The cardenolide UNBS1450 is able to deactivate nuclear factor κB–mediated cytoprotective effects in human non–small cell lung cancer cells

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Abstract

Non–small cell lung cancers (NSCLC) are associated with very dismal prognoses, and adjuvant chemotherapy, including irinotecan, taxanes, platin, and Vinca alkaloid derivatives, offers patients only slight clinical benefits. Part of the chemoresistance of NSCLCs results from the constitutive or anticancer drug-induced activation of the nuclear factor–κB (NF–κB) signaling pathways. The present study shows that human A549 NSCLC cells display highly activated cytoprotective NF–κB signaling pathways. UNBS1450, which is a cardenolide belonging to the same class of chemicals as ouabain and digoxin, affected the expression and activation status of different constituents of the NF–κB pathways in these A549 tumor cells. The modifications brought about by UNBS1450 led to a decrease in both the DNA-binding capacity of the p65 subunit and the NF–κB transcriptional activity. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylenetrazolium bromide colorimetric assay, we observed in vitro that UNBS1450 was as potent as taxol and SN38 (the active metabolite of irinotecan) in reducing the overall activity. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylenetrazolium bromide colorimetric assay, we observed in vitro that UNBS1450 was as potent as taxol and SN38 (the active metabolite of irinotecan) in reducing the overall activity. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylenetrazolium bromide colorimetric assay, we observed in vitro that UNBS1450 was as potent as taxol and SN38 (the active metabolite of irinotecan) in reducing the overall activity.

Introduction

Because adjuvant chemotherapy (including camptothecin, taxane, platin, and Vinca alkaloid derivatives) has only a limited therapeutic effect, non–small cell lung cancers (NSCLC) are the leading cause of death from cancer in most developed countries, and are characterized by an overall 5-year survival rate as low as 15% (1, 2). Most tumor cells (including NSCLC) are naturally resistant not only to apoptotic-related cell death (type I programmed cell death), but are also resistant to nonapoptotic types such as necrosis, autophagy (type II programmed cell death), senescence, mitotic catastrophe, and paraptosis (3–5). In addition to mechanisms enabling them to resist cell death, NSCLCs are also able to resist various cytotoxic insults because they possess a large set of intracellular signaling pathways that counteract chemotherapeutic insults including the constitutive activation of the phosphatidylinositol-3-kinase (6), Akt (7, 8), and the nuclear factor–κB (NF–κB) signaling pathways (8, 9), all of which are interlinked (8, 10). In fact, the constitutive or drug-induced activation of the NF–κB signaling cascade constitutes one of the major pathways by which tumor cells escape cytotoxic insults (11–13).

NF–κB is a collective designation for a family of highly regulated dimeric transcription factors regulating the expression of genes encoding cytokines and chemokines, factors involved in tumor promotion/proliferation, angiogenesis, and a plethora of antiapoptotic proteins (11–13). Virtually all vertebrate cells express at least one of the five Rel/NF–κB members, p50/p105 (NF–κB1), p52/100 (NF–κB2), c-Rel, p65 (RelA), and RelB, which are assembled into homodimers and heterodimers binding a common DNA sequence motif known as the κB (11–13). The most commonly encountered dimer in mammalian cells is the p65/p50 dimer (11–13). In most normal cells, Rel/NF–κB dimers are retained in the cytoplasm as an inactive complex by means of direct binding with specific inhibitors, i.e., IκB proteins (11–13). Various signals can lead to the phosphorylation and the subsequent ubiquitin proteasome–mediated degradation of the IκB proteins with the resultant translocation of the active Rel/NF–κB complex into the nucleus (11–13). In contrast, a large number of tumor cells display constitutively high levels of nuclear NF–κB activity due to the hyperactivation of the NF–κB signaling pathways or to inactivating mutations in the regulatory IκB subunits (11–13). Experimental data involving NF–κB inhibition/deactivation as an important new approach in the treatment of various malignancies have shown that the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, and the suppression of apoptosis seems to lie at the heart...
of the ability of NF-κB to promote oncogenesis and resistance to cancer therapy (13). In our present study, we investigated whether a cardenolide (such as ouabain and digitoxin, belonging to the so-called group of cardiotonic steroids; refs. 14, 15) could deactivate a constitutively activated NF-κB signaling pathway in the experimental A549 NSCLC model. In vivo orthotopic xenografts of human A549 NSCLC cells developing in the lungs of immunodeficient mice clearly metastasize into the brains and livers of the host mice and display a broad panel of mechanisms enabling them to resist chemotherapeutic insults including cyclooxygenase-2, prostaglandin E synthetase, ornithine decarboxylase, the lung-related resistance protein, and cyclooxygenase-2, prostaglandin E synthetase, ornithine enabling them to resist chemotherapeutic insults including
cardiotoxic steroids with the sodium pump are independent
The signaling pathways rapidly elicited by the interaction of
these proteins and organizes cytosolic cascades of signaling proteins
sodium pump interacts with neighboring membrane pro-
cardenolides belong (27). In addition to pumping ions, the
cardiotonic steroids; refs. 14, 15) could deactivate a constitutively
activity can be decreased or even inhibited by one class of
sodium pump is an integral membrane protein found in the
cells of all higher eukaryotes and is responsible for trans-
nmole/L (with a half-log concentration increases). The
experiments were carried out in sextuplicate.

