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Inhibitory Activity of Camptothecin Derivatives Against Acetylcholinesterase in Dogs and Their Binding Activity to Acetylcholine Receptors in Rats

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Abstract—A camptothecin derivative, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11), shows a potent antitumour activity in experimental tumour models and in clinical trials. However, CPT-11 induced early diarrhoea and vomiting at high dose levels in clinical studies and showed an acetylcholine-like action on the guinea-pig ileum and trachea. In the present study, we investigated the activities of camptothecin derivatives in inhibiting acetylcholinesterase (AChE) and in binding to muscarinic acetylcholine receptors (AChR). CPT-11 inhibited AChE and binding of the specific ligand to AChR with respective 50% inhibition concentrations of 0·2 and 5 μM. These inhibitions were induced by camptothecin derivatives having an amino group at the C-10 position (or the C-4 position of hexacyclic derivatives), but were not or were only slightly induced by the others. Early defecation and vomiting in dogs were observed after intravenous injection of DU-6596 and DU-6888, two hexacyclic derivatives having the aminomethyl group at the C-4 position, and of CPT-11. DU-6174, however, which has a hydroxy group at this position, induced no early defecation and little vomiting. Plasma concentrations of CPT-11, DU-6596 and DU-6888 after intravenous treatment at doses causing such early adverse effects were maintained for 1 h or longer at levels sufficient to inhibit AChE. These results suggest that the inhibition of AChE by camptothecin derivatives with an amino group at the C-10 position (or the C-4 position) relates to the early defecation or diarrhoea and vomiting.

CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, is a derivative of camptothecin, a plant antitumour alkaloid isolated from *Camptotheca acuminata* by Wall et al (1966). Hsiang et al (1989) first demonstrated that camptothecin inhibits type I DNA topoisomerase (Topo I). This enzyme is now considered an important target of anticancer drugs. Camptothecin itself showed poor efficacy and severe toxicity in clinical trials (Gottlieb & Luce 1972; Moertel et al 1972; Muggia et al 1972). However, CPT-11 has exhibited high antitumour activity against experimental tumours (Kunimoto et al 1987; Matsuzaki et al 1988; Tsuruo et al 1988; Kawato et al 1991a) and in clinical studies (Ohno et al 1990; Fukuoka et al 1992). Negoro et al (1991) reported that the main adverse effects of this compound in phase I clinical studies were myelosuppression and gastrointestinal toxicity including diarrhoea and vomiting. Diarrhoea comprised both early and delayed types. Vomiting and delayed diarrhoea are induced by many other anticancer drugs, but early diarrhoea which occurs during and immediately after infusion is a rare toxicity.

Takayanagi et al (1989) reported that CPT-11 has an acetylcholine-like action against guinea-pig ileal and tracheal preparations. Acetylcholine or cholinergic agents are able to increase gastrointestinal motility (Gerring 1989) and stimulate chloride secretion (Isaacs et al 1976; Browning et al 1977; Donowitz 1983), and these activities may cause diarrhoea (Ooms & Degryse 1986). Moreover, cholinergic receptors may play an important role in the mediation of vomiting (Peroutka & Snyder 1982; Leslie et al 1990).

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In the present study, we investigated the inhibitory activity of camptothecin derivatives against acetylcholinesterase (AChE) and their binding activity to acetylcholine receptors (AChR) in rats. Furthermore, to evaluate the possibility that these activities are involved in early gastrointestinal toxicity (defecation or diarrhoea and vomiting), we examined the gastrointestinal toxicity and plasma concentration of these compounds in dogs.

Materials and Methods

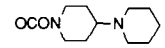
Chemicals and enzymes

CPT-11 and SN-38 were supplied by Yakult Honsha Co. Ltd (Tokyo, Japan). All other camptothecin derivatives were totally synthesized by Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Molecular structures of these compounds are listed in Table 1. Compounds with and without an amine group were used as their hydrochloride and sodium salts, respectively, or in the Topo I assay in solution in dimethyl sulphoxide. Acetylthiocholine iodide (ATChI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and atropine were purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). L-[Benzilic-4,4′-^{3}H(N)]-quinuclidinyl benzilate ([^{3}H]QNB) and AChE were purchased from NEN Research Products (Boston, MA, USA) and Sigma Chemical Co. (St Louis, MO, USA), respectively. Topo I of Ehrlich ascites tumour cells was prepared as described by Kawato et al (1991b).

Evaluation of AChE inhibition

AChE activity was determined essentially as described by Ellman et al (1961). Briefly, thiocholine, which was produced from ATChI by AChE, reacted with DTNB forming a yellow

Table 1. Molecular structures and inhibitory activities against AChE, binding of QNB to AChR, and Topo I of camptothecin derivatives.

