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Synthesis and antitumor activity of bis-camptothecin ester of succinic acid

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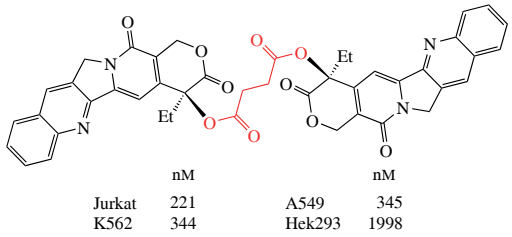
New succinic diester containing two residues of antitumor alkaloid camptothecin has been synthesized. Its cytotoxicity and effect on cell cycle and induction of apoptosis were explored using modern methods of flow cytometry, as well as *in vitro* studies of the inhibitory activity against human topoisomerase I.



Keywords: camptothecin, succinic diester, hybrid molecules, cytotoxicity, cell cycle, apoptosis, topoisomerase I inhibitors, antitumor activity, flow cytometry.

Camptothecin **1** is a pentacyclic alkaloid isolated in 1966 from the bark and stem of the Chinese tree *Camptotheca acuminata*.[1] The bark of this tree is used in traditional Chinese medicine to treat psoriasis, stomach ailments and colds.[2] Camptothecin possesses insecticidal, fungicidal and virucidal activity.[3–5] The main mechanism of action of camptothecin and its derivatives is the effect on the DNA-Top1 covalent complex resulting in the formation of a DNA-Top1-CPT ternary complex from which the enzyme Top1 cannot dissociate. Furthermore, this ternary complex inhibits replication and transcription and leads to the formation of double-stranded DNA breaks as the internal ligase activity of topoisomerase I is also blocked.[6,7] Unfortunately, there are a number of problems associated with the clinical use of both camptothecin and its derivatives. Camptothecin being highly hydrophobic has poor pharmacokinetic properties due to its low solubility in water, which makes its clinical use as an intravenous injection difficult.[8]

The efficacy of camptothecin is also limited due to rapid hydrolysis of the lactone ring *in vivo*, high toxicity, and rapidly acquired resistance in mammalian cells.[9,10] Topotecan, irinotecan and belotecan (Figure 1) are three clinically approved semi-synthetic derivatives of camptothecin designed to increase its aqueous solubility. Topotecan contains a 9-positioned basic amine substituent that allows the drug to form an ammonium salt, thereby increasing its aqueous solubility at physiologic pH.[11] Conversely, the solubility of irinotecan in water is attributed to the primary side chain at C-9. Irinotecan is a prodrug that undergoes hydrolysis *in vivo* to form 7-ethyl-10-hydroxy-camptothecin also known as SN-38, an active metabolite with remarkable antitumor efficacy.

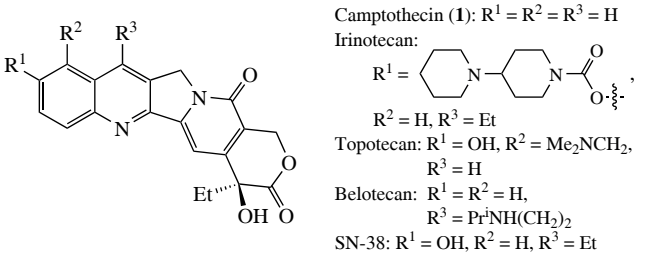
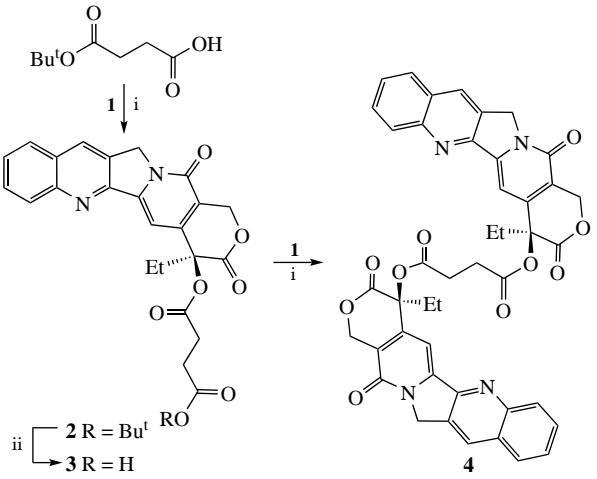


Figure 1 Camptothecin and its derivatives irinotecan, topotecan, belotecan, and SN-38.

