Cathepsin B Cleavable Novel Prodrug Ac-Phe-Lys-PABC-ADM Enhances Efficacy at Reduced Toxicity in Treating Gastric Cancer Peritoneal Carcinomatosis

An Experimental Study

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BACKGROUND: Doxorubicin (Adriamycin) is effective in gastric cancer treatment, but with severe dose-dependent toxicities. A novel prodrug of doxorubicin (Ac-Phe-Lys-PABC-ADM) is designed to deliver free doxorubicin relying on cathepsin B and reduce side effects. The authors examined the antitumor effect and toxicities of Ac-Phe-Lys-PABC-ADM against gastric cancer peritoneal carcinomatosis. METHODS: SGC-7901 gastric cancer cell line was used for the study. The in vitro study investigated the effects of doxorubicin and Ac-Phe-Lys-PABC-ADM on cell growth dynamics and cell cycle. The in vivo study investigated the efficacy and toxicity of Ac-Phe-Lys-PABC-ADM on a nude mice model of peritoneal carcinomatosis, with doxorubicin as positive control. RESULTS: In the in vitro study, Ac-Phe-Lys-PABC-ADM had a lower dose-dependent inhibitory effect on SGC-7901 cells. In the in vivo study of control, doxorubicin, and Ac-Phe-Lys-PABC-ADM groups, the median experimental peritoneal carcinomatosis indexes were 6, 1.5, and 1, respectively (P = .004); the body weights were 24.32 ± 1.40 g, 18.40 ± 2.97 g, and 23.61 ± 0.80 g, respectively (P = .000). Biochemical studies showed that Ac-Phe-Lys-PABC-ADM had significantly lower toxicities on the bone marrow, liver, kidney, and particularly heart. Histopathological studies of the control, doxorubicin, and Ac-Phe-Lys-PABC-ADM groups found significant myocardium toxicities in 3, 7, and 4 animals, respectively. CONCLUSIONS: Ac-Phe-Lys-PABC-ADM could be an effective molecular targeting drug to treat gastric cancer peritoneal carcinomatosis with enhanced efficacy and reduced toxicity. Cancer 2011;000:000–000. © 2011 American Cancer Society.

KEYWORDS: cathepsin B, gastric cancer, peritoneal carcinomatosis, Doxorubicin, prodrug.

INTRODUCTION

Gastric cancer (GC) is among the most common malignancies in developing countries, where it ranks second in terms of incidence rate and third in terms of mortality rate among the male population, and ranks fourth in terms of both incidence rate and mortality rate among the female population, according to the most recent global statistics.1 GC is also the third leading cause of cancer mortality in China,2 where >80% of GC has already become clinically advanced by the time of surgical exploration, so that curative treatment is no longer possible.3 It has long been recognized that GC tends to have lymphatic, hematogenous, or intra-abdominal metastasis because of its pathophysiological heterogeneity. To some extent, extended lymphadenectomy, regional radiotherapy, and adjuvant antitumor chemotherapy could prolong overall survival. This has been proven by some large-scale international studies, including the INT-0116 trial, the MAGIC trial, and the

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However, these studies also show that regardless of whether patients receive surgery alone or surgery combined with perioperative or postoperative chemoradiotherapy, locoregional recurrence, especially abdominal metastasis, is the most common pattern of cancer recurrence.

GC is prone to intra-abdominal metastasis, mainly because of free cancer cells in the peritoneal cavity. Free GC cells in peritoneal lavage fluid are detected in up to 24% of stage IB and 40% of stage II or III GC patients. Peritoneum seeded with free cancer cells forming peritoneal carcinomatosis is a characteristic feature of GC spread. As a result, >30% of advanced GC patients have developed peritoneal carcinomatosis when diagnosed, and 60% of all GC patients die of peritoneal carcinomatosis.

During the development of peritoneal carcinomatosis, GC cells secrete enzymes to facilitate cancer cells seeding and colonization on the peritoneum. Cathepsin B is among the key enzymes in this critical process, overexpressed in GC as well as other cancers, and actively involved in cancer invasion. Conversely, its expression is extremely low in normal cells, and it is inactive or loses activity as soon as it is dispersed in aqueous media away from cells. Thus, cathepsin B has long been considered a candidate target in cancer therapy.

