Antiulcer Activities of Glycyrrhetinic Acid Derivatives in Experimental Gastric Lesion Models

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Glycyrrhetinic acid (Ia) and eighteen related derivatives were examined for antiulcer activity using stress-induced gastric lesions (restraint plus water immersion at 25 °C) in mice and rats as screening tests. Among the compounds tested, dihemiphthalate derivatives of 18α- or 18β-olean-12-ene-3β,30-diol (IV, IIId), 18β-olean-9(11)12-diene-3β,30-diol (VIc), and olean-11,13(18)-diene-3β,30-diol (VIIc) showed potent inhibition of gastric lesion formation at a dose of 12 or 25 mg/kg (*p.o.*); carbenoxolone sodium (Ib) significantly suppressed the lesion formation at a dose of 500 mg/kg (*p.o.*). Further evaluation of the antiulcer activity was carried out mainly for compound IIId. Compound IIId (*p.o.*) prevented the formation of indomethacin-induced or 0.6 N HCl-induced gastric lesions; the latter antiulcer effect was noted even in the combined treatment with indomethacin, suggesting that the effect occurs independently of endogenous prostaglandins. In contrast, compound IIId had no preventive effect against Shay rat ulcer when intragastrically (i.g.) administered; further, no antisecretory effect was seen by i.g. application in pylorus-ligated rats. Administration of compound IIId for 2 weeks accelerated the healing rate of acetic acid-induced gastric ulcer in rats. No significant change in urine excretion was observed after its consecutive administration for 3 d. These results suggest that dihemiphthalate derivatives (IIId, IV, VIc, VIIc) may produce a strong antiulcer activity, probably by strengthening some gastric mucosal defensive mechanism.

Keywords glycyrrhetinic acid; antiulcer activity; stress ulcer; cytoprotection; gastric secretion; dihemiphthalate compound; deoxoglycyrrhetol; carbenoxolone; screening test

Glycyrrhetinic acid (Ia), the aglycone of glycyrrhizin isolated from licorice root (*Glycyrrhiza* spp. (Leguminosae)), is known to have antiinflammatory,[2)] antiallergic[3)] and antiulcer[4)] activities. Among glycyrrhetinic acid derivatives, carbenoxolone sodium (Ib), the sodium salt of the 3β-*O*-hemisuccinate of glycyrrhetinic acid, has been extensively studied and reported to have antiulcer effects in animals[4,5)] and man.[6)] It has been suggested that the antiulcer activity of carbenoxolone is exerted by strengthening the defensive mechanisms of gastric mucosa,[7)] but its clinical usefulness may be limited by the occurrence of side effects related to pseudoaldosteronism.[8)] The 11-oxo-$\Delta^{12(13)}$ system in ring C of glycyrrhetinic acid has been found to be associated with mineral corticoid-like activity, because of metabolic competition with the 3-oxo-$\Delta^{4(5)}$ system in ring A of cortical steroids at the active site of the reducing enzyme.[9)] Thus, chemical modification has been attempted to prepare glycyrrhetinic acid derivatives with a view to eliminating the pseudoaldosteronism while retaining or enhancing the therapeutic activities.[10)] The antiulcer activities of our samples, including deoxoglycyrrhetol (IIIa) and related compounds, were reported preliminarily.[10a)]

In this paper, we report that, of eighteen glycyrrhetinic acid derivatives, some dihemiphthalates of triterpenes derived from glycyrrhetinic acid have potent antiulcer activities in several experimental gastric ulcer models.

Experimental

Male ddY mice and male Wistar rats were used in this study.

Stress-Induced Gastric Lesions Mice weighing 20—22 g and rats weighing 240—260 g were deprived of food but allowed free access to water for 18 h prior to the experiment. The animals were placed in a stress cage and immersed to the level of the xiphoid process in a water bath (25 °C) for 6 h.[11,12)] Drugs were given orally (*p.o.*), subcutaneously (s.c.), or intraperitoneally (i.p.) 30 min before water immersion. At the end of stress, the mice were killed by dislocation of the neck and the rats by an overdose of ether. The stomach of each animal was removed and slightly inflated by injecting 1% formalin solution for 10 min. Subsequently, the stomach was incised along the greater curvature, and the length of each lesion in the glandular portion was measured under a dissecting microscope. The sum of the length (mm) of all lesions for each animals was used as a parameter of erosion severity.