	Structures			IC50 (μM)[a]		
					QNB binding[c]	
Code No.	R_1	R_2	R_3	AChE[b]	to AChR	Topo I[d]
CPT-11		H	CH_2CH_3	0·2	5·2	>1000
SN-38	OH	H	CH_2CH_3	>20	>100	0·4
SKF 104864	OH	$CH_2N(CH_3)_2$	H	>20	>100	9·2
DT-5124		H	CH_2CH_3	<0·2	n.d.	n.d.
DV-7152	H	H	CH_2NH_2	>20	n.d.	n.d.
	Structures					
	R_1	X				
DU-6174	OH	CH_2		>20	>100	0·3
DU-6596	CH_2NH_2	S		0·7	18	1·2
DU-6888	CH_2NH_2	CH_2		0·6	24	1·3
DV-7663	CH_2NHCH_3	CH_2		0·7	28	4·6
DV-7705	$CH_2N(CH_3)_2$	CH_2		0·4	28	18
DV-7881	$CH_2N(CH_3)_3$	CH_2		0·7	18	13
DV-7925	CH_2NH_2	S=O		1·6	73	n.d.
DT-5532	$OCH_2CH_2N(CH_3)_2$	CH_2		<0·2	n.d.	n.d.
DV-7188	$NHCNHNH_2$	CH_2		1·0	n.d.	n.d.
DV-7222	$OCH_2CH_2NH_2$	CH_2		0·7	17	n.d.

[a] Concentrations inducing 50% inhibition. [b] AChE activity was determined by spectrophotometry using ATChI as a substrate, essentially as described by Ellman et al (1961). [c] [^{3}H]QNB binding to AChR was measured with a liquid scintillation counter as described in Materials and Methods. [d] Topo I activity was quantified as described by Kawato et al (1991b). n.d.: not done.

colour. The rate of colour production was measured by spectrophotometry at 412 nm. The concentration of each test compound required to induce a 50% reduction in the colour production rate (IC50) was estimated from the dose–response curve.

Evaluation of binding to AChR

Synapse membrane crude fraction was prepared as described by Zukin et al (1974) from female Sprague-Dawley rats aged 7 weeks (Shizuoka Laboratory Animal Center, Hamamatsu, Japan). One hundred microlitres of this fraction, 200 μL of 5 nM [^{3}H]QNB, and 100 μL of test compound solution were added to 50 mM NaCl and 50 mM Tris–HCl (pH 7·1). This reaction mixture (1 mL) was incubated for 30 min at room temperature (25°C) then filtered through a Whatman GF/B filter (Whatman International Ltd, Maidstone, UK). Radioactivity on the filter was measured with a liquid scintillation counter (Liquid Scintillation System, Model LSC-903, Aloka Co. Ltd, Tokyo, Japan). Complete (100%) inhibition of [^{3}H]QNB binding to the synapse membrane was determined in the presence of 10 μM atropine. The concentration of each test compound inducing a 50% reduction in the [^{3}H]QNB binding (IC50) was evaluated from the dose-response curve. This value is considered to reflect the binding activity of the compound to muscarinic AChR.

Assay of Topo I inhibition

Assay of Topo I activity and calculation of the concentration of each test compound required for 50% inhibition of Topo I activity (IC50) were performed as previously described (Kawato et al 1991b).

Observation of defecation and vomiting in the dog

Male dogs (Laboratory Research Enterprise Beagle), aged 2 years, were purchased from Kasho Co. Ltd (Tokyo, Japan). They were given a single intravenous dose of a test compound dissolved in physiological saline, and were observed for 4 h. The number of episodes of defecation and vomiting was recorded.

Determination of plasma concentration

Plasma of dogs was sampled at 0·5, 1, 2, 4, and 8 h after the intravenous injection of DU-6596 or DU-6888 at a dose of 2 mg kg^{-1} or CPT-11 at 10 mg kg^{-1} in physiological saline. DU-6596 and DU-6888 in dog plasma were separated by HPLC using a TSK gel ODS 80T$_M$ column (Tosoh Corp., Tokyo, Japan) with an eluting solution (20% CH_3CN and 0·02 M HCl). CPT-11 was also separated by HPLC (Nucleosil 5C$_{18}$ column; Macherey-Nagel, Germany) with 50% CH_3CN, 25% C_2H_5OH, and 0·2% $(NH_4)_2CO_3$. DU-6596, DU-6888, and CPT-11 were quantified by fluorospectrometry at respective excitation wavelengths of 367, 355, and 370 nm, and at respective emission wavelengths of 528, 429, and 430 nm (Fluorescence Spectrophotometer, Model F1000 and Model 650-10LC; Hitachi Ltd, Tokyo, Japan).