Materials and Methods

Compounds

The drugs were purchased as follows: taxol (Paclitaxel; S.A. Bristol-Myers Squibb, Brussels, Belgium), irinotecan (Campto, Aventis, Brussels, Belgium), SN-38 (7-ethyl-10-hydroxycamptotecin; Aventis), oxaliplatin (Oxaliplatin; Inter-Chemical, Ltd., Shen Zhen, China), cisplatin (Platinol; S.A. Bristol-Myers Squibb), carboplatin (Paraplatin; S.A. Bristol-Myers Squibb). The UNBS1450 was obtained by means of chemical hemisynthesis, as detailed elsewhere (26).

Cell Lines

The A549 (ATCC code CCL-185) cell line was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in MEM supplemented with 5% fetal bovine serum, in a mixture of 0.6 mg/mL glutamine (Life Technologies-Invitrogen SA, Merelbeke, Belgium), 200 IU/mL penicillin (Life Technologies-Invitrogen), 200 IU/mL streptomycin (Life Technologies-Invitrogen), and 0.1 mg/mL gentamicin (Life Technologies-Invitrogen). The trypsin-EDTA, the fetal bovine serum, and the cell culture media and their supplements were obtained from Life Technologies-Invitrogen. The fetal bovine serum was heat-inactivated for 1 hour at 56°C. The cells were incubated at 37°C, in sealed (airtight) Falcon plastic dishes (Nunc, Invitrogen SA, Merelbeke, Belgium) in a 5% CO2 atmosphere.

In vitro Overall Growth Determination

Overall cell growth was assessed by means of the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, Bornem, Belgium) assay, as detailed elsewhere (26). The tumor cells were incubated for 72 hours in the presence (or absence – control) of the various drugs. The drug concentrations ranged between 10⁻⁹ and 10⁻³ mol/L (with a half-log concentration increases). The experiments were carried out in sextuplicate.

Figure 1. Comparison of the chemical structures of one well-known cardenolide, i.e., ouabain (A), with 2'-oxovoruscharin (UNBS1244; B) and its hemisynthetic derivative, UNBS1450 (C).
Western Blotting Analyses

Cell extracts were prepared by the lysis of subconfluent A549 cells in the SDS-PAGE loading buffer [1× SDS sample buffer, 62.5 mmol/L Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mmol/L DTT, 0.01% w/v bromophenol blue]. The A549 cell lysates were loaded onto a denaturing polyacrylamide gel (5–12%) and blotted onto a PolyScreen-polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA). The proteins analyzed (see below) were immunodetected by specific affinity-purified primary antibodies (in TBS containing 0.1% Tween 20 and 5% fat-free dry milk powder or bovine serum albumin) in conjunction with a secondary antibody, in the form of IgG conjugated with horseradish peroxidase. Control experiments included the omission of the incubation step with the primary antibodies (negative control). Equal loading was verified after the Ponceau red coloration of the membranes. The integrity and quantity of the extracts was assessed by means of tubulin immunoblotting. The proteins submitted to Western blotting analyses were detected by means of primary antibodies provided by (a) CST Technologies (Westburg, Leusden, the Netherlands): I-βtub (1:1,000), phospho-I-βtub (1:500), ubiquitin (1:500); (b) AbCam (Cambridge, United Kingdom): cdc34 (1:500), tubulin (1:3,000); (c) BD Transduction Laboratories (Erembodegem, Belgium): p65 (1:500); and (d) Santa Cruz (Tebu-Bio, Boechout, Belgium): I-βIκB (1:100). The secondary antibodies used were obtained as follows: anti-mouse and anti-goat IgG from Pierce (PerbioScience, Erembodegem, Belgium), anti-rabbit from NEN Life Science Products, and anti-rat from AbCam. Blots were developed using the Pierce Supersignal Chemiluminescence system (PerbioScience).

