Ampelopsin sodium exhibits antitumor effects against bladder carcinoma in orthotopic xenograft models

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The aim of this study was to establish xenograft models of tumor in mice bladder and evaluate the antitumor efficacy of ampelopsin sodium (Amp-Na). A total of 2×10^6 human bladder carcinoma EJ cells and murine sarcoma 180 cells were instilled into the bladder of BALB/c nu/nu mice and Swiss mice after preconditioning to establish the tumor model. Mice bearing orthotopic tumors were treated with Amp-Na by intravenous, intraperitoneal, or intravesical instillation. In addition, the pharmacokinetics property of Amp-Na was investigated in normal BALB/c mice. Our results showed that Amp-Na was excreted mainly through the urine, where it existed at a high concentration. Amp-Na significantly inhibited the proliferation of EJ and sarcoma 180 cells both *in vivo* and *in vitro* and this can be at least partially attributed to the cell cycle arrest induced by Amp-Na. This study suggests that the use of Amp-Na is an attractive chemotherapeutic modality for bladder cancer patients. *Anti-Cancer Drugs* 23:590–596 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Bladder cancer is the fourth most common cancer among men and the eighth most common cancer among women in the USA. In the USA alone, there are 70 530 new cases of bladder cancer each year, with 14 680 associated deaths [1]. Transitional cell carcinoma accounts for more than 90% of bladder cancer cases. The majority of transitional cell carcinomas are superficial at the time of diagnosis and most of them have a propensity for recurrence after initial transurethral resection of bladder tumor. Therefore, there is an urgent need to develop effective strategies to control bladder cancer. Chemoprevention and chemointervention refer to the administration of chemical entities either as individual agents in their pure chemically defined forms or as naturally occurring constituents of the diet such as fruits, vegetables, common beverages, and several herbs and plants [2,3]. Intravesical administration of Bacillus Calmette–Guerin (BCG) after transurethral resection is by far the most effective treatment for superficial bladder cancer [4–6]. However, BCG therapy would cause obvious side effects, and approximately one-third of patients fail to respond.

In the present series of trials, Adriamycin (doxorubicin, ADM) intravesical instillation (i.i.) therapy was found to be effective against tumors of papillary morphology. The rate of complete disappearance increased in proportion to the concentration of ADM and the duration of retention of the drug. However, ADM caused local side effects dependent on both the drug concentration and the duration of drug retention [7–10]. Carboplatin, paclitaxel, and irinotecan are potent anticancer chemotherapy drugs commonly used in the treatment of many solid tumors. As a second-generation analog of cisplatin, carboplatin shares many structural and pharmacologic features with cisplatin, and yet, it has an improved toxicity profile. These characteristics have made carboplatin an ideal candidate for chemotherapy [11–14].

Among several classes of the chemical agents, ampelopsin has received increased attention in the last few years. Ampelopsin is an active component extracted from the root of the Chinese medicinal herb Radix *Ampelopsis cantoniensis* or *Ampelopsis grossedentata*, which has detoxification and anti-inflammation functions [15,16]. The structural analysis, extraction, and purification of ampelopsin have been well established [17]. Several recent studies by others and us have shown that ampelopsin possesses anticancer effects on various human cancer cell lines including hepatic, lung, prostate, breast, colon, and skin cancers *in vitro* and on transplanted B16 mouse melanoma *in vivo* [18–20].

However, the clinical use of ampelopsin requires repeated large dose application because it is insoluble and unstable, with a short half-life in the body. To improve the solubility and stability, ampelopsin is prepared as a sodium salt [ampelopsin sodium (Amp-Na)], which is more suitable for the clinical use of drug formulation. In this study, we evaluated the antitumor effects of Amp-Na on bladder cancer cells *in vitro* and bladder cancer xenograft *in vivo*. Furthermore, we investigated the pharmacokinetics property of Amp-Na in mice. Our results suggest that Amp-Na is a promising agent for the chemotherapy of bladder cancer.

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Materials and methods

Cell culture and reagents

The human bladder carcinoma cell line EJ and murine sarcoma 180 (S180) were obtained from the American Type Culture Collection (Rockville, Maryland, USA); EJ cells were cultured in RPMI-1640 medium (GIBCO, Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin sodium, and 100 μg/ml streptomycin sulfate, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. S180 cells were maintained as ascites in Swiss Webster mice by weekly passage. Amp-Na was a gift from Taihe Pharmaceutical (Guangdong, China). Carboplatin (CBP) and Adriamycin (ADM) were purchased from Qilu Pharmaceutical (Shandong, China) and Zhejiang Hisun Pharmaceutical (Zhejiang, China), respectively.

Animals

Female athymic nude mice (BALB/c nu/nu) aged 6–7 weeks were purchased from the Shanghai Institute of Materia Medica (Shanghai, China), housed in sterile cages under laminar airflow hoods in a specific pathogen-free room with a 12-h on/off light cycle, and fed autoclaved chow and water. BALB/c and Swiss mice (6–7 weeks old, weighing 18–22 g) were purchased from Lanzhou University (Lanzhou, China). The animals were maintained in an air-conditioned barrier-system animal room with an ambient temperature of 25 ± 2°C, a relative humidity of 50 ± 10%, and a 12-h on/off light cycle. The study protocols were in accordance with the regulations of Good Laboratory Practice for Non-clinical Laboratory Studies of drug issued by the National Scientific and Technologic Committee of People's Republic of China. All experiments were carried out according to institutional ethical guidelines on animal care.

