Exosomal formulation of anthocyanidins against multiple cancer types

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Over the last two decades, berries and berry bioactives, particularly anthocyanins and their aglycones anthocyanidins (Anthos) have demonstrated excellent anti-oxidant, anti-proliferative, apoptotic and anti-inflammatory properties. However, their physicochemical and pharmacokinetic limitations such as, low permeability, and poor oral bioavailability are considered as unfavorable properties for development as drugs. Therefore there is a need to develop systems for efficient systemic delivery and robust bioavailability. In this study we prepared nano-formulation of bilberry-derived Anthos using exosomes harvested from raw bovine milk. Exosomal formulation of Anthos enhanced antiproliferative and anti-inflammatory effects compared with the free Anthos against various cancer cells \textit{in vitro}. Our data also showed significantly enhanced therapeutic response of exosomal-Anthos formulation compared with the free Anthos against lung cancer tumor xenograft in nude mice. The Anthos showed no signs of gross or systemic toxicity in wild-type mice. Thus, exosomes provide an effective alternative for oral delivery of Anthos that is efficacious, cost-effective, and safe, and this regimen can be developed as a non-toxic, widely applicable therapeutic agent.

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Introduction

Many plant bioactives suffer from notoriously poor bioavailability and instability \cite{1-4} and therefore have failed to shine their full potential despite high doses. Several strategies have been investigated in order to achieve clinical efficacy of drugs with poor bioavailability and stability. Lately, new concepts from the field of nanotechnology have been utilized to overcome the challenge of poor absorption especially for oral delivery and enhancing the outcome of chemopreventives which is often referred to as ‘nano-chemoprevention’ \cite{5,6}. Nano-formulations of agents such as curcumin \cite{7,8}, EGCG \cite{9,10} and resveratrol \cite{11,12} have been explored as polymeric micelles, liposome/phospholipid, nano-emulsions, nanogels, solid lipid nanoparticles, polymer conjugates, etc. Although, some successful liposomal formulations of drugs, \textit{e.g.}, doxorubicin (DoxilR) have made to clinic, others have suffered from limitations of reproducibility and/or toxicity \cite{13}. Unmodified liposomes are unstable \textit{in vivo} with short blood circulation time, and lack of target selectivity \cite{14}. Other limitations of liposomal system include opsonization, change of pharmacokinetics in multiple-dosing regimen, etc. \cite{15}. Polymer-based delivery systems offer advantages of linking various ligands, but its cost-effective production is nowhere in the vicinity. Moreover, there are serious toxicity concerns with the long-term use of polymeric nanoparticles \cite{16}. Exosomes as nano-carriers have the potential to overcome these limitations \cite{17,18}. We have exploited exosomes from bovine milk as biological nanoparticles to deliver agents of both hydrophilic and hydrophobic nature \cite{19}. Additionally, milk exosomes as drug delivery vehicle has positive attributes of being in abundance, cost-effective, scalable and biocompatible.

Berries and berry bioactives, particularly the colored pigments, anthocyanins and their aglycones anthocyanidins (Anthos), are
well known for their anti-oxidant, anti-proliferative, apoptotic and anti-inflammatory properties [20–27]. Anthocyanins are pigments responsible for red and blue hues of many fruits (e.g., berries), vegetables (e.g., purple potatoes), flowers (hibiscus) and grains (purple rice). Blueberry, blackberry, bilberry, raspberry, strawberry are especially rich sources of dietary anthocyanins [28]. Over 600 structurally distinct anthocyanins have been identified in nature [29,30]. The diversity of anthocyanins comes from the number and the position of: (i) hydroxyl (OH) and methoxyl in part, be hydrolyzed to Anthos [42,43]. Other dietary berry-treated animals, suggesting that anthocyanins may, glucuronides) in the plasma as glucuronides has been reported in instability. Presence of conjugated Anthos (Cy and Pe monohybrids) are relatively more hydrophobic in nature which facilitates their passively diffusion across the mucosal epithelium [41]. However, these attributes make anthocyanins poorly bioavailable with limited absorption by passive diffusion. The absorption of anthocyanins across the intestine wall requires either a specific active transport mechanism or needs to be hydrolyzed to their aglycones form by the action of specific enzymes such as β-glucosidase, β-glucuronidase, and α-rhamnosidase by the gut intestinal microflora [40]. In contrast, aglycones lacking sugar moieties are relatively more hydrophobic in nature which facilitates their passively diffusion across the mucosal epithelium [41]. However, at the same time they exhibit considerable chemical instability. Presence of conjugated Anthos (Cy and Pe mono-glucuronides) in the plasma as glucuronides has been reported in dietary berry-treated animals, suggesting that anthocyanins may, in part, be hydrolyzed to Anthos [42,43]. Other flavonoids have also been indicated to undergo similar hydrolysis by intestinal β-glucosidases [44], resulting in the release of their aglycone form. These observations suggest the aglycons of anthocyanins might have a role in biological activity and contribute to protective effects rendered by berry intake.

