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Original article

Selection of cholesterol absorption inhibitors devoid of secondary intestinal effects

F Marquet [1], F Abou El Fadil [1], B Boubia [2], C Guffroy [2], D Pansu [1, 3], M Descroix-Vagne [1*]

[1] *Inserm U 45, Hôpital É-Herriot, 69437 Lyon cedex 3;*
[2] *Laboratoires Fournier, 50, rue de Dijon, BP 90, 21121 Daix;*
[3] *École pratique des hautes études, Hôpital É-Herriot, 69437 Lyon, cedex 3, France*

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Summary — The digestive tolerance of cholesterol absorption inhibitors, which requires a constant improvement, was the main purpose of this study. Given the known hypocholesterolemic and antiatherosclerotic properties of some steroid glycosides, we synthesized a series of sterol derivatives by coupling some phytosterols known to interact with sterol absorption and also to be poorly absorbed to a cationic group. The first derivative was a potent inhibitor of cholesterol absorption and a potent hypocholesterolemic agent in different animal models, but was responsible for severe gastro-intestinal side-effects. In order to control the tolerance of the newly synthesized compounds, cholesterol and taurocholate absorption were measured in the jejunum and in the ileum, respectively. The intestinal water and ionic transport and the estimation of histological changes in the intestinal mucosae were determined simultaneously. The in-situ isolated loop technique, in anaesthetized rats, allowed the simultaneous control of these three parameters which were used to select the best derivative, inhibitor of cholesterol absorption devoid of any deleterious effect, as seen via a three-dimensional representation. The results showed that it was possible to obtain a specific cholesterol absorption inhibitor without secretory and deleterious effects and suggested that the amphiphilic characteristics of the molecules were responsible for their deleterious effects on digestive tract.

cholesterol / absorption / inhibitor / intestine / taurocholate

Résumé — Sélection d'inhibiteurs de l'absorption du cholestérol dépourvus d'effets secondaires intestinaux. La tolérance digestive des inhibiteurs de l'absorption du cholesterol mérite d'être améliorée, elle a été le premier but de la présente étude. En utilisant les propriétés hypocholestérolémiantes et antiathéroscléreuses reconnues de certains stéroïdes glycosidiques, nous avons synthétisé une série de dérivés stérols en couplant à un groupement cationique des phytostérols qui agissent sur l'absorption des stérols et qui sont peu absorbés. Le premier dérivé synthétisé a

* Correspondence and reprints
Tel: (33) 04 72 34 82 41; fax: (33) 04 72 11 75 76; e-mail: descroix @cismsun.univ-lyon1.fr

été un inhibiteur puissant de l'absorption du cholestérol et un agent hypocholestérolémiant actif sur différents modèles animaux, mais a induit de sévères effets secondaires intestinaux. Pour contrôler la tolérance des produits synthétisés, leur effet sur l'absorption du cholestérol et du taurocholate a été mesuré dans le jéjunum et l'iléon, respectivement, en même temps que leur action sur les transports intestinaux d'eau et d'électrolytes et sur l'intégrité macroscopique et microscopique de la muqueuse intestinale. La technique des anses ligaturées in situ, chez le rat anesthésié, a permis la mesure simultanée de tous ces paramètres qui ont été utilisés pour sélectionner le meilleur dérivé, inhibiteur de l'absorption du cholestérol dépourvu d'effet secondaire, que la représentation en trois dimensions met en évidence. Les résultats montrent qu'il est possible d'obtenir un inhibiteur de l'absorption du cholestérol dépouvu d'effets sécrétoires et délétères sur les muqueuses intestinales. Ils suggèrent que les caractéristiques amphiphiliques des molécules seraient responsables de leurs propriétés agressives sur le tractus digestif.

cholestérol / absorption / inhibiteur / intestin / taurocholate

INTRODUCTION

A causative link between elevated plasma cholesterol and coronary heart disease has been firmly established (Lipid Research Clinics, 1984b; Pedersen, 1994). Studies in human have established a positive correlation between plasma LDL cholesterol level and the efficiency of intestinal cholesterol uptake (Kesäniemi and Miettinen, 1987; Kesäniemi et al, 1987; Gylling and Miettinen, 1995). Non-systemic agents, which interfere with the re-uptake of bile salts and cholesterol within the lumen of the intestinal tract, reduce the level of serum cholesterol in a causal relationship (Stredronsky, 1994; Homan and Krause, 1997). Bile salt sequestrants such as cholestyramine have provided impressive evidence of their efficacy in cardio-vascular disease prevention (Brensike et al, 1984; Lipid Research Clinics, 1984a). However, the high dosages, between 12 and 24 g/day, as well as their poor palatability and their gastro-intestinal side-effects limit their use.

