

# *Acta Medica Okayama*

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*Volume 56, Issue 5*

2002

*Article 2*

OCTOBER 2002

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## Camptothecin induces urokinase-type plasminogen activator gene-expression in human RC-K8 malignant lymphoma and H69 small cell lung cancer cells.

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# Camptothecin induces urokinase-type plasminogen activator gene-expression in human RC-K8 malignant lymphoma and H69 small cell lung cancer cells.\*

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## Abstract

We previously reported that anthracyclines, which could generate reactive oxygen species (ROS), could induce the urokinase-type plasminogen activator (uPA) gene expression in human RC-K8 malignant lymphoma cells and in H69 small cell lung cancer (SCLC) cells. In screening other uPA-inducible anti-cancer agents, we found that camptothecin (CPT) and its derivative, SN38, could induce uPA in RC-K8 and H69 cells. CPT and SN38, which are also used for the treatment of lymphoma and SCLC, significantly increased the uPA accumulation in the conditioned media of both cells in a dose-dependent manner. The maximum induction of uPA mRNA levels was observed 24 h after stimulation. Pretreatment with pyrrolidine dithiocarbamate (PDTC), an anti-oxidant, inhibited the CPT-induced uPA mRNA expression. Thus, CPT induces uPA through gene expression, and, therefore, CPT may influence the tumor-cell biology by up-regulating the uPA/plasmin system.

**KEYWORDS:** CPT, SN38, uPA, RC-K8, H69

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\*PMID: 12530505 [PubMed - indexed for MEDLINE]

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## Original Article

**Camptothecin Induces Urokinase-type Plasminogen Activator Gene-Expression in Human RC-K8 Malignant Lymphoma and H69 Small Cell Lung Cancer Cells**Misako Shibakura<sup>a</sup>, Kenji Niiya<sup>b</sup>, Toru Kiguchi<sup>b</sup>,  
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We previously reported that anthracyclines, which could generate reactive oxygen species (ROS), could induce the urokinase-type plasminogen activator (uPA) gene expression in human RC-K8 malignant lymphoma cells and in H69 small cell lung cancer (SCLC) cells. In screening other uPA-inducible anti-cancer agents, we found that camptothecin (CPT) and its derivative, SN38, could induce uPA in RC-K8 and H69 cells. CPT and SN38, which are also used for the treatment of lymphoma and SCLC, significantly increased the uPA accumulation in the conditioned media of both cells in a dose-dependent manner. The maximum induction of uPA mRNA levels was observed 24 h after stimulation. Pretreatment with pyrrolidine dithiocarbamate (PDTC), an anti-oxidant, inhibited the CPT-induced uPA mRNA expression. Thus, CPT induces uPA through gene expression, and, therefore, CPT may influence the tumor-cell biology by up-regulating the uPA/plasmin system.

**Key words:** CPT, SN38, uPA, RC-K8, H69

**U**rokinase-type plasminogen activator (uPA) catalyzes the conversion of zymogen plasminogen to plasmin and plays a central role in the fibrinolytic process. uPA is also related to tumor progression, and high levels of uPA suggest a poor prognosis of the cancer [1]. uPA is suggested to be one of the acute phase reactants whose synthesis is regulated by pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and IL-1. These pro-inflammatory cytokines are also shown to generate reactive oxygen species (ROS) in human cells [2, 3]. Previously we demonstrated that IL-1 $\beta$  and lipopolysaccharide (LPS), along with hydrogen peroxide,

induced uPA gene expression in human malignant cells [4, 5]. Furthermore, anthracycline anti-cancer agents, which generate ROS, also induced uPA gene expression in both RC-K8 and H69 cells [6, 7]. ROS, such as super-oxide and hydrogen peroxide, work as signaling messengers, activating transcription factors such as NF- $\kappa$ B and AP-1, and inducing a number of gene expressions [8, 9]. NF- $\kappa$ B and AP-1 elements are also identified in the uPA promoter region [10], and, therefore, NF- $\kappa$ B and/or AP-1 transcription factors appear to be involved in the IL-1- and TNF $\alpha$ -induced-uPA expression [11, 12]

Similar to anthracyclines, CPT and its derivative SN38 also generate ROS [13, 14]. CPT is a cytotoxic agent that inhibits the nuclear enzyme Topoisomerase I (Topo I), and produces a complex with Topo I, also

Received January 24, 2002; accepted April 22, 2002.

