

Antiproliferative effect of nitrosulindac (NCX 1102), a new nitric oxide-donating non-steroidal anti-inflammatory drug, on human bladder carcinoma cell lines

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are potent antitumoral agents but their side effects limit their clinical use. A novel class of drugs, nitric oxide-donating NSAIDs (NO-NSAIDs), was found to be safer and more active than classical NSAIDs. This study explored the effect of the NO-donating sulindac derivative, NCX 1102, on three human urothelial epithelial carcinoma cell lines (T24, 647V, and 1207) and primary cultures of normal urothelial cells. Cytotoxicity, antiproliferative effect, cell cycle alterations, morphological changes, and apoptosis were investigated after treatment with NCX 1102 in comparison with the native molecule. After treatment, there was a cytotoxic effect (with IC₅₀ at 48 h of 23.1 μM on 647V, 19.4 μM on T24, and 14.5 μM on 1207) and an antiproliferative effect on all three cell lines with NCX 1102 but not with sulindac. No effect was detected on normal urothelial cells. Flow cytometric analysis showed a differential NCX 1102-induced accumulation of cells in various phases of the cell cycle, depending on cell line and concentration. NCX 1102 induced an occurrence of multinucleated cells in all cell lines and mitotic arrest in 647V and 1207. NCX 1102-treated T24 and 647V cell lines showed a significant difference of apoptotic cell amount when compared to controls. Our results demonstrated a greater antiproliferative potency of NCX 1102 compared to its parent molecule sulindac, and suggested that this new NO-NSAID may have therapeutic impact in the management of bladder cancer. [Mol Cancer Ther. 2004;3(3):291–298]

Introduction

Bladder cancer is the fourth cause of cancer in man (1, 2) and survival of high-risk bladder cancer remains problematic when first-line curative measures have failed. There is also a need to improve local therapy for urothelial cell carcinoma (UCC) (3) of the bladder with intermediate risk. New molecules, acting directly on tumoral cells by inhibiting their growth and inducing apoptosis, are needed to improve the efficiency of the current therapies.

It has been shown that while cyclooxygenase 2 (COX-2) is not expressed in normal bladder urothelium, its expression is up-regulated in bladder transitional cell carcinoma but also in carcinoma *in situ* (CIS) and preneoplastic lesion (3, 4), as well as in cultured bladder cancer cell lines (5). Moreover, the expression levels of COX-2 seem correlated with tumor grade and tumor invasion (6).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been found to be antiproliferative and/or pro-apoptotic agents against various types of cancer including genitourinary tumors (7, 8). The antitumoral and anti-inflammatory effects of NSAIDs was first thought to be due to inhibition of the activity of cyclooxygenase 1 (COX-1) (9) and COX-2 (10), thereby reducing the level of prostaglandins. However, NSAIDs are also able to down-regulate cytosolic phospholipase A₂ (cPLA₂) mRNA expression and consequently reduce the availability of arachidonic acid, the substrate of cyclooxygenases (11). Moreover, COX-independent pathways could also play a role, considering the recently demonstrated activation of a specific NSAID-activated gene (*NAG-1*) in the study of cells lacking both COX-1 and COX-2 isoenzymes (12).

Unfortunately, the use of NSAIDs leads to several side effects, like gastrointestinal (13) or renal lesions (14), hence limiting their use. Coupling a nitric oxide (NO)-donating group to NSAID molecules has been shown to reduce their side effects on the gastrointestinal tract (15, 16) and on the kidney (17, 18) due to the cytoprotective properties of the NO (19, 20). The resulting new chemical entities (NO-NSAIDs) show higher anti-inflammatory and antipyretic properties with respect to the native molecule (21); moreover, most of its anticancer properties were enhanced in comparison with the native NSAID (22, 23).

NO is synthesized by normal and neoplastic urothelial cells (24, 25). It has been suggested that low concentrations of endogenous NO increase cell proliferation (26) while higher concentrations (from inducible origin) of NO induce cytotoxicity and apoptosis (27). Intravesical inoculation of Bacillus Calmette-Guerin (BCG), an effective therapy against bladder cancer, induces a marked increase in NO production by urothelial cells (28). On the other hand, studies using exogenous NO donors, like sodium

Received 7/31/03; revised 10/22/03; accepted 12/12/03.

Grant support: INSERM, Faculté de Médecine de Créteil, Université Paris 12 Val de Marne, Association Claude Bernard, Association Certus.

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nitroprusside (SNP) and other NO-generating molecules, showed that NO has antiproliferative and/or pro-apoptogenic properties on varying tumor cells, *in vivo* and *in vitro* (29, 30).

Because they combine the antiproliferative and pro-apoptotic properties of both their NO and NSAID moieties, NO-NSAIDs are potent antitumoral agents.

We decided to investigate the effects of a new NO-donating sulindac derivative, NCX 1102, on cell viability and proliferation of human bladder carcinoma cell lines, to elaborate new strategies for bladder cancer therapy, based on this innovative compound.

Materials and Methods

Cell Lines

Three human bladder carcinoma cell lines (T24, 1207, and 647V) were grown as monolayer in RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 5% fetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). Cells were seeded at a density of 20,000 cells/cm² and incubated at 37°C with 5% CO₂ and 95% humidity.

