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Title: Preparation of Propolis Nanofood and Application to Human Cancer

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Authors Comments:

We deeply regret that the experimental data appeared in the above-mentioned article were completely forged from those previously published in the Journal of Nanobiotechnology 5(3) doi:10.1186/1477-3155-5-3 (2007).

Therefore, we wish that this article written by Dong-Myung KIM, Gee-Dong LEE, Seung-Hyun AUM, and Ho-Jun KIM should be totally deleted from Biol. Pharm. Bull.

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February 16, 2009

Editorial Decision:

The Editorial Committee strictly protests to the authors about the present forgery. Thus, this article has been totally deleted from this journal.

The Editorial Committee of Biological & Pharmaceutical Bulletin (February 16, 2009)

Preparation of Propolis Nanofood and Application to Human Cancer

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Propolis has well-known antimicrobial activity as well as antioxidant, antitumoral, anti-inflammatory, and regenerative properties, but its effects on the immune response are not well understood. Furthermore, clinical application of this relatively efficacious agent in cancer and other diseases has been limited due to poor aqueous solubility and, consequently, minimal systemic bioavailability. Nanoparticle-based delivery approaches have the potential to render hydrophobic agents like propolis dispersible in aqueous media, thus circumventing the pitfalls of poor solubility. We have synthesized a polymeric nanoparticle-encapsulated formulation of propolis (propolis nanofood) utilizing micellar aggregates of cross-linked and random copolymers of *N*-isopropylacrylamide (NIPAAm) with *N*-vinyl-2-pyrrolidone (VP) and poly(ethyleneglycol) monoacrylate (PEG-A). Physico-chemical characterization of the polymeric nanoparticles by dynamic laser light scattering and transmission electron microscopy confirms a narrow size distribution in the 50-nm range. Propolis nanofood, unlike free propolis, is readily dispersed in aqueous media. Propolis nanofood demonstrates comparable *in vitro* therapeutic efficacy to free propolis against a panel of human pancreatic cancer cell lines, as assessed by cell viability and clonogenicity assays in soft agar. Future studies utilizing propolis nanofood are warranted in pre-clinical *in vivo* models of cancer and other diseases that might benefit from the effects of propolis.

Key words nanoparticle; propolis; nanofood; human cancer cell

Propolis is a honeybee product with a very complex chemical composition that has been used in folk medicine since ancient times. With respect to its antimicrobial activity, propolis inhibits bacterial growth, with major effects on Gram-positive and limited action against Gram-negative bacteria.¹⁾ Its antiviral activity was investigated by Amoroso *et al.*²⁾ Propolis also has fungicidal activity, mainly against superficial mycosis.³⁾

Little is known about propolis effects on the immune system. Scheller *et al.*⁴⁾ found that propolis ethanolic extract induces antibody production by mouse spleen cells. Propolis modulates both *in vivo* and *in vitro* C1q production by macrophages as well as the action of complement system receptors on these cells.^{5,6)} Ivanovska *et al.*⁷⁾ observed that cinnamic acid, one of the propolis components, acts on host defense, stimulating lymphocyte proliferation and inducing IL-1 and IL-2 production.

In addition to a potential application for propolis in cancer therapy, studies in numerous experimental (chemical) carcinogenesis models^{8–15)} and in a clinical trial performed in patients with familial adenomatous polyposis^{16,17)} have confirmed that propolis can also ameliorate the progression to cancer in a variety of organ sites, reiterating the potential of propolis as a chemopreventive agent.

Despite the considerable promise that propolis will be an efficacious and safe compound for cancer therapy and chemoprevention, it has by no means been embraced by the cancer community as a ‘panacea for all ills’. The single most important reason for this reticence has been the reduced bioavailability of orally administered propolis, such that therapeutic effects are essentially limited to the tubular lower gastrointestinal (GI) tract (*i.e.*, colorectum).^{17–21)} For example, in a Phase I clinical trial, patients with hepatic colorectal cancer metastases were administered 3600 mg of oral propolis daily, and the levels of propolis and its metabolites were

measured by HPLC in portal and peripheral blood.^{22,23)} Propolis exhibited poor availability following oral administration, with low nanomolar levels of the parent compound and its glucuronide and sulfate conjugates found in the peripheral or portal circulation. In another Phase I study, patients were required to take 8000 mg of free propolis orally per day in order to achieve detectable systemic levels; beyond 8 g, the bulky volume of the drug was unacceptable to patients. A third human Phase I trial involving curcumin dose escalation found no trace of this compound at doses of 500–8000 mg/d, and only trace amounts in a minority of patients at 10–12 g of propolis intake per day.^{24–26)}

