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13. ABSTRACT (Maximum 200) The goal of this project is to develop Bowman-Birk protease inhibitor (BBI), a soybean polypeptide, as a chemopreventive agent for breast cancer. In order to achieve this goal, we proposed the following two specific aims in our original application: (1) using an <i>in vitro</i> mammary gland culture system to demonstrate the anti-transformation activity of both BBI and its palmitic acid conjugate (Pal-BBI), and (2) using a mouse model to demonstrate the advantages of Pal-BBI in oral delivery of BBI. During the first year, we have made important progress in both directions. First, we have established the cultured mammary gland system in our laboratory and have used this system to demonstrate the transformation induced by dimethylbenz[a]anthracene (DMBA). This transformation assay will allow us to further investigate the protective effects of BBI and Pal-BBI on the carcinogenesis of mammary glands. Secondly, studies have been carried out on the comparison between the oral absorption of BBI and Pal-BBI. Preliminary results indicate that Pal-BBI, when used with a lipid formulation, can be absorbed via the gastrointestinal tract as an intact polypeptide. Further improvement on the oral delivery of Pal-BBI will be carried out during the second year of this project. In addition to the two specific aims, we have also developed an alternative method for the preparation of Pal-BBI by using partially reduced BBI to generate free sulfhydryl groups from cystinyl disulfide bonds for conjugation. Unlike the previous method that used chemically modified BBI as a starting material, this new method not only simplifies the preparation procedure, but also ensures a release of unmodified BBI from Pal-BBI in the target tissues.				
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FOREWORD

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(5) INTRODUCTION

This report covers the progress of the project, "Breast Cancer Prevention by a Soybean Protein", from July 15, 1996 to July 14, 1997. In the original application, we proposed to investigate chemopreventive effects of Bowman-Birk protease inhibitor (BBI), which is an 8 kDa polypeptide isolated from soybean, on breast cancer formation. It has been shown that BBI can suppress cellular transformation and carcinogenesis *in vitro* and to reduce incidence of a variety of malignant tumors *in vivo* (1). Even though epidemiological studies indicate that vegetarians, especially those who consume legumes as the major source of food, have a significantly lower rate of breast cancer incidence (2), the chemopreventive effect of BBI on breast cancer has not been demonstrated. Our specific aims in this project are: (a) using cultured mouse mammary glands to demonstrate that BBI is an effective soybean component which can prevent the transformation induced by a chemical carcinogen, dimethylbenz[a]anthracene (DMBA), (b) using a newly developed lipidization method to prepare a BBI-palmitic acid conjugate (Pal-BBI) and to demonstrate the enhanced chemopreventive effects of the conjugate due to the increase of the lipophilicity of BBI, and (c) using Pal-BBI as a lipophilic polypeptide to investigate the gastrointestinal (GI) absorption of BBI and to develop an oral delivery formulation for breast cancer prevention.

Progress of this project is presented in two sections, corresponding to the two technical objectives as described in the Statement of Work in the original application, namely, (a) to demonstrate breast cancer preventive effect of BBI or Pal-BBI by using mouse mammary gland cultures, and (b) to obtain preliminary results on oral delivery of Pal-BBI in mice.

(6) BODY

I. Technical Objective 1

1. Preparation of Pal-BBI (Objective 1, Task 1)

In our original proposal, we planned to prepare Pal-BBI by using the procedure described in our previous report, which involved the preparation of a 2-pyridyl 3-propionate disulfide succinimidyl ester (SPDP)-modified BBI and subsequently SPDP-modified BBI was reduced to generate sulfhydryl groups for the conjugation reaction. We recognized that several shortcomings were associated with this procedure. First, it is difficult to control the number of SPDP incorporated into each BBI molecule. The number of SPDP, and the subsequent sulfhydryl groups, per each BBI in this preparation represents only an average of a mixture product. Furthermore, the reduced form of Pal-BBI prepared from this procedure are chemically modified BBI molecules rather than the native BBI. In addition, the yield of the final product was inconsistent and was usually very low because this method involves many steps for the preparation of Pal-BBI.