NF-κB DNA Binding Assay

NF-κB DNA binding activity was assessed with the Trans-AM NF-κB family transcription factor assay kits (Active Motif Europe, Rixensart, Belgium) developed by Renard et al. (29) as a new sensitive assay to estimate the amount of activated NF-κB in whole-cell protein extracts. This ELISA-like test measures the level of the active form of NF-κB contained in cell extracts specifically able to bind to an oligonucleotide containing the NF-κB consensus site (5’-GGGACTTTCC-3’) attached to a 96-well plate (29). Whole cell lysates were prepared and 10 μg extracts were added to the 96-well plates. The binding of NF-κB to the DNA was visualized by incubation with anti-p50, anti-p52, anti-p65/Rel-A, and anti-Rel-B antibodies that specifically target activated NF-κB, followed by a secondary antibody conjugated with horseradish peroxidase. Antibody binding was determined as absorbance values at 450 nm.

Luciferase Reporter Assay

Although a luciferase reporter plasmid construct (pNF-κB-Luc) containing five NF-κB binding sites was purchased from Stratagene (Amsterdam, the Netherlands), the plasmid construct containing three NF-κB (p3NF-κB-Luc) binding sites was kindly provided by Dr. Corinne Grangette (Laboratoire de Bactériologie des Ecosystèmes, Institut Pasteur, Lille, France). Clones of A549 tumor cells stably containing the NF-κB-Luc constructs were obtained by cotransfection with the NF-κB-Luc plasmid and pSV2neo (30) in a molecular ratio of 10:1. The A549 tumor cells were plated 24 hours after transfection and the isolated clones were selected by cultivating the A549 cells in MEM supplemented with 700 μg/mL of the neomycin analogue G418 (Geneticin, Life Technologies-Invitrogen). Selected A549 clones were analyzed for luciferase activity by the luciferase assay system (Promega, Leiden, the Netherlands). Briefly, 10⁴ A549 cells were plated in 96-well plates and, 24 hours after plating (see Results), the cells were cultivated in MEM supplemented with 10 nmol/L UNBS1450 for various experimental times. The cells were rinsed with PBS, lysed in 20 μL of lysis buffer (Promega), and frozen at −20°C. The A549 cell lysates were tested for luciferase activity with 40 μL of luciferase reagent on a TD-20/20 luminometer (Turner Designs, Promega) with an integration time of 30 seconds.

In vivo Orthotopic Grafting of A549 NSCLC Human Cells

The potential in vivo UNBS1450-related therapeutic effects were determined on human A549 orthotopic xenografts by administering UNBS1450 chronically by i.p. or p.o. injections thrice a week for 4 consecutive weeks and at different concentrations of the maximum tolerated dose (MTD) varying from (MTD)/32 to (MTD)/8 (see Results). The MTD for UNBS1450 was determined by administering it acutely (i.e., in one single i.p. or p.o. dose) to healthy mice (i.e., not grafted with tumors). The survival periods and the weights of the animals were recorded for up to 28 days after the injection of the compound. Seven different doses of UNBS1450 (i.e., 2.5, 5, 10, 20, 40, 80, 120, and 160 mg/kg) were used to determine the MTD index. The MTD index is defined as the concentration killing at least one mouse in a group of three after a minimum of 28 days. Acute UNBS1450 MTDs via the i.p. and the p.o. route were 120 and >160 mg/kg, respectively.

