Biochemical Engineering Journal 33 (2007) 159–167

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Source of isopentenyl diphosphate for taxol and baccatin III biosynthesis in cell cultures of *Taxus baccata*

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Received 27 July 2005; received in revised form 21 June 2006; accepted 20 October 2006

Abstract

To achieve a better understanding of the metabolism and accumulation of taxol and baccatin III in cell cultures of *Taxus*, three cell lines (I, II and III) of *T. baccata* were treated (on day 7) with several concentrations of fosmidomycin (100, 200 and 300 μM), an inhibitor of the non-mevalonate branch of the terpenoid pathway, or mevinolin (1, 3 and 5 μM), an inhibitor of the mevalonate branch, in both cases in presence and absence of 100 μM methyl jasmonate (MeJ). They were compared with lines treated only with the elicitor MeJ as well as an untreated control with respect to growth, viability and production of taxol and baccatin III. The results show that the cell line type was an important variable, mainly for taxane accumulation. The blocking effect of fosmidomycin on taxane production was significantly greater than that of mevinolin in all the cell lines, clearly suggesting that the isopentenyl diphosphate (IPP) used for the taxane ring formation was mainly formed via the non-mevalonate pathway. However, the significant reduction in the content of taxol (on average 3.8-fold) and baccatin III (on average 4.3-fold) in line I when treated with the elicitor together with mevinolin concentrations of 5 and 1 μM, respectively, also suggests that both non-mevalonate and mevalonate pathways are involved in the biosynthesis of the two taxanes as a result of cytosolic IPP and/or other prenyl diphosphate transport to the plastids. The observation that the inhibitory effect of fosmidomycin or mevilonin on taxol and baccatinn III yield does not interfere with methyl jasmonate elicitation is discussed.

Keywords: *Taxus baccata*; Cell lines; Taxol; Baccatin III; Isopentenyl diphosphate; Fosmidomycin; Mevinolin; Methyl jasmonate

1. Introduction

Taxol is a highly substituted, polyoxygenated cyclic diterpenoid characterised by the taxane ring system, which presents a very intense antitumoral activity. As the supply of taxol from nature is very limited, methods have been developed for the semisynthesis of taxol from related taxanes, such as baccatin III [1], present in substantial quantities (from 0.2 to 1 g/kg) in the leaves of the European yew, *Taxus baccata*, and other yews (e.g. Himalayan yew, *Taxus wallichiana*), which is a renewable starting material. However, this methodology requires the continued use of precursors from natural sources and significant amounts of solvents, both of which present environmental problems.

Another alternative for obtaining taxol and its synthetically useful progenitors is plant cell culture. Cell suspension [2–5] or cell immobilised systems [6,7] can provide an environmentally friendly way to produce taxol and enhance its productivity. The complex chemical structure of taxol means that total chemical synthesis is not commercially viable at present, and an efficient and economical supply of the drug must rely on biological production systems for the foreseeable future [8]. Up-regulation of the taxol biosynthetic pathway by overexpression of selected genes in *Taxus* cells can potentially address the supply issue. In all cases, improving the biological production yields of taxol depends critically upon a detailed understanding of the biosynthetic pathway.

In plants, isopentenyl diphosphate (IPP), the universal precursor of terpenoids, is synthesized either via the classical cytosolic mevalonate pathway or via the non-mevalonate plastid 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erytritol 4-phosphate (MEP) pathway [9]. Whereas the IPP derived from

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doi:10.1016/j.bej.2006.10.016

the mevalonate pathway is generally used in the biosynthesis of sesquiterpenes, phytosterols and triterpenes, the IPP derived from the MEP pathway is employed in the biosynthesis of monoterpenes, diterpenes and tetraterpenes. According to Eisenrich et al. [10], the IPP involved in the biosynthesis of the taxane ring, which has a diterpenic structure, is formed through the non-mevalonate pathway. However, other work [11,12] shows that when *Taxus* plants are supplied with labelled mevalonate, high rates of radioactively labelled taxol are obtained. Furthermore, our recently obtained results using a *Taxus media* cell suspension supplemented with mevalonate suggest that both biosynthetic routes could be involved [4]. Therefore, although many researchers have shown that diterpenes are synthesized via the non-mevalonate pathway in different systems [13,14], it might be a mistake to accept this as the general route. According to Adam and Zapp [15], a distinct division between the mevalonate and non-mevalonate pathways may not always exist for a given end-product because the biosynthesis of certain plant terpenoids appears to involve both routes. The involvement of the two pathways may be a result of the transport of prenyl diphosphate intermediates between the different sites of terpenoid biosynthesis [16,17].

