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Camptothecin: Discovery and Developments

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ABSTRACT

Camptothecin (CPT), a monoterpene indole alkaloid, is a promising plant based metabolite known for its anti-tumour activity. CPT is regarded as one of the most promising anticancer drugs of the twenty first century. This compound was isolated through bioassay guided fractionation of various extracts and through chromatographic fractions. Their unique structures were elucidated by nuclear magnetic resonance, mass spectrometry and X-ray analysis. CPT uniquely inhibits an enzyme, topoisomerase I, involved in DNA replication thus inhibiting cell division. CPT and its analogs singly or combined with cisplatin showed efficacy against solid tumors, breast, lung and colorectal cancer, which hitherto have been unaffected by most cancer chemotherapeutic agents.

KEYWORDS: Camptothecin, Secondary metabolites, Anticancer agents, Isolation guided by bioassay.

ABBREVIATIONS: *CPT, camptothecin; ACPT, O-acetyl-CPT; HCPT, 10-hydroxy CPT; MACPT, 9-methoxy-20-O-acetyl-CPT; MCPT, 9-methoxyCPT; N. foetida, Nothapodytes foetida; C. acuminata, Camptotheca acuminata; O. pumila, Ophiorrhiza pumila; TSB, β-subunit of tryptophan synthase; TDC, tryptophan decarboxylase; SSS, strictosidine synthase; 10-HGO, 10-hydroxygeraniol oxidoreductase; DXP, 1-deoxy-D-xylulose-5-phosphate; MECS, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; G10H, geraniol-10-hydroxylase; SLS, secologanin synthase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; T-I, Topoisomerase I.*

INTRODUCTION

Camptothecin is a monoterpene indole alkaloid was first isolated from a Chinese deciduous tree *Camptotheca acuminata* (Nyssaceae) (1). Irinotecan and topotecan, two water soluble derivatives of camptothecin (CPT), have been approved by the Food and Drug Administration (FDA) of the United States of America for treating colorectal and ovarian cancer (2-4). CPT Later it was isolated from a variety of plant species including *Merriliodendron megacarpum,* and *Nothapodytes nimmoniana* (family Icacinaceae), *Ophirrohiza mungos* and *O. pumila* (family Rubiaceae), *Eravatamia heyneana* (family Apocynaceae) and *Mostuea brunonis* (family Loganiaceae) (5-9). Among these, the highest concentration of CPT (about 0.3% dry weight) has been reported from *N. nimmoniana* (5). Natural products chemists and phytochemists have always been impressed by the fact that compounds found in nature display an almost unbelievable range of diversity in terms of their structures and physical and biological properties. Most of these compounds are secondary metabolites whose functions in plants, fungi and marine organisms are still not widely understood. Currently, it is believed that many of these compounds act in defense of the harmful effects of toxins, carcinogens or mutagens found in the plant or attack by external predators (10-12).

Phytochemical screening for cortisone precursors

During the period 1950-1959, M. E. Wall, director of a large program at the Eastern Regional Research Laboratory (ERRL), USDA, Philadelphia, PA, which involved a screening study of thousands of plants, searching for steroids that would be cortisone precursors. The plant collections were conducted by botanists under the auspices of the Plant Introduction Division of the USDA. The joint effort of chemists and botanists proved to be a good model for future natural products program. The survey included not only quantitative data for steroidal sapogenins, but also qualitative analysis for sterols, alkaloids, tannins, and flavonoids (13). Thousands of the plant alcoholic extracts, particularly of the more unusual plants, were saved. One of the extracts so saved and stored was prepared from the leaves of *Camptotheca acuminata*, a tree that is native to China, growing in relatively warm areas of southeastern provinces of this country, such as Szechwan, Yunnan, and Kwangsi (14). This plant was introduced several times into the USA, and eventually a few trees were grown at a USDA Plant Introduction Garden in Chico, CA, from which this sample was procured for a detailed account of the introduction of *C. acuminata* into the USA and California. Site of accumulation of CPT among their natural sources were shown in Table 1.

