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Camptothecin

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ABSTRACT :

Camptothecin was identified in the early 1960s through extensive natural product screening conducted by the National Cancer Institute (NCI). Due to its poor solubility in water, camptothecin lactone was administered as a sodium salt during phase I clinical trials. Although initial studies showed some promising responses, further assessments of this compound uncovered significant and unpredictable toxicities, including hemorrhagic cystitis and diarrhea. The lactone ring of camptothecin can reversibly open in a pH-dependent manner, resulting in a carboxylate form that exhibits markedly diminished activity both in vivo and in vitro. Under physiological conditions, the carboxylate form is the predominant species; however, the precise equilibrium position in vivo is influenced by additional factors such as protein binding, metabolism, and elimination. Camptothecin exerts its effects by targeting a complex formed between the nuclear enzyme topoisomerase I and DNA, representing a novel approach in cancer chemotherapy. Topoisomerase I primarily facilitates the relaxation of DNA necessary for transcription and replication. The transient covalent complexes that arise from the interaction between the enzyme and the 3' end of a nicked DNA strand are stabilized in the presence of camptothecin, leading to collisions with replication forks. This results in the stalling of the fork and the formation of permanent double-strand breaks, which are believed to contribute to the antiproliferative effects of camptothecin. Resistance to camptothecin in cell cultures is generally attributed to a decrease in the quantity and activity of topoisomerase I. Single-point mutations in the enzyme's gene have been identified in several resistant variants. Camptothecin is not a favorable substrate for P-glycoprotein, and its intracellular accumulation remains largely unaffected in cells exhibiting a multidrug-resistant phenotype. Several water-soluble and active derivatives of camptothecin have been synthesized, with CPT-11 and topotecan being the most advanced in development.

KEYWORDS: Camptotheca acuminata, camptothecin, traditional Chinese medicine, nanoparticles, topoisomerase-1, and theranostics.

Introduction

Camptothecin (CPT) is a cytotoxic alkaloid initially extracted from the plants Camptotheca acuminata and Nothapodytes foetida. It is recognized as the third anticancer drug to be commercially prescribed, following Taxol and vinblastine. The remarkable antiproliferative properties of CPT stem from its exceptional ability to inhibit the activity of eukaryotic topoisomerases, thereby obstructing the DNA relaxation process in tumor cells while exhibiting minimal effects on normal cells. In eukaryotic organisms, Topoisomerase I is covalently bonded to the 3'-end of single-stranded DNA, whereas Topoisomerase II is associated with the 5'-ends of cleaved double-stranded DNA, facilitating the relaxation of DNA supercoiling necessary for normal DNA replication and transcription. Both Topoisomerases I and II induce single and double-strand breaks, allowing for the relaxation of DNA strands and catalyzing the religation of cleaved DNA. Treatment with Camptothecin inhibits the activity of these topoisomerases, thereby halting DNA replication and disrupting the cell cycle, ultimately leading to cell death. The biosynthetic pathway of CPT and its rate-limiting enzymes have been previously documented. Currently, Camptotheca acuminata serves as the primary source of CPT; however, the yield from the bark and leaves of this plant is typically less than 0.4%. Given the low yield of CPT and the high demand, coupled with the geographical limitation of this plant to Asia, there is a significant industrial challenge in meeting the pharmaceutical requirements for this essential compound. Recently, the metabolic capabilities of endophytic fungi associated with medicinal plants have shown promise for the industrial production of CPT due to their rapid growth and the controllability of fermentation processes. The endophyte Entrophospora infrequens, derived from Nothapodytes foetida, has been identified as the first producer of CPT.Numerous endophytic fungi are found within various plant species. Specific isolates, such as Aspergillus terreus from Ficus elastica, Cestrum parqui, and Cinnamomum camphora, have been identified as significant contributors to camptothecin (CPT) production. Additionally, A. flavus, an endophyte of Astragalus fruticosus, and Penicillium chrysogenum, an endozoic fungus associated with Cliona sp., have also been recognized as promising CPT-producing isolates. Nonetheless, the industrial application of fungi for CPT production is often hindered by a decline in CPT yield due to subculturing and storage processes. Various strategies have been employed to restore the biosynthetic capabilities of these fungi, including metabolic engineering to enhance the expression of rate-limiting genes, co-cultivation with the host plant's microbiome, and the incorporation of different plant extracts. However, the quest for novel fungal endophytes residing in medicinal plants with established ethnopharmacological properties, which also demonstrate stable CPT production, remains a significant challenge. Furthermore, it is essential to assess the antiproliferative and inhibitory effects of purified CPT on topoisomerases.