In order to overcome these problems, which is critical for the success of this project in the future, we have developed a new method for the preparation of Pal-BBI. In this method, the endogenous disulfide bonds in BBI molecule will be selectively reduced (3), and conjugated with N-palmitoyl cysteinyl 2-pyridyl disulfide (CPD) as described previously (4) to produce the final Pal-BBI. When reduced to remove the fatty acid moieties, this conjugate product will regenerate the original BBI molecule rather than derivatives. The new method of Pal-BBI preparation is described briefly as following.

BBI (10 mg, Sigma) was dissolved in 1 ml of 0.1 M borate buffer, pH 9, and 7.8 mg of NaBH₄ was added slowly at 25°C. A small aliquot of the reaction mixture was

pipetted at different time intervals and diluted in 20% acetone solution, and the number of free sulfhydryl groups was determined by using Ellman's reagent. When the Ellman reaction indicated that about 3 sulfhydryl groups were generated per each BBI, the reaction was stopped by the addition of 5% acetone, and, subsequently, a 3-fold molar excess of CPD was added to the reduced BBI solution. The conjugation reaction was monitored by measuring the increase of 2-thiopyridine generation at 343 nm. The final product was purified by two chromatographic procedures, first using an LH-20 column eluted with DMF to remove excess CPD, and subsequently, a Sephadex G-50 column to separate conjugated and free BBI. The final yield of purified dipalmitic acid conjugated BBI was 7.5 mg.

Pal-BBI prepared by this new method has been characterized and was similar to the previously reported conjugate in the serum binding (5)(Fig. 1). The cellular uptake of the new conjugate in Caco-2 cell monolayers was slightly higher than that of the previous preparation (4) (Table 1).

2. Mouse mammary gland culture

As pointed out by the reviewers in the Peer Review Panel Report of our proposal, we did not have previous experience in the mouse mammary gland culture. Therefore, this section demanded most of our time at the beginning of the project. The progress of this section is limited by two factors, namely, the requirement of a large number of mice and a long period of time (33 days) for each experiment.

(A) Mammary gland culture (Objective 1, Task 2)

Hormone (β -estradiol and progesterone) primed 3-4 week old BALB/c mice were used for mammary gland explanting as described by others (6). The first problem encountered at the beginning of this project was the contamination of cultures. The contamination was mostly due to the relatively long exposure of the tissues to the non-sterile environment under the dissecting microscope outside the sterile hood. This problem was finally solved by acquiring a suitable laminar hood in our laboratory for the dissection process. Another problem that we encountered at the beginning of the project was the short survival of the cultured organs. This problem was most likely due to the dehydration of the tissues during the isolation process, and was finally solved by shortening the dissecting process and maintaining the newly isolated glands in the culture medium as soon as possible.

(B) Cytotoxicity (Objective 1, Task 3)

Because there was a large range of DMBA concentrations used in carcinogenesis assays reported by others, experiments were carried out to determine the optimal concentration to be used in our culture system. Concentrations of DMBA were tested from 0.001 to 3.0 $\mu\text{g/ml}$. It was found that concentrations lower than 0.1 $\mu\text{g/ml}$ of DMBA did not show any mutagenic effect. On the other hand, 3 $\mu\text{g/ml}$ of DMBA was too toxic to the culture gland, as indicated by a low viable organs in the culture. Therefore, a concentration of 2 $\mu\text{g/ml}$ of DMBA in DMSO was finally selected for the assay system.

(C) Mammary gland culture (Objective 1, Task 3)

In order to reduce the number of animal used in each experiment, attempts were made to use more than just the second pair (thoracic) of mammary glands in each mouse as described in the literature (6). It was found that the first pair (cervical) of mammary glands were also suitable to be used for the assay. Cervical mammary glands of the mouse are easy to be dissected because they are not covered by other tissues and, unlike the mammary glands of the third pair and below, do

not attach with a large fat tissue. By using the first and the second pairs, we were able to obtain four glands per each mouse. Mammary glands were dissected and cultured on sterile Dacron rafts (6) as described in the original proposal.

