Vincristine and Vinblastine Anticancer *Catharanthus* Alkaloids: Pharmacological Applications and Strategies for Yield Improvement

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Abstract *Catharanthus roseus* L. is a potent medicinal plant belonging to Apocynaceae family. In a number of countries, different parts of it are traditionally used in the treatment of various diseases, e.g. diabetes, menstrual irregulations, hypertension, cancer, etc. The high added-value of this plant is because of its enormous pharmaceutical features, which are because of its more than 130 terpenoid indole alkaloids (TIAs), some of which exhibiting imperative pharmacological activities. The most striking biological activity investigated is the antitumour effect of dimeric alkaloids such as that of anhydrovinblastine, vinblastine and vincristine, which are under study either in preclinical phase or are being used presently. The great pharmacological importance of these indole alkaloids contrasts with their small amounts in the plant, making their extraction a very expensive process. To overcome this problem, researches have looked for alternative sources and have been trying the strategies to produce them in higher amounts. Using biotechnological approaches, intensive research on the biosynthesis of TIAs and on the regulation of their biochemical pathways has been developed with the aim to increase the production of these high added-value compounds. This chapter is focused on the pharmaceutical application of the antitumour alkaloids and on the various strategies which improve the production of these alkaloids; it also analyses the beneficial effects that these compounds exert on human health.

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1 Introduction

Natural products, beneficial in human healthcare, were the basis of the first pharmaceutical practice. These plant products continue to play an important role in modern therapy too. The term 'natural products' is applied to materials derived from plants, microorganisms, invertebrates and vertebrates. They may serve as raw materials for chemical or biological modification to new products, which may lead to new therapeutic agents for new organic synthesis and fundamental metabolic studies. Approximately, 25–50% of the current pharmaceuticals are derived from plant products that show lesser side effects compared to the synthetic drugs (Upadhyay 2011). In fact, the synthetic drugs, after getting inside the body, generate many biochemical alterations and cause cross-reactivity inside the body fluids in addition to widely inhibiting bio-membrane functioning in human beings. Today, consumers prefer herbal medicines over the synthetic drugs as they believe that the former may be safer than the later. This has resulted in 'explosion' of researches in the field of identification, distribution and variations of plant species, the development of new and improved tests useful in the therapeutic evaluation of drugs, new procedures for the isolation, separation, identification and structural elucidation, and exploration of new types of organic synthesis with regard to plant products. The National Cancer Institute (NCI) of the US Public Health Service has recognized the value of plants as the sources of potential anticancer agents. In 1960, NCI initiated a systematic effort to collect and screen the plants for anticancer properties in collaboration with United States Department of Agriculture (USDA). Between 1960 and 1982, some 35,000 plants were collected by USDA from more than 60 countries; later, their pharmaceutical constituents were screened by NCI against a range of animal tumour systems.

Out of several anticancer plants, periwinkle (*Catharanthus roseus*) is considered as a unique plant in cancer therapy. It accumulates in its leaves the dimeric terpenoid indole alkaloids vincristine (VCR) and vinblastine (VBL), which may be regarded as the first natural drugs used in prevention and cure of cancer; these alkaloids are still included among the most valuable chemotherapeutic agents in the treatment of human neoplasma (Sottomayor and Ros Barceló 2005). Both of these anticancer alkaloids disrupt the spindle formation in mitotically dividing cells, thereby preventing the uncontrolled growth of malignant tumours (Fig. 1).

Catharanthus roseus L. is a perennial tropical medicinal plant belonging to the family Apocynaceae, which comprises eight species, of which seven (*Catharanthus coriaceus*, *C. lanceus*, *C. longifolius*, *C. ovalis*, *C. roseus*, *C. scitulus* and *C. trichophyllus*) are endemic to Madagascar and one (*C. pusillus*) is reported from India. Basically, *C. roseus* is an ornamental plant with beautiful pink flowers and nicely shaped leaves. However, it is also recognized as an important remedial plant with enormous pharmaceutical uses in human healthcare. In fact, it is nothing less than a biochemical factory, producing more than 130 different TIAs, some of which exhibit

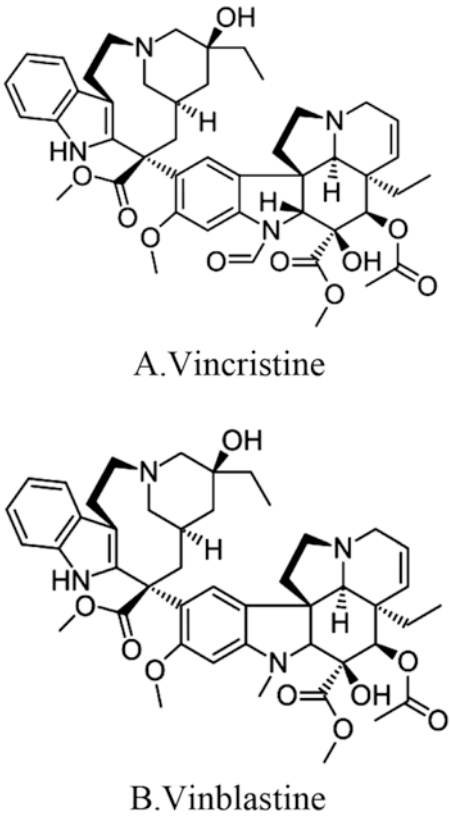

Fig. 1 Figures of vincristine and vinblastine

potent pharmacological activities in favour of human health (Van der Heijden et al. 2004). Vincristine and vinblastine alkaloids are commercial TIAs used in cancer chemotherapy, and together with many related semi-synthetic compounds are collectively named the *Vinca* alkaloids, as *Vinca rosea* is old name of *C. roseus*. Currently, vincristine, vinblastine, vinorelbine and vindesine have been used in clinical trials although only vincristine, vinblastine and vinorelbine have been approved for therapeutic exploitation in the United States (Rowinsky 2003). Fluorinated analogue of vinorelbine, called as vinflunine, has been approved for cancer chemotherapy in Europe (Bennouna et al. 2008; Schutz et al. 2011). Vincristine and vinblastine also exhibit strong antimicrobial activity (Grellier et al. 1999). Apart from anticancer alkaloids mentioned above, *C. roseus* also produces ajmalicine and serpentine alkaloids, which are monoterpenic indole compounds used as antihypertensive and anti-neuroinflammatory agents. Besides, yohimbine alkaloid of *C. roseus* is used in the treatments of erectile dysfunction, while vindolicine alkaloid is used for the development of antidiabetic drugs.

C. roseus anticancer compounds, vincristine and vinblastine, are derived from the coupling of catharanthine and vindoline alkaloids. Since there is very low recovery of these compounds from *C. roseus* plant with expensive extraction procedure, scientists have a large interest to increase their production through various biological

techniques. Low levels of vincristine and vinblastine are mainly associated to the spatial separation of biosynthetic sites where these compounds are produced in the plant. Mainly, it pertains to the high degree of specialization in leaf cells where the assembly of specific steps of the TIA biosynthetic pathway occurs (Yu and de Luca 2013). Factually, catharanthine is accumulated almost exclusively in the wax exudates on the leaf surface, whereas vindoline is produced in specialized internal leaf cells, suggesting that an involvement of transport processes is needed for their coupling to take place (Roepke et al. 2010). Recently, an ABC transporter, CrTPT2, has been identified with its primary function in enhancing the transport; hence, its role in accumulation of catharanthine in the leaf epidermal surface has been expected (Yu and de Luca 2013). However, the physical separation of catharanthine and vindoline observed by Yu and De Luca (2013) is probably a limiting factor in very young leaves, where anhydro-vinblastine (AVBL) was actually shown to be absent (Naaranlahti et al. 1991), but definitely not in developed leaves, where the dimer AVBL was repeatedly reported to be in abundance (Balsevich and Bishop 1988; Goodbody et al. 1998; Sottomayor et al. 1998; Carqueijeiro et al. 2013). Further, Carqueijeiro et al. (2013) demonstrated that catharanthine, vindoline and AVBL were accumulated in the vacuoles of mesophyll cells by a specific proton antiport system, which is dependent on the transtonoplast pH gradient generated by V-H^+-ATPase and V-H^+-PPase system, using vacuoles isolated from leaves of adult plants.

