行政院國家科學委員會專題研究計畫　成果報告

設計合成水溶性喜樹鹼用於癌症的治療

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行政院國家科學委員會補助專題研究計畫成果報告

Design and synthesis of water-soluble of camptothecin for cancer therapy

設計合成水溶性喜樹鹼用於癌症的治療

計畫類別：√個別型計畫　　□整合型計畫

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共同主持人：

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中文摘要

為了增加喜樹鹼之溶解度；以及增加它對腫瘤有選擇性的毒殺作用，設計了 10-羥基喜樹鹼之葡萄糖醛酸的前驅藥物 (**10-HCG**)，並將合成出來，**10-HCG** 是將 10-羥基喜樹鹼藉一個芳香環為空間棒來連線葡萄糖醛酸。

溶解度實驗証實，**10-HCG** 在 pH 4.0 的水溶液下有很好的溶解度(1.84 mg/ml)。安定性試驗証實，**10-HCG** 於 37℃的血清中 48 小時，仍然安定，可見它不受血清中酵素的水解。體外細胞毒殺試驗証實，**10-HCG** 的細胞毒性比原藥物(10-羥基喜樹鹼)低。當同時加入β-glucuronidase 時，則此前驅藥物的毒性則與原藥物相同。 β-glucuronidase 酵素水解試驗証實，**10-HCG** 受β-glucuronidase 水解葡萄糖醛酸後，形成一個帶負電荷的氧，此電子藉 1,6-脫去反應，即可釋放出 10-羥基喜樹鹼及 quinone methide。

由以上的這些實驗証實，**10-HCG** 具備了前驅藥物結合抗體連接酵素之治療模式所具備的條件，所以 **10-HCG** 可提供當此治療模式之候補藥物，以增加喜樹鹼在腫瘤治療上的新選擇藥物。

ABSTRACT

10-Hydroxycamptothecin glucuronide was designed and synthesized with the aim to improve the water solubility of camptothecin and selectivity toward tumors.

10-Hydroxycamptothecin was connected to glucuronic acid by an aromatic spacer via ether linkage.

10-HCG exhibited high aqueous solubility (1.84 mg/mL) at pH 4.0. The prolonged stability of 10-HCG in human serum indicates that the ether linker is resistant to nonspecific cleavage and confirms that β-glucuronidase activity is low in human serum at physiological pH values. 10-HCG displayed reduced toxicity compared with 10-hydroxycamptothecin to a variety of human tumor cells, differential toxicity between parent drug and prodrug, and susceptibility to β-glucuronidase hydrolysis. 10-HCG is activated by β-glucuronidase-mediated cleavage, leading to a 1,6-elimination reaction that release 10-hydroxycamptothecin and quinone methide. A good withdrawing group ($-NO_2$) in ortho position of the aromatic ring may accelerate electron transfer in 1,6-elimination reaction.

Glucuronide prodrugs of 10-hydroxycamptothecin have been synthesized and shown to possess the necessary prerequisites to be considered as candidates for cancer prodrug monotherapy and antibody-directed enzyme prodrug therapy.

KEY WORD: antibody-directed enzyme prodrug therapy, camptothecin.

INTRODUCTION

Chemotherapy plays an important role in cancer therapy. A major limiting factor in cancer chemotherapy is the toxicity of cytotoxic agents to normal tissues (1). Attempt to solve this problem have led to tumor targeting approach. The development of prodrugs can be activated selectively in tumor tissue (2). Prodrugs can be transformed to form the pharmacologically active species either by metabolism or by enzymatic hydrolysis after administration (3). Ideally, the activation of a prodrug should be restricted in the site of treatment. Antibodies to tumor-associated proteins have been used in the development of antibody-directed enzyme prodrug therapy (ADEPT). This concept was developed and described by Bagshawe and Senter (4-7). In this approach, monoclonal antibodies are employed to target an enzyme to cancer cells. ADEPT can activate subsequently administered prodrug. Selective activation of prodrugs at neoplastic cells can increase the concentration of active drug in tumors (8-9), reduce systemic toxicity (10), and allow bystander killing of antigen-negative cancer cells (11-12).

20(S)-Camptothecin (**1**), an antitumor alkaloid first isolated from *Camptotheca acuminata* (*Nyssaceae*) by Wall and co-workers in 1966 (13), inhibits the activity of topoisomerase I and displays antitumor activity in various experimental tumor models (14). Camptothecin, however, is difficult to formulate due to its poor water solubility. Several research teams have synthesized camptothecin derivatives aimed at preserving the antitumor properties of the parent compound while improving its safety and water solubility (15-19). Two water soluble derivatives, topotecan (**2**) and irinotecan (CPT-11, **3**), are approved for clinical use. We have employed an alternate strategy to improve the water solubility of camptothecin and increase its tumor cell selectivity based on the enzyme activation of prodrugs at tumor cells.