(D) Transformation assay (Objective 2, Task 3)

In a typical transformation assay, cultured mammary glands were divided in two groups, i.e., the control and the transformation groups. Mammary glands in the control group were treated with only DMSO, the solvent of DMBA; and those in the transformation group were treated with 2 $\mu\text{g/ml}$ of DMBA in DMSO. Mammary glands were treated on day 3 and 4 only and then cultured in promoting medium and lactogenic medium for a total of 10 days before changed to regression medium. After 14 days in the regression medium, mammary glands were fixed in ethanol:glacial acetic acid, 3:1, for 60 min, washed in 70% ethanol for 15 min, double-distilled water for 5 min, and then stained in Alum Carmine. After overnight staining, fixed glands were washed consecutively with 70%, 95%, and 100% ethanol each for 15 min, and then dehydrated in toluene for 15 min. The final mammary glands were mounted on slides and examined under a dissecting microscope.

As shown in Fig. 2, control mammary glands maintained only the regression ductal structures and lobulo-alveolar vestiges (Fig. 2A). However, in DMBA-treated glands, numerous nodule-like alveolar lesions (NLAL) were found (Fig. 2B). When used a higher magnification under the microscope, NLAL could be seen as densely stained nodules with an irregular shape (Fig. 2C). At 2 $\mu\text{g/ml}$ of DMBA, 7 of the 8 viable glands (87.5%) showed the presence of NLAL.

II. Technical Objective 2

1. Oral delivery of BBI and Pal-BBI (Objective 2, Task 1, 2 and 3)

BBI and Pal-BBI were iodinated with ^{125}I using a modified chloramine T method. Female CF-1 mice, 2 to 3 weeks old, weighing 20-25 g each, were used in these studies. The mice were fasted for 16 hr prior to all of the experiments described here. A dose of 3 mg/kg of labeled BBI or Pal-BBI in 0.2 ml, which was about 1×10^6 cpm of radioactivity, was given to each mouse orally via a gavage needle. Formulations used included PBS, olive oil, and Intralipid (Pharmacia). At various time intervals after gavaging feeding, 3 animals from each group were sacrificed and their blood (200 ml), kidneys, lungs, liver, stomach, intestine and colon were collected. After rinsed extensively in ice-cold phosphate buffered saline (PBS), the radioactivity in each organ was counted in a gamma counter. The weights of the organs were recorded and used to adjust the concentration of BBI or Pal-BBI in each organ. The nature of the accumulated radioactivity in the blood of the animals was determined by Sephadex G-50 gel filtration column. Briefly, 0.2 ml of blood was diluted with 0.8 ml of water and centrifuged at 3000 rpm for 5 min. The supernatant was then eluted from the column using PBS. Fractions (1 ml) were collected and the radioactivities were determined. Results from the blood analysis demonstrated that intact BBI was detected only in the blood of mice received the olive oil, but not in PBS, formulation (Fig. 3).

A comparison of the olive oil formulation for the oral delivery of BBI and Pal-BBI indicated that the stomach retention was higher for Pal-BBI than BBI in the first 6 hr post-feeding. Similarly, the biodistribution of Pal-BBI was also higher than that of BBI in all tissues except the kidneys within first 6 hr. However, no significant difference was detected after 8 hr post-feeding (Fig. 4).

III. Table 1

Table 1. Cellular Uptake of ^{125}I -BBI and ^{125}I -Pal-BBI in Cultured Caco-2 Cell Monolayers

	Cellular Uptake (ng BBI/mg protein/hr, N=3, $\pm$ S.D.)	
	<u>Serum-free medium</u>	<u>10% fetal bovine serum</u>
BBI	1.96 $\pm$ 0.71	0.86 $\pm$ 0.05
Pal-BBI	416 $\pm$ 33	65.0 $\pm$ 18
BBI/Pal-BBI	1/212	1/75

Confluent monolayers of Caco-2 cells grown on plastic surfaces were treated with ^{125}I -labeled BBI or Pal-BBI for one hour. Cells were detached from the plastic surfaces by trypsinization and washed three times with ice-cold PBS. The final cell pellets were counted in a gamma counter. The amount of BBI associated with cells was calculated based on the specific radioactivities of ^{125}I -BBI and ^{125}I -Pal-BBI, and was presented as ng BBI per mg of cell protein.

IV. Figure legends

Fig. 1. Separation of Pal-BBI and BBI by Sephacryl S-200 gel filtration. A mixture of ^{125}I -Pal-BBI, which was prepared by the new method, and ^{125}I -BBI was incubated in a medium containing 10% of fetal bovine serum. After 10 min at 37°C, the mixture was loaded onto a Sephacryl S-200 column (40 ml) and eluted with PBS. Fractions (1-ml) were collected and radioactivity of each fraction was determined by using a gamma counter. The recovery of the total radioactivity from the column was higher than 90%. The absorbency at 280 nm of each fraction was also measured (dotted line) as an indication of the position of serum albumin. The binding of Pal-BBI to a large serum protein is identical to that of the previous reported Pal-BBI (5).

