Evaluation of the anticancer activity of enzymatically synthesized Baccatin III: an intermediate precursor of Taxol®

Balendra Sah¹ · Madhuree Kumari¹ · Kamalraj Subban¹ · Jayabaskaran Chelliah¹

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Abstract

Baccatin III is an important precursor for the synthesis of clinically important anticancer drug Taxol. Previously, we have characterized a key enzyme of 10-deacetylbaccatin III-10- β -O-acetyltransferase (DBAT) which catalyses the 10-deacetylbaccatin III into baccatin III in taxol biosynthesis. Here, in the present study, we have evaluated and compared the cytotoxic properties of the enzymatically synthesized baccatin III (ESB III) with standard baccatin III in different human cancer cell lines, namely human cervical cancer (HeLa), human lung cancer (A549), human skin cancer (A431) and human liver cancer cells (HepG2). Among the various cancer lines tested, HeLa was more susceptible to ESB III with IC₅₀ of 4.30 μ M while IC₅₀ values for A549, A431 and HepG2 ranged from 4 to 7.81 μ M. Further, it showed G2/M phase cell cycle arrest, production of reactive oxygen species and depolarised mitochondrial membrane potential. In addition, annexin V-FITC staining was performed which showed the apoptotic cell death of HeLa cells, when treated with ESB III. Hence, ESB III was capable to show anticancer activities by inducing apoptotic cell death which could further be used for the semisynthesis of taxol in future.

Keywords Paclitaxel · Baccatin III · 10-Deacetylbaccatin III-10- β -O-acetyltransferase (DBAT) · Cytotoxic · Apoptotic activity

Introduction

Taxol is a clinically successful anticancer drug, which was obtained from *Taxus* species plant (Miao et al. 2018). The increasing demand for taxol and the constraint in supply have given rise to a search for an alternative to taxol production. To meet the supply of taxol, it is prepared on a commercial scale either by semisynthesis from 10-deacetylbaccatin III (10-DAB), which is a renewable advanced intermediate from the needles of *Taxus baccata* or by fermentation of the Chinese yew *Taxus chinensis* (Suffness 1995; Chow et al. 2007). A report suggested that 10-DAB can be produced in high yield from endophytic fungi (Li et al. 2015). Altogether,

the literature suggests that 10-DAB can be easily obtained from plants and fungi for the semisynthesis of taxol. Further, different groups suggested that 10-DAB production can be increased through the optimization of culture conditions, genetic breeding, and metabolic engineering of the endophytic fungi and *Taxus* species plants (Lin et al. 2018; Han et al. 2014; Gabetta et al. 1995). However, in the case of baccatin III, no notable yield in *Taxus* plants and endophytic fungi has been observed (Sonia et al. 2011; Zaiyou et al. 2013). Baccatin III contains the core taxane ring leading to the cytotoxic properties (Miller et al. 1999) against a variety of cancer cell lines (Pengsuparp et al. 1996). Thornburg et al. (2017) carried out enzyme engineering and showed that precursor can be directly converted into taxol using an enzyme cascade. There are several enzymatic steps involved in the semisynthesis of taxol (Patel 1998), among which conversion of 10-deacetylbaccatin III into baccatin III is one of the key steps in *Taxus* spp. plants and it is catalyzed by 10-deacetylbaccatin III-10- β -O-acetyl transferase (DBAT) (Walker and Croteau 2000; Li et al. 2017; Huang et al. 2020). Recently, we have also characterized a *dbat* gene and recombinant DBAT enzyme (Sah et al. 2017, 2019) from

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✉ Jayabaskaran Chelliah
cjb@iisc.ac.in

¹ Department of Biochemistry, Indian Institute of Science, CV Raman Rd, Bangalore 560012, India

taxol-producing endophytic fungus (Fig. 1). However, to date, the anticancer activity of the enzymatically synthesized baccatin III has not been analysed. Therefore, the objective of the present study is to investigate the anticancer properties of enzymatically synthesized baccatin III (ESB III).