HCl-Induced Gastric Lesions Rats weighing 200—220 g were fasted for 24 h and deprived of water for 3 h prior to the experiment. Drugs were orally administered 30 min before HCl challenge. Sixty minutes following oral administration of 1 ml of 0.6 N HCl, the animals were killed by an overdose of ether.[13)] The stomachs were removed and incised along the greater curvature. Gastric lesions were measured in millimeters and the total lesion length per stomach was calculated. In another series of experiments, indomethacin, 8 mg/kg s.c., was injected 90 min before oral administration of drugs.

Indomethacin-Induced Gastric Lesions Rats weighing 210—230 g were deprived of food for 48 h and then given indomethacin orally at 30 mg/kg. The animals were killed 20 h later, and the stomach of each was examined for lesions developed in the glandular portion. The sum of the length (mm) of all lesions for each rat was used as a parameter of erosion severity. Drugs were given orally 30 min before indomethacin dosing.

Shay Rat Ulceration Rats weighing 180—200 g were deprived of food for 48 h and the pylorus was ligated under ether anesthesia by the method described by Shay *et al.*[14)] The animals were killed 15 h later by an overdose of ether and the stomach was removed. The gastric contents were collected, centrifuged and titrated for acidity. The pepsin activity was determined by the method described by Berstad.[15)] The stomach was incised along the greater curvature and the surface area of each lesion (mm^2) in the forestomach was measured, summed and graded into five degrees as an ulcer index as follows:

lesion area (mm^2)	1—12	13—25	26—37	38—50	>51 or perforation
ulcer index	1	2	3	4	5

Drugs were given intragastrically (i.g.) or intraduodenally (i.d.) 10 min after pylorus ligation.

Acetic Acid-Induced Gastric Ulcer Rats weighing 200—240 g were used. Under ether anesthesia, a polyethylene mold (7.5 mm in diameter) was tightly placed upon the serosal surface of the gastric wall at the junction of the body of the glandular stomach and the antrum of the anterior wall. Acetic acid (100%, 0.07 ml) was poured into the mold and allowed to remain for 60 seconds. After removal of the acetic acid by aspiration with a syringe, the abdomen was closed.[16)] The animals were maintained on Oriental rat chow and water *ad libitum*, and were killed by an overdose of ether on the 15th day after the operation. The ulcerated areas (mm^2) were measured under the dissecting microscope as an ulcer index.

Drugs were given orally twice a day from one day after operation for 14 consecutive days.

Gastric Secretion Rats weighing 200—220 g were deprived of food but allowed free access to water for 24 h prior to the experiment. Under ether anesthesia, the pylorus was ligated by the method of Shay *et al.*[14] Drugs were given i.g. or i.d. immediately after pylorus ligation. The rats were killed 4 h later by an overdose of ether. Gastric contents were collected and analyzed for volume, acidity and peptic activity. Peptic activity was determined by the method described by Berstad.[15]

Urine Excretion Rats weighing 200—250 g were used in this experiment. Drugs were given orally twice a day for 3 d. The animals were deprived of food but allowed free access to water for 20 h after the last administration and placed in an individual metabolic cage. Urine was collected in a test tube for 6 h between 10:00 and 16:00 on the 4th day of the experiment. The rats were killed by an overdose of pentobarbital sodium, and urine in the bladder was also taken by suction with a syringe, then added to the test tube. The collected urine was analyzed for volume, sodium concentration and potassium concentration. Urinary concentrations of sodium and potassium were determined by using a flame photometer (Hitachi 170-50A). The concentration ratio of sodium to potassium was also calculated.

Drugs Glycyrrhetinic acid and its derivatives (Chart 1) were prepared according to Shibata *et al.*[10b] These compounds were suspended in 1.4% (v/v) Tween 80. Phthalic acid monosodium salt, phthalic acid disodium salt (Nakarai) and atropine sulfate (Wako) were dissolved with saline. Sucralfate (Chugai) was suspended in saline. Indomethacin (Sigma) was suspended in 0.3% carboxymethyl cellulose. Prostaglandin E_2 (Sigma) was dissolved in ethanol and then diluted in saline.

Statistics Statistical analysis was carried out by one-way analysis of variance (ANOVA) coupled with the Dunnet test.

