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Molecules of Interest

Camptothecin, over four decades of surprising findings

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Abstract

Camptothecin (CPT) is a modified monoterpene indole alkaloid produced by *Camptotheca acuminata* (Nyssaceae), *Nothapodytes foetida*, *Pyrenacantha klaineana*, *Merrilliodendron megacarpum* (Icacinaceae), *Ophiorrhiza pumila* (Rubiaceae), *Ervatamia heyneana* (Apocynaceae) and *Mostuea brunonis* (Gelsemiaceae), species belonging to unrelated orders of angiosperms. From the distribution of CPT and other secondary metabolites, it has been postulated that the genes encoding enzymes involved in their biosynthesis evolved early during evolution. These genes were presumably not lost during evolution but might have been "switched off" during a certain period of time and "switched on" again at some later point. The CPT derivatives, irinotecan and topotecan, are used throughout the world for the treatment of various cancers, and over a dozen more CPT analogues are currently at various stages of clinical development. The worldwide market size of irinotecan/topotecan in 2002 was estimated at about $750 million and at $1 billion by 2003. In spite of the rapid growth of the market, CPT is still harvested by extraction from bark and seeds of *C. acuminata* and *N. foetida*. All parts of *C. acuminata* contain some CPT, although the highest level is found in young leaves (~4–5 mg g^{-1} dry weight), approximately 50% higher than in seeds and 250% higher than in bark. The development of hairy root cultures of *O. pumila* and *C. acuminata*, and the cloning and characterization of genes encoding key enzymes of the pathway leading to CPT formation in plants has opened new possibilities to propose alternative and more sustainable production systems for this important alkaloid.

Keywords: Camptothecin; Monoterpene indole-alkaloids; *Camptotheca acuminata*; *Ophiorrhiza pumila*; Metabolic engineering

1. Introduction

Alkaloids are a diverse group of low molecular weight, nitrogen-containing molecules found in about 20% of plant species. Many of the ~16,000 alkaloids for which structures have been described function in the defense of plants against herbivores, microbes, viruses and competing plants (Wink, 2003). The potent biological activity of some alkaloids has traditionally been exploited by humans for hunting, execution, warfare, and the treatment of diseases. Plant-derived alkaloids currently in clinical use include the anticancer agents camptothecin (CPT), taxol, vincristine, and vinblastine, the analgesics codeine and morphine, the gout suppressant colchicine, the muscle relaxant (+)-tubocurarine, the antiarrythmic ajmalicine, the antimalarial quinine, the antiamoebic emetine, the antibiotic sanguinarine, the sedative scopolamine, and the topical analgesic capsaicin to mentioned some of the most representative examples (Raskin et al.,

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2002). Most alkaloids are derived from the amino acids Phe, Tyr, Trp, Lys, and Orn. The monoterpenoid indole-alkaloids (TIAs), which form a large class of complex compounds, are derived from Trp and terpenoid precursors (De Luca and St. Pierre, 2000).

CPT is a potent antitumor TIA first isolated by Monroe E. Wall and Mansukh C. Wani in 1958 from extracts of *Camptotheca acuminata* ("Xi Shu" or tree of joy), a deciduous tree native to China and Tibet, which has been extensively used in traditional Chinese medicine. CPT has a unique pentacyclic structure (Fig. 1). Since sophisticated analytical techniques for structural determination were not available in the 1960s, its structure was determined via single-crystal X-ray analysis of an analogue, CPT iodoacetate (Wall et al., 1966; for an historical review see Oberlies and Kroll, 2004). Early assessments identified the importance of the 20*S* chiral carbon for activity, and also noted a dynamic equilibrium between the close-ring lactone, and open-ring carboxylic acid forms (Fig. 1). The success of CPT in preclinical studies in tumors of both colonic and gastric origin, led to clinical investigations. Due to the negligible water solubility of CPT, these trials were initiated using the water-soluble sodium salt. The lesser efficacy of the open-ring salt form, accompanied by unpredictable and severe levels of tox-

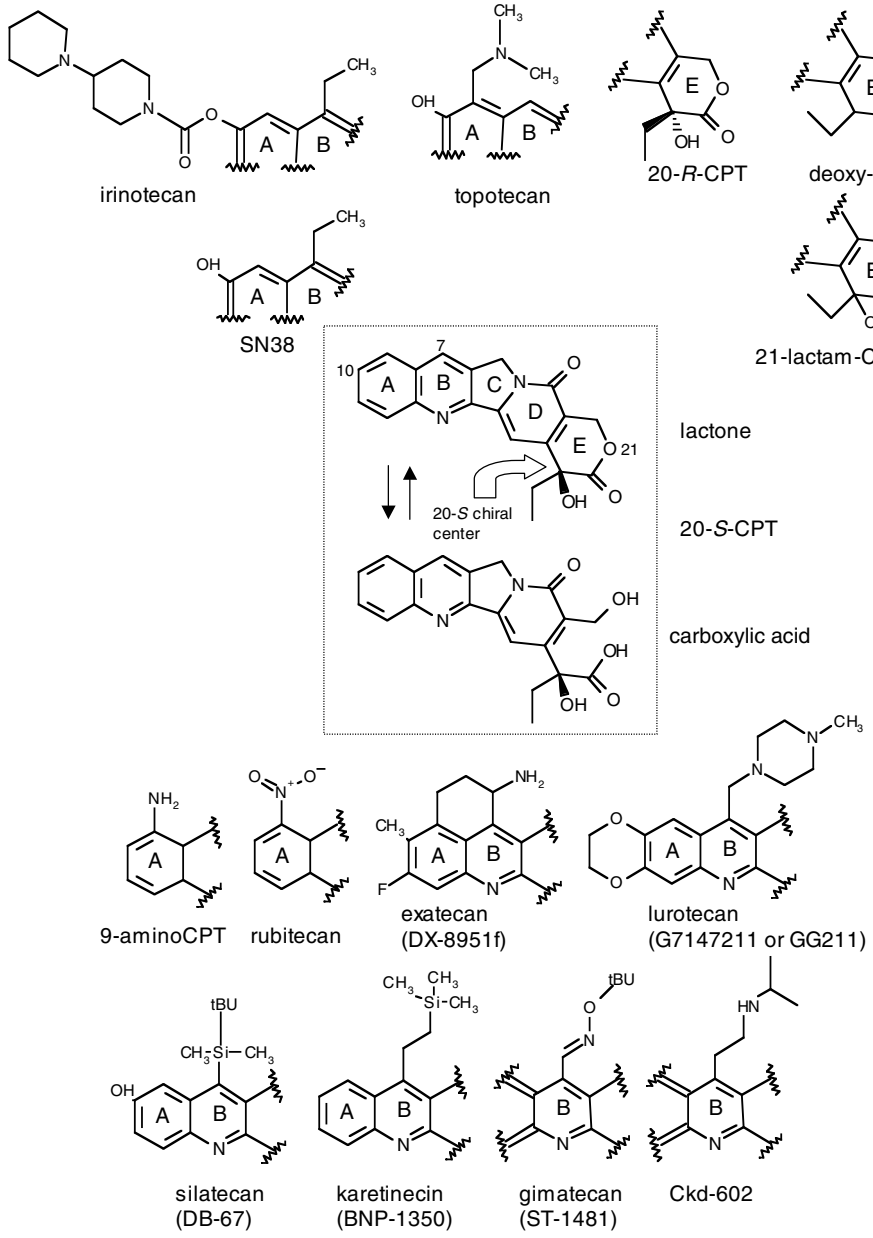

Fig. 1. Structure of CPT illustrating the 20*S* chiral center, and the dynamic equilibrium between the lactone and carboxylic acid forms. The structure of some of the derivatives that are commercially available or in clinical trials is also shown.