Materials and methods

Chemicals and reagents

Standard baccatin III, Dulbecco's modified Eagle's medium (DMEM), 2, 4-dinitrophenol (2, 4-DNP), and propidium iodide, were purchased from Sigma-Aldrich, India. Bovine serum albumin (BSA), Tris base, UV₂₅₄ TLC plates, dimethylsulfoxide (DMSO), hydrogen peroxide, and organic solvents like methanol and chloroform were procured from Merk-Millipore. Penicillin, MTT, and gentamycin were purchased from SRL–Ranbaxy. The plastic items for the animal tissue culture were purchased from Corning and Thermo Scientific, USA. Annexin V-FITC apoptosis detection kit was procured from BD Bioscience, USA.

Enzymatic synthesis of baccatin III

As described previously (Sah et al. 2019), ESB III was prepared from *Lt*DBAT enzyme. Briefly, assay buffer containing *Lt*DBAT enzyme, 10-DAB acetyl-CoA and reaction mixture was incubated at 30 °C for 1 h. Five such independent assays were performed and the reaction was stopped by adding a double volume of chloroform to the reaction mixture. Then the reaction mixtures were pooled together, dried in a rotatory evaporator and the residue was dissolved in methanol. Further, the residue was purified by preparative thin-layer chromatography (PTLC) using a solvent system of chloroform: methanol (7:1, v/v) and a correspondent band to standard baccatin III was scraped and dissolved in high-performance liquid chromatography (HPLC)-grade methanol. The purified ESB III was characterized by liquid chromatography–electron spray ionization–tandem mass spectroscopy analysis (Sah et al. 2019) and obtained ESB III was used for anticancer activity.

Cell lines and maintenance

HeLa (human cervical cancer), HepG2 (human liver cancer cells), A549 (human lung cancer cells), and A431 (human skin carcinoma) cells were procured from National Centre for Cell Science, Pune, India. All the cell lines were grown and maintained in DMEM medium supplemented with 10% FBS, penicillin (100 UI/ml) and streptomycin (100 UI/ml) at 37 °C in a humidified atmosphere with 5% CO₂. Cell culture experiments were performed under aseptic condition.

MTT assay

The cytotoxic activity of ESB III was carried out by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The different human cancer cell lines, namely HeLa, HepG2, A549 and A431 (1×10^5 cells/ml) were cultured separately in a 96-well plate containing Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum and antibiotics. EBS III was dissolved in the DMEM medium with 0.4% DMSO at a final concentration of 1, 3 and 5 μ M. The HeLa, HepG2, A549 and A431 cells were treated with EBS III and standard baccatin III for 24 h. The HeLa cells were treated at different time points (12, 24, 48 and 72 h) with the respective concentration of ESB III. After the mentioned incubation time, 10 μ l of the MTT solution was added to each well and the cells were incubated for 3 h. Subsequently, the medium was discarded, 100 μ l of DMSO was added to dissolve the formazan crystal and the optical density (OD) was measured by a microplate reader (VERSAmax, USA) at 595 nm with 655 nm as a reference (Mosmann 1983). All the experiments were conducted in triplicate and the percentage of cell viability and cell death was calculated.

Cell viability assay using propidium iodide

HeLa cells (1×10^5 cells/ml) were seeded in 24-well plates, incubated in a CO₂ incubator overnight to get acclimatized, the cells were treated with different concentrations (1, 3 and 5 μ M) of ESB III and standard baccatin III for 24 h.

Fig. 1 Schematic diagram representing the enzymatic conversion of 10-deacetylbaccatin III (10-DAB) into baccatin III in the presence of acetyl coenzyme A and 10-deacetylbaccatin III-10- β -O-acetyltransferase (DBAT) enzyme (Sah 2018)

The treated, untreated cells were harvested separately, resuspended in 100 μ l of PBS and PI stain (1 μ g/ml) was added in each sample just before the acquisition of data in FACS verse (Becton Dickinson, USA). The untreated cells served as control. The acquired data were analyzed on BD FACS DIVA™ software.