Discovery of anti-tumor activity in extracts of *Camptotheca acuminata*

In addition to screening the plants collected for various chemical constituents, some extracts were tested for antibiotic, antitumor, and antiviral activity. In 1957, after a visit by the late Dr. Jonathan Hartwell from the Cancer Chemotherapy National Service Center (CCNSC), considered by most natural products scientists to be the pioneer worker in the field of plant antitumor constituents, it was agreed to send him 1000 ethanolic plant extracts for testing for antitumor activity. Almost a year later the astonishing result came back that the *Camptotheca* extracts were the only ones to have high activity. Distribution of CPT in plant species belonging to unrelated orders and families of angiosperms was

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Table 1: Sites of accumulation of the anti-tumor alkaloid CPT and its natural derivatives in natural sources (15).

Species	Tissue analyzed	Sample origin	Camptothecinoids content (µg/gram dw)
C. acuminata	Young leaves	Texas, USA	CPT 4000-5000, HCPT 20-30
	Seeds		CPT 3000, HCPT 25
	Bark		CPT 2000, HCPT 2-90
	Root		CPT 400, HCPT 13-20
	Young fruit		CPT 842
	Old fruit		CPT 2362
C. lowreyana	Young leaves	Texas, USA	CPT 3913-5537
	Old leaves		CPT 909-1184
C. yunnanensis	Young leaves	Texas, USA	CPT 2592-4494
	Old leaves		CPT 590
E. heyneana	Bark	India	CPT 1300, MCPT 400
N. foetida	Stem wood	Okinawa, Japan	CPT 1400-2400
	Stem	Taiwan	ACPT 0.24
	Shoot	Mahabaleshwar, India	CPT 750, MCPT 130
	Plantlet culture		MCPT 7
	Callus		MCPT 1
	Stem	Godavari, India	MCPT 2.5
	Callus	Ooty, India	CPT 9.5, MCPT traces
	Cell	Satara, India	CPT 1.1, MCPT 0.81
M. megacarpum	Leaves	Guam	CPT 530, MCPT 170
M. brunonis	Entire plants	Lope, Gabon	CPT-20-O-β-glucoside100
O. mungos	Entire plants	Colombo	CPT 12, MCPT 10.41
O. pumila	Leaves	Japan	CPT 300-400
	Young roots		CPT 1000
P. klaineana	Stem	Ghana	CPT 4.8, MCPT 1.6

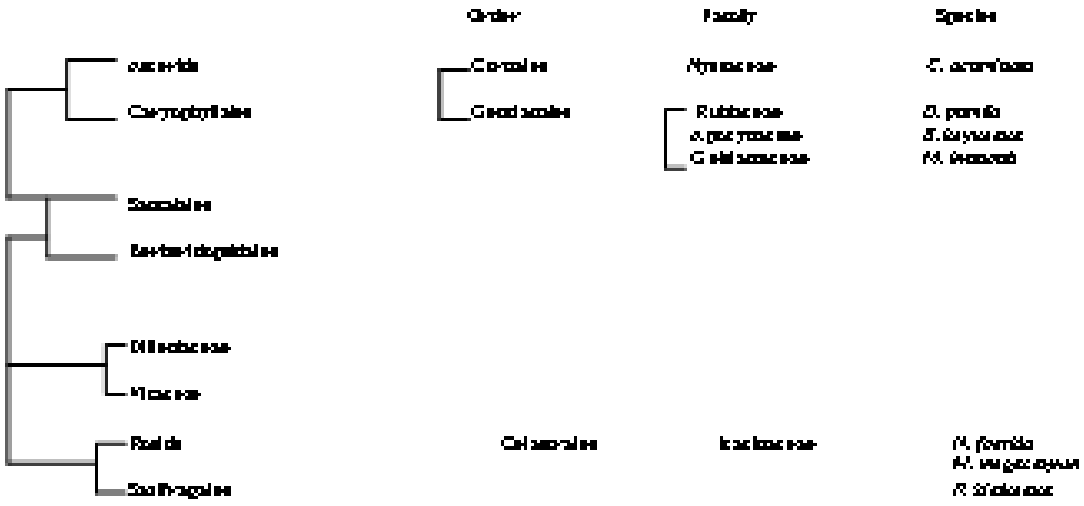

Fig. 1. Distribution of CPT in plant species belonging to unrelated orders and families of angiosperms (15).