Considering that IPP is not only an essential precursor but also the first intermediate in taxane biosynthesis, it is important to elucidate its source in this process in order to know to what extent the cytosolic mevalonate and plastid MEP (non-mevalonate) pathways contribute to the formation of the taxane ring system of taxol and baccatin III in *Taxus* cell cultures. In this context, it is of interest that mevinolin and fosmidomycin are two inhibitors that block the mevalonate and non-mevalonate pathways, respectively. Mevinolin (6α-methylcompactin) is a compound belonging to the statins group, which competitively inhibits the binding of natural substrate hydroxymethylglutary-CoA (HMGCoA) to the active site of the enzyme hydroxymethylglutary-CoA reductase (HMGR), and, consequently blocks the synthesis of cytosolic IPP [18]. Fosmidomycin, an antibiotic, herbicidal and antimalarial compound, is an inhibitor of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DOXP reductoisomerase) blocking the plastid non-mevalonate pathway [19].

In this study, using three different cell lines of *T. baccata*, the dependence of methyl jasmonate-induced and non-induced taxol and baccatin III production on IPP derived from the mevalonate and non-mevalonate pathways was detected by selectively blocking the IPP biosynthesis with specific inhibitors. The effects of the inhibitors were analysed in terms of cell growth, cell viability and content of taxol and baccatin III.

2. Materials and methods

2.1. Cell lines and culture conditions

The cell lines I–III of *T. baccata* used in this study were established from stable callus lines as reported earlier [20]. The lines were maintained in 175-ml flasks (Sigma, St. Louis, MO) in the dark at 25 ± 0.2 °C and 100 ± 1 rpm in a shaker-incubator (Adolf Kühner AG, Schweiz). Every 10–12 days, 1 ± 0.2 g of cells were used as inoculum in 10 ml of Gamborg's B5 medium [21] with 0.5% sucrose + 0.5% fructose, 2 mg l^{-1} of NAA and 0.1 mg l^{-1} of BAP (growth medium), which has previously been demonstrated as optimum for *T. baccata* cell growth [22]. All flasks were capped with Magenta B-Caps (Sigma).

In order to obtain high levels of taxol and baccatin III in our cell lines, which was necessary to more clearly discern the effect of the specific inhibitors fosmidomycin and mevinolin, and considering that the taxol production in *Taxus* cell cultures takes place mainly when the lineal growth phase has finished and the culture is in its stationary growth phase [23,24,4], 2 ± 0.2 g wet weight of cells grown for 12 days in the growth medium (the length of time necessary for them to enter the stationary growth phase) were transferred to 10 ml of B5 medium with 3% sucrose, 2 mg l^{-1} of Picloram and 0.1 mg l^{-1} of kinetin (production medium), which had previously been selected as optimum for both taxol and baccatin III yield of *T. baccata* cells [22]. They were then cultured for 28 days either with 100 μM methyl jasmonate (MeJ), or without the elicitor (control), or with different concentrations of fosmidomycin (100, 200 and 300 μM) or mevinolin (1, 3 and 5 μM) in presence and absence of the elicitor. Prior to application, methyl jasmonate (Sigma) was dissolved in ethanol [25], and both fosmidomycin (Invitrogen) and mevinolin (Fluka) were dissolved in cultivation medium. All compounds were sterilized by filtering through 0.22 μm sterile filters (Millipore) and added at 7 days of culture of the cell lines in the production medium to give the final concentrations considered. For analysis, six flasks from each treatment were harvested also at day 7 and then at days 14, 21 and 28.

2.2. Biomass accumulation and viability assay

Fresh weight was determined by suction filtering of suspension cultures using Miracloth filters (Calbiochem, CA). Then the cells were lyophilised to obtain dry weight and analysed to determine their content of taxol and baccatin III. Cell viability was studied by the fluorecein diacetate staining technique [26]. The cells (0.2 g wet weight) were incubated in fresh production medium (5 ml) containing fluorecein diacetate (0.1 mg ml^{-1}) for 30 min and then observed in a fluorescence microscope.

2.3. Taxol and baccatin III measurements

Taxanes were extracted from lyophilised cells and the culture medium as described by Cusidó et al. [20]. Quantification of paclitaxel and baccatin III was performed by high performance liquid chromatography (HPLC) as described in our paper [27]. Criteria for identification included retention time, UV spectra, co-chromatography with standard and peak homogeneity by photo-diode array detector when spiked with authentic standard. Taxol and baccatin III were provided by Hauser Chemicals (USA).