Efficacy of a nitric oxide–releasing nonsteroidal anti-inflammatory drug and cytotoxic drugs in human colon cancer cell lines in vitro and xenografts

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Abstract

We previously showed that NCX 4040 inhibits in vitro and in vivo tumor growth and induces apoptosis in human colon cancer cell lines. On the basis of these results, NCX 4040 antitumor activity in combination with 5-fluorouracil (5-FU) or oxaliplatin was evaluated in vitro and in vivo in human colon cancer models. The cytotoxicity of different NCX 4040 and 5-FU or oxaliplatin combination schemes was evaluated on a panel of colon cancer lines (LoVo, LoVo Dx, WiDr, and LWZ) by the sulforhodamine B assay, and apoptosis was assessed by flow cytometry. NCX 4040 and 5-FU combination was always additive in vitro regardless of the scheme used. Sequential NCX 4040 → oxaliplatin treatment produced a strong synergism in three cell lines, with a ratio index ranging from 3.7 to 4. The synergistic effect was accompanied by apoptosis induction (up to 40%). In the in vivo experiments, xenografted mice were treated with the sequential combination of NCX 4040 and oxaliplatin, and apoptosis was evaluated immunohistochemically in excised tumors. Furthermore, in WiDr xenografts, this sequence caused a significantly higher reduction (~60%) in tumor growth compared with single-drug treatments and produced extensive apoptotic cell death (15.3%), significantly higher (P < 0.01) than that observed in untreated tumors (2.7%) or in tumors treated with NCX 4040 (5.1%) or oxaliplatin (5.7%) alone. These data show that NCX 4040 sensitizes colon cancer cell lines to the effect of antitumor drugs and suggests that their combination could be useful for the clinical management of colon cancer. [Mol Cancer Ther 2006;5(4):919–26]

Introduction

Colorectal cancer is one of the most frequently diagnosed cancers worldwide and is the second leading cause of death from cancer in western countries (1). Despite advances in surgery and early detection, overall survival for advanced patients has improved only marginally in the last few decades. In fact, whereas 5-year overall survival is now >90% for patients with stage I disease, it gradually decreases for stages II (60–85%) and III (25–65%), and does not exceed 7% for those with stage IV disease (2). 5-Fluorouracil (5-FU), for many years the only drug approved for the treatment of colorectal cancer, led to a modest improvement (10–15%) in median survival with respect to supportive care (3–7). The subsequent introduction of leucovorin to modulate 5-FU produced response rates of 20% to 25% and the 5-FU/leucovorin regimen became the gold-standard first-line therapy for advanced disease from the late 1980s to 2002 (7–10).

The addition of the topoisomerase I inhibitor, irinotecan, or the platinum analogue, oxaliplatin, to the 5-FU/leucovorin regimen further improved response rates and progression-free and overall survival of patients with metastatic colon cancer. In particular, the oxaliplatin/5-FU/leucovorin treatment markedly increased response rates and progression-free survival with respect to treatment with only 5-FU/leucovorin (11–12).

Moreover, recent phase III clinical trials have shown the superiority of first-line oxaliplatin and 5-FU/leucovorin over an irinotecan/5-FU/leucovorin regimen in terms of time to progression and overall survival (13), and, in a second-line setting, in terms of response rates and progression-free survival (14). Similar results have been obtained in an adjuvant setting (15).

However, notwithstanding the significant progress made in the treatment of colorectal cancer during the past 5 years, other novel therapeutic strategies are needed to further improve the management of patients with this disease.

Several studies have focused on the in vitro and in vivo efficacy of different nonsteroidal anti-inflammatory drugs (NSAID), especially the selective cyclooxygenase-2 inhibitor celecoxib, in combination with 5-FU and/or irinotecan on colon cancer cell growth (16–22). Only one study has...
been published to date on the efficacy of oxaliplatin and celecoxib combination (23). Notwithstanding the increasing amount of preclinical data, the few clinical trials designed to verify the effectiveness and safety of celecoxib/irinotecan/5-FU regimens in patients with advanced colon cancer have produced contrasting results (24–26), underlining the need for further investigation.