icity associated with treatment, including hemorrhagic cystitis and myelotoxicity, resulted in suspension of the trials (Pizzolato and Saltz, 2003 and references therein). The discovery that the primary cellular target of CPT is type I DNA topoisomerase (topo I) was the breakthrough that revived interest in the drug in the mid-1980s (Hsiang et al., 1985). Advances in the medicinal chemistry of CPT during the late 1980s and early 1990s resulted in semi-synthetic, water-soluble analogues, which are used clinically, and over a dozen of new derivatives are currently under clinical development at various stages. The clinical application of the CPTs (García-Carbonero and Supko, 2002; Zunino and Pratesi, 2004), as well as the chemical synthesis of new derivatives (Bailly, 2003; Du, 2003; Thomas et al., 2004) has been discussed in several excellent reviews.

Here we describe the distribution of CPT and its metabolites, the present understanding of its mechanism of action, the biosynthetic route leading to CPT formation in plants with emphasis in the enzymes/genes involved in the pathway, and we conclude with strategies that might aid in the development of alternative and more sustainable sources of this family of outstanding antitumor agents.

2. Distribution of CPT and its metabolites

Since secondary metabolites are often similar within members of a clade, their occurrence or absence might be taken as an indication of common descent and thus relatedness. Chemotaxonomists have compared the known distribution patterns of CPT with the phylogenetic system of classification of the angiosperms, finding surprising results (Wink, 2003 and references therein). Quite often, even allelochemicals of high structural specificity and complexity occur simultaneously in unrelated families of the plant kingdom. The multifunctional TIA CPT is an illustrative example of this kind of metabolites. As shown in Fig. 2, CPT has been isolated from samples of the following unrelated orders and families of angiosperms: Order Celastrales: *Nothapodytes foetida* (Aiyama et al., 1988), *Pyrenacantha klaineana* (Zhou et al., 2000), and *Merrilliodendron megacarpum* (Arisawa et al., 1981; Icacinaceae): Order Cornales: *C. acuminata* (Wall et al., 1966), *C. lowreyana*, and *C. yunnanensis* (Li et al., 2002; Nyssaceae); Order Gentianales: *Ophiorrhiza mungos* (Tafur et al., 1976), *O. pumila*, *O. filistipula* (Saito et al., 2001; Rubiaceae), *Ervatamia heyneana* (Gunasekera et al., 1979; Apocynaceae), and *Mostuea brunonis* (Dai et al., 1999; Gelsemiaceae).

The inevitable conclusion drawn from these observations is that the expression of secondary metabolites of a given structural type has almost invariably arisen on a number of occasions in different branches of the plant phylogenetic tree. It is likely that in some cases, the genes that encode the enzymes for the production of a given structure or structural skeleton have evolved early during evolution. These genes are not lost during phylogeny but might be "switched off". On the other hand such genes might be "switched on" again at some later point (Wink and Witte, 1983; Wink, 2003).

Table 1 summarizes the information generated by multiple research teams regarding the sites of accumulation of CPT and its concentration in multiple natural sources. The most abundant natural derivatives isolated

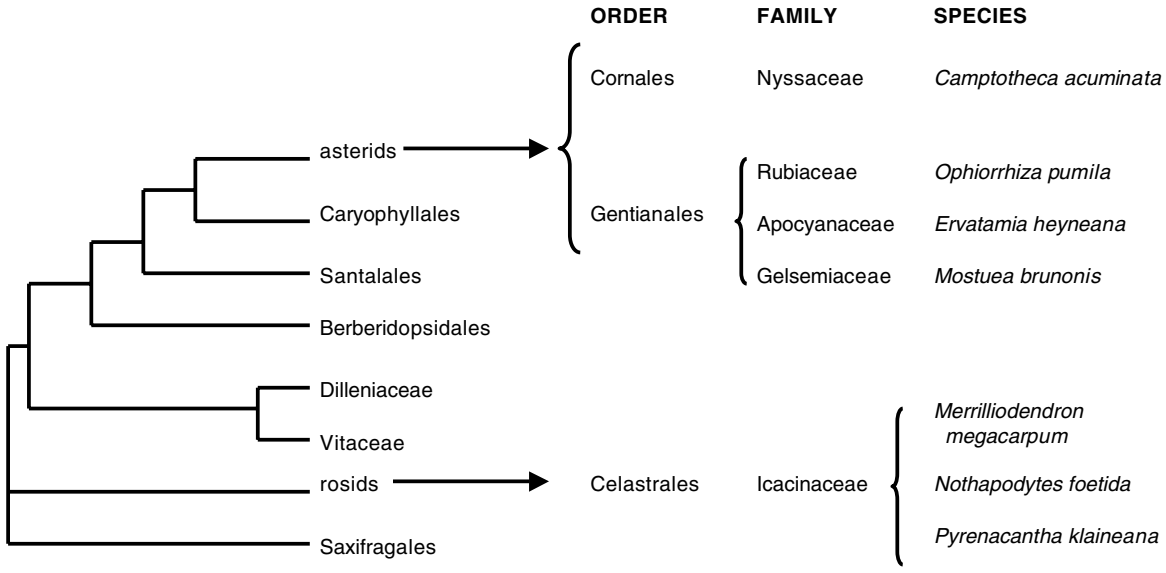

Fig. 2. CPT has been identified in plant species belonging to unrelated orders and families of angiosperms (based on recent reviews of Gentianales and angiosperm phylogeny, Backlund et al. (2000) and Hilu et al. (2003), respectively).

Table 1
Sites of accumulation of the antitumor alkaloid CPT and its natural derivatives in several natural sources