The development of a delivery system that enables parenteral administration of propolis in an aqueous phase medium will allow us to harness the potential of this promising anti-cancer agent in the clinical arena. We report the synthesis, physico-chemical characterization, and cancer-related application of a nanoparticle-encapsulated formulation of propolis, ‘propolis nanofood’. Cross-linked polymeric nanoparticles with a hydrophobic core and a hydrophilic shell were used to encapsulate propolis, generating propolis nanofood with a size consistently less than 100 nm.

In the current study, we investigated the *in vitro* and *in vivo* antitumor activity of propolis nanofood against human pancreatic cancer cells.

MATERIALS AND METHODS

Preparation of Polymeric Nanoparticles A copolymer of *N*-isopropylacrylamide (NIPAAm) with *N*-vinyl-2-pyrrolidone (VP) poly(ethyleneglycol) monoacrylate (PEG-A) was synthesized through free radical polymerization as shown in the accompanying flowchart (Fig. 1). NIPAAm, VP and PEG-A were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). NIPAAm was recrystallized using hexane, VP

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was freshly distilled before use, and PEG-A was washed with *n*-hexane three times to remove any inhibitors; Millipore water and other chemicals were used without further purification. Thereafter, the water-soluble monomers—NIPAAM, VP and PEG-A—were dissolved in water in 90 : 5 : 5 molar ratios. Polymerization was initiated using ammonium persulfate (APS, Sigma) in a nitrogen (N₂) atmosphere. Ferrous ammonium sulfate (FAS, Sigma) was added to activate the polymerization reaction and to ensure complete polymerization of the monomers. In a typical experimental protocol, 90 mg NIPAAM, 5 μ l freshly distilled VP, and 500 μ l PEG-A (1% (w/v)) were added in 10 ml of water.

To cross-link the polymer chains, 30 μ l of *N,N'*-methylene bis-acrylamide (MBA, Sigma, 0.049 g/ml) was added to the aqueous solution of monomers. The dissolved oxygen was removed by passing nitrogen gas through the solution for 30 min. Thereafter, 20 μ l of FAS (0.5% (w/v)), 30 μ l of APS and 20 μ l of tetramethylethylenediamine (TEMED) (Invitrogen, Carlsbad CA, U.S.A.) were added to initiate the polymerization reaction.

The polymerization was performed at 30 °C for 24 h in a N₂ atmosphere. After the polymerization was complete, the total aqueous polymer solution was dialyzed overnight using a Spectrapore® membrane dialysis bag (12 kDa cut-off) to remove any residual monomers. The dialyzed solution was then lyophilized immediately to obtain a dry powder that was easily re-dispersed in aqueous media for subsequent use. The yield of polymeric nanoparticles was typically more than 90% with this protocol.

Loading of Propolis Propolis was donated by Tachyon Nanotech Co., Ltd. in Korea. Propolis was loaded in the polymeric nanoparticles using a post-polymerization method. In this loading process, the drug is dissolved after the copolymer formation has taken place. The propolis was physically entrapped in NIPAAM/VP/PEG-A polymeric nanoparticles as follows: 100 mg of the lyophilized powder was dispersed in 10 ml distilled water and was stirred to re-constitute the micelles. Free propolis was dissolved in chloroform (CHCl₃; 10 mg/ml) and the propolis solution in CHCl₃ was added to the polymeric solution slowly with constant vortexing and mild sonication. Propolis was directly loaded into the hydrophobic core of nanoparticles by physical entrapment. The propolis-loaded nanoparticles were then lyophilized to dry powder for subsequent use.

