Cancer Chemother Pharmacol (2000) 45: 183–191 © Springer-Verlag 2000

ORIGINAL ARTICLE

Gabriel F. Eilon · Jirong Gu · Lewis M. Slater
Kaoru Hara · John W. Jacobs

Tumor apoptosis induced by epoxide-containing piperazines, a new class of anti-cancer agents

Received: 11 June 1999 / Accepted: 3 September 1999

Abstract *Purpose*: The overall purpose of this study was to determine the potential efficacy of epoxide-containing piperazines as a new class of anti-cancer agents. Two representative compounds, specifically NCO-700, a 4-trimethoxyphenyl-substituted epoxide-piperazine, and TOP-008, a 4-phenylpropenyl-substituted epoxide-piperazine were tested in cytotoxic assays with human breast and prostate cancer cell lines. A second objective was to determine if these two compounds had anti-cancer activity in vivo when tested against xenograft tumors in nude mice or human tumors grown under the kidney capsule in mice. A final objective of this study was to establish if NCO-700 and TOP-008 achieved cancer cell killing through an apoptotic mechanism. *Methods*: The anti-proliferative activity of NCO-700 and TOP-008 were tested in a 7 day cell-survival assay utilizing a number of well characterized breast (HS-578T, T47D, MCF-7) and prostate (DU-145, PC-3, LNCaP) cancer cell lines. In vivo studies with the two compounds were performed, in nude mice bearing DU-145 xenograft tumors, and in normal mice in which DU-145 prostate cancer cells and HS-578T breast cancer cells were grown as solid tumors in the subrenal capsules of the animals. Apoptotic cell death of cancer cells was determined by a number of established techniques that detect apoptosis, including the confocal laser microscopy of treated cells and mitochondrial leakage assays utilizing the cationic dye, JC-1. Finally, the activation of the caspase cascade, enzymes that carry out apoptosis in mammalian cells, was examined in treated cells by immunoblot assays. *Results*: NCO-700 and TOP-008 displayed cytotoxicity to HS-578T human breast cancer cells, with ED_{50} values in the 3–6 μM range. Cytotoxicity to androgen receptor-negative human prostate cancer cells (PC-3 and DU-145 cells) occurred with ED_{50} values in the 5–20 μM range. Cytotoxicity to hormone receptor-positive breast and prostate cancer cell lines occurred at 10 to 20-fold higher concentrations of the two compounds. When human prostate (DU-145) or breast cancer (HS-578T) cells were grown as solid tumors in the subrenal capsules of mice, significant anti-tumor activity of NCO-700 was observed at 20 mg/kg and 50 mg/kg body weight respectively, for prostate and breast tumors. In nude mice bearing DU-145 prostate tumor xenografts, 50 mg/kg doses of the two compounds either stopped (TOP-008) tumor growth or slowed (NCO-700) growth. The mechanism of cytotoxicity was shown to be through apoptosis, (a) by confocal microscopy studies revealing nuclear fragmentation, (b) by mitochondrial studies revealing disruption of the mitochondrial membrane and release of the cationic dye, JC-1, into the cytoplasm and (c) by protein immunoblot assays indicating that over a 6 h period, TOP-008 induced a significant accumulation of the pro-apoptotic protein, bak, in the mitochondrial fraction of HS-578T human breast cancer cells, accompanied by activation, at 2.5 h, of caspase-3. *Conclusions*: These studies indicated that the epoxide-containing piperazines, as exemplified by NCO-700 and TOP-008, were effective anti-cancer agents when tested in vitro and in vivo against human breast and prostate tumors. Our studies also indicated that TOP-008 induced the initiation of the caspase cascade leading to apoptosis. Previous toxicology studies in rodents and dogs, as well as a Phase I study in humans, showed NCO-700 to be a well-tolerated, non-toxic compound. Taken together with our current findings, these results suggest that this class of compounds has the potential to be relatively safe, new chemotherapeutic agents for refractory breast and prostate cancers.

Key words Epoxide-containing piperazines · Apoptosis · Chemotherapeutics

G.F. Eilon · J.W. Jacobs (✉)
Hitachi Chemical Research Center,
1003 Health Sciences Road West, Irvine, CA 92612, USA
Tel.: +1-949-7252734; Fax: +1-949-7252727

L.M. Slater
Department of Medicine,
University of California, Irvine, Calif., USA

K. Hara
Nippon Chemiphar Co. Ltd., Tokyo, Japan

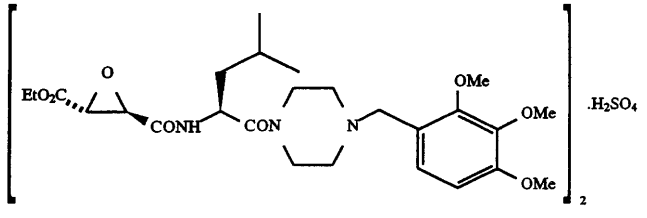
Abbreviations *NCO-700* Bis[ethyl(2R,3R)-3-[(*S*)-3-methyl-1-[4-(2,3,4-trimethoxyphenylmethyl)piperazin-1-ylcarbonyl]butylcarbamoyl]oxirane-2-carboxylate]-sulfate · *TOP-008* Bis[ethyl(2R,3R)-3-[(*S*)-3-methyl-1-[4(3-phenyl-2-propenyl)piperazin-1-ylcarbonyl]butyl-carbamoyl]oxirane-2-carboxylate]sulfate

Introduction

The epoxide-containing piperazines are protease inhibitors which were developed over 10 years ago for use in cardiovascular diseases [6, 13, 17]. Compound NCO-700 (see Fig. 1 for structure) has been the most extensively studied member of this class. The compound is a potent and irreversible inhibitor of the lysosomal cysteine protease, cathepsin B [8], as well as of the calcium-activated neutral proteases (calpains) [7]. In a number of animal studies the compound was shown to salvage myocardial tissue following ischemic injury. Extensive toxicology studies in rodents and dogs, as well as a preliminary safety study in humans, showed NCO-700 to be a relatively safe, well-tolerated compound (personal communication, N. Miyake, Nippon Chemiphar Co. Ltd., Japan). As described in the present report we have discovered in this class of compounds a new activity, namely the induction by NCO-700 and the structurally-related derivative, TOP-008, of apoptosis in selected breast and prostate tumors.