Results

Inhibitory activity of camptothecin derivatives against AChE

The IC50 values are summarized in Table 1. The value for CPT-11 was approximately 0·2 μM. All derivatives with an amino group at the C-10 position (or the C-4 position of hexacyclic derivatives) showed inhibitory effects on AChE with IC50 values of 1·6 μM or less. Among these compounds, DV-7925, in which oxygen is bound to sulphur in the F ring, exhibited the weakest activity. Derivatives with no amino group at the C-10 (C-4) position had almost no effect on AChE (IC50 >20 μM).

Table 2. Frequency of defecation and vomiting within 4 h after a single intravenous injection of camptothecin derivatives into dogs.

Compound	Dose (mg kg^{-1})	Dog no.	Defecation	Vomiting
Saline	—	1	0	0
		2	0	0
CPT-11	20	1	2	1
		2	2	6
DU-6596	2	1	1	7
		2	2	6
	20	1	1	6
		2	1	2
DU-6888	2	1	2	9
		2	3	7
	20	1	0	0
		2	4	5
DU-6174	20	1	0	1
		2	0	1

Binding activity of camptothecin derivatives to AChR

All of the tested compounds with an amino group at the C-10 (C-4) position inhibited the binding of [^{3}H]QNB to AChR with IC50 values of 5–73 μM (Table 1). Among these compounds, DV-7925 was the least active in this test. The other tested compounds showed less effect on [^{3}H]QNB binding to AChR (IC50 >0·1 mM). These results indicate that derivatives having an amino group at the C-10 (C-4) position may bind to AChR.

Effect of camptothecin derivatives on Topo I

The inhibitory effect of some of these derivatives on Topo I activity was examined. The most active derivative was DU-6174 (IC50 0·3 μM), a compound which exhibited little effect on AChE activity or on binding of [^{3}H]QNB to AChR (Table 1).

Defecation and vomiting after intravenous injection of camptothecin derivatives

As shown in Table 2, CPT-11 at 20 mg kg^{-1} induced defecation and vomiting within 4 h. DU-6596 and DU-6888 also caused these adverse effects at doses of 2 and 20 mg kg^{-1}. In addition to gastrointestinal toxicity, miosis, lacrimation, and salivation were observed after treatment with these compounds. The number of episodes of defecation and vomiting induced by these was greater than that induced by DU-6174 and by physiological saline.

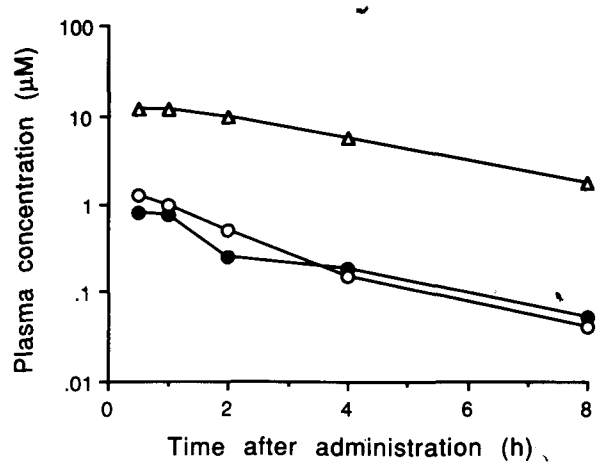


FIG. 1. Time courses of plasma concentrations of CPT-11, DU-6596, and DU-6888. The dogs were treated intravenously with 10 mg kg^{-1} CPT-11 (△), 2 mg kg^{-1} DU-6596 (●), or 2 mg kg^{-1} DU-6888 (○). The plasma was sampled from the dogs at 0·5, 1, 2, 4, and 8 h after treatment.

Plasma concentration of camptothecin derivatives

Fig. 1 demonstrates the time course of plasma concentration of derivatives after intravenous injection into dogs. Plasma concentration of CPT-11 at 10 mg kg^{-1} was maintained at 5 μM or more for 4 h after administration. Plasma concentrations of DU-6596 and DU-6888 after a single dose of 2 mg kg^{-1} were greater than 0·7 μM for 1 h. Even at 4 h after injection, both were detected at approximately 0·2 μM.

