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Urease-induced alkalinization of extracellular pH and Its antitumor activity in human breast and lung cancers

Wah Yau Wong,[1] Carl I. DeLuca,[1] Baomin Tian,[1] Iain Wilson,[1] Sharon Molund,[1] Nalini Warriar,[2] Manjapra V. Govindan,[2] Donald Segal,[3] and Heman Chao[1,3,]

[1]Sensium Technologies Inc. 8308-114 Street, Rm 1034, RTF Bldg, Edmonton, Alberta, T6G 2V2, Canada

[2]Laval University and Centre De Recherche, L'Hôtel-Dieu de Québec, 9 McMahon, Québec, QC, G1R 2J6, Canada

[3]Helix BioPharma Corp., 3-305 Industrial Parkway South, Aurora, Ontario, L4G 6X7, Canada

Correspondence to: Heman Chao, Helix BioPharma Corp., 3-305 Industrial Parkway South, Aurora, Ontario, L4G 6X7, Canada
Telephone: +1 (905) 841-2300. Fax: +1 (905) 841-2244. Email: hchao@helixbiopharma.com

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Jack bean urease catalyzes the decomposition of urea into ammonia, which in turn increases the pH of the surrounding medium. Based on these two properties, we have investigated the antitumor effects of urease *in vitro* and *in vivo* on human lung and breast cancer cell lines either by the enzyme itself or in combination with other chemotherapeutic drugs. First, through the generation of toxic ammonia, urease exerted direct cytotoxicity on A549 and MDA-MB-231 tumor cells with LC50 of 0.22 and 0.45 U/ml, respectively. The cytotoxic effects could effectively be blocked using the reversible urease inhibitor acetohydroxamic acid. Complete protection was observed at dose ≥ 2 mM. In addition, nude mouse xenograft models demonstrated that intratumoral urease injections (1 - 10 U/dose) inhibited A549 and MCF-7 tumor growth *in vivo*. Second, when combined with weak-base anticancer drugs, urease provided indirect antitumor effects via pH augmentation. Alkalinization of extracellular pH by urease (2 U/ml) and urea (≥ 2 mM) was found to enhance the antitumor efficacy of doxorubicin (50 μM) and vinblastine (100 μM) significantly.

Key words: urease, ammonia, cytotoxicity, mouse xenograft, weak-base chemotherapeutics

INTRODUCTION

It has been observed that solid tumors produce a local microenvironment that is more acidic than normal tissues (1,2). Although intracellular pH (pHi) is normal to slightly alkaline, the extracellular pH (pHe) can be as low as 6.2 (3). This phenomenon may impact the efficacy of small molecule chemotherapeutics by altering their partitioning coefficient between the extracellular and intracellular compartments due to charge state alterations in the drug (4,5). It has been demonstrated that the efficacy of certain weak-base drugs such as mitoxantrone, daunorubicin and doxorubicin is enhanced by raising pHe, while the efficacy of weakly acidic drugs such as 5-fluorouracil, chlorambucil and cyclophosphamide is enhanced by lowering the pHe (5). Treatment regimens have been designed to modulate pHe by bicarbonate infusion to raise pHe or the converse where a hyperglycemic state is induced so as to exacerbate the low local pHe (6,7). Another method to obtain a local pH increase is through the action of an enzyme, specifically urease. Ureases are urea degrading enzymes which occur in a range of organisms from bacteria to plants converting urea into the more toxic ammonia at very high conversion rates > 10^{14} and lead to a local rise in pH from the generated ammonia (8-10). In this study we

use the urease from jack beans (*Canavalia ensiformis)* to obtain a two pronged approach to cell killing. The pH effect will enhance uptake of chemotherapeutic drugs while the ammonia produced is in itself a cytotoxic compound (10,11). The toxicity of ammonia likely arises from its ability to interfere with metabolic enzymes as well as competing with other cations for membrane ion channels (11). These effects may be more pronounced in cancer cells and other rapidly growing cells due to the high levels of glutaminolysis which occurs for energy production and to produce building blocks for purine synthesis (11,12).

MATERIALS AND METHODS

Materials

Urease from *Canavalia ensiformis* (jack beans) was obtained from BioVectra Ltd. (PEI, Canada) and further purified by acid precipitation and ion exchange chromatography to remove the two major contaminants, canavalin and concanavalin A. The purity of the enzyme was > 96% as determined by SDS polyacrylamide gel electrophoresis, HPLC and mass spectrometry. One unit of urease is defined as the production of 1 µmole of ammonia per minute at 25°C and pH 7.6.