Researchers have also looked for alternative sources and strategies to produce TIAs in high amounts in *C. roseus* plant. In fact, the low levels of the TIAs with anticancer activity found in plants have stimulated an intense interest in research efforts aiming to obtain in vitro *C. roseus* cultures with a higher production of these TIAs. Technologically, Zhao and Verpoorte (2007) showed that although *C. roseus* cells can be cultivated in bioreactors, the TIA biosynthesis is extremely low, which prevents their industrial production. To increase the anticancer alkaloids production, several approaches were tried (Zhao and Verpoorte 2007) using *C. roseus* cell cultures, using genetic modification or metabolic engineering, the most promising biotechnological tools for high production of these compounds (Van der Heijden et al. 2004) (Table 1).

The biosynthetic route for indole alkaloids has been studied by De-Luca and Cutler (1987). Because of the presence of cytotoxic *bis*-indole alkaloids of therapeutic importance, the production of vincristine, vinblastine and vindesine has become one of the main fields of interest in modern cell biotechnology (Zhao and Verpoorte 2007). The plant produces the active dimeric alkaloids in low concentrations (0.0005%), where nearly 500 kg of dry leaves of *C. roseus* are used to isolate 1 g of vinblastine (Van der Heijden et al. 2004) and 2 tons of macerated leaves produced 1 g of the alkaloid as the active principle, which is the amount required for the treatment of a child with leukaemia for 6 weeks (Karthikeyan et al. 2008). Because of the large number of alkaloids the *C. roseus* plant contains, the isolation of vincristine and vinblastine in the laboratory is very costly. Although all parts of the plant produce alkaloids (leaves, stems and roots) in different proportions (Soleimani et al. 2013), the maximum concentrations are found in the cortex of the roots, particularly when blooming (Jaleel et al. 2008). Wide arrays of different alkaloid subclasses have been identified, viz. vincosan, corynanthean, vallesiachotaman,

Table 1 Alkaloids isolated from different parts of *C. roseus* L. (Junaid et al. 2010)

Alkaloid	Extracted from (plant part)
β-Carboline	Leaf
Tryptamine,*N,N*-dimethyl	Cell suspension culture
Apparicine	Leaf, flower
Ammocalline	Plant extract, root
Anthirine	Plant extract, cell suspension culture
Akuammicine	Plant extract, leaf, root, callus culture, cell suspension culture, shoots
Iochrovicine	Leaf
Pericyclivine	Plant extract, leaf
Pleiocarpamine	Cell suspension culture
Cavincine	Plant extract, leaf, root, callus culture, hairy root
Iochnerine	Cell suspension culture
Tubotaiwine	Callus culture, cell suspension culture
Rosicine	Leaf
Catharanthine	Plant extract, leaf, flower, seedlings, callus culture, cell suspension culture, shoots
Tabersonine	Plant extract, leaf, seedlings, seed, callus culture, cell suspension culture
Venalstonine	Root
Akuammicine,12-Hydroxy	Cell suspension culture
Perivine	Plant extract, leaf, flower, root, callus culture, cell suspension culture
Vinervine	Cell suspension culture
Coronaridine	Flower
Vincadifformine	Cell suspension culture
Cyclolochnerine,21-Hydroxy	Callus culture, cell suspension culture, shoots, hairy root
Iochneridine	Leaf, callus culture, cell suspension culture, hairy root
Alstonine	Root, callus culture
Serpentine	Leaf, root, seedlings callus culture
Cathenamine	Plant extract
Vallesiachotamine	Callus culture, cell suspension culture
Isovallesiachotamine	Callus culture, cell suspension culture
Ajmalicine	Callus culture, cell suspension culture
Ajmalicine,19-*epi*,3-*iso*	Plant extract, callus culture, cell suspension culture
Ajmalicine, 3-*epi*	Plant extract, callus culture ,cell suspension culture
Akuammigine	Cell suspension culture
Akuammiline *O*-Deacetyl	Leaf, callus culture
Iochnericine	Plant extract, leaf, cell suspension culture
Minovincine	Plant extract
Preakuammicine	Seedlings

(continued)

Table 1 (continued)

Alkaloid	Extracted from (plant part)
Rosamine	Leaf
Tabersonine,19-Hydroxy	Cell suspension culture
Tetrahydroalstonine	Plant extract, flower, root, callus culture
Vindolinine, −Oxide	Plant extract, cell suspension culture
Vindolinine,19-*epi*,N-Oxide	Cell suspension culture
Fluorocarpamine, N-Oxide	Plant extract, leaf
Perividine	Plant extract
Isositsirikine, 19,20-Cis-16 (R)−	Plant extract, cell suspension culture
Isositsirikine, 19,20-Trans-16 (R)−	Plant extract, cell suspension culture
Isositsirikine, 19,20-Trans-16 (S)−	Plant extract, leaf, cell suspension culture
Minovincinine	Cell suspension culture
Sitsirikine	Plant extract, leaf, callus culture, cell suspension culture, shoots
Yohimbine	Plant extract, leaf, root, callus culture, cell suspension culture, hairy root
Sitsirikine,Dihydro-	Plant extract, leaf, root, callus culture, cell suspension culture
Perimivine	Plant extract, root
Tabersonine,11-Methoxy	Plant extract, flower
Almalicine, 7-Hydroxy-Indolenine	Callus culture
Ajmalicine *pseudo*-Indoxyl	Callus culture
Akuammiline,10-Hydroxy- Deacetyl	Callus culture
Epimisiline,19(S)	Hairy root
Horhammericine	Cell suspension culture, shoots
Mitraphylline	Flower, callus culture
Vincoline	Plant extract, leaf
Vindolinine	Plant extract, leaf, cell suspension
Vindolinine,19-*epi*	Plant extract, leaf, cell suspension culture
Vincolidine	Plant extract, leaf
Akuammine	Plant extract
Lochnerinine	Plant extract, leaf, cell suspension culture
Lochrovidine	Plant extract
Tabersonine,19-Hydroxy-11-Methoxy	Plant extract
Iochrovine	Plant extract
Vindolidine -Deacetyl-	Plant extract
Akuammiline	Plant extract, cell suspension culture
Horhammericine, 11-Methoxy	Cell suspension culture, shoots
Vincarodine	Plant extract, leaf
Vinosidine	Root
Vindoline, Deacetoxy-	Cell suspension culture, leaf, seedlings
Tabersonine,19-Acetoxy-11-Hydroxy-	Plant extract, leaf, cell suspension culture
Vindoline, Deacetyl-	Plant extract, leaf

(continued)

Table 1 (continued)