Fig. 2. Cultured mouse mammary glands after development and regression. (A) A control gland treated with only DMSO. The presence of regressed lobulo-alveolar structures and the absence of NLAL can be seen. (B) A gland treated with DMBA (2 $\mu\text{g}/\text{ml}$). At least 7 NLAL are present in this gland. (C) A high magnification of the right end of the gland in (B). The densely stained, non-regressed NLAL can be easily recognized.

Fig. 3. The oral bioavailability of ^{125}I -Pal-BBI given in PBS or olive oil vehicles. CF-1 mice were administered with 3 mg/kg of Pal-BBI in PBS or olive oil. The animals were sacrificed at 3 hr post-dosing, and the organs were removed and assayed for the accumulated radioactivity. The results are expressed as % dose/g tissue $\pm$ SEM (bars) for different organs (panel A). Blood samples (0.2 ml) were diluted with 0.8 ml of water and analyzed using a Sephadex G-50 column (10 ml size) (panel B).

Fig. 4. The bioavailability of ^{125}I -Pal-BBI and ^{125}I -BBI given orally in the olive oil delivery vehicle. Pal-BBI or BBI (3 mg/kg) was administered to the animals in an olive oil solution. The animals were sacrificed at 3 (A), 6 (B), and 8 (C) hr post-administration and the organ associated radioactivity was determined for each animal. The results are presented as % dose/organ $\pm$ SEM (bars) for the blood, kidneys, lungs, liver, stomach, intestinal, and colon.

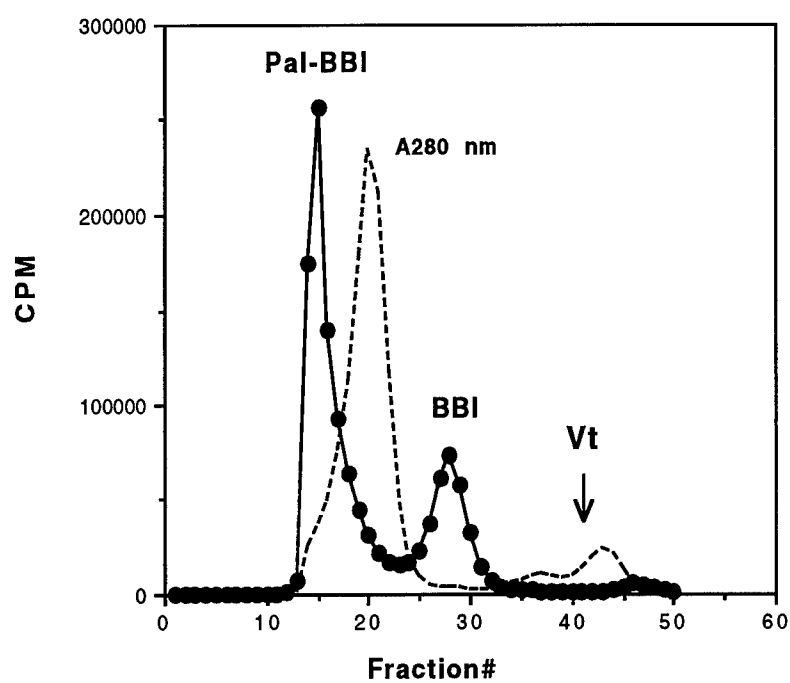


Fig. 1

Fig. 2

(A)



(B)



(C)