Protease inhibitors, such as NCO-700, have been examined in cancer therapy mainly as anti-metastatic agents [3, 5, 11]. These agents have been shown to inhibit tumor cell-derived proteases that are thought important for the cancer cell to escape the vasculature and invade the sub-endothelial space and outlying tissues. However, there are few reports on the ability of such protease inhibitors to induce programmed cell death. It is known from a number of studies that induction of apoptosis is a common mechanism underlying the activity of several chemotherapeutic agents [2, 9]. Apoptosis is characterized by numerous biochemical and morphological changes in the cell, including fragmentation of nuclear DNA into large particles, condensation of the nuclear structure and disruption and leakage of components through the mitochondrial membrane. Anti-cancer agents which can induce the tumor cell to enter apoptosis, at doses below which they display cytotoxicity to normal cells, could be of high clinical value. The epoxide-containing piperazines, as reported in the present study, are able to induce apoptosis in selected tumors at doses well below those at which toxicity is observed in normal cells, making this class of compounds attractive for development as new chemotherapeutic agents.

NCO-700 Bis[ethyl (2R,3R)-3-[(S)-3-methyl-1-[4-(2,3,4-trimethoxyphenylmethyl) piperazin-1-ylcarbonyl]butylcarbamoyl]oxirane-2-carboxylate] sulfate



TOP-008 Bis[ethyl (2R,3R)-3-[(S)-3-methyl-1-[4-(3-phenyl-2-propenyl)piperazin-1-ylcarbonyl]butylcarbamoyl]oxirane-2-carboxylate] sulfate

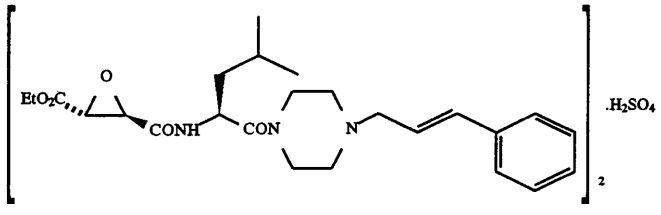


Fig. 1 Chemical structures of the two epoxide-containing piperazines, NCO-700 and TOP-008, used in this study

Materials and method

Compounds studied

The epoxide-containing piperazines NCO-700 and TOP-008 (Fig. 1), were synthesized by Nippon Chemiphar (Tokyo, Japan), and were generously provided for use in the present studies. The compounds were dissolved in water prior to use for both in vitro and in vivo studies.

Cell lines

All human breast and prostate tumor cells were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). The cells were cultured in DMEM (GIBCO/BRL Life Technologies, Grand Island, N.Y.) supplemented with 10% defined fetal bovine serum (FBS) (HyClone, Logan, Utah) and 0.5 μg/ml gentamicin (Sigma Chemicals, St. Louis, Mo.). Cells were incubated at 37 °C in the dark in a humidified atmosphere. All testing was done on cells that were in passage 3–12. Cells were tested routinely for the presence of mycoplasma (MA Biosciences, Rockville, Md.). Estrogen or testosterone supplementation at a concentration of 30 ng/ml was performed with hormone-dependent breast or prostate cancer cells.

Cell viability assays

Cell viability was determined by the commercially available Cell Titer 96 Aqueous Proliferation Assay (Promega, Madison, Wis.). This assay utilizes the conversion of MTS-tetrazolium by metabolically active cells to a stable, colored compound which can be detected spectrophotometrically at 490 nm. For overnight MTS assays, tumor cells were seeded at a rate of 6000 cells per well in 96-well-plates (Corning Costar, Cambridge, Mass.) and cultured for 24 h. Test compounds were then added, and the plates were incubated for 16 h at 37 °C. The assay was then performed according to instructions provided by the supplier of the kit, and the absorbance of the wells was determined by a Softmax Microplate Reader (Molecular Devices) at 490 nm wavelength.

For 7 day cell-survival assays, cells were seeded at a level of 400 cells per well 24 h prior to addition of the test compound. Cells were incubated at 37 °C for 7 days with daily media changes. On day 7 the medium was changed to one which did not contain phenol red, prior to addition of the MTS-reagent and the reading of absorbance on the 96-well plate reader.

Apoptosis assays

JC-1 flow cytometry assay

This assay detects changes in the integrity of the mitochondrial membrane, an early event in apoptosis. The assay employs a fluorescent cation, JC-1, which emits red fluorescence when sequestered in the mitochondrial membrane of healthy cells, and emits a green fluorescence when released into the cytoplasmic compartment of the cell. The JC-1 dye (Molecular Probes, Eugene, Ore.) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. Cells were seeded in T-25 cm^2 flasks (Costar) for 48 h, or until they reached 70–80% confluency, after which the medium was changed to one containing JC-1 at a final concentration of 1 μg/ml and the cells were incubated for an additional 30 min at 37 °C. The mixture containing the JC-1 dye was aspirated, fresh medium added and the cells then treated with the test compounds, from 0.25–5 h. After this, the cells were lightly trypsinized (0.05% trypsin-EDTA, Gibco) and washed twice with phosphate-buffered saline (PBS) prior to analysis on the FACScan to determine red and green fluorescence.

Confocal microscopy

Apoptosis occurring in vivo was investigated by the in situ labeling of nucleic acid (DNA and RNA) in cancer cells exposed to the test compounds, followed by visualization of the nucleic acid by confocal scanning laser microscopy [1, 16]. Athymic, nude mice, 20–22 g in weight, were inoculated with 10×10^6 DU-145 human prostate cancer cells, by injection into the peritoneal cavity. After 48 h, to allow establishment of the cancer cells as ascites tumors, NCO-700 or TOP-008 was administered, intraperitoneally to the test-animals at a single daily dose of 2 mg per mouse. The drug treatment was continued for 8 days, at which time cancer cells were removed from the animals and purified by flow cytometry by which tumor cells can be separated from normal mouse cells based on the DNA/RNA content of the cells [20]. The isolated cancer cells were subjected to scanning confocal laser microscopy. Cancer cells had been stained with acridine orange prior to confocal microscopy. Fluorescence distributions were plotted for individual cancer cells, with corresponding color intensities reflecting the intactness of the DNA within the cell; the highest values (white color) indicating compacted, highly-ordered nucleic acid, typical of healthy cells [16]. Approximately 50% of the cancer cells analyzed by this procedure were determined to be undergoing apoptosis. Microscopy was performed with a Meridian Instruments (Okemos, Mich.) Laser Scanning Confocal Microscope.