Alkaloid	Extracted from (plant part)
Iochnerinine	Leaf, root
Tabersonine, 19-Acetoxy-11- Methoxy	Cell suspension culture
Cathovaline	Leaf
Vindolidine	Plant extract, flower
Strictosidine Lactam	Cell suspension culture, shoots, hairy root
Vindoline	Plant extract, leaf, flower, seedlings, shoots
Akuammicine, Xylosyloxy-	Cell suspension culture
Strictosidine	Plant extract, leaf, root, seed, callus culture, cell suspension culture
Bannucine	Plant extract, leaf
Leurosivine	Leaf
Leurosine,17-Deacetoxy-	Plant extract
Vinblastine,4-Deacetoxy-	Plant extract, leaf
Vinblastine, Deacetyl-	Plant extract
Vinsedine	Seed
Leurosinine	Plant extract
Vinsedicine	Seed
Vinblastine,3,4-Anhydro-	Leaf, shoots
Vingramine	Seed
Vinblastine,4′-Deoxy-	Plant extract, leaf
Vinosidine	Plant extract
Vinblastine, N-Demethyl-	Plant extract
Vingrmine, Methyl-	Seed
Catharanthamine	Plant extract, leaf
Leurosine	Plant extract, leaf, shoots
Roseadine	Plant extract, leaf
Vincathicine	Plant extract, leaf
Roseamine	Plant extract
Vinblastine	Plant extract, leaf, flower, seedlings, cell suspension culture
Vinblastine,20′-*epi*-	Plant extract, leaf
Catharicine	Plant extract, leaf, flower
Catharine	Plant extract, leaf, shoot
Leurosine, 5′-Oxo-	Leaf
Carosine	Plant extract, leaf, flower
Leurosine,N B′-Oxide	Leaf
Vinamidine	Plant extract, leaf
Vincristine	Plant extract, leaf
Leurosidine, N B-Oxide	Plant extract
Vinblastine,14′-Hydroxy-	Plant extract
Vinblastine, 15'hydroxy-	Plant extract

(continued)

Table 1 (continued)

Alkaloid	Extracted from (plant part)
Neoleurocristine	Plant extract, leaf
Vindolidine	Plant extract
Leurosinone	Leaf
Neoleurosidine	Plant extract, leaf
Neoleurosidine, N B-Oxide	Plant extract, leaf
Vindolicine	Plant extract, leaf
Ammorosine	Root
Cathalanceine	Root
Cathindine	Leaf, root, cell suspension culture
Cavincidine	Plant extract, leaf, root, callus culture, cell suspension culture
Lochneririne	Leaf, root
Maandrosine	Plant extract, root
Perosine	Plant extract, leaf, root, callus culture
Rovindine	Plant extract, leaf
Vinaphamine	Plant extract, leaf
Vinaspine	Plant extract, leaf
Vincamicine	Plant extract, leaf

strychnan, aspidospermatan, plumeran, ibogan, eburnan and *bis*-indole alkaloids (Kisakurek and Hesse 1980). Up to 40 different *bis*-indole alkaloids have been found in *C. roseus*, many of which contain a moiety of plumerane (vindoline) and ibogaine (catharanthine). In relation to plant chemistry, *C. roseus* contains carbohydrates, flavonoids, saponins, phenol compounds, terpene indole alkaloids (Ataei-Azimi et al. 2008), anthocyanins, glucosides (Piovan and Filippini 2007), heart glycosides, steroids and mono-terpene glucosides (Van der Heijden et al. 2004). It has no tannins. Two flavonols have also been isolated and identified (Yadav et al. 2013) in addition to glycosidic flavonols that have been identified in seeds, stems, leaves and flowers of *C. roseus* (Ferreres et al. 2008). The extracts of the sprouts of *C. roseus* are used as a potential source of natural available antioxidants and with excellent pharmaceutical applications (Mallik et al. 2013).

The biological mechanism of action of the vincristine and vinblastine consists of the binding with the tubulin subunits of spindle apparatus during mitosis. These compounds inhibit the chromatin filaments drawn to their respective poles (Huxtable 1992), leading to the inhibition of cellular mitosis during the metaphase, and thereby starting the programmed cellular death or apoptosis (Leveque and Jehl 2007). Vincristine inhibits polymerization of the microtubules, producing an arrest in G2/M phase and inducing apoptosis (Casado et al. 2007). The semi-synthesis of vincristine and vinblastine, starting with the precursors and the organic synthesis (coupling of vindoline and catharanthine), is highly expensive and the production is poor in *C. roseus*. Therefore, alternative biotechnology strategies have been used to be able to increase the production of these secondary metabolites. They include the addition of biotic or abiotic inducers that stimulate the production of the metabolites in the biosynthesis pathway of the alkaloids.

2 Pharmacological Activities

The main secondary metabolites of *C. roseus* are terpene indole alkaloids (TIAs) with important applications in human medicine as mentioned above, which are presenting biological activities such as antitumour, anti-diabetes, anti-helminthic, antihypertensive, anti-diarrhoea, and antimicrobial actions and others. The Vinca alkaloids are generally known as compounds in the treatment of cancer (Moudi et al. 2013). These compounds repress cell growth because they alter the microtubular dynamics, which ultimately provokes apoptosis. Semi-synthetic compounds, similar to vincristine and vinblastine, have been developed to increase their therapeutic action (Nirmala et al. 2011). Vinblastine is used in particular for the treatment of Hodgkin's disease, besides lymphosarcoma, choriocarcinoma, neuroblastoma, carcinoma of breast and lung, and lymphocytic leukaemia (Junaid et al. 2010; Rai et al. 2014). Anhydrovinblastine, the direct precursor of vinblastine, also showed significant in vitro cytotoxic effect against human non-small cell lung cancer C4 besides that against human cervical carcinoma, human leukemic cells and A431 human carcinoma cells (Kutney et al. 2000). Vincristine is an oxidized form of vinblastine that arrests mitosis at metaphase and is very effective for treating acute lymphoblastic leukaemia in both children and adults. It is also used against Hodgkin's disease, Wilkins's tumour, neuroblastoma and reticulum cell sarcoma (Rowinsky 2003; Moudi et al. 2013). In addition, vincristine has also been used in the treatment of multiple non-malignant hematologic disorders like autoimmune and thrombotic thrombocytopenia, and hemolytic uremic syndrome sarcoma (Rowinsky 2003; Moudi et al. 2013). On the other hand, the cytotoxic effect of catharoseumine, which is a monoterpenic indole alkaloid isolated from the whole plant of *C. roseus*, was tested in different human tumour cell lines showing only a moderate cytotoxic effect against HL-60 cell line (Wang et al. 2012).

The antitumour alkaloids vincristine and vinblastine are used in malignant diseases; they are used in chemotherapy for leukaemia since they reduce the number of leukocytes in the blood (a high number of leukocytes indicate leukaemia) and in the treatment of Hodgkin's disease (Jaleel et al. 2008) characterized by being a monoclonal B cell neoplasia, and by the presence of abnormal cells called Reed–Sternberg cells (Jaffe et al. 2008). Vinblastine (vinblastine sulphate) is experimentally used for neoplasia treatment and for resistant pregnancy choriocarcinoma, a malignant neoplasia of the trophoblast, which is a highly aggressive and fetal lesion, since even when there is a timely diagnosis and it is appropriately treated with chemotherapy, it produces mortality in 10–15% of the cases (Cruz Ortíz et al. 2000). It is also effective in the treatment of advanced testicular tumours, breast cancer, Kaposi sarcoma and the Letterer–Siwe disease (Rocha and Leech 2002). Vincristine, formally known as leurocristine (vincristine sulphate), is used in leukaemia treatment in children. Vincristine is produced by the bonds of the terpene indole alkaloids: vindoline and catharanthine in the *C. roseus* plant (Evans et al. 2009). The use of the vinblastine and vincristine combined with chemotherapy has given 80% remission in Hodgkin's disease, 99% in acute lymphocytic leukaemia, 80% in Wilms' tumour in children,

70% in pregnancy corium cancer and the remission of 50% in Burkitt's lymphoma (Walts 2004; Amirjani, 2013). The indole alkaloid called amotin also has a strong anti-leukaemia activity (Taha et al. 2008).