Our earlier work comparing anti-proliferative effects with purified extracts at anthocyanin and Anthos levels revealed significantly greater activity of anthos over anthocyanins [21,45]. Data from Mukhtar and colleagues, and other laboratories have shown different pharmacological properties of anthocyanidins [46–48]. In addition, we discovered that a mixture of individual Anthos (Dp, Cy, Pe, Pt and Mv) produced synergistic anti-proliferative, anti-tumor and anti-inflammatory effects compared to the individual moieties [21]. We propose that nano-formulation of Anthos (ExoAnthos) will enhance therapeutic efficacy compared with the free Anthos against multiple cancer cell lines.

Materials and methods

Isolation of exosomes

Exosomes were isolated from pooled raw milk from dozens of mid-lactation, pasture-raised Jersey cows, procured from a local dairy as described previously [19,49]. Briefly, exosomes were isolated by a sequential centrifugation process. Milk was first centrifuged at 13,000 × g for 30 min to remove fat, cells and casein debris. Whey thus obtained was centrifuged at 100,000 × g for 60 min to remove larger microvesicles. Finally, the resulting supernatant was centrifuged at 135,000 × g for 90 min to obtain exosomal pellet and suspended in PBS. The protein concentration was determined using BCA kit (Thermo Scientific, Rockford, IL.). exosome solutions were stored in aliquots at ≤5 mg exosomal protein/mL at −80 °C until use. As indicated in our previous publication [19], exosomes isolated from several batches of milk isolated at different times resulted in similar yields and exosomal markers.

Isolation of berry Anthos

Native mixture of Anthos was isolated from standardized anthocyanin-enriched extract of bilberry (Indena, S.p.A., Milan, Italy) by extraction of anthocyanins in 1% HCl, conversion of anthocyanins to Anthos in the presence of 3 N HCl at 90 °C for 1 h, followed by extraction in 1- pentanol and precipitation of the Anthos with petroleum ether [50]. The isolated Anthos were finally purified by C18 Sep-Pak column which provided over 94% purity as analyzed by UPLC. The relative percentages of the individual anthocyanidins, as determined by UPLC against reference compounds, in the isolated Anthos and the original bilberry extract were similar. The relative anthocyanin mixture contains Dp, Cy, Mv, Pe and Pt in the ratio of 33:28:16:16:7 respectively.

Stock solution of the Anthos was prepared based on average molecular weight of the individual anthocyanidins present in the bilberry extract used. The average molecular weight was calculated based on the molecular weight of individual anthocyanidins and their proportions present in the mixture. The profile of bilberry Anthos has been previously shown [21].

Preparation of Anthos-loaded exosomes

To prepare exosomal formulation of Anthos (ExoAnthos), first Anthos were dissolved in a mixture of acetonitrile: ethanol (1:1 v/v). Anthos solution was then added to the exosomes suspended in PBS at 6 mg/mg protein concentration with simple mixing. The solvent concentration was maintained at ≤10% of the total re-actant mixture. The selection of the solvent volume was based on our previous observation in which this concentration did not significantly affect the quality attributes of the exosomes [19]. After mixing the Anthos solution with exosomes, the mixture was kept at room temperature for approximately 15 min, followed by low-speed centrifugation at 10,000 × g for 10 min to remove any unbound Anthos. Anthos-loaded exosomes were collected by centrifugation at 135,000 × g for 90 min. The Anthos pellet thus obtained was suspended in PBS, centrifuged (10,000 × g for 10 min) to remove any residual unbound agent and passed through 0.22 μm syringe filter to remove any microbial contamination. The formulation was stored in aliquots at −80 °C until use.