Immunoblot analyses

Protein immunoblot analyses of bak, caspase-3, and procaspase-3 were performed with antibodies obtained from Transduction Labs. The antibodies were utilized according to instructions from the supplier. Cytosolic and mitochondrial fractions of HS578-T human breast cancer cells were prepared by differential centrifugation [19]. Cancer cell homogenates were subjected to SDS-polyacrylamide gel electrophoresis in 12% gels, followed by transfer of the proteins to polyvinylidine difluoride (PVDF) membranes. The blot was probed with the first antibody at a 1:1000 dilution, followed by incubation with the secondary antibody probe labeled with horseradish peroxidase (Amersham).

In vivo assays

Subrenal capsule assay

The 6 day subrenal capsule implant assay has been described in detail [4, 14, 15]. Female BDF_1 mice weighing 18–20 g were anesthetized, the left kidney exteriorized and a small nick made in the kidney capsule for implantation of tumor cells. Approximately 1×10^6 human breast or prostate cancer cells were prepared as a fibrinogen-gelled pellet as described [15] and implanted beneath the renal capsule. The test compounds, dissolved in water, were administered 24 h later for a period of 5 days by daily intraperitoneal (IP) injection. On the 6th day the animals were killed and the size of the tumor implant measured in situ. The growth of the tumor was determined by subtracting the mean initial implant size from the mean final implant size as described [15]. Statistical significance in the subrenal capsule assay was determined by Student's *t* test [15].

Xenograft tumors in nude mice

Balb/C nude mice were housed in a barrier facility under a controlled 12 h light 12 h dark cycle. They received subcutaneous injections of approximately 10×10^6 DU-145 prostate cancer cells. The tumors were allowed to grow in the animals until a volume of approximately 75 mm^3, had been reached, which occurred at approximately 21 days after the initial injection of the tumor cells. Each treatment group consisted of 6 mice which received (i) daily IP injections of TOP-008 and NCO-700 at a dose of 50 mg/kg; (ii) daily IP injections of doxorubicin at a dose of 6 mg/kg; or (iii) no drug. Tumor size was measured with calipers every 2 days over a 15 day period. All the experiments on the mice were carried out according to NIH laboratory animal care guidelines.

Results

Cell growth

The anti-proliferative activity of NCO-700 and TOP-008 (see Fig. 1 for structures) was tested in 7 day cell-survival assays. As shown in Table 1, a number of human breast and prostate cell lines were examined and both compounds showed enhanced cytotoxicity to cell lines that did not display steroid hormone growth dependency. The highest potency was observed in the hormone-independent breast cancer cell line, HS-578T, with IC_{50} doses for both compounds being in the 3–10 μM range. In contrast, when hormone-dependent breast cancer cell lines, such as T47D or MCF-7 cells were tested, 10–20 times higher doses of NCO-700 or TOP-008 were required to achieve comparable activity. A similar pattern was observed in human prostate cancer cell lines where the hormone-independent cell lines, PC-3 and DU-145, were approximately ten times more

Table 1 Anti-proliferative activity of NCO-700 and TOP-008 in human breast and prostate tumor cells in a 7 day cell-survival assay

Cell line	Cell type[a]	Drug IC_{50} (dose, μM)[b]	
		NCO-700	TOP-008
HS-578T	Breast (ER−)	7.5	2.8
T47D	Breast (ER+)	>100	55
MCF-7	Breast (ER+)	72	68
DU-145	Prostate (And−)	11.5	4.5
PC-3	Prostate (And−)	10.0	8.0
LNCaP	Prostate (And+)	95	85

[a] Hormone-independent cell lines are designated ER− or And−; hormone-dependent cell lines are ER+ or And+

[b] Means of three determinations performed in duplicate. Standard deviations in all assays were <5%

sensitive to NCO-700 and TOP-008 than the hormone-dependent LN prostate cancer line. Representative dose response curves are shown in Fig. 2, where the cytotoxicity of TOP-008 to DU-145 human prostate cancer cells and HS578-T human breast cancer cells is presented.

Apoptosis assays

Confocal microscopy

Morphological changes in cells undergoing apoptosis were visualized by confocal microscopy, which was performed on cancer cells isolated by flow cytometry, from treated or untreated athymic mice. Cancer cell nucleic acid was stained with acridine orange, subjected to confocal microscopy, and the fluorescence distribution plotted for individual cancer cells. Figure 3 shows a representative experiment in which mice carrying human prostate cancer cells (DU-145) were subjected to treatment with TOP-008 at a daily IP dose of 2 mg per animal (approximately 100 mg/kg). Figure 3 (upper panel) shows a typical cell from the control mice, with the bright, spherical staining of nucleic acid in the nuclear compartment indicative of highly ordered nuclear material, characteristic of a healthy, dividing cell. In contrast, shown in the lower left panel of Fig. 3 is a typical cancer cell removed from the TOP-008 treated animals. Here the nuclear material in this cell is lobular, disordered and typical of cells undergoing apoptosis.

When fluorescence distribution patterns were computed for the cells shown in Fig. 3, there was a high percentage of highly ordered nucleic acid (DNA and RNA) in the cell obtained from an untreated animal (upper right panel, Fig. 3). The highly ordered DNA has a high color intensity value, and can be seen as the white area on top of the fluorescence distribution pattern. In contrast, the highly ordered DNA is absent in the cell obtained from amouse exposed to TOP-008 (lower right panel, Fig. 3). Similar results were seen in cancer cells removed from animals treated with NCO-700 (data not shown).