As detailed elsewhere (16), orthotopic A549 xenografts were obtained by grafting 2 × 10⁶ human A549 cells through the thorax into the left-hand-side of the lungs of nude mice. All the orthotopic grafts were done under anesthesia [saline; Rompun (Bayer, Leverkusen, Germany); Imalgene (Merial, Lyon, France); 5/1/1, vol/vol/vol]. Highly reproducible tumor developments (100%) were obtained from the A549 tumor grafting in each experiment. The end point of the A549-related experiments was the recording of the survival periods of each of the A549-NSCLC–bearing nude mice. For ethical reasons, each animal was submitted to euthanasia (in a CO₂ atmosphere) when it had lost 20% of its weight as compared with its weight at the time of the tumor graft. As detailed elsewhere, autopsies and histology were done on each mouse to confirm the presence of the tumor development (16). All the in vivo experiments described in the current study were done on the basis of authorization no. LA1230509 of the Animal Ethics Committee of the Belgian Federal Department of Health, Nutritional Safety, and the Environment.
Statistical Analyses
Survival analysis was done by using Kaplan-Meier curves and the Gehan generalized Wilcoxon test. All the statistical analyses were carried out using Statistica software (Statsoft, Tulsa, OK).

Results
Human A549 NSCLC Cells Display Constitutively High Levels of NF-κB Activity
Figure 2A shows that A549 tumor cells (black columns) display basal RelA/p65 DNA-binding activity as high as in the tumor necrosis factor-α-stimulated Raji cells (gray columns) used as the positive control in the colorimetric NF-κB DNA binding assay (29) employed here. High basal levels of NF-κB activity were also observed in stably transfected clones of A549 cells containing three (Fig. 2B) or five (Fig. 2C) NF-κB binding sites in luciferase reporter constructs. The high basal levels of NF-κB activity in A549 tumor cells were further outlined by the fact that TNF-α, one of the most potent NF-κB inducers, less than doubled the NF-κB activity in these A549 cells, even when used at 100 ng/mL (Fig. 2B and C).

UNBS1450 Affects the Expression and Activation Status of NF-κB in Human A549 NSCLC Cells
UNBS1450-mediated effects in connection with expression and phosphorylation status of the different constituents of the NF-κB pathways in the A549 tumor cells were characterized at a 10 nmol/L dose, which, in its turn, constitutes the IC50 value in terms of the concentration that characterized at a 10 nmol/L dose, which, in its turn, one of the most potent NF-κB pathway inducers, less than doubled the NF-κB activity in these A549 cells, even when used at 100 ng/mL (Fig. 2B and C).

This dose is at least 100 times lower than the doses reported in other studies whose purpose is to characterize the influences of various cardenolides on the NF-κB signaling pathways (18, 19). The treatment of A549 tumor cells with 10 nmol/L UNBS1450 for 24 hours did not trigger off any cell death processes, a point which was determined by means of flow cytometry (data not shown). We chose to analyze the effects of UNBS1450 over periods as long as 24 hours because we were interested in the ability of this compound to deactivate constitutively activated NF-κB activity and not in its ability to counteract the rapid NF-κB activation resulting from proinflammatory stimulation which occurs within minutes.

Although UNBS1450 (10 nmol/L) was the major factor in inducing the accumulation of IκBα in the A549 tumor cells (Fig. 3A), it seemed to leave the levels of expression of IκBα unchanged (Fig. 3B). In contrast, UNBS1450 decreased IκBα phosphorylation between 4 and 8 hours following its addition to the A549 tumor cell culture medium (Fig. 3C). UNBS1450 (10 nmol/L) also decreased the levels of expression of cdc34 (Fig. 3D). cdc34 is a ubiquitin-conjugating enzyme (E2) involved in IκBα ubiquitination and its subsequent proteasomal degradation (31). In addition, 10 nmol/L UNBS1450 also led to a decrease in the accumulation of the βTrCP protein in the A549 tumor cells (data not shown). βTrCP is a ubiquitin ligase (E3) that forms a complex with Skip1 and Cullin1; this complex is involved in IκBα ubiquitination (32). Furthermore, 10 nmol/L of UNBS1450 induced an increase in the accumulation of ubiquitinated proteins in the A549 tumor cells by ~ 8 to 10 hours following its addition to the culture medium of these cells (Fig. 3E).