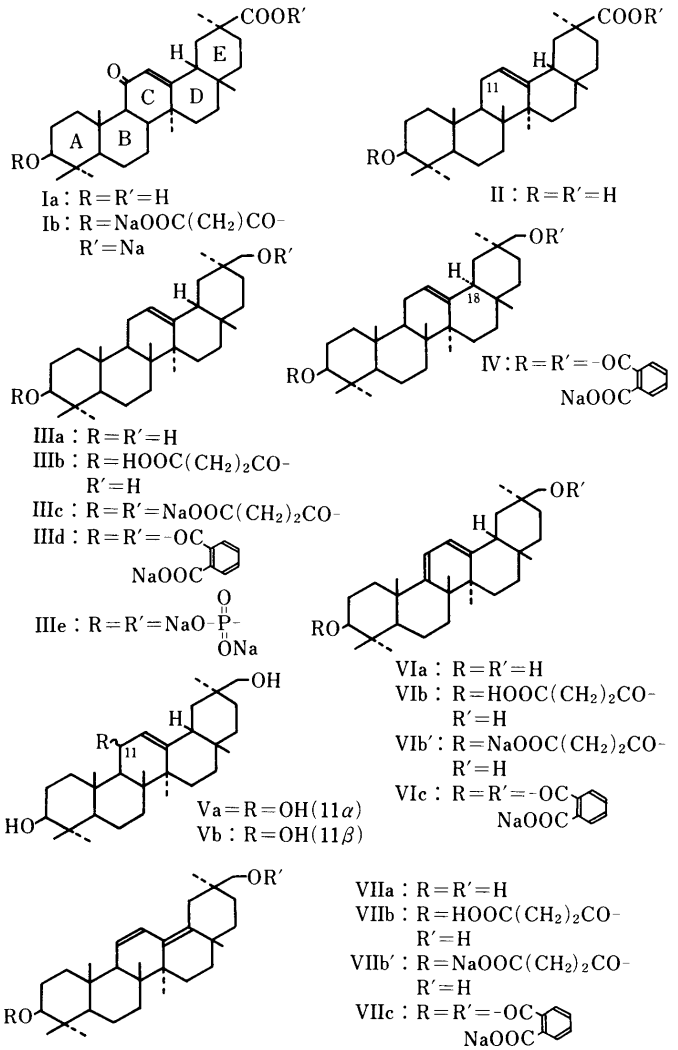

Chart 1. Structures of Glycyrrhetinic Acid Derivatives

Results

Effect of Glycyrrhetinic Acid Derivatives on Stress-Induced Gastric Lesions in Mice and Rats The effect of glycyrrhetinic acid derivatives on stress-induced gastric lesions in mice or rats was routinely tested for assessing anti-ulcer activity (Tables I and II). Glycyrrhetinic acid and the homoannular diene (VIa) and heteroannular diene (VIIa) compounds did not significantly affect the gastric lesion formation in stressed mice. In contrast, deoxoglycyrrhetol (IIIa) was effective in reducing stress-induced gastric lesions. Monohemisuccinates (VIb, VIb', VIIb, VIIb') of the homoannular diene and heteroannular diene compounds showed moderate inhibition of stress ulceration in mice. The dihemisuccinate (IIIc) of deoxoglycyrrhetol definitely prevented the formation of gastric lesions induced by stress at an oral dose of 200 mg/kg in rats. Furthermore, sodium dihemiphthalate derivatives (IIId, IV, VIc, VIIc) of 18β- or 18α-deoxoglycyrrhetol and the homoannular diene and heteroannular diene compounds produced a marked inhibition of stress-induced gastric lesions at an oral dose of 12 or 25 mg/kg. When administered i.p., compound IIId also prevented the lesion formation. However, phthalic acid monosodium salt and phthalic acid disodium salt, which are partial structures of the dihemiphthalate derivatives, were ineffective in preventing stress-induced lesion formation in rats. Oral administration of carbenoxolone sodium, the 3β-*O*-hemisuccinate derivative of glycyrrhetinic acid, significantly inhibited formation of stress-induced lesions at a dose of 500 mg/kg in rats.

