

5-Methyltryptophan Resistant Cells of *Catharanthus roseus*

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Several cell lines resistant to 5-methyltryptophan were selected from wild type cells of different *Catharanthus roseus* suspension cultures. The resistant cells had up to 30 times the normal levels of free tryptophan. Despite the increased pool size of tryptophan anthranilate synthetase activity of resistant cells was as sensitive to inhibition by L-tryptophan as wild type cells. The overproduction of tryptophan did not lead to intensified accumulation of tryptamine nor of indole alkaloids. This was supported by a low conversion of tryptophan to tryptamine in vivo and in vitro. The overproduction of one of the primary precursors was evidently not sufficient to stimulate the rate of indole alkaloid synthesis in *Catharanthus* cells.

Plant cell cultures normally accumulate very low levels of natural products. By using sensitive analytical methods, however, it has been demonstrated in a few cases that one can detect variants in wild type populations which synthesize and accumulate higher amounts of secondary compounds [1, 2]. To facilitate this analytical screening it would be highly advantageous to have a preselecting system for good producing strains. Recently, Teuscher suggested to select for regulatory mutants to establish high yielding cell lines [3]. For plant cells such selection systems are unknown. However, some results on the induction of secondary metabolite pathways in microorganisms may give hints how to develop such systems. Tryptophan has a stimulatory effect on ergoline alkaloid biosynthesis and is an inducer of the first enzyme of the secondary pathway [4]. Some other examples showing the importance of the primary precursors for the induction of antibiotic biosynthesis were reviewed by Drew and Demain [5]. Such observations

have led to the assumption that a better supply with the primary precursors may be one significant difference between producing and non producing strains [6].

Plant cell lines resistant to various amino acid analogs are known to overproduce the corresponding natural amino acids [7]. For some of these resistant cell lines secondary effects have been reported. 5-Methyltryptophan (5-MT) resistant cell lines of *Daucus carota* oversynthesizing tryptophan (trp) became auxin-autotrophic which may be due to increased synthesis of indole acetic acid by these cells [8]. Cell strains resistant to *p*-fluorophenylalanine have been selected from tobacco and sycamore cultures. These cell lines accumulated higher levels of phenylalanine derived secondary products rather than phenylalanine [9, 10].

Catharanthus roseus cultures are known to synthesize certain indole alkaloids [1]. Tryptophan or more precisely tryptamine is one of the primary precursors [11]. The objective of this study was to select for cell lines overproducing trp and to look whether a stimulatory effect of the higher trp level can be observed on the accumulation of indole alkaloids.

Some of the results were presented at the poster session of the Fourth International Congress of Plant Tissue and Cell Culture in Calgary, Canada 1978.

Abbreviations: trp, tryptophan; 5-MT, 5-methyltryptophan; MCW, methanol:chloroform water; MS, Murashige-Skoog; 2,4-D, 2,4-dichlorophenoxy acetic acid; BAP, benzylamino purine; IAA, indole acetic acid.

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Materials and Methods

Plant material

Suspension cultures of *Catharanthus roseus* v. Purple were initiated from stem pieces of various sterile



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grown seedlings. The cultures were maintained on MX-medium = MS salts with 2,4-D ($1.8 \mu\text{M}$) as sole hormone. The very fine suspensions were normally transferred every 8–10 days.

Selection of resistant cell lines

Growth of wild type cells (inoculum 0.5 g cells/100 ml medium) was inhibited by 1 mg 5-MT/l. The first screening was carried out on liquid MX-medium with 10 mg 5-MT/l. After 1 month growth was observed in 9 of 60 flasks. These 9 lines were grown for 2 months (4 transfers) on MX-medium containing 10 mg 5-MT/l. These resistant cell lines were subjected to another more rigorous selection on a medium with 100 mg 5-MT/l. Wild type cells are denoted as CP-1, CP-4, CP-6 and CP-7. The cell lines CP-1-1, CP-4-3, CP-4-5, CP-6-4 and CP-7-2 were resistant to 10 mg 5-MT/l. From these cells the higher resistant strains were selected as CP-1-10, CP-4-30, CP-4-50, CP-6-40 and CP-7-20.