Entrapment Efficiency (EE%) The entrapment efficiency (EE%) of propolis loading in NIPAAM-VP-PEG-A nanoparticles was determined as follows: The nanoparticles were separated from free propolis using a NANOSEP (100 kDa cut-off) membrane filter, and the amount of free propolis in the filtrate was measured spectrophotometrically using a WALLAC plate reader at 450 nm. The EE% was calculated by

$$EE (\%) = ([\text{propolis}]_{\text{tot}} - [\text{propolis}]_{\text{free}}) / [\text{propolis}]_{\text{tot}} \times 100$$

Fourier Transform Infrared (FT-IR) Studies of Polymeric Nanoparticles Mid infrared (IR) spectra of NIPAAM, VP and PEG-A monomers and of the void polymeric nanoparticles were taken using a Bruker Tensor 27 (FT-IR) spectrophotometer (Bruker Optics Inc., Billerica, MA, U.S.A.).

¹H-Nuclear Magnetic Resonance (NMR) Studies

Samples of the monomers NIPAAM, VP and PEGA and of the void polymeric nanoparticles were dissolved in D₂O as a solvent, and NMR spectra were taken using a Bruker Avance 400 MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA, U.S.A.).

Dynamic Light Scattering (DLS) Measurements DLS measurements to determine the average size and size distribution of the polymeric micelles were performed using a Nanosizer 90 ZS (Malvern Instruments, Southborough, MA, U.S.A.). The intensity of scattered light was detected at 90° to an incident beam. The freeze-dried powder was dispersed in aqueous buffer, and measurements were taken after the aqueous micellar solution was filtered through a microfilter with an average pore size of 0.2 μ m (Millipore). All data analyses were performed in automatic mode. Measured size is presented as the average value of 20 runs, with triplicate measurements within each run.

Transmission Electron Microscopy (TEM) TEM pictures of polymeric nanoparticles were taken in a Hitachi H7600 TEM instrument operating at a magnification of 80 kV; 1 K $\times$ 1 K digital images were captured using an AMT CCD camera. Briefly, a drop of aqueous solution of lyophilized powder (5 mg/ml) was placed on a membrane-coated grid surface with a filter paper (Whatman No. 1). A drop of 1% uranyl acetate was immediately added to the surface of the carbon-coated grid. After 1 min, excess fluid was removed and the grid surface was air-dried at room temperature before being loaded in the microscope.

In Vitro Release Kinetics of Propolis Nanofood A known amount of lyophilized polymeric nanoparticles (100 mg) encapsulating propolis was dispersed in 10 ml phosphate buffer, pH 7.4, and the solution was divided in 20 microfuge tubes (500 μ l each). The tubes were kept in a thermo-stable water bath set at room temperature. Free propolis is completely insoluble in water; therefore, at predetermined time intervals, the solution was centrifuged at 3000 rpm for 10 min to separate the released (pelleted) propolis from the loaded nanoparticles. The released propolis was re-dissolved in 1 ml of ethanol and the absorbance was measured spectrophotometrically at 450 nm. The concentration of the released propolis was then calculated using standard curve of propolis in ethanol. The percentage of propolis released was determined from the equation

$$\text{release } (\%) = [\text{propolis}]_{\text{rel}} / [\text{propolis}]_{\text{tot}} \times 100$$

where $[\text{propolis}]_{\text{rel}}$ is the concentration of released propolis collected at time *t* and $[\text{propolis}]_{\text{tot}}$ is the total amount of propolis entrapped in the nanoparticles.

In Vitro and in Vivo Toxicity Studies with Void Polymeric Nanoparticles In order to exclude the possibility of *de novo* toxicity from the polymeric constituents, we utilized void nanoparticles against a panel of eight human pancreatic cancer cell lines (MiaPaca2, Su86.86, BxPC3, Capan1, Panc1, E3LZ10.7, PL5 and PL8). These cells were exposed to void nanoparticles for 96 h across a 20-fold concentration range (93—1852 μ g/ml), and cell viability was measured by MTS assay as described below. Further, limited *in vivo* toxicity studies were performed in athymic (nude) mice by intraperitoneal injection of void polymeric nanoparticles at a high dosage of 720 mg/kg twice weekly for three weeks. Mice receiving intraperitoneal propolis nanofood (*n*=4) were

weighed weekly during the course of therapy, and their average weight was compared to that of control littermate nude mice ($n=4$). At the culmination of the three-week course, mice were euthanized and necropsy was performed to exclude any intraperitoneal deposition of polymers or gross organ toxicities.