Formulation characterization

Particle size and poly dispersity index (PDI) analysis

Exosome and ExoAnthos formulations were diluted (1 mg/ml) in PBS and analyzed for particle size and PDI using a Zetasizer (Malvern Instruments Ltd, Malvern, Worcestershire, UK).

Drug loading analysis

The practical load of Anthos in the ExoAnthos formulation was determined by measuring the protein and Anthos concentrations. Briefly, 50 μl of the ExoAnthos formulation was mixed with 0.95 ml acetonitrile to extract the Anthos and precipitate the proteins. The precipitated proteins were separated by centrifugation measuring the protein and Anthos concentrations. Briefly, 50 μl of the ExoAnthos formulation was mixed with 0.95 ml acetonitrile to extract the Anthos and precipitate the proteins.

UPLC analysis

Samples (15 μl) containing Anthos were analyzed by UPLC using a Shimadzu Premier C18 reverse-phase column (250 × 4.6 mm i.d., 5 μm). Mobile phase A, water; formic acid: acetonitrile (87:10:3) and B, water; formic acid: acetonitrile (40:60). The column was maintained at 40 °C with a flow rate of 0.6 ml/min. The gradient condition was 0–5 min, 5%; B: 5–15 min, 15%; B: 15–20 min, 25%; B: 20–30 min, 35%; B: 30–40 min, 45%; B: 40–45 min, 100%; B: 45–50 min, 5%. Anthos were detected at 250 nm by PDA-UV and total Anthos concentration was calculated against a standard curve. Reference anthocyanidins used were purchased from Chromadex (Irvine, CA).

 Atomic force microscopy (AFM) for morphology analysis

The morphology of the exosomes and ExoAnthos were determined by AFM. Exosomes and ExoAnthos were diluted to 10 μg/ml using deionized water and then 2 μl of the respective samples was placed on a silica wafer and air dried for 30 min. AFM (Asylum MF-3D, Oxford Instruments, Goleta, CA) images were captured in
tapping mode using aluminum-coated silicon probes. Topographic height, amplitude and phase retraces were imaged with a fixed force (<1 nN) at a scanning rate of 1 Hz. The images were processed using iQOR software (WaveMetrics, Inc., Portland, OR).

Storage stability
Stability of the exosomes and ExoAnths stored at −80 °C was examined. For ExoAnths twelve days after the storage, triplicate samples were thawed and the aliquots were divided in two parts: one part was used for particle size and PDI measurement after appropriate dilution as described in Section Formulation characterization while the other part was analyzed for Anthos content by UPLC.

In vitro studies
Cell culture
Human lung cancer (A549 and H1299), breast cancer (MDA-MB-231 and MCF7), pancreatic (PANC1 and Mia PaCa2), Prostate (PC3 and DU145), colon (HCT116) and ovarian (OVCAC432) cell lines were obtained from American Type Cell Culture (ATCC, Manassas, VA) or provided by colleagues at the University of Louisville Health Sciences, Louisville, KY. All cancer cell lines were supplemented with 10% fetal bovine serum and 1% antibiotics (Penicillin/Streptomycin). The A549 and PC3 cells were grown in F-12 K media. The MCF7, PANC1 and Mia PaCa2 received Dulbecco’s Modified Eagle’s Medium (DMEM). Media for MCF7 cells was also supplemented with 0.2 units/ml insulin. MDA-MB-231 cells were grown in L-15 media. The H1299 and OVCAC432 received RPMI media. DU145 were maintained in Eagle’s Minimum Essential Medium (EMEM) and HCT116 received McCoy’s Medium. All cell lines were maintained at 37 °C in a humidified chamber at 5% CO2 except MDA-MB-231 which were grown in absence of CO2.

Antiproliferative activity
The antiproliferative activity of the Anthos, ExoAnths and exosomes alone against various cancer cell lines was assessed by MTT assay, as described elsewhere [51]. Briefly, cells were seeded in a 96-well plate and treated with either Anthos, ExoAnths or exosomes alone at various concentrations for 72 h. At the end of treatment period the medium was replaced with media containing MTI (0.5 mg/ml) and incubated for 2 h, followed by solubilization of formazan crystals with DMSO at time and spectrophotometric measurement at 570 nm. IC50 values were calculated using GraphPad Prism statistical software (version 4.03; La Jolla, CA). Two-way ANOVA followed by Bonferroni post-test was used for xenograft study. Data in the xenograft studies is expressed as mean ± standard error of mean (SEM) (n = 8–10).