Other agents, including polysaccharide fibers such as chitosan (Maezaki et al, 1993), psyllium (Abraham and Mehta, 1988) and pectin (Jensen et al, 1997), phytosterols (Jones et al, 1997; Lees et al, 1997), steroid betain such as stigmastanyl phosphorylcholine (Cassal et al, 1988), saponins (Molgaard et al, 1987) and sucrose polyesters (Peters et al, 1997) have also demonstrated their capacity to decrease cholesterolemia by lowering cholesterol or bile salt absorption. Because their site of action is restricted to the intestine, these agents are more likely to generate fewer side-effects compared to systemically active compounds. Unfortunately, a common characteristic of most of these non-systemic agents, however, is the large quantities that must be ingested in order to achieve a therapeutic effect. For example, active hypocholesterolemic doses in human described in the previously cited studies, are per day up to 24 g for cholestyramine, 21 g for psyllium, 15 g for pectin, 40 g for alfalfa seeds and 15–50 g for olestra. Stimastanyl-phosphorylcholine accumulated in plasma membrane as a result of its strong interaction with membrane phosphorylcholine (Habiger et al, 1992) and saponins induced gut mucosal damages (Story et al, 1984; Johnson et al, 1986). Thus, the development of more effective inhibitors of cholesterol absorption that could be given in lower doses, combining absence of gastro-intestinal side-effects and improved patient compliance, should provide an attractive alternative for regulating plasma cholesterol levels in the general population.

Taking into account the hypocholesterolemic and antiatherosclerotic properties of some steroid glycosides (Cayen, 1971; Malinow et al, 1987), we synthesized a series of sterol derivatives by coupling some phytosterols known to interact with sterol absorption (Cayen and Dvornik, 1979; Uchida et al, 1984; Heinemann et al, 1991), and to be poorly absorbed (Cayen et al, 1979; Bhattacharyya and Lopez, 1979), to a cationic group, the latter having the potential of interacting with biliary salts. One of the first representatives of this series was F1 (LF 7-0165c) (Boubia et al, 1994a). This compound was 10–20 times more potent than cholestyramine as inhibitor of cholesterol absorption and hypocholesterolemic agent in different animals (Boubia et al, 1994b). However, the development of this compound had to be stopped due to the gastro-intestinal side-effects observed in the dog. The effects included severe diarrhea, emesis and anorexia. In order to understand the mechanism of these symptoms, we used the in-situ isolated loop technique in anaesthetized rats. This model allows simultaneous analysis of the effect of the compound on water and ionic transport, the histological alterations of the intestinal mucosa as well as the cholesterol and taurocholate absorption in the jejunum and the ileum, respectively. This study demonstrated that LF 7-0165c induced water and electrolyte secretion by altering the intestinal epithelium directly, even after the indomethacin inhibition of prostaglandin secretion. The in-situ loop method was developed to establish the relationship between tolerance and activity for some derivatives, potent inhibitors of cholesterol absorption as determined by the dual isotope ratio method of Zilversmit (1974). Molecules without deleterious effect were then selected. The results suggested that the amphiphilic characteristics of the molecules were responsible for their noxious effect on the intestinal mucosa.

METHODS

Materials

Diosgenin was obtained from Marcel Quarr (France) and recrystallized in our laboratory. Tigogenin and diosgenin derivatives, as well as their analogues, were synthesized by the Fournier Drug Discovery Department. Cetyltrimethylammonium (CTAB) came from Jansen (Belgium). Cholestyramine was obtained from Rohm and Haas (France). Digitonin, cholesterol, sodium taurocholate and indomethacin were from Sigma (USA), polyethylene glycol (PEG 4000) from Prolabo (France), [3H]-PEG 4000, [^{14}C]-cholesterol and [3H]-cholesterol were from NEN (USA), tauro[carbonyl-^{14}C]-cholic acid, sodium salt was from Amersham (UK). All other reagents were of analytical grade.

Inhibition of intestinal cholesterol absorption in the rat

The intestinal absorption of cholesterol in the rat was determined by a modification of the dual isotope ratio method described by Zilversmit (1972), Zilversmit and Hughes (1974). Fed Wistar rats (Iffa-Credo, France) (220–240 g, light from 3 pm to 3 am, with a 7-day adaptation period) were simultaneously treated in the middle of the dark period, under light anaesthesia: 1) with an intravenous dose of [^{14}C]-cholesterol (13 μg, 1.8 μCi/rat in 250 μL propanediol 40%, ethanol 10%, benzoic acid 2.5%, Na benzoate 2.5%, benzilic alcohol 1.5%); 2) with an intragastric dose of [^{3}H]-cholesterol (0.1 μg, 17 μCi/rat in 1 mL of 3% arabic gum and 2% Tween 80); 3) with an intra-gastric dose of the tested compound in suspension in 3% arabic gum (5 mL/kg body weight, BW) or the excipient alone for control.