At the neurologic level, the ajmalicine and serpentine are drugs used in treating depression and anxiety; these are also effective as anti-stress drugs (Taha et al. 2009; Hedhili et al. 2007). The supplements based on active ingredients of *C. roseus* such as vincamine are used for the prevention and treatment of cerebrovascular disorders and failures, vertigo, ischemic deficiencies and headaches, because they help oxygenate and increase brain glucose levels (Vas and Gulyas 2005); besides preventing abnormal clotting, they also increase the levels of serotonin, a brain neurotransmitter; the deficiency of serotonin produce schizophrenia, phobia, migraine and bulimia. On the other hand, vincamine is now known to increase the memory retention properties and it is effective in the treatment of vascular dementia (Gayatri and Chakravarthy 2013). Anhydrous vinblastine is used in the treatment of lung and cervix cancer (Kutney et al. 1998). The catharanthine isolated from *C. roseus* is cytotoxic in P-388 and KB human cancer cell lines (Wong et al. 2013). Furthermore, ajmalicine is used in the treatment of circulatory disorders and as an antihypertensive agent since it acts as an antagonist of the α1-adrenergic receptor, known as alpha blocker (Roquebert and Demichel 1984), with a preferential action on α2-adrenergic receptors (Chung et al. 2007).

2.1 How Do Alkaloids Execute Cancer Cell?

Drugs that interrupt mitotic progression, which are commonly referred to as 'antimitotics', are used extensively for the treatment of cancer. Currently, all such drugs that have been approved for clinical use target microtubules, with the taxanes and *Vinca* alkaloids showing much success against a number of cancers. Taxol (pacific yew tree), which is originally derived from the bark of *Taxus brevifolia*, is commonly used in the treatment of breast and ovarian cancers. *Vinca* alkaloids, such as vincristine, are often used in combination therapies to treat haematological malignancies (Jordan and Wilson 2004). Investigating the effects of these agents on microtubule dynamics has revealed much about their mechanism of action. The *Vinca* alkaloids interact with β-tubulin at a region adjacent to the GTP-binding site known as the *Vinca* domain (Rai and Wolff 1996). Within a concentration range that blocks proliferation, the *Vinca* alkaloids bind to tubulin at the plus-tip of microtubules. At the lower end of this range, this inhibits microtubule dynamics without altering polymer levels, whereas at higher concentrations, it induces microtubule depolymerization (Jordan et al. 1991). In both situations, mitotic spindle formation is disrupted, and cells therefore fail to complete a normal mitosis (Jordan et al. 1991). At very high concentrations (above 10 μM), vinca alkaloids can induce the aggregation of tubulin into paracrystals; however, this does not occur at clinically relevant concentrations (Jordan and Wilson 1999). Taxol steady the microtubules and dampens the dynamics of the polymer, thereby reducing depolymerization (Schiff et al. 1979). In mammalian cells, low concentrations of taxol stabilize microtubules, whereas higher concentrations increase polymerization (Jordan et al. 1993). Taxanes bind β-tubulin, but only when the monomer is

incorporated into a microtubule. The binding site for taxol is on the inner face of the polymer, and the drug can bind the length of the polymer. Drug binding is thought to stabilize the structure of the polymer by inducing a conformational change, which enhances the affinity of the interaction between tubulin molecules (Nogales, 2000). Stabilization of microtubules by taxol binding prevents normal formation of mitotic spindles (Jordan et al. 1996). On entry into mitosis, chromosomes can attach taxol-stabilized microtubules; however, the lack of microtubule dynamics means that tension is not produced across sister chromatids (Kelling et al. 2003), and prevents correct chromosome bi-orientation. This leads to chronic activation of the spindle assembly checkpoint (SAC), which in turn leads to mitotic arrest (Musacchio and Salmon 2007). Although the mechanisms by which antimitotic drugs elicit a mitotic arrest are now well understood, relatively little is known about how cells respond to this prolonged cell-cycle delay. Recently, however, several studies have taken a new approach, using high content imaging and live-cell analysis to monitor the long-term behaviour of cells in response to antimitotic drugs. In this commentary, we focus on the recent studies and on how they have advanced our understanding of how cancer cells respond to antimitotic drugs, at least in cell culture. We also discuss the relevance of these new findings to the clinical use of both classical and novel antimitotic agents (Fig. 2).

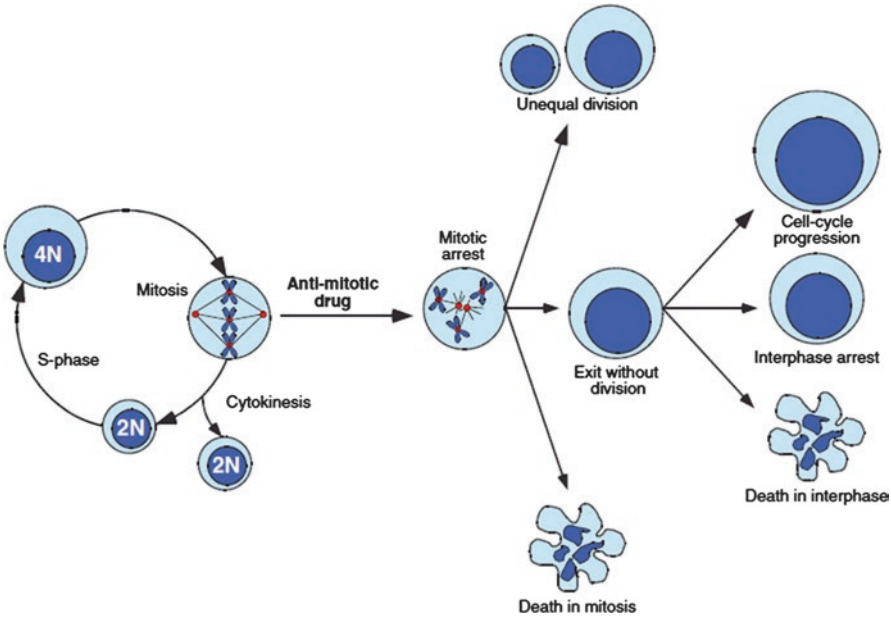

Fig. 2 Cell fate in response to antimitotic drug treatment. When cells are exposed to an antimitotic agent (*Vinca* alkaloids), they are arrested in mitosis due to chronic activation of the spindle assembly checkpoint. They then undergo one of several fates. Cells might die directly in mitosis, or divide unequally to produce aneuploid daughter cells. Alternatively, cells might exit mitosis without undergoing division. In this case, cells might then die in interphase, arrest in interphase indefinitely or enter additional cell cycles in the absence of division (from Gascoigne and Taylor 2009)

2.2 Clinical Use of Antimitotics

Although microtubule toxins have shown great success in the clinical therapy, two factors—namely resistance and toxicity—have limited their effectiveness. Patient resistance to classic antimitotic agents is commonly observed (McGrogan et al. 2008). Some patients respond well to treatment, but others rapidly acquire resistance and show little improvement. Toxicity is also a major limitation: in addition to killing tumour cells, antimitotic agents affect the division of normal cells, which therefore manifests as myelosuppression. Furthermore, because vincristine also disrupts microtubule dynamics in non-dividing cells such as peripheral neurons, neuropathies can also develop. Myelosuppression is reversible and therefore clinically manageable. By contrast, neurotoxicities are more problematic because they can often cause permanent damage (Rowinsky et al. 1993). To minimize neurological side effects, new agents are being developed that disrupt mitosis without interfering with microtubule dynamics in non-dividing cells. The rationale of this approach is that such drugs should prevent assembly of the mitotic spindles, and thereby retain antitumour activity, but they should not induce neuropathies. Frontrunners in this new class of therapeutics are inhibitors of the Eg5 kinesin, a motor protein that is required for the separation and movement of chromosomes to two opposite poles during mitosis. Agents are also being developed that inhibit mitotic kinases such as Aurora A and polo-like kinase 1 (Plk1), both of which also play a role in spindle formation (Bergnes et al. 2005; Keen and Taylor 2004; Strebhardt and Ullrich 2006). One of the most advanced new agents is the Eg5 inhibitor Ispinesib, which has entered phase II trials for metastatic melanoma, hepatocellular carcinoma and cancers of the head and neck (Kwon et al. 2008; Lee et al. 2008; Tang et al. 2008). Encouragingly, minimal neurotoxicity has been observed during these trials. However, the key question now is whether Ispinesib, or any of the other new antimitotic agents including taxol or *Vinca* alkaloids, will have clinical efficacy. To achieve this, it will be important to identify which tumours are most likely to respond to these agents. In turn, this requires an understanding of the basic mechanisms by which antimitotic agents kill tumour cells.