Cell lines T24 (31) and 647V (32) were obtained from the Molecular Biology Department of University of California at Los Angeles; 1207 was established in our laboratory (33).

Primary Culture of Normal Bladder Cells (Explants)

Fresh normal urothelium was obtained from nephro-urectomy samples as previously described (34). Briefly, the mucosal cell layer was stripped from the muscle layer and submucosa under an operating microscope, and then spread on a coated Cyclospore membrane (Becton Dickinson, Le Pont de Claix, France) with the basement membrane facing the support. The membranes were placed in six-well tissue culture plates and the compartments were filled with standard culture medium refreshed every 2 days. Standard medium consisted in a 1:1 mixture of DMEM and Ham's F-12 medium, supplemented with 10% heat-inactivated fetal bovine serum, 5 µg/ml insulin and 5 µg/ml transferrin, 50 nM hydrocortisone, 5 ng/ml sodium selenite, 10 µM HEPES, and 100 units/ml penicillin/streptomycin. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 95% humidity.

Reagents

NCX 1102 [(Z)-5-fluoro-2-methyl-1-[4-(methylsulfinyl)phenyl]methylene]-1H-indene-3-acetic acid 4-(nitrooxy)butyl ester] and sulindac were provided by NicOx S.A. (Sophia Antipolis, France). Stock solutions at 100 mM of both compounds were prepared in DMSO (Sigma, Saint-Quentin Fallavier, France) and stored at -20°C. Maximal final concentration of DMSO was 1 µl/ml (0.1% v/v).

Methyl Thiazolyl Tetrazolium Assay

Cells were seeded in 96-well tissue culture plates (Becton Dickinson) and incubated for 24 h, before addition of different concentrations of sulindac or NCX 1102. The different media were changed every day. The amount of living cells was counted daily for 4 days using the methyl thiazolyl tetrazolium (MTT) method (35). Briefly, cells were incubated for 2 h at 37°C with 1 mg/ml MTT (Sigma).

Then, MTT was discarded and replaced by isopropanol. Absorbance (A) was measured on an EL800 universal microplate reader (Bio-Tek instruments, Winooski, VT) at a wavelength of 550 nm. IC₅₀ was defined as the NSAID concentration entailing a 50% reduction of absorbance in comparison with control.

Tritiated Thymidine Incorporation (³HdThd)

After plating in 24-well tissue culture plates (Becton Dickinson) and 24 h incubation, cells were treated with sulindac or NCX 1102 for 24 h at different concentrations. A 2-h pulse of 2 µCi/ml [³H]thymidine was then applied to the cells. Thereafter, cells were incubated for 10 min at 4°C in 10% trichloroacetic acid (TCA), washed three times with PBS (pH 7.4), and lysed in 200 µl of 0.2 M NaOH/1% SDS solution. Radioactivity incorporated in the cells was measured by scintillation counting for 1 min on Beckman LS 6000SC scintillation counter (Beckman Coulter, Roissy, France). GI₅₀ was defined as the concentration inhibiting growth by 50% compared to control.

Cell Cycle Analysis

After 24 h contact with sulindac or NCX 1102, 1 × 10⁶ cells were treated for DNA staining with propidium iodide using the DNA-Prep Coulter Reagents kit (Beckman Coulter) according to the manufacturer's instructions and analyzed for cell cycle distribution using a Coulter epics elite flow cytometer (excitation: 488 nm, emission: 635 nm). Data recording was made using Expo2 software (Beckman Coulter) and cell cycle data were analyzed using Multicycle Wincycle software (Phoenix Flow Systems, San Diego, CA).

Morphological Changes

Changes in cell morphology after incubation with sulindac or NCX 1102 were examined using a variation of May Grünwald Giemsa (MGG) staining using the Kit RAL 555 (RAL, Martillac, France).

Semi-thin sections were prepared as follows: cultures in Petri dishes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), post-fixed for 1 h in 0.1 M phosphate-buffer 1% osmium tetroxide at pH 7.4, dehydrated several times in increasing concentrations of ethanol and embedded in Epitoke 812 (Merck Eurolab, Fontenay-sous-bois, France). Semi-thin sections were stained using the Richardson staining: sections were treated for 5 min in 1% aqueous periodic acid, rinsed briefly, dried, and stained for 6 min at 50°C with a mixture of 1% methylene blue in 1% borax solution and 1% aqueous Azur II.

Detection of Apoptosis

Apoptosis was assessed by DNA fragmentation analysis using the *in situ* end-labelling (ISEL) technique previously described (36). Briefly, cells were grown on glass lab-tek chamber slides (Nalge Nunc, Naperville, IL), and subconfluent cultures were exposed to sulindac, NCX 1102, or control medium for 24 h. Cells were fixed in 4% paraformaldehyde for 5 min, permeabilized in cold acetone for 5 min, and rehydrated in PBS. Cells were then pretreated by heating in 2× SSC (0.3 M NaCl and 30 mM Na-citrate, pH 7) at 80°C for 20 min, rinsed in distilled water, and digested with 0.1 unit/ml proteinase K in Tris-HCl (pH 7.4) for 20 min at 37°C, followed by washing in Tris-HCl (pH 7.4).