It has also been reported that in vitro nitric oxide–donating NSAIDs are more effective than conventional NSAIDs in reducing proliferation and in inducing apoptosis in human colon cancer cell lines (27, 28). The additional molecular mechanisms can probably be attributed to the NO group. In fact, it has been suggested that low concentrations of endogenous NO increase cell proliferation, whereas high concentrations of inducible origin induce cytotoxicity and apoptosis (29).

Moreover, their in vivo administration has been shown to inhibit the growth of human colon cancer xenografts (28) and prevent intestinal carcinogenesis (30). This chemical class maintains the chemopreventive properties of classic NSAIDs against colorectal cancer and exerts a protective effect on gastric mucosa, thus permitting long-term treatment (31).

Based on this evidence, and in an attempt to improve the response of human colon cancer to chemotherapy, we evaluated the antitumor activity of NCX 4040, a novel nitric oxide–donating NSAID, in combination with conventional antineoplastic drugs in a panel of human colon cancer lines in vitro and in xenografted immunosuppressed mice.

**Materials and Methods**

**Cell Lines**

The in vitro study was done on four established colon adenocarcinoma cell lines. LoVo and WiDr cell lines were obtained from American Type Culture Collection (Rockville, MD). LRWZ cells were isolated in our laboratory and derived from a patient with a histologically proven diagnosis of colon adenocarcinoma, and the multidrug-resistant LoVo Dx, derived from LoVo cells, was kindly provided by Dr. Mario Colombo (Istituto Nazionale Tumori, Milan, Italy).

Colon tumor cell lines were maintained as a monolayer at 37°C and subcultured weekly. Culture medium was composed of DMEM/HAM F12 (1:1) supplemented with FCS (10%), glutamine (2 mmol/L), nonessential amino acids (1%; Mascia Brunelli s.p.a., Milan, Italy), and insulin (10 μg/mL; Sigma, Milan, Italy). LoVo Dx cells were maintained by continuous exposure to 0.2 μmol/L doxorubicin (Pharmacia, Milan, Italy). Cells were used in the exponential growth phase in all experiments.

**Drugs**

For in vitro experiments, NCX 4040 [2-(acetyloxy)benzoic acid 4-(nitroxy-methyl)phenyl ester, NicOx SA, Sophia Antipolis, France] was solubilized in DMSO (Carlo Erba, Milan, Italy) to a concentration of 50 μmol/L. For in vivo administration, NCX 4040 was dissolved in a vehicle solution composed of DMSO diluted in 5% dimethylsulfoxide to a concentration of 1 mg/mL. 5-FU (Roche, Milan, Italy) and oxaliplatin (Sanofi-Synthelabo, Paris, France) were diluted to 50 and 5 mg/mL in saline solution (0.9%), respectively, divided into aliquots, and stored at −70°C. Drug stocks were freshly diluted in culture medium immediately before each in vitro and in vivo experiment. The final DMSO concentration never exceeded 1% and this condition was used as a control in each experiment.

**In vitro Studies**

**Chemosensitivity Assay.** The sulforhodamine B assay was used according to the method of Skehan et al. (32). Briefly, cells were collected by trypsinization, counted, and plated at a density of 5,000 per well in 96-well flat-bottomed microtiter plates (100 μL cell suspension per well). Experiments were run in octuplet and each experiment was repeated thrice. The absorbance of treated cells was determined at a wavelength of 540 nm by means of a colorimetric plate reader.

**Single-Drug Exposure.** 5-FU and oxaliplatin were tested at scalar concentrations of 2, 20, and 200 μmol/L and 0.08, 0.8, and 8 μmol/L, respectively. An exposure time of 24 hours for each agent was chosen from the dose-response survival curves of single drugs (data not shown) as the time that produced the maximum effect. The cytotoxic effect was evaluated immediately after the end of drug exposure.