Enzyme assays

a) To measure anthranilate synthetase, 5 g cells were homogenized with quartz sand and 3 ml of 0.2 M Tris-HCl, pH 8.0, 200 μM dithioerythritol, 200 μM EDTA and 60% glycerin. After centrifugation for 10 min at $20,000 \times g$, the supernatant was layered on a Sephadex G 25 column buffered with 0.1 M Tris-HCl, pH 7.6, 100 μM dithioerythritol, 100 μM EDTA and 10% glycerin, and was then centrifuged as described [9]. Enzyme activity and inhibition by L-tryptophan were measured as published [12].

b) For the measurement of trp decarboxylating activity, 5 g cells were homogenized with quartz sand and 3 ml 0.1 M Tris-HCl, pH 7.3, 1 mM pyridoxal phosphate. After centrifugation at $20,000 \times g$ the supernatant was saturated with a solution of 65% ammonium sulfate. The centrifuged precipitate was taken up in 1 ml 0.05 M Tris-HCl, pH 9.0, 1 mM pyridoxal phosphate, and was eluted from a Sephadex G 25 column by centrifugation [9]. The enzyme test has been described by Gibson *et al.* [13].

Chemical measurements

Freeze dried cells were extracted with $\text{CH}_3\text{OH} : \text{CHCl}_3 : \text{H}_2\text{O}$ (MCW) 12 : 5 : 3 (v/v/v) 3 times. The combined extracts were evaporated to dryness and taken up in H_2O which was then extracted 3 times with CHCl_3 . The organic phase was concentrated

and chromatographed on Silica gel plates with benzene : acetone : diethylamine 7 : 2 : 1 (v/v/v). Serpentine was eluted with CH_3OH and determined fluorometrically [14]. Tryptamine was eluted with water and measured colorimetrically [15]. The water phase was layered on a Dowex-50 column (H^+) and the amino acids were eluted by 0.3 N NH_3 . The amino acid fraction was taken to dryness, solved in 1 ml H_2O and trp was measured colorimetrically [15].

Feeding experiments

Cells in log phase were incubated with DL-[methylene- ^{14}C]tryptophan for 6 hours. Cell extracts were prepared as described and radioactivities of the various extracts and compounds were measured in a scintillation counter.

Results

Free tryptophan content

All wild type cells of various *Catharanthus roseus* suspension cultures (only a few of them are shown in Table I) had similar pool sizes of free trp. The usual amounts were 0.5–1.0 μM trp/g dry weight from cells harvested at the beginning stationary phase. All resistant cell lines had higher contents of trp. How-

Table I. Determination of tryptophan and serpentine in 10 d old cultures on MX-medium ($1.8 \mu\text{M}$ 2,4-D). Cells grown on MX were transferred to MX^+ -medium ($1 \mu\text{M}$ 2,4-D, 10 μM Kinetin, 1 μM IAA) and MX^A -medium (1 μM IAA, 5 μM BAP). Serpentine content was measured 10 d after the change in the medium composition.

Cell Line	Tryptophan Serpentine			
	MX	MX	MX^+	MX^A
	[$\mu\text{g/g}$ dry weight]			
CP-1	277	0.31	0.46	0.48
CP-1-1	889	0.36	0.81	2.88
CP-1-10	6095	2.88	—	—
CP-4	111	10.44	47.0	15.62
CP-4-3	547	1.04	2.04	4.04
CP-4-30	2662	0.10	—	—
CP-4-5	1137	3.09	7.72	24.72
CP-4-50	2490	0.45	—	—
CP-6	178	1.01	—	—
CP-6-4	472	0.48	—	—
CP-6-40	2232	0.14	—	—
CP-7	112	21.23	30.64	42.51
CP-7-2	1008	2.12	2.18	2.06
CP-7-20	1288	0.14	—	—