Cell proliferation assay

The effect of Amp-Na on cell proliferation was evaluated by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [21]. Briefly, EJ cells were seeded at 6×10^3/well in 96-well plates. Following 24 h of culture, the medium was removed and replaced with 100 μl fresh complete medium containing 50.0, 63.0, 80.0, and 100.0 μg/ml Amp-Na. The control group was treated with the vehicle control. After incubation for 48 or 72 h, 10 μl MTT (5 g/l) was added to each well and incubated at 37°C for 4 h, and then 100 μl SDS (10%, w/v, in 0.01 mol/l HCl) was added to each well and mixed thoroughly to dissolve formazan crystals at 37°C. Cell proliferation was determined by measuring the absorbance at a wavelength of 570 nm after 10 min of shaking at room temperature in a Microplate Reader (EL × 800 Instruments; Bio-TEK, Seattle, Washington, USA). The percentage of cell proliferation was calculated using the formula: absorbance of the test group/absorbance of the control group.

High-performance liquid chromatography analysis of Amp-Na concentration in the urine

Twenty-five BALB/c mice were given a single intravenous (i.v.) dose of 200 mg/kg Amp-Na and were divided into five cages (five mice per cage). Urine was collected at 0.17, 0.33, 1, 2, 4, 6, 8, 12, and 24 h postdose. The excreted volume was measured and urine samples were stored at 4°C. The concentration of Amp-Na in 200 μl urine samples was detected using the high-performance liquid chromatography (HPLC) method as previously described [22]. Briefly, the HPLC system consisted of a model M 45 HPLC pump and a model 712 WISP auto-sampler (Waters, Mississauga, Ontario, Canada), a variable wavelength UV detector model SPD-10A UV–vis (Shimadzu, Japan), set at 290 nm for detection. Analysis was conducted on a 15 cm × 4.6 mm i.d. 5-μm C18 analytical reverse-phase column (Chromatography Sciences Co., St-Laurent, Quebec, Canada). The mobile phase consisted of acetonitrile: 2% acetic acid (1 : 9, v/v), pumped at a flow rate of 1.0 ml/min. The temperature of the column was maintained at 25°C.

High-performance liquid chromatography analysis of Amp-Na concentration in the serum

The female BALB/c mice were anesthetized with a single dose of an intraperitoneal (i.p.) injection of 30 mg/kg sodium phenobarbital. The mouse bladder was catheterized through the urethra using a 20-gauge disposable anesthesia epidural catheter under sterile conditions. The bladder was then drained and mice were given a single dose of 260 mg/kg Amp-Na (100 μl) or mice were administered with i.v. (without anesthetizing). After the drug administration ($n = 5$ mice per time point), blood samples (approximately 0.5–1.0 ml) were withdrawn through the orbital vein of each animal at 0, 10, 20, 30, 45, and 60 min postdose and collected in heparinized tubes. They were centrifuged at 2000 *g* for 5 min and plasma was harvested and kept at −20°C until analyzed. The concentration of Amp-Na in 200 μl plasma samples was detected by HPLC as described above.

Tumor implantation and treatment

EJ or S180 cell suspension was implanted into mice following the protocols previously described [23,24]. Briefly, healthy female BALB/c, nu/nu, or Swiss mice were anesthetized with a single dose of i.p. of sodium phenobarbital (30 mg/kg body weight). The mouse bladder was catheterized through the urethra with a 20-gauge disposable anesthesia epidural catheter under sterile conditions. To facilitate tumor seeding, the bladder mucosa were conditioned with an acid rinse. Conditioning consisted of an intravesical administration of 100 μl 0.1 N HCl solution for 15 s and neutralization with 100 μl 0.1 N KOH for 15 s. The bladder was then drained and flushed with sterile PBS. Immediately after bladder conditioning, 2×10^6 EJ or S180 cells suspended in 100 μl serum-free RPMI 1640 medium were instilled and left indwelling for

at least 1 h. The mice were turned 90° every 15 min to facilitate whole bladder exposure to the tumor cell suspension. After 1 h, the catheter was removed and the mice were allowed to void the suspension spontaneously. On day 2, the mice were divided into five groups (9–11 animals per group): pH 6.0 PBS only; 40 mg/kg CBP or 2 mg/kg ADM; 160 mg/kg Amp-Na; 200 mg/kg Amp-Na; and 260 mg/kg Amp-Na. All drugs were diluted with pH 6.0 PBS and administered i.v., i.p., or i.i. On day 21 (for EJ cells) and day 14 (for S180 cells), animals were sacrificed and tumors were excised from each mouse and weighed. The efficacy of the treatment was expressed as best tumor growth inhibition [25,26]. The *T*/*C* value was calculated as follows: $\%T/C =$ (median tumor weight of treated tumors/median tumor weight of control tumors) $\times$ 100.

