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Research paper

Bromelain nanoparticles protect against 7,12-dimethylbenz[a] anthracene induced skin carcinogenesis in mouse model

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ABSTRACT

Conventional cancer chemotherapy leads to severe side effects, which limits its use. Nanoparticles (NPs) based delivery systems offer an effective alternative. Several evidences highlight the importance of Bromelain (BL), a proteolytic enzyme, as an anti-tumor agent which however has been limited due to the requirement of high doses at the tumor site. Therefore, we illustrate the development of BL loaded poly (lactic-co-glycolic acid) NPs that show enhanced anti-tumor effects compared to free BL. The formulated NPs with a mean particle size of 130.4 ± 8.81 nm exhibited sustained release of BL. Subsequent investigation revealed enhanced anti-tumor ability of NPs in 2-stage skin tumorigenesis mice model. Reduction in average number of tumors (~2.3 folds), delay in tumorigenesis (~2 weeks), percent tumorigenesis (~4 folds), and percent mortality rate as well as a reduction in the average tumor volume (~2.5 folds) in mice as compared to free BL were observed. The NPs were found to be superior in exerting chemopreventive effects over chemotherapeutic effects at 10 fold reduced dose than free BL, validated by the enhanced ability of NPs (~1.8 folds) to protect the DNA from induced damage. The effects were also supported by histopathological evaluations. NPs were also capable of modulating the expression of pro-apoptotic (P53, Bax) and anti-apoptotic (Bcl2) proteins. Therefore, our findings demonstrate that developed NPs formulation could be used to improve the efficacy of chemotherapy by exerting chemo-preventive effects against induced carcinogenesis at lower dosages.

1. Introduction

Cancer is one of the leading causes of morbidity and accounts for almost 7.6 million deaths annually worldwide [1]. Amongst cancers of all types, instances of skin cancer are reportedly increasing as their early detection is comparatively easier [2]. Surgery is often the most frequent approach to remove a localized tumor, but in the later stage of a metastasized cancer, use of chemotherapeutic agents, radiotherapy, or a combination of both is required to completely eliminate the tumor but does not work in most of the cases [3]. Chemotherapy usually involves long term chronic exposure to chemotherapeutic drugs, which leads to various physiological complications, severe/moderate distress and cytotoxicity [4,5].

Recently, topical application of anti-cancer drugs has gained tremendous importance as they offer several benefits such as ease of availability, application and reduced systemic side effects with no drug degradation in the gastrointestinal tract [6,7]. Thus, topical administration of anti-cancer drugs provides an attractive alternative to increase drug targeting and penetration of drugs in sufficient levels in the tumor tissues [8]. Contrary to this, reports suggest that the stratum corneum, the outermost layer of the epidermis, acts as a skin barrier, preventing entry of the majority of drugs into the viable skin [9]. In addition, the ability of tumor cells to develop multidrug resistance due to P-glycoprotein on their surface results in exudation of drug from tumor cells thus increasing the difficulties associated with conventional chemotherapy [10,11]. Several formulations have, therefore, been developed to overcome these barriers and to reach skin malignancies by favoring drug penetration into the deep layers of the epidermis [12,13]. In this regard, scientists have generated great interest in the nanoparticles (NPs) based delivery systems owing to their ability to have enhanced penetration and retention (EPR) effect at the tumor site [14,15]. Several reports have demonstrated the ability of NPs to release anti-cancer drugs into cells without triggering the P-glycoprotein pump as they internalize into the target cells by

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endocytosis [16,17]. In addition, NPs also provide protection to the drug against degradation and therefore, sustained release of drug molecules from the NPs surface could be achieved at the tumor site with high therapeutic efficiency and low cytotoxicity. Moreover, nano-carriers can reduce skin irritation by avoiding direct contact of the drug with the skin surface [18]. Various nano-carriers based on liposomes, biodegradable polymers, lipids, chitosan, dextrans, etc. have been studied extensively for the topical application of anti-cancer drugs such as 5-fluorouracil [19–21].

Recent studies strongly indicate that certain commonly consumed dietary photochemicals have potent cancer protective effects against a variety of human cancers [22]. More than 25% of drugs used during the last 20 years are directly derived from plants, while the other 25% are the chemically altered natural products [23]. The advantage of using such compounds for cancer treatment is that they are relatively non-toxic/less toxic at therapeutic doses. Several molecules, including paclitaxel (obtained from the bark of the Pacific yew tree), vincristine (from *Catharanthus roseus*), topotecan, curcumin (from Indian spice turmeric), epigallocatechin gallate (from green tea), etc. have been shown to possess excellent anti-cancer properties against a variety of human cancers and some of them are already in clinical use [24]. Among the various natural phytochemicals, Bromelain (BL), which belongs to a family of sulfhydryl proteolytic enzymes obtained from the pineapple plant (*Ananas comosus*), has been shown to possess anti-proliferative and anti-metastatic activities in tumor models *in vitro* and *in vivo* [25,26]. Presumably, BL is known to impart its anti-cancerous activity via immune, inflammatory, and homeostatic pathways, as well as it exerts its influence on a variety of molecules involved in cell signaling [26]. Although the mechanism of anti-cancer activity of BL is known, its therapeutic efficacy is low due to necessity of high doses of the drug at the tumor site.

