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Carboxyamido-triazole (CAI) Reverses the Balance between Proliferation and Apoptosis in a Rat Bladder Cancer Model

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Abstract. *Carboxyamido-triazole (CAI) is an orally bioavailable calcium influx and signal transduction inhibitor that has been shown to be anti-invasive, anti-angiogenic and anti-metastatic in different human tumors including transitional cell carcinoma. This study was undertaken to further evaluate the activity of CAI in a rat bladder cancer model. A transitional cell carcinoma (TCC) was chemically induced by intravesical installation of methyl-nitrosurea (MNU) in the bladder of female Fischer 344 rats. First, a toxicity study was performed which revealed no side-effects of CAI in the animals up to a dose of 250 mg/kg CAI. For treatment, a dose of 100 mg/kg CAI dissolved in PEG-400 vehicle was chosen. Oral administration of CAI continuously daily for 4 weeks (group A), 3 days/week over 6 weeks (group B), or intravesically twice a week for 6 weeks (group C) caused a reduction of spontaneous development of TCC. Lower stage and grade of tumors were seen in all CAI-treated animals. Under CAI treatment, the apoptotic rate in tumors increased, whereas the proliferation rate decreased, as shown by TUNEL assay and KI-67-immunhistochemistry, respectively. The highest efficacy was seen in group B, with 5 out of 10 animals tumor-free. Intravesical application (group C) resulted in 3 out of 10 animals tumor-free. Normal urothelium was not affected by CAI. This animal model confirms the anti-tumor effect of CAI and shows induction of apoptosis and growth inhibition in bladder cancer by the drug.*

Abbreviations: MNU, N-methyl.-N-nitrosurea; PBS, phosphate-buffered saline; TCC, transitional cell carcinoma.

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Metastatic bladder cancer is a cancer entity in which chemotherapeutic regimens yield only limited survival responses (1). Therefore, developing innovative agents targeting molecular pathways without severe adverse toxic effects is an important objective for future cancer therapy. Among the first in a new class of drugs, which inhibit intracellular calcium-dependant signaling events, is carboxyamido-triazole (CAI). Its proposed mechanism of action includes transmembrane signal transduction inhibition *via* calcium channel-mediated transmembrane and intracellular signaling transduction pathways (2). Regulation of intracellular calcium plays an important role in the proliferative and apoptotic processes including the maintenance and regulation of signal transduction pathways (3-5). CAI was found to have growth inhibitory, apoptosis inducing and anti-angiogenetic effects on a broad array of human tumor cell lines (6-11). Based on our previous findings (12), we evaluated, in an animal model, the activity of CAI on transitional cell carcinoma (TCC) *in vivo*. A TCC was chemically induced by direct intravesical installation of methyl-nitrosurea (MNU) in the bladder of female Fischer 344 rats to study the toxicity and activity of CAI.

Materials and Methods

Chemicals. One g of MNU (Sigma, St. Louis, MO, USA) was dissolved in 100 ml of normal saline. The animals received 0.15 ml (1.5 mg) of this solution. CAI was dissolved in non-toxic PEG 400 to produce the desired concentrations. Aliquots of stock solution were stored at -20˚C.

Animal model. Animal experiments were carried out in accordance with the principles of laboratory animal care and protection of animals after approval by German law (Approval no: 37/9185.811/920 and 23.203.2-bn 29). The model utilized was initially described by Hicks and Wakefield (13) and modified by Steinberg (14) and our group as described (15). One hundred and ten female Fischer 344 rats (aged 6-7 weeks and weighing 120-150 g) were purchased from Charles River (Sulzfeld, Germany) and

Figure 1. *Incidence of transitional cell carcinoma in MNU-treated rats 10 - 18 weeks after initiation of instillation.*

	10th week	12th week	14th week	16th week	18th week
Incidence	2/8	3/8	5/8	7/8	7/8
Percentage	25%	37%	62%	87%	87%

housed 5 rats per cage at a temperature of 23˚C in a controlled dark-light-rhythm of 12 h under pathogen low conditions. Water and standard laboratory diet were provided *ad libitum*. All rats used in the experiments were acclimatized for 2 weeks under routine laboratory conditions before starting any experiments. The animals were anesthetized with ether inhalation narcosis prior to instillation of the 0.15 ml MNU solution *via* a 22-gauge angiocatheter (Portex, Germany) into the bladder every other week for a total of 4 doses over 6 weeks. The animals remained anesthetized for approximately 45 min after catheterization to avoid spontaneous micturation.