**Drug Combination Exposure.** An exposure time of 24 hours was used in simultaneous drug treatments. In sequential drug schemes, cells were exposed to each drug for 24 hours, and the first drug was washed out before adding the second one. Specifically, the medium was removed from all the wells using a multichannel pipette. Fifty microliters of fresh culture medium were then added to all the wells and once again removed and discarded. Finally, fresh culture medium with or without the second drug was added to the treated and control samples, respectively, up to a final volume of 200 μL/well. The cytotoxic effect was evaluated immediately after the end of drug exposure in both simultaneous and sequential treatment schemes. In all combination experiments, 5-FU and oxaliplatin were tested at the same concentrations used for single-drug exposure (i.e., 2, 20, and 200; and 0.08, 0.8, and 8 μmol/L, respectively), whereas NCX 4040 was used at a fixed concentration of 10 μmol/L.

**Drug Interaction Analysis.** We used Kern et al.’s (33) method, subsequently modified by Romanelli et al. (34). In brief, the expected cell survival (Sexpobs) was defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) and the observed cell survival (Sobs) for the combination of A and B were used to construct an index (RI): RI = Sexp / Sobs. An RI of ≤0.5 indicated the absence of synergism or antagonism. Synergism was defined as any value of RI >1.5. In all experiments, the SD did not exceed 10%. Therefore, only differences of ≥0.5 from unity in RI values were considered significant (P < 0.05).

**Apoptosis Analysis.** Apoptosis was evaluated by flow cytometric analysis according to the terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL)
assay procedure as previously described (28). Briefly, after a 24-hour exposure to either 10 μmol/L NCX 4040 or 8 μmol/L oxaliplatin, or to a 24-hour NCX 4040 or 24-hour oxaliplatin treatment, cells were trypsinized, fixed, stained with propidium iodide, and analyzed by FACSVantage flow cytometer (Becton Dickinson, San Diego, CA). Positivity to the TUNEL assay was also evaluated by fluorescence photomicroscope (Axioscope 40, Carl Zeiss S.p.A., Milan, Italy) according to the instructions of the manufacturer (In situ cell death detection kit, fluorescein; Roche Diagnostic GmbH, Mannheim, Germany). Finally, the cell-permeable DNA dye 4',6-diamidino-2-phenylindole and a fluorescence photomicroscope (Zeiss, Axioscope 40) were used to visualize chromatin condensation and/or fragmentation typical of apoptotic cells.

**In vivo Studies**

**Tumor Growth Inhibition.** CD-1 male nude (nu/nu) mice, 6 to 8 weeks old and weighing 22 to 24 g, were purchased from Charles River Laboratories (Calco, Italy). All procedures involving animals and their care were conducted in conformity with institutional guidelines, which are in compliance with national (D.L. no. 116, G.U., Suppl. 40, February 18, 1992; Circolare no. 8, G.U., July 1994) and international laws and guidelines (EEC Council Directive 86/609, OJ L 358. 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). In vivo experiments were done on WiDr, LoVo, and LoVo Dx cells, but not on LRWZ cells, which failed to show tumorigenic ability. Cells in the exponential growth phase were harvested, washed with medium, and resuspended in cold medium without FCS. Viable cells (5 × 10⁷), as evaluated by the trypan blue exclusion test, were concentrated in 0.2 mL serum-free medium and injected into the hind leg muscles of the mice. Treatment was initiated 6 days after tumor implant, by which time there was a tumor mass of ~300 mg. Each experimental group included at least six mice. The administration schedule of the two drugs was chosen on the basis of toxicologic and pharmacokinetics studies. NCX 4040 was dissolved in a vehicle solution of DMSO diluted in 5% dimethylcellulose and administered p.o. at 10 mg/kg 5 d/wk for 4 consecutive weeks. Oxaliplatin was diluted in water and injected i.p. in a single administration (10% of the lethal dose, LD₁₀) at a concentration of 10 mg/kg. 5-FU was dissolved in saline solution (0.9%) and injected i.p. at 19 mg/kg/d for 5 consecutive days (35). Each treatment started on the 6th day after tumor cell implant. NCX 4040 was administered in the morning and oxaliplatin or 5-FU in the afternoon.