It was established in the MAGIC trial that the anthracycline-contained regimen is a useful chemotherapy for GC. Doxorubicin (Adriamycin) is a typical representative of anthracyclines. Although doxorubicin is an important drug in chemotherapy, its toxicities are also well known, such as cardiac toxicities and bone marrow suppression. To retain the therapeutic effect while reducing the side effects, we designed a smart prodrug of doxorubicin, Ac-Phe-Lys-PABC-ADM (Fig. 1A). In this modified doxorubicin, Phe-Lys is a dipeptide specific for cathepsin B, and PABC () is a self-immolative spacer. The prodrug is inactive when there is little cathepsin B activity, such as in normal tissues and peripheral blood, thus avoiding the side effects on normal tissue. During cancer invasion, activated cathepsin B is overexpressed on the exterior membrane of the invading cancer cells, which cleaves the Phe-Lys dipeptide at the Lys-PABC bond. Then the exposed PABC spacer can self-hydrolyze upon deacylation, and free doxorubicin molecules are released, resulting in direct killing of the invading cancer cells.

The in vitro release study of Ac-Phe-Lys-PABC-ADM showed that the half-life of doxorubicin release at 37°C was 16 minutes in cathepsin B solution, but no

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**Figure 1.** Ac-Phe-Lys-PABC-ADM structure and its in vitro effects on SGC-7901 cells are shown. (A) Chemical structure of Ac-Phe-Lys-PABC-ADM hydrochloride is shown. The molecular formula is C52H59N5O16HCl. The red bars divide the structure into 5 parts, Ac (acetyl), Phe (phenylalanyl), Lys (lysyl), PABC (para-aminobenzyloxycarbonyl), and ADM (doxorubicin), respectively. In cathepsin B-free medium, the whole molecule remains intact and stable. In cathepsin B-rich environment, the Phe-Lys dipeptide is cleaved by cathepsin B, exposing PABC spacer, which is hydrolyzed automatically, ultimately releasing free doxorubicin. (B, C) In vitro effects of Ac-Phe-Lys-PABC-ADM and doxorubicin on SGC-7901 gastric cancer cells are shown. Ac-Phe-Lys-PABC-ADM had a dose-dependent inhibitory effect on cell growth, but it did not significantly affect the cell cycle. D, day; PADM, Ac-Phe-Lys-PABC-ADM.
Changes were observed over 6 to 7 hours in human plasma. However, the prodrug has not yet been evaluated in a real life cancer model. This in vitro and in vivo study was to evaluate this prodrug for targeted treatment of peritoneal carcinomatosis from GC.

MATERIALS AND METHODS

Agents and Cells
Ac-Phe-Lys-PABC-ADM was synthesized (by Y.-P.H.) according to the previously reported chemical process. The molecular weight of Ac-Phe-Lys-PABC-ADM hydrochloride is 1045.50. In terms of equivalent mole content, 1.8 mg Ac-Phe-Lys-PABC-ADM hydrochloride is equivalent to 1 mg doxorubicin hydrochloride (molecular weight, 579.99). Other agents were obtained commercially, including Doxorubicin Hydrochloride for Injection (Pharmacia, Milan, Italy), RPMI-1640 medium (HyClone, Logan, Utah) and standard newborn bovine serum (Zhengzhou Ben BioTech, Zhengzhou, China) for cell culture, propidium iodine (PI) agents kit (Beckman Coulter, Fullerton, Calif) for flow cytometric analysis, rabbit anticathepsin B polyclonal antibody (Lot No.3190-100; BioVision, Mountain View, Calif), and peroxidase-conjugated AffiniPure goat antirabbit IgG (H + L) (Lot No.88,813; Jackson ImmunoResearch, West Grove, Pa) for immunohistochemical study. The human gastric adenocarcinoma cell line SGC-7901 was cultured in RPMI-1640 medium supplemented with 10% standard newborn bovine serum in the 5% CO₂, saturated humidity, 37°C incubator (Shel Lab, Cornelius, Ore).