Unless specified, all compounds were administered at 50 mg/kg (BW). Animals were then fasted and re-fed 8 h later. Seventy-two hours after administration of the isotopes, the ratio of the two labels in plasma was determined as a means to calculate the absorption of the trace amount of [^{3}H]-cholesterol. Mean values from eight treated animals were compared to mean values from eight control animals to calculate the percent inhibition of intestinal cholesterol absorption.

Measurement of intestinal water and ion movements in the rat

The protocol has been described in detail elsewhere (Chikh-Issa et al, 1992). Male Sprague-Dawley rats, weighing 200 ± 25 g (from Iffa-Credo, St Germain s/Arbresle, France) were fed a standard chow (AO4,UAR, Villemoisson-sur-Orge, France). Forty hours before the experiment, food was withdrawn and animals were allowed free access to water. The animals were anaesthetized with an intraperitoneal injection of 3.6 mg sodium pentobarbital/100 g BW.

A median laparotomy was performed and two 10-cm-long intestinal loops were prepared. The jejunal loop began 3 cm distal to the ligament of Treitz, and the ileal loop ended proximal to the ileo-coecal junction.

Next, 1 mL of the test-solution was injected into each loop and an additional suture was placed on the injection site to avoid any leakage. The loops were replaced in the abdomen, which was then sutured. After 1 h, the animals were killed by intravenous injection of anaesthetic, the loops were exteriorized and their contents were collected and centrifuged. The volume of the supernatant was measured before being used for all determinations. The test solution contained:

NaCl 60 mM, KCl 5 mM, $CaCl_2$ 1.2 mM, HCO_3Na 10 mM, mannitol 40 mM (for iso-osmolarity), sodium taurocholate 50 mM, cholesterol 100 µg/mL, arabica gum 3%, polyethylene glycol (PEG 4000) 5 g/L, [^{3}H]-PEG 4000, 3.2 kBq/mL (non-absorbable marker for content recovery determination).

The test solution injected in the jejunum also contained 0.16 kBq/mL of [^{14}C]-cholesterol (for the determination of cholesterol absorption), while that for ileum administration contained 0.16 kBq/mL of tauro[carbonyl-^{14}C]-cholic acid (for the determination of taurocholate absorption). The test solution was sonicated for 1 min.

Tested coumpounds were suspended in the test solution which was sonicated again for 30 s. Unless specified, all compounds were administered at 2 mg/loop in 1 mL test solution (about 10 mg/kg BW per loop and 20 mg/kg BW per rat, for the two loops). The difference in the duration of the Zilversmit method (72 h) and the two ligated loop technique (1 h) does not allow a full equivalence in the doses used, but the doses were comparable for each protocol, in order to achieve a true comparison of the activity of the drugs. Cholesterol and taurocholate, completely sequestered from the test solution by cholestyramine, could not be determined.

Na^+ and K^+ concentration in the supernatant were determined by flame photometry, Cl^- by coulometric titration, bicarbonate by alcali–acid titration and radioactivity by liquid scintillometry.

The significance of the differences between control and treated groups were determined by variance analysis. A *t*-test was calculated using the common variance.

Histological observation, quantification of the intestinal injury

The intestinal loops were collected at the end of each experiment. A 2-cm-long segment was opened, placed on a cork board, fixed in Bouin solution and embedded in paraffin. Several 4-µm-thick longitudinal sections were disposed on one slide, deparaffined and stained with hematoxylin, eosin and saffron. Quantitative examination was performed on color photographs (Microscope Leitz Laborlux S, final magnitude: × 125), by two observers separately. The scale of the lesions was determined after the study of all individual samples obtained from the jejunal and ileal control loops, maintained in contact with the test solutions containing cholesterol and taurocholate, respectively, for 60 min. Two types of alterations were taken into account: the decrease in the height of the intestinal wall and the morphological injury of the epithelium, lamina propria and vascular system.

The decrease in the height was recorded as follows: –0.5 cm = 0.5; –1 cm = 1; –1.5 cm = 1.5; –2 cm = 2; –2.5 cm = 3; –3 cm = 4; –4.5 cm = 6. The morphological injury included: i) *the desquamation* (absence = 0; visible nuclei in the mucus and in the intestinal lumen = 1; cellular fragments in the mucus = 2; breakdown of the mucosa = 3; large breakdown of the mucosa with villus axis in contact with the lumen = 4), ii) *the mucus secretion* (none = 0; mucus layer at the top of the villi = 1; layer of 5 mm = 2; layer of 10 mm = 3; layer thicker than 10 mm = 4), iii) *the vasodilatation* (vessels in the sub-mucosa not seen = 0; vessels visible = 1; dilated vessel = 2; several dilated vessels = 4), iv) *haemorrhage* (none = 0; one area = 1; several areas = 2).