Species	Tissue analyzed	Sample origin	Camptothecinoids content (micrograms per gram dry weight)	Reference
Camptotheca acuminata Decaisne	Young leaves	Texas, USA	CPT 4000–5000 HCPT 20–30	López-Meyer et al. (1994)
	Seeds		CPT 3000 HCPT 25	
	Bark		CPT 1800–2000 HCPT 2–90	
	Roots		CPT 400 HCPT 13–20	
	Young leaves	Texas, USA	CPT 2421–3022	Li et al. (2002)
	Old leaves		CPT 482	
	Young fruit		CPT 842	
	Old fruit		CPT 2362	
	Hairy roots	Texas, USA	CPT 1000 HCPT 150	Lorence et al. (2004)
	Callus	Shangai, China	CPT 2040–2360 HCPT 80–100	Wiedenfeld et al. (1997)
	Cell cultures		CPT 2.5 –4	Sakato et al. (1974);van Hengel et al. (1992)
Camptotheca lowreyana Li	Young leaves	Texas, USA	CPT 3913–5537	Li et al. (2002)
	Old leaves		CPT 909–1184	
Camptotheca yunnanensis Dode	Young leaves	Texas, USA	CPT 2592–4494	Li et al. (2002)
	Old leaves		CPT 590	
Ervatamia heyneana (Wall) T. Cooke	Wood and stem bark	India	CPT 1300 MCPT 400	Gunasekera et al. (1979)
Nothapodytes foetida (Wight) Sleumer	Stem wood	Okinawa, Japan	CPT 1400–2400 dCPT 19	Aiyama et al. (1988)
	Stem	Taiwan	ACPT 0.24	Wu et al. (1995)
	Shoot	Mahabaleshwar, India	CPT 750 MCPT 130	Roja and Heble (1994)
	Plantlet culture		MCPT 7	
	Callus		MCPT 1	
	Stem	Godavari, India	MACPT 2.5	Srinivas and Das (2003)
	Callus	Ooty, India	CPT 9.5 MCPT traces	Ciddi and Shuler (2000)
	Cell culture	Satara, India	CPT 1.1 MCPT 0.81	Fulzele et al. (2001)
Merriliodendron megacarpum (Hemsl.) Sleumer	Leaves and stem	Guam	CPT 530 MCPT 170	Arisawa et al. (1981)
Mostuea brunonis Didr.	Entire plant	Lope, Gabon	CPT-20-*O*-β-glucoside 100 Deoxypumiloside 100 Strictosamide 600	Dai et al. (1999)
Ophiorrhiza mungos Linn.	Entire plant	Colombo, Ceylan	CPT 12MCPT 10.41	Tafur et al. (1976)
Ophiorrhiza pumila Champ.	Leaves	Japan	CPT 300–400	Saito et al. (2001)
	Young roots		CPT 1000	
	Hairy roots		CPT 1000	
	Entire plant	Kagoshima, Japan	CPT 300–510 MCPT 70–140 Chaboside 300–690	Yamazaki et al. (2003b)
	Hairy roots		CPT 240	
	Cell cultures	Japan	None	Kitajima et al. (1998)
Pyrenacantha klaineana Pierre ex Exell & Mendoca	Stems	Ankasa Game Reserve, Ghana	CPT 4.8 MCPT 1.6	Zhou et al. (2000)

CPT = camptothecin, ACPT = *O*-acetyl-CPT; dCPT = (*20S*)-18,19-dehydro CPT; HCPT = 10-hydroxy CPT; MACPT = 9-methoxy-20-*O*-acetyl-CPT; MCPT = 9-methoxy CPT.

to date are 10-hydroxy-CPT (HCPT) and 9-methoxy CPT (MCPT), although the plasticity of the biosynthetic pathway is illustrated by the variety of minor CPT-related alkaloids found in all CPT-producing plants. Both HCPT and MCPT are water-soluble derivatives with the characteristic antitumor activity of the parental molecule. All parts of *C. acuminata* contain some CPT. An important finding from our work was that the highest level of CPT is found in young leaves (~4–5 mg g^{-1} dry weight), approximately 50% higher than in seeds and 250% higher than in bark, the tissues previously used for commercial CPT production (López-Meyer et al., 1994). This observation has been confirmed by other groups, while studying other members of the genus *Camptotheca* (Li et al., 2002) and *O. pumila* (Yamazaki et al., 2003b). It has been also reported that CPT content increases with heavy shade (Liu et al., 1997), while declines with leaf, branch, and tree age, and with time during the growing season (Liu et al., 1998).

Immature leaves serve as major sinks of photosynthates and sites of production for some phytohormones. They are nutrient-rich and tender in physical structure, features that make them attractive to herbivory and pathogens. Many species defend their juvenile developmental stages metabolically. It is likely that the 5–6-fold of the CPT content in young *C. acuminata* leaves compared to mature ones and the peak of CPT production in young seedlings (López-Meyer and Nessler, 1997; Lu and McKnight, 1999), represent a chemical defense mechanism deployed by young leaves and seedlings to deter attacks by herbivores, pathogens, or both. Although the role of CPT as a defense chemical has not been directly tested, there are indirect lines of evidence indicating its involvement in plant defense. The first is the lack of any observed insect or pathogenic damage to *C. acuminata* plantations in the USA (Liu et al., 1998). The second is the reported poisoning of goats feeding on *Camptotheca* leaves (Cao et al., 1986). *Camptotheca* trees are known to be relatively tolerant to termite attacks, although the resistance mechanism involved is not known. Interestingly, a patent application for the use of CPT and its analogues in termite control was filed in the USA (Li, 2002). In addition, CPT has been reported to inhibit the sprouting of potato and root growth of radishes (Wang et al., 1980).

3. CPT as an anticancer agent

The first-generation of CPT analogues Camptosar (irinotecan or CPT-11) and Hycamtin (topotecan) are water-soluble derivatives with an intact lactone ring that were approved for use by the US Food and Drug Administration (FDA) in 1996. These drugs are marketed in the United States by Pharmacia (Pfizer) and GlaxoSmithKline, respectively. Topotecan has modest activity in patients treated previously with ovarian and small cell lung cancer and is currently approved for use in the USA as second-line therapy in these diseases. Preliminary evidence for activity against hematological malignancies is also promising. Irinotecan is a pro-drug that undergoes enzymatic conversion to the biologically active metabolite 7-ethyl-10-hydroxy-CPT. Irinotecan became commercially available in Japan in 1994, where its approved indications were cancers of the lung (small-cell and non-small cell), cervix and ovaries. Later, it was approved in Europe in 1995 as a second-line agent for colon cancer. It is presently the treatment of choice when used in combination with fluoropyrimidines as first-line therapy for patients with advanced colorectal cancer or as a single agent after failure of 5-fluorouracil-based chemotherapy. Encouraging preliminary results suggest that irinotecan may have an increasing role in the treatment of other solid tumors, including small and non-small cell lung cancer, cervical cancer, ovarian cancer, gastric cancer, and malignant gliomas (García-Carbonero and Supko, 2002). The combined sales of irinotecan and topotecan in 2003 were expected to reach nearly $1 billion (Oberlies and Kroll, 2004).

Besides continued studies on topotecan and irinotecan, much effort has also been spent on development of new CPT analogues. Fig. 1 shows some important analogues that have entered clinical trials as anticancer drug candidates.

It is important to note that the structural features of CPT that are essential for activity include the 20(*S*)-hydroxyl (Wang et al., 1999), the pyridone moiety of the D-ring, the lactone moiety of the E-ring, and the planarity of the five-membered ring system. Hence, the C–D–E rings of CPT cannot be altered without severely affecting its efficacy. In contrast, modifications to the 9, 10, and 11 positions of the A-ring and the 7 position of the B-ring, are generally well tolerated and in many cases enhance the potency of the CPT analogue in both in vivo and in vitro studies (Redinbo et al., 1998 and references therein). Recently, a new family of CPT derivatives with an expanded seven-membered lactone E-ring has been developed. This class of analogues, known as homocamptothecins (hCPTs), has enhanced plasma stability, and reinforce the inhibition of topo I compared with conventional six-membered CPTs (Bailly, 2003).