Results
Characteristics of Anthos-loaded exosomes
Exosomes isolated from bovine milk had average size of 79 ± 5.2. Exosomes loaded with Anthos exhibited insignificant increase with particle size of 83 ± 17 nm as measured by zetasizer; the PDI of Exo and ExoAnths was found to be 0.39 and 0.23, respectively (Fig. 1A). The practical drug loading was found to be −20% in ExoAnths formulation. Surface morphology determination by AFM (Fig. 1B) revealed that the spherical shape of exosome particles remained unaltered after Anthos loading in ExoAnths formulations. The size of the exosomes and ExoAnths determined by AFM analysis was, in fact, somewhat smaller (50–60 nm and 80–90 nm, respectively) compared with the size measured with zetasizer. Analysis of exosomes stored as frozen aliquots at −80 °C were found to essentially retain the Anthos stability as evident by the unaltered HLPC profiles (Fig. 2). There was no effect of storage on particle size and PDI as well.

Anti-proliferative effects of exosomes alone on cancer cells
We first determined the anti-proliferative effects of milk exosomes per se (in the absence of any drug) on cancer cell lines of lung, prostate, colon, breast, pancreatic and ovarian by MTT assay. The treatment of these cell lines with 50 μg/ml exosomal proteins for 72 h resulted in 8–47% cell growth inhibition (Fig. 3). Survival of lung cancer cells A549 and H1299 was reduced to 66% and 76%; breast cancer cells MCF7 and MDA-MB-231 showed cell survival of 55% and 76%, respectively; both pancreatic cancer cells Panc1 and Mia Paca2 showed nearly 65% cell survival; prostate cancer cell line DU145 was the most sensitive with 53% and PC3 cell were least affected with 92% survival; while survival of ovarian cancer cells OVCA432 and colon cancer cells HCT116 was reduced to nearly 68% in each case. These findings indicate intrinsic anti-cancer activity of milk exosomes, which might be of additional benefit when exosomes are employed as vehicles to deliver anti-cancer drugs. We earlier reported exosomes alone had no cytotoxic effects on normal bronchial epithelial cells at 50 μg/ml exosomal protein concentration [19]. These protective effects are presumably related to some endogenous factors, e.g., therapeutic activity of complex of human milk-derived α-lactalbumin and oleic acid (HAMLET) [53] and bovine milk-derived α-lactalbumin and oleic acid (BAMLET) [53–55] trigger tumor cell death.

Anti-proliferative effects of Anthos and ExoAnths on cancer cells
Next we assessed the anti-proliferative effects of Anthos alone and in exosomal formulation on various cancer cell lines. The findings indicate that irrespective of the cancer cell type ExoAnths has significantly higher anti-proliferative effects (−4–60 fold decrease in IC50 Values) compared with the free Anthos (Fig. 4). ExoAnths also exhibited better dose-dependent anti-proliferative effects than Anthos alone. The IC50 values shown in Table 1 were derived from a range of concentrations up to 500 μM for Anthos and 100 μM for ExoAnths depending on cancer cell type. The observed enhanced

Clark, CA) and liver and kidney function enzymes. Electrolyte analysis was done by using ion-selective electrode while other biochemical parameters were analyzed spectrophotometrically by using ANOVA. Immuno Analyzer (Beckman Coulter, Inc., Brea, CA).

Statistical analysis
Statistical analysis was performed with Graph Pad Prism statistical software (version 4.03; La Jolla, CA). Two-way ANOVA followed by Bonferroni post-test was used for xenograft study. Data in the xenograft studies is expressed as mean ± standard error of mean (SEM) (n = 8–10).

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in vitro anti-proliferative effects could be attributed to increased stability of the Anthos in media when loaded on to exosomes and higher cell uptake; part of the anti-proliferative effects presumably resulted from the intrinsic activity of exosomes.