Incorporation of nucleotides was performed by incubation in a buffer containing 1 mM dATP, dGTP, and dCTP; 1 mM biotin-16-dUTP; and 50 units/ml Klenow polymerase for 20 min at 15°C. Cells were stained using ABC peroxidase Kit Elite standard (Vector AbCys, Paris, France) and revealed by peroxidase substrate fast 3,3'-diaminobenzidine (DAB) (Sigma).

Nuclear morphological changes were observed by fluorescent microscopy after 10 min staining with 0.1 µg/ml Hoechst 33342 dye (Molecular Probes, Eugene, OR) diluted in PBS. The criteria used to identify apoptosis included nuclear shrinkage, chromatin condensation, and apoptotic bodies.

Statistics

Statistical analysis was performed using statview (SAS Institute Inc., Cary, NC). Differences were considered statistically significant if the *P* was <0.05 as calculated using the unpaired Student *t* test when appropriate.

Results

NCX 1102 Has a Cytotoxic Effect on Human Bladder Carcinoma Cell Lines while Sulindac Has No Effect at the Same Concentration

The NO-NSAID, NCX 1102, produced a time- and concentration-dependent decrease in the number of living cells in all three bladder carcinoma cell lines as shown in Fig. 1. After 24 h treatment with 30 µM NCX 1102, T24 appeared to be the most sensitive cell line, because its cell viability was 40% while 647V and 1207 cell lines showed 60% in cell viability in the same conditions. Accordingly, IC₅₀ was 24.4, 35.9, and 37.9 µM, respectively, for the three cell lines. After 48 h treatment, the IC₅₀ was lower than 30 µM for all three cell lines: 19.4, 23.1, and 14.5 µM with T24, 647V, and 1207 respectively. Cell line 1207 was more sensitive to NCX 1102 than the two other cell lines. The 647V cell line was clearly the most resistant to NCX 1102 with a viability close to 60% after 72 h incubation with 10 µM NCX 1102 while the viability was about 30% in the two other cell lines. Contrasting with NCX 1102, sulindac did not affect the viability of either cell lines.

NCX 1102 Has No Detectable Cytotoxic Effect on Normal Urothelium Explants

After 24 h treatment with NCX 1102, cell death was found in all three tumoral cell lines, as shown by the presence of floating cells in the culture medium, as observed by light microscopy. This effect is depicted in Fig. 2 (representative of two distinct experiments) after treatment with 15 and 100 µM NCX 1102. In contrast, in normal urothelial explants, no cell death was observed after 48 h, even at the highest concentration of NCX 1102 (100 µM) tested. These results showed that NCX 1102 was much more effective in killing tumoral cells than normal urothelial cells, emphasizing the selective cytotoxicity of this compound.

NCX 1102 Inhibits Cell Proliferation

As an effect of NCX 1102 on cell viability was detected after 24 h, we selected this time to study the effect of the NO-NSAID on cell proliferation. In addition to the cytotoxic effect, NCX 1102 showed a concentration-dependent anti-

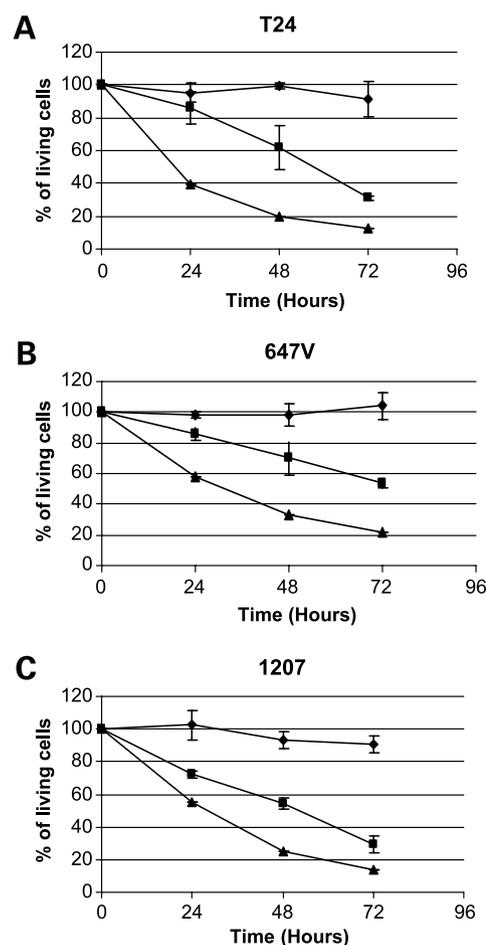


Figure 1. Effect of NCX 1102 on cell viability. T24 (A), 647V (B), and 1207 (C) cell lines were cultured in the presence of 10 µM sulindac (◆) and 10 (■) or 30 (▲) µM NCX 1102. Cell viability was evaluated using MTT assay and the percentage of living cells was determined in comparison with control medium (medium with 1 µl/ml DMSO). Points, means of three different experiments done in duplicate; bars, SD.

proliferative effect on the three human bladder cell lines with the concentration of 5 µM (Fig. 3). The calculated GI₅₀ at 24 h was 10 µM with the cell line 1207, 13.3 µM with T24, and 18.8 µM with 647V. These findings confirm that 647V is more resistant to the NO-NSAID than T24 and 1207. Sulindac did not display an inhibitory effect on any cell line at both tested concentrations 10 and 30 µM (data not shown).