4. Mechanism of action of CPTs, poisoning of topoisomerase I

Topoisomerases (topo) are ubiquitous enzymes that solve topological problems generated by key nuclear processes such as DNA replication, transcription, recombination, repair, chromatin assembly, and chromosome segregation. There are two types of topoisomerases: topo I and topo II. Topo I catalyzes changes in

the linking number of DNA (the number of times one strand of DNA crosses the other) by breaking and resealing phosphodiester bonds. In contrast to topo II, which induces both single- and double-strand breaks, topo I mediates only single-strand breaks. The topo I-mediated reaction can be divided into four steps: (1) enzyme–DNA binding, (2) DNA single-strand cleavage by reversible trans-esterification, in which the 5′ oxygen of a phosphodiester bond is exchanged by the hydroxyl group of tyrosine 723 of human topo I (through which the protein becomes covalently linked to the 3′ terminus of the cleaved DNA strand), (3) single-strand passage, and (4) re-ligation of the cleaved DNA strand. Topo I is the cellular target of CPT and its derivatives (Hsiang et al., 1985). CPT binds only weakly to normal B-DNA under physiological conditions, and it does not bind to topo I alone. CPT induces topo I-linked DNA breaks by preventing DNA re-ligation (Avemann et al., 1988).

The intermediate in topo I-linked DNA breakage is referred to as a ''cleavable complex'' because it is readily reversible to a non-covalent enzyme–DNA complex before or after topoisomerization of the DNA. By stabilizing the cleavable complex, CPT transforms the normally useful enzyme topo I into an intracellular, cytotoxic poison, and hence, CPTs are named topoisomerase ''poisons'' to distinguish them from conventional enzyme inhibitors. It has been demonstrated that these covalent cleavage complexes stabilized by CPT act as physical barriers to DNA synthesis and kill cells as a result of replication fork collision (Svejstrup et al., 1991). The efficacy of CPTs as anticancer agents may be related to the fact that drug-mediated cleavage complexes stabilized during S phase are much more toxic than those stabilized during G1 or G2 phases (Horwitz and Horwitz, 1973).

The precise sequence of events that follow the drug-induced DNA damage leading to cell death has not been fully elucidated. In vitro studies have shown that CPT-induced DNA damage abolishes the activation of the $p34^{cdc2}$/cyclin B complex and results in cell cycle arrest at the G2 phase. It has also been observed that treatment with CPTs can induce transcription of the *c-fos* and *c-jun* early response genes, which occurs in association with inter-nucleosomal DNA fragmentation, a characteristic of programmed cell death (apoptosis). In addition, non-cytotoxic concentrations of CPTs can induce differentiation of human leukemia cells. Other reports have suggested that CPTs may also have an anti-angiogenesis effect (reviewed in García-Carbonero and Supko, 2002). Recently, it has been shown that topo I is catalytically active in cleaving the G-rich telomeric strands in vitro in the presence of CPT and that endogenous topo I–DNA covalent complexes are present in chromosomal telomeric DNA in CPT-treated cells. These results suggest that telomeric DNA damage by topo I may be one of the early events in CPT-induced apoptosis (Kang et al., 2004). Mechanisms of resistance to CPT and its derivatives are not well understood and could arise from poor drug trafficking to tumors, efficient clearance of drug-induce lesions, and mutations in the target enzyme topo I (reviewed in Rasheed and Rubin, 2003).

Cross-linking studies have suggested the interaction of CPT with both, topo I and the DNA, forming a ternary complex that stabilizes the trans-esterification intermediate (Hertzberg et al., 1990; Pommier et al., 1995). The structure of this ternary complex is of great interest for elucidation of the detailed mechanism of action of CPT and development of new topo I poisons. Three binding models of CPT to the cleavable complex have been proposed by Pommier (Fan et al., 1998) and Hol (Redinbo et al., 1998) with their coworkers in 1998, and by Kerrigan and Pilch (2001). Remarkable progress has been made during the last six years on the understanding of the interaction between the CPT molecule, the DNA helix and specific residues of the topo I enzyme. None of these binding models is able to provide explanation for all of the mutations that have been found in CPT-resistant topo I (Staker et al., 2002; Chrencik et al., 2004).

A recent in vitro study on the subcellular localization of topotecan and gimatecan, has shown that topotecan gets sequestered mainly to the mitochondria, whereas the more lypophilic, gimatecan exhibits lysosomal localization (Croce et al., 2004). The increase persistence of DNA damage in gimatecan-treated cells is consistent with the interpretation that lysosomes represent a store of active drug. These results indicate that apart from intracellular accumulation, subcellular distribution plays a role in CPTs cytotoxic potency and contributes to their pharmacological features as illustrated in Fig. 3.

Continued development of topo I poisoning drugs is essential, as topo II poisons (i.e. deoxirubicin, etoposide, mitoxantrone, amsacrine) fall out of favor owing to their risk of secondary, therapy-related leukemias, a long-term side effect not shared by the CPTs.

5. Other pharmacological activities of CPT

Although the antitumor activity of CPT and its derivatives has been the focus of research for groups in both the industry and academic arenas, CPTs have also been studied as potent inhibitors of replication, transcription, and packing of double stranded DNA-containing adenoviruses, papovaviruses, and herpesviruses, and the single-stranded DNA-containing autonomous parvoviruses (reviewed in Pantazis et al., 1999). CPT inhibits viral functions by poisoning topo I, the host cell enzyme required for initiation and completion of viral functions. If properly developed, CPTs could prove to be powerful antiviral drugs for several DNA viruses,