Monroe E. Wall (1916-2002) :

was born in Newark, New Jersey, in 1916. He earned his B.S., M.S., and Ph.D. degrees from Rutgers University. In 1941, he began his career with the United States Department of Agriculture, where he gained national acclaim as a government scientist specializing in steroid chemistry from 1941 to 1960. In 1960, Dr. Wall transitioned to the Research Triangle Institute (RTI) to establish a chemistry research group, eventually becoming the Vice President of Chemistry and Life Sciences in 1971. He is widely recognized for his significant contributions to natural product research, particularly for the discovery and development of taxol and camptothecin. Dr. Wall retired from administrative duties in 1981 and dedicated his remaining years to research until his passing at the age of 85, just two weeks before his death.

Monroe Doctrine: "Recruit talented individuals, provide them with excellent resources, conduct rigorous scientific research, exert effort, and maintain this commitment."

Mansukh C. Wani

Dr. Wani, originally from Nandurbar, India, earned his B.S. and M.S. degrees from the University of Bombay. He later moved to the United States, where he completed his Ph.D. in chemistry at Indiana University under the guidance of Professor Ernest Campaign. Following a postdoctoral fellowship at the University of Wisconsin-Madison, he joined RTI in 1962 at the invitation of Dr. Wall. Together, they pioneered the development of two highly effective anticancer agents, taxol and camptothecin, which have positively impacted the lives of millions globally. Dr. Wani continues to contribute to RTI, overseeing the work of junior researchers.

Background:

Camptothecin is an alkaloid derived from the stem wood of the Chinese tree, Camptotheca acuminata. This compound specifically inhibits the nuclear enzyme DNA topoisomerase, type I. Various semisynthetic analogs of camptothecin have shown significant antitumor properties.

Camptothecin & Its Derivatives for Cancer Therapy:

Camptothecin (CPT) is a pentacyclic quinoline alkaloid initially extracted from the woody plant Camptotheca acuminata. Due to their selective action as topoisomerase I inhibitors that stabilize topoisomerase I cleavage complexes, camptothecin and its derivatives are regarded as promising candidates for cancer treatment.

Origin of Camptothecin:

In 1966, Monrece E. Wall and Mansukh C. Wani from the National Cancer Institute successfully isolated a pentacyclic monoterpene alkaloid from the woody plant Camptotheca acuminata, which is native to Southwest China. Between 1967 and 1970, subsequent research demonstrated that this alkaloid exhibited significant antitumor properties in vitro against HeLa cells, a cervical cancer cell line, as well as L1210 cells, which are derived from mouse lymphocytic leukemia, and various rodent models. Additionally, it showed effectiveness against several malignancies, including gastric cancer, rectal cancer, and leukemia. This discovery sparked extensive scientific investigation into Camptothecin and related compounds, leading to the identification of numerous Camptothecin-like substances. However, various challenges have hindered the effective clinical application of Camptothecin. In 1985, Hsiang and his team revealed that Camptothecin and its derivatives specifically target Topoisomerase, forming a reversible bond with the Topo-DNA cleavable complex. This interaction results in the creation of a CPT-Topo 1-DNA ternary complex, which inhibits both DNA replication and transcription, ultimately causing cancer cell death. Compared to Topo II inhibitors, Topo I inhibitors demonstrate higher potency and a wider anti-tumor spectrum, thereby reigniting interest in the specific inhibition of Topoisomerase I by Camptothecin.