Table I. Effect of Glycyrrhetinic Acid Derivatives on Stress-Induced Gastric Lesions in Mice

Compound	Dose (mg/kg, *p.o.*)	No. of mice	Inhibition (%)
Ia	200	9	33
Ia	200	8	10
II	200	8	6
IIIa	100	8	29
	200	8	48[a]
IIIc	200	8	73[b]
	500	8	86[b]
IIId	200	8	74[b]
	500	9	64[b]
IIIe	200	9	−47[a]
	500	9	−28
Va	200	10	7
	500	10	−1
Vb	200	8	−18
	500	8	12
VIa	200	7	−5
	500	8	10
VIb	200	6	−10
	500	7	67[a]
VIb'	200	9	27
	500	9	51[a]
VIIa	200	8	−29
	500	9	−15
VIIb	200	9	18
	500	10	45[a]
VIIb'	200	8	3
	500	9	55[b]
Atropine sulfate	10[d]	10	96[c]

Values of inhibition are expressed as percent of the control. Compounds were given 30 min before water immersion. *a*) $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$. *d*) Subcutaneously injected.

TABLE II. Effect of Glycyrrhetinic Acid Derivatives on Stress-Induced Gastric Lesions in Rats

Compound	Dose (mg/kg)	No. of rats	Inhibition (%)
Ib	200 *p.o.*	9	7
	500 *p.o.*	9	67[a]
IIIb	200 *p.o.*	7	−16
	400 *p.o.*	8	8
IIIc	80 *p.o.*	7	14
	200 *p.o.*	10	48[a]
	500 *p.o.*	9	66[a]
IIId	6 *p.o.*	8	14
	12 *p.o.*	8	76[a]
	25 *p.o.*	8	98[c]
	50 *p.o.*	8	96[c]
	4 i.p.	4	29
	10 i.p.	10	48[a]
	25 i.p.	5	70[b]
IV	25 *p.o.*	10	76[a]
	50 *p.o.*	10	89[b]
VIb′	200 *p.o.*	9	−6
	500 *p.o.*	8	66[a]
VIc	6 *p.o.*	10	0
	12 *p.o.*	10	63[a]
	25 *p.o.*	10	80[b]
VIIb′	200 *p.o.*	10	6
	500 *p.o.*	8	52[a]
VIIc	6 *p.o.*	10	35
	12 *p.o.*	10	80[b]
	25 *p.o.*	10	86[b]
Phthalic acid sodium	12 *p.o.*	8	14
Phthalic acid disodium	12 *p.o.*	8	−12
Atropine sulfate	10 s.c.	6	98[c]

Values of inhibition are expressed as percent of the control. Compounds were given 30 min before water immersion. *a*) $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$.

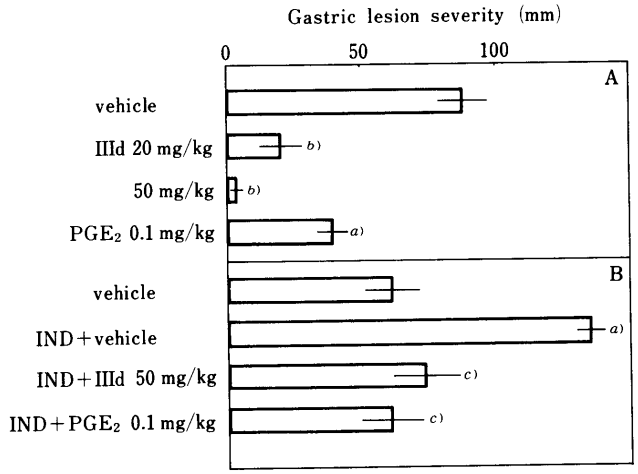

Fig. 1. Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on HCl-Induced Gastric Lesions in Rats

Panel A, the experiments in rats not pretreated with indomethacin. Panel B, the experiments in rats pretreated with indomethacin (8 mg/kg s.c.). Values represent the mean ± S.E. ($n=8$). *a*) $p<0.01$; *b*) $p<0.001$ *vs.* the vehicle control. *c*) $p<0.05$ *vs.* the indomethacin control. IND, indomethacin. Drugs were administered orally.

Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on HCl-Induced Gastric Lesions in Rats The dihemiphthalate compound (IIId) markedly protected the gastric mucosa against HCl-induced necrotic lesions at oral doses of 25 and 50 mg/kg (Fig. 1). Indomethacin significantly aggravated the HCl-induced lesion formation. However, oral pretreatment with compound IIId 30 min prior to HCl instillation definitely attenuated the indomethacin aggravation of the HCl-induced gastric lesions.