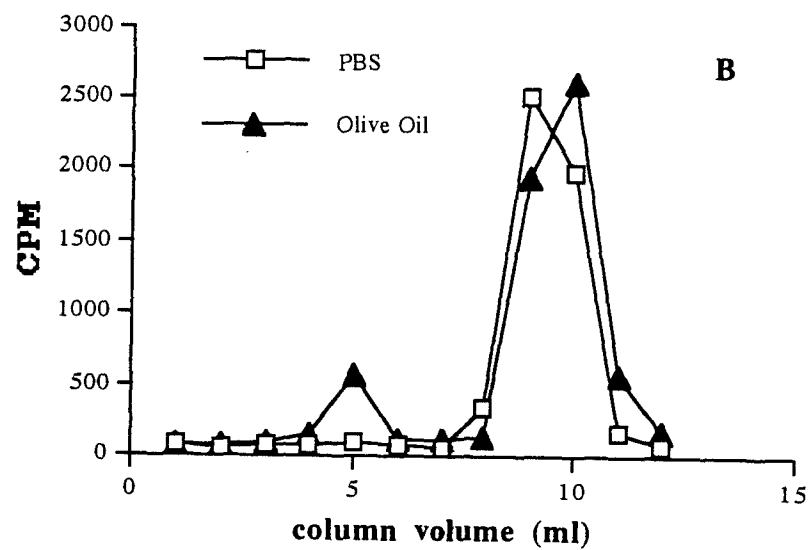
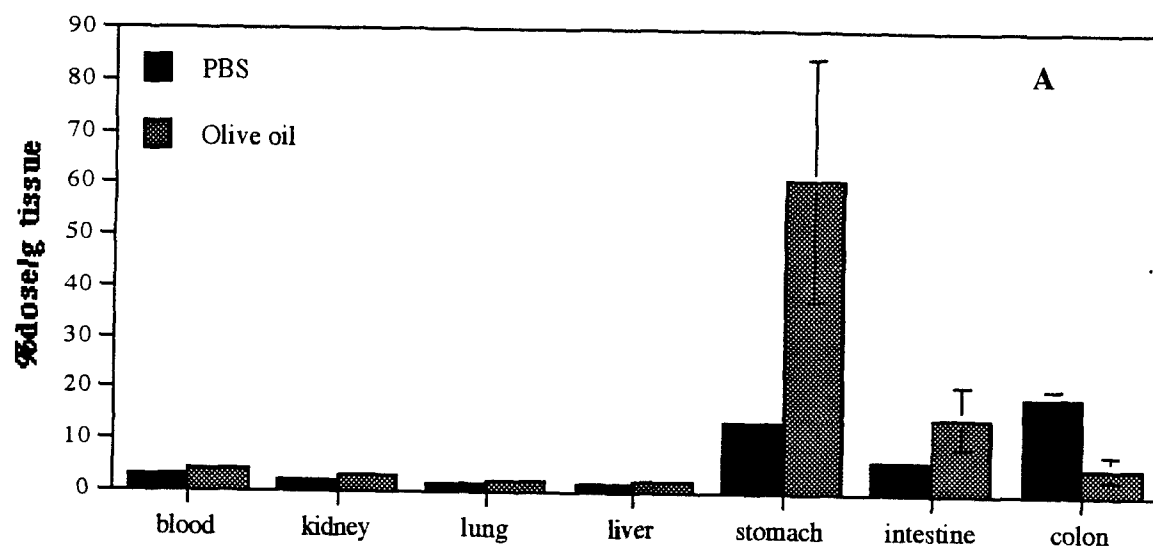


