Carboxyamido-triazole (CAI), a Signal Transduction Inhibitor Induces Growth Inhibition and Apoptosis in Bladder Cancer Cells by Modulation of Bcl-2

FRANK G.E. PERABO1, ANDREAS WIRGER2, STEFAN KAMP1, HEIKE LINDNER1, DORIS H. SCHMIDT1, STEFAN C. MÜLLER1 and ELISE C. KOHN3

1Department of Urology, University Hospital, Bonn University, 53105 Bonn;
2Department of Urology, Medical University, 23562 Luebeck, Germany;
3Laboratory of Pathology, National Cancer Institute, Bethesda, MD, 20892, U.S.A.

Abstract. Pro- and anti-apoptotic factors and intracellular signaling pathways are targets for therapeutic development of anticancer agents. Carboxyamido-triazole (CAI) is an inhibitor of transmembrane calcium influx and intracellular calcium-requiring signal transduction pathways. The present study investigates the effects of CAI on human transitional cancer cell (TCC) viability and apoptosis, and evaluates whether apoptotic resistance may be overcome pharmacologically. Both well-differentiated (RT4, RT112/grade 1) and poorly-differentiated (T24/grade 3; SUP/grade 4) human TCC lines were shown to express Fas. Upon exposure to agonistic monoclonal Fas antibody, only well-differentiated TCC lines underwent apoptotic cell death. CAI exposure reduced cell viability and caused an at least additive anti-apoptotic effect in combination with the Fas antibody in the Fas-insensitive TCC lines. Under the same conditions under which CAI treatment augmented Fas-mediated apoptosis, it was shown to reduce intracellular bcl-2 quantity. This response to CAI indicates that apoptotic cell death is enhanced by the reduction of bcl-2 protein expression. We suggest that the antitumor effect of CAI is at least partially based on restoring a pathway of apoptosis. It may cause transformation of cell homeostasis that leads to the alteration of apoptotic mechanisms, thus allowing highly malignant tumor cells to re-enter the physiological course of cell elimination.

Bladder cancer is a commonly occurring cancer with more than 50,000 new cases diagnosed every year and approximately 10,000 deaths (1). Advanced or metastatic bladder cancer has a poor prognosis and while responses to chemotherapy are observed, cures are rare (2). Chemotherapy effects are at least partly caused by induction of apoptotic cell death (3); however, some bladder cancer cells show intrinsic resistance to drug-induced apoptosis. In tumor cell escape mechanisms, Fas, a mediator of apoptosis, plays an important role (4,5). Since apoptosis is dependent upon regulation of both, pro- and anti-apoptotic factors as well as balanced intracellular signaling pathways, these factors and signaling pathways are logical targets for the therapeutic development of new anticancer drugs. Carboxyamido-triazole (CAI) is an inhibitor of transmembrane calcium influx and intracellular calcium-requiring signal transduction pathways (6-8). Structure-function analysis has linked the inhibition of calcium influx by CAI with inhibition of calcium-dependent signaling, inhibition of proliferation, experimental metastasis and angiogenesis (9-12). CAI has been shown to have growth inhibitory effects on a broad array of human tumor cell lines (6,11-13). In this study, we investigated the response of human transitional cell carcinoma cell lines (TCC) to CAI in comparison to and in combination with apoptosis-inducing anti-Fas-mAb. We examined if CAI induces growth inhibition and apoptosis in bladder cancer cells, and evaluated whether apoptosis-resistance of bladder cancer cells can be overcome by CAI treatment. Furthermore, this study explored, as possible underlying mechanisms of CAI activity, the bcl-2 expression, which is involved in the control of apoptosis and which regulates and is regulated by Ca2+ (14-16).