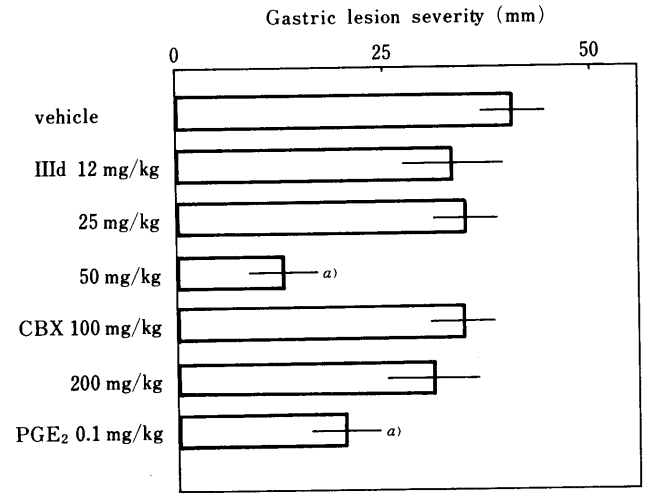

Fig. 2. Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Indomethacin-Induced Gastric Lesions in Rats

Values represent the means ± S.E. ($n=8$). *a*) $p<0.001$ compared with the vehicle control. CBX, carbenoxolone sodium. Drugs were administered orally.

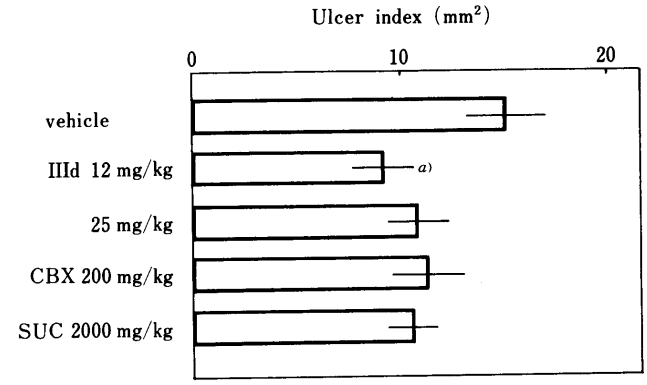

Fig. 3. Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Acetic Acid-Induced Gastric Ulcer in Rats

Values represent the mean ± S.E. ($n=12$). *a*) <0.05 compared with the vehicle control. CBX, carbenoxolone sodium; SUC, sucralfate. Drugs were administered orally for 14 d.

Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Indomethacin-Induced Gastric Lesions in Rats Oral administration of compound IIId significantly inhibited the formation of indomethacin-induced gastric lesions at a dose of 50 mg/kg (Fig. 2). Carbenoxolone sodium, 200 mg/kg *p.o.*, provided no protection against gastric mucosal lesions induced by indomethacin.

Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Shay Rat Ulceration Intragastric administration of compound IIId did not affect the Shay ulceration seen in the forestomach at doses of 20 and 50 mg/kg (Table III). On the other hand, its intraduodenal administration produced a slight inhibition of the Shay ulceration.

Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Acetic Acid-Induced Gastric Ulcer in Rats The consecutive administration of compound IIId, orally twice a day for 14 d, produced a significant acceleration of the ulcer healing at 12 mg/kg, although not in a dose-related fashion (Fig. 3). Sucralfate, 2000 mg/kg *p.o.*, tended to promote the healing of the acetic acid-induced ulcer.

Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Gastric Secretion in Rats Gastric juice was

TABLE III. Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Shay Rat Ulceration

	Treatment	mg/kg	No. of animals[a]	Ulcer index[b]	Volume[c] (ml/100 g)	Acid output[c] (μeq/100 g)	Pepsin output[c] (mg/100 g)
I.	Intragastric route						
	Control		3/8	4.9±0.1	4.5±0.8	221± 91	5.4±1.0
	IIId	20	4/8	4.3±0.5	5.2±0.8	520±117	5.9±1.0
		50	5/8	4.1±0.6	5.8±1.3	533±169	6.1±1.3
	Atropine sulfate	20	8/8	1.0±0.0[e]	3.7±0.4	559± 91	4.6±0.5
II.	Intraduodenal route						
	Control		3/8	4.3±0.5	5.5±1.6	510±195	6.4±2.0
	IIId	20	7/8	2.3±0.6[d]	6.1±0.2	540± 30	7.9±0.4
		50	5/8	2.9±0.7	6.0±0.4	690±135	7.7±0.4
	Atropine sulfate	20	8/8	2.1±0.6[d]	5.4±0.4	555± 45	7.1±0.5

Values represent the mean±S.E. *a*) Surviving animal number *vs.* total animal number. *b*) The mean values from the total animal number. *c*) The mean values from the surviving animal number. *d*) $p<0.05$ *vs.* the control. *e*) $p<0.01$ *vs.* the control.