Table I. Net fluxes measured in jejunal loop after 1 h.

Jejunum	*N*	*Water (mL/h)*	*Na (μEq/h)*	*Cl (μEq/h)*	*K (μEq/h)*	*HCO_3 (μEq/h)*	*pH*	*Cholesterol (mg/h)*
Controls								
M	50	–0.25	–13.8	–13.4	–6.3	–0.5	6.71	–59.8
SEM		0.02	3.2	3.0	0.2	0.3	0.2	1.2
F1 2 mg		***	***	***	***	***	***	***
M	19	0.19	55.6	28.7	–2.2	13.7	7.06	–36.5
SEM		0.07	11.2	9.0	0.5	2.4	0.05	1.9
F2 2 mg		***	***	***	***	***	***	***
M	19	0.24	60.8	42.9	–0.6	13.7	7.35	–33.0
SEM		0.03	4.6	3.0	0.4	0.9	0.02	1.5
F3 2 mg		***	***	***	***	***	***	***
M	10	0.02	26.5	18.3	–4.4	6.2	7.03	–30.2
SEM		0.05	7.9	6.6	0.3	1.8	0.04	1.0+
F4 2 mg		***	***	***		***	***	***
M	10	0.14	46.9	29.3	–5.3	12.9	7.21	–37.0
SEM		0.04	5.8	4.4	0.3	1.2	0.02	2.7
F5 2 mg		***	***	***		***	***	***
M	10	0.17	61.4	31.1	–5.2	18.1	7.10	–24.8
SEM		0.06	8.7	6.2	0.3	1.4	0.03	3.0
F6 2 mg		***	***	***			*	***
M	10	0.03	25.0	25.1	–5.8	0.8	6.64	–28.7
SEM		0.01	2.5	1.8	0.2	0.3	0.04	0.7
F7 2 mg								
M	10	–0.26	–13.5	–13.6	–5.6	–2.8	6.69	–58.0
SEM		0.06	8.5	8.1	0.4	0.5	0.04	2.4
F8 2 mg							***	***
M	10	–0.19	–1.8	–3.7	–5.2	1.0	6.61	–39.1
SEM		0.03	4.8	4.3	0.2	0.5	0.03	2.2
F8 4 mg					*		***	***
M	10	–0.26	–12.4	–11.5	–5.0	0.9	6.54	–43.7
SEM		0.04	5.1	4.6	0.6	0.5	0.02	1.8
Tiqueside 2 mg		***	***	***	**	**		***
M	10	0.00	22.2	17.1	–4.4	3.5	6.75	–24.9
SEM		0.04	5.4	4.4	0.3	1.3	0.04	1.7
CTAB 2 mg		***	***	***	***	***	***	***
M	10	0.22	55.5	41.2	–3.2	15.5	7.26	–29.9
SEM		0.05	8.3	5.1	0.7	2.3	0.05	1.9
Diosgenin 2 mg		*	**		***			
M	10	–0.41	–40.8	–23.1	–8.3	–1.9	6.74	–64.3
SEM		0.11	15.1	10.0	0.9	0.9	0.02	6.5
Digitonin 2 mg		***	***	***	**	***	***	***
M	10	0.02	28.1	26.4	–4.8	5.1	6.88	–32.1
SEM		0.05	8.1	6.1	0.4	1.0	0.05	1.9

–, Absorption, +, secretion. The controls and the treated groups were compared with analysis of variance and the significance of the *t*-test is given as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
The initial K concentration in the test solution was 12 μEq/mL due to the addition of arabica gum.

The presence of sub-epithelial lacunae, which indicate water absorption or reabsorption after induced secretion (Madara and Pappenheimer, 1987), were not included in the evaluation of the lesion grade.

RESULTS

In the control animals, water and ion net fluxes resulted in a net absorption in both intestinal segments. Only the bicarbonate ion was absorbed in the jejunum and secreted in the ileum. Cholesterol and taurocholate were also absorbed in the jejunum and ileum, respectively (tables I and II).

F1 (fig 1), whose structure consists of a diosgenin moiety and an ammonium chloride substitute, was able to inhibit cholesterol absorption (–36% with the method of Zilversmit at a dose of 50 mg/kg, –39% with the jejunal loop technique at 2 mg/loop), confirming previous results (Boubia et al, 1994b). F1 induced a strong stimulation of jejunal (table I) and ileal (table II) secretions. The decrease in cholesterol absorption in the jejunum was accompanied by a decrease in taurocholate absorption in the ileum (30%). Furthermore, F1 produced alterations in the jejunal and ileal morphology as evaluated by the lesion score, including several parameters of intestinal histology (tables III and IV). Figure 2 shows, in the same rats, the simultaneous jejunal variations of Na movement (negative for absorption, positive for secretion), lesion grade, and cholesterol absorption in three dimensions, as compared to control rats.