Animals
Male BALB/c nude mice, 5 to 6 weeks old, were from Beijing HFK Bio-Technology (Beijing, China; animal quality certificate No. SCXK[Jing] 2009-0004) and maintained in an Animal Biosafety Level 3 Laboratory at the Animal Experimental Center of Wuhan University. After 3 days of adaptation, the animals were used for in vivo study, and the protocols were approved by the animal care committee of Wuhan University.

In Vitro Cell Growth and Cell Cycle Study
SGC-7901 cells at exponential growth phase were plated in 24-well culture plates (Corning Inc., Corning, NY) at a density of 1 × 10⁵/well. After 48 hours, cell numbers were determined by direct cell counting of 3 wells, which was designated as cell number of day 1. Then the cells were divided into 6 groups, treated with normal saline (100 μL), doxorubicin (0.5 μg/mL), doxorubicin (1.0 μg/mL), Ac-Phe-Lys-PABC-ADM (0.9 μg/mL), Ac-Phe-Lys-PABC-ADM (1.8 μg/mL), and Ac-Phe-Lys-PABC-ADM (3.6 μg/mL), respectively, at 3 wells per group. Cells in each group were harvested and counted daily from day 2 to day 7. The cell growth curve was plotted.

For cell cycle study, SGC-7901 cells at exponential growth phase were treated for 24 hours with normal saline (25 μL), doxorubicin (0.25 μg/mL), and Ac-Phe-Lys-PABC-ADM (0.45 μg/mL). The cells were treated after a standard protocol and stained with PI kit. Cell cycle analysis was performed by flow cytometry with FC 500 (Beckman Coulter).

In Vivo Pilot Dosage Study
Because this was the first animal study, it was necessary to establish a workable dose range. Therefore, we first performed a pilot dosage study on 4 nude mice, which were divided into groups A (n = 2) and B (n = 2). The reported median lethal dose (LD₅₀) of doxorubicin was 13.2 mg/kg when given intraperitoneally (i.p.)²¹ and 12.0 mg/kg when given intravenously for mice.²² Therefore, we performed this tentative study based on these dosages. For group A, we used 24.0 mg/kg of Ac-Phe-Lys-PABC-ADM, which is equivalent to the LD₅₀ of doxorubicin for i.p. injection. The dosage of group B (36.0 mg/kg) was 1.5 times higher than that of group A. The mice were observed daily, and body weight was recorded every 3 days. If the status of mice was stable in 7 days, additional administrations were given. After 4 consecutive administrations, the mice showed signs of toxicity. They were sacrificed, and the heart, liver, and kidney were obtained for histopathology study. The blood was used for biochemical studies, including cardiac, hepatic, and renal functions.

In Vivo Efficacy Study
SGC-7901 cells (5 × 10⁶/0.2 mL per mouse) were injected i.p. into 29 nude mice on day 0. On day 8, the mice were randomized into 3 groups: control group (normal saline 10 mL/kg, i.p., n = 9), doxorubicin group (doxorubicin 2.0 mg/kg, i.p., n = 10), and Ac-Phe-Lys-PABC-ADM group (Ac-Phe-Lys-PABC-ADM 7.2 mg/kg, i.p., n = 10). Treatment was conducted on days 8, 12, 16, 20, 24, 28, 32, and 36, respectively. The total dosages were based on a previous report²³ and the above pilot dosage study. The mice were observed daily, and body weight was recorded every 4 days. On day 40, all animals were euthanized, and autopsy was conducted. An experimental peritoneal carcinomatosis index system was developed to...
evaluate the efficacy that took into consideration tumor nodule sizes, distributions, and the characteristics of ascites. In this system, the abdominal cavity of the mouse was divided into 4 regions: region I, subdiaphragm; region II, the liver, spleen, stomach, and affiliated ligaments; region III, small intestine, colon, mesenterium, and abdominal wall; and region IV, pelvic cavity, urogenital system, and rectum. The detailed scoring criteria were modified from a similar reporting system on a rat peritoneal carcinomatosis model24 and set as follows: score 0, no tumor nodules throughout the region; score 1, nodule size ≤2 mm in greatest diameter; score 2, nodule size >2 mm and up to 5 mm; score 3, nodule size >5 mm. If bloody ascites occurred, it was set as score 1. The sum of all the scores was the experimental peritoneal carcinomatosis index of the animal (ranging from 0 to 13).