Urea, trypsin, phenazine ethosulfate (PES), sodium nitroprusside, sodium hypochlorite solution, phenol, acetohydroxamic acid (AHA), doxorubicin hydrochloride and vinblastine were purchased from Sigma Chemical Co. (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corp. (Madison, WI). Cell culture medium, serum, and antibiotics were obtained from Invitrogen Life Technologies (ON, Canada). Female athymic nude mice were supplied by Charles River Laboratories (Wilmington, MA). Buffers used in the experiments: Dulbecco's phosphate-buffered saline (D-PBS) and modified Krebs Ringer buffer (KRB) containing NaCl (98.3 mM), KCl (4.73 mM), KH_2PO_4 (1.19 mM), $MgSO_4$ (1.19 mM), $NaHCO_3$ (3.57 mM), D-Glucose (11.7 mM), Na_2HPO_4 (11.1 mM) and NaH_2PO_4 (2.77 mM) at pH 7.2 or 6.8.

Cell culture and viability assay

Human breast cancer cell lines (MDA-MB-231 and MCF-7) and lung cancer cell line (A549) were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM/F12 (MDA-MB-231 and A549) or MEM (MCF-7) containing 10% fetal bovine serum and 50 U/ml penicillin and 50 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2.

Colorimetric MTS assay was employed to determine cell viability. MTS and PES were prepared as 2 and 1 mg/ml stock solutions, respectively, in D-PBS. After the cells were treated with test articles, the medium in the plate was replaced with 100 µl/well of plain medium, followed by the addition of 20 µl/well of MTS mix (MTS:PES at 20:1, vol/vol). The plate was incubated at 37°C for 1-2 hours in a humidified, 5% CO_2 atmosphere. The absorbance of soluble formazan produced by cellular reduction of MTS was measured at 490 nm with reference at 630 nm using an ELx808IU Microplate Reader (Bio-tek Instruments Inc., Winooski, VT).

In vitro cytotoxicity of urease

A549 cells ($1.0x10^5$ cells/ml) or MDA-MB-231 cells ($1.5x10^5$ cells/ml) were seeded into 96-well tissue culture plates (Becton Dickinson Labware, NJ) by transferring an aliquot of 100 µl of the cell suspension to each well. The plate was incubated at 37˚C overnight. After incubation, the medium in the wells was removed using a multichannel pipette. Urease and urea at various dilutions were prepared in pre-warmed KRB, pH 7.2 and 50 µl of each was added to corresponding wells. After incubation for two hours at 37˚C, buffer from each replicate was pooled and subjected to pH measurement and total ammonium determination. MTS cell viability assay was then performed on the plate.

The total ammonium present in the incubation buffer was determined by Berthelot's Indophenol reaction (13). In brief, fresh phenol solution (Solution A) was prepared by dissolving 165 mg of phenol and 132 mg of NaOH pellets in 10 ml of water, and then 66 µl of sodium nitroprusside solution (10 mg/ml) was added. Fresh Solution B was prepared by adding 80 µl of sodium hypochlorite solution (10-13% chlorine) to 10 ml of water. Urease activity in the pooled sample was quenched by adding 50 µl of 1 N HCl to 100 µl of sample. The acidified samples were then diluted 200 times and transferred to a 96-well microplate at 100 µl/well, followed by 50 µl/well of Solution A and Solution B. After incubation at 37°C for 15 min, the plate was read at 630 nm using the microplate reader. The amount of ammonium ions present in the sample was determined from the NH_4Cl standard curve.