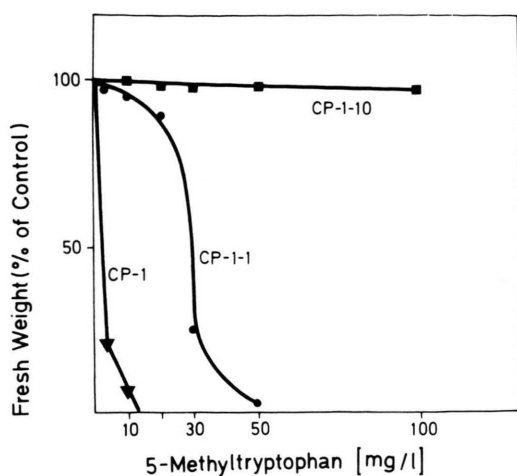


Fig. 1. Effect of increasing concentrations of 5-MT on the growth of 5-MT-sensitive and -resistant *Catharanthus roseus* cells. Fresh weights on 5-MT free medium were between 13.5 and 15.2 g by the various lines.

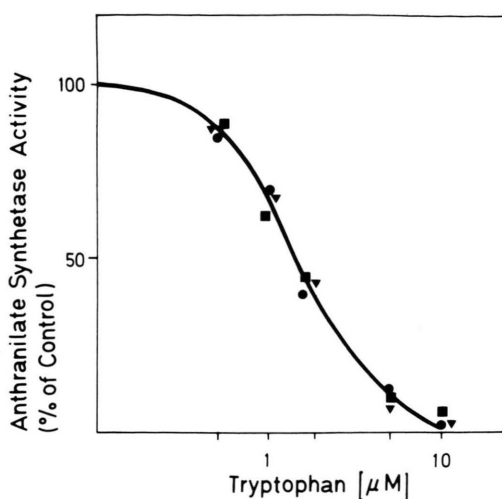


Fig. 2. Inhibition of anthranilate synthetase activity of 5-MT-sensitive and -resistant cell lines by L-tryptophan. ● = CP-1, ▼ = CP-1-1, ■ = CP-1-10.

ever, the lower resistant cell lines showed decreasing pools of free trp after several passages on 5-MT free medium. The amounts of trp given in Table I were measured after 3–4 months growth in the absence of 5-MT. The decrease in trp pool size was accompanied by a significant loss in resistance by some lines. Therefore the lower resistant cell lines were kept for nearly 2 months on medium with 100 mg 5-MT/l (2 transfers). This resulted in very resistant strains which did neither lose their resistance nor the tremendously increased pool sizes of trp when grown for several months in the absence of 5-MT (Fig. 1/ Table I). In some cases the pool size of trp was increased more than 30-fold. Since the selection procedure from low to high resistant cell strains took only a relative short time one can assume that during the screening on 100 mg 5-MT/l only non-resistant cells had to be eliminated which had survived the first 5-MT treatment and had diluted more and more the resistant cells.

Anthranilate synthetase

Tryptophan biosynthesis seems to be feedback regulated at the anthranilate synthetase in higher plants [12]. A lessened feedback control of this enzyme by L-trp was found in various 5-MT resistant tobacco and carrot cell lines causing an overproduction of trp [7]. Since the resistant cell lines of *Catharanthus roseus* had such increased levels of trp (Fig. 1) anthranilate synthetase was expected to have an al-

tered control. Anthranilate synthetase of *Catharanthus roseus* was indeed very sensitive to feedback inhibition by L-trp. Already a concentration of 1–2 μM trp caused 50% inhibition and 10 μM inhibited the enzyme activity completely (Fig. 2). However, no differences were found in the feedback inhibition pattern between sensitive and resistant cell strains. Sixteen resistant cell lines overproducing trp were checked for an altered anthranilate synthetase.