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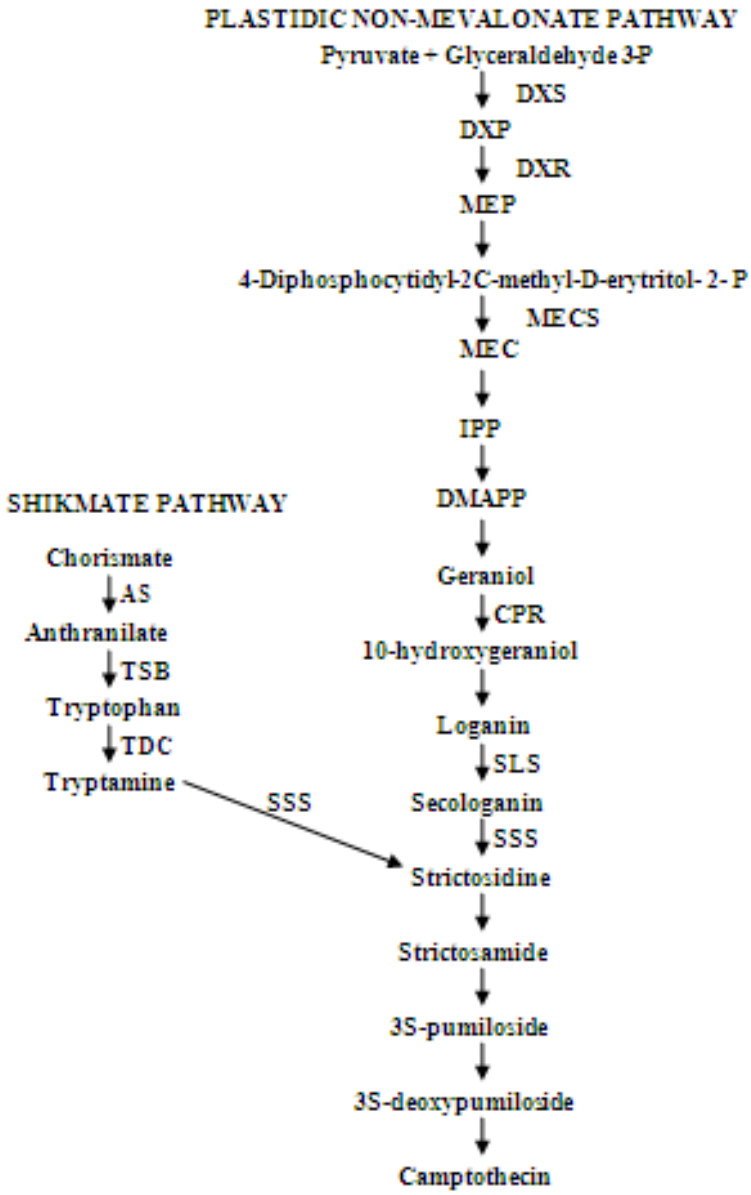

Fig. 2. Biosynthetic pathway of camptothecin in plants (15).

shown in Figure 1. Biosynthetic pathway of camptothecin in plants was shown in Figure 2.