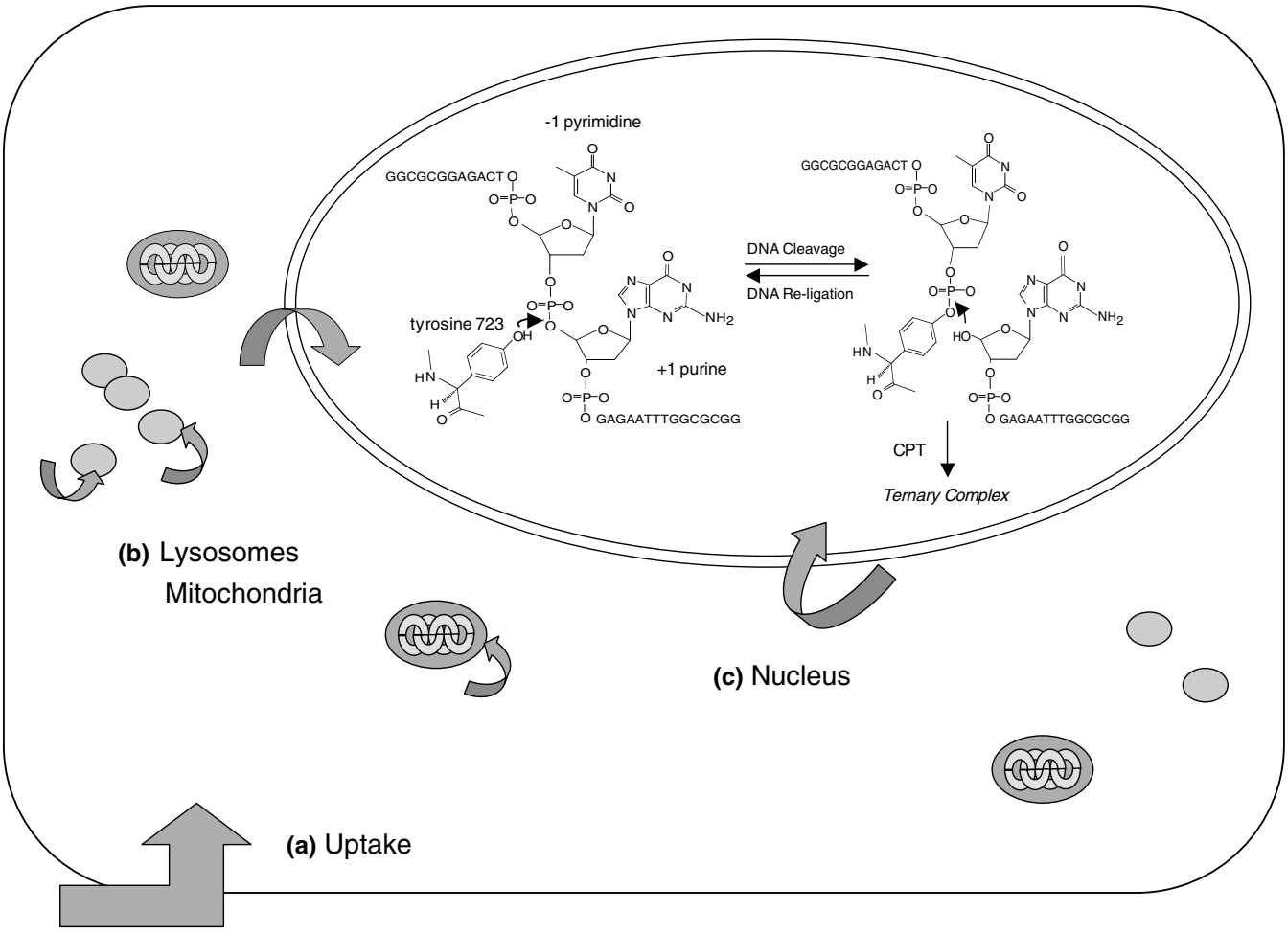

Fig. 3. Mechanism of action of CPT, a topoisomerase I "poison". Relevant events in determining the cytotoxic potency of CPT and its derivatives are: (a) uptake, (b) lysosomal or mitochondrial sequestration and, (c) nuclear localization and stabilization of the "cleavable complex".

which are causative agents for a large number of diseases.

In the mid-1990s, CPT was also shown to have promising activity against parasitic trypanosomes and *Leishmania* (Bodley and Shapiro, 1995). More recently, researchers at the National Cancer Institute screened 2000 diverse compounds for functional inhibition of the hypoxia-inducible factor 1 (HIF-1), a master regulator of the cancer cell's ability to survive under oxygen deprivation. Only four compounds exhibited HIF-1 inhibitory activity, and three of these were CPTs (Rapisarda et al., 2002). Hence, these drugs may have other desirable activities against solid tumors that are independent of topo I poisoning.

6. Biosynthesis of CPT

Several investigators have recognized plant cell cultures as a tool to unravel the biosynthetic pathway that leads to CPT formation in plants and as a potential system for its production. The first tissue culture study of *C. acuminata* was reported by Sakato et al. (1974). Those cultures produced 0.002 mg CPT g^{-1} dry weight, whereas the whole plant contains from 0.2 to almost 5 mg g^{-1} dry weight, depending on the tissue analyzed (López-Meyer et al., 1994). Later reports of in vitro CPT production by *C. acuminata* range from 0.004 mg g^{-1} dry weight in cell suspensions (van Hengel et al., 1992) to ≈2 mg g^{-1} dry weight in callus cultures (Wiedenfeld et al., 1997). These callus cultures were also reported to contain HCPT, from trace amounts up to 0.08–0.1 mg g^{-1} dry weight (Wiedenfeld et al., 1997), compared with the 0.002–0.09 mg g^{-1} dry weight HCPT found in the bark of the plant (López-Meyer et al., 1994).

Calluses and cell suspensions of *N. foetida* were found to produce small amounts of CPT and MCPT (Roja and Heble, 1994; Ciddi and Shuler, 2000; Fulzele et al., 2001), but again the level of alkaloid production was 100- to 1000-fold lower than that from soil-grown plants. There is a single report on the establishment of