Fig. 3

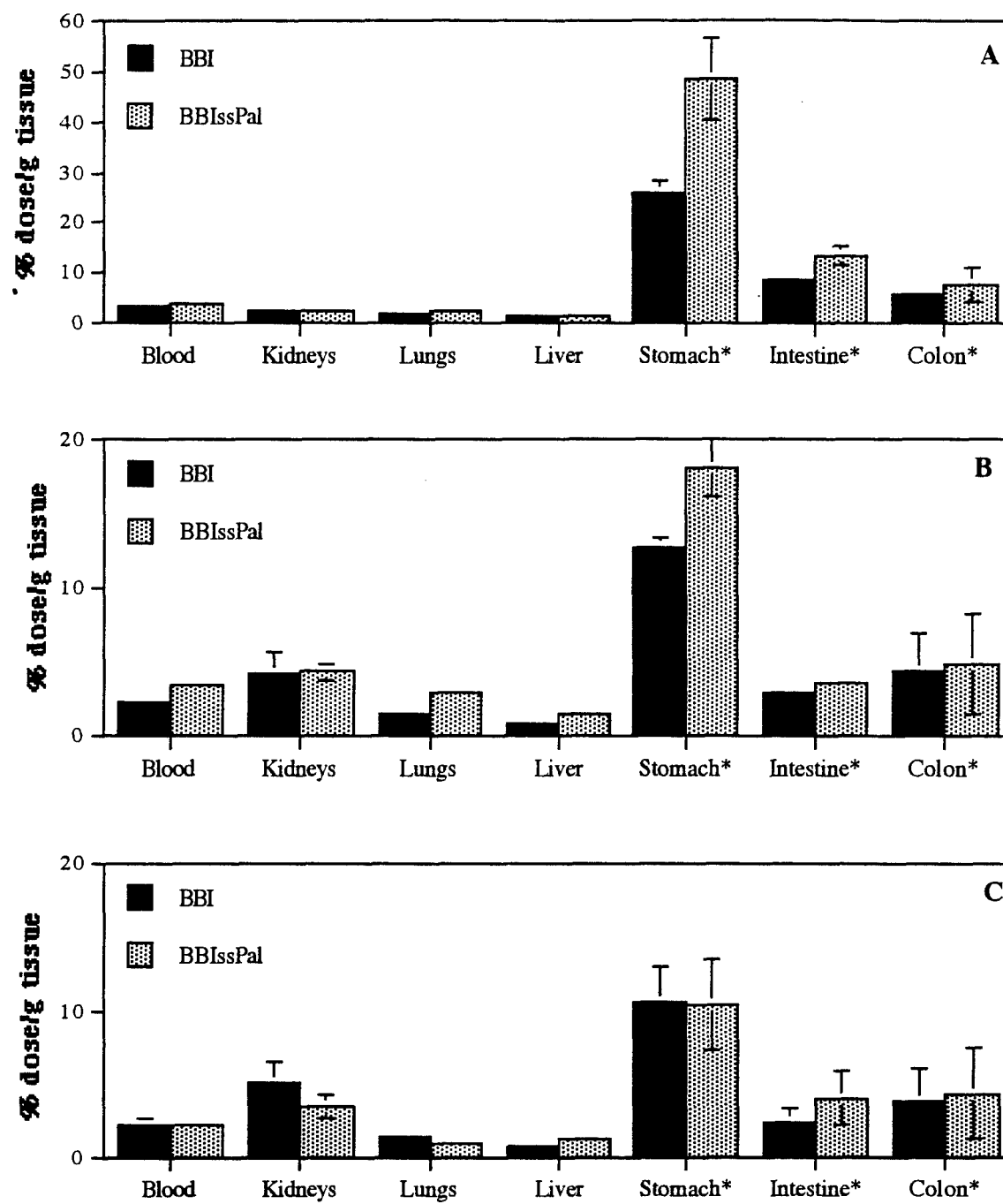


Fig. 4

(7) CONCLUSIONS

During the first year of this project, we have developed a new method for the preparation of Pal-BBI with both a higher yield and a higher purity than the previous method. Furthermore, this conjugate can regenerate the original BBI, rather than chemically modified BBI molecules, upon reduction of the disulfide linkages. This development is important for obtaining consistent results in the future investigation.

Another important progress of this project is the establishment of the cultured mouse mammary gland system in our laboratory. Even though this progress is slower than our original plan, we have finally solved several critical problems and been able to identify transformed nodules in DMBA-treated glands. Currently, experiments are under way to demonstrate the anti-transformative effects of BBI and Pal-BBI. Since each transformation assay with cultured mammary glands takes at least 33 days to complete, problems encountered at the beginning of the project caused a delay of about two months on the schedule proposed in the Statement of Work. However, with our accumulated experience from the first year and improved knowledge of the techniques in the culture of mammary glands, we are still optimistic that this project can be finished within the original two-year plan.

Interesting results were observed in the oral delivery of BBI in the animal model. These findings suggest that a lipid formulation may be favorable for the GI absorption of Pal-BBI. Currently, experiments are under way to test different lipid formulations for the oral delivery of Pal-BBI. One of the most promising carriers for the lipidized peptides is the solid lipid nanoparticle system (7). It is hoped that, with an optimal lipid formulation, the GI absorption of Pal-BBI can be further enhanced. If such an enhanced absorption can be achieved, it will help not only for the development of BBI into a practical chemopreventive agent for breast cancer, but also for the application of many polypeptides as therapeutic drugs.

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