2.3 Postmitotic Response

Although the model of ‘competing-networks’ described above provides a useful framework by which to describe the decision of a cell to either die during mitosis or exit mitosis, it does not easily explain the variety of behaviours that are observed following mitotic exit—the postmitotic response. Several fates have been described for cells that exit mitosis in the presence of an antimitotic drug, including cell-cycle arrest, apoptosis and cell-cycle progression. The molecular factors that govern these fates are not well understood, but p53 protein appears to be involved in this regard. Substantial evidence supports the theory that p53 restrains cell-cycle progression

following exit from a prolonged mitotic arrest (Lanni and Jacks 1998). It is unclear whether a p53-dependent response is induced during mitosis, or by a de novo signal that arises after mitotic exit. One possibility is that damage or stress that has accumulated during mitosis does not always trigger an apoptotic response during mitosis due to the inhibitory action of Cdk1 on caspase-9 (Allan and Clarke 2007). Following mitotic exit, and the loss of this Cdk1-mediated inhibition, the apoptotic threshold might fall to its interphase set-point. In turn, the pre-existing damage signal is then recognized, leading to execution of the apoptotic programme. Thus, it is likely that the fate of the cell in response to drug treatment is determined not only by events occurring during a mitotic arrest, but also by the consequences of these events after mitotic exit, as well as by additional signalling pathways that are active during interphase.

3 Approaches in the Enhancement of Pharmaceutically Active Compounds

Due to the pharmaceutical importance and the low content in the plant of vincristine and vinblastine, *Catharanthus roseus* turns into an important model system for biotechnological studies on plant secondary metabolism. Researchers are focusing their attention to enhance the alkaloids yield by various ways (chemically, enzymatically, synthetically or by cell culture method). The plant cell can be cultured at large scale (Verpoorte et al. 1991), but the yield of alkaloids production is too low and limits commercial applications. In recent times, however, two strategies have been commonly used for the enhancement of alkaloids.

(a) In vitro cultivation of shoot via organogenesis and somatic embryogenesis, callus or suspension by the optimization of media, phytohormones, temperature, pH, light, aeration, etc. In addition, high cell density culture, elicitor's treatment, mutagenesis, bioreactors and immobilization are also practised to improve alkaloids yield.
(b) Genetic engineering and overexpression of biosynthetic rate limiting enzymes in alkaloid biosynthesis pathways.

4 In Vitro Studies

In tissue culture, the response of culture has been influenced by a number of factors which in turn regulate alkaloids yield. The yield of alkaloids in suspension culture is directly influenced by the surrounding environmental conditions and genetic constitution of the concerned plant material. Over the years efforts have been made in numbers for optimization of culture media for better biomass and alkaloids production, some patents have also been filed (Van der Heijden et al. 1989; Ganapathi and

Kagri 1990; Moreno et al. 1995; Mujib et al. 2002). Carbon sources and inorganic compounds play a significant role in indole alkaloid production. It was earlier reported that nitrogen and phosphate both promoted growth but had an adverse effect on alkaloids yield (Knobloch and Berlin 1980; Van Gulik et al. 1993). The inhibitory effect of nitrogen on alkaloid production has not always been observed (Drapeau et al. 1987). The effect of nitrogen on alkaloids production is dependent on carbon availability to the cells which makes the carbon-to-nitrogen ratio (C/N ratio) an important factor to be taken into account. By the determination of the cellular C/N ratio, Rho and Andre (1991) identified three distinct growth phases: an active growth phase, an accumulation phase and a biomass decline phase (endogenous metabolism). They also noticed that phosphate (0.56 mM), nitrate (12.97 mM) and low concentration of ammonia were beneficial for maximum growth and increased alkaloids production. Similarly higher concentration of sucrose (carbon source) only enhanced the biomass; the optimized glucose (500 mM), ammonium and phosphate (0–12 mM) were previously used for higher alkaloids yield (Schlatmann et al. 1992). Medium composition and day's interval had direct effect on induction and accumulation of indole alkaloids (Junaid et al. 2009). A medium added with 6% sucrose is favourable for both biomass and alkaloids production in *Catharanthus* (Scragg et al. 1990). Liquid medium with 3–6% maltose was also found to be highly effective for production of somatic embryos (Junaid et al. 2006). It has been reported that agitated liquid media added with BAP (1.0 mg/L) was very productive for large-scale plant regeneration (Mujib et al. 1995). Alteration in macro- and micronutrient of MS medium (Murashige and Skoog 1962) has also been used to promote growth and subsequent alkaloid production (Smith et al. 1987a). Surface methodology (Tuominen et al. 1989) has been used for the rapid biomass growth and increase in ajmalicine production in hairy root cultures. Similar results in cell suspension culture have been noticed (Schlatmann et al. 1992). Hairy root culture is a unique system, often used for root-specific indole alkaloids production (Toivonen et al. 1992). Recently, Batra et al. (2004) have observed an increase in growth and in the yield of terpenoids indole alkaloids (ajmalicine and serpentine) when left and right termini-linked Ri T-DNA gene integration was made in hairy root cultures of *C. roseus*.

4.1 Phytohormones

The role of plant growth regulators in alkaloids production of *C. roseus* has been extensively studied, but the response varies with genetic makeup of the used explant, type and quantity of phytohormones (Ganapathi and Kagri 1990; Smith et al. 1987a). The cytokinin applied exogenously either alone or in combination with auxins to suspension cultured cells enhanced alkaloids accumulation in tumorous and non-tumorous cell lines (Kodja et al. 1989; Decendit et al. 1992). The enzyme peroxidase plays a significant role in alkaloids biosynthesis however, the addition of 2,4-D in the culture medium reduced the peroxidase activity (Liman et al. 1998).

Hirata et al. (1990) reported an increase in vindoline and catharanthine concentration by adding to the MS medium an amount of 0.1 mg/L of BAP and 0.1 mg/L of NAA. Exogenously supplied cytokinin increased ajmalicine and serpentine content in untransformed callus from cotyledons (Garnier et al. 1996). At the protein level, it was shown that endogenously produced cytokinin did not mimic the effect of exogenously applied cytokinin in *Catharanthus* (Carpin et al. 1997a), and they also noticed that the protein pattern of Ipt transgenic callus lines was insensitive to exogenously used cytokinin. A28 KD polypeptide and simultaneous ajmalicine accumulation was noted on omission of 2,4-D in medium and by the use of NaCl treatments. (Carpin et al. 1997b; Ouelhazi et al. 1993). In a separate study, Alam et al. (2012) worked out that the plants of two cultivars (Rosea and Alba) of *C. roseus* L. G. Don were sprayed with various PGRs viz. IAA, IBA, NAA, BAP, KIN, TDZ, GA_3, SA, HBR and TRIA, at the rate of 10^{-7} M at 60 days after planting (DAP). Application of HBR, KIN and GA_3 resulted in the ameliorative effects on total alkaloid content. Of the various PGRs, application of GA_3 increased vincristine content. It is reported that cultivar Rosea gave higher yield of foliage and roots and that of alkaloids compared to Alba (Idrees et al. 2010). Further, Alam (2013) studied the effect of GA_3 on the content of *C. roseus* alkaloids in addition to that on the yield of different plant parts like leaf, stem and root was taken into consideration separately. The content of total alkaloids (%) in all plant parts and the major alkaloids i.e. vincristine, vinblastine and vindoline were significantly increased by the influence of GA_3 treatments. The yield of total alkaloids in these plant parts was augmented accordingly owing to significant increase in total herbage yield of the plant. Further, he examined that EBL application positively influenced the content and yield of leaf and root alkaloid significantly. Further, Alam et al. (2016) find out the ameliorative effect of EBL on *C. roseus* leaf and root alkaloids content.