Cell cycle analysis

The HeLa cells were seeded in 12-well plates at a cell density of 1×10^5 cells/ml and allowed to adhere overnight. Subsequently, the HeLa cells were treated with different concentrations (1 and 3 μ M) of ESB III and standard baccatin III for 24 h. The cells were harvested, washed twice with ice-cold PBS and fixed in ice-cold 70% (v/v) ethanol overnight at -20°C . Then after washing with PBS, the cells were incubated with RNase A (5 μ g/ml) at 37°C for 3 h. The cells were stained with PI for 30 min at 37°C and the samples were analyzed by FACS verse (Becton Dickinson, USA). Ten thousand events were acquired for each sample. The percentage of different cell cycle phase was calculated with the aid of BD FACS DIVA™ software.

Annexin V-FITC/PI assay

The HeLa cells were seeded in 24-well plates at a cell density of 1×10^5 cells/ml and allowed to grow overnight. Later, the cells were treated with different concentrations of ESB III (1, 3 and 5 μ M) and standard baccatin III incubated at 37°C with 5% CO_2 for 24 h. Subsequently, untreated, treated cells were harvested and washed with ice-cold PBS. The cell pellets were resuspended in 400 μ l of an Annexin V-FITC binding buffer and were subsequently stained with 1 μ l of Annexin V-FITC, with and without 3 μ l of PI (50 μ g/ml). The data were acquired on BD FACS Verse (Becton Dickinson, USA) and analyzed using FACS DIVA software analyzed using quadrant statistics (Miller 2004).

Analysis of mitochondrial membrane potential (MMP or $\Delta\Psi\text{m}$)

To determine the changes in mitochondrial membrane potential, JC-1 staining was performed as described by Cossarizza and Salvioli (2000). Briefly, the HeLa cells were seeded in 24-well plates at a cell density of 1×10^5 cells/ml and allowed overnight. Subsequently, the cells were treated with different concentrations of ESB III (1, 3, and 5 μ M) and standard baccatin III for 24 h. The treated cells were stained with 0.2 μ M JC-1 dye and incubated in darkness at 37°C for 15 min. The stained cells were washed with ice-cold PBS twice and immediately analyzed by flow cytometry (FACS Calibur flow cytometer, Becton Dickinson, USA) using excitation at 488 nm with emission at 530/30 and 585/42 nm.

Untreated cells were taken as the negative control where cells treated with 2,4-dinitrophenol (2,4-DNP) served as the positive control. JC-1 monomers emit at 535 nm (FL-1 channel-green fluorescence) and J aggregates emit at 590 nm (FL-2 channel-red fluorescence).

Evaluation of intracellular reactive oxygen species (ROS) production

The HeLa cells were seeded in 24-well plates at a cell density of 1×10^5 cells/ml and allowed to grow overnight. The cells were treated with different concentrations of ESB III (1, 3 and 5 μ M) and standard baccatin III for 24 h. After treatment, the cells were washed with PBS and stained with 50 μ M of DCFH-DA for 15 min at 37°C in the dark. The intracellular ROS production in HeLa cells was evaluated after staining with DCFH-DA (Eruslanov and Kusmartsev 2010). Untreated cells were taken as the negative control whereas cells treated with 800 μ M hydrogen peroxide served as the positive control. Fluorescence generated due to the hydrolysis of DCFH-DA to DCFH by non-cellular esterase and subsequent oxidation of DCFH by peroxidases was measured by flow cytometry for green fluorescence at 500 nm using a FACS Calibur (Becton Dickinson, USA) equipped with CellQuest Pro software (Eruslanov and Kusmartsev 2010).