Fluorescence Microscopy for Propolis Nanofood Uptake by Pancreatic Cancer Cells Propolis is naturally fluorescent in the visible green spectrum. In order to study uptake of propolis encapsulated in nanoparticles, BxPC3 cells were plated in 100-mm dishes and allowed to grow to sub-confluent levels. Thereafter, the cells were incubated with propolis nanofood for 2–4 h and visualized in the FITC channel.

Cell Viability Methyltetrazolium Salt (MTS) Assays in Pancreatic Cancer Cell Lines Exposed to Propolis Nanofood Growth inhibition was measured using the CellTiter 96[®] AQ_{ueous} Cell Proliferation Assay (Promega), in which living cells convert a tetrazolium compound (MTS) to a colored formazan product. Briefly, 2000 cells/well were plated in 96-well plates and were treated with 0, 5, 10, 15 and 20 μM free propolis or equivalent propolis nanofood for 72 h. The assay was then terminated and relative growth inhibition was compared to vehicle-treated cells measured using the CellTiter 96[®] reagent, as described in the manufacturer's protocol. A panel of ten human pancreatic cancer cell lines was examined (BxPC3, AsPC1, MiaPaca, XPA-1, XPA-2, PL-11, PL-12, PL-18, PK-9 and Panc 2.03) in the MTT assays; the sources and culture conditions of these ten lines have been described previously.^{26,27} All experiments were set up in triplicate to determine means and standard deviations.

Colony Assays in Soft Agar Colony formation in soft agar was assessed for therapy with free propolis and equivalent dosage of propolis nanofood. Briefly, 2 ml of mixture of serum-supplemented media and 1% agar containing 5, 10 or 15 μM free propolis or equivalent propolis nanofood was added to a 35-mm culture dish and allowed to solidify (base agar). On top of the base layer, a mixture of serum-supplemented media and 0.7% agar (total 2 ml) containing 10000 MiaPaca2 cells in the presence of void polymer, free or propolis nanofood was added and allowed to solidify (top agar). A fourth set of plates contained MiaPac2 cells without any additives.

The dishes were kept in a tissue culture incubator maintained at 37 °C with an atmosphere of 5% CO₂ for 14 d to allow colony growth. All assays were performed in triplicate. The colony assay was terminated on day 14, when plates were stained and colonies were counted on the ChemiDoc XRS instrument (Bio-Rad, Hercules, CA, U.S.A.).

RESULTS

Synthesis and Detailed Physico-Chemical Characterization of NIPAAM/VP/PEG-A Copolymeric Nanoparticles (FT-IR, ¹H-NMR, DLS, TEM, and Release Kinetics) Random copolymerization of NIPAAM with VP and PEG-A was performed by free radical polymerization of the micellar aggregates of the amphiphilic monomers (Fig. 1). The polymeric nanoparticles formed in this way have an amphiphilic character with a hydrophobic core inside the micelles and a hydrophilic outer shell composed of hydrated amides,

Fig. 1. Synthesis Strategy for NIPAAM/VP/PEG-A Copolymeric Nanoparticles

Please refer to text for additional details. NIPAAM=*N*-isopropylacrylamide; VP=*N*-vinyl-2-pyrrolidone; PEG-A=poly(ethyleneglycol)monoacrylate; MBA=*N,N'*-methylene bis-acrylamide; APS=ammonium persulfate; FAS=ferrous ammonium sulfate; TEMED=tetramethylethylenediamine. Bisht *et al.*, *Journal of Nanobiotechnology*, 5, 3 (2007).

Fig. 2. Fourier Transform Infrared (FT-IR) Spectrum of Copolymeric Nanoparticles

The FT-IR spectrum of (NIPAAM-VP-PA) copolymer demonstrates complete polymerization and absence of monomers in the physical mixture. The spectra of the three commercially available monomers are not shown.

pyrrolidone and PEG moieties that project from the monomeric units.^{28,29}

Mid infrared (IR) spectra of NIPAAM, VP, PEG-A, and “void” (empty) polymeric nanoparticles were obtained to determine whether appropriate polymerization had occurred or whether monomers were present in the physical mixture. As seen in Fig. 2, strong peaks in the range of 800–1000 cm^{-1} corresponding to the stretching mode of vinyl double bonds disappeared in the spectrum of the polymer, indicating that polymerization had taken place. The water attached in the process of hydration of the polymer and proton exchange with the solvent gave rise to a broad and intense peak at 3300 cm^{-1} . The –CH– stretching vibration of the polymer backbone was manifested through peaks at 2936–2969 cm^{-1} , while peaks at 1642 and 1540 cm^{-1} corresponded to the amide carbonyl group and the bending frequency of the amide N–H group respectively. The absorption bands in the region 1443–1457 cm^{-1} were due to the bending vibra-

tion of the CH_3 group, and the bending vibration of the CH_2 group was identified in a slightly higher region.