Tumor weight was calculated from caliper measurements according to the method of Geran et al. (36). The antitumor efficacy of treatments was assessed as (a) percent tumor weight inhibition, calculated as \( \left[ \frac{1}{C_0} \times \frac{\text{mean tumor weight of treated mice}}{\text{mean tumor weight of controls}} \right] \times 100 \); (b) tumor growth delay, evaluated as \( T - C \), where \( T \) and \( C \) are the median times for treated and control tumors, respectively, to achieve equivalent size.

**Apoptosis Analysis.** Tumors were excised from untreated mice and from mice treated with oxaliplatin, NCX 4040, or the two-drug combination. The antibody used to detect apoptotic cells specifically recognizes the large fragment (17 kDa) of activated, but not full-length, caspase-3, (cleaved caspase-3 antibody, dilution 1:200, Cell Signaling, Danvers, MA). Tissue sections were treated with epitope retrieval solution [0.01 mol/L citrate buffer (pH 6.0)] in a water bath at 98.5°C for 40 minutes, followed by a 20-minute cooling period at room temperature. Immunoreactivity for active caspase-3 was evaluated quantitatively by counting at least 1,000 tumor cells at ×40 magnification. Apoptotic bodies, occurring as distinct spots that probably originated from the same apoptotic cell, were considered as one apoptotic cell. For all determinations, negative controls were obtained by omission of primary antibody. The apoptotic index was calculated as the percentage of apoptotic tumor cells out of a total of 1,000 neoplastic cells. All samples were evaluated blindly using light microscopy by two independent observers.

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**Figure 1.** In vitro cytotoxic effect of 5-FU and oxaliplatin in colon cancer cell lines LoVo (■), LoVo Dx (○), WiDr (△), and LRWZ (▲). Points, average of at least three independent experiments done in octuplet; bars, SD.
Statistical Analysis. The statistical difference in tumor weight among the different groups was determined by Student’s t test assuming unequal variances. The Bonferoni test was used to determine significant differences in apoptotic index between group means in an ANOVA setting. Apoptosis results are expressed as mean percentages ± SD. Differences were considered statistically significant when \( P < 0.05 \).

Results

In vitro Studies

Effect of Drugs Used Singly or in Combination. The panel of cell lines showed poor sensitivity to chemotherapeutic agents normally used for colorectal cancer in clinical practice such as 5-FU and oxaliplatin (Fig. 1). In fact, after a 24-hour exposure to 5-FU, the IC50 had not been reached at any concentration tested or in any cell line used. Conversely, oxaliplatin induced a maximum cell kill of 22% in LoVo Dx cells after a 24-hour exposure at the highest concentration (8 \( \mu \text{mol/L} \)), whereas cell kill in LoVo, WiDr, and LRWZ lines ranged from 2% to 6% at the drug concentrations tested.

The interaction between NCX 4040 and oxaliplatin or 5-FU was investigated using different treatment schedules. The type of interaction between NCX 4040 and 5-FU was always additive, regardless of the scheme used (Table 1). Similarly, the concomitant exposure of cells to NCX 4040 and oxaliplatin, or to oxaliplatin followed by NCX 4040, produced an additive interaction only. Conversely, exposure to 10 \( \mu \text{mol/L} \) NCX 4040 followed by treatment with oxaliplatin was found to be strongly synergistic in three cell lines, with RI values ranging from 3.7 in LoVo to 4 in WiDr and LRWZ lines (Fig. 2; Table 1). The same sequence produced only an additive interaction in NCX 4040 Dx cell line.