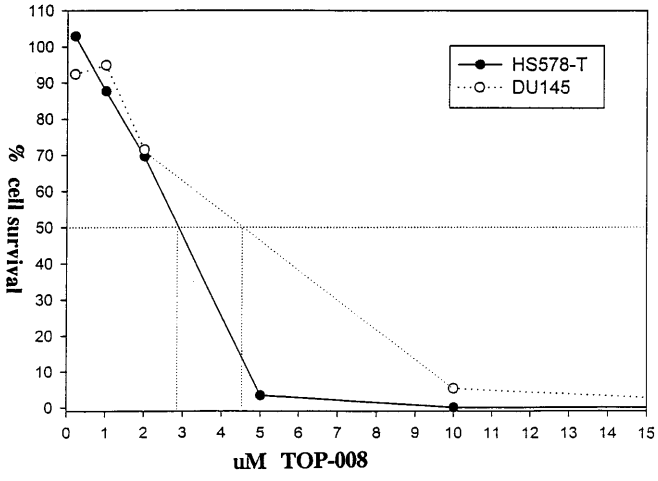


Fig. 2 Dose-dependent effect of TOP-008 on the survival of HS-578T human breast cancer cells (*solid line*) and DU145 human prostate cancer cells (*dashed line*) in a 7 day cell-survival assay. IC_{50} values are determined at 50% cell-survival (*vertical dashed lines*). For clarity, error bars have been omitted from the figure, but each datum point represents the mean percent cell-survival derived from three separate determinations performed in duplicate. Standard deviations (±) values were in the 2–9 range for each datum point

Flow cytometry assay of JC-1: a mitochondrial marker of apoptosis

Mitochondrial membrane depolarization is an early event in apoptosis. Recent studies have indicated that upon induction of apoptosis, mitochondria lose the ability to sequester charged cations and release cytochrome C from internal stores [10, 19]. An assay to detect these early changes in mitochondrial function employs a fluorescent cation, JC-1, which emits a red color when sequestered in the mitochondria of healthy cells, and emits a green color when it is located in the cytoplasmic compartment of the cell. Cells undergoing apoptosis are no longer able to sequester the JC-1 cation in the mitochondria, and are detected as green-colored cells by flow cytometry. Figure 4 shows the results of the JC-1 flow cytometry assay after treatment of human breast (HS578-T, Fig. 4A) or human prostate (DU-145, Fig. 4B) cancer cells with 25 μM TOP-008 for increasing lengths of time.

It can be seen that both breast and prostate cancer cells, treated with TOP-008, lose the ability to sequester the JC-1 cation dye in the mitochondrial compartment. In both cell lines there is a time-dependent increase in green versus red fluorescence. To aid quantification the scans in Fig. 4 have been divided arbitrarily into quadrants. At time 0, both breast and prostate tumor cells sequester more than 90% of the dye in the mitochondria, whereas after 5 h this value drops to 33% for prostate cancer cells and 22% for breast cancer cells. When similar experiments were performed on untreated breast and prostate cancer cells 90% (± 5%) of the JC-1 dye remained in the mitochondria over the 5 h time-course of the experiments (data not shown). These data add further support to the theory that apoptosis is the primary mechanism whereby the epoxide-containing piperazines exert their anti-tumorigenic activity.

Bak and caspase-3 immunoblot assays

In an attempt to identify molecular targets for the pro-apoptotic activities of the epoxide-containing piperazines, immunoblot analyses were performed to determine if activation or accumulation of proteins known to induce apoptosis occurred in treated cells. Figure 5A shows that in HS-578T human breast cancer