4.2 pH and Temperature of Culture Medium

In vitro biomass and alkaloid production are directly influenced by the pH values of the medium; pH values with a range of 5.5–6.5 did not have much effect on alkaloids yield. The value 5.5 was found as the optimum pH for serpentine production (Doller et al. 1976). It has been reported that alkaloids produced by suspension culture were stored in vacuole and simultaneously storage capacity changed in accordance with the changes of pH in the medium and vacuole (Neumann et al. 1983). Low and higher values of pH were used to release intracellular alkaloids into the culture medium (Asada and Shuler 1989). It is quite known that the optimized pH value (5.5–5.8) occasionally fluctuates during culture time and influences in vitro responses including alkaloid yield. For in vitro study temperature range from 20 to 30 °C has been considered the best for comparatively better biomass production and growth of cultures, but contradictory data have been reported about the alkaloids yield. Temperature in low range had inhibitory (Morris 1986), stimulatory (Courtosis and Guern 1980), or no effect (Scragg et al. 1988a, b) on alkaloid

yield. In the tested cell lines under different temperature range (20, 25 and 30 °C), the highest serpentine production was recorded at 25 °C and no effect was recorded at temperature 17, 23 and 32 °C (Scragg et al. 1988a, b), while in hairy root culture low temperature enhanced the alkaloid yield (Toivonen et al. 1992).

4.3 Light and Aeration

Light is an important factor for both ex vitro and in vitro morphogenetic study. Its presence, absence, time and intensity directly influence the anabolic and catabolic processes, particularly with regard to secondary metabolism (Seibert and Kadkade 1980; Morris 1986). Most of the study of the effect of light was observed on serpentine and ajmalicine, where serpentine content was directly related to the intensity of light in *Catharanthus roseus* (Lounasmaa and Galambos 1989). Same was true for vindoline (De Luca et al. 1986); however, another alkaloid catharanthine was decreased in the absence of light. It has also been reported that light did not affect alkaloid yield but it affected the accumulation site (Drapeau et al. 1987). However, 15 h per day light exposure, instead of 24 h, improved the serpentine accumulation. On the contrary, dark-grown culture was much better in comparison to light grown regarding serpentine and ajmalicine content; in comparison to dark-grown culture, the alkaloid content decreased in light-grown culture from 79 to 14% regarding serpentine and from 78 to 18% regarding ajmalicine. Gradual transfer of dark-grown culture of *C. roseus* towards the light increased the serpentine content; however, continuous exposure of light decreased serpentine level (Scragg et al. 1988a, b). It has been optimized that 12 h is the best light period for better callus growth and alkaloid production (Hirata et al. 1990); however, dark period more than 12 h decreased the alkaloid contents. It was investigated that an increased chloroplast number and enhanced chlorophyll accumulation in response to light influenced the serpentine production (Loyola-Vargas et al. 1992). Besides, exposure of monochromic light such as blue (450 nm) or red (670 nm) did not affect growth and alkaloid accumulation; these variable wavelengths showed constant ajmalicine and serpentine synthesis which, however, decreased to some extent under white light (Hirata et al. 1990; Loyola-Vargas et al. 1992). Different types of gases, mainly CO_2 and ethylene, are usually evolved within the culture. In many cases, these gases reduce O_2 level in close vessels, inhibit plant culture growth as well as secondary metabolism. High dissolved oxygen and improved gaseous permeability at aerated condition stimulated secondary metabolism as observed by Schlatmann et al. (1994), as ajmalicine production was increased with high oxygen level. Improved oxidative metabolism at rich O_2 level is believed to be the reason for better product conversion. Aeration has been provided in culture to influence the alkaloids synthesis and to make it more efficient modern stirring devices have been employed along with traditional shake flask (Tom et al. 1991; Mohamed and Scragg 1990; Leckie et al. 1991; Lee and Shuler 1991). Different types of fermenters have also been used such as shikonin and ginseng; the two important secondary metabolites have been

commercially produced by the use of fermenters. Several researchers (Paynee et al. 1988) have suggested the use of bioreactors in secondary metabolites production in plant cell culture of *C. roseus*. An impeller with a speed of 100 rpm was most appropriate for the accumulation of alkaloids**;** however, higher impeller speed increased the callus/suspension growth. Hoopen et al. (1994) studied the rate of ajmalicine production by using different vessels including shake flask and bioreactors. They found that biomass was not affected by different culture vessels; however, ajmalicine production was decreased with overfeeding of biomass in the shake flask and fermenter.

4.4 Elicitor's Effect

New groups of triggering factors, which are better known as elicitors, have been reported to stimulate the secondary metabolites (Eilert et al. 1986). The substance used as elicitors may be of biotic or abiotic in origin. Biotic elicitors include microbial filtrates (e.g. yeast, *Pythium* and other fungal filtrates), while abiotic elicitors comprise simple inorganic and organic molecules (e.g. vanadyl sulphate, oxalate, UV irradiation, etc.). It has been reported that addition of *Pythium aphanidermatum* filtrate -+*increased the accumulation of phenolic compounds instead of alkaloids production (Seitz et al. 1989). Effect of different concentrations of *Pythium vexans* extract was studied by Nef et al. (1991), who noticed that low elicitor concentration increased the serpentine production but no effect was observed on catharanthine yield. Addition of nicotinamide (8.2 mM) in *C. roseus* cell lines was used to enhance the anthocyanin accumulation (Berglund et al. 1993). The extract of *Pythium aphanidermatum* in hormone-free cell lines responded well and induced synthesis of enzymes (TDC and anthranilate synthase), which catalyse the biosynthesis of several intermediates and subsequent accumulation of tryptamine (Moreno et al. 1995). Several inorganic compounds (e.g. sodium chloride, potassium chloride and sorbitol) had also a positive effect on catharanthine accumulation (Smith et al. 1987b). Addition of vanadyl sulphate to cell suspension culture increased catharanthine, serpentine and tryptamine production but the event was concentration dependent (Tallevi and DiCosmo 1988); at 25 ppm, catharanthine and ajmalicine were primarily accumulated, and at 50–75 ppm, only tryptamine accumulation was noticed. Moreover, the effect of heavy metal was studied where addition of copper (200 μm) increased total indole alkaloid accumulation which was correlated with decreased tryptamine concentration. In addition, several stress factors (e.g. fungal elicitor, vanadyl sulphate and potassium chloride) were used and it was found that the alkaloids accumulation was concentration dependent (Kargi and Potts 1991). Adding the optimal concentration (29, 1.45 and 145 mg g^{-1} by dry weight) of fungal elicitor, vanadyl sulphate and potassium chloride into medium increased the alkaloids accumulation; however, higher concentration had toxic effects and resulted in the loss of cell viability. Twofold increase in alkaloids yield was noticed by adding tryptophan, fungal elicitor and vanadyl sulphate to the culture production medium