Materials and Methods

Tumor cell lines and culture conditions. The human transitional cell carcinoma cell lines RT4 (grade 1), RT112 (grade 1), T24 (grade 3)
and SUP (grade 4) were obtained from ATCC (Rockville, MD, USA). All tumor cell lines were maintained in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acids, 2 mM L-Glutamine, 100 units/ml Penicillin G, 100 µg/ml Streptomycin and a vitamin solution (Gibco BRL, Life Technologies Inc., Frederick, MD, USA). For experiments, 1x10^6 tumor cells were seeded in 25-cm² culture flasks (Costar, Cambridge, MA, USA) and incubated for 48 h to allow maximum confluence, thus providing optimal cell-cell contact. Cells were routinely subcultured by trypsinization [0.05% Trypsin, 1mM EDTA] (Gibco BRL).

**Antibodies and Reagents.** Inducing anti-Fas mAb ([Mouse IgG1, clone DX2] Calbiochem, Cambridge, MA, USA), FITC-labeled anti-Fas mAb ([Mouse IgG1κ, clone DX2] Pharmingen, San Diego, CA, USA), anti-bcl-2-FITC (Boehringer Mannheim, Germany), anti-bcl-2 (Boehringer Mannheim, Germany), Bcl-2 ELISA (Endogene, Woburn, MA, USA), Annexin-V-FITC and propidium iodide [PI] (Boehringer, Ingelheim, Germany), unconjugated IgG1 mAb (Cymbus, Hants, UK), and FITC/PE-conjugated IgG1 mAb (Dako, Denmark) were used in the study. CAI was resuspended in DMSO (Sigma, St. Louis, MO, USA). Aliquots of stock solution were stored at -20°C.

**Treatment Scheme.** Inducing anti-Fas-mAb (1 µg/ml), CAI (0.1-10 µM) alone or in combination were added to culture medium of cells in log-phase growth for the indicated periods of time. Vehicle control experiments were performed with DMSO.

**Cytotoxicity Assay.** The MTT (Micro culture Tetrazolium) assay was used to assess cell viability after exposure to inducing anti-Fas-mAb, CAI, or combination of both. In brief, trypsinized tumor cells were resuspended in medium at 5x10^5 cells/ml after verifying cell viability by trypan blue dye exclusion. One hundred µl of cell suspension were distributed into each well of a 96-well flat-bottomed microtiter plate and each plate was incubated for 24 h to allow adherent cell growth. Following incubation, the medium was removed and 200 µl of the different reagent solutions were distributed and incubated for 24, 48 and 72 h. Thereafter, 20 µl MTT dye working solution (Sigma) was added and incubated for 4 h. The supernatant in the wells was decanted and replaced by 200 µl Z-propanol, supplemented with 0.05 N HCl to dissolve the reactive dye. The absorbance (A) values of each well were read at 550 nm using an automatic multwell spectrophotometer (340 ATTC SLT, Crailsheim). The growth inhibition percentage was calculated using the background-corrected absorbance as follows: % Growth Inhibition = [(1-A of experimental well)/A of positive control well]*100. Each experimental data point represents average values obtained from six replicates; each experiment was performed in duplicate.

**Flow Cytometric Analysis of Cell Surface Fas.** The expression of cell surface Fas was determined by flow cytometry in non-permeabilized cells. Briefly, cells were incubated in normal medium for 24 h and then harvested. 3x10^5 cells were washed in cold calcium- and magnesium-free PBS and centrifuged at 400 x g for 10 min. Thereafter, the pellet was resuspended in 50 µl FACS buffer (PBS, 5% fetal calf serum, 0.02% sodium citrate) on ice, containing 6 µl of FITC-labelled anti-Fas or 5 µl of FITC-conjugated irrelevant mouse IgG1 mAb serving as isotype control. After washing in PBS once more, the cells were immediately analyzed using a FACSscan flow cytometer (Becton Dickinson, San Diego, CA, USA). A minimum of 10,000 events were acquired for each sample.