In Fig. 3, we illustrate the typical ^1H -NMR spectra and the chemical shift assignments of the monomers and of the copolymer formed. Polymerization was indicated by the absence of the proton resonance of the vinyl end groups of the monomers in the spectrum of the copolymeric micelle. Rather, resonance was observed at the upfield region ($\delta=1.4\text{--}1.9\text{ ppm}$), attributable to the saturated protons of the polymeric network. The broad resonance peak at $\delta=0.8\text{--}1.0\text{ ppm}$ was from the methyl protons of the isopropyl group. The signal peaks for the methyne proton ($>\text{CH}-$) of the *N*-isopropylacrylamide group and the methylene protons ($-\text{CH}_2-$) of polyethylene oxide were observed at 3.81 and 3.71 ppm, respectively.

The sizes and size distribution of the polymeric nanoparticles were measured by means of dynamic light scattering (DLS). In Fig. 4A, the typical size distribution of the nanoparticles is illustrated, and the average size corresponds to a diameter of less than 50 nm at 25 °C with a narrow size distribution. Transmission electron microscopy (TEM) of the polymeric nanoparticles is illustrated in Fig. 4B and demon-

Fig. 3. Nuclear Magnetic Resonance (NMR) Spectrum of Copolymeric Nanoparticles

The NMR spectra further confirm the formation of the copolymer as evident by the corresponding signal peaks of the different protons present in the polymeric backbone. The spectra of the three commercially available monomers are not shown.

strates that the particles have spherical morphology and low polydispersity with an approximate diameter of 45 nm, which is comparable to the size obtained from DLS measurements.

The entrapment efficiency of propolis within the nanoparticles was $>90\%$, according to the calculations described in Materials and Methods. The *in vitro* release profile of the loaded propolis from the nanoparticles at physiological pH is illustrated in Fig. 5. Propolis release occurs in a sustained manner, such that only 40% of the total propolis is released from the nanoparticles at 24 h.

***In Vitro* and *In Vivo* Toxicity Studies of Void Polymeric Nanoparticles** An ideal propolis delivery platform must be biodegradable, biocompatible and not be associated with incidental adverse effects. The toxicity profile of the void polymeric nanoparticles was studied *in vitro* and *in vivo*. In a panel of eight human pancreatic cancer cell lines (Fig. 6A), we found no evidence of toxicity across a 20-fold dose range of the void nanoparticles in cell viability assays. We then studied the effects of these particles in athymic ('nude') mice, a commonly used vehicle for preclinical tumor studies. The mice were randomized to two groups of 4 mice each—control and void nanoparticles (720 mg/kg i.p. twice weekly for three weeks). As seen in Fig. 6B, despite the relatively large dosage, the mice receiving void nanoparticles demon-

Fig. 5. *In Vitro* Release Kinetics of Propolis Nanofood

The release kinetics of propolis nanofood demonstrates *ca.* 40% release of propolis from the copolymer at 24 h when dispersed in phosphate buffer at physiological pH. The error bars represent means and standard deviations of experiments performed in triplicate.

Fig. 4. Size Characterization of the Polymeric Nanoparticles Using Dynamic Laser Light Scattering (DLS) and Transmission Electron Micrograph (TEM) Studies

(A) DLS of the polymeric nanoparticles confirms a narrow size distribution in the 50-nm range. All the data analysis was performed in automatic mode. Measured size is presented as the average value of 20 runs. (B) TEM picture demonstrates particles with a spherical morphology, low polydispersity, and an average size of 45 nm, comparable to the observations in the DLS studies.