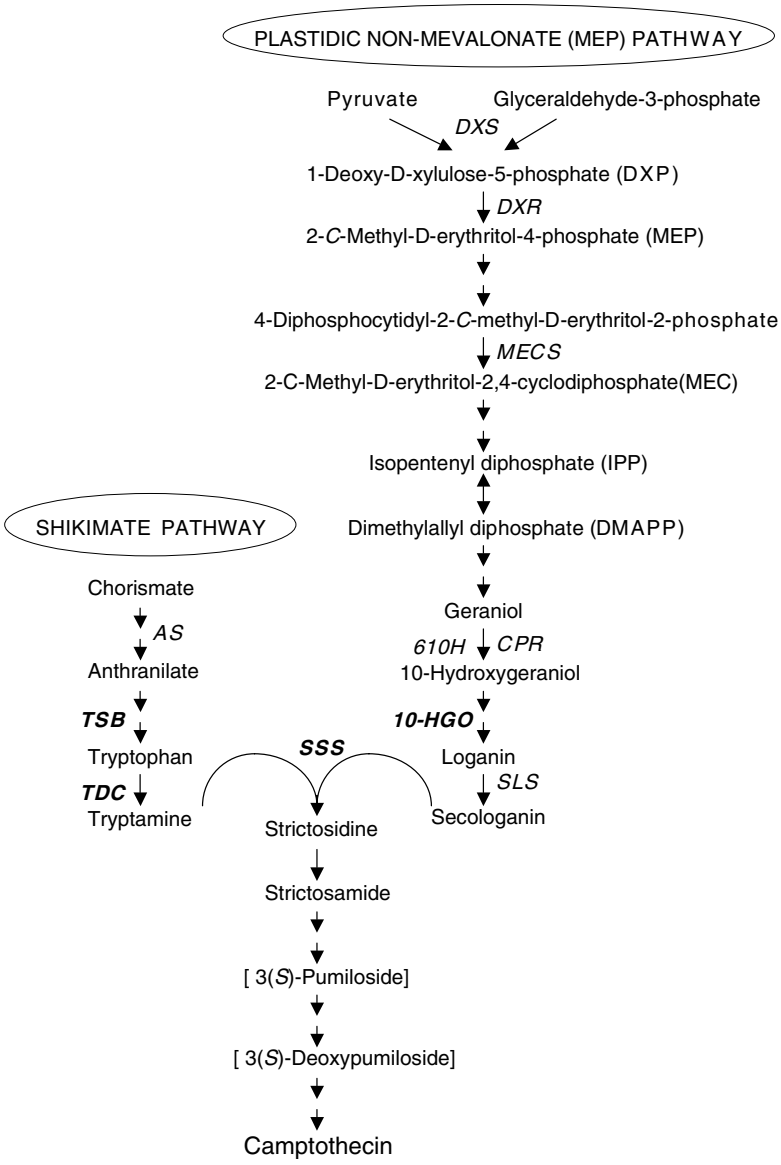

Fig. 4. Biosynthetic pathway for TIAs in CPT-producing plants. Multiple arrows indicate multiple steps between intermediates. The enzymes that have been cloned and characterized in either *C. acuminata* or *O. pumila* are shown in bold: TSB (β-subunit of tryptophan synthase), TDC (tryptophan decarboxylase), SSS (strictosidine synthase), and 10-HGO (10-hydroxygeraniol oxidoreductase). TSB is abundant in vascular tissues (cambium, primary xylem and primary phloem; Lu and McKnight, 1999). Other enzymes involved in this pathway already cloned in *C. roseus*, the best characterized TIAs-producing model, are also shown: 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS); DXP reductoisomerase (DXR); 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MECS); geraniol-10-hydroxylase (G10H), and secologanin synthase (SLS). In *C. roseus*, *dxs*, *dxr*, *mecs*, and *g10h* are expressed in the internal phloem parenchyma, while *sls*, *tdc* and *sss* are expressed in the epidermis (Burlat et al., 2004).

O. pumila cell cultures, however, the authors were not able to detect CPT or its derivatives (Kitajima et al., 1998). All these studies indicate that CPT biosynthesis and accumulation, as in the case of many other alkaloids, are under the strict control of cell development and environmental factors (De Luca and St. Pierre, 2000).

All TIAs, including CPT, are derived from the common precursor strictosidine, which is the product of a condensation reaction between the indole tryptamine and the terpenoid secologanin catalyzed by the enzyme strictosidine synthase (Kutchan, 1995) (Fig. 4). Early feeding experiments with radiolabeled precursors confirmed that the tryptamine moiety is completely incorporated into the CPT molecule (Sheriha and Rapoport, 1976). Strictosidine is then converted to strictosamide via intermolecular cyclization and this compound is a precursor of CPT as proven by incorporation of the radiolabeled precursor. The remaining details and precise intermediates between strictosamide and CPT are not completely defined. It has been postulated that CPT could be formed from strictosamide by three trans-

formations: (1) oxidation-recyclization of the B- and C-rings, (2) oxidation of the D-ring and removal of the C-21 glucose moiety, and (3) oxidation of ring E (Hutchinson et al., 1979). A presumed precursor of CPT was isolated from the polar fraction of a large-scale extraction of C. *acuminata* (Carte et al., 1990). 3(*S*)-Pumiloside, 3(*S*)- and 3(*R*)-deoxypumiloside, plausible CPT precursors, have been found in *O. pumila* (Aimi et al., 1989; Kitajima et al., 1997; Yamazaki et al., 2003b).

Because Trp biosynthesis is required for both primary and secondary metabolism in *C. acuminata*, how this part of the CPT pathway is expressed and regulated was of some interests. Trp biosynthesis begins with the conversion of chorismate to anthranilate by anthranilate synthase. After production of the intermediates 5-phosphoribosylanthranilate and indole glycerol phosphate, the α-subunit of Trp synthase (TSA) produces indole, which is then condensed with Ser by the β-subunit of Trp synthase (TSB) to form the final product (Fig. 4). The entire Trp pathway has been localized to the plastid (Zhao and Last, 1995), but all the enzymes are encoded by nuclear genes. Lu and McKnight (1999) cloned and characterized the TSB from *C. acuminata*. *Tsb* mRNA and protein were detected in all *C. acuminata* organs examined, and their abundance paralleled that of CPT. Within each shoot organ, TSB was most abundant in vascular tissues, especially the cambium, primary xylem, and primary phloem. *Tsb* was also highly expressed in *C. acuminata* during early seedling development at a stage corresponding to peak accumulation of CPT, consistent with the idea that Trp biosynthesis and the secondary TIAs pathway are coordinately regulated.

Once Trp is formed, tryptophan decarboxylase (TDC, E.C. 4.1.1.28) converts it to tryptamine, and therefore plays a role in TIAs biosynthesis by linking primary and secondary metabolism. TDC has been extensively studied in *Catharanthus roseus* cultures. The activity of this enzyme correlated with TIAs accumulation in suspension cell lines treated with biotic and abiotic elicitors (Eilert, 1987) or transferred to alkaloid production medium (Merrillón et al., 1986). TDC is also highly expressed in developing seedlings of *C. roseus* (De Luca et al., 1998; Alvarez-Fernández et al., 1989), and exogenous application of the signal molecule methyl jasmonate (MeJa) enhances TDC activity and alkaloid accumulation during germination (Aerts et al., 1994). In *Catharanthus* TDC is encoded by a single-copy gene (De Luca et al., 1989). *Tdc* mRNA accumulates after auxin starvation (Goddijn et al., 1992) and exposure to fungal elicitors (Pasquali et al., 1992), which suggest that the effect of elicitation of TDC occurs at the transcriptional level.

In contrast to *C. roseus*, two genes encode TDCs in *C. acuminata* (López-Meyer and Nessler, 1997). When expressed in *Escherichia coli*, the products of *tdc1* and *tdc2* could decarboxylate Trp, but were inactive against Tyr, Phe, and 3,4-dihydroxyPhe (DOPA). *Tdc1* is developmentally regulated, having its highest expression level in the apex, young stem, and bark, tissues which also contain the highest level of CPT. Expression of *tdc1* also increased during seedling development and was correlated with alkaloid accumulation during germination. *Tdc2* was only expressed when induced in *Camptotheca* leaf discs and cell suspension cultures treated with fungal elicitor or MeJa, treatments which did not affect *tdc1* expression. These data suggest that *tdc1* may be part of a developmentally regulated chemical defense system in *C. acuminata*, while *tdc2* serves as part of the defense system induced during pathogen challenge.

Recently, a cDNA encoding a functional TDC was isolated from *O. pumila* hairy roots. The deduced OpTDC showed high amino acid identity when compared to *C. acuminata* and *C. roseus* TDCs (71% and 67%, respectively). DNA hybridization experiments indicate that there are at least two copies of the *tdc* gene in the *O. pumila* genome. The highest *tdc* expression occurred in hairy roots, followed by the root and the stem. No expression was observed in the leaves of the plant (Yamazaki et al., 2003a).

For the formation of isopentenyl diphosphate (IPP), the precursor of terpenoid biosynthesis, the mevalonate (MVA) pathway has been known since the 1950s (reviewed in Cane, 1999). For many years the MVA route was thought to be the only source of building blocks for all plant isoprenoids. Recently, the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway, in which IPP is formed from 1-deoxy-D-xylulose-5-phosphate by condensation of glyceraldehyde-3-phosphate and pyruvate, was found to be present in many eubacteria, green algae, and plastids of plants (reviewed in Rohmer, 1999). Both isoprenoid pathways are operative simultaneously in higher plants. The enzymes of the MVA route are located in the cytoplasm, where they supply precursors of triterpenes, sesquiterpenes, and sterols. The enzymes of the MEP pathway carry putative plastid targeting signals and are believed to be located in plastids where they produce precursors for monoterpenes, some sesquiterpenes, diterpenes and carotenoids (Eisenreich et al., 2001; Rohdich et al., 2001). However, it has also been shown that the compartmental separation of the two pathways in plant cells is not absolute (Schwartz and Arigoni, 1999). In a recent quantitative NMR spectroscopic study using radiolabeled precursors and *C. roseus* cell cultures, it was shown that the cross-talk between MVA and MEP pathways cannot be explained in detail by a simple two-compartment model and requires further studies of the complex regulatory mechanisms involved (Schuhr et al., 2003).