**Assessment of Apoptosis by Annexin-V and Propidium Iodide.** To evaluate the extent and time course of apoptosis after incubating cells with agents, Annexin-V and PI were used in a double-stain technique. Cells were counterstained with PI as a vital dye to distinguish between early (Annexin V-positive, PI-negative) and late apoptosis (Annexin V-positive, PI-positive), and necrosis (Annexin V negative, PI positive), respectively. Following the procedure described above, controls and 3x10^5 treated cells (all attached and detached cells in supernatant) were resuspended in 200 µl of culture medium without phenol red, incubated with 5 µl FITC-conjugated Annexin-V and 10 µl PI for 20 min. at room temperature in the dark, then immediately analyzed by flow cytometry. A minimum of 10,000 events was acquired for each sample.

**DNA Fluorescence Microscopy.** Two well-Permanox chamber slides (Nalge Nune, Naperville, IL, USA) containing 2x10^3 cells/chamber were incubated for 24 h in 2 ml normal medium/chamber to allow adherent cell growth. Thereafter, the medium was removed and 2 ml medium with reagents was added. Cells were incubated for 48 h. After removing the medium and washing with PBS, cells were treated with RNase A 10 µg/ml, and stained with propidium iodide 50 µg/ml diluted in 0.1% sodium citrate with 0.1% Triton X-100 for 30 min at 4°C in the dark. The cells were then analyzed by fluorescence microscopy (Olympus BH2).

**Intracellular Flow Cytometric Analysis of bcl-2.** We used intracellular flow cytometry to determine the intracellular amount of bcl-2. In short, the cells were harvested by trypsinization, 3x10^5 cells were washed in cold calcium- and magnesium-free PBS and centrifuged at 460 x g for 5 min. Thereafter, fixation of the cells was performed with paraformaldehyde (4% in PBS) for 12 min at 4°C, and then washed two times with PBS. Permeabilization was achieved by washing the fixed cells two times with a Saponin buffer (0.1% in a 0.01 M HEPES/PBS). Thereafter, staining of the cell suspension was performed with 10 µl anti-bcl-2-FITC for 30 min at room temperature in the dark. Cells were washed once each with Saponin buffer and PBS then resuspended in 50 µl FACS buffer and analyzed within an hour. Unlabelled cells and isotype IgG-FITC-stained control cells were analyzed with each experiment. A minimum of 10,000 events were acquired for each sample. Bcl-2 expression was determined by subtracting the mean fluorescence of the unspecific isotype staining from the mean fluorescence of the FITC-labelled bcl-2-antibody.

**Bcl-2 ELISA.** Bcl-2 content of lysed cells was measured with commercially available enzyme-linked immunosorbent assays (ELISA) (Endogene). A monoclonal antibody recognizing an epitope of bcl-2 was precoated onto the wells of the microtiter plate. Standards and diluted samples were introduced into the wells and immediately the corresponding horseradish peroxidase (HRP)-conjugated antibody was added. Following incubation, unbound enzyme-conjugated antibodies were removed by washing and substrate solution was added to the wells. A colored product was...
formed in proportion to the amount of bcl-2 in the sample. The reaction was stopped with sulphuric acid and the absorbance was measured photometrically by an ELISA reader at 450 nm (SLT, Crailsheim). The concentrations of bcl-2 were calculated by means of comparison with a standard curve of recombinant bcl-2.

**Western Blot for bcl-2.** Cells were harvested by trypsin, washed with ice-cold PBS and lysed in lysis buffer (10mM Tris-HCl pH 7.4, 10 mM magnesium chloride, 5 mM EDTA and 1% Triton-X-100) supplemented with 1mM PMSF (protease inhibitor phenylmethylsulphonyl fluoride) (Boehringer), 200 µg aprotinin (Sigma) and 10µM leupeptin (Boehringer) for 20 min at 4°C. The lysate was centrifuged (14,000 rpm) for 10 min at 4°C, and its protein content was determined using a protein assay kit (BioRad Laboratories). Equal amounts of protein were heated in denaturizing loading buffer at 95°C for 10 min, then subjected to SDS-PAGE (12.5%) and transferred to a nitrocellulose transfer membrane (Millipore). Membranes were blocked with 5% non-fat dry milk in TBS (50mM Tris, pH 7.5, 150 mM sodium chloride) and incubated overnight at 4°C with anti-bcl-2-mAb diluted in 2% TBS Tween 20. Immunological complexes were visualized by enhanced chemiluminescence (Amersham Life Sciences) using horseradish peroxidase-conjugated goat-antimouse IgG (Dako).