A first series of substitutions were made to replace the hydrophilic ammonium group by another hydrophilic moiety (fig 1). The substitution by a Zwiterion (F2) increased the inhibition of cholesterol absorption (–59% with the method of Zilversmit, –45% with the loop technique), but also amplified the hypersecretion and the deleterious effects (fig 3). We determined that this effect was not a result of a PGE_2 release in the intestinal mucosa by treating the rats with indomethacin (50 mg/kg, per os, 1 h before the start of the experiment). Neither the hypersecretion nor the mucosal alteration were modified by such a treatment (data not shown). The substitution of ammonium chloride by an uncharged sugar residue (F3) led to a decrease in the deleterious side-effects. The increase in carbon chain length (F4) did not modify the percentage of cholesterol absorption inhibition nor the severity of the side-effects. β-Tigogenin cellobioside (Tiqueside) and the derivative amido diosgenin (F5) were the most potent inhibitors of cholesterol absorption (respectively, –64 and –62% with the technique of Zilversmit, –59 and –58% with the loop technique), but they both showed the secretory and morphological side-effects.

A study of reference products was made in order to define which sterol moiety could be responsible for the observed side-effects (tables I–III). Diosgenin (fig 4) showed no inhibitory effect on cholesterol absorption (with both methods, at the tested dose) and did not induce any side-effects on secretion and morphology. In contrast, digitonin inhibited cholesterol absorption and produced jejunal secretion and mucosal degradation. CTAB also induced such side-effects.

The substitution of the tigogenin moiety by another hydrophobic, non-steroidic structure (F6, fig 1) did not suppress the side-effects. The addition of an hydrophilic moiety at the opposite end of the ammonium moiety suppressed the inhibitory capacity on cholesterol absorption and the secretory and caustic effects (F7). The best improvement was obtained by dimerization of the cationic derivatives of diosgenin or tigogenin similar to F1, which led to molecules such as F8 (fig 1). Despite being less potent than F1 for inhibiting cholesterol absorption, as demonstrated by the method of Zilversmit (–36%, at the dose of 100 mg/kg), F8 displayed notable activity toward cholesterol absorption by the loop

Table II. Net fluxes measured in ileal loop after 1 h.

Ileum	*N*	*Water (mL/h)*	*Na (μEq/h)*	*Cl (μEq/h)*	*K (μEq/h)*	*HCO_3 (μEq/h)*	*pH*	*Taurocholate (mg/h)*
Controls								
M	47	–0.23	–19.6	–41.8	–5.0	40.7	8.16	–18.0
SEM		0.02	3.1	1.2	0.3	1.8	0.02	0.5
F1 2 mg		***	***	***	***	***	**	***
M	24	0.40	87.9	36.2	–0.5	54.2	7.96	–12.8
SEM		0.10	14.0	7.6	0.7	4.8	0.07	0.8
F2 2 mg		***	***	***	**		***	***
M	29	0.13	41.0	17.0	–3.7	33.9	7.90	–10.1
SEM		0.03	4.8	2.7	0.3	2.3	0.02	0.6
F3 2 mg		*				*		***
M	10	–0.05	1.9	–30.0	–5.4	53.0	8.12	–11.9
SEM		0.04	5.5	2.7	0.2	3.6	0.03	0.8
F4 2 mg		***	***	***			***	***
M	10	0.30	69.7	29.4	–4.6	44.6	7.55	–10.0
SEM		0.02	4.3	2.2	0.4	1..9	0.05	0.7
F5 2 mg		***	***	***	***	*	***	***
M	9	0.26	97.6	21.7	–1.0	53.5	7.72	–10.1
SEM		0.06	9.1	6.6	0.3	2.8	0.03	0.3
F6 2 mg		***	***	***			***	***
M	10	0.22	69.4	34.1	–5.1	33.7	7.49	–12.7
SEM		0.05	8.7	4.7	0.4	2.5	0.02	0.9
F7 2 mg		**		***			***	**
M	10	0.05	–10.0	–4.7	–5.1	45.6	7.86	–14.1
SEM		0.08	8.6	6.4	0.4	9.0	0.04	0.8
F8 2 mg					*			
M	10	–0.16	–4.6	–36.6	–3.1	49.2	8.05	–18.5
SEM		0.08	9.9	3.9	0.6	5.2	0.06	1.1
F8 4 mg							*	
M	10	–0.25	–16.8	–45.3	–3.7	43.4	7.96	–20.2
SEM		0.04	5.2	3.1	0.3	2.7	0.05	0.7
Tiqueside 2 mg		***	***	***		***	***	***
M	10	0.02	26.4	–15.5	–4.4	54.8	7.82	–11.2
SEM		0.05	6.8	4.2	0.3	3.2	0.04	1.4
CTAB 2 mg		***	***	***			***	*
M	10	0.35	78.8	5.0	–4.8	41.4	7.54	–15.6
SEM		0.06	8.4	8.4	0.4	2.4	0.05	0.9
Diosgenin 2 mg				**	**			
M	10	–0.30	–30.4	–51.5	–6.5	39.4	8.20	–18.7
SEM		0.05	6.2	2.3	0.5	4.0	0.05	1.0
Digitonin 2 mg		***	***	***		*	***	
M	10	0.18	52.2	20.7	–5.4	48.4	7.80	–17.1
SEM		0.03	3.7	2.9	0.2	1.8	0.02	0.4

