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Protein stability, conformational change and binding mechanism of human serum albumin upon binding of embelin and its role in disease control

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ABSTRACT

Here, we present the inclusive binding mode of phytochemical embelin, an anticancer drug with human serum albumin (HSA) established under physiological condition. Also, to understand the pharmacological role of embelin molecule, here, we have studied the anti-cancer activity of embelin on human cervical cancer cell line (HeLa cell line), which revealed that embelin showed dose dependent inhibition in the growth of cancer cells and also induces 26.3% of apoptosis at an IC_{50} value of 29 μM. Further, embelin was titrated with HSA and the fluorescence emission quenching of HSA due to the formation of the HSA–embelin complex was observed. The binding constant of this complex is $5.9 \pm .01 \times 10^4\ M^{-1}$ and the number of bound embelin molecules is approximately 1.0. Consequently, molecular displacement and computational docking experiments show that the embelin is binding to subdomain IB to HSA. Further evidence from microTOF-Q mass spectrometry showed an increase in mass from 66,563 Da to 66,857 Da observed for free HSA and HSA + embelin complex, signifying that there is robust binding of embelin with HSA. In addition, the variations of HSA secondary structural elements in presence of embelin were confirmed by circular dichroism which indicates partial unfolding of protein. Furthermore, the transmission electron micrographs established that complex formation leads to aggregation of HSA plus embelin. Molecular dynamics simulations revealed that the stability of the HSA–embelin complexes and results suggests that at around 3500 ps the complex reaches equilibration state which clearly contributes to the understanding of the stability of the HSA–embelin complexes.

1. Introduction

Embelin (2,5-dihydroxy-3-undecyl-[1,4]benzoquinone) is a plant-based benzoquinone derivative amphiphilic in nature, having an alkyl chain and a dihydroxybenzoquinone unit. The molecular formula and weight of this compound are $C_{17}H_{26}O_4$ and 294.39 Da, respectively (Fig. 1, insert). Embelin can be extracted from plants and this substance like phenolic lipids is considered to be secondary metabolites which are not essential for cell growth [1]. The alkyl chain increases cell permeability, and embelin itself has been found to inhibit X-linked inhibitors of apoptosis proteins (XIAP) and these XIA-proteins tend to interact with caspase-9, an important initiator of apoptosis, thus helping cancer cells to become immortal by blocking their programmed cell death [2]. Previous studies revealed that embelin displayed various biological activities like free radical scavenging, wound healing, and anti-inflammatory, antitumor, analgesic, antibacterial, antihelmintic and antifertility effects [3]. It also exhibits the inhibition of endothelial mitochondrial respiration and impairs neoangiogenesis during tumor growth and wound healing [4]. Recently studies showed that it is also a cardioprotective agent [5]. The action of embelin, a naturally occurring plant benzoquinone with male fertility regulating potential was investigated and showed changes in the concentrations of testosterone, luteinising hormone and progesterone associated upon administration of embelin [6]. Few reports on embelin derivatives show significant antimitotic activity [7]. Given the potential uses of embelin, the current work is to investigate the interaction mechanism with HSA and to explore its pharmacological importance.

Human plasma, having various proteins, includes human serum albumin (HSA) and α-1-acid glycoprotein (AGP). These two plasma protein have high affinity to bind a wide range of ligands and metabolites [8,9]. HSA is an abundant plasma protein in the human blood circulation system, and it is synthesized in the liver. The presence of HSA is about 60%, whereas AGP is 3% of the total plasma protein [10]. HSA is a single polypeptide chain of 585 amino acids with an average molecular weight of 66.5 kDa, and is a major soluble protein constituent of the circulatory system. It comprises 17 disulfide bridges involved in stabilization of protein [11,12]. This protein is very widely used as a model system for pharmacokinetics studies due to its unique ability to transport fatty acids, drugs, organic and inorganic ions and nutrients. HSA is a monomeric multi domain heart shaped globular protein, is the most common

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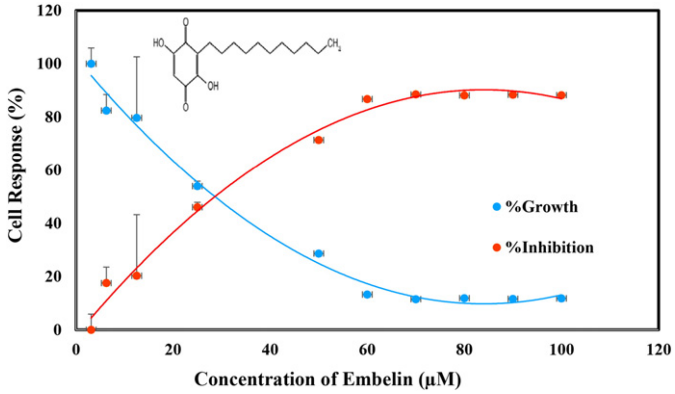

Fig. 1. Cytotoxic study was carried out using HeLa cell lines and cell growth was measured by the MTT assay. Insert shows the structure of embelin and the molecular formula $C_{17}H_{26}O_4$ and 294.39 Da, respectively.