Drug Delivery Technology Enhances Camptothecin-derived Medications:

To improve the effectiveness of medications, reduce toxicity, and enhance safety, drug delivery systems are frequently employed in the field of oncology. Various technologies, such as liposomes, antibody-drug conjugates, dendrimers, and micelles, have been developed to tackle the challenge of bioavailability associated with camptothecin-based drugs.

Liposomes:

In 1983, Yakult Honsha Co. Ltd. in Japan successfully discovered and synthesized irinotecan, a semisynthetic derivative of the quinoline alkaloid camptothecin. Initially, it demonstrated significant efficacy against a variety of experimental cancers, leading to the initiation of clinical phase trials. By 1994, binotecan was approved in Japan for the treatment of ovarian, lung, and cervical cancers, with subsequent approvals in the USA (1996) and Europe (1995). Unlike traditional (non-liposomal) formulations of this topoisomerase 1 inhibitor, liposomal irinotecan (nal-IRI; Onivyde, also referred to as pegylated liposomal irinotecan) was designed to maximize antitumor efficacy while minimizing drug-related toxicity. Liposomes, which consist of phospholipid bilayers enclosing an internal aqueous compartment, facilitate the effective delivery of both hydrophobic and hydrophilic drugs. They provide a protective barrier that prevents the encapsulated substance from undergoing structural changes or chemical degradation. Furthermore, the covalent attachment of PEG molecules can enhance the systemic circulation of the drug. Additionally, liposomal formulations are being developed for

belctecan and SN-38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of irinotecan following carboxylase conversion, which exhibits significantly greater topoisomerase inhibition than irinotecan itself. Neopharm has developed the SN-38 liposome known as L LE-SN38 (Liposome-Entrapped SN38). The primary technology of this product, tetraglyceride cardiolipin, interacts closely with lipophilic drugs and stabilizes the liposomal phospholipid membrane, thereby significantly enhancing stability.The utilization of both in vitro and in vivo methods facilitates the injection of the poorly soluble SN-38 for effective drug delivery.

Key aspects concerning the function of camptothecin in cancer therapy are as follows:

Inhibition of Topoisomerase I:

Camptothecin exerts its effects by inhibiting topoisomerase I, creating a stable complex with both the enzyme and DNA. This interaction obstructs the religation of DNA strands, resulting in DNA breaks. Given that cancer cells undergo rapid division and replication, they exhibit heightened sensitivity to disruptions in DNA replication, rendering them more vulnerable to the action of camptothecin.

Induction of DNA Damage:

The disruption of topoisomerase I activity leads to the generation of both single-strand and double-strand DNA breaks. This damage initiates cell cycle arrest and apoptosis (programmed cell death) in cancer cells, thereby effectively curtailing their proliferation.

Development of Derivatives:

Although camptothecin has demonstrated significant anti-cancer efficacy, its clinical application has been hindered by challenges such as limited solubility and adverse side effects. Consequently, derivatives of camptothecin, including irinotecan and topotecan, have been formulated and are utilized as chemotherapeutic agents for various malignancies.

Irinotecan (Camptosar):

This agent is employed in the treatment of colorectal cancer and other solid tumors, being converted into its active form, SN-38, within the body.

Topotecan (Hycamtin):

This drug is indicated for the treatment of ovarian cancer, small cell lung cancer, and cervical cancer.

Clinical Use:

Irinotecan and topotecan are typically administered intravenously in clinical environments. They are frequently combined with other chemotherapy agents to enhance their therapeutic effectiveness.

Side Effects:

The clinical application of Camptothecin derivatives is linked to various side effects, including myelosuppression (a decrease in blood cell counts), diarrhea, and other gastrointestinal complications. Effectively managing these side effects is a crucial component of cancer treatment.

It is essential to recognize that while Camptothecin and its derivatives have demonstrated effectiveness against certain cancer types, they are integral to standard cancer therapies and must be administered under the guidance of healthcare professionals. The utilization of these agents is determined by the specific cancer type and stage, with treatment plans tailored to the individual patient's overall health and circumstances.