**Results**

**Expression and regulation of cell surface Fas.** Flow cytometric analysis of Fas expression was performed on all TCC cells because lack of Fas receptor expression can be one mechanism of apoptosis resistance (17). As shown in Figure 1, high Fas expression was found in all bladder cancer cell lines. We then further examined whether treatment of the bladder carcinoma cell lines with CAI influences expression of Fas-receptor. Thereby, no modulation of Fas could be demonstrated on any of the cell lines (data not shown).

**Cytotoxicity of inducing anti-Fas-mAb and CAI.** Some monoclonal antibodies (mAb) against Fas have been shown to induce apoptosis in cells expressing Fas-receptor (18). We investigated the effect of an agonistic monoclonal antibody (inducing Fas-mAb) on cell viability with the MTT assay in all cell lines. First, inducing Fas-mAb (1 µg/ml) was added to culture medium and incubated with cells in log-phase for 24 h, 48 h and 72 h. A cell loss of 50-60% after 72 h was observed in RT4 and RT112 cells after treatment with the Fas-mAb in comparison to normal cell viability (p<0.05) (Figure 2a-b). On the contrary, T24 and SUP showed only a limited 15-20% decrease in cell viability when treated with the Fas-mAb (Figure 2c-d). We then incubated all bladder cancer cell lines with 0.1 - 10.0 µM CAI alone and in combination with inducing Fas-mAb. No cytotoxic response was seen in concentrations of 0.1 and 1.0 µM CAI. After treatment of RT4 and RT112 cells with 10 µM CAI, a reduction of cell growth of 50 - 60% (p<0.05) was found at 72 h (Figure 2a-b); however, between 24 - 48 h, the cytostatic response was weaker than the one observed with Fas-mAb. In T24 and SUP cells, only a limited effect on cell viability was achieved by CAI alone, again similar to the response of cells to Fas-mAb exposure (Figure 2c-d). However, in these Fas-insensitive T24 and SUP cells, the combined treatment of CAI with Fas-mAb resulted in an additive cytotoxic effect which led to a 2-fold decrease of cell viability beginning 48 h after exposure (p<0.05). In the Fas-sensitive cell lines RT112 and RT4, too, a significant difference of combined treatment in comparison to single agent exposure was found although the difference was less than additive in the period from 48 - 72 h. Vehicle control experiments with DMSO did not show any effect.

**Apoptosis induction of anti-Fas-mAb and CAI.** After having shown the additive effect of CAI on Fas-mediated cell loss, we evaluated induction of apoptosis in the cell lines. Apoptosis induction was quantified by flow cytometric analysis after Annexin V/PI staining and additionally confirmed by fluorescence microscopy of PI-stained cells. After exposure to Fas-mAb, RT4 and RT112 cells underwent significant apoptosis (p<0.05) in comparison to untreated cells in the observed period, whereas T24 and SUP cells remained to a greater extent vital over a period of 72 h (Figure 3a-d). Incubation of TCC cells with 10 µM CAI induced apoptosis to a higher, but not significant, extent than Fas. Again, there was a difference in apoptosis induction by CAI with regard to Fas-sensitivity of cell lines. The Fas-insensitive cell lines T24 and SUP responded 10 - 30% less than the Fas-sensitive cell lines RT4 and RT112 to apoptosis induction by either Fas or CAI alone. When CAI and Fas-mAb were co-administered, a more than 70% induction rate of apoptosis was observed in RT4 and RT112. After 72 h, only few viable tumor cells remained. In Fas-insensitive T24 and SUP cells, incubation with combined CAI 10 µM and Fas-mAb led to an induction of apoptosis of 59% in T24 and 46%, in SUP, respectively, after 72 h (Figure 3a-d). Treatment with DMSO vehicle did not result in induction of apoptosis.