Flow cytometry analysis

EJ tumors obtained from the orthotopic xenograft were dissociated into single-cell suspensions after filtration through 400 mesh. The single-cell suspension was washed in cold PBS (pH 7.4), fixed in 70% ethanol/30% PBS, and stored at 4°C until processing. 1×10^6 cells were washed twice in cold PBS and incubated for 30 min at 4°C in the dark with a fluorochrome DNA staining solution (1 ml) containing 40 μg propidium iodide and 0.1 mg ribonuclease A. The stained cells were analyzed by flow cytometry for cell cycle profiling as described previously [27].

Statistical analysis

Data were presented as mean ± SD. The one-way analysis of variance test, followed by the Student–Newman–Keuls test was used to assess the statistical difference among independent groups. Tumor growth inhibition was expressed as percentages. *P*-value less than 0.05 was considered statistically significant. Pharmacokinetic parameters of Amp-Na were calculated using noncompartmental analysis (WinNolin version 5.3; Pharsight, California, USA). The observed peak plasma concentration (C_{max}) and the time-to peak concentration (T_{max}) were recorded. Absolute bioavailability (*F*) after i.i. doses was calculated as the $AUC_{i.i.}/AUC_{i.v.}$ ratio.

Results

Pharmacokinetics of Amp-Na in the female BALB/c mice

The concentration of Amp-Na in the urine of healthy female BALB/c mice at different time intervals after i.v. of 200 mg/kg Amp-Na was detected by HPLC (Fig. 1a). The cumulative percent of Amp-Na excreted in the urine was calculated as shown in Fig. 1b. The cumulative percent of Amp-Na excreted in the urine after 1 and 12 h was 21.28 and 21.54%, respectively.

The plasma concentration–time profiles and the main pharmacokinetic indices of Amp-Na after i.v. and i.i. administration are shown in Fig. 1c and Table 1. The peak plasma concentration, after i.i. administration, occurred approximately 30 min postdose. The absolute bioavailability for i.i. doses was 69.10%.

Amp-Na inhibits the proliferation of bladder carcinoma cells *in vitro*

MTT assay showed that Amp-Na significantly inhibited the proliferation of EJ cells in a time-dependent and concentration-dependent manner (Fig. 2). Notably, strong inhibition of cell growth was observed at 80 and 100 μg/ml doses of Amp-Na following treatment for 48 and 72 h.

Antitumor activity of Amp-Na *in vivo*

To further evaluate the in-vivo antitumor effect of Amp-Na, first, we established a bladder cancer xenograft. Single-cell suspensions of 2×10^6 S180 cells instilled into normal (nonconditioned) bladders did not result in tumor establishment. In contrast, S180 cells instillation into bladder with preconditioned mucosa led to the establishment of bladder tumor. The microscopic and gross characteristics of the bladder tumors are shown in Fig. 3.

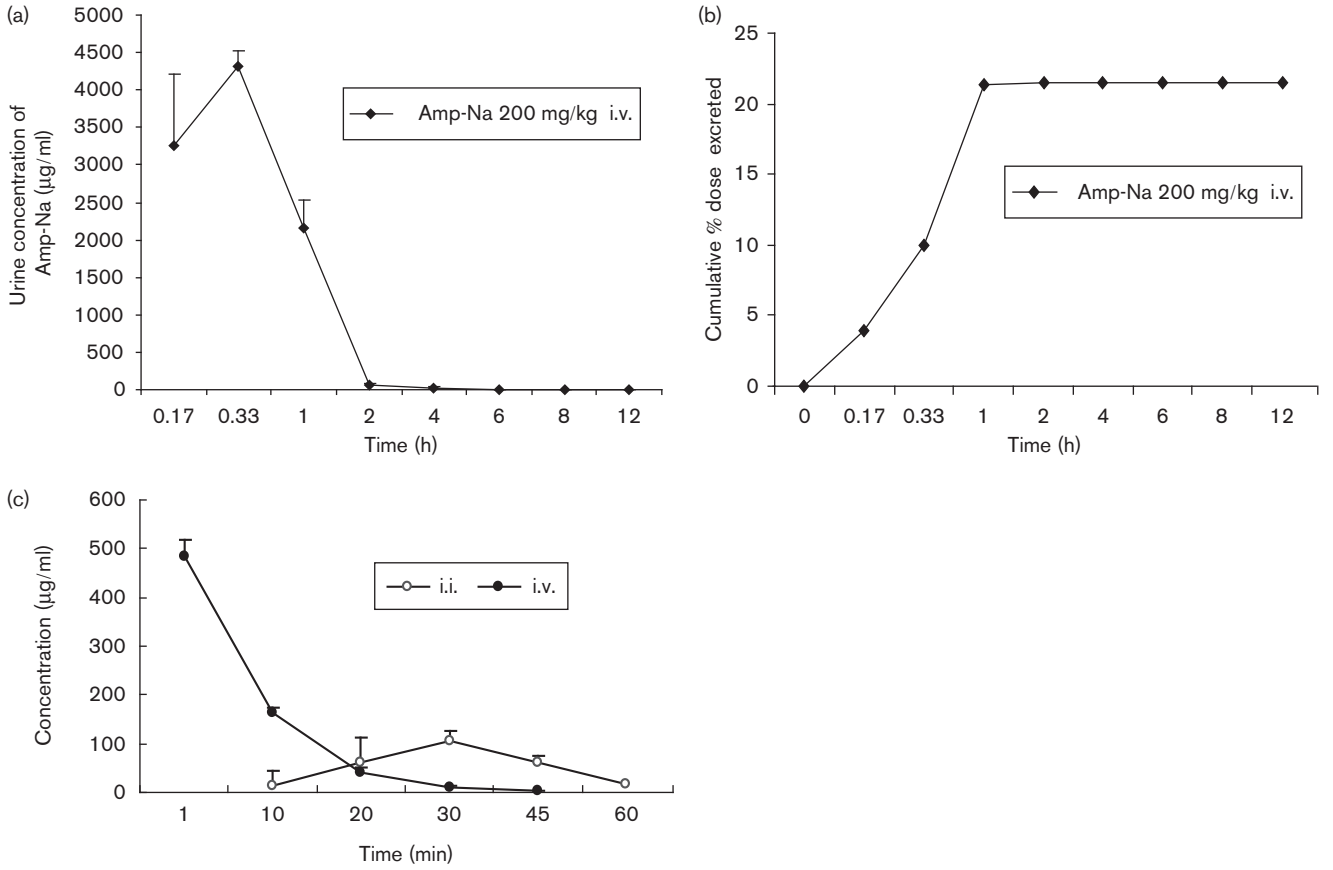
Next, S180-xenografted and EJ cell-xenografted mice were treated with Amp-Na for 2–3 weeks, respectively, according to the regimen. Antitumor activity was evaluated by tumor weight. In the S180 cell-xenografted models, the inhibition of tumor growth by Amp-Na at 160, 200, and 260 mg/kg through i.v. or i.i. was 59.14 ± 13.81 51.96 ± 7.35, and 48.68 ± 10.66, or 46.58 ± 14.27, 45.40 ± 9.74, and 39.08 ± 7.66%, respectively. In the EJ cell-xenografted models, the inhibition of tumor growth by Amp-Na at 160, 200, and 260 mg/kg through i.p. or i.i. was 74.01 ± 12.01, 57.31 ± 11.41, and 55.64 ± 9.93, or 53.00 ± 5.63, 46.69 ± 4.70, and 43.21 ± 6.56%, respectively (Fig. 4). In both tumor models, Amp-Na showed profound antitumor potential compared with the negative controls, but its antitumor effects were not as strong as CBP or ADM.