Depending on the Cell Type, NCX 1102 Leads to Cell Accumulation in Different Cell Cycle Phases

The inhibition of proliferation induced by NCX 1102 was associated with a concentration-dependent accumulation of cells in different phases of the cell cycle (Fig. 4, A–C) and the occurrence of sub-G₀-G₁ population of cells. After 24 h, 15 µM NCX 1102 produced an accumulation of cells in G₂-M phases of the cell cycle (Fig. 4, B and C) and the occurrence of a sub-G₀-G₁ peak in 1207 and 647V cell lines (Fig. 4, D–G). The T24 cell line also displayed a large sub-G₀-G₁ peak (not shown) but no cell cycle arrest was detected (Fig. 4A). With 30 µM NCX 1102 for 24 h, T24 and 647V cell lines showed an accumulation in G₂-M phases while

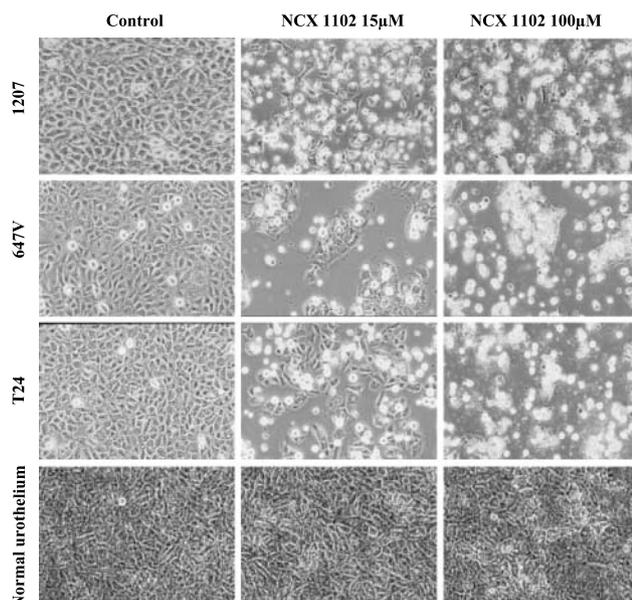


Figure 2. Effect of NCX 1102 on normal urothelium and tumoral cell lines. Cells were observed by light microscopy in the presence of 15 or 100 μM NCX 1102 or vehicle. The pictures show the effect of NCX 1102 after 24 h for the tumoral cell lines T24, 647V, and 1207 and 48 h for the normal urothelial explant at a $\times 200$ magnification.

1207 showed an accumulation in the S and G₂-M phases of the cell cycle (Fig. 4, A–C). Moreover, no sub-G₀-G₁ was detected in any cell line. Sulindac did not display any effect on the cell cycle at used concentrations.

NCX 1102 Induces Changes in Cell Morphology

To examine morphological changes, cells were stained after 24 h treatment with NCX 1102, at the concentrations used to study the cell cycle modifications (15 and 30 μM). Treated cells exhibited a more heterogeneous morphology and the occurrence of multinucleated cells was not observed in untreated or in sulindac-treated cells (Fig. 5A). On the other hand, NCX 1102 induced mitotic arrest in 647V and 1207 cell lines as demonstrated by the presence of cells in mitosis in semi-thin sections (Fig. 5B). Moreover, nucleus staining with Hoechst showed the occurrence of cells with brighter nuclei after treatment with NCX 1102 compared to untreated cells, corresponding to cells in the G₂-M phases with more condensed and greater amount of DNA (Fig. 6A).

NCX 1102 Induces Different Rate of Apoptosis, Depending on Cell Line

To determine whether cell death could be due to apoptosis, we looked for apoptotic cells after exposure of the different cell lines to NCX 1102. Nuclear morphology analysis confirmed the presence of apoptotic cells (Fig. 6A), which were also detected by ISEL and the Richardson staining on semi-thin sections after treatment with 15 μM NCX 1102 (Fig. 7). Cell line T24 was the most responsive cell line to NCX 1102-induced apoptosis (13.4% after 24 h). The amount of apoptosis observed with the 647V cell line was very low (2.1% after 24 h), but was significantly different from that of the control ($P = 0.002$). Cell line 1207 was the least sensitive cell line to NCX 1102-induced

apoptosis, showing no significant difference in the apoptotic rate compared to the control (Fig. 6B). When compared to untreated cells, sulindac displayed a slightly significant enhancement of apoptotic induction after 48 h on T24 cell line; from 4% to 5.3% ($P = 0.015$).

Discussion

Effectiveness of NO-NSAIDs in inhibiting cell growth and inducing apoptosis has already been demonstrated on colon cancer cell lines (37–39) and on various cultured human cell lines from pancreas, prostate, lung, and tongue cancers (40). In our laboratory, we tested different NO-donating NSAID derivatives for their cytotoxic activity on bladder cancer cells (data not shown) and select the NO-donating derivative of sulindac, NCX 1102, as a representative of these agents. Here we show that NCX 1102 has an important effect on cell kinetics.