A small family of genes encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the enzyme that catalyzes the conversion of HMG-CoA to mevalonate (*hmg1*, *hmg2*, and *hmg3*), has been isolated and

characterized in *C. acuminata*. This irreversible reaction is considered to be the key regulatory step controlling the flux through the MVA pathway. In *Camptotheca*, *hmg1* transcripts were detected only in young seedlings and not in vegetative organs of older plants. Using deletions and translational fusions of the *hmg1* promoter and β-glucuronidase (GUS), it was shown that a 165-bp fragment of this promoter is sufficient to confer developmental regulation as well as wound induction and MeJa suppression of expression in transgenic tobacco. In vegetative tobacco tissues, GUS staining was localized to the epidermis of young leaves and stems, particularly in glandular trichomes. Roots showed intense staining in the cortical tissues in the elongation zone and light staining in the cortex of mature roots; expression of *hmg1::GUS* was also observed in sepals, petals, pistils, and stamens of developing flowers, with darkest staining in the ovary wall, ovules, stigmas, and pollen (Burnett et al., 1993). In *Camptotheca*, transcript levels for two family members were highest in the shoot apex, dry seeds (*hmg1*), and bark (*hmg3*) which are the tissues containing the highest levels of CPT and HCPT, respectively. Levels of *hmg3* mRNA correlated with the accumulation of HCPT during germination. In *C. acuminata* leaf disks, *hmg1* mRNA increased in response to wounding, and this induction was suppressed by MeJa, in agreement with results previously obtained in tobacco (Burnett et al., 1993). In contrast, wounding and MeJa did not affect *hmg2* and *hmg3* transcript levels in C. *acuminata* (Maldonado-Mendoza et al., 1997). These results showed that members of the *C. acuminata* HMGR family are differentially expressed in various tissues under different physiological conditions, which may contribute to a certain degree to the regulation of TIAs in this species.

Secondary metabolites, such as the terpenoid moiety of TIAs in *C. roseus*, are primarily derived from the MEP pathway (Contin et al., 1998; Chahed et al., 2000; Veau et al., 2000). Recently, feeding studies with *O. pumila* hairy roots and [1-^{13}C]-glucose, assisted by the Atomic Reconstruction of Metabolism (ARM) software, have shown that the secologanin moiety of CPT was synthesized via the MEP pathway, while the quinoline skeleton from tryptophan was labeled as predicted by the ARM program via the shikimate pathway (Yamazaki et al., 2004). In *C. acuminata*, there have been no studies to evaluate the contribution of the MVA and MEP routes to CPT production, thus contribution of enzymes involved in the MVA pathway (i.e. HMGR) to TIAs biosynthesis in this species needs further assessment.

As shown in Fig. 4, strictosidine synthase (SSS, E.C. 4.3.3.2) is the enzyme that mediates the condensation of tryptamine with the iridoid glucoside secologanin to yield strictosidine (Kutchan, 1995). The cDNA encoding SSS was first isolated from *Rauvolfia serpentina* (Kutchan et al., 1988) and subsequently from *C. roseus* (McKnight et al., 1990). Those *sss* genes have been heterologously expressed in *E. coli*, yeast, insect cells and tobacco (Kutchan et al., 1988; Kutchan, 1989; McKnight et al., 1991). Interestingly, two independent groups have failed to detect SSS activity in *C. acuminata* and *N. foetida* (López-Meyer et al., 1994; Yamazaki et al., 2003b) or isolate homologs of the *C. roseus sss* cDNA in *C. acuminata* libraries (López-Meyer and Nessler, unpublished results). In a recent study using a *C. acuminata* CG1 cell line that does not produce alkaloids, the Verpoorte group has been able to detect SSS (Silvestrini et al., 2002). The activity of the enzyme in 11-day old cells was 0.1 pkat mg^{-1} of protein, which is considerably lower compared to the typical SSS values in cell cultures of *C. roseus* (i.e. from 10 to 400 pkat mg^{-1} of protein). The SSS activity in the *C. acuminata* plant was also low (i.e. 1.1 pkat mg^{-1} protein in the leaf). Feeding studies with strictosidine revealed that this precursor was easily biotransformed by two enzymes (i.e. a hydroxylase and a dehydrogenase) in hydroxystrictosidine and didehydrostrictosidine), but CPT was never detected (Silvestrini et al., 2002).

A cDNA encoding a functional SSS has been isolated from *O. pumila*. The deduced amino acid sequence of OpSSS exhibited 51% and 55% identity with SSS from *C. roseus* and *R. serpentina*, respectively. OpSSS is most likely located in the vacuole according to predictions by pSORT, where the SSS product was localized in *C. roseus* (McKnight et al., 1991). Southern blot experiments suggest that SSS in encoded by a single-copy gene in the *O. pumila* genome. The highest *Opsss* expression occurred in hairy roots, followed by the root and the stem, whereas no expression was detected in leaves. The *Opsss* expression pattern paralleled with one observed for *Optdc*, suggesting coordinate expression of these genes for CPT biosynthesis. The expression of *Opsss* was repressed by salicylic acid, yeast extract, and NAA (Yamazaki et al., 2003a).

In *C. roseus* aerial organs, TIAs appear to accumulate in specialized cells, the laticifer-idioblast system (St-Pierre et al., 1999). Recent studies showed that the last two steps in the biosynthesis of vindoline, one moiety of the active dimers vinblastine and vincristine, occur in these TIA-accumulating cells. Interestingly, the central steps of this pathway, involving the formation of tryptamine and secologanin, and their subsequent condensation to form strictosidine, occur in the epidermis (St-Pierre et al., 1999; Irmler et al., 2000). Using Northern blot and in situ hybridization experiments, it has been shown that three MEP pathway genes (*dxs*, *dxr*, *mecs*, Fig. 4) and the gene encoding geraniol 10-hydroxylase (*g10h*), a cytochrome P450 monooxygenase involved in the first committed step in the formation of monoterpenoids display identical cell-specific expression patterns. These results indicate a new level of complexity

to the multicellular nature of TIAs biosynthesis. Intermediates are proposed to be translocated from the internal phloem parenchyma to the epidermis and, ultimately, to laticifers and idioblasts during TIAs biosynthesis (Burlat et al., 2004). Similar studies in CPT-producing plants are necessary to gain a better understanding of the regulation of the pathway(s) leading to the formation of this important alkaloid. Such studies will help in explaining apparent discrepancies, i.e. lack of expression of the biosynthetic genes *tdc* and *sss* in *O. pumila* leaves (Yamazaki et al., 2003a), but CPT accumulation in that tissue (Yamazaki et al., 2003b).

