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Original Article

Exosomal formulation of anthocyanidins against multiple cancer types

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ABSTRACT

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 *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.

Introduction

Many plant bioactives suffer from notoriously poor bioavailability and instability [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' [5,6]. Nano-formulations of agents such as curcumin [7,8], EGCG [9,10] and resveratrol [11,12] have been explored as polymeric micelles, liposome/phospholipid, nano-/micro-emulsions, nanogels, solid lipid nanoparticles, polymer conjugates, etc. Although, some successful liposomal formulations of drugs. [e.g., doxorubicin (DoxilR)] have made to clinic, others have suffered from limitations of reproducibility and/or toxicity [13]. Unmodified liposomes are unstable *in vivo* with short blood circulation time, and lack of target selectivity [14]. Other limitations of liposomal system include opsonization, change of pharmacokinetics in multiple-dosing regimen, etc. [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 [16]. Exosomes as nano-carriers have the potential to overcome these limitations [17,18]. We have exploited exosomes from bovine milk as biological nanoparticles to deliver agents of both hydrophilic and hydrophobic nature [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

Abbreviations: miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; AFM, atomic force microscopy; Anthos, anthocyanidins; ExoAnthos, Exosomal formulation of Anthos; Cy, cyaniding; Dp, delphinidin; Pt, petunidin; Pe, peonidin; Pg, pelargonidin; Mv, malvidin; EMSA, Electrophoretic Mobility Shift Assay (EMSA); UPLC, ultra high-performance liquid chromatography; TNF-α, tumor necrosis factor-α; PDI, poly dispersity index.

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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 (OCH_3) groups ii) type of sugars, and (iii) type of aliphatic or aromatic acids attached to the sugar. There are approximately 17 Anthos of which 6 occur widely, namely, cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pe), pelargonidin (Pg), and malvidin (Mv) [28].

Accumulating evidence from our laboratory [20–24] and other researchers [25–27] strongly indicate its preventive and therapeutic activity against various cancer types [31,32]. There have been few attempts towards translation of berry bioactives in clinical trials using berry powder [33–35]. However, the amount of berry powder that needs to be consumed daily for therapeutic response is not readily translatable/practical; in addition nearly 60% berry powder is composed of sugars, which further limit target patient population. Berry extracts [36] and anthocyanins on other hand address these concerns with some positive effects on pre-neoplastic lesions or cancers of the oral cavity, esophagus and colon, however, it still faces the challenge of poor oral bioavailability and stability [2,36–39].

Anthocyanins are highly water-soluble molecules. Glycosylation confers increased stability and water solubility and acylation of the sugar residues further improves anthocyanin stability [30]. 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 monoglucuronides) 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 antiproliferative, anti-tumor and anti-inflammatory effects compared to the individual moieties [21]. We propose that nano-formulation of Anthos will not only increase its stability and bioavailability resulting in enhanced therapeutic effects but will also facilitate clinical translation by overcoming limitations associated with the use of whole berries, berry powder and berry extracts. In this study, we investigated if exosomal 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 of ≤6 mg exosomal proteins/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 anthocyanidin 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/ml protein concentration by simple mixing. The solvent concentration was maintained at ≤10% of the total reaction 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 ExoAnthos 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 exosomal proteins. The precipitated proteins were separated by centrifugation (10,000 × g for 10 min), and supernatant containing the Anthos was analyzed by UPLC. The pellet was suspended in PBS and exosomal proteins were analyzed by BCA method.

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 40:10:50 were used at 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% B. Anthos were detected at 520 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