Indole alkaloid accumulation

All wild type cells were able to synthesize and accumulate at least low levels of serpentine (0.3–20 $\mu\text{g/g}$ dry weight) on MX-medium (Table I). In most cell lines ajmalicine was found in similar concentrations. Table I clearly shows that the resistant cell strains generally accumulated lower levels of serpentine. 2,4-D is known to reduce the capacity for indole alkaloid synthesis in *Catharanthus* cells [1]. Therefore several sensitive and resistant cell lines were transferred to media with hormone compositions known to stimulate the biosynthesis of these alkaloids [1]. Higher levels of serpentine were already found in cells grown on such media for the first time (Table I). Even higher amounts were found after the second transfer on MX⁺ and MX^A-medium. However, cell growth ceased on these media after the second or third transfer. In some cases resistant cells responded better than wild type cells to the altered hormone composition. However, it cannot be gener-

Table II. Various *Catharanthus* cell lines were incubated with 0.7 μCi DL-[methyl- ^{14}C]tryptophan for 6 h. Radioactivity in the medium is given as percentage of total radioactivity. MCW-extracts are given as percentage of total radioactivity and as percentage of radioactivity taken up in brackets. Activity in single compounds were measured after scraping off the spots from TLC-plates.

Cell Line	Medium	Distribution of Radioactivity [%]			
		MCW-Extracts	Tryptophan	Tryptamine	Serpentine
CP-1	35.1	19.3 (29.9)	72.3	3.1	0.1
CP-1-1	42.0	22.6 (39.1)	89.3	1.3	0.05
CP-4	35.3	33.4 (51.7)	90.4	0.9	0.05
CP-4-5	25.6	44.0 (59.2)	91.3	0.7	0.05

alized that cell lines overproducing trp were more stimulated for indole alkaloid production.

Tryptamine synthesis

The oversynthesized trp was evidently not diverted into indole alkaloids. To elucidate the biochemical reason for this result tryptamine contents of sensitive and resistant cell strains were compared. Tryptamine was found to be comparably low (5–8 $\mu\text{g/g}$ dry weight) in sensitive and resistant cells. Increased pools of trp did not increase tryptamine pools. For two cell lines (CP-4 and CP-4-5) tryptamine, tryptophan, serpentine and “tryptophan decarboxylase” activity were measured during a growth cycle. Since

both cell lines showed the same tendencies, only the results of CP-4-5 are shown (Fig. 3). Tryptamine levels rose in the stationary phase while the trp pool was steadily increasing. However, this increase in tryptamine did not parallel with an increase in indole alkaloids. Serpentine and ajmalicine levels per gram fresh weight remained unchanged during the whole growth cycle as found by others, too [1]. In resistant and sensitive cell lines a trp decarboxylating activity was measured (0.1–0.3 nmol 5-hydroxytryptamine/mg protein \cdot min). This activity increased 3-fold in CP-4 and CP-4-5 cells during the stationary phase (Fig. 3). The high levels of trp did not stimulate trp decarboxylase activity in resistant cell lines.

The low conversion from tryptophan to tryptamine was confirmed by feeding experiments with [^{14}C]trp. Between 30 to 60% of the radioactivity taken up remained soluble in sensitive and resistant cell lines (Table II). More than 70% of the soluble (MCW) radioactivity was always recovered as trp. Only 0.5–3% of the label was found in tryptamine. Serpentine was very poorly labelled and no other predominant peaks were detected.