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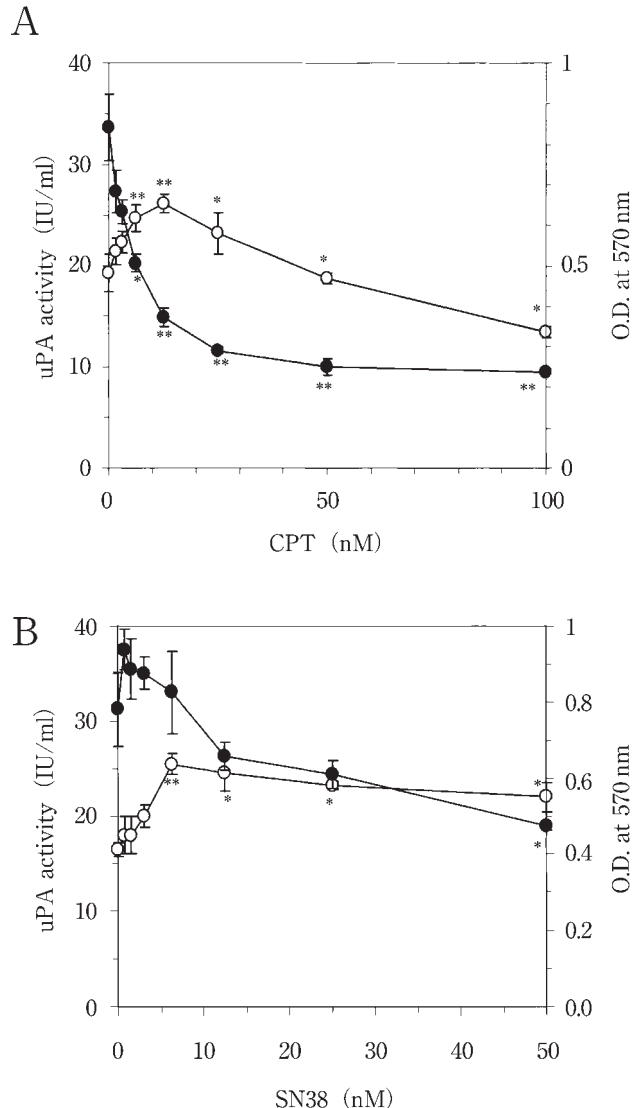
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stabilizing the replication system. CPT and SN38 appear to be effective for various solid tumors [15], and both are now used for SCLC and lymphoma as a salvage treatment [16, 17]. Therefore, we examined the effects of CPT on uPA expression in RC-K8 malignant lymphoma and H69 SCLC cells.

## Materials and Methods

**Cell culture.** Human RC-K8 malignant lymphoma [18] and H69 small cell lung cancer cell line (American type culture collection, Rockville, MD, USA), were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Whittaker Bioproducts, Walkersville, MD, USA), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, as previously described [7]. In the stimulating experiment with CPT, the cells were washed once with phosphate buffered saline, resuspended in serum-free RPMI-1640 (at approximately  $1 \times 10^6$ /ml), and cultured in a 24-well culture plate (500  $\mu$ l/well) with or without CPT or SN38. After 48 h, 20  $\mu$ l of the cell mixture was transferred to a 96-well plate containing 80  $\mu$ l of RPMI-1640 and 10  $\mu$ l of the MTT solution in each well, and then the cell density was determined by the colorimetric MTT assay, as described by Mosmann [19]. The uPA activities in the culture supernatant were measured using a synthetic uPA-substrate S-2444 and a plasminogen-containing fibrin plate (fibrin zymography), as previously described [7]. A high molecular weight form of two-chain uPA was demonstrated in both the RC-K8 and H69 conditioned media at and after 24 h; therefore, the enzymes were all active. No activation step was necessary to measure the uPA activity.