Combined drug assays

A549 and MDA-MB-231 plates were prepared as described above. Urease, urea and the two weak-base anticancer drugs, doxorubicin and vinblastine, were prepared in pre-warmed KRB, pH 6.8. After the medium in the plate was removed, 50 µl of urease, urea

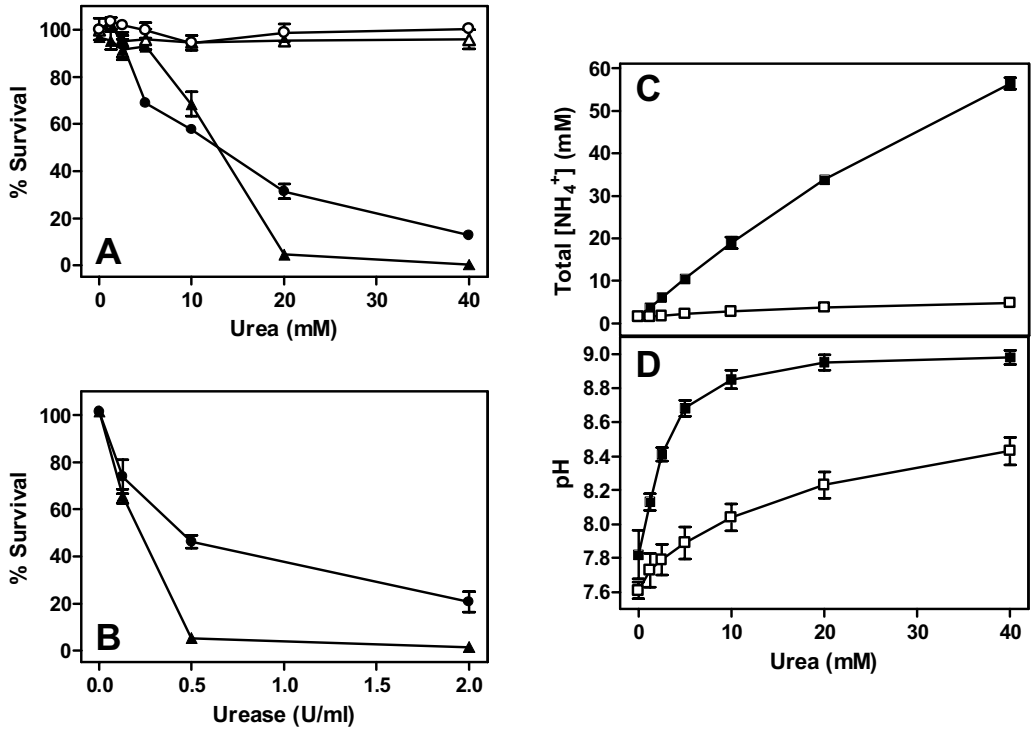

Figure 1. Urease induced cytotoxicity on A549 and MDA-MB-231 cells and augmented the pH and ammonium content of the culture medium. *A*, A549 (▲) and MDA-MB-231 (●) cells were treated with 2 U/ml of urease in the presence of 0 – 40 mM of urea and incubated at 37°C for 2 hours. Viability of the treated cells began to drop as the urea level increased. Urea alone has no effects on A549 (Δ) and MDA-MB-231 (○) controls. *B*, dose-response curve of urease on the viability of A549 and MDA-MB-231 cells. The tumor cells were subjected to urease treatment in the presence of 20 mM urea for 2 hours. A549 cells (▲) were more susceptible to urease than MDA-MB-231 cells (●). *C* and *D*, total ammonium and pH measured in pooled incubation buffer collected from *A*. Hydrolysis of urea by urease (■) caused an increase in ammonium content (*C*) and pH (*D*) as compared to the controls (□). Values are means ± S.D. of 4 replicates from 3 experiments.

and drug solutions were added subsequently into corresponding wells. The final concentration of doxorubicin and vinblastine were 50 μM and 100 μM, respectively. After the plates were incubated overnight (~18 hours) at 37°C, MTS cell viability assay was performed and the buffer pH was measured.

In vivo animal studies

A549 xenografts: Female athymic nude mice (7-9 weeks old) were injected subcutaneously in the right lateral thorax with 5 x 10^6 human A549 lung cancer cells. When tumors reached 100-200 mg, the tumor-bearing animals were randomly selected and sorted into four groups. Group 1 contained ten untreated control mice. Group 2 contained ten positive control mice treated with the reference chemotherapeutic agent Cisplatin. Groups 3 and 4 contained 15 mice each that received one of two different concentrations of urease (1 U or 4 U per injection). Each animal received five doses of injection scheduled at 48 hours between treatments (q2dx5). Urease was administered intratumorally, whereas Cisplatin was administered via an intravenous tail vein injection. Twenty-four hours after the fifth urease administration, five mice from each group were sent for necropsy and tumor collection. In addition, five mice from Groups 2-4 were euthanized and tumors were collected, dissociated, and cultured for viability testing. The remaining five animals from each group continued on study until study termination. Tumor size was recorded using calibrated hand-held Vernier calipers. Throughout the study, the length (L) and width (W) of any tumors that developed were measured in millimeters. The tumor weight in mg was calculated using the formula: (L x W^2)/2. Individual animal weights were taken twice weekly. The experimental protocol was covered under Charles River Laboratories Institutional Animal Care and Use Committee (IACUC).