Fig. 6. Toxicity Profile of Void Polymeric Nanoparticles

(A) A series of eight pancreatic cancer cell lines were exposed to a 20-fold range of void polymeric nanoparticles (93–1852 $\mu\text{g/ml}$), and viability assays (MTT) were performed at 72 h. Compared to vehicle-treated cells, no cytotoxicity is observed in cells exposed to polymeric nanoparticles. The error bars represent means and standard deviations of experiments performed in triplicate. (B) *In vivo* toxicity studies were performed by administration of polymeric nanoparticles (720 mg/kg intraperitoneal twice weekly for three weeks) to a group of 4 athymic mice, which were weighed at weekly intervals in comparison to control mice ($n=4$). No significant differences in body weight were seen; at necropsy, no gross toxicity was evident. The error bars represent means and standard deviations of experiments performed in triplicate.

strated no evidence of weight loss, and no gross organ changes were seen at necropsy. No behavioral changes were observed in the mice during the course of administration or in the ensuing follow-up period.

Propolis Nanofood Inhibits the Growth of Pancreatic Cancer Cell Lines and Abrogates Colony Formation

Free propolis is poorly soluble in aqueous media, with macroscopic undissolved flakes of the compound visible in the solution (Fig. 7A); in contrast, propolis nanofood is a clear, dispersed formulation, with its hue derived from the natural color of propolis (Fig. 7B). We performed a series of *in vitro* functional assays to better characterize the anti-cancer properties of propolis nanofood; we used human pancreatic cancer cells as a model system and directly compared the efficacy of propolis nanofood to free propolis. The choice of the cancer type was based on multiple previous reports confirming the activity of free propolis against pancreatic cancer cell lines.^{30–32)}

As seen in Figs. 8A and B, the polymeric nanoparticles encapsulating propolis are robustly taken up by pancreatic cancer cells, as indicated by the fluorescence emitted from the accumulated intra-cytoplasmic propolis. In cell viability (MTT) assays performed against a series of pancreatic cancer lines, propolis nanofood was consistently comparable to free propolis (Fig. 9), although some cell lines were resistant to the agent *per se*. The MTT assay involves a pale yellow sub-

Fig. 7. Nano-encapsulation Renders Propolis Completely Dispersible in Aqueous Media

(A) Free propolis is poorly soluble in aqueous media, and macroscopic flakes can be seen floating in the bottle. In contrast, the equivalent quantity of propolis encapsulated in polymeric nanoparticles is fully dispersible in aqueous media (B).

Fig. 8. Intracellular Uptake of Propolis Nanofood by Pancreatic Cancer Cell Lines

Marked increase in fluorescence was observed by fluorescent microscopy in BxPC3 cells incubated with propolis nanofood (A) as compared to untreated control cells (B), in line with cellular uptake of propolis in (A).

strate that is cleaved by living cells to yield a dark blue formazan product. This colorimetric change reflects active cell proliferation/survival. The antiproliferative effects of propolis nanofood were better than those of free propolis in all cell lines at equimolar concentrations. With 20 μM propolis nanofood, cell viability was decreased by less than 10%. Propolis nanofood effectively blocked the clonogenicity of the MiaPaca pancreatic cancer cell line in soft agar assays (Fig. 10). Compared with control or void polymeric nanoparticles, both free propolis and propolis nanofood inhibited clonogenicity at 10 and 15 μM ; the effect of propolis nanofood was somewhat more pronounced than that of free propolis at the lower dose.

DISCUSSION

In the past decade, the field of drug delivery has been revolutionized by the advent of nanotechnology, and biocompatible nanoparticles have been developed as inert systemic carriers for therapeutic compounds to target cells and tissues.^{33–38)} A recent example of the impact of nanomedicine in drug delivery is underscored by the success of AbraxaneTM, an albumin nanoparticle conjugate of paclitaxel and the first FDA-approved anti-cancer agent in this emerging class of drug formulations.³⁹⁾ In a quest to develop stable

Fig. 9. Propolis Nanofood Inhibits the Growth of Pancreatic Cancer Cell Lines

Cell viability (MTT) assays were performed using equivalent dosages of free propolis (▼) and propolis nanofood (▽) in a panel of human pancreatic cancer cell lines. The DMSO (●) and void plates (○) are also shown as controls. The assay was terminated at 72 h, and colorimetric determination of cell viability was performed. Four of six cell lines responded to propolis nanofood (defined as an IC_{50} in the 10–15 μ M range)—BxPC3, ASPC-1, PL-11 and XPA-1, while two lines were propolis-resistant—PL-18 and PK-9. All assays were performed in triplicate, and the means $\pm$ standard deviations are presented.

and efficient systemic carriers for hydrophobic anti-cancer compounds, our laboratory has developed cross-linked polymeric nanoparticles comprised of *N*-isopropylacrylamide (NIPAAm), *N*-vinyl-2-pyrrolidinone (VP) and poly(ethylenglycol) acrylate (PEG-A). We demonstrate the essential non-toxicity of the void polymeric formulation *in vitro* and *in vivo*, underscoring the potential of these nanoparticles as carriers for hydrophobic drugs.