**Morphological changes and apoptosis after treatment with CAI.** To verify the apoptosis induction, TCC cells were incubated with CAI for 24 h, 48 h and 72 h. Thereby, the cells underwent the classical morphological changes of apoptosis. The cells showed shrinkage, vacuolization of the cytoplasm and fragmentation, chromatin condensation and apoptotic bodies (Figure 4). No such changes were seen in DMSO vehicle-treated cells.

**Expression and regulation of bcl-2.** The finding that CAI might restore apoptosis and Fas sensitivity in T24 and SUP cells led us to evaluate bcl-2 as a putative mechanism of this effect. Our results demonstrate bcl-2 expression in the TCC cell.
lines differed depending on the histological grade of the cell line. In the experiments, TCC cells were again exposed for 24, 48 and 72 h to 10 μM CAI. A reduction of bcl-2 expression, seen in the decrease of the mean fluorescence of bcl-2, was found in cultures of three cell lines, RT112, RT4 and T24 (Figure 5). There was no detectable pattern and time-dependency of the decrease in bcl-2 expression between the different cell lines. In RT112 cells, the decrease of bcl-2 expression approximated 30-40% in the observed period. In RT4, there was initially no reduction of bcl-2 expression, but from 48 to 72 h, bcl-2 expression more than doubled from 14.9 to 44.6%. In T24, there was an initial high decrease of bcl-2 expression of 45.8%, which then dropped from 36.6% at 48 h to 24.9% at 72 h. In SUP cells we found no reduced bcl-2 expression, but in fact an increase during CAI treatment over the time. After 24 h, first a slight decrease of bcl-2 of 17.4%, but then an increase of 26.6% after 48 h and an increase of 13.5% after 72 h (Figure 5) was seen. The apparent decrease in bcl-2 expression was confirmed by ELISA of cell lysates. In the cell lines RT4, RT112 and T24 the decrease of the total bcl-2 content could be confirmed, whereas cell line SUP again showed almost no reduction of bcl-2 (Figure 6). Additionally, in T24 and SUP cells, the bcl-2 protein was isolated and subjected to Western blotting after 48-h treatment with CAI. The intensity of the bcl-2 band was minimal in the T24 cells, whereas SUP demonstrated no change in the bcl-2 protein band (Figure 7).

Discussion
Loss of physiological signal transduction and deregulation or disruption of apoptotic pathways contribute to the malignant behavior of the tumor cell and often make successful drug therapy difficult. Carboxyamido-triazole (CAI), an inhibitor
of non-voltage-operated calcium entry and calcium influx-mediated pathways, has been evaluated as a promising, effective drug. It was shown to have antiproliferative, anti-invasive and anti-angiogenetic properties in preclinical studies (6,11-13) and showed clinical efficacy in various trials (19-24). The data presented here demonstrate that the mere expression of Fas is not sufficient to trigger a significant amount of apoptosis in poorly-differentiated TCC cells. We further show an anti-proliferative effect of CAI on bladder cancer cells, dependent on Fas sensitivity, which results in a significant reduction of the cell viability in grade 1 tumor cells. Highly malignant grade 3 and 4 tumor cells respond to a lesser extent to CAI treatment. The combination of CAI with anti-Fas during treatment has an additive inhibitory effect on cell viability and apoptosis induction in the Fas-insensitive bladder cancer cell lines. We could show a correlation between cytotoxicity as determined by the MTT assay and apoptosis as assessed by FACS analysis. This observation, and the fact that treatment with CAI leads to induction of apoptosis even in Fas-sensitive and -insensitive bladder cancer cells, indicates the ability of this signal transduction inhibitor to modulate apoptosis regulating mechanisms independently of specific activation through the Fas system. We did not find a modulation of Fas-receptor expression due to CAI treatment as a mechanism for overcoming the Fas-resistance. However, we could demonstrate that treatment of TCC cells with CAI resulted in a decrease in bcl-2 expression in all except one cell line. Under the same conditions under which CAI treatment augmented Fas-mediated TCC apoptosis, it was shown to reduce intracellular bcl-2 quantity. Flow cytometry, immunoblot analysis and ELISA indicated a reduction in bcl-2 protein in all TCC cell lines in response to CAI treatment except SUP. This finding correlates with the lowest apoptosis-inducing effect of CAI in the SUP cell line. The observation of apoptotic cell death after CAI treatment may indicate that CAI is able to restore a pathway of apoptosis induction. It may cause a transformation of cell homeostasis that leads to the alteration of pro- and anti-apoptotic mechanisms, thus allowing highly malignant tumor cells to reenter the physiological course of cell elimination.