Assessment of tumor incidence and progression. An initial tumor induction study was performed prior to the therapeutic trial to confirm the time to maximum tumor induction. In brief, 40 animals were divided into 5 groups of 8 rats each. MNU instillation was performed as just described and rats were sacrificed beginning at week 10 at 2-week intervals (weeks 10, 12, 14, 16 and 18) following the initial instillation of MNU. The bladders were fixed and completely examined for pathological changes.

Treatment schedules. For the toxicity evaluation study, 10 animals received daily doses of CAI starting at 50 mg/kg/week escalating to 250 mg/kg/week orally as well as intravesically for a total of 5 weeks. For the efficacy studies, a dose of 100 mg/kg CAI dissolved in PEG-400 vehicle was administered, beginning on the 16th week after tumor initiation, orally daily for 4 weeks (group A), or 3 days/week over 6 weeks (group B) or intravesically twice a week for 6 weeks (group C) in groups of 10 animals. As a control group, rats with tumor were treated by intravesical instillation of PEG-400 vehicle alone in the same schedules. The animals were sacrificed 2 days after the end of therapy by carbon dioxide narcosis and their organs harvested. The urinary bladder was excised *in toto*. The liver, lungs and abdomen were inspected for pathological conditions and, if suspicious, removed for further histological examination. The kidneys and ureters were dissected and inspected for pyonephrosis, stones and upper tract tumors. All organs were fixed in isopentane and then frozen in liquid nitrogen. Sections were cut transversely through the midportion of the bladder, and 4-μm sections were taken from each half to adequately sample the entire bladder. All sections were reviewed by two investigators and the section which appeared to have the greatest amount of change from normal urothelium was selected for histological grading and staged by the World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder (16). Hyperplasia, dysplasia and atypia were not considered as tumor. Comparison of treatment results was done by assessing stage and grade, as well as by evaluation of proliferation and apoptosis by MIB-1 (KI-67 antigen) immunostaining and TUNEL assay, respectively.

MIB-1 immunostaining. All tumor specimens were stained with MIB-1 to assess the proliferation fraction. Five-μm sections were incubated with blocking solution (Dako, Denmark), and then incubated for 30 min with anti-Ki-67, clone MIB-1 (Dianova, Hamburg, Germany) in a dilution of 1:20. These sections were thereafter covered with normal swine serum to reduce non-specific staining. The sections were then processed at room temperature as follows: PBS wash, 30-min incubation with biotinylated rabbit-antigoat IgG 1:50 (Dako), PBS wash, 30-min incubation with ABComplex/HRP (avidin and biotinylated horseradish peroxidase) (Dako), PBS wash, followed by staining with diaminobenzidine tetrahydrochloride (DAB) solution (Fluka, Germany). Finally, the sections were counterstained with hematoxylin and examined.

TUNEL assay. Apoptotic cells in the urothelium and stroma of the specimen were detected by an *in situ* cell death detection kit (Boehringer Mannheim, Germany), based on the terminal-deoxynucleotidyltransferase-mediated dUTP nick-end labelling method (TUNEL). Frozen tissue was cut into 5-μm sections, then dried and fixed in 3% paraformaldehyde. After washing the sections, blocking solution (Dako) to block endogenous peroxidase was added for 15 min at room temperature and, after washing again with PBS, immersed in terminal deoxynucleotidyltransferase reaction mixture containing enzyme and fluorescein-labelled dUTP at 37˚ C for 1 h. Then the anti- fluorescein antibodies, F_{ab} fragments from swine, conjugated to horseradish peroxidase were applied to the sections for 15 min to detect the labelled nucleotides. Binding was localized with diaminobenzidine and the sections were slightly counterstained with hematoxylin. Apoptotic cells in the sections were counted by microscopic examination with the hot spot procedure as described below.

Analysis and statistics. For analysis of MIB-1 and TUNEL staining, areas with pronounced apoptotic or proliferative activity were identified and examined at 200-fold magnification by light microscopy, as described by Weidner (17). With a counting frame of 0.0092 mm^2, all positive as well as all negative nuclei were counted. From this counting, percentages were calculated. The counting procedure was repeated with 5 hot spots. All sections were analyzed blinded by two different investigators. The results of the MIB-1 staining and TUNEL assay were statistically evaluated by the Wilcoxon *U*-test. A p value of 0.05 or less was considered significant.