Statistical analysis

The values were represented as the mean of three replicates and standard deviations (means $\pm$ SD). All statistical analysis was calculated by Microsoft Excel (Redmond, W A) and GraphPad Prism software 5.03 package.

Results

Cytotoxic activity of ESB III in different human cancer cell lines

Four different human cancer cells, viz. HeLa, HepG2, A549, and A431 were used to determine the cytotoxic effects of ESB III and compared with standard baccatin III using the MTT assay. As shown in Fig. 2a, ESB III showed a significant dose-dependent inhibition of cell growth in HeLa cells followed by HepG2, A549 and A431. HeLa cells treated with ESB III showed the least IC_{50} value of 4.46 μ M while the highest IC_{50} value 7.81 μ M was observed with the A431 cell line (Supplementary Table 1). Further, a time-dependent increase in cytotoxicity of ESB III was also observed with HeLa cells in three different concentrations (1, 3 and 5 μ M). Maximum cytotoxicity was observed after treatment with 5 μ M of ESB III (Fig. 2b).

Fig. 2 a Cytotoxic activity of enzymatically synthesized baccatin III (ESB III) and standard baccatin III against human skin cancer (A431), human lung cancer (A549), human liver cancer (HepG2) and human cervical cancer (HeLa) cell lines. (A431, A549, HepG2, and HeLa). Different concentrations of ESB III and standard baccatin III were treated to cells for 24 h and cytotoxicity was determined

by MTT assay. **b** Cytotoxic activity of enzymatically synthesized baccatin III (ESB III) in human cervical cancer (HeLa) cell line at different time intervals. Cytotoxicity was determined by MTT assay with different concentrations of ESB III and standard baccatin III were treated to cells for 12, 24, 48 and 72 h

Evaluation of cytotoxicity of ESB III in HeLa cancer cells

A live-dead cell assay was carried out on HeLa cells while treated with ESB III. The percentage of dead HeLa

cells were found to be 30.6, 45.5, and 56.5% when treated with 1, 3 and 5 μ M of ESB III, respectively (Fig. 3a). A similar result was also found with standard baccatin III as shown in Fig. 3b. This observation confirmed that ESB

Fig. 3 a Live and dead analyses of HeLa cells by PI staining while treated with different concentrations of enzymatically synthesized baccatin III (ESB III) and standard baccatin III. **b** Bar diagram showing the percentage of dead cells observed during PI live/

dead staining. PI-based live/dead staining was performed by flow cytometry analysis upon treatment with different concentrations of ESB III and standard baccatin III

III induced dose-dependent cytotoxicity on HeLa cells similar to standard baccatin III.

ESB III induces G2/M phase cell cycle arrest in HeLa cells

The results showed the distribution of the cell population and the DNA content in untreated and treated cells with different concentrations of ESB III and standard baccatin III. The various stages of the cell cycle (sub-G0, G1, S, and G2/M) are shown in Fig. 4a; approximately 40% cell populations were found in the G2/M phase in ESB III-treated cells whereas 25% of cells were found in G2/M of the cell cycle in the untreated cells. Standard baccatin III-treated cells resulted in 34% of cells in the G2/M phase. Hence, the results suggested that the percentage of the cell population in the G2/M phase was increased, in comparison to the control on treatment with ESB III in a dose-dependent manner and similar results were found with standard baccatin III (Fig. 4a–c).

ESB III induces mitochondrial membrane depolarization in HeLa cells

HeLa cells treated with three different concentrations of ESB III for 24 h showed a loss in MMP in a dose-dependent manner as shown in Fig. 5. HeLa cells showed 27.1, 42 and 65.2% of mitochondrial membrane potential (MMP) loss when treated with 1, 3 and 5 μM ESB III respectively. The results obtained with ESB III were comparable with standard baccatin III, where 22.7, 49 and 55% of MMP loss were observed after treatment with 1, 3 and 5 μM standard baccatin III, respectively (Fig. 5a, b). Here, 2, 4 DNP served as positive control and a significant amount of MMP loss was observed in cells treated with 2, 4 DNP.

