action of NCX-4016 in CR cells.

**Discussion**

NCX-4016 belongs to a new class of nonsteroidal antiinflammatory drugs (NSAIDs) capable of releasing NO. Unlike conventional NO donors that release NO in aqueous solutions, NCX-4016 releases NO only after intracellular enzymatic conversion (11, 13). The drug has been shown to release sustained amounts of NO in vivo (14, 15). Although the use of NSAIDs is limited by their gastrointestinal (GI) and renal toxicity, NO-releasing NSAIDs were shown to exert antiinflammatory and analgesic effects that were at least as potent as those of the parent drug, without causing GI tract toxicity (16–19). The in vivo metabolism of these drugs has been established reasonably well in a rat model (14, 20), except that the precise mechanism by which NO is released from the nitro moiety has yet to be understood. NCX-4016 is metabolized by esterases in the liver cells and in plasma to salicylic acid and 3-(nitrooxymethyl)phenol, which is rapidly metabolized to 3-hydroxybenzylalcohol and NO (20). The carbon atom of 3-(nitrooxymethyl)phenol can react with GSH to form 1-glutathionyl-3 methylheptadien-2-hydroxybenzene. This process is enzymatically mediated by GSH transferase and is dependent on the availability of GSH (20). Napoli et al. (21, 22) showed that NCX-4016 reduced experimental restenosis, an effect associated with lower vascular smooth muscle cell proliferation. The slow and sustained release of NO seems to be the reason for its nontoxic nature, safe profile, and ability to intervene with the pathological process.

The EPR spectroscopy analysis offered direct evidence that NO was released from NCX-4016 in the CR cells upon treatment with the drug. The absence of an NO adduct signal in cells not treated with NCX-4016 indicated the participation of certain cellular enzyme(s) in the metabolic conversion of NCX-4016. NO was released slowly and continued beyond 90 min of treatment. This is in sharp contrast to many other NO donors that release NO over a short duration (<10 min) and without requiring any enzymatic conversion. The in vivo and in vitro metabolism of NCX-4016 has been recently investigated in rat venous blood by using EPR spectroscopy (14). It was shown that NCX-4016 undergoes rapid metabolism, with the formation of salicylic acid and a nitro moiety, wherein the latter is metabolized to release NO via some unknown mechanism. NO-Fe(II)-Hb, formed by NO reaction with heme proteins, was detectable in the venous blood 1 h after dosing, and its formation was maximal at 4–6 h after dosing (10, 14). However, the exact mechanism and kinetics of the metabolic conversion of NCX-4016 in the cellular environment are not known at present.

The antiproliferative effects of cisplatin on the two currently studied cell lines were reminiscent of results described above. Cisplatin was cytotoxic to HOCCs treated with NCX-4016, and the cytotoxicity of NCX-4016 in CR cells was also comparable with that in the CS cells (Fig. 4), suggesting that NCX-4016 was not subject to the thiol resistance that was observed with cisplatin. The antiproliferative nature of NCX-4016 could be attributed to its ability to generate sustained amounts of NO in the cells. This was further confirmed by the observations that short-term (1 h) treatment of CR cells with NCX-4016 or with SIN-1 failed to induce any significant antiproliferative effect. Furthermore, the fact that cytotoxicity was only observed with NCX-4016 but not with aspirin [acetylsalicylic acid (ASA)] suggested that aspirin failed to contribute to the observed cell killing by the drug.

A recent study reported that NCX-4016 and a few other related NO NSAIDs exerted remarkable cytotoxicity in human cancer cell lines of adenomatous and squamous origins with a wide variety of tissue targets (13). NCX-4016, in particular, was consistently the most potent cytotoxic agent in all cell lines studied, and its effect, unlike that of aspirin, was cyclooxygenase-independent.

In general, it was observed that NCX-4016 was more effective than cisplatin in inducing the cytotoxic effect. This was evident from the current study with CR, CS, and CHO cells (Fig. 6). The efficacy and dose-dependent effect of NCX-4016 in the cancer cells revealed that cellular GSH had no effect on the action of the drug, because a similar extent of cytotoxicity was observed in the two cell lines. It was apparent that NCX-4016 was capable of overcoming the thiol-mediated cytoprotection in CR cells. The thiol data from CR cells suggested that an ∼50% reduction in GSH levels was achieved upon treatment with NCX-4016 (Fig. 7). The thiol depletion could cause cells to bypass their refractory nature and could sensitize them to further treatment with cisplatin. This was achieved by pretreating CR cells with NCX-4016 for 6 h and then treating them with cisplatin for 1 h. The results indicated that substantially enhanced cell killing was obtained by pretreating CR cells with NCX-4016 (Fig. 6). Assuming that NCX-4016 caused a 30% killing of CR cells (Fig. 6) and that cisplatin could cause a 32% cell killing of CS cells, which was presumably similar to the NCX-4016-pretreated...
(thiol-depleted) CR cells, we estimated the effective cell killing to be 52%. This corresponded to a survival of 48%, which agreed closely with the observed survival of 48% in the combined treatment with NCX-4016 and cisplatin of CR cells. Overall, the results suggested a dual role for NCX-4016 in CR cells: anti-proliferative effect and thiol depletion. The thiol depletion creates an opportunity for cisplatin for further cell killing. This observation has important implications in the development of treatment modalities for ovarian cancer.

There are, however, still several questions that remain to be answered. The involvement of GSH in the bioactivation/bioconversion and anti-proliferative action of NCX-4016 in HOCCs has not been clearly established by the present study. The mechanism of the cytotoxic effect of NCX-4016 may be entirely different from that of cisplatin. However, the present results from cells in culture will be useful in studies with animals, in vivo.