Apoptosis. TUNEL analysis was done to assess whether apoptosis induction was responsible for the synergistic effect observed after treatment with the combination NCX 4040–oxaliplatin. In WiDr cells, NCX 4040 or oxaliplatin used singly did not induce apoptosis, whereas sequential treatment caused a marked apoptosis, with ~40% of cells positive to TUNEL assay (Fig. 3). Furthermore, in LoVo cell line, where NCX 4040 as a single agent produced 90% of apoptotic cells, an increment to 98% was observed after exposure to NCX 4040–oxaliplatin (data not shown).

In vivo Studies

Effect of NCX 4040 Combined with 5-FU or Oxaliplatin on Xenografts. The experiments were done in vivo on three lines, WiDr, LoVo, and LoVo Dx. The LRWZ line was not analyzed in vivo as it is not tumorigenic in mice.

The WiDr cell–derived tumor (Fig. 4A) was extremely resistant to oxaliplatin. In fact, tumor growth after administration of the platin analogue was not significantly

### Table 1. Interaction between NCX 4040 and 5-FU or oxaliplatin

<table>
<thead>
<tr>
<th>Combination of NCX 4040* with</th>
<th>RI mean value</th>
<th>LoVo Dx</th>
<th>LoVo</th>
<th>WiDr</th>
<th>LRWZ</th>
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<tr>
<td>5-FU</td>
<td></td>
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<tr>
<td>5-FU + NCX 4040 (24 h)</td>
<td>0.7 ± 0.01</td>
<td>0.6 ± 0.05</td>
<td>0.6 ± 0.05</td>
<td>0.7 ± 0.06</td>
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<tr>
<td>5-FU (24 h)–NCX 4040 (24 h)</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± 0.03</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.07</td>
<td></td>
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<tr>
<td>NCX 4040 (24 h)–5-FU (24 h)</td>
<td>0.9 ± 0.005</td>
<td>0.6 ± 0.05</td>
<td>0.7 ± 0.1</td>
<td>1.1 ± 0.04</td>
<td></td>
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<tr>
<td>Oxaliplatin^x</td>
<td></td>
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<tr>
<td>Oxaliplatin + NCX 4040 (24 h)</td>
<td>0.9 ± 0.02</td>
<td>0.9 ± 0.0</td>
<td>0.7 ± 0.01</td>
<td>0.6 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin (24 h)–NCX 4040 (24 h)</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.04</td>
<td>0.6 ± 0.06</td>
<td>0.7 ± 0.02</td>
<td></td>
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<tr>
<td>NCX 4040 (24 h)–oxaliplatin (24 h)</td>
<td>1.2 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td>4.0 ± 0.8</td>
<td>4.0 ± 0.5</td>
<td></td>
</tr>
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</table>

^xNCX 4040 [10 \( \mu \text{mol/L} \)].

^zType of interaction: RI \( \geq 1.5 \), synergistic; RI <1.5 to >0.5, additive; RI ≤0.5, antagonistic interaction (see Materials and Methods).

^b5-FU [2, 20, and 220 \( \mu \text{mol/L} \)].

^xOxaliplatin [0.08, 0.8, and 8 \( \mu \text{mol/L} \)].
different to that observed in untreated mice. Treatment with NCX 4040 caused a 40% reduction in the tumor mass, which was significantly higher than that observed in the untreated (P = 0.002) and oxaliplatin-treated groups (P = 0.017). When mice were treated with the NCX 4040 and oxaliplatin combination on the 6th and 13th day after tumor cell injection, a greater growth inhibition (60%) was observed compared with oxaliplatin-treated (P = 0.0004) or NCX 4040–treated (P = 0.019) mice. The efficacy of the drug combination was further confirmed by the delay caused in tumor growth. Specifically, no tumor growth was observed from days 10 to 18, after which time the tumor began to grow again, but with a significantly longer delay (21 days) than that observed after NCX 4040 (10 days, P = 0.00015) or oxaliplatin treatment (4 days, P < 0.0001). A reduced antitumor activity was observed when treatment with oxaliplatin was delayed.