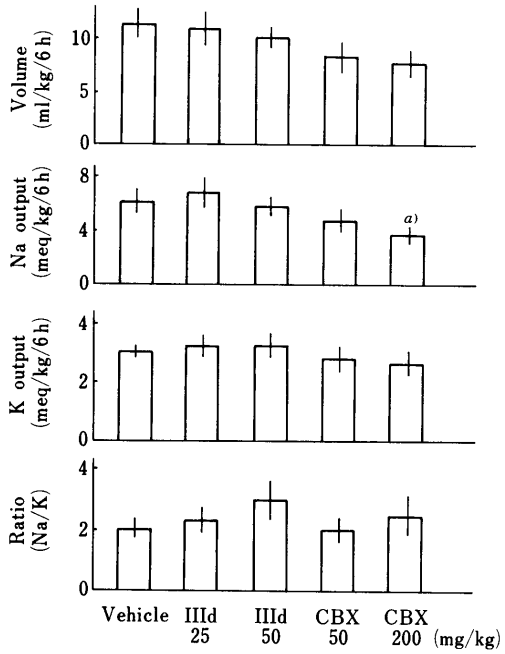

Fig. 4. Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Urine Excretion in Rats

Values represent the mean±S.E. ($n=11$—12). *a*) $p<0.05$ compared with the vehicle control. CBX, carbenoxolone sodium. Drugs were administered orally. Urine was collected for 6 h.

collected for 4 h in pylorus-ligated rats. Intragastric administration of compound IIId had no significant effect on gastric volume, acid output or pepsin output (Table IV). In contrast, intraduodenal administration of compound IIId produced a significant inhibition of all secretory parameters such as gastric volume, acid output and pepsin output.

Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Urine Excretion in Rats The oral administration of compound IIId at doses of 25 and 50 mg/kg for 3 d had no significant change in urinary parameters such as urinary volume, sodium concentration, potassium concentration and concentration ratio of sodium to potassium (Fig. 4). Carbenoxolone sodium did not greatly affect urinary parameters such as urinary volume and concentration ratio of sodium to potassium at doses of 50 and 200 mg/kg. No significant difference was noted in body weight among all animal groups treated with vehicle, IIId and carbenoxolone sodium.

TABLE IV. Effect of 18β-Olean-12-ene-3β,30-diol Di-*O*-hemiphthalate (IIId) on Gastric Secretion in Pylorus-Ligated Rats

Treatment	mg/kg	No. of animals	Volume (ml/100 g)	Acid output (μeq/100 g)	Pepsin output (mg/100 g)
I. Intragastric route					
Control		8	3.1±0.5	277±58	2.9±0.5
IIId	8	8	3.9±0.2	395±28	3.1±0.2
	16	8	3.3±0.2	324±28	2.8±0.1
II. Intraduodenal route					
Control		6	4.0±0.3	653±84	3.7±0.3
IIId	8	6	2.2±0.5[a]	359±97[a]	2.1±0.4[a]
	16	6	1.0±0.2[b]	116±33[c]	1.0±0.2[a]

Values represent the mean±S.E. *a*) $p<0.05$ *vs.* the control. *b*) $p<0.01$ *vs.* the control. *c*) $p<0.001$ *vs.* the control.

As for the acute toxicity, the LD_{50} of compound IIId in mice was 78.9 mg/kg, i.v. (the up-and-down method), and rats survived following oral administration of compound IIId at 1000 mg/kg.