In a previous study, we have synthesized 9-aminocamptothecin glucuronide (**4**, 9-ACG)(20). 9-ACG was stable in both aqueous solution and human plasma, 9-ACG was over 80 times more soluble than 9-aminocamptothecin in aqueous of solution at pH 4.0. 9-ACG was 25-60 times less toxic than 9-aminocamptothecin to five human cell lines. The strong antitumor activity observed after combined treatment with β-glucuronidase and 9-ACG in human cell lines to produce similar cell killing as 9-aminocamptothecin. The *in vivo* toxicity of 9-ACG in BALB/c mice was dose-, route-, sex-, and age-dependent (21). 9-ACG was significantly less toxic to female than to male mice but the difference decrease with age. 9-ACG cured a high percentage of CL1-5 human lung cancer xenograft with efficacy that was similar to or greater than 9-aminocamptothecin. The potent antitumor activity of 9-ACG suggests

that this prodrug should be further evaluated for cancer treatment.

However, the synthetic cost of 9-ACG was expensive, because 9-aminocamptothecin was prepared from 10-hydroxycamptothecin by three steps. To circumvent this shortcoming, we also designed and synthesized 10-hydroxycamptothecin glucuronide (10-HCG), in which 10-hydroxycamptothecin was connected to glucuronic acid by an aromatic spacer via an ether linkage. 10-HCG is activated by β-glucuronidase-mediated cleavage, leading to a 1,6-elimination reaction that release 10-hydroxycamptothecin and quinone methide. A good withdrawing group ($-NO_2$) at ortho position of the aromatic ring may accelerate electron transfer in 1,6-elimination reaction. Here we describe a novel strategy for drug release involving the fragmentation of glucuronide benzyl ether derivatives.

Biological data

10-hydroxycamptothecin was poorly soluble in aqueous solution at both acid and neutral pH (44uM at pH4.0 and 120.5uM at pH 7.0). In contrast, **10-HCG** was 61 times more soluble at pH4.0 and 35 times more soluble at pH 7.0 (Table 1).

Table 1. Aqueous solubility of drugs.

Drugs	pH 4.0	pH 7.0
10-hydroxycamptothecin	44 μM	120.5 μM
10-HCG	2.67 mM	4.23 mM

Incubation of **10-HCG** at 37 ^{o}C in 95% human plasma revealed that the prodrug was stable for at least 48 h (figure 1).

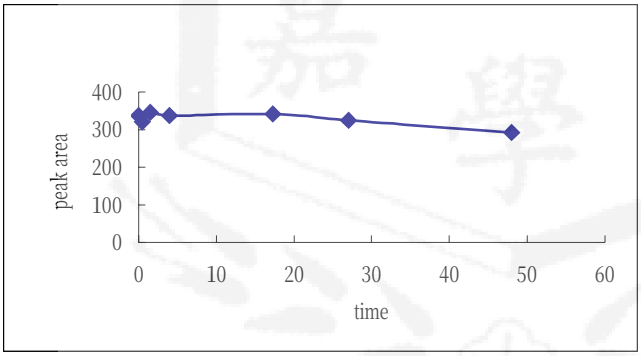

Figure 1. Stability of 10-hydroxycamptothecin glucuronide in 95% human plasma.

The cytotoxicity of 10-hydroxycamptothecin and **10-HCG** to four human tumor cell lines was determined by measuring [^{3}H] thymine incorporation into cellular DNA after 48 h of drug exposure. Comparison of IC_{50} values revealed that **10-HCG** was 10-fold less toxic than 10-hydroxycamptothecin. Simultaneous addition of β-glucuronidase (5 μg/mL) and **10-HCG** to tumor cells resulted in a cytotoxic effect similar to 10-hydroxycamptothecin alone (figure 2, table 2), indicating efficient enzymatic cleavage of the glucuronide functional group and release of 10-hydroxycamptothecin.

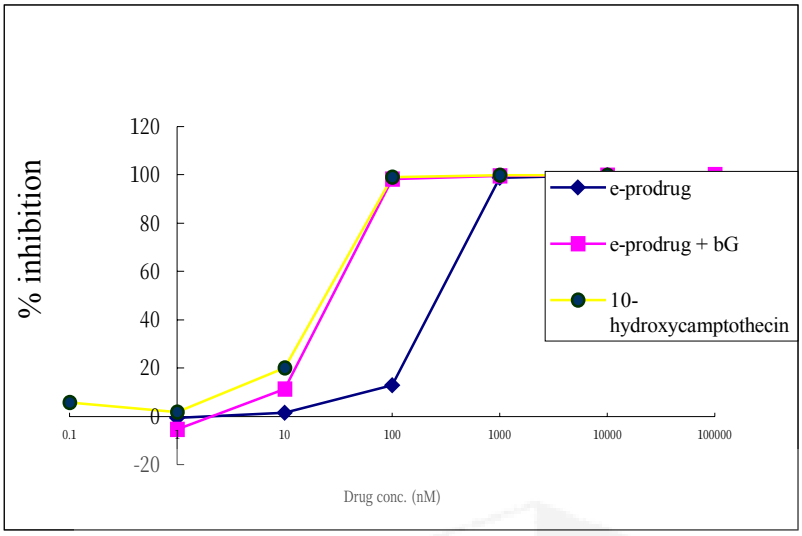

Figure 2. Cytotoxicity of drugs to human colorectal carcinoma cells (HT29).
HT29 cells were exposed to drugs with or without β-glucuronidase (βG) for 48h before the incorporation of [^{3}H] thymidine into cellular DNA was measured.
Results represent mean values of triplicate determinations.