Results

Tumor induction and histopathological progression of MNU-treated rat bladders. We found that 1.5 mg intravesically administered MNU on the schedule described induced transitional cell cancer of the urinary bladder in 87% (7 out

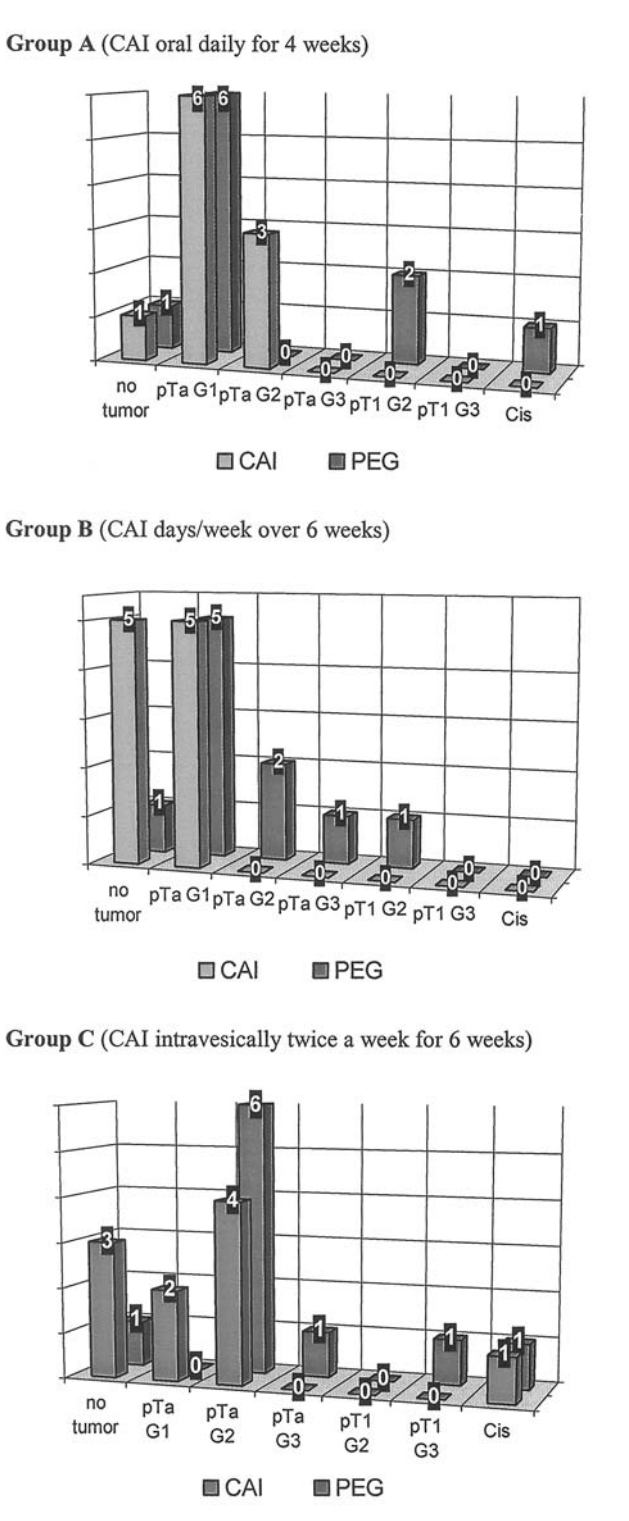

Figure 1. *Stage and grade of CAI (100 mg/kg dissolved in PEG-400)-treated rats in comparison to PEG-treated rats. Depicted are the total animal numbers. The efficacy results show for all examined groups (oral daily for 4 weeks [group A], 3 days/week over 6 weeks [group B] and intravesically twice a week for 6 weeks [group C]) no significant change, but a trend towards lower stage and grade.*