Treatment with MG132, a proteasome inhibitor, was used as a control because proteasome inhibition also results in the accumulation of ubiquitinylated proteins, a feature observed here in the case of MG132 (Fig. 3E).
UNBS1450 Decreased the DNA Binding Capacity of the p65 Subunit

UNBS1450 (10 nmol/L) decreased the levels of the p65/RelA subunit protein accumulation (Fig. 4A). A colorimetric NF-κB DNA-binding assay with cell lysates from untreated and UNBS1450-treated A549 cells at two doses (10 and 100 nmol/L) further revealed that the UNBS1450 treatment of the A549 tumor cells resulted in a dose- and time-dependent decrease in p65 DNA-binding activity (Fig. 4B).

UNBS1450 Decreased the NF-κB Transcriptional Activity

The data in support of an UNBS1450-induced down-regulation of the NF-κB activity presented in Fig. 3 and in Figs. 4A and B were further confirmed by the use of luciferase reporter constructs containing three and five NF-κB binding sites stably transfected into A549 tumor cells (Fig. 4C). Indeed, 10 nmol/L UNBS1450 induced marked time-dependent decreases in the NF-κB reported luciferase activity in the four different A549 tumor cell clones used in the present study.

In vitro Antitumor Effects of UNBS1450 on the Overall Growth of Human A549 NSCLC Cells

UNBS1450 displayed in vitro antiproliferative activities with respect to the A549 tumor cell populations, similar to those effects observed with taxol or SN38, the active metabolite of irinotecan, but its effects were significantly more pronounced than those observed with various platinum derivatives (Fig. 5A).

In vivo Antitumor Effects of UNBS1450 in A549 Orthotopic Xenografts

UNBS1244, the novel cardenolide that we identified in C. procera, had an acute MTD level of 10 mg/kg when injected i.p. in healthy mice. This value is similar to those observed with respect to ouabain (5 mg/kg) and digitoxin (10 mg/kg; ref. 26). In sharp contrast, whereas both UNBS1244 and UNBS1450 showed in vitro IC₅₀ values of ~10 nmol/L with respect to the mean inhibition of the overall growth levels of 57 human cancer cell lines (26), UNBS1450 showed an acute i.p. MTD value of 120 mg/kg. Thus, although UNBS1450 is much better tolerated in vivo than its mother compound, UNBS1244, the in vitro antiproliferative effects of both compounds are identical.

Orthotopic xenografts of human A549 NSCLCs into nude mice are resistant to taxol and oxaliplatin, and only weakly sensitive to irinotecan (16). However, UNBS1450 markedly increased the survival of A549 NSCLC orthotopic xenograft-bearing mice when given i.p. chronically (12 times) at 10 or 20 mg/kg (Fig. 5B). There are no statistically significant differences between the 10 and the 20 mg/kg dose schedules. Chronic i.p. administrations of UNBS1450 at 5 mg/kg also contributed weak, but nevertheless significant, increases in these survival periods (Fig. 5B).

At higher doses, i.e., 40 mg/kg, chronic i.p. treatment was associated with slight toxic effects, i.e., a transient loss of weight in the A549 xenograft-bearing mice, but without the deaths that occurred with a chronic i.p. treatment of 80 mg/kg (data not shown). The experiments illustrated in Fig. 5B were reproduced 6 months later with identical results (data not shown). Furthermore, when chronically administered 12 times p.o. at 80 mg/kg, UNBS1450 was able to bring about a beneficial therapeutic effect in the case of the orthotopic A549 model (Fig. 5C).

One can wonder what happened to A549 orthotopic xenografts in vivo following UNBS1450 treatment. Based on the in vitro data reported above and on the data relying on an in vivo experiment carried out with the s.c. NCI-H727 NSCLC xenograft model, we hypothesize that UNBS1450 decreased the growth rates of both the primary xenografts and brain and liver metastases. Indeed, we treated s.c. NCI-H727 NSCLC xenografts with UNBS1450 exactly as we did here with respect to the A549 NSCLC orthotopic xenograft model: we observed that UNBS1450 decreased by ~50%
The levels of the sodium pump α and β subunits can be markedly modified in cancer cells as compared with their normal counterparts (36–38). In fact, it seems that poorly differentiated carcinoma cells show a reduced expression of the β1 sodium pump subunit, a feature that correlates with an increased expression of the Snail transcription factor, which is known to down-regulate E-cadherin (37). In addition, the down-regulation of the β1 sodium pump subunit and E-cadherin by Snail are associated with events leading to epithelial to mesenchymal transition (37), which is an important step in the metastatic processes in lung cancers (39, 40).