Toxicities Study
Potential toxicities to major organ systems were evaluated. On days 16, 24, and 32 of the efficacy study, 80 μL of blood was obtained from tail veins for routine hematological study by Sysmex KX-21 automated hematology analyzer (Sysmex, Kobe, Japan). On day 40, blood was collected for biochemical study, including alanine aminotransferase (ALT), aspartate aminotransferase, blood urea nitrogen, creatinine, creatine kinase, creatine kinase-MB, and lactate dehydrogenase by Aeroset Clinical Chemistry Analyzer (Abbott Laboratories, Abbott Park, Ill). At autopsy, major organs including the heart, liver, kidneys, spleen, and lungs were examined for any toxic changes. Any organs involved by the tumor and the tumor nodules were formalin fixed, paraffin embedded, and cut at 5 μm thickness for histopathological study after hematoxylin and eosin staining.

Immunohistochemical Study
To determine the cathepsin B level in this tumor model, we performed immunohistochemical studies on tumor tissue from control mice, after the detailed procedure developed in our group.25

Statistical Analysis
The data were analyzed on SPSS software version 13.0 (SPSS Inc., Chicago, Ill). The differences in body weight and blood routine among different groups were tested using analysis of variance at each time point, and the
differences between every 2 groups were analyzed using the least significant difference test. Because of the small sample size, the experimental peritoneal carcinomatosis index and blood biochemistry analysis could not fit a normal distribution of continuous data; they were given as median and range. Therefore, the 2-sided nonparametric Kruskal-Wallis H test was used to analyze the differences among the 3 groups, and Mann-Whitney U test was used to analyze the difference between every 2 groups.26

P < .05 was considered to be statistically significant.

Figure 3. The impact of Ac-Phe-Lys-PABC-ADM and doxorubicin on the general status of nude mice is shown. (A) Nude mice in the Ac-Phe-Lys-PABC-ADM group had similar body weight to those of the control group throughout the whole study period. In comparison, nude mice in the doxorubicin group showed progressive decreases in body weight after 4 doses of intraperitoneally doxorubicin delivery. (B) Animal status at the study endpoint is shown. Note 1 nude mouse in the doxorubicin group died on day 36 because of severe toxicity. *P < .05. ADM, doxorubicin; PADM, Ac-Phe-Lys-PABC-ADM.
RESULTS

Ac-Phe-Lys-PABC-ADM Showed Lower Inhibition on Cell Growth and Cell Cycle In Vitro

As shown in Figure 1B, both doxorubicin and Ac-Phe-Lys-PABC-ADM could inhibit cell growth in a dose-dependent fashion, compared with control. At the endpoint (day 7), the inhibition rates of doxorubicin (0.5 μg/mL), doxorubicin (1.0 μg/mL), Ac-Phe-Lys-PABC-ADM (0.9 μg/mL), Ac-Phe-Lys-PABC-ADM (1.8 μg/mL), and Ac-Phe-Lys-PABC-ADM (3.6 μg/mL) were 80.2%, 96.3%, 38.0%, 33.0%, and 62.3%, respectively. Figure 1C shows that doxorubicin had a significant effect on the cell cycle. In doxorubicin-treated cells, a prominent apoptosis peak was observed. In comparison, Ac-Phe-Lys-PABC-ADM-treated cells did not show significant apoptosis, and the DNA content distribution was similar to that of the control group.

Ac-Phe-Lys-PABC-ADM Had Higher Maximum Tolerated Dose

In the pilot dosage study, the actual total dosage delivered was 96.0 mg/kg for group A and 144.0 mg/kg for group B. There were no obvious changes in body weight among all the 4 animals for the first 3 injections. After the fourth administration (36.0 mg/kg for group A and 54.0 mg/kg for group B), however, persistent body weight decreases were observed. On the basis of these results, we set the ceiling dosage of <96.0 mg/kg for the formal in vivo test. No obvious damages were observed according to histopathological study of major organs. The maximum tolerated dose (MTD) of Ac-Phe-Lys-PABC-ADM is >4× higher than that of doxorubicin.