Externalizations of phosphatidylserine in HeLa cells treated with ESB III

The treatment of HeLa cells with ESB III and standard baccatin III, the cells get distributed into four different populations. There is an increase in apoptotic events with

Fig. 4 Effect of enzymatically synthesized baccatin III (ESB III) and standard baccatin III on cell cycle phase distribution. **a** Percent of HeLa cells in different phases of cell cycle after treatment with ESB III and standard baccatin III at different concentrations. **b** The bar diagram represents the distribution of cells at different phases of the

cell cycle following treatment with standard baccatin III and **c** ESB III. The percentage of cells in each cell cycle phase was evaluated by propidium iodide-based staining by flow cytometry in the HeLa cells after treatment with ESB III and standard baccatin III

Fig. 5 Effect of enzymatically synthesized baccatin III (ESB III) on mitochondrial membrane potential (MMP). **a** Scatter plot showing HeLa cells treated with different concentrations of ESB III and std. baccatin III. **b** Bar diagram representing the ratio of red to

green fluorescence. Cells treated with different concentrations of ESB III and std. baccatin III, and were stained with JC-1 to detect depolarization of mitochondrial membrane using flow cytometry

an increase in the concentration of ESB III as shown in Fig. 6. The results showed that when cells were treated with 1 μM ESB III, 20% of cells were in early apoptosis while 8% of cells were in late apoptosis. When cells were treated with 3 μM ESB III, 49% of cells wherein early apoptosis and 20.4% in late apoptosis. The treatment of 5 μM of ESB III resulted in 58.8% of early apoptotic and 21.6% of late apoptotic cells (Fig. 6a). When compared to the standard baccatin III also showed 18, 33 and 57% of early apoptotic and 7.8, 17 and 20% of the late apoptotic stage in a dose-dependent manner (Fig. 6b).

Enzymatically synthesized baccatin III induces ROS generation in HeLa cells

An increase in ROS production was observed in a concentration-dependent manner after treatment with ESB III and standard baccatin III (Fig. 7). A treatment of 1, 3 and 5 μM of ESB III resulted in approximately a seven-, eight- and tenfold increase in ROS production, respectively, with respect to the control. Standard baccatin III of the similar concentration resulted in a 7-, 8- and 11-fold increase in ROS production. H₂O₂ as a positive control for ROS production (Fig. 7).

Discussion

Baccatin III is one of the important intermediate precursors used for the semisynthesis of taxol, thus making it essential to evaluate the anticancer activity of ESB III. Therefore, the present study, for the first time, reports in vitro anticancer activity of ESB III prepared from a recombinant *Lt*DBAT enzyme (Sah et al. 2017, 2019). Earlier, the enzymatic synthesis of baccatin III was reported by different methods like enzyme immobilization and biotransformation, but no report of anticancer activity (Patel et al. 2000; Han et al. 2014) of ESB III was available in the literature. However, recombinant DBAT enzymes cloned from several *Taxus* spp. plants have the potential to convert 10-DAB into baccatin III and this was confirmed by chromatography and spectroscopy methods (Li et al. 2017; Walker and Croteau 2000). In the earlier study carried by the authors' group, the purified recombinant *Lt*DBAT enzyme was used to catalyze 10-DAB into baccatin III and the molecular mass of parent fragment and daughter fragments of ESB III was similar to standard baccatin III (Sah et al. 2019). Further, to investigate the cytotoxic activity of ESB III, four different human cancer cell lines were selected viz. HeLa, HepG2, A451, A431, and the cytotoxicity was compared with standard baccatin III. Among the cancer cell lines, the HeLa cell line was the