Finally, to investigate the mechanism by which Amp-Na exhibits antitumor effects against bladder cancer cells *in vivo*, EJ tumors were taken from orthotopic xenografts and dissociated into single-cell suspensions for cell cycle profiling analysis. Flow cytometry analysis showed that Amp-Na induced an accumulation of EJ bladder cancer cells in the G2/M phase at the dose of 160 mg/kg and induced an accumulation of EJ bladder cancer cells in the S phase at higher doses of 200 and 260 mg/kg (Fig. 5). These results provide in-vivo evidence that Amp-Na inhibits the proliferation of bladder cancer cells.

Discussion

Although the technique of orthotopic bladder tumor implantation has been used widely to establish an in-vivo bladder cancer model, the rate of success ranges from 50 to 90% [28,29]. To our knowledge, in the current study, we have established a bladder xenograft model of murine S180 for the first time. In addition, we increased the rate

Fig. 1



Pharmacokinetics of ampelopsin sodium (Amp-Na) in the mice. (a) Urine concentration of Amp-Na in healthy female BALB/c mice, (b) cumulative percent of Amp-Na excreted in the urine of healthy female BALB/c mice after its intravenous (i.v.) administration, and (c) mean Amp-Na plasma concentration vs. time curve after single i.v. and intravesical instillation (i.i.) administration of 260 mg/kg Amp-Na in healthy female BALB/c mice.

Table 1 Main pharmacokinetic indices of ampelopsin sodium after the administration of 260 mg/kg ampelopsin sodium to the female BALB/c mouse

	Route of administration	
Pharmacokinetic indices	i.v.	i.i
T_{max} (min)	Not determined	30
C_{max} (μg/ml)	484.75	105.14
AUC_{0-t} (μg h/ml)	4855.71	3132.33
$AUC_{0-\infty}$ (μg h/ml)	4898.81	3385.09
$t_{1/2}$ (min)	6.34	11.01
F (%)	–	69.10

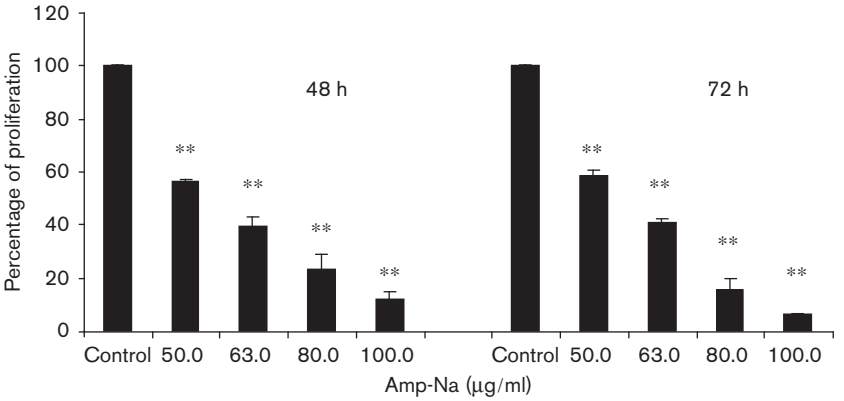
AUC, area under the curve; i.v., intravenous; i.i., intravesical instillation.

of human orthotopic bladder tumor formation to 100% for EJ cells by pulsing with 20% supernatant of murine S180 ascites that are rich in growth factors. This model produces superficial tumors that closely mimic human bladder carcinoma and thus provides better opportunities for the evaluation of antitumor activity of antineoplastic agents.