Table 2. Cytotoxicity of drugs to human tumor cells.

Cell lines/ IC_{50} (nM)[a]/drug	10-Hydroxycamptothecin	10-HCG	10-HCG plus βG[b]
Hep G2	5.4	56.5	6.6
Colo 205	9.1	94.2	10.2
HT 29	8.8	97.8	10.0
H 928	6.9	91.1	11.1

[a] Concentration of drugs that inhibited incorporation of [3H] thymidine into cellular DNA of human hepatocellular (Hep G2), colorectal (Colo 205), colorectal (HT 29), or lung (H 928) carcinoma cells by 50% after 48 h are indicated. Values represent means of 1-3 experiments performed in triplicate with coefficients of variation of <10%.
[b]β-glucuronidase (βG, 5 μg/mL) was added with drugs.

Biologic test:

HPLC analysis.

Drugs were analyzed by high-pressure liquid chromatography (HPLC). Briefly, 20 μL of sample was injected onto a reversed phase column (Hypersil C18, 4.6 mm inside diameter, 250 mm length, 5 μm particule size) using a mobile phase (1 mL/min) of 45% MeOH and 25 mM phosphate buffer (pH 2.55). Eluted compounds were detected on a Gilson model 121 Fluorometer (excitation: 397 nm, emission: 482 nm). Peak areas were analyzed with Beckman System Gold software. Calibration curves were obtained by plotting the peak area of standard as a function of drug concentration. The retention times of 10-hydroxycamptothecin and 10-hydroxycamptothecin glucuronide were 8.8 and 14.6 min, respectively. The recoveries of 10-hydroxycamptothecin and 10-hydroxycamptothecin glucuronide from 95% human plasma were greater than 90 %.

Drug solubility

Drug solubility were determined in βG buffer (100 mM acetic acid, 50 mM bis-tris, 50 mM triethanolamine, pH 7.0) or phosphate buffer (100 mM, pH 4.0) by equilibrating an excess of solid compound in 0.25 mL of buffer at 25 ^{o}C for 24 h.

The samples were filtered through a 0.2 μm Millipore filter, diluted in HPLC mobile phase, and analyzed by HPLC.

Prodrug stability in 95% human serum

Drug stability was determined in 95% human plasma at 37 ^{o}C for 2 days. Aliquots were taken, the sample was neutralize by 12 mM phosphoric acid, and extracted three times by equal volume of ethyl acetate. This ethyl acetate layer was dryness. This residue was soluble in HPLC mobile phase, filtered through a 0.2 μm Millipore filter, and analyzed by HPLC.

***In Vitro* Cytotoxicity**

Exponentially growing tumor cells at a density of 2-3 x 10^4 cells/well in RPMI medium containing 10% bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin were incubated in a 96-well microtiter plate for 72 h (37^{o}C, 5% CO2, humidity) with various concentrations of drug. 10-Hydroxycamptothecin was dissolved in DMSO such that the final concentration of DMSO in wells did not exceed 0.5%. The prodrug was dissolved in medium. Control wells consisted of cells exposed to either medium or 0.5% DMSO in medium. βG, added at 1 μg/well in some experiments, was not toxic by itself to cells. Triplicate wells were prepared for each drug concentration and for the controls. After 48 h, cells were pulsed for 12 h with [^{3}H]

thymidine (1 μCi/well) in complete medium. Medium was removed, and the wells were washed once with PBS before trypsinized cells were harvested and counted for radioactivity in a Topcount liquid scintillation counter. The coefficient of variation for triplicate determinations was <10%. IC_{50} values were calculated from interpolation of logarithmic dose-response curves.

Discussion

We evaluated the properties of **10-HCG** suitable for cancer prodrug monotherapy and ADEPT including resistance to nonspecific cleavage, differential toxicity between parent drug and prodrug, and susceptibility to β-glucuronidase hydrolysis. The prolonged stability of 10-HCG in human serum indicates that the ether linker is resistant to nonspecific cleavage and confirms that β-glucuronidase activity is low in human serum at physiological pH values.

10-HCG exhibited high aqueous solubility (2.03 mg/mL) at pH 4.0. Formulation of camptothecin at acidic pH is important to prevent opening of the lactone ring and formation of the inactivate carboxylate form of camptothecin. The solubility of 10-HCG compares favorably than topotecan (1.02 mg/ml at pH 5). The high solubility of the glucuronide prodrug (10-HCG) at pH 7.0 (3.2 mg/mL) indicates that the prodrug will not precipitate after intravenous administration.

10-HCG displayed reduced toxicity compared with 10-hydroxycamptothecin to a variety of human tumor cells as found for other glucuronide prodrugs.

In summary, glucuronide prodrugs of 10-hydroxycamptothecin have been synthesized and shown to possess the necessary prerequisites to be considered as candidates for cancer prodrug monotherapy and antibody-directed enzyme prodrug therapy.

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