**Northern blot analysis.** The total RNA (10  $\mu$ g) was isolated from the cells by the acid guanidinium thiocyanate-phenol-chloroform method, and was subjected to Northern blot analysis as described by Sambrook *et al.* [20]. The cDNA probe for uPA was labeled with  $^{32}$ P-dCTP by the random primed DNA labeling technique. The mRNA levels were quantitated by counting the radioactivity using the BAS 2000 imaging analyzer (Fuji Film Co., Tokyo, Japan). As a control for differences in the RNA sample loading, the filters were rehybridized with a radiolabeled  $\beta$ -actin cDNA probe.



**Fig. 1** The effects of CPT and SN38 on uPA accumulation and cell density in RC-K8 cells. RC-K8 cells were cultured in the presence of varying concentrations of CPT (A) and SN38 (B) for 48 h, and the uPA activity and cell density were measured using a synthetic uPA substrate (S-2444) and the MTT assay, respectively, as described in Materials and Methods. Each experiment was conducted at least twice in triplicate, and the results were reproducible. Open circles and closed circles indicate uPA activity and cell density, respectively. Means  $\pm$  SD of triplicate values are shown. \* $P < 0.05$  and \*\* $P < 0.01$  compared to controls.

## Results

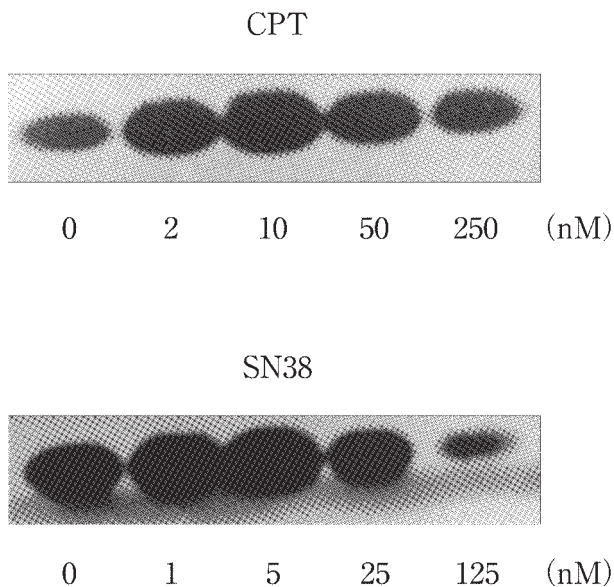
**CPT and SN38 induced uPA accumulation in RC-K8 cells.** The cells were treated with CPT and SN38 for 48 h, and the uPA activities in the super-

natant were measured by the uPA-substrate, S-2444. The accumulation of uPA in the conditioned media increased approximately 1.5-fold after treatment with 10 nM of CPT and 5 nM of SN38, respectively (Fig. 1, panels A and B). Fibrin zymography also revealed the induction of uPA by both of the drugs (Fig. 2). However, the uPA accumulation caused by the CPT- or SN38-stimulation was lower than that caused by the anthracyclines.