At the cellular level, CPT has been localized in the mesophyll and sub-palisade layers of young leaves and in the palisade and one cell layer beneath the palisade layer in older *Camptotheca* leaves. At the subcellular level, CPT has been localized in the vacuoles of young and older leaves (Nolte, 1999). The precise mechanism of transport and storage of CPT and its precursors remains unclear. One possible mechanism of transport of such an insoluble compound is its conversion into a more water-soluble form such as a glucoside, e.g. chaboside (Aimi et al., 1990) and transport to other parts of the plant (Yamazaki et al., 2003b).

7. Methods of CPT production

Although CPT itself is not used clinically due to its cytotoxicity, CPT derivatives are being used for the treatment of cancer throughout the world. These semi-synthetic derivatives are made from natural CPT, which is obtained by extraction from bark and seeds from intact plants. However, extraction from plants is problematic because of a shortage of natural resources and subsequent environmental concerns. Thus, alternative and more sustainable methods for CPT production are needed.

We have developed two methods to increase the amount of CPT harvested available for derivatization. One way is through the clonal propagation of elite cultivars via shoot bud culture, which insures that highly productive trees can be rapidly propagated (Jain and Nessler, 1996). Another production method is the repeated harvest of *C. acuminata* young leaves, without the destruction of the tree. Under greenhouse conditions, a six-week harvest interval of the young leaves produced a high amount of CPT per gram fresh weight (175 mg CPT 12 weeks^{-1} 2 m^{-2}; Vincent et al., 1997).

Since alkaloid biosynthesis and accumulation are under the strict control of cell developmental and environmental factors (De Luca and St. Pierre, 2000), establishing cultures of cell types suitable for biosynthesis and accumulation are a requirement for the in vitro production of CPT. Root cultures often exhibit a remarkable ability to synthesize a diversity of plant secondary metabolites (Flores et al., 1999). Hairy root cultures induced by transformation with the soil-borne pathogen *Agrobacterium rhizogenes* are an attractive experimental system as they are long-term aseptic root clones, genetically stable, with growth rates comparable to those of the fastest growing cell suspension cultures. Saito et al., 2001) described hairy root cultures of *O. pumila* that produce and partially secrete CPT (~1 mg g^{-1} dry weight). These authors could stimulate secretion of the alkaloid by adding Diaion HP-20, a polystyrene resin to the medium, which reversibly absorbs the alkaloid and from which it can be easily recovered. These cultures have been scaled up to a 3-l bioreactor for production of CPT (Sudo et al., 2002), and have been a key tool for allowing these investigators to make considerable progress on the understanding of the CPT biosynthetic pathway in *O. pumila* (Yamazaki et al., 2003a,b; Yamazaki et al., 2004). *C. acuminata* hairy roots have also been established (Lorence et al., 2004). These cultures produce and secrete CPT and HCPT into the medium. Remarkably, these cultures were able to synthesize the alkaloids at levels equal to, or sometimes greater than, the roots *in planta*, i.e., 1.0 and 0.15 mg g^{-1} dry weight for CPT and HCPR, respectively. These results from *C. acuminata* hairy roots and those of Saito et al., 2001) in *O. pumila*, strongly suggest that the differentiated phenotype of the culture is an essential requirement for high production levels of CPT.

8. Prospects for CPT research

The suite of genes encoding enzymes involved in the CPT biosynthetic pathway already available [*tdc1*, *tdc2*, *hmg1*, *hmg2*, *hmg3*, *tsb* and 10-hydroxygeraniol oxidoreductase or *hgo* (Gorman and McKnight, Texas A&M University, unpublished results) from *C. acuminata*; *tdc*, *sss* and a *P450* from *O. pumila* (Yamazaki et al., 2003a)], the hairy root cultures, and the recent progress in the understanding of the complexity of the regulation of similar routes in other TIA-producing plants are all tools that will allow the development of more sustainable sources and processes for CPT production.

Ectopic expression of specific transcription factors can redirect the metabolic differentiation of plant cells by acting simultaneously and coordinately on different events, including the regulation of the expression of genes that encode biosynthetic enzymes and proteins that are necessary for metabolite storage and differentiation of appropriate subcellular compartments (Memelink et al., 2001; Gantet and Memelink, 2002; Vom Endt et al., 2002).

A short *sss* promoter sequence called the JERE (jasmonate and elicitor-responsive element) is responsible for elicitor-responsive and jasmonate-responsive gene expression (Menke et al., 1999). Using the JERE as bait

in yeast one-hybrid screening a cDNA that encodes ORCA2 (octadecanoic-responsive *Catharanthus* AP2-domain protein 2) was isolated. ORCA2 is a transcription factor of the plant-specific AP2/ERF (APETALA2/ethylene-responsive factor) family that is characterized by the presence of an AP2/ERF DNA-binding domain. Transcription of *ORCA2* is rapidly induced by jasmonate and ORCA2 activates *sss* expression by interacting with the JERE (Menke et al., 1999). A closely related TIA regulator gene, called *ORCA3*, was isolated by T-DNA activation tagging in cultured *C. roseus* cells (van der Fits and Memelink, 2000). ORCA3 also binds to the JERE and activates *sss* expression. *ORCA3* expression is also induced by jasmonate, which indicates that the functions of ORCA3 and ORCA2 might overlap to regulate jasmonate-responsive expression of alkaloid biosynthetic genes. Ectopic expression of ORCA3 in cultured *C. roseus* cells induced several genes in primary and secondary metabolism leading to TIAs biosynthesis, including *sss* and *tdc*, and resulted in increased TIAs production upon feeding of secologanin (van der Fits and Memelink, 2000).

In addition, the *sss* promoter contains an element that is conserved in plants, called the G-box located adjacent to the JERE element. The G-box is an active *cis* -regulatory element in *planta*. A yeast one-hybrid screen using the G-box as bait isolated G-box binding factors (CrGBFs) of the basic leucine zipper class and MYC-type bLHL transcription factors (Chatel et al., 2003). CrGBF1 and CrGBF2 were shown to repress *sss* expression (Sibéril et al., 2001). They also bind in vitro to a G-box like element in the *tdc* promoter (Sibéril et al., 2001), which indicates that CrGBFs could coordinately regulate several TIA biosynthetic genes. Inactivating CrGBF transcription factors might de-repress TIAs biosynthesis, particularly the terpenoid branch of the pathway. Combined with ORCA3 over-expression, this would activate TIAs biosynthesis (Gantet and Memelink, 2002).

Constitutive expression of the cold-responsive AP2/ERF domain protein DREB1A/CBF3 under control of the cauliflower mosaic virus 35S promoter resulted in transgenic *Arabidopsis* plants with a dwarf phenotype (Shinozaki and Yamaguchi-Shinozaki, 2000). We have obtained similar results by expressing the *C. acuminata* ORCA3 transcription factor in *Arabidopsis*, under the control of the 35S promoter (Lorence and Nessler, unpublished results). Experiments using transcription factors homologs to the ORCA2, ORCA3, CrGBFs, and CrMYC from *C. roseus* might be the starting point of new breakthroughs in CPT research. Modular constructs where these transcription factors can be "switch on" at specific moments or in specific tissues could offer particular advantages for driving high levels of alkaloid expression under controlled environmental conditions (Broun, 2004).

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