LoVo tumors (Fig. 4B) showed moderate sensitivity to oxaliplatin with a tumor weight inhibition of ~25% compared with untreated mice and a 7-day tumor growth delay. In contrast, treatment with NCX 4040 produced a tumor reduction of ~45% and a growth delay of 21 days, both significantly different from results obtained in untreated (P < 0.0001 and P = 0.0019, respectively) and oxaliplatin-treated (P = 0.027 and P = 0.0013) mice. The combination of oxaliplatin and NCX 4040 on days 6 and 13 caused the highest tumor reduction (60%), which was significantly different from that produced by NCX 4040 alone (P = 0.0026). Moreover, the NCX 4040 and oxaliplatin scheme produced a longer duration of tumor regression lasting from the 14th to the 32nd day after tumor implant, and a significantly longer tumor regrowth delay (36 days) than that observed in mice treated with NCX 4040 (P = 0.0019) or oxaliplatin alone (P < 0.0001). The delayed treatment with oxaliplatin elicited a reduced antitumor efficacy.

In LoVo Dx tumors (Fig. 4C), exposure to NCX 4040, oxaliplatin, or their combination produced a growth inhibition similar to that observed in the non–anthracycline-resistant cell line, with a significantly greater inhibition (35%) compared with untreated tumors (P = 0.008). The combination of the two drugs proved to be even more active, with a 50% tumor weight reduction compared with untreated tumors (P = 0.0014). The higher efficacy of the drug combination was further confirmed by the 21-day delay in tumor growth, which was significantly longer than that observed in oxaliplatin-treated (7 days, P = 0.0008) or NCX 4040–treated (8 days, P = 0.0005) tumors. As previously observed for the other tumor cell lines, the combination was more effective when oxaliplatin was given on the 6th and 13th day after tumor cell implant.

The antitumor effect of 5-FU and NCX 4040 in combination was also evaluated and was found ineffective in controlling colon cancer growth of WiDr, LoVo Dx, and LoVo tumor-bearing mice, confirming in vitro results (data not shown).
Apoptosis. The induction of apoptosis was investigated in WiDr tumors by analyzing caspase-3 activation immunohistochemically using an antibody that specifically recognizes the activated form of caspase-3. A strong reaction of caspase-3 was observed in both the cytoplasm and nucleus of the apoptotic cells. Caspase-3-positive cells displayed the typical morphology of apoptotic cells with condensed and fragmented chromatin and a shrunken cytoplasm (Fig. 5). The apoptotic index was significantly higher in mice treated for 24 hours with oxaliplatin (5.1 ± 0.6) or NCX 4040 (5.7 ± 1.1) than in untreated mice (2.7 ± 0.95, \( P < 0.05 \)). A further, significant increase (15.3 ± 0.9, \( P < 0.01 \)) was observed when a 24-hour exposure to NCX 4040 was followed by a 24-hour exposure to oxaliplatin.

Discussion

We previously showed that the nitric oxide–donating NSAID, NCX 4040, but not its parental compound, aspirin, is capable of reducing the in vitro and in vivo growth of different colon cancer lines, thus indicating its potential for treating colon cancer. In particular, a 10 \( \mu \)mol/L concentration of NCX 4040 inhibited cell growth by \( \sim 20\% \) in LoVo Dx cells and by \( \sim 50\% \) in all the other lines tested (28). Based on these findings, we evaluated the antitumor activity of NCX 4040 in combination with 5-FU or oxaliplatin, both of which are widely used for the clinical management of colorectal cancer (2). Several methods have been proposed to evaluate the interaction between drugs, as critically analyzed by Zoli et al. (37). However, most of them, including the classic isobologram method (38, 39), Steel and Peckham’s (40) isobologram method, the isobologram method subsequently improved by Kano et al. (41), and the median effect principle according to Chou and Talalay (42) and Kanzawa et al. (43), are not suitable for drugs with a low cytotoxic effect or without dose-response curves. Our results indicate that 5-FU and oxaliplatin were not particularly effective in reducing proliferation in the cell lines tested. Thus, we used Kern et al.’s method modified by Romanelli et al. because it is the only correct method to evaluate the type of interaction between the two drugs when one or both has a low cytotoxic effect or no dose-response curve.