The constitutive activation of NF-κB helps a variety of tumors, including NSCLCs, to resist natural or chemotherapeutically induced cell death (11–13). Major efforts to develop NF-κB inhibitors for anticancer purposes are consequently taking place in fundamental research and in the pharmaceutical industry. It is therefore very important to point out the differences in the (de)activation of NF-κB signaling pathways in immune responses and in cancer. Whereas proinflammatory stimuli induce the rapid activation of early response genes, the hyperactivation of the NF-κB signaling pathways in cancers affects the expression of the different genes involved in regulating the “hallmarks of cancer” (11–13, 41). Consequently, the mechanisms and their kinetics required for the down-regulation of the NF-κB signaling pathways are different in immune responses and in cancers. As reported in the Introduction, cardenolides are able to deactivate the NF-κB signaling pathways (17, 18) and have a number of antitumor effects on lung cancers (20, 21). In the present study, we clearly show that A549 tumor cells exhibit a high basal level of NF-κB activity, and that UNBS1450 is able to deactivate this NF-κB activity in vitro a few hours after the addition of UNBS1450 to the A549 NSCLC cell culture medium. This UNBS1450-induced deactivation of the NF-κB pathways occurs at several levels, including both the inhibitory I-κB portion of the NF-κB signaling pathway and its stimulatory p65/Rel-A NF-κB portion. UNBS1450-induced effects on the inhibitory I-κB protein level involve (a) the up-regulation of inhibitory protein expression (as observed for I-κBβ; Fig. 3A), (b) the down-regulation of the Na⁺ and K⁺ concentrations) include modifications to the Src kinase, the epidermal growth factor receptor, and the Ras and p42/p44 mitogen-activated protein kinase activity (27, 28), all of which are associated with major roles in biological NSCLC behavior (33–35). In fact, there are two sodium pump pools within the plasma membrane with two different functions, i.e., one being the standard enzyme pool as an energy-transducing ion pump whose partial inhibition by ouabain initiates the increase in intracellular calcium concentration ([Ca²⁺]), and the other, the signal-transducing pool of the enzyme, which, through protein-protein interactions, leads to the activation of a signaling intermediate and an increase in the intracellular reactive oxygen species (27, 28). The sodium pump consists of two subunits in equimolar ratios in the shape of α and β subunits, with three α and three β subunit isoforms (27, 28).

The signaling pathways that are rapidly elicited by the interaction of cardenolide (including ouabain, whose chemical structure is depicted in Fig. 1) with the sodium pump (and that are independent of changes in intracellular Na⁺ and K⁺ concentrations) include modifications to the Src kinase, the epidermal growth factor receptor, and the Ras and p42/p44 mitogen-activated protein kinase activity (27, 28), all of which are associated with major roles in biological NSCLC behavior (33–35). In fact, there are two sodium pump pools within the plasma membrane with two different functions, i.e., one being the standard enzyme pool as an energy-transducing ion pump whose partial inhibition by ouabain initiates the increase in intracellular calcium concentration ([Ca²⁺]), and the other, the signal-transducing pool of the enzyme, which, through protein-protein interactions, leads to the activation of a signaling intermediate and an increase in the intracellular reactive oxygen species (27, 28). The sodium pump consists of two subunits in equimolar ratios in the shape of α and β subunits, with three α and three β subunit isoforms (27, 28).
The phosphorylation of NF-κB transcription factors is regulated not only by protein serine/threonine kinases, but also by protein serine/threonine phosphatases. Compared with the well-established mechanisms for the phosphorylation of NF-κB transcription factors (that relate to the direct consequence of proinflammatory stimulations, for example), the mechanisms underlying the dephosphorylation of these transcription factors are still poorly understood. Four major classes of protein phosphatases have been described, including PP1, PP2A, PP2B (calcineurin), and PP2C (42). Although PP2B is calcium-dependent, and PP2C is magnesium-dependent, PP1 and PP2A are not dependent on divalent cations (42). PP1 and PP2A are widely expressed in mammalian cells and are involved in the regulation of signaling pathways by a mechanism of phosphorylation/dephosphorylation with a variety of protein kinases (42). Increases in [Ca^{2+}]\_i result in the activation of many calmodulin-dependent enzymes such as calcineurin, for example (43). This phosphatase could participate in a site-specific dephosphorylation of IκBα (phospho-Ser32) resulting in NF-κB deactivation, as is observed in astrocytes (44). Because the interaction of cardenolides with the sodium pump induces an increase in [Ca^{2+}]\_i (22), the dephosphorylation of IκBα observed upon treatment with UNBS1450 (Fig. 3 C) could, at least partly, relate to the activation of calcineurin, a feature that we are currently analyzing.