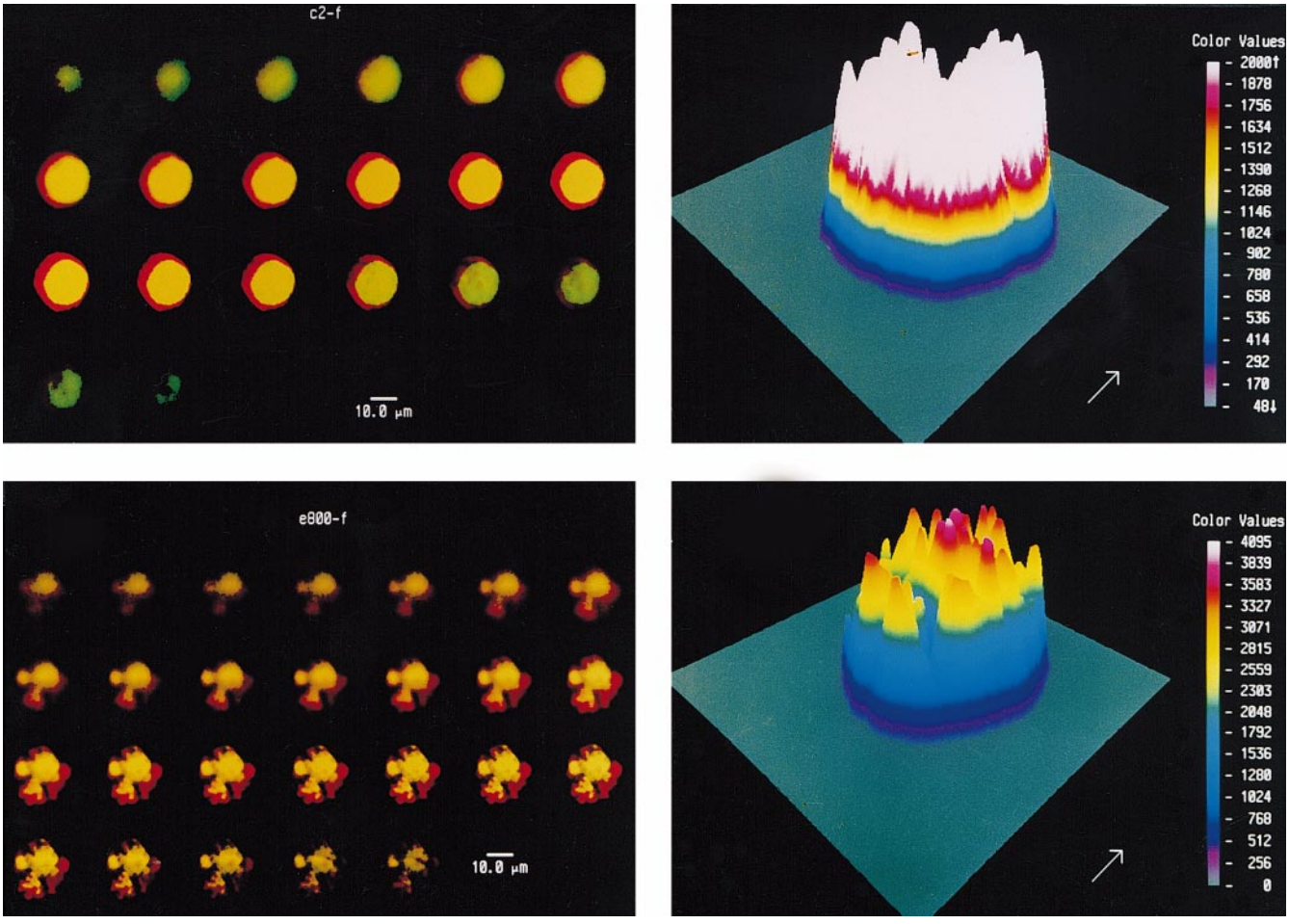


Fig. 3 Confocal microscopy of a human prostate (DU-145) cancer cell stained with acridine orange, following exposure to TOP-008. The cells were grown as ascites tumors in mice and the animals received a daily 100 mg/kg dose of TOP-008 for 8 days. *Upper left panel*, a tumor cell from an untreated animal; *lower left panel*, a tumor cell from a treated animal. Fluorescent distribution patterns for the cell from an untreated mouse (*upper right panel*) and a mouse treated with TOP-008 (*lower right panel*)

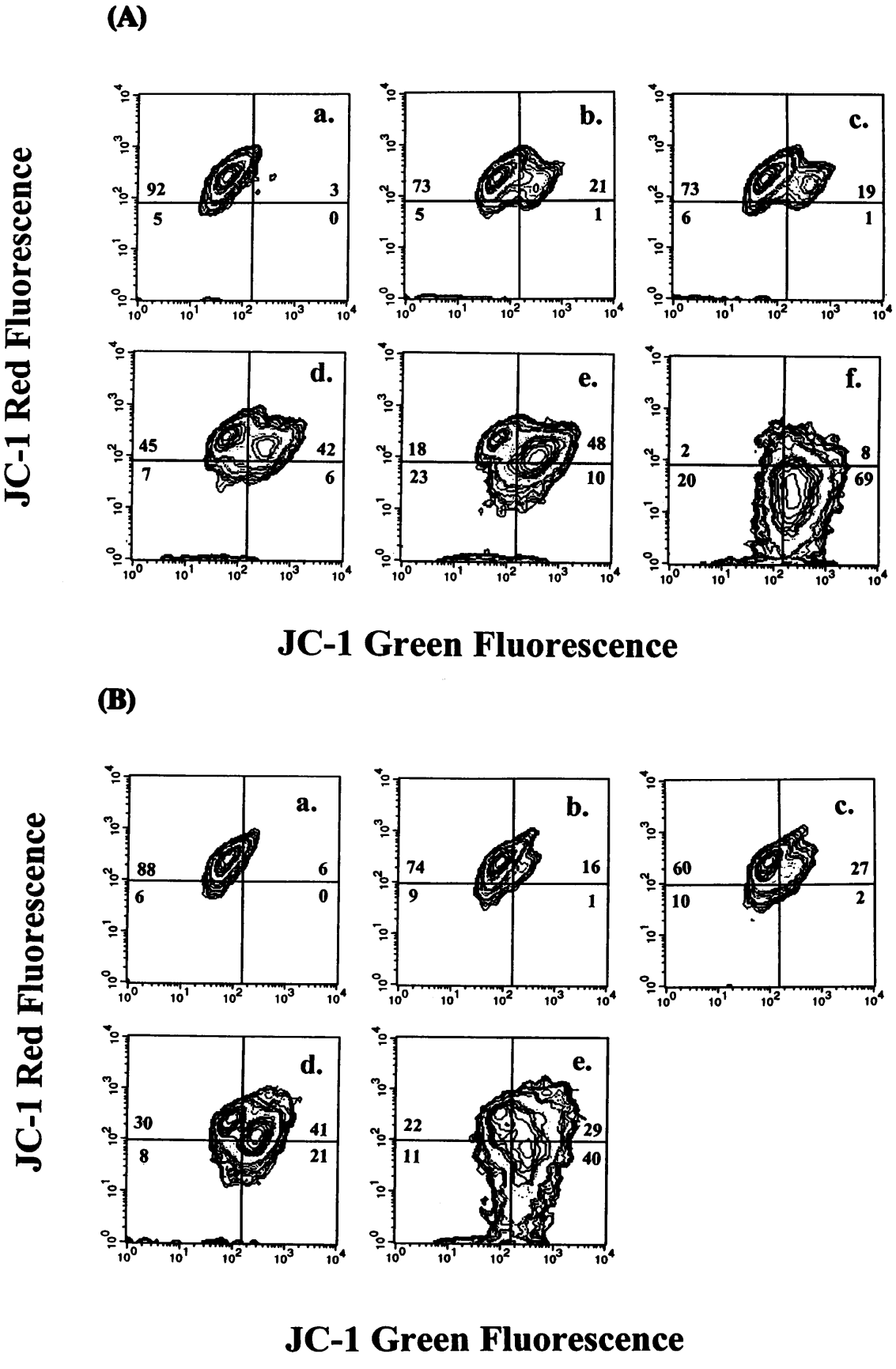
cells treated with 25 µM TOP-008 for 6 h, a significant accumulation of bak occurs in the mitochondrial fraction of the cells. Similar results were observed when DU-145 prostate cancer cells were treated with 50 µM TOP-008 (data not shown). Bak is a member of the pro-apoptotic family of proteins including Bax and Bik [12] that through its interactions with Bcl-2 on the mitochondrial membrane surface leads to the activation of the caspase cascade.

Consistent with the finding of increased accumulation of bak in mitochondrial fraction of breast cancer cells, we also saw activation of caspase-3 after addition of TOP-008 to the cells. Figure 5B shows a protein immunoblot analysis of cytosolic fractions prepared from TOP-008 treated breast cancer cells that reveals a steady decrease of procaspase-3 (designated CPP32 in Fig. 5) over the 6 h time-course of the experiment, and a peak appearance of caspase-3 (designated as p20 in Fig. 5) at 2.5 h after drug addition.