CAMPTOTHECIN

Discovery of camptothecin

Wall became intensely interested in discovering the nature of the compound(s) responsible for the antitumor activity of *C. acuminata* extracts. For administrative reasons it was not possible to do this in the USDA, and hence in July 1960 he left the USDA and established a natural products group at the Research Triangle Institute with support from the National Cancer Institute (NCI). By 1963 a sizable sample of approximately 20 kg of the wood and bark of the tree was made available to him, and in 1964, Dr. M.C. Wani joined Wall's group, commencing fruitful 30 year collaboration.

Use of L1210 assay for fractionation

A typical biodirected isolation was initiated, in which the fractionation of the sample would be directed entirely by the antitumor assay. By this time it was known that the crude extracts of *C. acuminata* were very active in the L1210 mouse leukemia life prolongation assay. This was, and still is, highly unusual. Most of the hundreds of plant extracts investigated by our group and by others were never sufficiently active in L1210 to conduct fractionations in this system. However, this was the case for camptothecin (CPT) and aroused very high

interest on the part of NCI. In general, after conducting a particular phase of the isolation, it would often take 3 months or more before the results were received. This was primarily due to the fact that the L1210 assay is based on life prolongation. In the case of highly active extracts the study would have to be carried out for 30 days or although it was a slow process, our group persisted, working at the same time on other bioassay directed plant programs (16).

Fractionation of *Camptotheca acuminata*

Extraction of almost 20 kg of dry plant material, which consisted of the wood plus wood bark of the tree. It should be noted that this sample was obtained from the Plant Introduction Station in Chico, CA, or in some cases from trees growing nearby in California that were made available. Our procedure involved a continuous hot extraction with heptane. The residual plant material was extracted with hot 95% ethanol, and after concentration, the aqueous ethanolic residue was extracted with chloroform. Only the chloroform fractions were active with yield 222 gm (16).

Craig countercurrent partition

Several procedures involving chromatography, particularly on alumina, were tested on a small scale. They were all unsuccessful for reasons unknown to the group at the time, although it is now known that CPT is adsorbed very tightly on alumina, and that this is not an appropriate chromatographic agent for CPT. The Craig countercurrent partition procedure was then tested. This methodology was designed and brought to a high pitch of perfection by the late L. C. Craig, who received the Nobel Prize for his work (17, 18). Various types of countercurrent equipment were available in the early 1960s. Most of this equipment has now become museum pieces, as more convenient and compact equipment is available. The beauty of the Craig partition technique was that it involved liquid-liquid partition and avoided harsh treatments that could cause changes in a substance of unknown constitution. Most of the chloroform phase after concentration, was subjected to Craig countercurrent distribution. In this large separatory funnels using a chloroform-carbon tetrachloride-methanol-water partition system. All the fractions were analyzed by both the *in vivo* L1210 mouse life prolongation assay and by a 9KB *in vitro* cytotoxicity assay. There was reasonable correlation between the two assays. Tubes 2-6 were judged to contain the most active material. In *in vivo* life prolongation assay, the combination of the lowest dose with the largest T/C activity is the basis for selecting active fractions. In the cytotoxicity assay it is simply the lowest ED_{50} dose that will inhibit the growth of the 9KB cells. We had thus removed about 80% of the total weight while concentrating the most active fractions in 20% of the original weight of the chloroform extracts. By comparison with a pure sample obtained later but again given the standard L1210 assay, it can be estimated that the most active fractions contained between 1% and 2% of the active compound. It should be noted that 9KB activity was reasonably correlated with the L1210 assay. When fractions 2-6 were combined and the solvent partially concentrated, a yellow precipitate was formed and collected by filtration. This material was subsequently further purified by chromatography on a silica gel column and crystallization. As stated above, the pure compound is very active in L1210. Doses as low as 0.5 mg/kg gave an appreciable life prolongation. A dose of 4 mg/kg was the maximum range prior to occurrence of toxicity.