Simultaneous exposure to 5-FU and NCX 4040 produced an additive interaction only, regardless of the treatment scheme. Furthermore, simultaneous treatment with NCX 4040 and oxaliplatin, or oxaliplatin followed by NCX 4040, showed the same additive interaction. Conversely, treatment with NCX 4040 followed by the platin analogue produced a strong synergistic interaction in all but LoVo Dx cell line.

The inability of tumor cells to undergo apoptosis is a recognized pathway of drug resistance for most chemotherapeutic drugs, including oxaliplatin and 5-FU (44–46). In the present study, we showed that the synergistic effect on WiDr cell survival elicited by treatment with NCX 4040→oxaliplatin is associated with a marked increase in apoptosis, suggesting that this schedule may sensitize colon cancer cells to cytotoxic drugs by activating the apoptotic program. The enhanced cytotoxic effect and apoptotic index observed in WiDr cells after sequential NCX 4040→oxaliplatin treatment may be due to the ability of the two agents to induce different perturbations in the cell cycle. It has also been reported that treatment with oxaliplatin produces an arrest of cells in G2-M phase (44). These observations would seem to indicate that, whereas cells treated with either drug singly are able to overcome the block in the different phases of the cell cycle, the NCX 4040→oxaliplatin combination induces an irreversible perturbation, resulting in apoptotic cell death.

The synergistic interaction produced in vitro by sequential NCX 4040→oxaliplatin treatment was investigated in immunosuppressed mice injected with all but the LRWZ cell line, which did not show tumorigenic ability. It was observed that NCX 4040 was effective in reducing the

![Figure 4](https://example.com/figure4.png)
growth of tumors derived from all three cell lines (LoVo, LoVo Dx, and WiDr), reproducing in vitro results. Conversely, whereas oxaliplatin was less effective than NCX 4040 in LoVo-derived tumors and completely ineffective in WiDr-derived tumors, it caused tumor growth inhibition similar to that observed after NCX 4040 treatment in LoVo Dx xenografts. This could be responsible for the higher efficacy of the combination observed in xenografts compared with the in vitro system, where the effect of the combination was additive. Moreover, the apparent discordance between in vitro and in vivo results can be hypothetically attributed to several factors related to the experimental system, and, in particular, to the role of the microenvironment in the in vivo models. When mice were treated with NCX 4040 followed by oxaliplatin, a marked increase in the antitumor response was observed. In particular, NCX 4040 was found to sensitize WiDr tumors to the effect of oxaliplatin treatment. One of the possible mechanisms of action underlying the synergistic interaction may be the oxidative stress and the consequent NCX 4040–induced DNA damage, which adds to and enhances the damage caused by oxaliplatin. It is interesting to note that the antitumor activity of sequential NCX 4040–oxaliplatin administration did not produce toxicity in the mice models. Our studies on in vitro cell lines and xenografts showed that the efficacy of the oxaliplatin and NCX 4040 combination in reducing tumor growth was associated with the induction of apoptosis. Furthermore, our work identifies the most effective schedule in vitro and in vivo of the two drugs, whose combination has never been investigated before.

In conclusion, our results show that NCX 4040 is capable of sensitizing colon cancer to the effects of antineoplastic agents, such as oxaliplatin, and that this combination could be potentially useful for the treatment of human colon cancer.

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