Ac-Phe-Lys-PABC-ADM Retained a Better Antitumor Effect Than Doxorubicin

In the full-scale study, the actual total dosage of Ac-Phe-Lys-PABC-ADM could inhibit cell growth in a dose-dependent fashion, compared with control. At the endpoint (day 7), the inhibition rates of doxorubicin (0.5 μg/mL), doxorubicin (1.0 μg/mL), Ac-Phe-Lys-PABC-ADM (0.9 μg/mL), Ac-Phe-Lys-PABC-ADM (1.8 μg/mL), and Ac-Phe-Lys-PABC-ADM (3.6 μg/mL) were 80.2%, 96.3%, 38.0%, 33.0%, and 62.3%, respectively. Figure 1C shows that doxorubicin had a significant effect on the cell cycle. In doxorubicin-treated cells, a prominent apoptosis peak was observed. In comparison, Ac-Phe-Lys-PABC-ADM-treated cells did not show significant apoptosis, and the DNA content distribution was similar to that of the control group.

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As shown in Figure 1B, both doxorubicin and Ac-Phe-Lys-PABC-ADM could inhibit cell growth in a dose-dependent fashion, compared with control. At the endpoint (day 7), the inhibition rates of doxorubicin (0.5 μg/mL), doxorubicin (1.0 μg/mL), Ac-Phe-Lys-PABC-ADM (0.9 μg/mL), Ac-Phe-Lys-PABC-ADM (1.8 μg/mL), and Ac-Phe-Lys-PABC-ADM (3.6 μg/mL) were 80.2%, 96.3%, 38.0%, 33.0%, and 62.3%, respectively. Figure 1C shows that doxorubicin had a significant effect on the cell cycle. In doxorubicin-treated cells, a prominent apoptosis peak was observed. In comparison, Ac-Phe-Lys-PABC-ADM-treated cells did not show significant apoptosis, and the DNA content distribution was similar to that of the control group.

The hematological effects of Ac-Phe-Lys-PABC-ADM were observed. On the basis of these results, we set the ceiling dosage of <96.0 mg/kg for the formal in vivo test. No obvious damages were observed according to histopathological study of major organs. The maximum tolerated dose (MTD) of Ac-Phe-Lys-PABC-ADM is >4× higher than that of doxorubicin.

Ac-Phe-Lys-PABC-ADM Retained a Better Antitumor Effect Than Doxorubicin

In the full-scale study, the actual total dosage of Ac-Phe-Lys-PABC-ADM (57.6 mg/kg) was 2× that of doxorubicin (16 mg/kg) in terms of equal mole content. As shown in Figure 2, both Ac-Phe-Lys-PABC-ADM and doxorubicin reduced the experimental peritoneal carcinomatosis index compared with control. The median (range) experimental peritoneal carcinomatosis index scores were 6 (1-10) for the control group, 1.5 (0-6) for the doxorubicin group, and 1 (1-4) for the Ac-Phe-Lys-PABC-ADM group, respectively (Fig. 2A, B; P = .004; doxorubicin vs control, P = .008; Ac-Phe-Lys-PABC-ADM vs control, P = .003). Lung metastasis was found in 1 nude mouse in the control groups (Fig. 2C), but there was no lung metastasis in any mouse of the Ac-Phe-Lys-PABC-ADM and doxorubicin groups.