Anti-inflammatory effects of Anthos and ExoAnthos

The nuclear factor-kappa B is a classic pro-inflammatory signaling molecule, as it plays a key role in the expression of pro-inflammatory genes such as cytokines, chemokines, and adhesion molecules [56]. We have earlier reported anti-inflammatory activity of Anthos [21]. Here we evaluated the effect of Anthos alone and in exosomal formulation against TNFα-induced NF-κB activity in lung (H1299) and breast (MCF7) cancer cells. The TNFα treatment resulted in nearly 6-fold increase in the levels of NF-κB. Although Anthos exhibited ability to inhibit TNFα-induced NF-κB activity this effect was markedly enhanced when Anthos were delivered in exosomal formulation (Fig. 5). In case of the H1299 cells although, Anthos alone at 200 μM completely knocked out TNFα-induced NF-κB levels, lower doses of Anthos had no effect on cells. When H1296 cells were treated with ExoAnthos there was dose-dependent inhibition of NF-κB. On other hand, the MCF7 cells also showed inhibition of NF-κB activity; however, this effect was not concentration-dependent. Overall, ExoAnthos exhibited greater dose-dependent inhibition of NF-κB activity compared to the free Anthos.

In vivo antitumor efficacy of Anthos and ExoAnthos

We determined the antitumor efficacy of Anthos and ExoAnthos using athymic nude mice bearing subcutaneous lung cancer A549 xenografts and compared with exosomes alone. We did not observe any gross toxicity due to Anthos or ExoAnthos, as no significant change was observed in the body weight, diet consumption and the animal movement compared with the vehicle treatment. We have earlier shown modest anti-tumor effects when animals were treated with exosomes alone in comparison with vehicle (PBS) treatment [19]. In the current study compared to treatment with vehicle (PBS), Anthos (10 mg/kg) showed a slight but insignificant inhibition of the tumor growth. However, ExoAnthos (5 mg/kg Anthos and 50 mg exosomal proteins/kg) showed a significant growth inhibition (Fig. 6). Noteworthy is that the Anthos concentration in ExoAnthos formulation at one half of the free Anthos was effective, while the free Anthos was ineffective. The growth inhibition became obvious after four weeks of ExoAnthos treatment with significant higher (p < 0.01) inhibition in subsequent weeks.

Toxicity study

Systemic toxicity of Anthos was evaluated using wild-type mice which received the Anthos (oral gavage: 8 mg/kg, 5 times a week or 40 mg/kg weekly dose). Here we purposely used somewhat higher dose in comparison with anti-tumor study (weekly dose of 30 mg/
kg as free Anthos or 15 mg/kg in exosomal formulation). We have recently demonstrated non-toxicity of milk exosomes [19] hence comparisons were made to vehicle (PBS) control group. No signs of gross toxicity due to the Anthos were observed, as there was no significant change in the body weight, diet consumption and the animal movement compared with the vehicle treatment (data not shown). The effect of the Anthos on the hematological and serum biochemical parameters of liver and kidney functions were examined after three weeks of the treatment. As shown in Supplementary Fig. 1, no difference in leukocytes, erythrocyte counts as well as liver and kidney function enzymes was observed in Anthos-treated animals and their levels were well within the normal physiological range of age-matched control animals.

### Discussion

Accumulating evidence indicate therapeutic effects of phytochemicals especially for the colored pigments from berries that exist as glycoside of anthocyanidins. Several studies examined the molecular mechanisms for the health benefits [28,38,41]. However their physicochemical and pharmacokinetic limitations such as, low permeability, and poor oral bioavailability [23,39,40] is widely accepted. There are mixed views in the literature on absorption and bioavailability of anthocyanins and Anthos. Glycosylation and acylation are considered the two structural features that dramatically affect anthocyanin absorption and influences bioavailability [30]. Some studies demonstrated that anthocyanins are mostly absorbed in their intact glycoside forms, while other studies indicated that they are first metabolized by the gut microflora, and held in their aglycone anthocyanidins forms [40,43]. A search for clinical trials conducted with anthocyanins/anthocyanidins at ClinicalTrials.gov show at least 72 completed and ongoing studies that have made attempts to understand the bioavailability, pharmacokinetics and health effects. These studies indicate a serious interest for clinical translation of anthocyanins as a viable therapeutic agent. However, their propensity to undergo extensive metabolism and instability are considered unfavorable properties that limit its suitability for development as potential drugs.