MCF-7 xenograft: Female athymic nude mice were

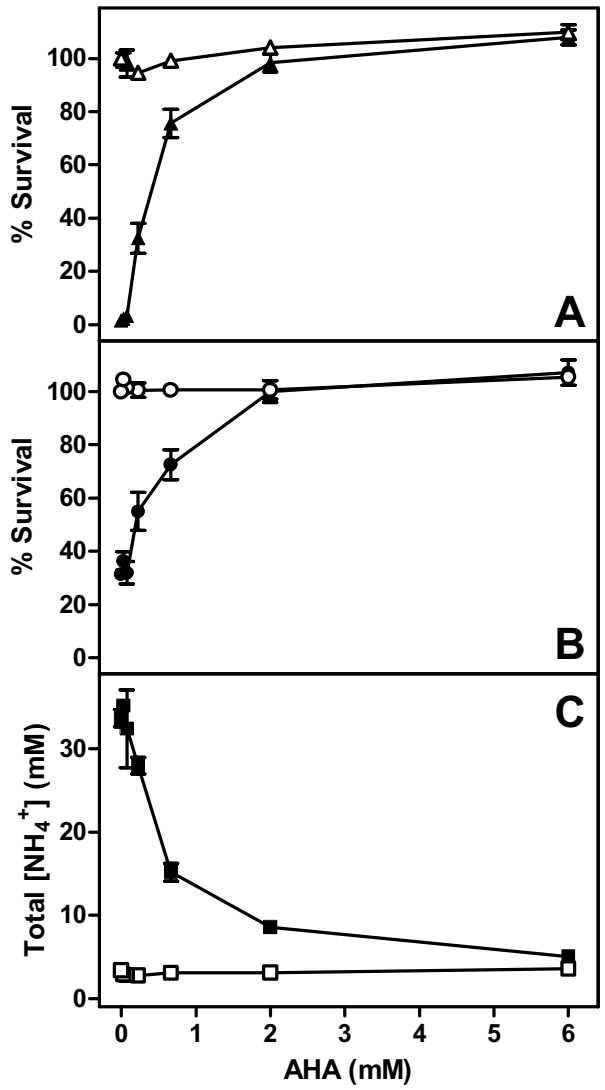

Figure 2. Protective effects of acetohydroxamic acid on urease cytotoxicity. *A*, A549 cells (▲) and *B*, MDA-MB-231 cells (●) treated with 2 U/ml of urease and 20 mM of urea were protected from the cytotoxic effects by addition of acetohydroxamic acid to the incubation buffer. AHA alone at concentrations up to 6 mM was not toxic to both cell lines (no urease controls: Δ, A549; ❍, MDA-MB-231). Complete protection was observed at dose ≥ 2 mM. *C*, AHA inhibited ammonium production by urease (■), which corresponds to an increase in survival rate of both cell lines as shown in *A* and *B*. Higher amounts of AHA (6 mM) can reduce the ammonium level close to that of the control (□). Values are means ± S.D. of 4 replicates from 3 experiments.

injected subcutaneously with $1.8x10^6$ MCF-7 breast tumor cells. When tumor size reached approximately 9 mg, eight mice were injected intratumorally with two doses of urease (10 U/injection) at 48 hour intervals (q2dx2). Five mice were treated with saline as control. The condition of all animals and tumor size were monitored 24 hours after inoculation and then every other day. Animal care was in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). At the end of the observation period, tumors were excised and prepared for histology analyses.

RESULTS

Cytotoxic effects of urease

Human tumor cell lines A549 and MDA-MB-231 were susceptible to the enzymatic activity of jack bean urease. The survival rate of both cell lines decreased as the cells were treated with 2 U/ml of urease for 2 hours with an increasing amount of urea in the medium (Fig. 1 A). Urea alone (up to 40 mM as tested) was not toxic to the cells during the 2-hour incubation period. In the presence of 2 U/ml of urease, the LC_{50} (concentration required to cause 50% drop in tumor cell viability) of urea was found to be 13 mM for both cell lines (Fig. 1 A). Furthermore, the cytotoxic effects were also dependent on the availability of urease. With fixed urea concentration at 20 mM in the medium, the LC_{50} of urease were found to be 0.22 and 0.45 U/ml for A549 and MDA-MB-231 cells, respectively (Fig. 1 B).