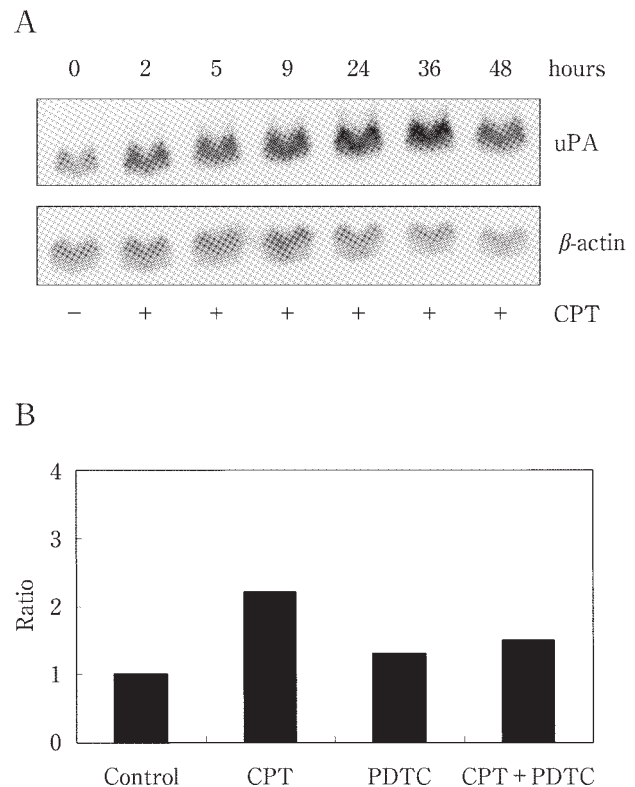
**Effects of CPT on uPA mRNA levels.** To determine whether the CPT-induced uPA accumulation was a result of the activation of the gene expression, we examined the effects of CPT on the uPA mRNA levels. The total RNA was extracted as indicated in the Materials and Methods section, and 10  $\mu$ g of total RNA were applied to Northern blot analysis. The peak induction of uPA mRNA was seen 24 h after CPT stimulation (Fig. 3, panel A), and it was approximately two-fold of non-stimulated control. To determine the involvement of ROS in the CPT-induced uPA expression, 0.5 mM of PDTC, an anti-oxidant drug, was added to the RC-K8 cultured medium before stimulation with CPT. Pretreat-

ment with PDTC resulted in a complete inhibition of the CPT-induced uPA mRNA accumulation (Fig. 3, panel B), suggesting that ROS may be involved in the CPT-induced uPA gene expression.

**SN38-induced uPA accumulation in RC-K8 and H69 cells.** CPT and SN38 induced uPA in H69 as well as in RC-K8 cells, as shown in Fig. 4, panels A and B. CPT also induced uPA mRNA expression 24 h after stimulation in H69 cells; however, it was a very weak expression, in parallel with the uPA activities (data not shown).



**Fig. 2** Dose-dependent stimulating effects of CPT and SN38 on uPA accumulation in RC-K8 cells detected by fibrin zymography. Twenty micro liters of one of the RC-K8 cultured media stimulating varying concentrations of CPT and SN38 for 48 h were applied to each lane of 7.5% polyacrylamide running gel for SDS-PAGE, and zymography was then performed. High molecular weight forms (50–54 kDa) of uPA were primarily observed in the conditioned medium.



**Fig. 3** Time-course of CPT-induced uPA mRNA levels and effects of PDTC on CPT-induced uPA mRNA levels in RC-K8 cells. The uPA mRNA levels were measured by Northern blotting. A  $\beta$ -actin cDNA probe was used as an internal control. The total RNA (10  $\mu$ g) was extracted from the RC-K8 cells collected at the times shown following exposure to 10 nM of CPT. (B) The RC-K8 cells were pretreated with 0.5 mM of PDTC for 1 h and further cultured in the presence of 10 nM of CPT for 24 h. The total RNA (10  $\mu$ g) was isolated and analyzed by Northern blotting. The mRNA levels were quantitated by counting the radioactivity of the bands using a BAS 2000 imaging analyzer, as described in Materials and Methods. The Y-axis indicates the relative intensity of the radioactivity compared with that of the untreated control.

## Discussion

We previously reported that the anthracycline anti-cancer agent induced the up-regulation of uPA gene expression in human RC-K8 malignant lymphoma and H69 small lung cell cancer cells [7]. In the present study, it is demonstrated that CPT and SN38 also induce the uPA expression in RC-K8 and H69 cells. ROS work as