First of all, we discovered that NCX 1102 was much more active than its native molecule. Indeed, sulindac had no

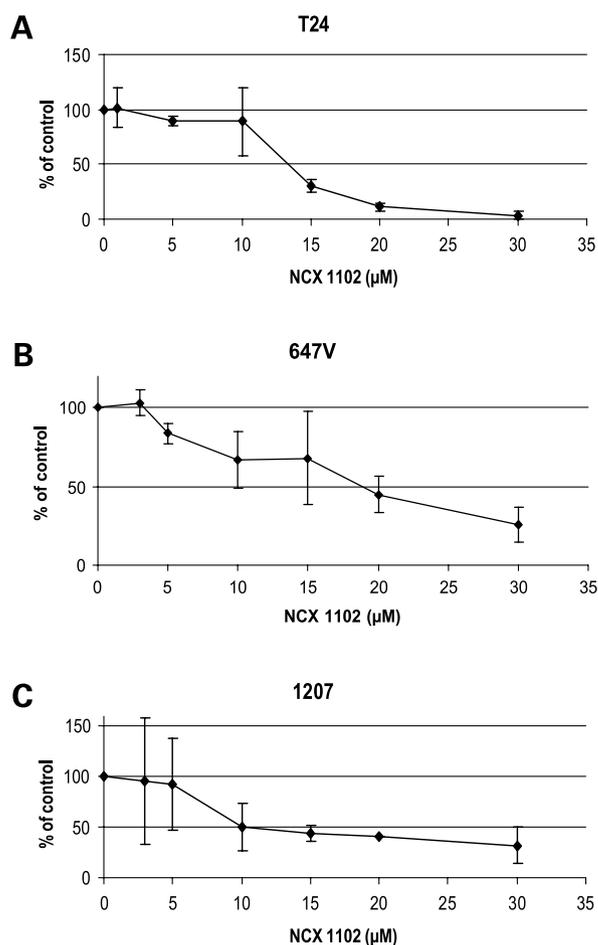
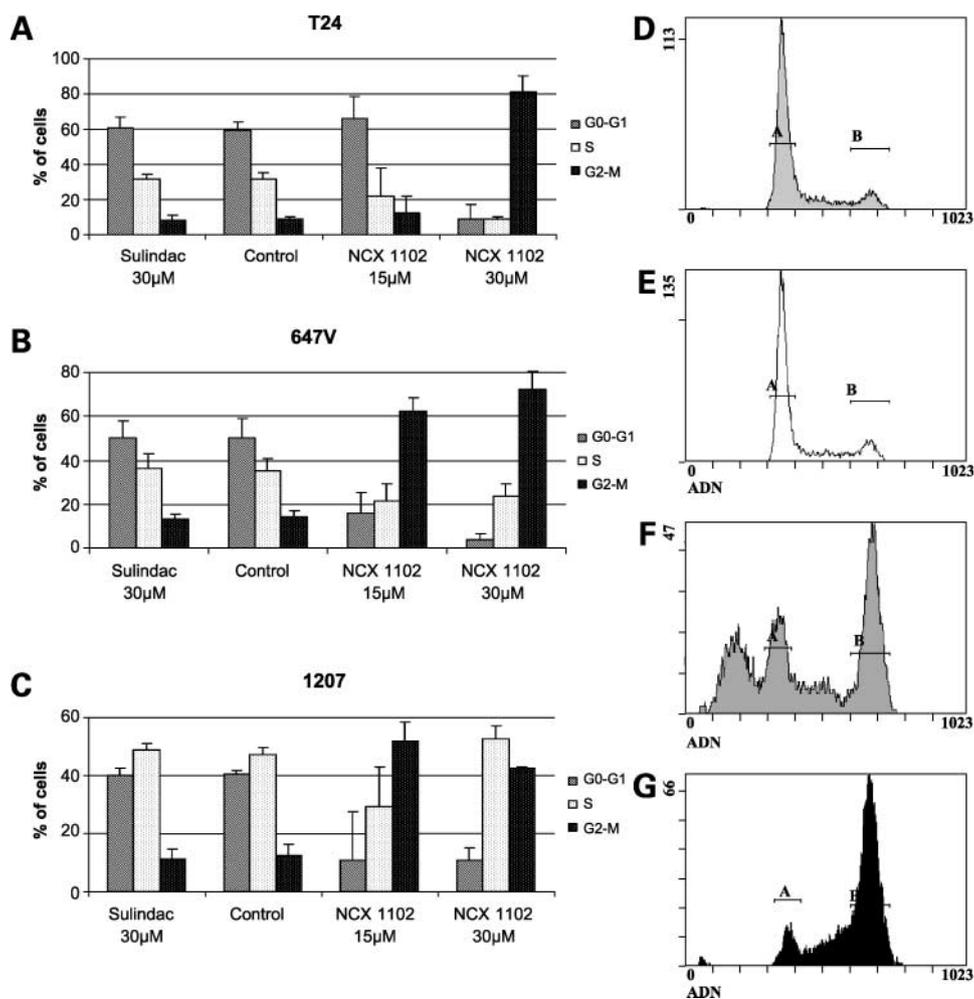


Figure 3. Effect of NCX 1102 on cell proliferation. After 24 h incubation with different NCX 1102 concentrations, cell proliferation of T24 (A), 647V (B), and 1207 (C) cell lines was assessed by ^3H thymidine incorporation. Points, means of at least two different experiments done in triplicate; bars, SD.