There are three primary challenges in the development of camptothecin-based pharmaceuticals:

- Camptothecin's unique structure results in poor lipid and water solubility, leading to limited druggability. Consequently, modifications are necessary to enhance water solubility. However, such modifications can result in rapid drug release, potentially causing elevated blood concentrations that may lead to severe toxic side effects, including diarrhea, hemorrhagic cystitis, and significant bone marrow suppression. It is crucial that structural modifications take into account the drug's release rate and stability to ensure both efficacy and safety.

- To develop low-toxicity, water-soluble camptothecin derivatives, specific active sites on their five-ring backbone are often altered, particularly the A, B, and E rings. These modifications aim to enhance water solubility, mitigate toxic side effects, and improve the stability of the lactone ring, with the most frequently studied modification sites being the 7, 9, 10, and 20 carbon positions.

- The first camptothecin derivative, 10-Hydroxycamptothecin (HCPT), was independently developed in China during the 1970s and garnered significant attention due to its consistent clinical efficacy. In the 1990s, a new generation of camptothecin drugs, including Topotecan (TPT) and Irinotecan (CPT-11), was successfully introduced, leading to the development of numerous similar drugs in subsequent years.

Materials and Methods

Collection of Plant Samples, Isolation, and Identification of Endophytic Fungi

In October 2021, leaves of Catharanthus roseus were collected from the campus of Zagazig University. The isolation of their endophytic fungi was performed using Potato Dextrose Agar (PDA) medium, following protocols established in our previous research [26-28]. The hyphal tips that emerged were purified, and the fungal isolates were identified based on their morphological characteristics [29-31].

The selected fungal isolate known for producing CPT was molecularly confirmed through sequencing of its ITS region [32, 33]. Genomic DNA was extracted using the CTAP method [12] and served as a template for PCR amplification with the primer pair ITSA (5'-GGAAGTAAAAGTCG TAACAAGG-3') and ITS5 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR reaction mixture consisted of 10 µl of 2x PCR master mix (i-Taq, Cat. # 25027), 1 µl of genomic DNA, and 1 µl of each primer (10 pmol/µl), resulting in a total volume of 20 µl. The PCR conditions included an initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 10 seconds, and extension at 72 °C for 30 seconds, concluding with a final extension at 72 °C for 2 minutes. The resulting amplicons were analyzed via agarose gel electrophoresis, sequenced, and the sequences were subjected to non-redundant BLAST searches. Alignment was performed using Clustal W [34], and phylogenetic analysis was conducted employing the neighbor-joining method with 50 bootstrap replications [35].

The production of CPT by the isolated endophytic fungi was evaluated through cultivation in potato dextrose broth (Cat.# DF0549-17-9) as referenced in studies 13, 18, and 23. Following a 10-day incubation period at 30 °C under static conditions, the cultures underwent filtration and were subsequently extracted using methylene chloride. The extracts were concentrated via rotary evaporation until oily residues were obtained. The crude extracts were then subjected to fractionation using 1 mm TLC (Silica gel 60 F Merck KGaA, Germany) with a solvent mixture of dichloromethane and methanol in a 9:1 v/v ratio. The TLC plates were illuminated at a specific wavelength, revealing spots that exhibited identical coloration and relative mobility to the authentic CPT (Cat.#7689- (3-4)). The intensity of the CPT spots was quantified using the Image 1 software, based on the known concentration of the authentic standard. CPT was extracted from the designated silica gel spots (12, 13, 24, 36), and its purity and concentration were determined through High Performance Liquid Chromatography (YOUNG In, Korea) utilizing a C18 column (Cat. 959963-902). The solvent system employed was a methanol/water mixture (60:40 v/v) at a flow rate of 1.0 ml/min for a duration of 20 minutes, with absorbance readings taken at a wavelength of 360 nm. The purity and concentration of the sample were evaluated based on the retention time and peak area corresponding to the authentic CPT.