Our in-vitro results clearly showed that Amp-Na significantly inhibited the proliferation of human bladder carcinoma EJ cells in a time-dependent and dose-dependent manner, consistent with our preliminary data on the in-vitro antitumor effects of Amp-Na against human lung adenocarcinoma cell line SPCA-1, A549, murine LLC, and S180 (Zhang *et al.* unpublished observation).

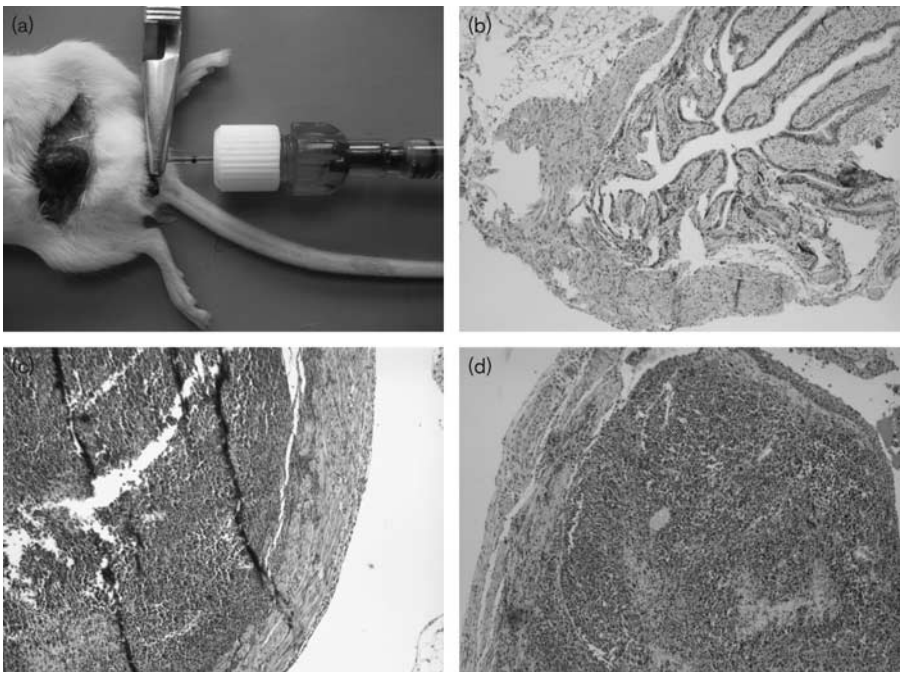
Intravesical ADM and BCG instillation are widely used as a topical therapy for superficial bladder tumors, and their efficacy has been confirmed by different groups [4–6,30,31]. In our current study, the antitumor effects of Amp-Na were evaluated on the basis of a more clinically relevant mouse orthotopic xenograft model. Through i.p., i.v., and i.i. administration, we examined the antitumor effects of Amp-Na on S180 and EJ orthotopic xenograft tumors in mice bladder. As expected, we observed that Amp-Na could significantly inhibit the growth of S180 and EJ xenografts in mice compared with the negative control. Furthermore, flow cytometry analysis showed that tumor cells derived

Fig. 2



Antiproliferative effects of ampelopsin sodium (Amp-Na) on human bladder carcinoma EJ cells. EJ cells were treated with Amp-Na at the indicated dose and for the indicated time. The proliferation of EJ cell was examined by an MTT assay and the inhibition rate was calculated. $^{**}P<0.01$ vs. control group ($n=6$). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Fig. 3