Figure 4. Flow cytometry analysis of bladder carcinoma cell lines exposed to sulindac or NCX 1102. **A–C**, cell cycle distribution of T24 (**A**), 647V (**B**), and 1207 (**C**) cells in different phases of cell cycle after 24 h treatment with 30 μ M sulindac and 15 or 30 μ M NCX 1102. *Columns*, means of three independent experiments. **D–G**, representative histograms of 647V cell line after 24 h treatment with 30 μ M sulindac (**D**), 1 μ l/ml DMSO (control) (**E**), 15 μ M NCX 1102 (**F**), or 30 μ M NCX 1102 (**G**). Labels **A** and **B** on histograms are cells in G₀-G₁ and G₂-M phases, respectively. A sub-G₀-G₁ peak is clearly seen in **F**.



effect at the same concentrations, whereas NCX 1102 exerted cytotoxic, antiproliferative activities and disturbed the cell cycle. The greater potential of NCX 1102 compared to its parent molecule was also observed on human colon cancer cell lines (37, 38, 40) and on human pancreatic, prostate, and tongue cancer cell lines (40).

Our results showed that NCX 1102 had a stronger cytotoxic activity on human bladder carcinoma cell lines than on other types of cancer cell lines. In the literature, IC₅₀ for NCX 1102 (48 h treatment) was found to be 65 μ M for a pancreatic carcinoma cell line, MIA PaCa-2; 65 μ M for a lung cancer cell line A549; 35 μ M for a tongue squamous cell carcinoma cell line SCC-25; 92 μ M on prostate cancer cell line LNCaP and about 30 μ M for colon carcinoma cell line HT-29 (37, 40). In our study, the IC₅₀ of bladder carcinoma cell lines after 48 h ranged from 14.5 to 23.1 μ M.

One of the mechanisms leading to the inhibitory activity of NCX 1102 is a strong reduction of growth of all three bladder tumoral cell lines, with a GI₅₀ of 10 μ M with the cell line 1207, 13.3 μ M with T24, and 18.8 μ M with 647V. These results are in agreement with data reported by Lavagna *et al.* (38), who demonstrated the growth-inhibiting potential of 7 μ M of NCX 1102 on human colon carcinoma cell lines

using a clonogenic assay. This inhibition of cell proliferation is likely due to an alteration of cell division, as demonstrated by an accumulation of cells in G₂-M phase for all three bladder carcinoma cell lines and the appearance of multinucleated cells at higher concentrations of NCX 1102. The effect of NCX 1102 on the cell cycle seems to be dependent on cell type since a G₀-G₁ to S block and a slight increase in proportion of cells in G₀-G₁ were observed in human colon cancer cell lines (37, 38), while arrest in G₂-M to G₀-G₁ transition was observed in pancreatic cancer cells (40).

As far as 647V and 1207 cell lines are concerned, G₂-M accumulation appears to be related to a mitotic arrest after treatment with NCX 1102 as depicted by morphological analysis of semi-thin sections. This effect was already reported for two other molecules with anti-inflammatory properties, mesalazine (41) and curcumin (42). Accumulation of cells in G₂-M, arrest in mitosis, and multinucleated cell, which could be the result of endoreplication or inhibition of cytokinesis, would suggest that NCX 1102 has an effect on the cytoskeleton.

The other mechanism whereby NCX 1102 may exert its activity is cell death. The presence of apoptotic cells was demonstrated by morphological analysis of cell nucleus in

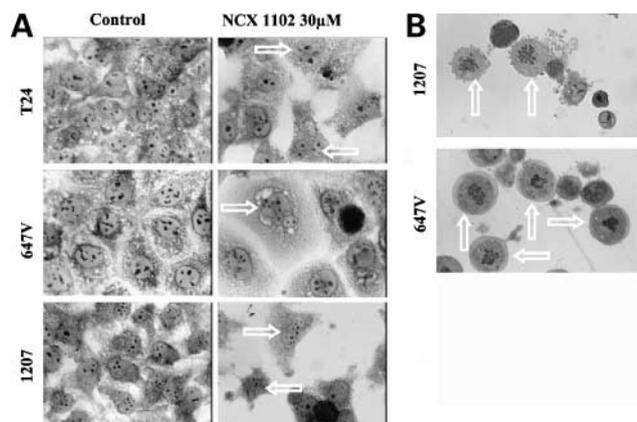


Figure 5. NCX 1102 induces changes in cell morphology. **A**, light microscopy analysis of MGG-stained cells revealed the occurrence of multinucleated cells (*white arrows*) in all three cell lines ($\times 400$ magnification). **B**, semi-thin sections of 647V and 1207 cells treated with $15 \mu\text{M}$ NCX 1102 for 24 h and stained with the Richardson technique. Mitotic arrested cells are shown by *white arrows* ($\times 400$ magnification).