The antitumor effects of urease were exerted through the hydrolysis of urea into ammonia (Fig. 1 C) with a corresponding elevation of pH (Fig. 1 D) due to protonation of ammonia in aqueous medium. Figures 1 C and D were the total ammonium and pH measured in the incubation buffer collected from experiments of Figure 1 A. It was found that urease cytotoxicity (Fig. 1 A) correlated well with a corresponding increase of ammonium content and pH in the incubation buffer (Fig. 1 C and D). The rise in pH of the control as shown in Fig. 1 D was probably due to autolysis of urea in aqueous medium resulting in the generation of a total of 3 to 5 mM of ammonium. However, separate experiments showed that pH alone was insufficient to cause any significant cytotoxicity on the two cell lines during a two-hour incubation window (data not shown). Therefore, it was the level of ammonium, or more specifically, availability of free ammonia that mediated the rapid cell killing effects. An augmented pH in turn increases the availability of free ammonia to the cells according to the following equation:

$$pH = pK + \log ([NH_3] / [NH_4^+]),$$
where pK = 9.3 at 37°C (11).

Acetohydroxamic acid is a potent reversible inhibitor of urease and is commercially available for the treatment of chronic urea-splitting urinary infection (14). When AHA was added to the culture buffer

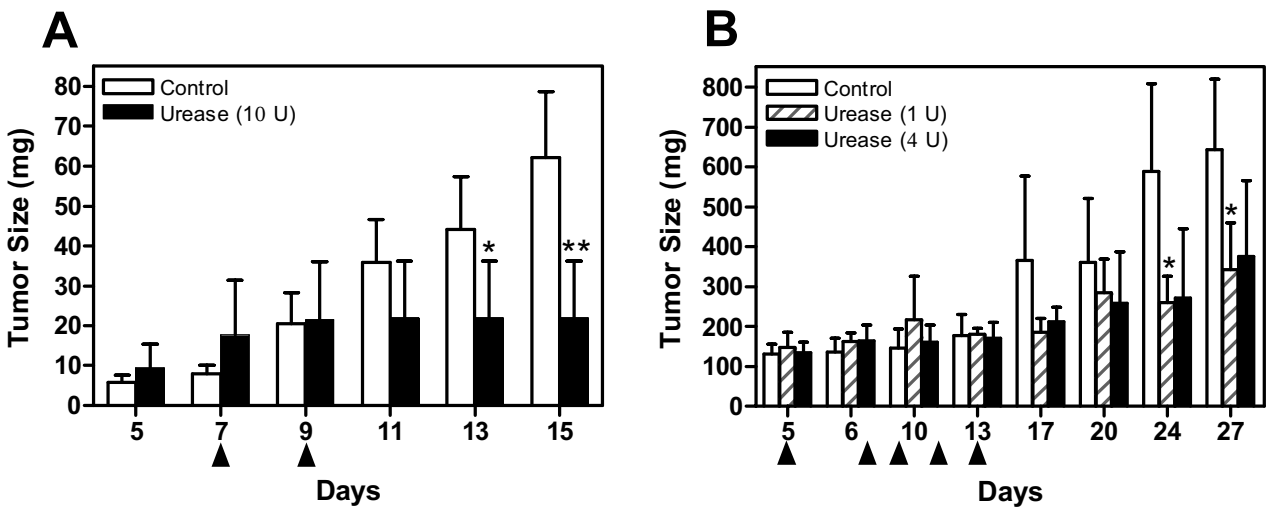

Figure 3. Growth inhibitory effects of urease on MCF-7 and A549 xenografts. *A*, urease inhibits the growth of established MCF-7 xenografts. The breast tumor stopped growing after the second injection of a high-dose urease (10 U/injection, solid bars) on day 9 as compared to the controls (open bars). Time of intratumoral injections are indicated by ▲ below the x-axis. *B*, effects of multiple low-dose (1 U/injection, hatched bars) and medium-dose (4 U/injection, solid bars) injections of urease on established A549 xenografts. Intratumoral injections were performed on days 5, 7, 9, 11 and 13 (▲). Delay of tumor growth was observed from day 17 onwards as compared to the controls (open bars). Significance was determined using the two-tailed unpaired Student's *t* test: $^{*}P < 0.05$ and $^{**}P < 0.005$.