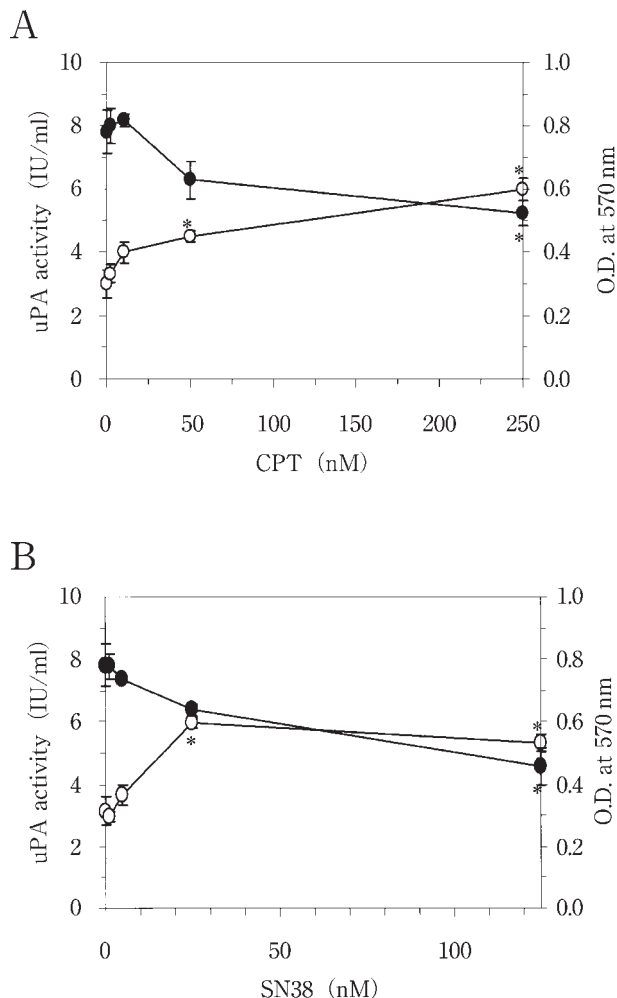
second messengers for the signal transduction system and activates transcriptional factors, such as NF- $\kappa$ B and AP-1 [8, 9]. It is also shown that CPT-11 and SN38 activate the NF- $\kappa$ B transcriptional factor in HT1080 cells, and that PDTTC inhibits the activation of NF- $\kappa$ B [21]. Ten nM of CPT induced the accumulation of cytochrome c in the cytosol (data not shown), and an antioxidant, PDTTC, inhibited the CPT-induced uPA mRNA expression. These observations suggest the involvement of ROS in the CPT-induced uPA expression.

The clinical findings demonstrated that the plasma levels of the metabolized CPT or SN38 concentrations were approximately 10 to 100 nM 48 h after intravenous infusion, when patients were treated with 350 mg/m<sup>2</sup> of CPT-11 [22]. As shown in Figs. 1, 2 and 4, these concentrations are sufficient to induce uPA in either RC-K8 or H69 cells. The role of uPA/plasmin system in tumor metastasis and invasiveness is well established. uPA-generated plasmin degrades the extracellular matrix as well as fibrin. uPA bound to uPA-receptor (uPAR) on tumor cell surface causes the focal proteolysis. The complex between uPA and uPAR induces the change of the receptor cluster on tumor cell surface, which causes a detachment from the extracellular matrix and a migration of tumor cells [1]. Furthermore, it has been suggested that the plasminogen/plasmin and matrix metalloproteinases (MMP) systems are functionally interactive and cooperate in extracellular matrix degradation. uPA converts plasminogen to plasmin and it activates MMPs, especially MMP-1, 3, 7, 8, 9, 12 and 13 [23]. Tumor cells detached from the extracellular matrix become more sensitive to anti-cancer agents [24]. Therefore, CPT-induced uPA may enhance anti-tumor effects of CPT by causing cell-detachment.

**Acknowledgments.** This work was supported in part by a Grant-in-Aid (12771476) for the Encouragement of Young Scientists (to M.S) from the Japan Society for the Promotion of Science.

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**Fig. 4** Effects of CPT (A) and SN38 (B) on uPA accumulation and cell density in H69 cells. H69 cells were cultured in the presence of varying concentrations of CPT and SN38 for 48 h, and uPA activity and cell density were measured using a synthetic uPA substrate (S-2444) and MTT assay, respectively, as described in Materials and Methods. Each experiment was conducted at least twice in triplicate, and the results were reproducible. Open circles and closed circles indicate uPA activity and cell density, respectively. Means  $\pm$  SD of triplicate values are shown. \* $P < 0.05$  compared to controls.



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