Table 1. Effects of ADM and PADM on Peripheral Blood Parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 16</th>
<th>Day 24</th>
<th>Day 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC, T/L</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>9.67 ± 0.25</td>
<td>9.74 ± 0.35</td>
<td>9.78 ± 0.41</td>
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<tr>
<td>ADM</td>
<td>9.34 ± 0.24a</td>
<td>8.89 ± 0.48a</td>
<td>9.06 ± 0.62a</td>
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<tr>
<td>PADM</td>
<td>9.60 ± 0.30b</td>
<td>9.51 ± 0.38b</td>
<td>9.40 ± 0.29</td>
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<tr>
<td>HGB, g/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>160.11 ± 4.43</td>
<td>154.89 ± 5.23</td>
<td>156.11 ± 4.70</td>
</tr>
<tr>
<td>ADM</td>
<td>151.60 ± 6.36a</td>
<td>144.20 ± 8.99a</td>
<td>150.00 ± 8.18a</td>
</tr>
<tr>
<td>PADM</td>
<td>157.80 ± 3.80b</td>
<td>151.80 ± 5.47b</td>
<td>150.00 ± 5.54b</td>
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<tr>
<td>PLT, G/L</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>1192 ± 69</td>
<td>1129 ± 185</td>
<td>1031 ± 322</td>
</tr>
<tr>
<td>ADM</td>
<td>1199 ± 166</td>
<td>1137 ± 263</td>
<td>1063 ± 260</td>
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<tr>
<td>PADM</td>
<td>1168 ± 221</td>
<td>1183 ± 249</td>
<td>948 ± 252</td>
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<tr>
<td>WBC, G/L</td>
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<tr>
<td>Control</td>
<td>8.68 ± 1.08</td>
<td>9.66 ± 2.45</td>
<td>9.72 ± 1.90</td>
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<tr>
<td>ADM</td>
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<td>7.80 ± 1.67a</td>
<td>7.84 ± 1.62a</td>
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<tr>
<td>PADM</td>
<td>8.54 ± 1.33</td>
<td>7.01 ± 1.54a</td>
<td>8.99 ± 1.57</td>
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<tr>
<td>LYM, G/L</td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>5.79 ± 0.82</td>
<td>5.79 ± 1.45</td>
<td>5.86 ± 1.25</td>
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<tr>
<td>ADM</td>
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<td>3.75 ± 0.93a</td>
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<td>NEU, G/L</td>
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<tr>
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<td>3.33 ± 0.97</td>
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<td>2.46 ± 0.36b</td>
<td>3.10 ± 1.04</td>
<td>3.32 ± 0.44</td>
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</table>

Abbreviations: ADM, doxorubicin; HGB, hemoglobin; LYM, lymphocyte; NEU, neutrophil; PADM, Ac-Phe-Lys-PABC-ADM; PLT, platelet; RBC, red blood cell; WBC, white blood cell; T/L, 10^12/L; G/L, 10^9/L; g/L, grams/Liter.
*P < .05, ADM and PADM vs control, at the same time point.
*P < .05, ADM and PADM vs control, at the same time point.
Ac-Phe-Lys-PABC-ADM group and the control group regarding the peripheral blood parameters.

**Ac-Phe-Lys-PABC-ADM Reduced Toxicities to Liver, Kidney, and Particularly Heart**

As shown in Figure 4, both Ac-Phe-Lys-PABC-ADM and doxorubicin had adverse effects on liver and kidney functions. For Ac-Phe-Lys-PABC-ADM, among the 4 major parameters studied, only ALT levels achieved statistical significance between the Ac-Phe-Lys-PABC-ADM and control groups. For doxorubicin, however, 3 of the 4 parameters achieved statistical significance between the doxorubicin and control groups. Histopathological study confirmed that Ac-Phe-Lys-PABC-ADM reduced liver and renal toxicities.

Potential cardiac toxicity was also studied in greater detail, as shown in Figure 5. Histopathological studies found significant myocardium toxicities in 3, 7, and 4 animals in the control, doxorubicin, and Ac-Phe-Lys-PABC-ADM groups, respectively. The Ac-Phe-Lys-PABC-ADM group has significantly lower myocardium toxicities than the doxorubicin group.

**Ac-Phe-Lys-PABC-ADM Works via the Cathepsin B Pathway**

As shown in Figure 6, prominent cathepsin B expression was observed in tumor cells, suggesting that Ac-Phe-Lys-PABC-ADM works via the cathepsin B pathway.