Protein stability is a key regulatory mechanism in the control of cell development, the cell cycle, cell growth and apoptosis, and a recent emerging theme for anticancer drug discovery and development logically relates to protein degradation. The ubiquitin system targets a wide array of short-lived regulatory proteins such as transcriptional activators, tumor suppressors and growth modulators, cell cycle regulators, and signal transduction pathway components (45). Protein degradation by the ubiquitin system involves two steps, i.e., (a) the covalent attachment of multiple ubiquitin molecules to the target protein and (b) the degradation of the tagged substrate by the 26S proteasome (45). Ubiquitin is covalently attached to substrate proteins by a protein complex usually including an activating enzyme (E1), a conjugating enzyme (E2), and a protein ligase (E3; ref. 45). UNBS1450-induced

![Figure 5](https://mct.aacrjournals.org/article/5/2/397/Figure5.large.jpg)
increases in the level of global ubiquitination therefore suggest that UNBS1450 could affect the ubiquitin-proteasomal protein degradation pathway. Moreover, the levels of expression of cd34, which is a conjugating enzyme (E2) involved in the degradation pathway of IκBα, were markedly decreased by UNBS1450 treatment in the A549 tumor cells.

All the effects that we report here with respect to the direct action of UNBS1450 on the cytoprotective effects of the constitutive NF-κB signaling pathways in A549 NSCLC cells are paralleled by marked in vitro and in vivo antitumor effects on these A549 tumor cells (Fig. 5).

In vitro UNBS1450-induced cell death in the A549 model resulted from a UNBS1450-mediated Hsp70 down-regulation, a process that led to massive caspase-independent tumor cell death involving lysosomal membrane permeabilization (data not shown). Nylandstedt et al. (46) already showed cell death processes that occur in relation to modifications in Hsp70 expression. In addition, Frese et al. (47) reported that a selective down-regulation of Hsp70 induces cell death in tumor but not in normal lung cells, a feature that could explain, at least partly, the high therapeutic index observed in vivo with respect to UNBS1450.

The potential therapeutic benefit contributed by anticancer drugs could also be eliminated by phosphatidylinositol-3-kinase/Akt signaling pathway. In our study, we observed no in vitro additive effects on A549 NSCLC cell mortality when combining UNBS1450 with phosphatidylinositol-3-kinase inhibitors (data not shown), a feature which suggests that the phosphatidylinositol-3-kinase-mediated signaling pathway does not seem to protect NSCLC cells against the UNBS1450-induced cell death–related effects.

Taken together, all these data therefore argue in favor of the use of certain cardenolides to combat a devastating disease like NSCLC. Indeed, apart from the direct antitumor effects evidenced here for UNBS1450 on very aggressive orthotopic xenografts of the human A549 NSCLC model, certain cardenolides could be used to sensitize NSCLC cells to antitumor agents through the deactivation of the cytoprotective effects caused by constitutively activated NF-κB signaling pathways in these tumor cells. In addition, it has already been proved that the enzyme activity or the level of expression of the sodium pump may contribute to the cellular uptake of cis-diaminedichloroplatinum(II) and determine the sensitivity of human lung cancer cells to cis-diaminedichloroplatinum(II) (48). It has also been proved that the sodium pump is active in both NSCLCs and small cell lung cancers, but that the importance of the enzyme as an active transporter of cis-diaminedichloroplatinum(II) may be limited only to NSCLC cells (49). We are currently investigating whether in vivo UNBS1450 is capable of improving the antitumor effects of taxol and oxaliplatin on A549 orthotopic xenografts. In parallel, UNBS1450 is entering the candidate drug development stage and should be in phase I clinical trials by 2006.

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