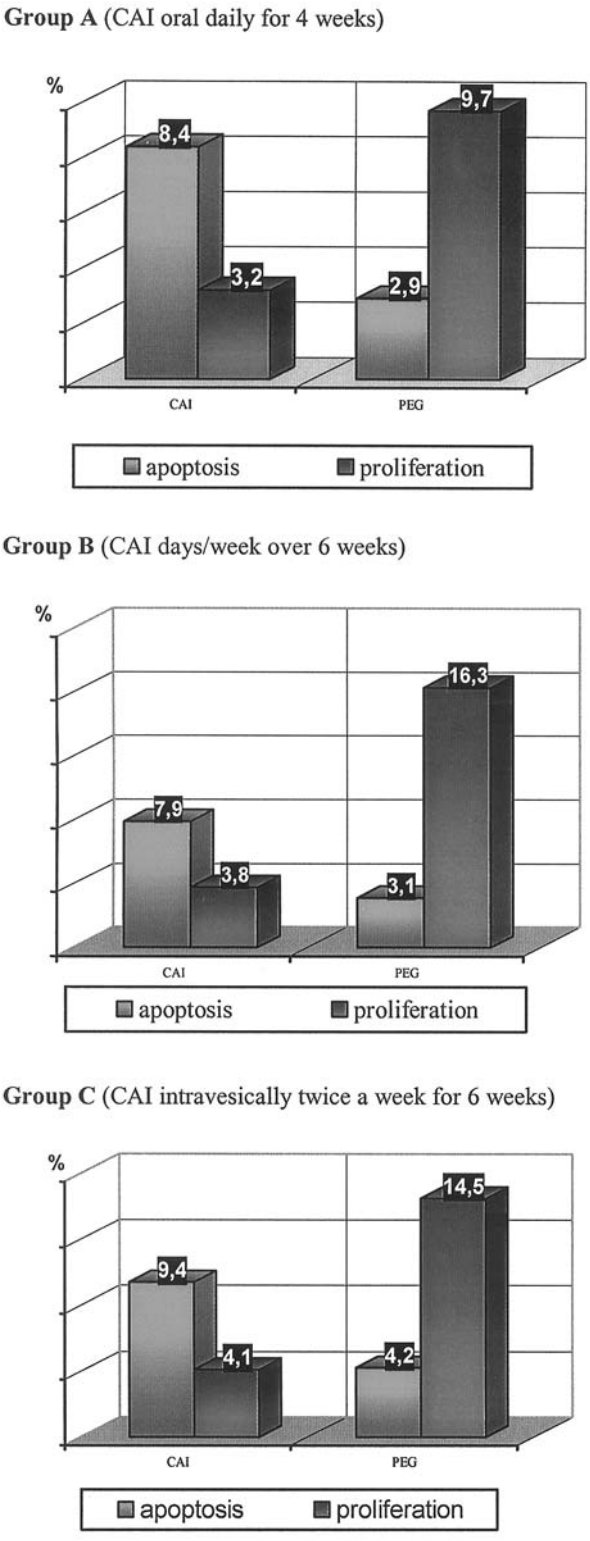

Figure 2. *Apoptosis and proliferation ratios in CAI (100 mg/kg dissolved in PEG-400)-treated rats in comparison to PEG-treated rats. The results show for all CAI groups (oral daily for 4 weeks [group A], 3 days/week over 6 weeks [group B] and intravesically twice a week for 6 weeks [group C]) an increase of apoptosis and a decrease of proliferation in the remaining tumor.*

of 8 rats) by the 16th week (Table I). The incidence of tumors rose with time beginning at week 10 and progressed from hyperplasia and atypia to carcinoma-*in-situ* and superficial papillary tumors and invasive tumors. No animal in this study or in our previous study developed squamous cell carcinoma (15).

Toxicity. The toxicity study with escalating doses of up to 250 mg/kg CAI daily revealed neither major nor minor side-effects (incl. hematuria) in the animals over a period of 5 weeks.

Efficacy. The efficacy results showed in the examined groups (oral daily for 4 weeks [group A], 3 days/week over 6 weeks [group B] and intravesically twice a week for 6 weeks [group C]) no significant change of stage and grade of the remaining tumors. However, a trend to lesser stage and grade was seen in all 3 groups (Figure 1). The best anti-tumor efficacy was found in group B, with 5 out of 10 animals tumor-free; in group C, 3 out of 10 animals were tumor-free. In group A, both the treatment and control group demonstrated one animal without tumor. Normal urothelium did not show any morphological changes after treatment with CAI. PEG-400 did not show any anti-tumor activity.