containing urease, it effectively inhibited the enzymatic activity of urease (Fig. 2 C) and restored the survival rate of A549 (Fig. 2 A) and MDA-MB-231 (Fig. 2 B) cells to normal level at dose ≥ 2 mM. This result suggested that the cytotoxicity of urease is solely due to its enzymatic activity.

In vivo antitumor effects of urease

Both MCF-7 and A549 xenografts were susceptible to intratumoral injections of urease. Growth of MCF-7 was completely stopped after the second injection of 10 U of urease (Fig. 3 A). Histological analysis of tumors excised from the treated mice showed that the tumor mass was dead and could not re-grow in culture medium.

In the case of A549 xenografts, delay in tumor growth was observed in mice treated with 5 injections of low dose (1 U) or medium dose (4 U) of urease (Fig. 3 B). Growth regressions of the urease-treated groups were similar to that of the positive control group treated with 6 mg/kg Cisplatin (data not shown). Tumor tissue slices showed significant necrotic area compared to the control. However, cell viability was not affected (> 82%) in dissociated tumors taken from mice treated with urease. There were no significant differences in body weight of the treatment and control groups in both xenograft studies.

Combined drugs studies

Krebs Ringer buffer at pH 6.8 was used to mimic the acidic extracellular environment of solid tumor. At this incubation condition, the raise in pH caused by the urease activity was lower than what was observed in Figure 1 D. However, the pH increase was sufficient to enhance the antitumor efficacy of the two weak-base anticancer drugs as tested. At low urea concentration (2 mM), urease significantly enhanced the antitumor activity of doxorubicin on A549 (Fig. 4 A) and MDA-MB-231 cells (Fig. 4 B), as well as that of vinblastine on MDA-MB-231 cells (Fig. 4 B). When concentration of urea was increased to 8 mM, urease enhanced the activity of both drugs on both cell lines (Fig. 4).

DISCUSSION

Ammonia production and pH elevation are two important characteristics of urease. In this study, we investigated the use of these two properties as means of cancer therapeutics. From our *in vitro* and *in vivo* studies, it was shown that sufficient amounts of ammonia produced by urease could mediate cell death on both human lung and breast cell lines (Fig. 1 and 3). In the unprotonated form, ammonia can freely diffuse through the cell membrane and incurs toxicity to mammalian