In vivo assays

Subrenal capsule assay

The subrenal capsule assay provides a convenient system to study the effect of drugs on the growth of tumor cells in vivo. Human cancer cells can be grown in the mouse subrenal capsule where for a limited period of time they escape the detection of the animal's immune system. The hormone-independent breast (HS578-T) and prostate (DU-145) tumors were implanted under the renal capsule of mice and treated with increasing doses of NCO-700 for 5 days. As shown in Fig. 6, there was a dose-dependent and highly significant decrease in tumor growth in both sets of animals. At the highest dose of NCO-700 tested, 40 mg/kg for the prostate tumors (Fig. 7A) and 100 mg/kg for the breast tumors (Fig. 6B), there was almost no tumor detected in the subrenal capsule when the animals were examined after 5 days of drug treatment. TOP-008 was also effective in preventing development of these two tumors in mice but at doses higher than those of NCO-700 (100 mg/kg; data not shown) and may reflect a lower bioavailability of this more hydrophobic compound when administered in water.


(A)
JC-1 Red Fluorescence
a.
92
3
5
0
b.
73
21
5
1
c.
73
19
6
1
d.
45
42
7
6
e.
18
48
23
10
f.
2
8
20
69
JC-1 Green Fluorescence
(B)
JC-1 Red Fluorescence
a.
88
6
6
0
b.
74
16
9
1
c.
60
27
10
2
d.
30
41
8
21
e.
22
29
11
40
JC-1 Green Fluorescence

◀

Fig. 4A,B Effect of TOP-008 on the mitochondrial sequestration of the fluorescent cation dye, JC-1, determined by flow cytometry analyses. **A** HS-578T human breast cancer cells were treated with 25 μM TOP-008 for 0, 15, 30, 60, 120 and 300 min (*panels a–f*) and the relative intensity of green versus red fluorescence was plotted. The JC-1 dye emits a red fluorescence when sequestered in the mitochondria. **B** DU-145 human prostate cancer cells were treated with 25 μM TOP-008 for 0, 30, 60, 120, 300 min (*panels a–e*) and the intensities were recorded. The plots of both figures were divided arbitrarily into quadrants with the % signal intensities shown in each quadrant

Nude mice studies

Figure 7A shows the effects of TOP-008 on the growth of human prostate cancer cells grown in nude mice. Drug treatment started at 21 days after tumor cell inoculation of the nude mice and continued for 15 days. The change in the size of the tumors is shown in Fig. 7A where closed circles depict the control animals and open circles the treated animals. Mice that were not treated with TOP-008 showed a steady increase in the size of their tumor mass, with tumor size increases of almost 50% reached by day 15. In animals that received 50 mg/kg of TOP-008, administered daily as an IP injection, tumor growth was completely halted. All animals were judged healthy, as assessed by body weight at the end of the experiment.

Figure 7B shows the effects of NCO-700 in similar experiments in nude mice bearing human prostate tumors. Although not as strong as that with TOP-008, there was a slowing of tumor growth, which was significant when compared with untreated controls. Animals treated with doxorubicin (6 mg/kg) showed no tumor shrinkage, and toxicity was seen, because two of the six animals died (data not shown). The relative potency of NCO-700 versus TOP-008 is reversed in the nude mice studies when compared with the subrenal capsule assay.

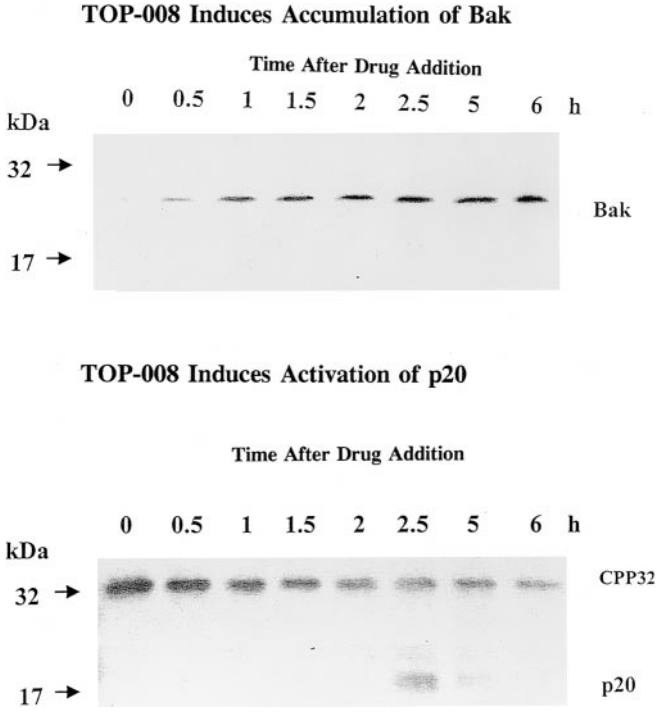


Fig. 5A,B TOP-008 induced the accumulation of bak and the activation of CPP32 in HS-578T human breast cancer cells. **A** Protein immunoblot analysis of the accumulation of bak in the mitochondria fraction of HS578-T cells treated with 25 μM TOP-008 for increasing periods of time. Molecular weight markers are shown on the *left hand side* of the figure. **B** Protein immunoblot analysis of levels of procaspase-3 (*designated CPP32*), and caspase-3, (*designated p20*), in the cytosolic fraction of HS-578T cells treated with 25 μM TOP-008 for increasing periods of time