The wound healing capability of UO-31 cells in response to the extracted CPT was evaluated. The cells were plated at a density of 5 x 10^5 cells in a 40 mm well and allowed to grow until they formed a confluent monolayer, approximately 60 k/cm². A scratch was then created in the monolayer, followed by rinsing the plate with PBS. The cells were subsequently treated with purified CPT at the IC_2g concentrations, using DMSO as a vehicle. The closure of the wound, attributed to cell migration, was observed using a phase-contrast microscope. The percentage of wound healing was calculated based on the area of the gap in the treated cells, normalized against the control cells.

For the analysis of apoptosis and cell cycle in tumor cells in response to the extracted CPT, the apoptosis of UO-31 cells was assessed according to the manufacturer's protocol (Cat #K101-25) using Annexin V-FITC Apoptosis detection. This technique is based on the externalization of phosphatidylserine (PS) from the inner leaflet of the plasma membrane, which can be detected by the fluorescent stain Annexin V through flow cytometry. Following seeding in a 12-well plate at a density of 2 x 10^5 cells per well, the UO-31 cells were treated with the retrieved CPT at the IC_2 concentrations and incubated for 48 hours. In accordance with the manufacturer's instructions, the cells were washed with phosphate-buffered saline, followed by annexin-binding buffer, Annexin V-FITC, and Pl. After a 15-minute incubation in the dark, flow cytometry was employed to analyze the binding of Annexin V-FITC (Ex, a nm; Em, 3 nm). Additionally, the cell cycle of UO-31 cells was evaluated using the Propidium Iodide (PI) Flow Cytometry Kit (Cat #. ab139418). After seeding in a 12-well microtiter plate and incubating for 12 hours at 37 °C, the UO-31 cells were treated with the extracted CPT at the ICs value and incubated for an additional 48 hours. Following collection, the cells were fixed in 1 milliliter of ice-cold 70% ethanol for two hours at 4 °C. Subsequently, 500 µl of PI with RNase was added, and the mixture was rehydrated with PBS and incubated inCells were analyzed at an excitation wavelength of 491 nm and an emission wavelength of 515 nm. Subsequently, the ratios of GD-G1, S, and G2-M cells were calculated.

Bioprocessing of CPT yield utilizing selected fungal isolates was conducted through a Plackett-Burman design. The physicochemical characteristics of the effective isolates were leveraged to optimize their CPT yield using the Plackett-Burman design. A total of nineteen variables, including malt extract, yeast extract, glucose, sucrose, salicylic acid, asparagine, glutamine, cysteine, tryptophan, glycine, phenylalanine, peptone, pH, incubation time, sodium acetate, citric acid, CaCl, NaCl, and methyl jasmonate, were processed to enhance the CPT yield from the tested fungi. The parameters were represented at high (+1) and low (-1) levels. The design is based on a first-order reaction model: $Y=\beta 0+\Sigma\beta iXi$, where Y denotes the CPT yield, Xi represents the independent variable, β0 is the linear coefficient, and ß0 is the intercept. The experiments were performed in biological triplicates, and the response was quantified as the mean CPT yield.

The metabolic biosynthetic stability of CPT production by potent fungal isolates was evaluated through storage and subculturing methods. The axenic culture of the CPT-producing fungus underwent nine successive subcultures, initiated with a plug inoculated on a PDA plate and incubated at 30 °C for a duration of eight days. The productivity of CPT was assessed by cultivating the fungus in optimized media under standard conditions, followed by extraction and quantification of CPT using thin-layer chromatography (TLC). The initial axenic culture of the CPT-producing isolate was preserved as a slope PDA culture at 4 °C and tested for CPT productivity over a period of ten months, with extraction and quantification performed as previously described.