Discussion

All 5-MT resistant cell lines of *Catharanthus roseus* became very likely resistant due to the overproduction of the natural amino acid trp. Anthranilate synthetase was expected to be the regulatory enzyme in this biosynthetic pathway. The enzyme from *Catharanthus* was found as sensitive to feedback inhibition by L-trp as the enzyme from tobacco and carrot cells [7, 12]. However, cell strains of *Catharanthus* accumulating low or high levels of trp showed the same feedback inhibition pattern. The trp pool of *Catharanthus* cells can evidently be enlarged without an

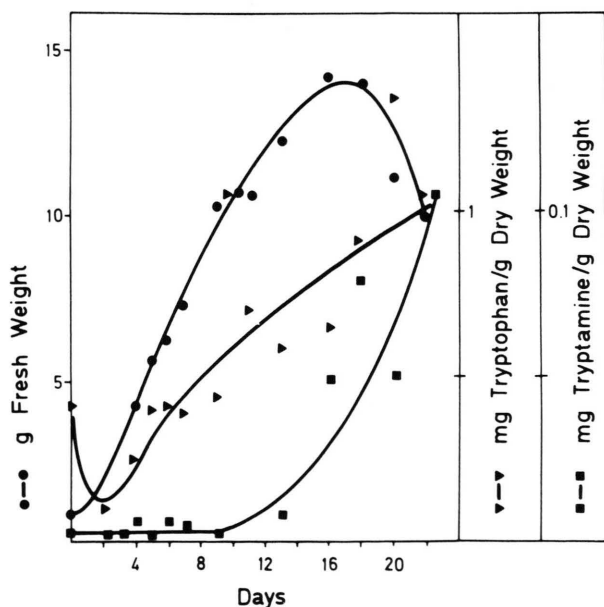


Fig. 3. Changes in free tryptophan (Δ --- Δ) and tryptamine (\blacksquare --- \blacksquare) during a growth cycle (\bullet --- \bullet) of strain CP-4-5.

alteration of the regulatory enzyme anthranilate synthetase. This is in contrast to other reports on 5-MT resistant cell lines [7]. In potato cell cultures two isozymes of anthranilate synthetase were detected [16]. The predominance of a feedback resistant form of anthranilate synthetase in 5-MT resistant strains was given as explanation for the 40-fold increase in trp. Since we were able to reproduce the different feedback inhibition patterns in 5-MT sensitive and resistant tobacco cells from Widholm's laboratory [12], some other observations may explain the higher levels of trp found in the resistant *Catharanthus* cells. Overproduced trp may rapidly be transported from the biosynthetic site and may not act as feedback inhibitor *in vivo*. In carrot cells up to 70% of the amino acid pools were located in vacuoles [17]. Pool sizes can also be manipulated without an altered control in the biosynthetic pathway. By adding the phenylalanine ammonia lyase inhibitor α -aminoxy- β -phenylpropionic acid [18] to tobacco cells we were able to increase the content of phenylalanine 17-fold (Berlin and Vollmer, unpublished). Thus, pool sizes measured as total amounts of one compound do not necessarily tell anything about regulatory control. It cannot be excluded, however, that another regulatory enzyme in the trp biosynthesis has been altered in the resistant *Catharanthus* cells.

Effects of exogenously added trp and tryptamine on indole alkaloid accumulation in *Catharanthus roseus* cultures have been described [1, 14, 20]. Added

trp or tryptamine had in most cases no effect on indole alkaloid biosynthesis. At higher concentrations the alkaloid production was severely inhibited. So far only one variant was described showing a 3-fold stimulation of alkaloid biosynthesis by adding L-trp to the culture medium [1]. The results clearly demonstrate that the cells have to be ready for increased alkaloid production. This, however, cannot be achieved by adding the precursors. The objective of this study was to manipulate cell cultures for alkaloid biosynthesis by selecting for cells with a higher rate of precursor biosynthesis. However, the internal stimulation of trp biosynthesis did not result in higher levels of indole alkaloids. A dysregulation in one precursor pathway was evidently not sufficient to induce the secondary pathway. It remains to be seen whether such alterations are a prerequisite for good producing strains. Anthranilate synthetase of a cell line accumulating 200 times the levels of indole alkaloids of our lines showed the same feedback inhibition pattern by L-trp as low producing lines (unpublished). A biochemical characterization of high and low producing strains may give more decisive clues how to select biochemically for high yielding strains.

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