(Kargi and Ganapathi 1991). Exposure of 2,2-*azobis* dehydrochloride (AAPH, an oxidative stress agent) and UV-B irradiation to *C. roseus* culture increased the nicotinamide and trigolline contents (Berglund et al. 1996). Simultaneously, phenylalanine ammonia lysate (PAL) activity was also increased. However, the increase in PAL activity (caused by addition of 2 μm of AAPH) was prevented by 0.1 mm 3-amino benzomide, which is an inhibitor of poly-(ADP-ribose) polymerase. This suggests that nicotinamide and its metabolites function as signal transmitter in response to the oxidative stress, since poly-polymerase has defensive metabolic functions. In shoot culture of *C. roseus*, the level of vinblistine and leurosine were increased in response to irradiation with near ultraviolet light (370 nm) (Hirata et al. 1991; Hirata et al. 1992); however, catharanthine and vindoline content were decreased. Leaves were more sensitive to dimeric alkaloid accumulation in comparison to shoot; however, exposure of near ultraviolet irradiation to whole plant of *C. roseus* led to increased accumulation of dimeric alkaloids (Hirata et al. 1993). Yeast extract induced the transcription of the biosynthetic gene encoding strictosidine (STR) in the cultured *C. roseus* cells and alkalinization of the culture medium; the active principle from yeast extract was partially purified and found to be of proteinaceous in nature (Menke et al. 1999). Age of culture is very important factor for the elicitors to be effective (Ramos-Valdivia et al. 1997); addition of elicitors is preferred after a few days of inoculation of the culture when the cells are rapidly dividing.

4.5 Mutagenesis

Mutagenesis plays a potent role in the alteration of the genetic constitution, which leads to produce new varieties. *Penicillium* is the most classic example, with many other successful cases. Process of mutagenesis in diploid plants is very complex. Mutagenesis enhances alkaloids yield but the route of biosynthesis and the necessary regulation procedure are not elucidated yet clearly. Therefore, mutation at target site in duplicate genome is really difficult. In spite of several limitations in this process, scientists have used mutagens. Berlin (1982) noted accumulation of higher level of phenolics in some p-fluorophenylalanine resistant cell lines of *Nicotiana tabacum* and *N. glauca*. In case of *C. roseus,* he noticed that a tryptophan-analogue resistant mutant accumulated catharanthine in both growth and production medium. Similarly several research groups used X-rays to produce increased serpentine alkaloid. Beside these examples, some successful reports are available in other group of crops where mutagenesis improved metabolic accumulation. In order to increase secondary metabolites production, high cell density culture feeding has been attempted with or without much success. Ajmalicine production was very low when inoculum potential was increased to 2:8 from 1:9 mg/g. Moreover, low-density cultures increased alkaloids yields (Moreno et al. 1993). It has also been remarked that low oxygen level and inadequate nutrient uptake are among the possible causes for low metabolic accumulation during high cell density culture. Isolation and selection of superior

lines from the heterogeneous cell populations help to improve the yield of alkaloids. These cells show genetic variability which was further diversified by the use of various mutagenic agents. Ajmalicine and serpentine level were increased in *C. roseus* by the selection of superior cell lines after mutagenesis (Zenk et al. 1977).

4.6 Bioreactor and Immobilization

In tissue culture, research for alkaloids production has been mainly focused on suspension culture, which requires a rotatory shaker. For large-scale production, however, large-sized culture vessel fermenter/bioreactor is most important. In both types of systems, a stirring device is provided for improved aeration (Drapeau et al. 1987; Kargi and Rosenberg 1987; Scragg et al. 1988a, b). In the device, there are several important vessels fitted with compressors, which provide filtered air. For plant culture growth and productivity, it is recommended that bioreactors with low shear-stress are much more suitable than those of high shear-stress. Bioreactors with improved mechanical designs are regularly introduced in bioreactors industry with innovated impeller, which helps to regulate shear agitation (Joilicoer et al. 1992). In *C. roseus,* immobilization of plants cells has been suggested for better accumulation of terpenoids (Hulst and Tramper 1989; Archambault et al. 1990). Immobilization not only maintains the cells viable for a longer period of time but also helps in extracellular alkaloids accumulation. Alginate-mediated immobilized cells enhanced the accumulation of tryptamide, ajmalicine and serpentine (Zenk et al. 1977; Majerus and Pareilleux 1986). The use of agar and agarose is found to be effective for long-term maintenance of cells. In the last few years, surface immobilization has been proposed using different types of matrices for large-scale production of alkaloids (Facchini et al. 1988; Facchini and DiCosmo 1990). In some other cases, negative influence of immobilization on cells was noticed (Archambault et al. 1990); gel or matrices, entrapment on polysaccharide sheet, is fairly successful in many plant systems and in *C. roseus*. Root of *C. roseus* contains a variety of secondary metabolites, which produce alkaloids. High rooting can be induced by genetic transformation using *Agrobacterium rhizogenes.* Induced roots grew with a faster rate in hormone-free medium with high accumulation of secondary metabolites in *C. roseus.* In transgenic *C. roseus* root, a significant increase in ajmalicine and catharanthine was noticed (Batra et al. 2004; Vazquez-Flota et al. 1997). Other groups used various types of bioreactors/fermenters to improve the growth of hairy roots, leading to better production of secondary metabolites (Davidou et al. 1989; Nuutila 1994). Although somatic embryogenesis (SE) has been reported in a wide variety of plant genera (Thorpe 1995; Mujib and Samaj 2006), it has been reported for the first time in *C. roseus* (Junaid et al. 2006). Earlier, a preliminarily study on plant regeneration from immature zygotic embryo was reported in *C. roseus* (Kim et al. 2004). The advantage of SE is that the initial cell populations can be used as a single cellular system and their genetic manipulation are easy and are similar to microorganisms.

5 Metabolic and Genetic Engineering in Alkaloids Biosynthesis

In alkaloids biosynthesis, the roles of several enzymes have been discussed in *C. roseus*: a few of them have been purified, identified, and characterized, and their encoding genes have also been cloned. The alkaloids biosynthesis is a very complex process that arises from the precursors tryptamine and secologanin. These two precursors are derived from two different pathways. Tryptamine is formed by the enzyme tryptophan decarboxylase (TDC), which has been reviewed earlier by various workers (Bentley 1990; Poulsen and Verpoorte 1992; Singh et al. 1991), while the strictosidine synthetase (SSS) helps in the coupling of tryptamine and secologanin to produce strictosidine (Madyastha and Coscia 1979; Inouye and Uesato 1986). The other enzymes such as geraniol 10-hydroxylase (G10H), NADPH-cytochrome P-450 reductase and anthranilate synthetase (AS) have the similar activities as TDC, which are involved in the biosynthesis of indole alkaloids (Poulsen et al. 1993). The TDC enzyme has been purified from cell suspension culture (Pennings et al. 1989) and ultimately its cDNA gene was established (Pasquali et al. 1992). The cytochrome P450 enzyme, geraniol-10-hydroxylase (G10H) and other enzymes have been studied extensively from intact plant of *C. roseus*. By HPLC study (Collu et al. 1999) and selection of a cell line with high G10H activity (Collu et al. 2001), the enzyme was purified to homogeneity (Collu et al. 1999). Based on the internal amino acid sequences obtained from the digested protein, gene was cloned and functionally expressed in yeast. The enzyme belongs to the CYP76B subfamily and is designated as CYP76B6. The activity of this enzyme was induced by treating the cells with the cytochrome P450 inducer Phenobarbital; it was decreased after treatment of the inhibitor ketoconazole (Contin et al. 1999). Besides, many other enzymes have been identified and characterized that metabolize strictosidine, which after undergoing several rearrangements produced cathenamine and ajmalicine (Hemscheidt and Zenk 1985; Stevens 1994). Another important enzyme is desacetoxyvindoline-4-hydroxylase (DAVH), active during vindoline biosynthesis; it was purified from intact plant of *C. roseus*. The native enzyme is a monomer and has a molecular weight of 45 KD with three isoforms (De Carolis and De Luca 1994). Recently, attention has been paid on the regulation of mevalonate biosynthesis that terminates with its end product strictosidine. Encoding genes and the enzymes of different steps of mevalonate pathway have been elucidated (Maldonado-Mendonza et al. 1992). After the formation of strictosidine, first step of alkaloid biosynthesis is the removal of sugar moiety from strictosidine to form an unstable aglycone. Two strictosidine β-glucosidases (SG) were partially purified and characterized from *C. roseus* cell cultures (Hemscheidt and Zenk 1980; Stevens 1994). Feeding of terpenoids precursors to *C. roseus* cell suspension cultures increased the alkaloids production (Naudascher et al. 1990; Facchini and DiCosmo 1991; Moreno et al. 1993). Addition of tryptophan (0.5 mM) to *C. roseus* cells resulted in high intracellular levels of tryptamine and an increase in STR activity but it did not influence ajmalicine accumulation much (Bongaerts 1998).