In an effort to enhance the metabolic biosynthetic capacity of CPT production by Alternaria brassicicola, various organic extracts from Catharanthus roseus, including methylene chloride, methanol, ethyl acetate, petroleum ether, and isopropyl alcohol, were added to the CPT production medium. Fresh leaves of C. roseus (10 g) were ground in 100 ml of solvents for 12 hours, after which the extracts were filtered, centrifuged at 5000 rpm, and concentrated to 20 ml. These plant extracts were introduced into three-day-old pre-fungal cultures at concentrations of 1, 5, and 10 ml per 50 ml of medium, followed

by a 15-day incubation period to determine CPT levels. Additionally, the impact of the indigenous microbiome from C. roseus leaves on the restoration of CPT productivity by A. brassicicola was investigated. The leaves were cut, surface sterilized, and added to three-day-old cultures of A. brassicicola grown on PDB medium, with incubation continued for 15 days before assessing CPT levels using high-performance liquid chromatography (HPLC). Surface-sterilized leaves of C. roseus were also inoculated into blank PDB media for further analysis.

Concentrations were utilized as a control in relation to the culture of Alternaria brassicicola that did not include plant parts.

Fungal Deposition

The isolate Alternaria brassicicola EFBI.-NV has been deposited in GenBank under the accession number #OR131587.1.

Statistical Analysis

The experiments were performed in triplicate, and the yield of CPT was reported as means ± standard deviation (SD). Significance was assessed using one-way ANOVA, followed by Fisher's Least Significant Difference post hoc test.

Results

Isolation, identification, and screening for CPT production from the endophytic fungi of Catharanthus roseus resulted in the acquisition of fifteen fungal isolates from C. roseus on PDA media. These were classified into five genera based on their morphological characteristics: Aspergillus, Cladosporium, Alternaria, Trichoderma, and Chaetomium. The isolates recovered from leaves (five isolates) and twigs (ten isolates) were cultivated in liquid PDB medium under standard conditions. Following incubation, CPT was extracted and quantified. According to the results (Table 1), the highest CPT yield was observed for Alternaria brassicicola (965 µg/L), followed by A. solani (905 µg/L), A. alternata (67.6 µg/L), and Trichoderma (38.5 µg/L). Notably, the endophytic fungal isolates from the leaves of C. roseus did not exhibit the ability to produce CPT, whereas the CPT-producing isolates were predominantly found in the plant twigs. This suggests the influence of certain chemical signals from the plant or the interaction of the plant's endogenous microbiome in the twigs rather than in the leaves. The most effective CPT-producing endophytic fungal isolate was identified based on its macromorphological and microscopical characteristics (Fig. 1). The fungal culture appeared olive-grey, transitioning to grey-black at maturity, with a velvety texture, long chain, branched conidiophores, and septate hyphae. The conidia exhibited light and dark green coloration, with ellipsoidal or ovoid shapes, and may possess several longitudinal or oblique septa. Camptothecin, a natural alkaloid, was initially isolated from the bark and stem.

The Camptotheca acuminata tree, native to China, is the source of camptothecin, a compound that, along with its derivatives, has shown significant anti-cancer properties in preclinical studies, particularly against solid tumors such as lung, breast, ovarian, and colon cancers. However, the clinical application of camptothecin is hindered by challenges such as poor solubility, toxicity, and limited biodistribution. To address these issues, nanotechnology-based drug delivery systems have been developed to enhance bioavailability and improve distribution following administration. Among these, polymeric micelles have been extensively studied to increase the solubility, stability, and effectiveness of camptothecin. These micelles feature a core-shell architecture, with a hydrophobic core that can effectively encapsulate hydrophobic drugs. The design of polymeric micelles can be optimized to achieve a high drug loading capacity, allowing for the transport of substantial amounts of hydrophobic drugs within their core. The outer shell is made of hydrophilic polymers, which play a crucial role in shielding the drug from the reticuloendothelial system (RES). This review examines recent advancements in the use of polymeric micelles for the delivery of camptothecin in cancer therapy.

Conclusions:

Natural products have historically been acknowledged as valuable sources for identifying potential treatments for various ailments, including cancer. Considerable advancements have been made in modifying natural camptothecin to mitigate its limitations and enhance its therapeutic efficacy. Several derivatives have received approval for clinical application in cancer therapy, while others are in various stages of preclinical or clinical trials. Nonetheless, the integration of diverse functional groups.

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