all three cell lines, but apoptosis was found to be significantly enhanced only in T24 and 647V cell lines, with respectively 13.4% and 2.1% of apoptotic cells after 24 h treatment with $15 \mu\text{M}$ NCX 1102. In the literature, the other most usual technique to detect apoptosis in cultured cells treated with NCX 1102, besides analysis of nuclei morphology by fluorescence microscopy, is to look for the presence of a sub- G_0 - G_1 peak by flow cytometry (37, 40). Our flow cytometry data showed a sub- G_0 - G_1 peak for all three cell lines treated for 24 h with $15 \mu\text{M}$ NCX 1102. Regarding the rate of apoptosis detected by morphological analysis, this peak likely reflects the occurrence of both cell debris and apoptotic cells. This hypothesis is supported by the lack of sub- G_0 - G_1 peaks on higher concentration of NCX 1102; this may be due to the loss of highly damaged cells after centrifugation and therefore not detectable by flow cytometry. Moreover, it can be noted that in T24 cell line exposed to $15 \mu\text{M}$ NCX 1102, this sub- G_0 - G_1 peak was larger than with the other cell lines, in agreement with the higher apoptotic rate for this cell line compared to the other two. The difference between the apoptotic induction rate and the high number of dead cells observed by light microscopy and the MTT assay after treatment with NCX 1102, especially for 647V, could be explained by the occurrence of a non-apoptotic cell death which may be the consequence of the G_2 -M arrest, in addition to the classical apoptosis. The existence of a non-apoptotic cell death was discussed by Lavagna *et al.* (38) after their observation that death of colon carcinoma cell lines treated with NCX 1102 was caspase 3-independent. This could explain the detection of the sub- G_0 - G_1 peak in treated 647V and 1207 cell lines with $15 \mu\text{M}$ NCX1102 in spite of their low apoptotic rate.

The effect of NCX 1102 differs between the cell lines. Cell accumulation in G_2 -M phases of the cell cycle, occurrence of multinucleated cells, and mitotic arrest were found in 647V and 1207 cell lines; this was associated with very low

apoptosis induction. On the contrary, the inhibitory effect of NCX 1102 on T24 cell line seemed to be mainly related to apoptosis induction, which corroborates the high sub- G_0 - G_1 peak observed by flow cytometry, reinforced by effects on cell division only at higher concentrations.

Our study demonstrated the potential of NCX 1102 to affect kinetics of human tumoral bladder cell growth by inducing growth inhibition, cell cycle disturbance, and cell death, and also pinpointed another very interesting feature of this new NO-NSAID by showing its little effect on normal bladder urothelium (obtained from explants). One of the explanations for this differential effect could be the over-expression of COX-2 in the urothelial carcinoma cell lines compared to the normal tissue. This hypothesis is supported by a study about celecoxib, another COX-2 inhibitor, which exerts an antiproliferative action on a human COX-2-over-expressing cholangiocarcinoma cell line while no significant antiproliferation was detected on a COX-2-deficient cholangiocarcinoma cell line used in the same study (43).

We showed that NCX 1102 has an effect on cell division and proliferation. Thus, another possible explanation for the selective effect of NCX 1102 could be related to the low proliferation rate of normal urothelium compared to transitional cell carcinoma (44).

As we tested several NO-donating molecules on bladder carcinoma cell lines, NCX 1102 showed to be the most active

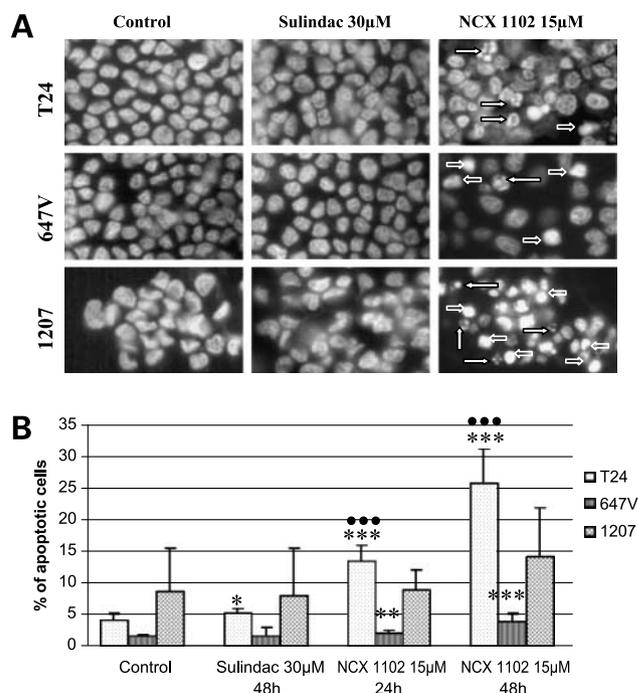


Figure 6. NCX 1102 induces apoptosis. **A**, detection of apoptosis by nuclear morphology analysis after staining with Hoechst 33342. Cells were previously treated for 24 h with $30 \mu\text{M}$ sulindac or $15 \mu\text{M}$ NCX 1102. *Full arrows* indicate apoptotic cells. *Empty arrows* indicate condensed cells corresponding to cells in G_2 -M phase of the cell cycle. **B**, NCX 1102-induced apoptosis. Values were obtained by counting apoptotic cells in at least 10 fields (at $\times 400$ magnification) for each assay. ***, $P < 0.0001$; **, $P = 0.002$; *, $P = 0.015$ compared to control with $1 \mu\text{l/ml}$ DMSO. ●●●, $P < 0.0001$ compared to $30 \mu\text{M}$ sulindac on T24 cell line.