As in other feedback inhibitions, product accumulation depends upon the product degradation and this phenomenon has been reported in cell suspension culture of *C. roseus*. It is now known that the precursor for alkaloids (tryptophan to tryptamide) was located in the cytosol whereas the enzyme SSS was localized in the vacuole (Stevens et al. 1993).

5.1 Coupling Methods for Alkaloids Biosynthesis

The *bis*-indoles are derived from the coupling of vindoline and catharanthine. Catharanthine is thought to be derived from strictosidine via 4,21-dehydrogeissoschizine, stemmadenine and dehydrosecodine rout, while vindoline is derived from strictosidine via stemmadenine and tabersonine pathway. This pathway (transformation of tabersonine to vindoline) has got orderly six reactions (De Luca et al. 1986; Balsevich et al. 1986). The enzyme anhydrovinblastine synthase couples catharanthine and vindoline to yield AVBL, which was purified and characterized from *C. roseus* leaves. This heme protein has a molecular weight of 45 KD and shows the peroxidase activity. During this enzymatic coupling, both the monomers were incubated with cultured *C. roseus* cells at 30 °C at acidic pH (tris buffer 7.0). Only after 3 h the chemical reaction produced vinblistine and anhydrovinblastine as major products along with other dimeric alkaloids. Vindoline and catharanthine were also non-enzymatically coupled to the dihydropyridinium intermediate (DHPI) under near-UV light irradiation with a peak at 370 nm in the presence of flavin mononucleotide. Subsequently, DHPI can be reduced to anhydrovinblastin (AVBL) with an overall yield of 50%, based on initial amount of vindoline. Vinblastine content was further improved up to 50% by using various compounds as stimulants (Bede and DiCosmo 1992). Similarly, vincristine can be isolated from vinblastine by chemical conversion. Two routes are employed; first route is the isolation of *N*-deformyl-VCR, which was further converted into vincristine by formylation. The second method involves a formylation of the *C. roseus* extract in which conversion of *N*-deformyl–VLB to VCR takes place, after which the material is oxidized. In both cases, vincristine was purified by column chromatography and then sulphated. It was also reported that $MnCl_2$ and FMN/FAD stimulated coupling process. However, in the absence of *C. roseus* cell suspension enzymes, ferric acid stimulated coupling process. The production of vinblastine through enzymatic coupling pathway is thought to be highly efficient and is likely to be used commercially very soon. Vindoline and *bis*-indole alkaloids are accumulated only in green tissue and are not found in root and cell suspension cultures (Endo et al. 1987). The developmental regulation of TDS, SSC and the enzymes involved in late steps of vindoline biosynthesis has been studied extensively (De Luca and Cutler 1987; Fernandez et al. 1989). In seedlings of *C. roseus*, transcription of these enzymes was not under strong developmental control where enzymes activities were modulated by tissue specific or light dependent factors. The concentration of vindoline, catharanthine and 3',4'-anhydrovinblastine (AVBL) are age-dependent

(Naaranlahti et al. 1991). Vinblastine was increased as seedlings matured, reaching a steady concentration when the plants became more than 3 months old. On an average, whole seedlings, young plants and mature plants contained 7, 11.5 and 12-μg/g dry weight VLB, respectively. After induction of shoot formation, the VLB contents increased rapidly to similar levels of in vitro seedlings (Datta and Srivastava 1997).

5.2 Subcellular Compartmentation

Subcellular compartmentation plays an important role in alkaloids metabolism. This process of metabolism involves the participation of plant cell to separate the enzyme from their substrates and end products. In this, alkaloids biosynthesis requires three cellular compartments, namely vacuole, cytosol and plastid (Meijer et al. 1993). The transformation of tryptophan into tryptamine takes place in cytosol (De Luca and Cutler 1987; Stevens et al. 1993) and that of SSS in vacuoles (Stevens et al. 1993; McKnight et al. 1991). SG was tightly bound to the tonoplast boundary (Stevens et al. 1993). Synthesis of strictosidine takes place inside the vacuole, which is later transported to the cytoplasm where its glucose moiety gets detached. Ajmalicine has the potentiality to move freely across the cell membrane and is accumulated into the vacuoles where it is converted into the serpentine using peroxidases (Blom et al. 1991); thus produced serpentine is stored in vacuole and cannot pass through the tonoplast. In cell suspension cultures, alkaloid accumulation seems to be restricted to certain cells (Asada and Shuler 1989). Permeability of cell plays a potent role to release plant products. There are several permeabilizing agents, like DMSO and Triton X-100, which are found to be very effective in *C. roseus* cell culture. Besides, for the release of secondary products, several other agents (e.g. chitosan, alginate beads, electroportion and ultra sonication) have been used with or without cell viability in other groups of plants. The cell membrane with active uptake mechanism has also been noticed in *C. roseus*. Most of the secondary products are generally accumulated intercellular; however, several compounds such as taxol and anthraquinones are identified in the media, which filtrate itself through membrane. For this extracellular product secretion, addition of resin XAD-7 enhanced the product adsorption in *Cinchona* (Ganapathi and Kagri 1990). The media provided with amberlite-type resin and XAD-7 resin adsorbed ajmalicine and catharanthine effectively in *C. roseus*.

6 Conclusion

C. roseus has been a research symbol because of the enormous number of phytochemical compounds, secondary metabolites and the therapeutic effects that they produce. The secondary metabolites of *C. roseus* are terpene indole alkaloids exhibited pharmacologic activity and with a number of applications in human medicine.

The plant has an extensive variety of properties: anticancer, anti-diabetes, anti-helminthic, antihypertensive, anti-diarrheic, antimicrobial, among others. The indole dimeric alkaloids, vinblastine and vincristine, have become important drugs in cancer chemotherapy due to their potent antitumour activity against several types of leukaemia and solid tumours. Remarkable example is vinblastine (a member of the iboga family of the indole alkaloids), which is produced by catharanthine, vindoline and catharanthine.

Because of the use of plants and in vitro cell cultures, the biosynthesis pathway has been determined, but not entirely clarified. Moreover, a considerable number of enzymes have been characterized and their particular cloned genes defined, with the production of the alkaloids being found as well regulated at the transcriptional level.

In order to increase the availability of alkaloids for therapeutic use, the production of biomass from in vitro cultures of calluses from leaves has taken place as biotechnological tool to augment the accumulation of alkaloids in *C. roseus*. The combination of various basal media as carbon sources, phytohormones and inducers of the biotic and abiotic type may provide positive ways for the rational technical development and the increase in production yields of several of these bioactive molecules in vitro. The in vitro cultures of calluses or cells in suspension could be used at a large industrial scale to obtain bioactive compounds that are of great significance in human health, and are envisaged as models to circumvent the limitations of other production systems.

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