Apoptosis and proliferation in the tumors after CAI treatment. After treatment with CAI, the rat bladder tumors in group A (oral continuously) showed an elevated apoptotic rate of 11.4 %, at the same time the proliferation rate decreased to 3.2 % in comparison to the control group where apoptosis and proliferation were 2.9 % and 9.7 %, respectively. In group B (oral intermittent), the remaining tumors showed, after CAI treatment, an apoptotic rate of 7.9 %, at the same time the proliferation rate decreased to 3.8 %. In the control group, PEG-treated bladder tumors showed a proliferation rate of 16.3 %, whereas the apoptotic rate was merely 3.2 %. On intravesical application (Group C), the treatment group demonstrated an induction of apoptosis of 9.4 % while the proliferative activity was 4.1 %. In the control group, again, cell increase was 14.5 % and cell death was 4.2 %. Taken together, after CAI treatment the remaining tumors demonstrated an increased rate of apoptosis up to 2.5-fold, whereas at the same time the tumor proliferation rate decreased 5-fold (Figure 2).

Discussion

CAI is an orally bioavailable calcium channel blocker inhibiting calcium influx and signal transduction. It has been shown to be anti-invasive, anti-angiogenic and anti-metastatic in different human tumors. Our earlier studies showed a growth inhibitory and apoptosis-inducing effect in transitional cell carcinoma cell lines (12). We wanted to further confirm the findings in an *in vivo* model of bladder cancer and to evaluate the efficacy and toxicity of the drug. The animal model used in this study has been previously characterized and produces transitional cell carcinoma in approximately three quarters of the animals. The model reflects the clinically observed tumors in humans; the tumors arise only from the urothelium, they are spontaneous and not implanted and are histologically equivalent to human TCC.

These lesions progress through early stages to invasive cancer and develop at discrete times and in high frequency, thereby allowing treatment to be initiated at a known stage of disease. The intravesical instillation of fractionated doses of MNU to induce bladder cancer provides a more controllable bladder cancer model than those using carcinogens in the diet or drinking water, since MNU acts directly on coming in contact with tissue (18). In our study, there was a progressive increase in the incidence of TCC in the animals beginning in the 10th week after the initiation of instillation and progressing to the 18th week, at which 87% of the animals had developed tumors. The treatment of rat urinary bladder cancer with CAI demonstrated best efficacy for intermittent treatment with 5/10 animals tumor-free. In intravesical treatment, 3/10 animal were tumor-free. A trend to lesser stage and grade was seen for all 3 groups. There seems to be no advantage in daily *versus* intermittent dosage of CAI. In fact, group B, with a longer period of treatment yielded higher response rates. Interestingly, the oral application was superior to the intravesical application. This could be due to the lipophilic nature of CAI, causing an insufficient contact between the drug and the tumor, but is advantageous for enteric resorption on oral intake. However, human clinical trials will have to further clarify the optimal dosage and scheduling. After CAI treatment, the rate of apoptosis was increased, whereas the tumor proliferation rate decreased. Normal urothelium was not affected and the PEG-400 vehicle caused no toxicity, nor did it have anti-tumor activity. This indicates that CAI inhibited not only tumor growth, but also malignant progression. These data are in concordance with the previously reported *in vitro* data that demonstrated significant inhibition of tumor growth and proliferation as well as induction of apoptosis in TCC cells on CAI treatment (12). We, therefore, suggest that the anti-tumor effect of CAI is *in vivo* mediated at the same time by induction of apoptosis and inhibition of tumor growth. Furthermore, the model shows that CAI is both safe and effective. Recent clinical phase I and II trials with oral formulations of CAI in various solid tumors have shown that the drug was well tolerated, with mostly grade 1 to 2 toxicity. Grade 3 events included fatigue (5%), vomiting (2%), neutropenic fever (2%) and neutropenia (2%). There were no grade 4 adverse events. Dose escalation to 300 mg/m^2 showed dose-limiting neurotoxicity. No additive or cumulative toxicity was observed in the patients (19-23).

Taken together, these results indicate that CAI could be a useful therapeutic agent for bladder cancer, inhibiting tumor growth, malignant progression and inducing apoptosis. CAI treatment may offer a new therapeutic option for bladder cancer and should be evaluated further in a clinical trial.

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