Despite well-accepted poor bioavailability of anthocyanins and Anthos, only limited attempts have been made to develop formulations to address this issue. Previously Stoner and Mallery groups developed freeze-dried black raspberry (FBR) aqueous gel for topical application on premalignant oral lesions. Although the treatment resulted in histopathologic regression of lesions, the chemopreventive benefit was not observed in all trial participants [36]. In another attempt, these groups formulated millicylindrical injectable implants of FBR ethanol extract using poly (l-lactic-glycolic acid) (PLGA) and poly (l-lactic acid) (PLA) for sustained delivery of chemopreventive FBR anthocyanins [37,57]. Although implantable vehicles alleviate concerns with multiple dosing regimens to address this issue. Previously Stoner and Mallery groups developed freeze-dried black raspberry (FBR) aqueous gel for topical application on premalignant oral lesions. Although the treatment resulted in histopathologic regression of lesions, the chemopreventive benefit was not observed in all trial participants [36]. In another attempt, these groups formulated millicylindrical injectable implants of FBR ethanol extract using poly (l-lactic-glycolic acid) (PLGA) and poly (l-lactic acid) (PLA) for sustained delivery of chemopreventive FBR anthocyanins [37,57]. Although implantable vehicles alleviate concerns with multiple dosing schedules associated with gel formulations, this is not patient friendly. Oral delivery of drug is the most patient friendly with highest compliance. Recently, alginate-based in situ gastroretentive gelling [58] and microencapsulation with maltodextrin, whey protein isolate and beet pectin [59–61] systems are explored to increase stability, modulate the release and increase the retention time of anthocyanins.

Based on our published [21,45] and unpublished findings indicating Anthos to have higher therapeutic effect both in vitro and in vivo compared to anthocyanins, we believe that Anthos may be the biologically-active moieties and a superior candidate to anthocyanins for drug development. Anthos have been investigated only in limited studies compared to anthocyanins due to unavailability of pure compounds in large quantities. We have developed simple solvent–solvent extraction-based method for the isolation of anthocyanidins from various sources and have chemically modified them to Anthos. We have developed a nanosized formulation of Anthos and have demonstrated their antiproliferative activity against various cancer cells in vitro.

**Fig. 3. Intrinsic activity of bovine milk-derived exosomes to inhibit growth of various human cancer cell types:** Cancer cell lines of lung, breast, ovarian, colon, pancreas and prostate were treated with 50 μg/ml exosomal proteins for 72 h and effect on cell growth inhibition was assessed by MTT assay and compared with untreated cells. Statistical analysis was performed using Student t-test to compare exosomes alone with vehicle treatment. *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001.
Fig. 4. Anti-proliferative activity of Anthos-loaded exosomes (ExoAnthos) against various cancer cell types: Cancer cell lines of lung, breast, ovarian, colon, pancreas and prostate were treated with various concentrations of bilberry-derived Anthos or ExoAnthos for 72 h and effect on cell growth inhibition was assessed by MTT assay.
of pure Anthos from enriched berry extracts [50]. Our experimental evidence also demonstrate synergistic activity of the native mixture of the five Anthos in bilberry, namely, Dp, Cy, Pu, Pe, Mv compared to individual entities [21]. Anthos are considered to be much prone to avid chemical decomposition than the glycoside counterparts. Therefore, they make a suitable candidate for nano-encapsulation to improve treatment efficacy.

Exosomes are biological nanoparticles that are drawing great attention as drug delivery vehicle due to their cell-based origins [62–65]. Attempts have been made in the recent past to isolate exosomes from both non-cancerous and cancer cells in culture and fruit juices for delivering the chemotherapeutic drugs, doxorubicin [66,67], paclitaxel [68,69] and natural agents such as curcumin [70], withaferin A [19] and celastrol [49]. Bulk isolation and safety concerns are the major limitations of such approaches. Our laboratory has recently shown that bovine milk-derived exosomes as a safe alternative source and demonstrated incorporation of both plant bioactives and chemotherapeutic drugs [19] for enhanced therapeutic attributes such as size and PDI was observed between Anthos-loaded and naked exosomes. We postulate that the lipid and protein nature might, in part, play a role in drug loading on exosome; however passive diffusion of the drug in to the lumen of the exosomes cannot be ruled out.