A further significant challenge associated with the utilization of camptothecin and its derivatives is the genetically determined drug resistance exhibited by cancer cells. Camptothecin-resistant HCT116 sublines with mutated Top1 gene (HCT116-SN6, HCT116-G7, HCT116-A2, and HCT116-SN50) have been previously established.[12] In recent years, there has been a significant accumulation of knowledge on the mechanisms of multidrug resistance in the context of chemotherapeutic drug use.[13,14]

Further research is required to gain a deeper understanding of the relationship between camptothecin modifications and the resistance that develops in numerous histologic tumor types during adjuvant therapy. The objective of this research was to develop novel chemotherapeutic agents with high antitumor potential and promising antiproliferative activity. Herein, a new compound consisting of two camptothecin molecules linked by succinic acid residue was synthesized (Scheme 1), and its biological activity was studied. Initially, standard esterification of *tert*-butyl hydrogen succinate with camptothecin **1** gave non-symmetric diester **3** whose deprotection afforded monosuccinate **3**. Its final esterification with the second molecule of camptothecin **1** produced the target diester **4**.

A comparative analysis of the cytotoxic effect of the newly synthesized compound **4** and those of camptothecin **1**, staurosporine, and the camptothecin derivative **3** was then performed (Table 1). The effect was evaluated in Jurkat acute lymphoblastic leukemia, K562 myeloblastic leukemia, A549 alveolar adenocarcinoma cell lines, and in HEK293 conditional

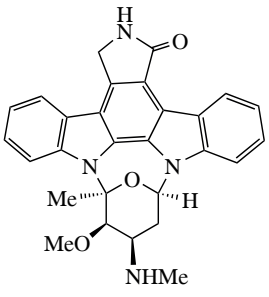


Scheme 1 *Reagents and conditions*: i, EDC·HCl, DMAP, DMF, room temperature, 12 h (for **2**, 92%) or 24 h (for **4**, 74%); ii, CF_3CO_2H, CH_2Cl_2, room temperature, 2 h (85%).

Table 1 A comparison of the cytotoxicity (CC_{50}, nM) of synthesized compounds **3** and **4** with that of staurosporine and camptothecin **1**.

Compound	Jurkat	K562	A549	HEK293
Staurosporine	44±4	49±5	82±5	176±14
Camptothecin **1**	488±61	594±44	699±43	2146±128
3	457±50	580±49	683±52	2376±143
4	221±24	341±28	345±24	1998±112

normal cell culture by flow cytometry using 7-aminoactinomycin D dye (7-AAD).[15] All compounds including camptothecin and staurosporine demonstrated the lowest cytotoxicity values on the conditionally normal HEK293 cell line. This is possible because these cells are a model of healthy tissue and are frequently utilized to examine various cytotoxic effects of vaccines, biological agents, and drugs. Significant differences in cytotoxicity were observed when CC_{50} values were compared between suspension (Jurkat and K562) and adherent (A549 and HEK293) cultures. Given the greater genome conservation and less susceptibility to genetic instability of the HEK293 cell line, any cytotoxic agents exhibited a tendency to be slightly less toxic to these cells than to cultures with marked tumor transformation.[16] Compounds **3** and **4** exhibited pronounced cytotoxicity in comparison to camptothecin **1** across a range of cell cultures (see Table 1). A comparison of the cytotoxicity of compounds **1**, **4** with that of staurosporine reveals that both exhibit greater cytotoxicity, which can be attributed to differences in the mechanisms of action of these compounds. It can be reasonably inferred that the action of compound **4** on cells is similar to that of the camptothecin **1** molecule, and is probably also characterized by the inhibition of the enzyme topoisomerase I. In contrast, the main mechanism of action of staurosporine is the inhibition of protein kinase by preventing ATP binding to the enzyme.[17]



Staurosporine

In view of the mechanism of action of camptothecin on the intranuclear enzyme topoisomerase I, the activity of compound **4** towards human topoisomerase I *in vitro*, specifically in the relaxation reaction of supercoiled plasmid DNA under standard conditions, was studied. Additionally, the capacity of this compound to interact with plasmid pHOT1 in the absence of topoisomerase I was ascertained. Two categories of interaction between the topo I enzyme and inhibitory compounds are known: inhibition of enzyme activity (catalytic inhibitory compounds, or CICs) and stimulation of the formation of cleavage complexes or 'topoisomerase poisons' (interfacial poisons, or IFPs). The CICs typically exert their effects on the enzyme in a non-specific manner, either by obstructing access to the DNA substrate or by intercalating into the DNA. Interfacial poisons (IFPs) are agents that typically inhibit the DNA chain re-ligation step resulting in the accumulation of DNA topoisomers. Camptothecin and related chemopreventive agents exemplify IFPs. Nevertheless, it is the specific or irreversible inhibition that is regarded as crucial for impeding tumor cell proliferation and tumor growth. The results of the inhibitory activity study indicate that in the relaxation reaction of supercoiled plasmid DNA with inhibited activity due to topoisomerase I action (Topogen, USA), the introduction of compounds **4** and **3** leads to formation of supercoiled form of plasmid pHOT1, implying inhibition of the topo I enzyme (see Online Supplementary Materials, Figure S7). Furthermore, the analysis of DNA samples of plasmid pHOT1 incubated with the tested compounds in the absence of topo I enzyme reveals that these compounds do not affect the process of DNA migration in agarose gel and do not intercalate into DNA.