Physical properties of CPT

The compound was a high melting substance, with a molecular weight of 348.111, obtained by high resolution mass spectrometry, corresponding to the formula $C_{20}H_{16}N_{204}$. It gives an intense blue fluorescence under UV and is optically active [a], +31.3°C. The compound gives the following qualitative reactions: negative phenol ($FeCl_3$); negative indole tests; negative Dragendorff and Mayer tests. No crystalline salts could be obtained with a variety of acids. The compound can not be methylated with diazomethane or dimethyl sulphate under a variety of conditions. The compound does not react with bicarbonate or carbonate, but can be quantitatively reconverted to the sodium salt with sodium hydroxide at room temperature. On acidification, the sodium salt regenerates CPT (19).

Structure of CPT

It was found that CPT can readily be converted to an acetate and a chloroacetate. The chloroacetate was converted to the corresponding iodoacetate by treatment with sodium iodide-acetone. The iodoacetate crystallized in orthorhombic crystals suitable for X-ray analysis (19). This structure was completely in accord with ultraviolet infrared spectra, nuclear magnetic resonance (NMR) spectra, and mass spectral data. The structure is unique. CPT has been shown to be related to the indole alkaloids, and the 6-membered ring B and a 5-membered ring C are formed by a ring expansion/ring contraction sequence of reactions. The pentacyclic ring structure is highly unsaturated. Some of the unique structural features involve the presence in ring E of α-hydroxy lactone system and in ring D of an unsaturated conjugated pyridine moiety. On treatment with alkali, the compound readily opens, forming an open lactone sodium salt (19). On acidification, the extremely water soluble sodium salt is relactonized readily. The parent compound, however, is extremely water insoluble and, indeed, is insoluble in virtually all organic compounds except dimethylsulfoxide, in which it exhibits moderate solubility.

Biological activity of CPT and analogs

The isolation and structure proof for CPT and some of the analogs obtained at an early stage, 10-hydroxy and 10-methoxy-CPT enabled extensive studies to be conducted on CPT and a few of its analogs (20). CPT was remarkably active in the life prolongation of mice treated with L1210 leukemia cells, showing activity in doses between 0.5 and 4.0 mg/kg in this L1210 mouse life prolongation assay. It showed activity of a similar order in the life prolongation assay for P388 leukemia. The compound was also very active in the inhibition of solid tumors that were being studied at this early stage, including the Walker WM tumor, which was completely inhibited by CPT. 10-Methoxy-CPT was found to be active but somewhat less active than CPT, whereas 10-hydroxy-CPT was the most active compound in the series and was more active than CPT in both L1210 and P388 leukemia life prolongation

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assays (20-22). Unfortunately, 10-hydroxy-CPT is found in nature in only very small quantities, probably amounting to about 10% of the CPT content. All these previous compounds were extremely insoluble in water, but it will be recalled that the lactone could be opened under mild conditions with sodium hydroxide or sodium methoxide, and it was found that this sodium salt of CPT was very soluble in water. It was not until much later that it was shown by definitive studies that this compound was only 10% as active as CPT in the P388 assay (21).

Early clinical trials

Encouraged by the broad scope of animal antitumor activity of CPT, a decision was made

by NCI to go to clinical trial with the sodium salt. In contrast to CPT, the sodium salt was water soluble, and hence was easily formulated for intravenous administration. In Phase I trial, Gottlieb and Luce (1972) made a study of 18 patients and found that there were partial responses in five of them (23). These responses were primarily in gastrointestinal tumors and of short duration. Toxicity relating mainly to dose limiting hematological depression was noted, along with some vomiting and diarrhoea. In another Phase I trial only two partial responses were found in ten evaluable patients (24). Because of the somewhat encouraging results obtained in the Phase I study of Gottlieb and Luce, a Phase II study was undertaken in 61 patients with adenocarcinomas of the GI tract, but only two patients showed objective partial responses (25). The drug has also been under study as a sodium salt in the People's Republic of China. Up to 1000 patients were put on trial, and effective results were reported in gastric cancer, intestinal cancer, head and neck tumors, and bladder carcinoma (26). These results are more promising than those in the US trial, but this may be due to the fact that in the USA only patients that had been treated with many other drugs previously and had become resistant were put under test.