**DISCUSSION**

In the multidisciplinary treatment of GC, chemotherapy plays an important role, because meta-analysis has shown that adjuvant chemotherapy could reduce the risk of death and improve overall survival for GC.27,28 In currently available chemotherapeutic regimens, anthracyclines are important drugs, as is well demonstrated in the MAGIC trial.4,29,30 Anthracyclines cause cell damage by intercalating into DNA, leading to chromatin unfolding and aggregation, which ultimately results in apoptosis.31

However, like many cytotoxic agents, anthracyclines can cause serious organ damage. With doxorubicin, toxicity to the heart and bone marrow are usually dose limiting, with the MTD far below the minimum curative dose. Therefore, strategies to shield the heart and marrow by excluding doxorubicin from them have long been a top
Another strategy has been to target the drug to the tumor by attaching it to some tumor-binding moiety, for example, a tumor-specific monoclonal antibody (MoAb) such as trastuzumab used in chemotherapy for human epidermal growth receptor 2-positive GC. 

Drawbacks to the use of MoAbs have been that 1) the tumor Ag that binds the MoAb is never completely tumor specific, so that some of the drug goes where it does harm; 2) foreign MoAbs are often immunogenic; and 3) MoAb therapy is very expensive.

In the present study, normal organs are protected by masking the cytotoxic drug doxorubicin with a simple dipeptide that renders it nontoxic. At the tumor, the mask is removed by cathepsin B, a ubiquitous proteolytic enzyme that is so destructive to tissue that normally it occurs only within cells, encased in lysosomes. Only tumor cells secrete cathepsin B externally, confined to their plasma membranes, for the purpose of penetrating basement membrane and extracellular barriers as they spread. The prodrug Ac-Phe-Lys-PABC-ADM is rapidly cleaved by cathepsin B at the Lys-PABC bond. The resulting PABC-doxorubicin decomposes at once to \textit{para}-amino-benzyl alcohol, CO\textsubscript{2}, and free doxorubicin. The PABC self-immolating linker \cite{33} is necessary because the cathepsin B’s active site cannot accommodate the bulky doxorubicin molecule, but the smaller PABC fits into the active site.

Free doxorubicin released right on the cancer cells penetrates them readily, killing them. To be sure, a certain portion of the free drug may drift away from the tumor, but the concentration ratio tumor/heart-bone marrow should be much higher than if the doxorubicin is given as the free drug, when the expected ratio is about 1. In this way, even without a positive targeting agent like a MoAb, it is possible to raise the MTD without significantly raising the minimum curative dose. The goal is to raise the MTD above the minimum curative dose, where cures become possible, but even short of that, a rise in the MTD/minimum curative dose ratio will enhance the effect.

This is the first study to systematically evaluate the in vitro and in vivo efficacy and side effects of Ac-Phe-Lys-PABC-ADM. The predominant in vitro finding was that doxorubicin and Ac-Phe-Lys-PABC-ADM had dose-dependent effects on cell growth, and that Ac-Phe-Lys-PABC-ADM could retard cell growth. In terms of pure doxorubicin content, an equal amount of Ac-Phe-Lys-PABC-ADM inhibits cell growth less than doxorubicin itself. This suggests that 1) cells in vitro do not produce
much external cathepsin B, so Ac-Phe-Lys-PABC-ADM is not converted to doxorubicin; and 2) Ac-Phe-Lys-PABC-ADM affects cell metabolism through a mechanism different from that of doxorubicin. Indeed, our flow cytometry study revealed that Ac-Phe-Lys-PABC-ADM mainly blocked the cell cycle at phase S. In comparison, there was a significant apoptosis peak in the doxorubicin-treated cells, suggesting that Ac-Phe-Lys-PABC-ADM inhibits cell growth by a cytostatic mechanism, but doxorubicin by a cytotoxic mechanism.

There are 2 requirements this program must meet: first, the masking group must be somatically stable, to prevent inappropriate release of free doxorubicin in nontumor locales; second, the release of free doxorubicin at the tumor must be fast enough to kill cancer cells. Both these requirements had been met in vitro, but not yet demonstrated in vivo.

We have now found that Ac-Phe-Lys-PABC-ADM is indeed both stable and effective in vivo. Free doxorubicin produced toxicity in the mice, evidenced by weight loss beginning on day 20, which was not seen in either the control or Ac-Phe-Lys-PABC-ADM groups up to day 40. This shows that the amount of free doxorubicin released from Ac-Phe-Lys-PABC-ADM by hydrolysis outside the tumor was small if any. However, although somatically stable, Ac-Phe-Lys-PABC-ADM released free doxorubicin efficiently at the tumor, with antitumor power equivalent to that of free doxorubicin.