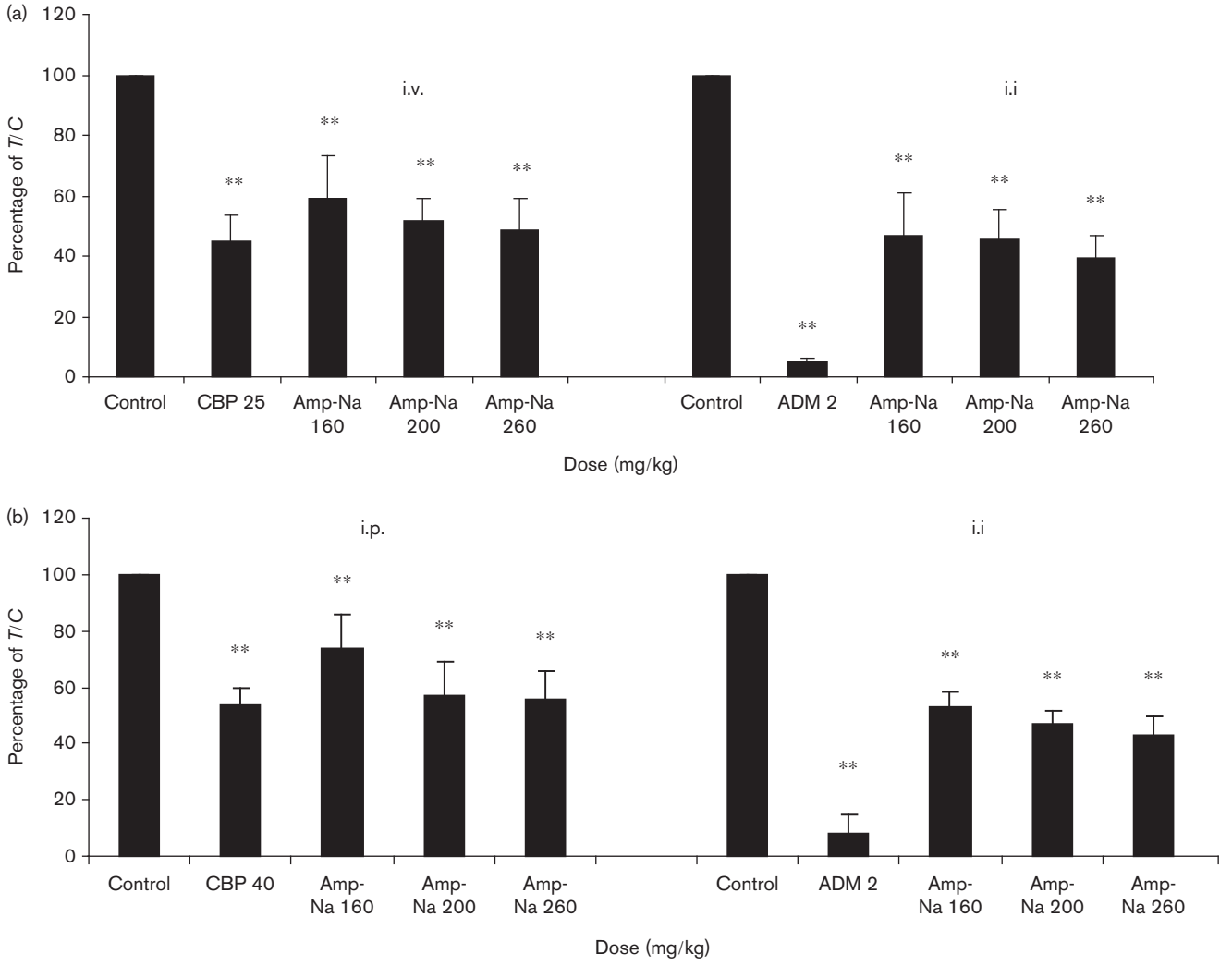


Microscopic and gross features of sarcoma 180 bladder tumors at different stages. (a) The method for the establishment of a xenograft model, (b) a normal mouse bladder. Hematoxylin and eosin (H&E) staining, magnification, ×100, (c) mouse bladder on day 7 after implantation, H&E staining, magnification, ×100, and (d) mouse bladder on day 14 after implantation. H&E staining, magnification, ×100.

from EJ orthotopic xenografts treated with Amp-Na were primarily arrested in the S phase, proving that the in-vivo antitumor activity of Amp-Na can at least partially be attributed to the inhibition of the proliferation of bladder cancer cells.

Pharmacokinetics analysis of Amp-Na in normal mice revealed that Amp-Na was excreted mainly through the urine, where it existed at a high concentration after a single dose i.v. 200 mg/kg Amp-Na, and the concentration of Amp-Na in the urine remained at an effective level

Fig. 4

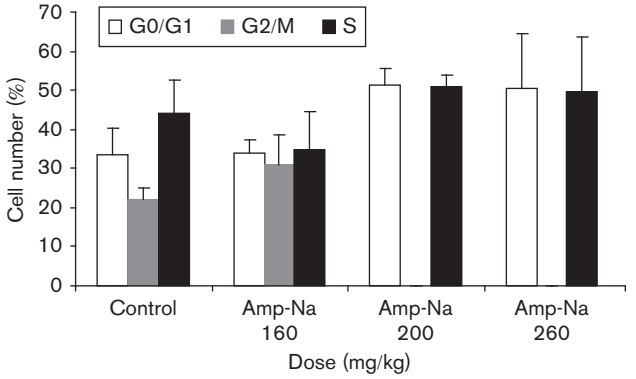


Antitumor effects of ampelopsin sodium (Amp-Na) *in vivo*. (a) Percentage of *T/C* in sarcoma 180-bearing mice. Sarcoma 180 tumor cells were transplanted in the bladder of Swiss mice. After 1 day, mice were randomized to receive vehicle (PBS), carboplatin (CBP) [25 mg/kg, intraperitoneal (i.p.), once per two days] or adriamycin (ADM) [2 mg/kg, intravesical instillation (i.i.), once a week], and Amp-Na [160, 200, and 260 mg/kg, once per two days intravenous (i.v.) or once a week i.i. for 14 days]. (b) Percentage of *T/C* in EJ-bearing mice. The EJ cells were orthotopically transplanted in the bladder of nude mice. After 7 days, nude mice were randomized to receive vehicle (PBS), CBP (40 mg/kg, i.p., once per two days) or ADM (2 mg/kg, i.i., once a week), and Amp-Na (160, 200, and 260 mg/kg, once a day i.p. for 14 days or once a week i.i. for 21 days one day after tumor implant). PBS was treated as a negative control and CBP or ADM was treated as a positive control. Each group included nine to 11 mice. At the end of the treatment, mice were sacrificed and tumors were harvested and weighed. All data were expressed as mean ± SD. $^{**}P < 0.01$ vs. the negative control group.