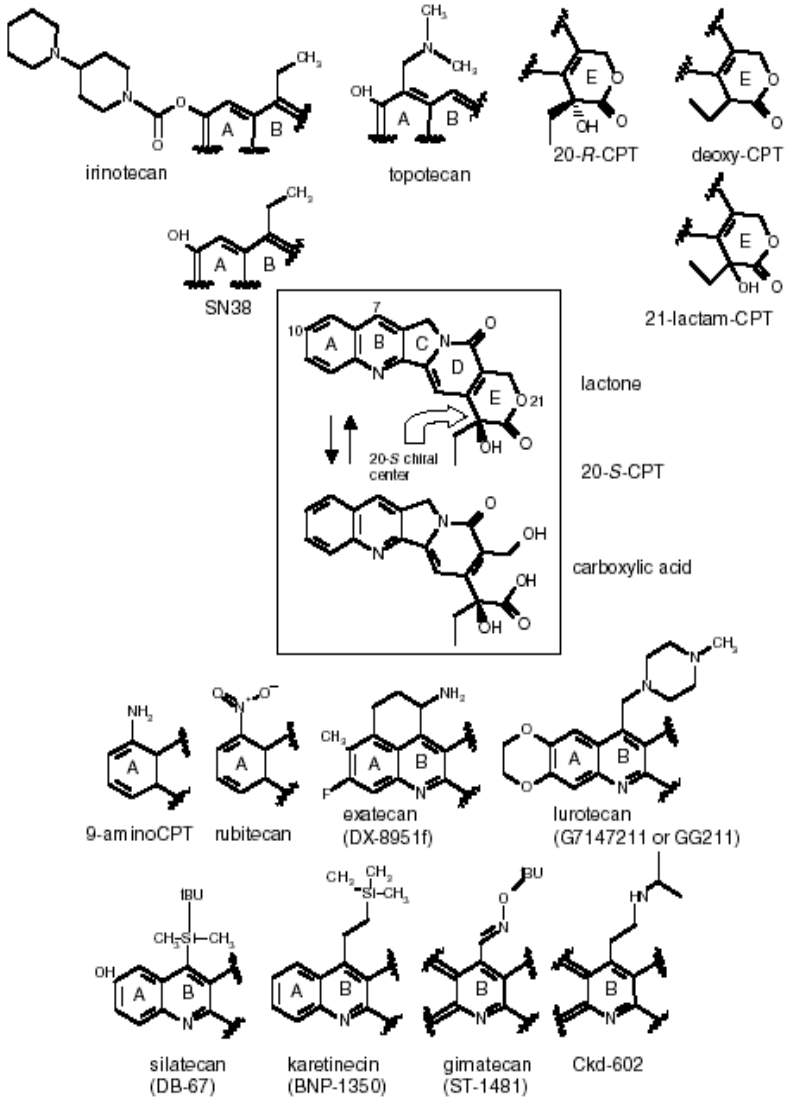

Fig. 3. Structure of CPT illustrating the 20S chiral center, and the dynamic equilibrium between the lactone and carboxylic acid forms.

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The structure of some of the derivatives that are commercially available or in clinical trials is also showed (15).

SAR studies

Although our work on CPT discovery may be considered to have nominally ceased by 1970, our interest in CPT and/or its analogs did not wane, and since some of this work has led to current clinical studies and will also undoubtedly lead to future clinical studies, some of the subsequent studies will be briefly reported. In 1969, the isolation of 10-hydroxy and 10-methoxy-CPT as minor components of *C. acuminata* was reported. It was later found that 10-hydroxy-CPT was much more active than CPT in a number of assays, and this stimulated synthetic efforts by both the SmithKline Beecham group and a Japanese pharmaceutical company, Daiichi, to prepare water soluble 10-hydroxy-CPT analogs. Oxidation of the ring B nitrogen with chloroperbenzoic acid led to considerable loss of activity.