circulating protein in the blood, and represents the major controlling element of fluid dispersal among body compartments, and the most pivotal factor in regulating plasma osmotic pressure. Furthermore, ligand binding ability enabled this HSA in carrying many endogenous and exogenous compounds composed of structurally three similar domains (I, II and III), divided into two subdomains A and B, respectively. It is known that aromatic and heterocyclic ligands bind to HSA within two hydrophobic pockets of subdomains IIA and IIIA, which are known as *Sudlow* site I and site II, respectively [13–15]. Also, it has been reported that subdomain IB is the third major drug binding region of HSA [16]. Although embelin presumes to have remarkable biological importance, its pharmacokinetics has not been addressed so far. Thus the investigation of embelin binding to HSA and AGP can provide useful information on the transport. Our group has recently showed that the natural and synthesized products, like coumarin derivatives, chitooligomers, asiatic acid and trimethoxy flavone, coumarintyramine, β-sitosterol and 7-hydroxy coumarin derivative possess robust binding to HSA in different domains, which leads to the HSA ligand complexation and results in the structural changes in the HSA [17–23]. Apart from this, HSA has many vigorous physiological functions in distribution and transport of many endogenous molecules like fatty acids, hemin and bilirubin [24–26] and numerous exogenous drug molecules like warfarin, digitoxin, ibuprofen, ferulic acid and quinidine with binding constants of 3.4×10^5, 0.4×10^5, 2.7×10^6, 2.2×10^4 and 1.6×10^3 M^{-1}, respectively [27]. Thus, it is known that HSA is a negative acute phase protein and AGP is a positive acute phase protein, hence, the binding studies with HSA and AGP play a positive role in understanding the pharmacokinetic behavior of drug at different biological states.

In the present study, to have a detailed understanding of the biological importance on cancer disease, we have used different cell lines and studied the apoptotic nature. Also, to unravel the binding mechanism and conformational changes of embelin with plasma proteins, here, we have used a different biophysical and computational approach.

2. Materials and Methods

2.1. Materials

For the preparation of stock solutions, fat free HSA and AGP (Purchased from Sigma Aldrich, USA) were dissolved in physiological aqueous solution of 0.1 M phosphate buffer pH 7.4 at a final concentration of 1 μM. Embelin was purchased from Natural remedies Pvt., Ltd., Bangalore, India, with purity of 99.2%. Its stock solution (2 mM) was prepared in 20:80 ethanol:water mixture. From our previous reports, a solution containing 20% ethanol has showed no effect [28] on HSA secondary structure. All the chemicals are purchased from Sigma Aldrich.

2.2. Cell Response Assay

The HeLa cell line was purchased from the National Center for Cell Science (NCCS), Pune, India. HeLa cells were grown at 37 °C in a humidified incubator under 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 200 IU/ml penicillin, 200 g/ml of streptomycin, and 1 mM sodium pyruvate. Culture medium was replaced every next day. After the cells were in confluence, the cells were subcultured followed by trypsinization and cell response assay was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining method] [29]. Cells were harvested from four-to five-day-old cultures and were seeded in 96-well plates at the density of 5×10^3 cells. HeLa cells were treated with embelin in increasing concentrations of 20, 40, 60, 80, and 100 μM for 48 h in a final volume of 100 μl. At the end of the treatment, 20 μl of MTT (5 mg MTT/ml in PBS) was added and the cells were incubated for 4 h. In control experiments, cells were grown in the same media without embelin. About 100 μl of DMSO was added to each culture and mixed by repeated pipetting to dissolve the reduced MTT crystals. Relative cell response was evaluated by measuring the optical density at 570 nm on a microplate reader (μ Quant Bio-tek Instruments, Inc.). Three independent experiments were carried in triplicates and mean ± SE ($n = 4$) was calculated and reported as the cell response (%) vs concentration (μM).

2.3. The Evaluation of Anti-cancer Activity by Apoptosis

The programmed cell death i.e. apoptosis was measured by using Annexin V-FITC detection Kit (Sigma cat no: APOAF). HeLa cells were treated with 29.1 μM of embelin for 24 h, after that cells were washed twice with PBS and resuspended in binding buffer (100 mM HEPES, pH 7.5) provided by Kit and stained with 5 μl of Annexin V-FITC, staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. It conjugates with fluorescent isothiocyanate (FITC) to label phospholipid, phosphatidylserine (PS) sites on the membrane surface and 10 μl of propidium iodide (PI) for 10 min to label the cellular DNA [30]. Flow cytometry (BD LSRFortessa) was done to determine the percent apoptosis induced by embelin.

2.4. Fluorescence Emission Spectra Measurements

LS-55 Spectrofluorimeter (PerkinElmer corporate, USA) was used to measure the fluorescence emission spectra. Room temperature fluorescence emission spectra were recorded with a wavelength range of 300–500 nm with an excitation wavelength of 285 nm, and slit width of 5.0 nm for both excitation and emission [28,31]. Here the concentration of HSA and AGP was fixed as 0.001 mM, and the concentrations of embelin varied from 0.001 to 0.009 mM suspended in 0.1 M phosphate buffer with pH 7.4 (physiological pH). Three independent experiments were performed and each time identical spectra were obtained. The binding constant was calculated using the maximum fluorescence intensity value at maximum emission wavelength (360 nm) for HSA and for AGP (340 nm). With increasing concentrations of embelin with HSA absorption at excitation (285 nm) and emission (360 nm) wavelengths introduces an inner filter effect, which may decrease the fluorescence intensity and result in a non-linear relationship between the observed fluorescence intensity and the concentration [Q] of the embelin. Such effect was corrected using the following equation.

$$\mathrm{Fcor} = \mathrm{Fobs}10(\mathrm{Aexc} + \mathrm{Aemi})/2. \quad (1)$$

Here, Fcor is the corrected fluorescence intensity, A_{exc} and A_{emi} represent the absorbance at the fluorescence excitation (285 nm) and emission (360 nm) wavelengths of HSA and (340 nm) of AGP, Fobs is the observed fluorescence [32]. Accurate fluorescence was observed