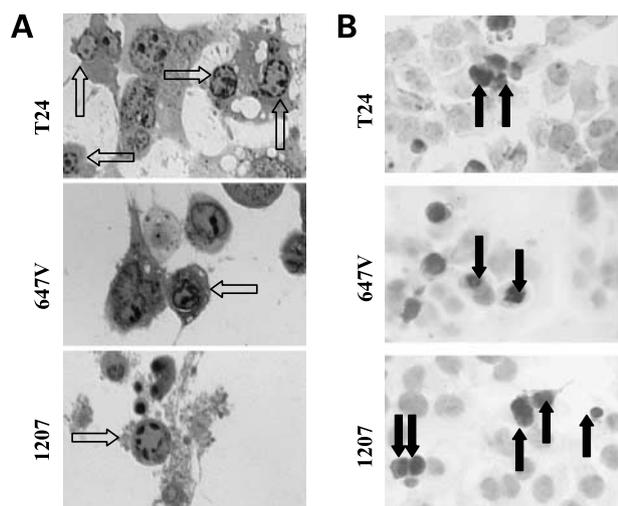


Figure 7. Detection of NCX 1102-induced apoptosis after 24 h treatment at 15 μM . **A**, cells with typical apoptotic morphology (empty black arrows) were observed in all three cell lines by light microscopy on semithin sections stained by Richardson technique. **B**, apoptotic cells (arrows) were stained by the ISEL technique.

one, even more active on 647V and 1207 cell lines than the classical NO donor sodium nitroprusside. Both molecules had an equivalent effect on T24 cell line (data not shown). However, induction of apoptosis in tumor cells by endogenous NO, from endogenous NO donors like glyceryl trinitrate (GNT) (45) or produced by induction of nitric oxide synthase (NOS) (46), has also been reported. Thus, the apoptotic and antiproliferative properties of NO could be an explanation of the great activity of NCX 1102 on tumoral cell lines compared to sulindac alone.

A xenograft model in severe combined immunodeficiency syndrome (SCID) mice mimicking human CIS has been recently established using 1207 and T24 UCC cell lines (47). It would be tempting to explore the efficiency of NCX 1102 in this model, to develop new possibilities for the conservative management of CIS of the bladder.

NCX 1102 could also be tested as adjuvant to already existent therapies. Indeed, one of the most currently effective treatments of superficial bladder cancer is the intravesical immunotherapy with the BCG, which is, unfortunately, associated with side effects, particularly inflammation. Combining an NSAID, and particularly a NO-donating NSAID to the BCG therapy would be of great interest. So far, the effect of NSAIDs (acetyl salicylic acid) on BCG has been studied, showing that NSAIDs display no lethal effect on the BCG viability (48) and that indomethacin was able to enhance the activity of lymphokine-activated killer (LAK) cells against human bladder tumor cells (49). In addition, down-regulation of prostaglandin E2 by COX inhibitors could enhance the BGC-induced activity of macrophages against murine bladder cancer cells *in vitro* (50). Moreover, BCG treatment up-regulates gene and protein expression of inducible and endothelial nitric oxide synthase in rat bladder, suggesting a role of NO in the antitumoral activity of the BCG (51).

The association of NCX 1102 and BCG therapy could be a very promising treatment in the management of patients with bladder carcinoma, and therefore NCX 1102 could be a good candidate to reduce the side effects of BCG therapy and improve the efficacy of the treatment.

Finally, NCX 1102 could also be considered as a possible chemopreventive agent. Several studies showed the chemopreventive potential of NSAIDs on bladder cancer *in vitro* and/or *in vivo* (52, 53), including sulindac (54, 55). Moreover, because COX-2 seems to be involved in the development of transitional cell carcinoma and preneoplastic lesions (3), it could be an interesting target for NSAID-mediated chemoprevention (56). However, NO-donating NSAIDs are known to be more active and safer than classical NSAIDs. In addition, the chemopreventive activity of NO (57) and also of a NO-donating aspirin derivative (NO-ASA) (22) have already been demonstrated in chemically induced carcinomas in rats.

According to all these facts, NCX 1102 is a promising compound as a new agent for the treatment and prevention of UCC of the bladder.

Acknowledgments

We thank Danielle Césari for her assistance on the flow cytometry assays, Maryvonne Ginat and José Courty for technical assistance, Ahmad Daher for providing normal urothelial explants, and Catherine Jones for the language revision.

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Mol Cancer Ther 2004;3:291-298.

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