All animals in the control group developed clinically significant peritoneal carcinomatosis, with tumor nodules seeding on the abdominal wall, diaphragm, liver capsule, small intestine surface, and mesenterium. Prominent angiogenesis was also observed in the abdominal tumor nodules. All these testify to the success of our model system. To more objectively evaluate efficacy, we established an experimental peritoneal carcinomatosis index system, similar to a clinical peritoneal carcinomatosis index system developed by Portilla et al. Compared with control, Ac-Phe-Lys-PABC-ADM and doxorubicin reduced the experimental peritoneal carcinomatosis index by 83.3% (5 of 6) and 75% (4.5 of 6), respectively. The results also suggest that Ac-Phe-Lys-PABC-ADM may have better antitumor effect than doxorubicin in this model system, where the dosage of Ac-Phe-Lys-PABC-ADM was twice that of doxorubicin.

Also encouraging is the finding that the antitumor efficacy of Ac-Phe-Lys-PABC-ADM was accompanied by significantly better general status, blood profiles, and organ system tolerability. Animals in the Ac-Phe-Lys-PABC-ADM group maintained body weight throughout the study similar to those in the control group (23.61 ± 0.80 g vs 24.32 ± 1.40 g), whereas there was significant body weight reduction in the doxorubicin group beginning at the halfway point (18.40 ± 2.97 g; Fig. 3). In terms of the routine peripheral blood test, both Ac-Phe-Lys-PABC-ADM and doxorubicin had a negative effect on bone marrow function. However, Ac-Phe-Lys-PABC-ADM had a much smaller impact on red blood cells and lymphocytes than doxorubicin (Table 1). Thus, the masking group apparently made Ac-Phe-Lys-PABC-ADM less toxic to the hemopoietic system. Biochemical studies also indicated that Ac-Phe-Lys-PABC-ADM had smaller toxicities to major organs such as the liver, the kidneys, and
particularly the heart. In addition, histopathology showed prominent tissue structure and cell morphology changes, in agreement with these biochemical alterations. All these results indicate that the toxicity of Ac-Phe-Lys-PABC-ADM is much reduced by the Ac-Phe-Lys-PABC masking group.

To test whether Ac-Phe-Lys-PABC-ADM achieved its effects via the cathepsin B pathway, we performed immunohistochemical studies, which clearly showed that the SGC-7901 tumor indeed produced a large amount of cathepsin B. As was previously shown, Ac-Phe-Lys-PABC-ADM was stable in human plasma, but rapidly cleaved in cathepsin B-rich medium. We can conclude with reasonable confidence that in this animal model system, Ac-Phe-Lys-PABC-ADM inhibited peritoneal carcinomatosis development and progression through the cathepsin B mechanism.

Apart from the promising results, this study also has limitations. First, it focused on only a single model system, which did not reveal the entire antitumor spectrum of Ac-Phe-Lys-PABC-ADM. Different model systems of gastric cancer could help evaluate this prodrug more extensively. Other solid tumors with high cathepsin B expression are also potential targets for this prodrug. Second, pharmacological and pharmacodynamic studies have not yet been completely conducted, even in this model system. As this study is an initial experiment primarily focused on in vivo efficacy, we assigned lower priority to pharmacologic and metabolic studies. Although the current in vivo dosage produced good results, the LD50 and optimal dosages are yet to be found. Third, the study suggests that Ac-Phe-Lys-PABC-ADM might have mechanisms of action yet unknown, different from those of doxorubicin.

In conclusion, this study is the first to systematically evaluate Ac-Phe-Lys-PABC-ADM in any animal model (peritoneal carcinomatosis). The results suggest that Ac-Phe-Lys-PABC-ADM is a promising prodrug with better antitumor efficacy and much reduced toxicities.

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**CONFLICT OF INTEREST DISCLOSURES**

The authors made no disclosures.

**REFERENCES**