Several studies, including our own, have reported anti-cancer effects of Anthos both in vitro and in vivo. In agreement with earlier reports we demonstrated in vitro anti-proliferative activity of the Anthos against six different cancer cell types. The observed anti-proliferative effect of the Anthos was further enhanced with exosomal formulation by a nearly 4–60-fold reduction in the IC50 values. Naked exosomes also exhibited intrinsic mild to modest anti-proliferative effects in all cancer cell lines tested; these findings were in-line with our previous observations [19]. However exosomes alone were relatively safe with no cytotoxic effects observed against normal cells [19]. This difference in response to milk exosomes by cancer and normal cells may be due to differential uptake, retention and response to internal payload of exosomes.

The activation NF-κB signaling pathway by inflammatory mediators like interleukins and cytokines has been reported in many cancers [56]. Anti-inflammatory effects of Anthos have been documented earlier by us [21]. Similarly, treatment with Anthos and ExoAnthos caused inhibition the TNFα-induced activation of NF-κB in cancer cells. ExoAnthos manifested significantly greater

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**Table 1**

IC50 values of Anthos and ExoAnthos of various cancer cell lines.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cell line</th>
<th>IC50 (μM)</th>
<th>ExoAnthos</th>
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<td>MDA-MB-231</td>
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<td>68</td>
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<tr>
<td>Lung</td>
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<td>A549</td>
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<td>Ovac432</td>
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**Fig. 5. Anti-inflammatory activity of Anthos-loaded exosomes (ExoAnthos) against cancer cells:** Lung and breast cancer cells were with indicated concentrations of Anthos or ExoAnthos for 24 h and effect on NFκB activity was assessed by EMSA. Panels on the bottom represent quantification of the band and expressed as fold change. Statistical analysis was performed using Student t-test to compare TNF-α + Anthos and TNF-α + ExoAnthos vs TNF-α alone group. **p ≤ 0.01 and ***p ≤ 0.001.

**Fig. 6. Anti-tumor activity of Anthos-loaded exosomes (ExoAnthos) against lung tumors:** Following inoculation with A549 cells, nude mice were treated oral gavage three times a week with Exo-Anthos (5 mg Anthos and 50 mg Exo protein/kg bw). Three other groups were treated i.p. with Exo alone (50 mg/kg) or Anthos (10 mg/kg). Data represent average ± SE of means (n = 7–8). Significance of ExoAnthos vs. Exo alone group is shown.
inhibition of NF-κB activation than the free Anthos. Oral delivery of EoAnthos also resulted in enhanced anti-tumor activity against lung cancer in nude mice. In view of experimental evidence suggesting improved stability of Anthos in exosomal formulation, we postulate that the enhanced anticancer effects of EoAnthos is likely due to higher stability, higher uptake and/or slow release of Anthos from the exosomes. In addition, other principles such as passive targeting by enhanced permeability and retention effect could also contribute to explain these results. Nevertheless, in-depth analysis of stability and release kinetics under various physiological conditions need to be performed rigorously to confirm the above findings.

Information on the toxicity of the Anthos against wild-type mice with a weekly dose of 40 mg/kg had no effect on the hematological and serum biochemical parameters of liver and kidney functions indicating that the Anthos were well tolerated. In addition we have established and reported safety of milk exosomes in our earlier studies [19]. Although oral exosomal formulation of Anthos needs to be examined for tolerance and toxicity under chronic conditions, in view of the safety profile of Anthos and exosomes alone in our prior studies there is little concern that EoAnthos would raise undesired effects.

In summary, our report demonstrates improved stability and anti-cancer effects of exosomal formulation of the Anthos against multiple cancer types. The greater efficacy exhibited by exosomal formulation of Anthos, in part, comes from the intrinsic activity of naked exosomes, which is a ‘bonus’ effect not common to traditional drug delivery vehicles. Encapsulation of Anthos onto/into milk exosomes can achieve higher drug efficacy with no toxic side effects. Thus, exosomes provide an effective alternative for oral delivery of Anthos that is efficacious, cost-effective and safe for the treatment of multiple cancers.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2017.02.004.

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