CPT reacted readily with amines, such as ethylamine, resulting in ring opening and lower activity. Of great interest was the fact that hydroxylation in ring A, at least at the 10 position, was compatible with activity, and indeed this compound, 10-hydroxy-CPT, has greater activity than CPT. Major reduction of anti-neoplastic activity was noted as a result of reactions involving the hydroxyl or lactone moiety in ring E. After acetylation of CPT, the resulting acetate is virtually inactive. Other reactions also point to the absolute requirement of the hydroxy group, as shown by the fact that after replacement of this group by chlorine, the resultant chloro analog and the corresponding reduction product, desoxy-CPT, are inactive in L1210 leukemia. Reduction of the lactone under mild conditions to give the lactol also results in complete loss of activity (16, 21).

Inhibition of the enzyme topoisomerase I

Interest in CPT and analogs remained at a low ebb until 1985, when it was discovered that CPT, by a unique mechanism, inhibited the enzyme topoisomerase I (T-I) (27). The enzyme has been implicated in various DNA transactions such as replication, transcription, and recombination. CPT and analogs bind to a complex formed by DNA with the T-I enzyme. With the mechanism now understood, the possibility opened for clinical use of CPT by virtue of its inhibition of T-I, which might be found in cancer cells and hence would inhibit tumor growth. Many new CPT analogs were synthesized at RTI (28, 29) and were tested for their T-I inhibition. The inhibition of T-I activity closely parallels *in vivo* mouse leukemia assays, as is shown in figure 4.

Finally, it was possible for our group to separate the racemic synthon by which we synthesized various CPT analogs into its corresponding 20(S) and 20(R) analogs, and we have been able to demonstrate that the 20(R) form is inactive, both in topoisomerase inhibition and in the *in vivo* assays (30). Another study conducted cooperatively involved the Stehlin Institute, NYU Medical School, Johns Hopkins University, and the Research Triangle Institute. Both 9-amino and 10, 11-methylenedioxy-CPT compounds were shown to have great potency in inhibiting human colon cancer xenografts in nude mice (31). It was also shown that a large number of compounds commonly used in cancer chemotherapy were totally ineffective. Comparison of in vivo and in vitro activity of CPT and its analogs was shown in table 2.

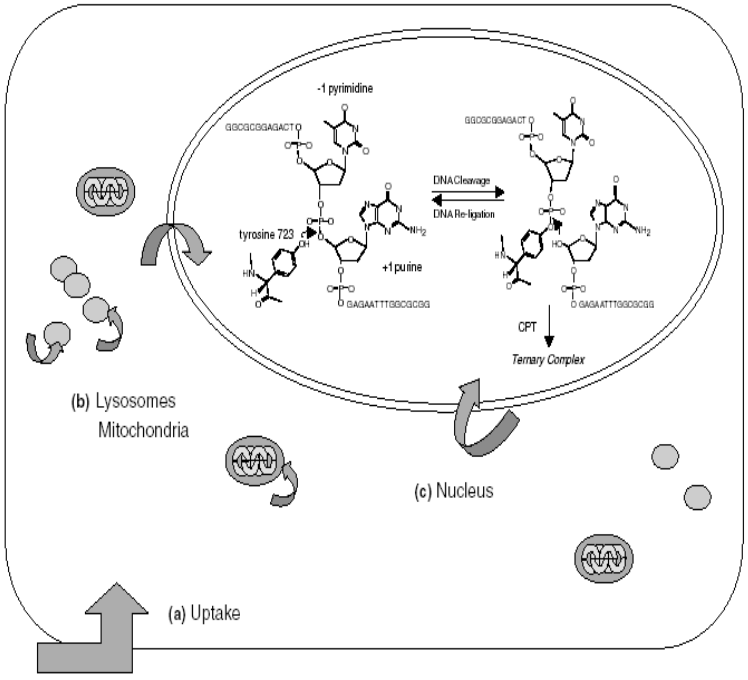

Fig. 4: Mechanism of action of CPT, a topoisomerase I ''poison''.