–, Absorption, +, secretion. The controls and the treated groups were compared with analysis of variance and the significance of the *t*-test is given as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
The initial K concentration in the test solution was 12 μE_2/mL due to the addition of arabica gum.

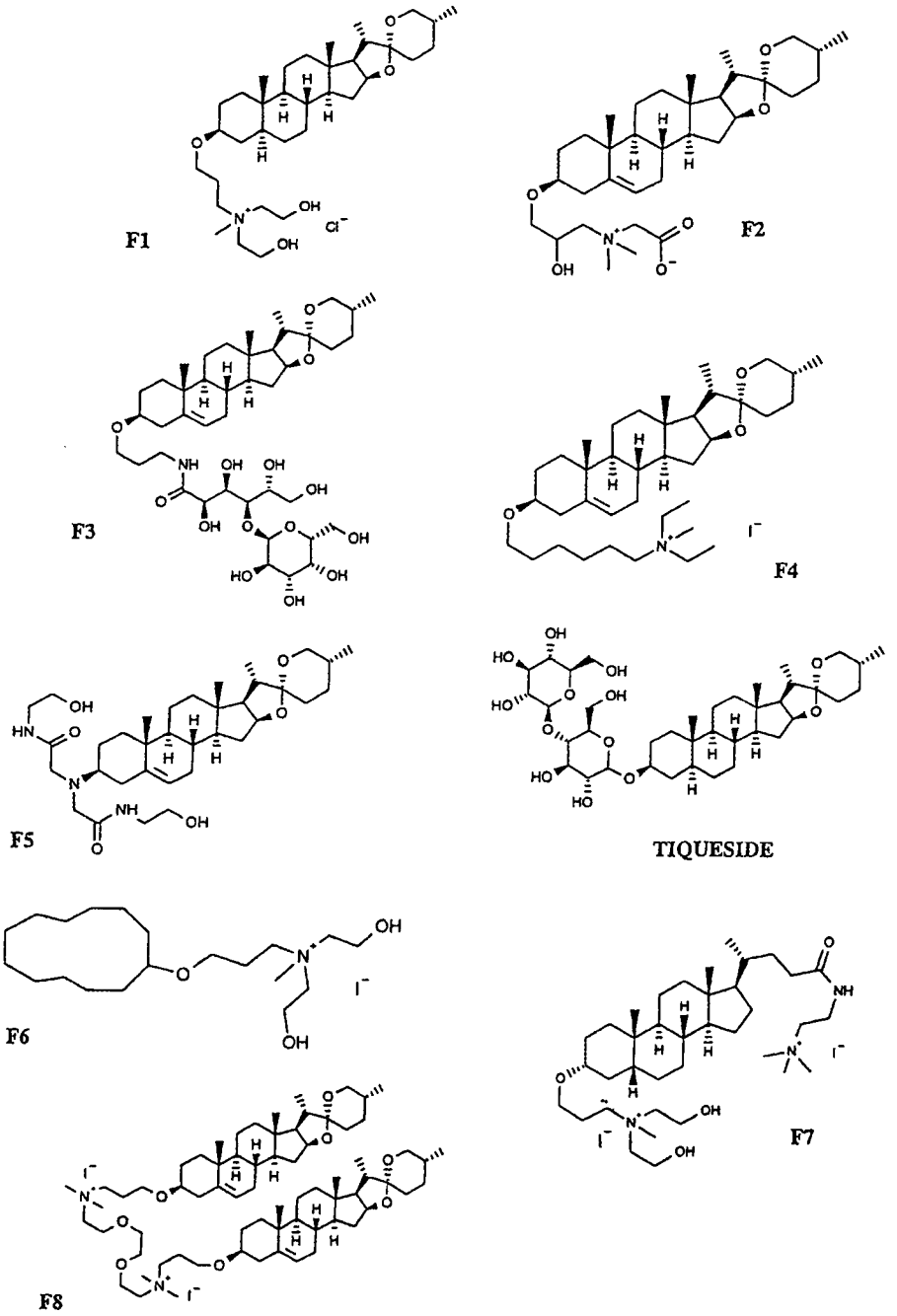

Fig 1. Chemical structures of the synthesized derivatives used in the present study.