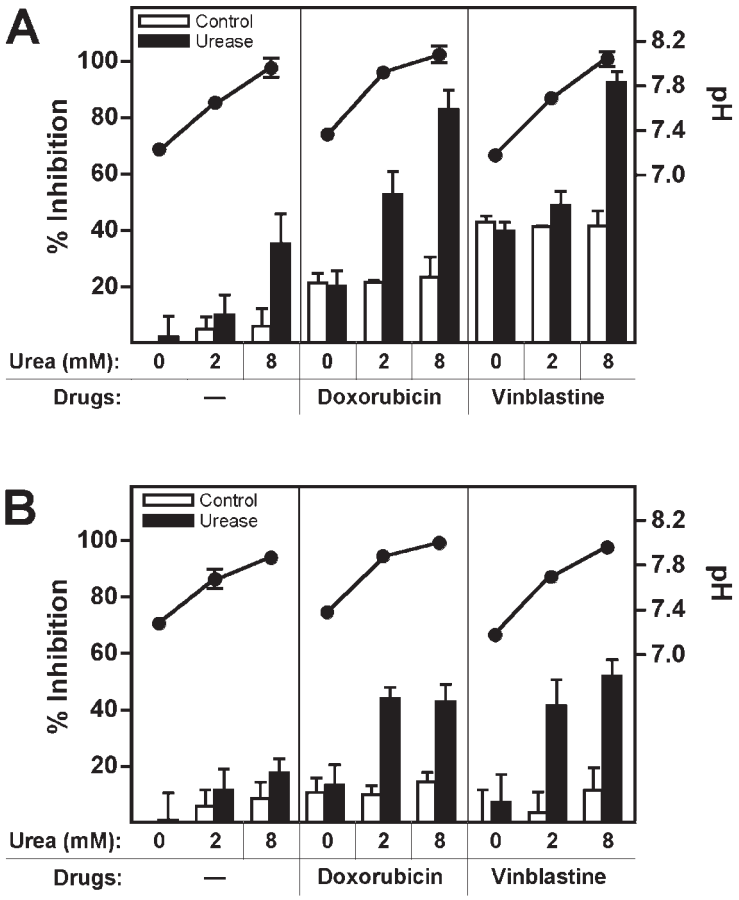

Figure 4. Effects of urease on the cytotoxicity of two weakly basic anticancer drugs, doxorubicin and vinblastine. *A*, lung tumor A549 and *B*, breast tumor MDA-MB-231 were treated with 2 U/ml of urease, 0 – 8 mM of urea and either 50 μM of doxorubicin or 100 μM of vinblastine at pH 6.8 overnight. The antitumor efficacies of the two chemotherapeutic agents were enhanced at the presence of urease (solid bars) and urea as compared to the control (open bars). The solid circle (●) indicates the pH of urease-treated group measured after treatment. Values are means ± S.D. of 4 replicates from 3 experiments.

cells. Once protonated, the ammonium ion cannot diffuse through the membrane but can only be transported by membrane pumps (11). At physiological pH, however, only 1% of ammonia is in the unprotonated form. It has been reported that ammonia inhibited cell growth (11,10) and induced S-phase arrest in gastric cell lines (15). The antitumor activity of urease depends directly on the availability of both the enzyme and urea, where the normal blood urea nitrogen level is in the range of 2.5 – 7.1 mM. Results from Figure 1 showed that short term *in vitro* urease treatment required much higher amount of urea than that of longer term *in vivo* treatment (Fig. 3). Multiple intratumoral injections of urease together with a constant supply of urea *in vivo* provided a steady source of ammonia to act on the cancer xenografts. This explains why long term *in vivo* urease treatment has a much lower urea requirement than that of the *in vitro* ones.

In 1972, Zimber and Visek (16) reported that urease injections caused suppression of Ehrlich ascites tumor growth in mice. They concluded that the antitumor activity was due to toxic ammonia released from endogenous urea by the enzyme since significant reduction in body weight of treated animals was also observed (16). However, in our present xenograft studies, no significant differences in body weight were found. The discrepancy may be mainly due to the way urease was prepared and a different type of mice were used. Although Zimber and Visek used crystalline jack bean urease in their studies, we found that significant amounts of canavalin and concanavalin A contaminants were still present in a commercial source of crystalline urease as detected by HPLC and mass spectrometry (data not shown). These contaminants were able to increase *in vitro* cytotoxicity of the enzyme (unreported

observation). Ideally, host toxicity caused by urease overdosage and subsequent ammonia intoxication can be protected through the use of AHA, especially when the enzyme is applied systemically or in proximity to organs that are susceptible to ammonia. In addition, AHA also provides a fine tuning mechanism that can be used to adjust the effective dosage of urease *in vivo*.

It is a well known fact that solid tumors have an acidic extracellular pH and a neutral-to-alkaline intracellular pH (2,1). This pH gradient across the tumor cell membrane creates a barrier that prevents weak-base chemotherapeutics from acting effectively against tumors (5). Alkalinization of tumors has been demonstrated as a possible remedy to enhance the uptake (6,17) and thus the efficacy of weak-base chemotherapeutics (2,6). Results from our *in vitro* studies showed that urease provides an effective way of tumor alkalinization (Fig. 1 D), which enhances the antitumor efficacy of weak-base anticancer drugs such as doxorubicin and vinblastine on both lung and breast cancer cells (Fig. 4). The combined treatment worked effectively at the lower range of physiological urea level (2 mM), suggesting the feasibility of applying the combined drug treatment *in vivo*. Future works are designed to attach a targeting moiety such as specific anti-tumor antibody to urease so as to increase the specificity of the reagent.

CONCLUSION

In summary, we present here a *de novo* approach of applying urease as a therapeutic to cancer. The enzyme can be used alone through the direct cytotoxicity of ammonia or in combination with other weakly basic chemotherapeutic agents whose efficacies are hindered by a higher intracellular/lower extracellular pH gradient in solid tumors.

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