Table 2: Comparison of in vivo and in vitro activity of CPT and its analogs.

CPT derivative	Inhibition of DNA relaxation of supercoiled DNA (%)	DNA scission (%)	Antitumor activity (I/C) x 100	ED_{50} (mg/ml)
S	52	48	197	10^{-2}
R	20	12	< 125	10^{-1}
RS	30	25	-	10^{-1}
20-deoxy-(R,S)	0	0	< 125	10^{-1}
10-OH-(S)	73	71	348	10^{-2}
11-OH-(R,S)	74	35	357	-
10-OCH_3-(R,S)	-	-	167	10^{-2}
10-NO_2-(R,S)	19	23	219	10^{-1}
11-NO_2-(R,S)	0	11	147	10^{0}
12-NO_2-(S)	0	10	151	-
9-NO_2-(S)	40	50	348	10^{-2}
9-NH_2-(S)	91	35	348	10^{-2}
21-Lactam-(S)	3	0	178	10^{-1}

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'' (15).

Clinical trials

CPT and a number of its analogs are now in active clinical trial. The compounds include CPT, 9-nitro and 9-amino-CPT, all of which were first discovered at RTI. These are all water insoluble and are administered orally or in very dilute solution. Two water soluble analogs of 10-hydroxy-CPT also discovered at RTI are in very active clinical study at present. These are CPT-11, a product of a Japanese pharmaceutical company, Daiichi, and topotecan, a product of the American pharmaceutical company, SmithKline Beecham. Finally, a CPT analog of novel structure that originated at Glaxo, also water soluble, is in initial trials. Basically, CPT and analogs are showing promising activity against a variety of solid tumors that have been refractory to any sort of treatment. Although further clinical studies involving dosage, administration forms, and single or combined therapy are under way, it is already apparent that significant objective responses have been found on treatment of many hitherto resistant solid tumors with CPT and analogs. Response rates of 20-30% were obtained with single agent trials with patients with non-small cell lung (NSMLC), cervical, ovarian, colorectal and breast cancers, while 30-40% was obtained with patients with small cell lung and acute T-cell leukemia/lymphoma. As high as 40-50% responses in Phase I trials of CPT analog plus cisplatin have been found in patients with NSMLC. Recently, some new CPT analogs prepared at RTI have been shown to have *in vitro* potent activity in antiparasitic studies with trypanosomes and against the organisms responsible for malaria, and also due to the inhibition of T-I in these organisms (16).

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* (32), 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 (33-35). A short sss promoter sequence called the JERE (jasmonate and elicitor responsive element) is responsible for elicitor responsive and jasmonate responsive gene expression (36). 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 (36). A closely related TIA regulator gene, called ORCA3, was isolated by T-DNA activation tagging in cultured *C. roseus* cells (37). 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 (37). 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 ß-LHL transcription factors (38). CrGBF1 and CrGBF2 were shown to

repress sss expression (39). They also bind *in vitro* to a G-box like element in the tdc promoter which indicates that CrGBFs could coordinately regulate several TIA biosynthetic genes (39). Inactivating CrGBF transcription factors might depress TIAs biosynthesis, particularly the terpenoid branch of the pathway. Combined with ORCA3 over-expression, this would activate TIAs biosynthesis (34). 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 (40). We have obtained similar results by expressing the *C. acuminata* ORCA3 transcription factor in *Arabidopsis*, under the control of the 35S promoter. 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 (41).

CONCLUSION

The authors have been delighted that initial discovery of more than 25 years ago of camptothecin, a novel natural product with excellent activity in a number of animal models has now reached the stage where it is available in adequate quantity for therapeutic use. Undoubtedly, there are other highly active natural products from plant, marine, and fungal sources as yet unknown which, when discovered, will have therapeutic utility. Cancer is not one, but several hundred diseases, and will require many different types of agents.

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