A comparative analysis of the apoptosis-inducing effect of compound **4** and camptothecin **1** shows that compound **4** induces apoptosis to a slightly lesser extent, exhibiting a more cytostatic effect than camptothecin. Following a 24-hour incubation period, the percentage of live cells in the presence of camptothecin is 38%, whereas in the presence of compound **4**, 55% of the cell population remains viable. Additionally, the proportion of cellular secondary necrosis in samples treated with compound **4** is 41.1%, in comparison to 56.1% for camptothecin (Figure 2). It should be noted that the action of compound **4** is slower than that of camptothecin, but the overall effect is similar.

In order to analyze the effect of compound **4** on the cell cycle during cytometric analysis, an appropriate gating strategy was implemented. This involved gating the main pool of cells based on direct (FSC) and lateral (SSC) light scattering, with the objective of removing small (debris) and large (aggregates) particles. The fluorescence intensity of propidium iodide staining

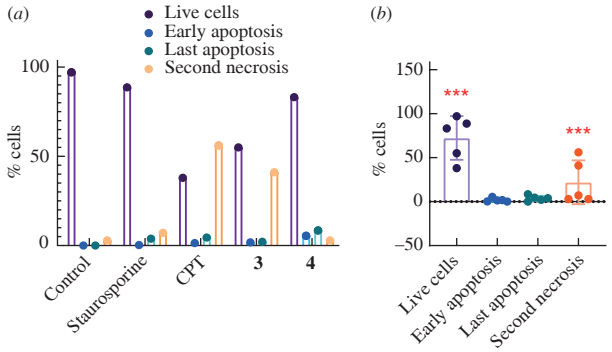


Figure 2 Analysis of the apoptosis-inducing activity of compound **4** in a Jurkat culture. (*a*) The data are presented as the average ±SD of three independent experiments. (*b*) A significant difference was observed when comparing the results with those obtained with camptothecin and the control (marked with ***, $P < 0.001$). The incubation period was 24 h, and all compounds were administrated at a concentration corresponding to the CC_{50}.

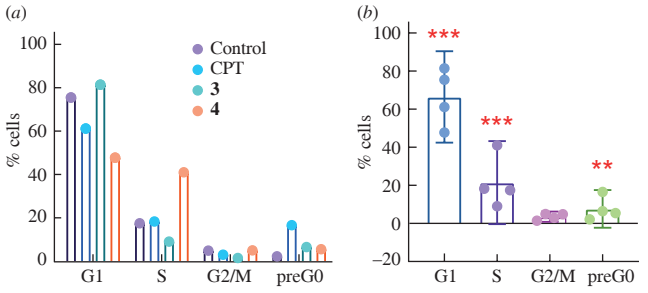


Figure 3 The cell cycle analysis of Jurkat cells treated with compound **4**. (*a*) The data are presented as the mean ±SD of three independent experiments, with an incubation period of 24 h. (*b*) All compounds were administered at a concentration corresponding to the CC_{50} (marked as ** with $P < 0.01$, and *** with $P < 0.001$, significant difference compared with camptothecin and control).

was evaluated by the histogram analysis. In the cell cycle histogram, the X-axis represents the fluorescence intensity in the PI channel, while the Y-axis depicts the number of events (number of cells). A minimum of 70000 events were recorded for each experimental sample at low flow rates. It is notable that there was an increase in the cell population in the G0/G1 phase. Conversely, there was a dramatic decrease in all cell populations in all other phases of the cell cycle. Specifically, there was a reduction in the S phase compared to camptothecin and the control (9.01, 18.29 and 17.44%, respectively), as well as a reduction in the number of cells in the mitosis phase (1.41, 2.95 and 4.85%, respectively). In other words, compound **4** functions as a cell cycle arresting agent in the G1 phase (Figure 3).

To conclude, the synthesized compound **4** has been found to elicit an effect on cancer cultures comparable to that of camptothecin, yet exhibits a heightened degree of cytotoxicity, selectively targeting distinct cell lines. This is likely attributable to its capacity to inhibit topoisomerase I, as distinct cell lines are understood to exhibit varying degrees of amplification of this enzyme. Additionally, compound **4** induced apoptosis in Jurkat culture cells and affected the cell cycle, resulting in the arrest of cells in the G1 phase.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7641.

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