(50 μg/ml) for more than 2 h. On the basis of the pharmacokinetic properties of Amp-Na, the bladder is a promising target for the antitumor effects of Amp-Na.

The absorption of Amp-Na through the mice bladder epithelium was investigated using the HPLC method. Our data indicate that Amp-Na could be absorbed by the mice bladder epithelium after i.i. administration. The absolute bioavailability was 69.10%. In addition, Amp-Na administered to the bladder had a significantly longer half-life than Amp-Na given i.v. (11.01 vs. 6.34 min). Therefore, the mice given i.i. therapy are exposed to a lower systemic level of Amp-Na but for a significantly longer period of time than i.v. chemotherapy, which would help avoid the side-effects following i.i. of Amp-Na. Indeed, our preliminary studies showed that Amp-Na causes little damage to the bladder mucosa of rats and beagle dogs, indicating the low systemic toxicity of Amp-Na (Zhang *et al.* unpublished observation). Nevertheless, it is possible that i.i. administration of chemotherapy may alter the immunologic milieu of the bladder. Further studies are necessary considering the importance of the practical implications, especially in the field of topical chemotherapy.

Fig. 5



The induction of cell cycle arrest in xenograft bladder cancer cells by ampelopsin sodium (Amp-Na). EJ cells were orthotopically transplanted in the bladder of nude mice. After 7 days, nude mice were treated once daily intraperitoneally for 14 days with Amp-Na (160, 200, and 260 mg/kg) or left untreated as a control. The tumors were harvested and a single-cell suspension was prepared for flow cytometry analysis. All data were expressed as mean ± SD ($n = 3$).

In summary, the promising results reported here suggest that further investigation of the mechanisms underlying the growth inhibition of bladder cancer by Amp-Na treatment is warranted. Although the low potency of Amp-Na relative to standard antitumor drug such as CBP and ADM presents a potentially serious obstacle to its clinical development, the advantages of Amp-Na lie in its low systemic toxicity and pharmacokinetic characteristics, in that the concentration of Amp-Na is high in the urine after i.v. administration. Thus, we propose that the use of Amp-Na is an attractive chemotherapeutic modality for patients with bladder cancer.

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Conflicts of interest

There are no conflicts of interest.