One of the major anti-apoptotic factors is bcl-2. The bcl-2 resides primarily in the outer mitochondrial membrane, nuclear envelope and endoplasmatic reticulum (25) and Ca\(^{2+}\) homeostasis is maintained in the endoplasmatic reticulum and mitochondria by bcl-2 (16,26). It seems possible that CAI might influence Ca\(^{2+}\) flux-associated processes involved in bcl-2 expression. Overexpression of bcl-2 preventing apoptosis have been described (27-29). Several diverse mechanisms affecting bcl-2 function can be imagined. Studies investigating Ca\(^{2+}\) compartmentation during apoptosis have shown the association between the calcium concentration inside the endoplasmatic reticulum \([\text{Ca}^{2+}]_{\text{ER}}\), a primary intracellular store for Ca\(^{2+}\), and the Ca\(^{2+}\) influx through the plasma membrane (30-32). Decreased ER calcium pools are known to stimulate Ca\(^{2+}\) influx (33), which may modulate important apoptosis signaling pathways. Preneoplastic syrian hamster embryo (SHE) cells, which exhibit a high propensity to undergo apoptosis, have decreased calcium entry at the plasma membrane resulting in decreased ER Ca\(^{2+}\) pools; elevation of the extracellular [Ca\(^{2+}\)] rescues these cells from apoptosis in low serum and blocks the loss of ER calcium (31). He et al. suggests that ER Ca\(^{2+}\) pool depletion may trigger apoptosis and bcl-2 overexpression preserves the ER Ca\(^{2+}\) pool, when extracellular Ca\(^{2+}\) and Ca\(^{2+}\) influx is low. Furthermore, low extracellular Ca\(^{2+}\) seems to reduce the anti-apoptotic action of bcl-2, which could indicate the maintenance of calcium homeostasis within the ER by bcl-2 (26). Nevertheless, there could be additional mechanisms, as CAI has been shown to inhibit c-fos expression in epithelial cell lines. The calcium-dependent expression of c-fos may modulate other genes responsible for the malignant behavior of tumor cells (34).

CAI has successfully been investigated in Phase I and II clinical studies of various cancers, including therapy refractory cancers, with only moderate side-effects. For therapy of bladder cancer, this drug might offer an interesting alternative. The next step in evaluating CAI should be an in vivo model of bladder cancer.

References

Figure 2a-d. Comparison of cell viability in RT4 (a), RT112 (b), T24 (c) and SUP (d) cells after 24-, 48- and 72-h incubation with 1 μg/ml inducing anti-Fas-mAb, 10 μM CAI alone and in combination as assessed by MTT assay. Difference in viability is compared with the cell growth of the untreated cell line. Values expressed as mean ± SD of three independent experiments.

Figure 3a-d. Comparison of apoptosis induction in RT4 (a), RT112 (b), T24 (c) and SUP (d) bladder cancer cells after 24-, 48- and 72-h incubation with 1 μg/ml inducing anti-Fas-mAb, 10 μM CAI alone and in combination as assessed by flow cytometric analysis of Annexin-V/PI staining. Values are expressed as mean ± SD of two independent experiments.
Figure 4. Fluorescence microscopy after PI staining. Morphological (shrinkage, vacuolization of the cytoplasm, fragmentation) and apoptotic alterations (chromatin condensation, apoptotic bodies) of T24 cells after (72h) treatment with CAI 10 μM. (A) untreated cells, (B) cells after 72-h incubation with CAI. Magnification x400.
Intracellular flow cytometry of bcl-2 expression