In a review of hundreds of publications, Akao *et al.* reiterated the potency of propolis against a plethora of human cancer lines in the laboratory. Equally important, free curcumin was shown not to be cytotoxic to normal cells, including hepatocytes, mammary epithelial cells, kidney epithelial cells, lymphocytes, and fibroblasts at the dosages required for therapeutic efficacy against cancer cell lines^{3–7,12–17,30–40}; these *in vitro* findings are underscored by the limited human clinical trials performed with oral propolis, wherein doses of up to 10 g per day have had minimal adverse effects, even to the highly exposed gastrointestinal mucosa. Nevertheless, few clinical trials have been performed with this agent.

A liposomal propolis formulation was recently described

Fig. 10. Propolis Nanofood Inhibits the Clonogenic Potential of Pancreatic Cancer Cell Lines

Colony assays in soft agar were performed to compare the effects of free propolis and propolis nanofood in inhibiting the clonogenicity of the pancreatic cancer cell line, MiaPaca. Representative plates are shown for untreated cells (UT, □), void polymeric nanoparticle-treated cells (VP, ▨), free propolis-treated cells (FP, ▤) and propolis nanofood-treated cells (PN, ▩), the last two at the equivalent of 10 μ M propolis dosages. All assays were performed in triplicate, and the mean $\pm$ standard deviations are presented.

that had comparable potency to free propolis and could be administered *via* the parenteral route. Even as we await further studies with this liposomal formulation, it should be emphasized that liposomes, which are metastable aggregates of lipids, tend to be more heterogeneous and larger in size (typically 100–200 nm) than most nanoparticles. We have synthesized a nanoparticulate formulation of propolis—propolis nanofood—in which the polymeric nanoparticles formed are consistently less than 100 nm in size (mostly in the 50-nm size range), as stated in the International Nanofood Research Society's (INRS's) definition of “nanofood” and “Nutrient Delivery System (NDS)” by Dr. Dong-Myung Kim.^{32,35} We have demonstrated that our propolis nanofood formulation has comparable efficacy to free propolis against pancreatic cancer cell lines *in vitro*, inhibiting cell viability and colony formation in soft agar.

The rationale for propolis nanofood is that an aqueous form of propolis can be administered more efficiently than normal propolis. The applicability of normal propolis in cancer chemotherapy has not been established, but propolis nanofood may advance its application. This article shows the fundamental function of propolis nanofood *in vitro*. There are two differences between normal propolis and propolis nanofood. First, propolis nanofood is more soluble than normal propolis. Second, propolis nanofood is more specific for cancer tissue because its macromolecular structure enhances permeability and the retention (EPR) effect. The EPR effect also provides a great opportunity for more selective targeting of lipid- or polymer-conjugated anticancer drugs, such as SMANCS and PK-1, to the tumor.^{41,42}

Figures 5–7 show that propolis nanofood is soluble and can release propolis under physiological conditions. The

specificity or distribution of propolis nanofood is only shown by *in vivo* analysis. Figures 9 and 10 show the antitumor activity of propolis nanofood *in vitro*; in future studies, we will evaluate the efficacy and toxicity in clinical trials that include PK analysis *in vivo*.

Propolis nanofood opens up avenues for systemic therapy of human cancers wherein the beneficial effects of propolis have been propounded. Future studies using relevant experimental models will enable us to address these scenarios in an *in vivo* setting. Propolis nanofood inhibits pancreatic cell growth in murine xenograft models; these effects are accompanied by a potent anti-angiogenic response and should facilitate the eventual clinical translation of this well-known but under-utilized therapeutic agent. No overt host toxicity is noted when maximal volumes are administered to mice. Taken together with the dismal outlook for patients with human pancreatic carcinoma, our observations suggest that propolis nanofood should be investigated in the clinical setting.

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