Discussion

CPT-11 demonstrated high antitumour activity and low toxicity in experimental chemotherapy against human tumour xenografts (Kawato et al 1991a). Although this compound was also effective in clinical trials, it caused gastrointestinal toxicity including diarrhoea and vomiting in addition to myelosuppression (Ohno et al 1990; Negoro et al 1991; Fukuoka et al 1992). In some patients, watery diarrhoea was induced during or immediately after intravenous infusion of CPT-11 at high doses. This early diarrhoea was clearly different from that usually observed with other cancer chemotherapies.

An acetylcholine action of CPT-11 on contraction of the ileum and trachea was reported by Takayanagi et al (1989), and it is thought that cholinergic action induces secretory diarrhoea through stimulation of chloride secretion (Ooms & Degryse 1986). Our study indicates that one of the mechanisms of the cholinergic action of CPT-11 is its inhibition of AChE activity, the IC50 value being approximately 0·2 μM. Kinetic analysis revealed that CPT-11 noncompetitively inhibited AChE activity with an unchanged apparent Michaelis constant (K_m) of 63–68 μM and an inhibition constant (K_i) of 0·2 ~ 0·3 μM. Negoro et al (1991) showed in clinical studies that peak plasma concentrations of CPT-11 after 90 min intravenous infusion at doses of 50–150 mg m^{-2} were 0·6–3 μg mL^{-1} (0·8–4 μM), while Morris et al (1980) demonstrated that intravenous administration of neostigmine, a potent AChE inhibitor, caused secretion of sodium chloride and water in the human jejunum. These observations suggest that the inhibitory activity of CPT-11 against AChE is involved in the early watery or secretory diarrhoea.

Under the conditions of our study, physostigmine, another potent AChE inhibitor, gave a K_i value of approximately 0·04 μM. Hemsworth & West (1970) indicated that the urethane group seems to be essential for the strong AChE inhibitory effect of physostigmine. SN-38, an active metabolite of CPT-11 (Kaneda et al 1990; Kawato et al 1991b,c), has no urethane structure and showed no inhibitory activity against AChE up to 20 μM. These observations suggested that the urethane group at the C-10 position of CPT-11 plays a major role in the inhibition of this enzyme. However, as Table 1 shows, this group is not essential to the inhibition of

AChE. All tested camptothecin derivatives with an amino group at the C-10 position (or the C-4 position of hexacyclic derivatives) possessed this inhibitory activity, while the other derivatives showed no or little activity. DV-7925, which has one oxygen atom binding to the sulphur (a sulphoxide) in the F ring of DU-6596, exhibited a lesser inhibitory effect on AChE than did DU-6596, and this oxygen atom may sterically affect the interaction between the enzyme and the aminomethyl group. These results indicate that an amino group at the C-10 (C-4) position is one of the principal determinants in the inhibition of AChE.

To estimate whether the inhibition of AChE correlated with the early diarrhoea, some of the camptothecin derivatives were injected into dogs. Animals given CPT-11, DU-6596, or DU-6888 showed early defecation instead of early watery diarrhoea, and vomiting. The plasma concentrations of these compounds were high enough to inhibit AChE for 1 h or longer after their injection, and we also observed miosis, lacrimation, and salivation, all typical symptoms arising from cholinergic action. It therefore appears that the increase in gastrointestinal motility (Gerring 1989) and the stimulation of appropriate sites such as the chemoreceptor trigger zone (Peroutka & Snyder 1982; Carpenter et al 1988) through the cholinergic action of these compounds cause the defecation and vomiting, respectively. However, vomiting was also induced by DU-6174 which showed no inhibitory activity against AChE, suggesting that a mechanism other than this cholinergic action participates in vomiting. These results suggest that CPT derivatives containing an amino group at the C-10 (C-4) position induce early diarrhoea or defecation, and vomiting in some cases, as a result of their inhibition of AChE.

Table 1 shows that CPT derivatives having an inhibitory effect on AChE may bind AChR, although the AChR binding activity seems to be weaker than the AChE inhibitory activity, and that DV-7925 was inferior to DU-6596 in both activities. It is therefore likely that these activities are related. Usually, inhibitors of QNB binding to AChR exhibit anti-emetic activity (Pedigo & Brizzee 1985), but if a compound acts as an agonist to AChR, it possesses cholinergic action. In the present study, it was not possible to evaluate the physiological significance of the binding of camptothecin derivatives to AChR.

The inhibition of Topo I is considered to be a dominant mechanism in the cytotoxicity of camptothecin derivatives (Eng et al 1988; Hsiang & Liu 1988; Hsiang et al 1989; Jaxel et al 1989). Our study demonstrates that Topo I inhibitory activity does not correlate with AChE inhibition. We therefore expect to be able to synthesize camptothecin derivatives with high antitumour activity and low early gastrointestinal toxicity.

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