References

1 Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. *CA Cancer J Clin* 2010; **60**:277–300.
2 Morse MA, Stoner GD. Cancer chemoprevention: principles and prospects. *Carcinogenesis* 1993; **14**:1737–1746.
3 Tyagi A, Agarwal C, Harrison G, Glode LM, Agarwal R. Silibinin causes cell cycle arrest and apoptosis in human bladder transitional cell carcinoma cells by regulating CDKI-CDK-cyclin cascade, and caspase 3 and PARP cleavages. *Carcinogenesis* 2004; **25**:1711–1720.
4 Herr HW, Wartinger DD, Fair WR, Oettgen HF. Bacillus Calmette-Guerin therapy for superficial bladder cancer: a 10-year follow-up. *J Urol* 1992; **147**:1020–1023.
5 Lamm DL, Thor DE, Harris SC, Reyna JA, Stogdill VD, Radwin HM. Bacillus Calmette-Guerin immunotherapy of superficial bladder cancer. *J Urol* 1980; **124**:38–40.
6 Wu Q, Mahendran R, Esuvaranathan K. Nonviral cytokine gene therapy on an orthotopic bladder cancer model. *Clin Cancer Res* 2003; **9**:4522–4528.
7 Ohe H, Watanabe H, Mishina T, Kojima M, Nakagawa S. Intravesical instillation of adriamycin for bladder tumors. *Cancer Chemother Pharmacol* 1992; **30 (Suppl)**:S45–S49.
8 Qian L, Ding H, Zhou J, Wang X, Shao P, Wu S, *et al*. Intravesical *N*-(4-hydroxyphenyl) retinamide and adriamycin induces apoptosis in bladder cancer. *Front Biosci* 2006; **11**:2045–2051.
9 Naito S, Kotoh S, Omoto T, Osada Y, Sagiyama K, Iguchi A, *et al*. Prophylactic intravesical instillation chemotherapy against recurrence after a transurethral resection of superficial bladder cancer: a randomized controlled trial of doxorubicin plus verapamil versus doxorubicin alone. *Cancer Chemother Pharmacol* 1998; **42**:367–372.
10 Naito S, Iguchi A, Sagiyama K, Ariyoshi A, Osada Y, Omoto T, *et al*. Significance of the preoperative intravesical instillation of doxorubicin and the oral administration of 5-fluorouracil in preventing recurrence after a transurethral resection of superficial bladder cancer. *Int J Urol* 1997; **4**:352–357.
11 DeVita VT Jr, Chu E. A history of cancer chemotherapy. *Cancer Res* 2008; **68**:8643–8653.
12 Briasoulis E, Golfinopoulos V, Karina M, Papakostas P, Pavlidis N, Fountzilas G. Phase I trial of weekly irinotecan and paclitaxel combined with carboplatin in patients with advanced cancer: a Hellenic Cooperative Oncology Group Study. *Anticancer Drugs* 2010; **21**:785–789.
13 Alberts DS, Dorr RT. New perspectives on an old friend: optimizing carboplatin for the treatment of solid tumors. *Oncologist* 1998; **3**:15–34.
14 Thiery-Vuillemin A, Dobi E, Nguyen T, Royer B, Montange D, Maurina T, *et al*. Duration: escalation study of oral etoposide with carboplatin in patients with varied solid tumors. *Anticancer Drugs* 2010; **21**:958–962.
15 Liu DY, Ye JT, Yang WH, Yan J, Zeng CH, Zeng S. Ampelopsin, a small molecule inhibitor of HIV-1 infection targeting HIV entry. *Biomed Environ Sci* 2004; **17**:153–164.
16 Kotake M, Kubota T. About ingredients from *Ampelopsis meliaefolia* Kudo (Haku-Tya). *Liebigs Annalen der Chemie* 1940; **544**:253–271.
17 Wang MX, Liu WZ, Huang AD, Luo LQ, Zhou AQ. Isolation and identification on the consistuents of flavonoids in Radix *Ampelopsis Cantoniensis*. *J Chin Med Mater* 1989; **12**:28–30, (in Chinese).
18 Liu DY, Luo M, Xie BF, Feng GK, Zhu XF, Liu ZC. Antitumor effects of ampelopsin. *Chin J Cancer* 2001; **20**:1372–1375, (in Chinese).
19 Liu DY, Qiu MQ, Liang TY. Extraction and isolation of Ampelopsin from *Ampelopsis Cantoniensis* Planch and its inhibitory effect on B16 melanoma. *Acad J SUMS* 1999; **20**:127–129, (in Chinese).
20 Tan GY, Zhang MH, Feng JH, Han AY, Zheng SS, Xie P. Effects of pretreatment by the flavanol ampelopsin on porcine kidney epithelial cell injury induced by hydrogen peroxide. *Agric Sci China* 2010; **9**:598–604.
21 Bi S, Liu JR, Li Y, Wang Q, Liu HK, Yan YG, *et al*. Gamma-tocotrienol modulates the paracrine secretion of VEGF induced by cobalt(II) chloride via ERK signaling pathway in gastric adenocarcinoma SGC-7901 cell line. *Toxicology* 2010; **274**:27–33.
22 Aghazadeh-Habashi A, Sattari S, Pasutto F, Jamali F. High performance liquid chromatography determination of glucosamine in rat plasma. *J Pharm Pharm Sci* 2002; **5**:158–161.
23 Chin J, Kadhim S, Garcia B, Kim YS, Karlik S. Magnetic resonance imaging for detecting and treatment monitoring of orthotopic murine bladder tumor implants. *J Urol* 1991; **145**:1297–1301.
24 Chong L, Ruping Y, Jiancheng B, Guohong Y, Yougang F, Jiansong W, *et al*. Characterization of a novel transplantable orthotopic murine xenograft model of a human bladder transitional cell tumor (BIU-87). *Cancer Biol Ther* 2006; **5**:394–398.
25 Bello E, Colella G, Scarlato V, Oliva P, Berndt A, Valbusa G, *et al*. E-3810 is a potent dual inhibitor of VEGFR and FGFR that exerts antitumor activity in multiple preclinical models. *Cancer Res* 2011; **71**:1396–1405.
26 Zhang B, Gao B, Dong S, Zhang Y, Wu Y. Anti-tumor efficacy and pre-clinical immunogenicity of IFNa2a-NGR. *Regul Toxicol Pharmacol* 2011; **60**:73–78.
27 Li Z, Liu W, Mo B, Hu C, Liu H, Qi H, *et al*. Caffeine overcomes genistein-induced G2/M cell cycle arrest in breast cancer cells. *Nutr Cancer* 2008; **60**:382–388.
28 Soloway MS, Masters S. Urothelial susceptibility to tumor cell implantation: influence of cauterization. *Cancer* 1980; **46**:1158–1163.
29 Gunther JH, Jurczok A, Wulf T, Brandau S, Deinert I, Jocham D, *et al*. Optimizing syngeneic orthotopic murine bladder cancer (MB49). *Cancer Res* 1999; **59**:2834–2837.
30 Banks MD, Pontes JE, Izbicki RM, Pierce JM Jr. Topical instillation of doxorubicin hydrochloride in the treatment of recurring superficial transitional cell carcinoma of the bladder. *J Urol* 1977; **118**:757–760.
31 Mishina T, Fujiwara T, Miyakoda K, Araki H, Kobayashi T, Saitoh M, *et al*. Adriamycin instillation therapy for bladder tumors. *Tohoku J Exp Med* 1979; **127**:339–343.