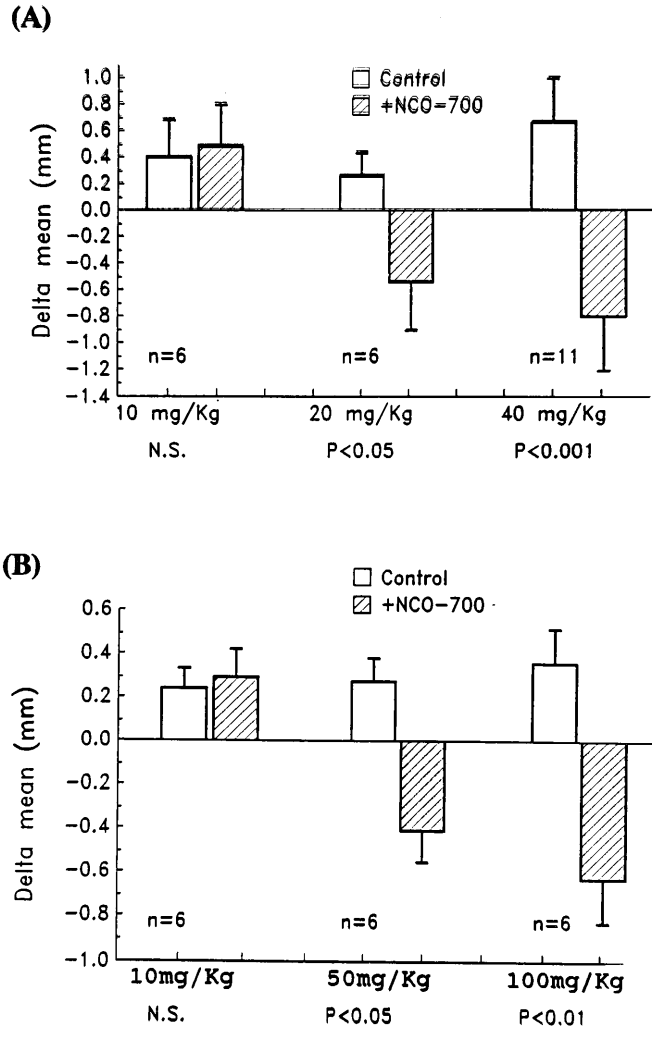


Fig. 6A,B Effect of NCO-700, in vivo, on the growth of human tumors in the subrenal capsule assay. **A** DU-145 prostate cancer cells were grown as tumors in the subrenal capsule of mice and treated with increasing doses of NCO-700. The relative size (delta mean) of the tumor was determined (see text). The $n = 6$ refers to the number of mice in each experimental group. A statistically significant reduction in tumor size was seen at the 20 mg and 40 mg/kg dose levels. **B** Effect of increasing doses of NCO-700 on the growth of HS-578T human breast cancer cells in the subrenal capsule of mice. A statistically significant reduction in tumor size was seen at the 50 mg and 100 mg/kg dose levels

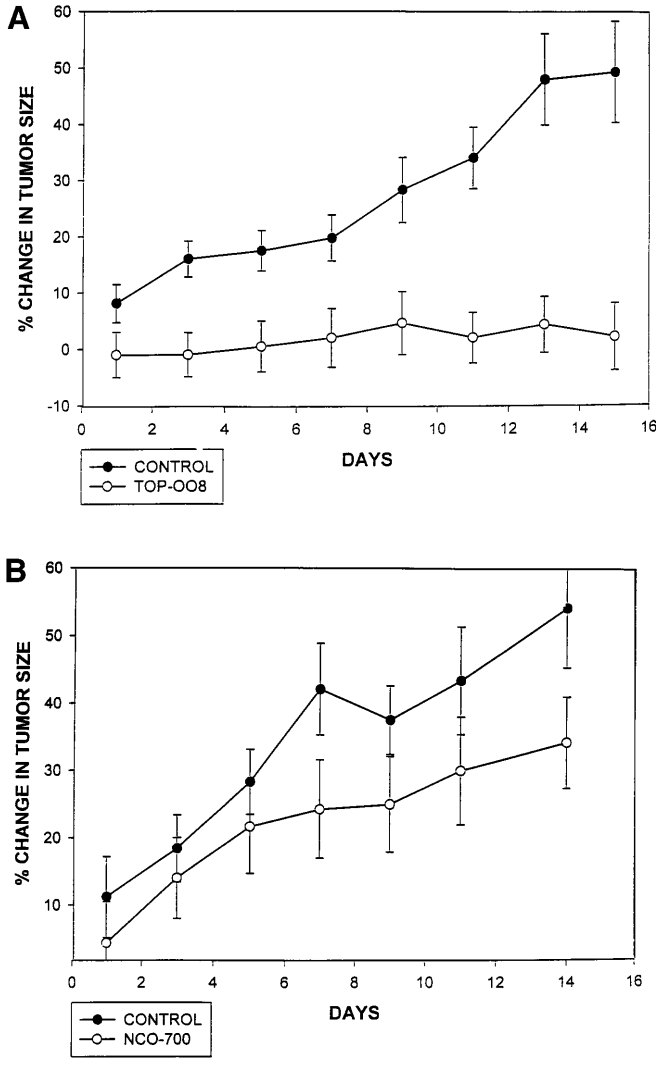


Fig. 7 Effect of 50 mg/kg doses of TOP-008 (**A**) and NCO-700 (**B**) on the growth of human prostate tumor xenografts in nude mice. Each group contained six treated and six untreated animals

The reasons for this are not known but probably reflect the differing bioavailability of the two drugs to the subrenal versus subcutaneous compartments where the tumors are grown.

Discussion

A previously unrecognized activity of the epoxide-containing piperazines, the induction of programmed cell death in selected tumor cells, has been demonstrated in the present study. Two representative compounds of this class, NCO-700 and TOP-008, which are structurally similar, were shown to have anti-cancer activity against human breast and prostate tumor cell lines. This was confirmed in vivo, where breast and prostate cells were grown in the subrenal capsules of mice, and in nude mice bearing xenograft human prostate tumors. Evidence of the induction of apoptosis in tumor cells was supported by a number of different studies, and included, morphological changes as visualized by confocal microscopy and changes in the integrity of the mitochondrial membrane as assessed by leakage of the mitochondrial dye, JC-1. Other studies (data not shown) which indicate that apoptosis is the mechanism for cellular toxicity by the epoxide-containing piperazines include DNA laddering, observed by gel electrophoresis in treated cancer cell lines, DNA fragmentation, assayed by the TdT-mediated dUTP nick end-labeling (TUNEL) method and rapid movement of cytochrome C out of the mitochondrial compartment.

Epoxide-containing cysteine protease inhibitors, such as NCO-700 and E-64, were previously examined as inhibitors of apoptosis, as it was thought that these compounds may inhibit caspases, cysteine proteases which activate the programmed cell death pathway [18]. However, no caspase-inhibiting activity was observed with E-64 [19] or NCO-700 (unpublished observations). It is also unlikely that the apoptosis-inducing activity of NCO-700 and TOP-008 is mediated through calpains, the calcium-activated cysteine proteases that are inhibited by these two compounds. We found no evidence that the activity of NCO-700 or TOP-008 was correlated with the level of calpain in cancer cells, and more importantly, more potent calpain inhibitors including E-64, calpain inhibitor 1 and N-CBZ (N-CBZ-Leu-Leu-Tyr-diazomethyl ketone) were cytotoxic to human breast cancer cells at doses ten times higher than those of NCO-700 or TOP-008 (data not shown).