Table III. The jejunal lesion grade (last column) was equal to the sum of the decrease in the height of the mucosa and the morphological injury.The height was determined on color photographs (Microscope Leitz Laborlux S, final magnitude: × 125).

Jejunum Dose/mL	*N*		*Height*	*Desquamation*	*Mucus*	*Vaso-dilatation*	*Haemorrhage*	*Lesion grade*
CONTROLS	20	M	9.4	0.30	0.80	0.45	0.50	
		SEM	0.2	0.15	0.09	0.14	0.14	
		SCORE	0		2.05			2.05
F1	10	M	7.67	1.30	1.00	0.50	0.70	
2 mg		SEM	0.24	0.30	0.21	0.22	0.21	
		SCORE	2		3.50			5.50
F6	10	M	6.92	1.60	2.50	0.50	0.50	
2 mg		SEM	0.34	0.27	0.22	0.17	0.17	
		SCORE	4		5.10			9.10
CTAB	10	M	5.52	2.80	2.40	0.60	1.00	
2 mg		SEM	0.27	0.29	0.34	0.22	0.47	
		SCORE	6		6.80			12.80
Cholestyramine	10	M	7.97	0.60	0.10	0.50	0.70	
20 mg		SEM	0.35	0.16	0.10	0.22	0.26	
		SCORE	1.5		1.90			3.40
CONTROLS	25	M	10.03	0.32	1.28	0.28	0.17	
		SEM	0.26	0.10	0.17	0.12	0.10	
		SCORE	0		2.05			2.05
F2	10	M	6.36	2.43	1.43	0.57	0.00	
2 mg		SEM	0.21	0.20	0.37	0.37	0.00	
		SCORE	4		4.48			8.43
Tiqueside	10	M	8.84	0.90	1.40	0.40	0.30	
2 mg		SEM	0.44	0.43	0.34	0.27	0.21	
		SCORE	1		3.00			4.00
Diosgenin	10	M	9.66	0.22	0.11	0.56	0.22	
2 mg		SEM	0.44	0.15	0.11	0.38	0.15	
		SCORE	0		1.11			1.11
Digitonin	10	M	7.88	1.88	1.38	1.75	0.00	
2 mg		SEM	0.32	0.52	0.32	0.53	0.00	
		SCORE	2		5.00			7.00
CONTROLS	10	M	8.21	0.60	0.70	0.40	0.10	
		SEM	0.29	0.27	0.15	0.16	0.10	
		SCORE	0		1.80			1.80
F8	10	M	8.03	0.40	0.70	0.70	0.00	
2 mg		SEM	0.29	0.16	0.21	0.21	0.00	
		SCORE	0		1.80			1.80
F8	10	M	8.90	0.50	0.50	0.20	0.30	
4 mg		SEM	0.25	0.17	0.17	0.13	0.15	
		SCORE	0		1.80			1.80

The decrease in the height was numbered: –0.5 cm = 0.5; – 1 cm = 1; –1.5 cm = 1.5; –2 cm = 2; –2.5 cm = 3; –3 cm = 4; –4.5 cm = 6. The morphological injury included: i) *the desquamation* (absence = 0; visible nuclei in the mucus and in the intestinal lumen = 1; cellular fragments in the mucus = 2; breakdown of the mucosa = 3; large breakdown of the mucosa with villus axis in contact with the lumen = 4), ii) *the mucus secretion* (none = 0; mucus layer at the top of the villi = 1; layer of 5 mm = 2; layer of 10 mm = 3; layer thicker than10 mm = 4), iii) *the vasodilatation* (vessels in the sub-mucosa not seen= 0; vessels visible = 1; dilated vessel = 2; several dilated vessels = 4), iv) *haemorrhage* (none = 0; one area = 1; several areas

Table IV. The ileal lesion grade (last column) was equal to the sum of the decrease of the height of the mucosa and the morphological injury.

Ileum Dose/mL	*N*		*Height*	*Desquamation*	*Mucus*	*Vaso-dilatation*	*Haemorrhage*	*Lesion grade*
Controls	26	M	8.20	0.38	0.93	0.54	0.04	
		SEM	0.22	0.10	0.13	0.16	0.04	
		SCORE	0		1.51			1.51
F2	10	M	6.85	2.80	2.60	0.80	0.20	
2 mg		SEM	0.30	0.25	0.16	0.33	0.20	
		SCORE	2		6.40			8.40
F3	10	M	8.06	0.67	1.33	0.00	0.10	
2 mg		SEM	0.45	0.29	0.33	0.00	0.10	
		SCORE	1		2.10			3.10
Tiqueside	10	M	7.67	1.90	1.40	0.40	0.00	
2 mg		SEM	0.44	0.38	0.31	0.27	0.00	
		SCORE	1.5		3.70			5.20
Diosgenin	10	M	9.32	0.33	0.44	0.56	0.22	
2 mg		SEM	0.23	0.17	0.24	0.38	0.15	
		SCORE	0		1.56			1.56
Digitonin	10	M	7.62	2.56	2.56	0.00	0.11	
2 mg		SEM	0.20	0.18	0.41	0.00	0.11	
		SCORE	1.5		5.22			6.72