<table>
<thead>
<tr>
<th></th>
<th>24 h ± SD</th>
<th>48 h ± SD</th>
<th>72 h ± SD</th>
<th>Percentage of bcl-2 decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT112</td>
<td>34.1 ± 4.2</td>
<td>36.70 ± 5.1</td>
<td>33.10 ± 7.1</td>
<td>39.9%</td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.5 ± 1.7</td>
<td>22.8 ± 1.8</td>
<td>22.60 ± 2.6</td>
<td>37.9%</td>
</tr>
<tr>
<td></td>
<td>37.9%</td>
<td>37.9%</td>
<td>31.7%</td>
<td></td>
</tr>
<tr>
<td>RT4</td>
<td>45.5 ± 5.6</td>
<td>48.5 ± 3.6</td>
<td>55.6 ± 4.4</td>
<td>30.8 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45.1 ± 4.5</td>
<td>41.3 ± 3.7</td>
<td>30.8 ± 3.1</td>
<td>14.9%</td>
</tr>
<tr>
<td></td>
<td>30.8 ± 3.1</td>
<td>30.8 ± 3.1</td>
<td>30.8 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>T24</td>
<td>45.5 ± 5.6</td>
<td>55.6 ± 4.4</td>
<td>30.8 ± 3.1</td>
<td>20.6%</td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45.5 ± 5.6</td>
<td>41.3 ± 3.7</td>
<td>30.8 ± 3.1</td>
<td>14.9%</td>
</tr>
<tr>
<td></td>
<td>30.8 ± 3.1</td>
<td>30.8 ± 3.1</td>
<td>30.8 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>SUP</td>
<td>28.8 ± 1.4</td>
<td>33.9 ± 3.3</td>
<td>34.6 ± 5.9</td>
<td>17.4%</td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.8 ± 2.2</td>
<td>42.9 ± 4.6</td>
<td>39.3 ± 2.9</td>
<td>+ 26.6%</td>
</tr>
<tr>
<td></td>
<td>39.3 ± 2.9</td>
<td>39.3 ± 2.9</td>
<td>39.3 ± 2.9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. The mean fluorescence of the bcl-2 expression in TCC cells after 24, 48 and 72 h of treatment with 10 μM CAI is shown as evaluated by intracellular flow cytometry. The levels of bcl-2 expression decreased in cultures treated with CAI. The depicted results represent the mean of three separate experiments ± SD.

ELISA of cell lysates for bcl-2 expression

<table>
<thead>
<tr>
<th></th>
<th>24 h bcl-2 (U/l)</th>
<th>48 h bcl-2 (U/l)</th>
<th>72 h bcl-2 (U/l)</th>
<th>Percentage of bcl-2 decrease/increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT112</td>
<td>102.2</td>
<td>106.9</td>
<td>98.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>24.8</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>RT4</td>
<td>162.3</td>
<td>151.9</td>
<td>164.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.6</td>
<td>55.2</td>
<td>32.3</td>
<td></td>
</tr>
<tr>
<td>T24</td>
<td>141.0</td>
<td>144.3</td>
<td>147.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.8</td>
<td>82.4</td>
<td>32.4</td>
<td></td>
</tr>
<tr>
<td>SUP</td>
<td>59.7</td>
<td>62.6</td>
<td>60.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>untreated cells</td>
<td>CAI-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>52.9</td>
<td>49.0</td>
<td>63.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6. Cell lysates of TCC cells show down-regulation of bcl-2 expression (U/l) after treatment with 10 μM CAI. Bcl-2 was measured with commercially available ELISA.

Figure 7. Immunoblot analysis of bcl-2 expression. Lysates of T24 and SUP cells were subjected to PAGE and blotted onto a nitrocellulose membrane after 48-h treatment with CAI 10 μM. T24 cells demonstrate almost no expression of bcl-2; SUP cells demonstrate unchanged expression of a Mr 28.5 kD protein.


Received June 17, 2004
Accepted July 13, 2004

2877