The exact biochemical target for the apoptosis-inducing activity of the epoxide-containing piperazines is still under study but preliminary evidence in human breast cancer cells indicates that TOP-008 permits the accumulation of the pro-apoptotic protein, bak, to occur in the mitochondrial fraction of treated cells (Fig. 5). This could then initiate apoptosis through the subsequent activation of the caspase cascade in the cytosol of the cell [12, 18]. Consistent with this mechanism, we saw activation of caspase-3 at 2.5 h after the addition of TOP-008 to breast cancer cultures (Fig. 5B).

One attractive feature of the development of the epoxide-containing piperazines into a new class of chemotherapeutic agents is the relatively low toxicity of this class of compounds. In a series of studies on acute and chronic toxicology, NCO-700 was shown to be a safe compound when administered to rodents, rabbits and dogs (personal communication, N. Miyake, Nippon Chemiphar Co. Ltd., Japan). For example, LD_{50} values for NCO-700 in mice were approximately 800 mg/kg (IP dose). No toxic effects, either acute or chronic, on any organs or tissues was observed as a result of daily IP injections of NCO-700 in doses of up to 200 mg/kg in mice. This value is well above the 50 mg/kg dose at which efficacy was seen with NCO-700 in the subrenal capsule model in the present study. Epoxide-containing piperazines are stable, water-soluble compounds with a good bioavailability, and a large number of simply-substituted derivatives of NCO-700 and TOP-008 have

been synthesized as part of earlier structure/activity studies. These derivatives can now be screened for cytotoxicity to different human tumors, to ascertain if the epoxide-piperazine calpain inhibitors represent a class of agents with broad tumor specificity. Finally, our present findings that NCO-700 and TOP-008 showed activity against hormone-independent breast and prostate tumor cell lines suggest that there is a suitable patient population upon whom initial clinical trials for these new anti-cancer compounds could be conducted.

Acknowledgements We acknowledge the skilled technical assistance of Sven Merten, Satish Kathuria, Anuradha Mathur, Paula Sweet and Marie Sutpecky. We thank Professor Jin-Yu Xie of the China Academy of Traditional Chinese Medicine (Beijing) for performing the confocal microscope studies, the scientists and managers of the Nippon Chemical Co. Ltd., (Tokyo) for the generous gift of epoxide-piperazine calpain inhibitors and for technical advice, and N. Chikazumi and S. Harada of Hitachi Chemical Co. Ltd. (Tokyo) for their support of this work.

References

1. Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H (1997) Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 89: 1881–1886
2. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hypothermia. Biochem Pharmacol 40: 2353–2362
3. Berquin IM, Sloane BF (1994) Cysteine proteases and tumor progression. Perspectives in Drug Discovery and Design 2: 371–388
4. Bogden AE, Haskell PM, LePage DJ, Kelton DE, Cobb WR, Esber HJ (1979) Growth of human tumor xenografts implanted under the renal capsule of normal immunocompetent mice. Exp Cell Biol 47: 281–293
5. Dimitroff CJ, Sharma A, Bernacki RJ (1998) Cancer metastasis: a search for therapeutic inhibition. Cancer Invest 16: 279–290
6. Haga N, Ishibashi T, Hara A, Abiko Y (1985) Effect of NCO-700, an inhibitor of protease, on myocardial pH decreased by coronary occlusion in dogs. Pharmacology 31: 208–217
7. Hara K, Takahashi K (1983) Inhibition of calcium-activated neutral protease of monkey cardiac muscle by epoxysuccinic acid derivatives. Biomed Res 4: 121–123
8. Hirao T, Hara K, Takahashi K (1984) Purification and characterization of cathepsin B from monkey skeletal muscle. J Biochem 95: 871–879
9. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L (1996) Mitotic block induced in HeLa cells by low concentration of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res 56: 816–825
10. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer (1997) The release of cytochrome C from mitochondria: a primary site for bcl-2 regulation of apoptosis. Science 275: 1132–1136
11. Lah TT, Kos J (1998) Cysteine proteinases in cancer progression and their clinical relevance for prognosis. Biol Chem 379: 125–130
12. Reed JC (1997) Double identity for proteins of the bcl-2 family). Nature 387: 773–776
13. Sashida H, Abiko Y (1985) Inhibition with NCO-700, a protease inhibitor, of degradation of cardiac myofibrillar proteins during ischemia in dogs. Biochem Pharm 34: 3875–3880
14. Stratton JA, Kucera PR, Micha JP, Rettenmaier MA, Braley PS, Berman ML, DiSaia PJ (1984) The subrenal capsule tumor implant assay as a predictor of clinical response to chemotherapy: 3 years of experience. Gynecol Oncol 19: 336–347
15. Stratton JA, Micha JP, Rettenmaier MA, Braley PS, DiSaia PJ (1984) Chemosensitivity testing of nonsolid tumors by the subrenal-capsule assay. Gynecol Oncol 17: 185–188
16. Terzis AJ, Thorsen F, Heese O, Visted T, Bjerkvig R, Dahl O, Arnold H, Gundersen G (1997) Proliferation, migration and invasion of human glioma cells exposed to paclitaxel (Taxol) in vitro. Br J Cancer 75: 1744–1752
17. Toyo-Oka T, Kamishiro T, Masaki M, Masaki T (1982) Reduction of experimentally produced acute myocardial infarction size by a new synthetic inhibitor, NCO-700, against calcium-activated neutral protease. Japanese Heart J 23: 829–834
18. Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Newcomb EW (1998) Bax cleavage is mediated by calpain drug-induced apoptosis. Oncogene 17: 1069–1078
19. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng T, Jones DP, Wang X (1997) Prevention of apoptosis by bcl-2: release of cytochrome C from mitochondria blocked. Science 275: 1129–1132
20. Zanker KS, Brandt B, Junker R, Griwatz C, Heidl S, Brinkmann O, Semjonow A, Assman J (1996) Isolation of prostate-derived single cells and cell clusters from human peripheral blood. Cancer Res 56: 4556–4561