Discussion

Carbenoxolone sodium, the sodium salt of 3β-*O*-hemisuccinate of glycyrrhetinic acid, has been used clinically for several decades in Europe for the treatment of peptic ulcers. It is reported that carbenoxolone is clinically as effective as cimetidine in healing ulcers.[17] The healing mechanism is still obscure. Carbenoxolone is not an antisecretory drug,[5] but stimulates mucus production and is cytoprotective to the gastric mucosa.[7] On the other hand, this drug possesses mineralocorticoid-like effects related to pseudoaldosteronism,[8] and it has been debated whether the mineralocorticoid properties are involved in the therapeutic activity.[18] At present, evidence is being accumulated to demonstrate that the antiulcer action of carbenoxolone is not related to its mineralocorticoid action.[19] In this study, we investigated the antiulcer activities of seventeen compounds which lack the 11-oxo-$\Delta^{12(13)}$ system in ring C of glycyrrhetinic acid, because this moiety was found to be closely related to the mineralocorticoid-like activity.[9]

The antiulcer activity of compounds was first screened using the mouse stress-induced gastric lesion model,[11] and then using the rat stress-induced gastric lesion model.[12] Of the compounds tested in this screening system, sodium

dihemiphthalate derivatives (IIId, IV, VIc, VIIc) of triterpenes derived from glycyrrhetinic acid produced a marked inhibition of stress-induced gastric lesions when given orally at doses of 12 and 25 mg/kg in rats; compound IIId appeared to be the most active among these four derivatives. Phthalic acid monosodium or disodium salt itself was ineffective in preventing the stress-induced gastric lesions. This fact suggests that possible metabolic generation of phthalic acid from these dihemiphthalate derivatives in the body is not directly associated with their antiulcer activity. Among hemisuccinate derivatives, the dihemisuccinate (IIIc) of deoxoglycyrrhetol was the most active in preventing stress-induced gastric lesions; the effective oral dose was 200 mg/kg. Carbenoxolone sodium, the hemisuccinate of glycyrrhetinic acid, was ineffective orally at lower doses than 500 mg/kg.

Further evaluation of the antiulcer activity of glycyrrhetinic acid derivatives was carried out for the sodium dihemiphthalate (IIId) of deoxoglycyrrhetol. Compound IIId exerted a cytoprotective effect against gastric lesion formation induced by 0.6 N HCl in rats with and without indomethacin treatment, suggesting that endogenous or mucosal prostaglandins could not be involved in the mechanism of action of this compound. Compound IIId, 50 mg/kg *p.o.*, also depressed the formation of gastric lesions induced by indomethacin in rats. This activity might be exerted through undefined mucosal defensive mechanisms including mucosal cytoprotection. In contrast, intragastric administration of compound IIId had no effect against Shay ulceration in rats; the compound was not antisecretory when administered i.g. in pylorus-ligated rats. It is generally accepted that Shay rat ulceration is effectively prevented by antisecretory drugs or antacids. Besides the prophylactic effects in acute experimental gastric lesion models, oral administration of compound IIId for 14 d accelerated the healing rate of acetic acid-induced gastric ulcer in rats, although not in a dose-related fashion.

Interestingly, compound IIId reduced gastric secretion and prevented the Shay ulceration when administered i.d., although oral administration of this compound had no effect. Intraduodenal administration of compound IIId was effective in preventing gastric lesion formation induced by stress. These findings suggest that direct contact of compound IIId with gastric mucosa is not necessary for its antiulcer action. However, the possibility remains that these antiulcer effects are exerted by a decrease in gastric secretion which results from accumulation of body fluid such as intestinal secretions and peritoneal fluid in the respective injected site of compound IIId. It was previously reported that stimulation of the active transport of sodium by carbenoxolone occurred in the human rectal mucosa.[20]

In addition to antiulcer activity, compound IIId and other dihemiphthalate derivatives (IV, VIc, VIIc) were reported to have antiinflammatory and antiallergic activities.[21] The mechanisms of these activities are suggested to involve inhibition of arachidonate metabolism, affecting prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4).[21c] Inhibition of PGE_2 and LTC_4 formation is more potent on topical application than on oral administration. On the other hand, the antiulcer effect of dihemiphthalate derivatives by the oral route would be expected at lower doses than the antiinflammatory or antiallergic effect. Oral administration of compound IIId for 3 d did not significantly affect any parameter of urine excretion, as compared with the vehicle control. Taking the above findings together, compound IIId and, probably, other tested dihemiphthalates may be suitable for development as antiinflammatory and antiallergic agents which simultaneously have antiulcer activity.

As for the antiulcer mechanism of these dihemiphthalates, it has been found that their topical application to the stomach stimulates mucus secretion in rats[22]; these results will be reported in detail elsewhere.

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References and Notes

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