Fig. 6 Annexin V-FITC and PI staining to evaluate the different stages of apoptosis in HeLa cells following enzymatically synthesized baccatin III (ESB III) and standard baccatin III treatment. **a** In each panel, the lower left quadrant shows cells, which are negative for both PI and annexin V-FITC, while upper left quadrant shows only PI-positive cells, which are necrotic (N-necrotic). The lower right

quadrant shows annexin positive cells (EA-early apoptotic) and the upper right quadrant shows annexin and PI-positive cells (LA-late apoptotic cells). **b** The bar diagram representing the comparison percentage of early and late apoptotic cells for ESB III and **c** standard baccatin III. All data of result from three independent experiments

most susceptible towards ESB III with IC_{50} value 4.46μ M and the cytotoxicity of ESB III and standard baccatin III was also comparable in the HeLa cell line at different hours of treatment. ESB III leads to cell death in a dose-dependent manner in HeLa cells.

Chakravarthi et al. (2013) reported that baccatin III induced G2/M arrest. Further, a cell cycle analysis revealed that ESB III induced cell cycle arrest in the G2/M phase of the cell cycle. G2/M cell cycle arrest is one of the key

mechanisms employed by many cytotoxic drugs to inhibit the growth of cancer cells (Shapiro and Harper 1999). Recent literature suggested that cytotoxic drugs including Xerophilus B and Flavokwain induce G2/M phase arrest in cancer cell lines (Yao et al. 2015; Jandial et al. 2017). To bring apoptotic death in the cancer cell a proper transmission of signals is essential but in cancer cells, the signalling mechanism is impaired and death is halted (Evan et al. 2001). It is well explained that during apoptosis, phosphatidylserine

Fig. 7 Effect of enzymatically synthesized baccatin III (ESB III) and std. baccatin III on the generation of ROS in HeLa cells. **a** HeLa cells were treated with different concentrations of ESB III and standard baccatin III. **b** Bar diagram representing the fold increase in ROS

after different treatment in comparison to control. Intracellular ROS production was observed by DCFH-DA dye after cells treated with ESB III and std. baccatin III by flow cytometry

from the inner to the outer phospholipid bilayer of the cell membrane is an early apoptotic phenomenon (Fadok et al. 1992; Martin et al. 1995). In HeLa cells, the externalization of phosphatidyl serine in upon treatment with ESB III was detected by Annexin V-FITC staining, which suggests that ESB III leads the cell death via the apoptosis phenomenon, similar to that in standard baccatin III.

Mitochondrial depolarization is an early event of apoptosis leading to different molecular changes for apoptotic cell death (Kitai et al. 2017). Chakravarthi et al. (2013) reported that baccatin III induced apoptotic cell death with the involvement of anti-apoptotic Bcl2 and a loss in mitochondrial membrane potential. ESB III leads to the loss of mitochondrial membrane potential in HeLa cells in a concentration-dependent manner as observed by JC-1 staining and this suggests that ESB III triggers the mitochondrial-dependent pathway of apoptosis, which is essential for the treatment of cancer. Alexandre et al. (2006) suggested that the ROS level gets elevated upon the treatment of cancer cells with an anticancer drug such as taxol and gelsolin associated with the apoptosis response. Moreover, the present study showed ESB III induces apoptosis accompanied by increased intracellular ROS similar to standard baccatin III as observed by DCFH-DA staining.

In conclusion, this is the first report on the ESB III, which reveals that strong anticancer property similar to standard

baccatin III on the cancer cell line. This finding can address the problem related to large-scale production of taxol intermediate precursor baccatin III in a short period without scarifying *Taxus* plants and ultimately this precursor can be used for the semisynthesis of taxol in future.

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Author contributions CJB and BS conceived and designed the experiments with inputs from all authors. BS performed the purification of ESB III and MS/MS. BS performed the cell-based experiment with help from MK. CJB, BS, MK, and KS analysed the data. BS wrote the manuscript. CJB, KS, and MK conducted a review of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical statement The authors declare that no human materials, human subject, or human data were used in this study.

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