In order to further increase the potency of BL against solid tumors, a concept of nano-chemoprevention has been made. We hypothesized that formation of safe and biocompatible BL encapsulated stable NPs would provide a prolonged release of BL at the tumor site, hereby coupled with the EPR effect exerted by NPs could offer more consistent biological results. Thus, in the present investigation, BL was encapsulated in an FDA approved biodegradable and biocompatible, poly (lactic-co-glycolic acid) (PLGA) polymer, formulating NPs named as, BL-PLGA NPs. Formulated BL-PLGA NPs were characterized and then evaluated for their anti-cancer efficacy in 7,12-dimethylbenz[a]anthracene (DMBA) induced and 12-O-tetradecanoylphorbol-13-acetate (TPA) promoted 2-stage skin tumorigenesis model in Swiss albino mice. The experiments were carried out to investigate the chemo-preventive and chemotherapeutic effects of BL-PLGA NPs and compared with that of free BL.

2. Materials and methods

2.1. Materials

Poly (lactic-co-glycolic acid) (PLGA) 50:50 (m.wt. 30–60 kDa), Bromelain from pineapple stem, poly vinyl alcohol (PVA) (m.wt. 30 kDa), sodium bicarbonate, sucrose, casein, 7,12-dimethylbenz [a]anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), sodium dodecylsulphate (SDS) and bicinchoninic acid (BCA) kit for protein estimation were purchased from Sigma–Aldrich (St. Louis, USA). Other chemicals and reagents were purchased locally and were of highest purity grade.

2.2. Preparation of BL-PLGA NPs

BL-PLGA NPs were prepared using double emulsion solvent evaporation technique, as described previously in the literature by our group [27]. The formulated NPs with 84 ± 3.4% yield were obtained and stored at 4 °C until further use.

2.3. Characterization of BL-PLGA NPs

2.3.1. Particle size analysis by Dynamic Light Scattering

The formulated NPs were evaluated for their mean particle size and distribution by Dynamic Light Scattering (DLS) technique (Zetasizer Nano-ZS, Malvern Instruments, UK), employing a nominal 5 mW He–Ne laser operating at 633 nm as described here. Briefly, BL-PLGA NPs were suspended in double distilled water at a concentration of 0.5 mg/mL and the particle size was measured. The measurements were carried out at 25 ± 2 °C with the following settings: 14 measurements per sample, refractive index of water: 1.33, viscosity for water: 0.89 cP. The particle size reported was the average of three samples. The particle size of the NPs was computed from the intensity–intensity correlation function using the Malvern software package based on the theory of Brownian motion and Stroke's equation.

2.3.2. Transmission Electron Microscopy

The morphology and size of the BL-PLGA NPs were evaluated using Transmission Electron Microscopy (TEM). Briefly, BL-PLGA NPs were suspended in double distilled water at a concentration of 0.5 mg/mL, and a drop of the suspension and a drop of 1% uranyl acetate were placed gently on a formvar-coated TEM grid surface. Q5 After 30 min of incubation, excess fluid was removed and the grid surface was allowed to air dry at 25 ± 2 °C. The particles were visualized under the microscope (FEI Company, Hillsboro, OR) operated at 80 kV and attached to a Gatan Digital Micrograph™ (Gatan Inc, Pleasanton, CA).

2.3.3. Encapsulation efficiency and drug loading

The encapsulation efficiency (% EE) and drug loading (% DL) of BL in the BL-PLGA NPs were evaluated as done previously [27].

2.3.4. In vitro release study

In vitro release profile of BL from BL-PLGA NPs was evaluated in phosphate buffer saline (PBS) at the physiological pH (7.2–7.4) and acidic pH (6.2–6.5). In brief, 10 mg BL-PLGA NPs were suspended in 1 mL of respective buffers and kept in an incubator shaker pre-maintained at 37 ± 0.5 °C with constant stirring. At predetermined time intervals, the suspension was centrifuged at 100*g* for 10 min at 4 °C; the supernatant was analyzed for total BL content by BCA assay. Equal amount of fresh buffer was added to the NPs pellet and the release study was continued. The amount of the BL release was then calculated using a previously drawn standard curve of BL in the same buffer.

2.3.5. Proteolytic activity of BL in BL-PLGA NPs: Casein Digesting Unit

The enzymatic activity of BL encapsulated in BL-PLGA NPs was measured by estimating its ability to act on its substrate, casein and measured Casein Digestion Units (CDU), as described earlier [27]. The experiment was carried out in triplicate.

2.3.6. Colloidal stability of BL-PLGA NPs

The colloidal stability of BL-PLGA NPs was examined by measuring change in their hydrodynamic diameter during storage using DLS technique. Briefly, 0.5 mg BL-PLGA NPs were dispersed in 1 mL of PBS pH 7.2–7.4 and 6.2–6.5, respectively. The NPs suspension was stored at 4 °C. At regular intervals of time, the particle size was measured by DLS using a Zetasizer Nano-ZS instrument. The experiment was carried out in triplicate.

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