In summary, NCX-4016, a nitroaspirin, belongs to a new class of NSAIDs. NCX-4016 releases sustained amounts of NO in cells and in vivo. We investigated the cytotoxic effect of this drug in two HOCC lines: CS cells, which responded to cisplatin treatment, and CR cells, which were refractory to cisplatin treatment apparently because of higher levels of intracellular thiols. The cells were treated with NCX-4016 and/or cisplatin and assayed for clonogenicity. EPR spectroscopy was used to confirm the release of NO in cells treated with NCX-4016. The data showed that treatment of the cancer cells with NCX-4016 for 6 h resulted in a significant reduction in the colony-forming ability of the cells. The CR cells subjected to NCX-4016 treatment showed a more pronounced reduction in the surviving fraction and cellular GSH levels, compared with those subjected to cisplatin treatment. CR cells pretreated with NCX-4016 and then treated with cisplatin showed significantly greater toxicity when compared with those treated with NCX-4016 or cisplatin alone. The thiol-depleting ability of NCX-4016 caused these cells to regain cisplatin sensitivity. Thus, NCX-4016 was a potent inhibitor of the proliferation of CR ovarian cancer cells as well as a sensitizer of CR cells for treatment with cisplatin in patients with recurrent ovarian cancer.

Materials and Methods

Reagents. Nitroaspirin [2-(acetyloxy)benzoic acid 3-nitrooxy-methyl phenyl ester; NCX-4016] was obtained from NicOx (Sophia Antipolis, France). GSH (L-glutamyl-L-cysteinyl-glycine), aspirin, DMSO, SIN-1 hydrochloride, SNAP, trypan blue, and crystal violet were obtained from Sigma. Cisplatin [cis-diaminedichloroplatinum(II)], ammonium iron(II) sulfate hexahydrate, and acetonitrile were purchased from Aldrich. Cell culture medium (RPMI medium 1640), FBS, antibiotics, sodium pyruvate, trypsin, and PBS were purchased from GIBCO/BRL.

The imidazoline biradical probe, used for thiol measurements, was a gift from Valery Khramtsov (Russian Academy of Science, Novosibirsk, Russia). MGD was synthesized and purified in our laboratory (23).

Cells. CR and CS HOCCs were obtained from Raj Sridhar (Howard University Medical School, Washington, DC). Cells were grown in RPMI medium 1640 supplemented with 10% FBS, 2% sodium pyruvate, and 1% penicillin/streptomycin. Cells were plated at a density of 5 × 10^4 and 1.2 × 10^6 in 60- and 100-mm dishes for drug treatment (NCX-4016 and cisplatin) and determination of GSH, respectively. Cells were grown up to 70% confluence in 75-cm² flasks for determination of intracellular NO release. Culture and drug treatment(s) of cells were carried out at 37°C in an atmosphere of 5% CO₂ and 95% air. Cells were routinely trypsinized (0.05% trypsin/EDTA) and counted by using a hemacytometer. Cell viability was determined by the trypan blue dye exclusion method.

Cell-Survival Assay. Cell survival was assessed by clonogenic assay. Cells at ~80% confluence were trypsinized, rinsed, seeded onto 60-mm dishes (5 × 10^4 cells per dish), grown for 24 h at 37°C, and treated afterward with cisplatin, NCX-4016, SIN-1, or aspirin for 1 or 6 h. After treatments, cells were washed twice with PBS, trypsinized, counted, and plated in 60-mm dishes in triplicate and incubated for 7 days. On day 7, the colonies were stained with crystal violet (in ethyl alcohol) and counted. Each experiment was repeated at least five times.

Cell colonies were counted by using a ColCount automated colony counter (Oxford Optronix, Oxford, U.K.). ColCount was designed to accurately reproduce the proportionality of manual counting methods and to yield highly comparable results of the surviving fraction and, hence, similar survival curves. It uses a high-resolution charge-coupled device camera to capture the image of stained cells in the entire dish. The image is then analyzed to identify (contour) the colonies and to perform a quantitative analysis. The technique provides detailed information on the radius, area, density, and distance to nearest neighbor of the colonies. Postprocessing options permit a variety of user-selectable parameters that can be used to set inclusion/rejection criteria. Colonies in the range of 0.1–1 mm were scored in all experiments.

GSH Assay. Cellular levels of GSH were determined by EPR spectroscopy, which was based on the application of an imidazoline biradical label (24). The label reacts with reduced thiol and shows a characteristic EPR spectrum, which can be quantified by using known amounts of GSH. Cells (2.5 × 10^6 cells per milliliter) were trypsinized, counted, and treated with the thiol-specific label (0.5 mM) at pH 7.0 for 7 min, after which their EPR spectra were measured. The concentration of GSH was determined from a standard curve prepared with known concentrations of GSH under similar conditions.

EPR Spectroscopy Measurements. CR and CS HOCCs grown to 70% confluence in 75-cm² flasks were trypsinized, and cell suspensions at 2 × 10⁷ cells per milliliter were prepared and then treated with 500 μM NCX-4016 for the desired lengths of time. Cell suspensions were incubated at room temperature. The NO spin trap (MGD):Fe(II) was prepared by mixing 1 mM Fe^{2+} [ammonium iron(II) sulfate hexahydrate] with 5 mM MGD and then was added to the cell suspension. The measurements were performed by using an x-band EPR spectrometer. The EPR spectra were measured by using 20-mW microwave power, 3-G modulation amplitude (1 G = 0.1 mT), 100-kHz modulation frequency, 16-msec time constant, 100-G scan range, and 30-sec scan time. SNAP was used as an authentic positive control for NO trapping.

Statistical Analysis. Data were expressed as mean ± SEM. Comparisons among groups were performed by using Student’s t test. The significance level was set at P < 0.05. This work was supported by National Institutes of Health Grants RR003048, CA78886, and CA102264.