Same legend as in table III.

technique (−35 and −39% at 2 and 4 mg/loop, respectively) (fig 2). Even when the dose was increased to 4 mg/loop, it did not induce any jejunal Na secretion (table I) and it was the only derivative to give a lesion grade identical to that of control rats (table III), as seen in figure 5 where representative samples of the jejunal tissue after 60 min contact with test solution alone (control, A), Tiqueside (B), F1 (C) and F8 (D) are shown. F8 did not induce any ileal secretion and ileal taurocholate absorption was not inhibited. Significant decreases in levels of ileal secretion and taurocholate absorption were even observed with the higher dose (table II). Figure 6 shows that F8 was the only derivative to inhibit jejunal cholesterol absorption without decreasing ileal taurocholate absorption. A linear relationship between ileal secretion of water and the decrease of taurocholate absorption for all compounds ($r = 0.661$) seemed to indicate an indirect inhibition of taurocholate, secondary to the incidence of mucosal lesions.

DISCUSSION

We synthesized a large series of quaternary ammonium diosgenin and tigogenin derivatives that were all potent inhibitors of cholesterol absorption in the rat as demonstrated by the Zilversmit method (Boubia et al, 1994b). The in-situ isolated intestinal loop technique in anaesthetized rats demonstrated that these cationic sterol derivatives, such as F1 and F4, when present in the jejunum or the ileum for 1 h, were able to induce water and electrolyte secretion with a desquamation of the cells and an erosion of the villi. These effects were observed at concentrations that approximated the intra-intestinal concentrations expected after the per os administration of a dose of an unabsorbed compound which

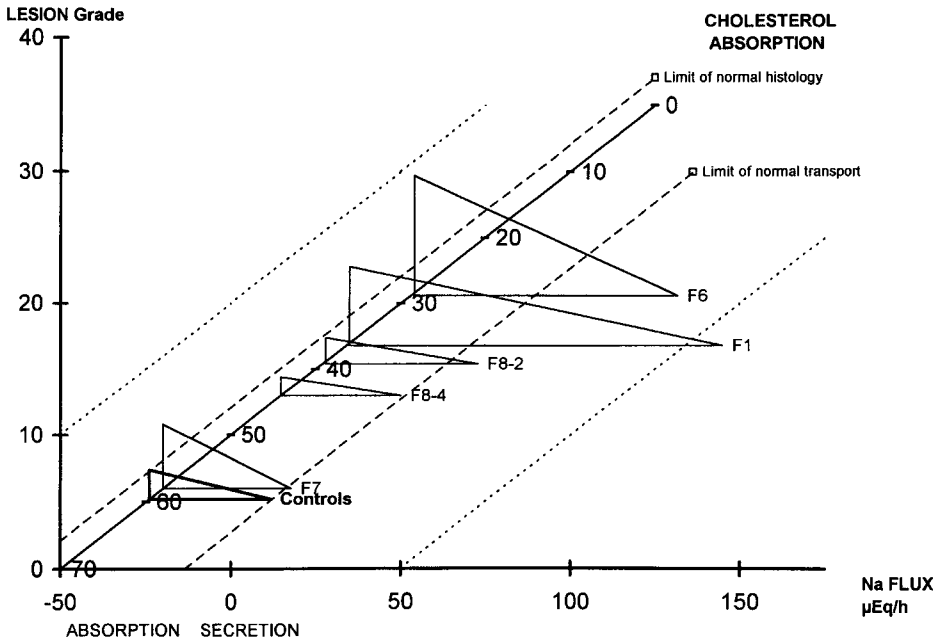

Fig 2. Three-dimensional representation of the three parameters for F1, F6 to F8 derivatives and controls. F8 was tested at two doses: 2 and 4 mg/mL and loop. Compounds were administered in suspension at a dose of 2 mg/mL of test solution. 1, Oblique: cholesterol absorption (in positive absolute value); 2, abscissa: Na jejunal absorption (negative values) or secretion (positive values), obtained after 1 h incubation and expressed as µEq; the right dashed line represents the Na absorption for the controls; 3, ordinate: lesion grade of jejunal mucosa after 1 h of incubation; the left dashed line represents the lesion grade for the controls.

inhibits cholesterol absorption by 50% in the rat. While no gastro-intestinal side-effects were clinically observed in rodents, the secretory and lesional properties of the compounds could be responsible for the gastro-intestinal secondary effects observed in the dog, a species characterized by a slow intestinal transit time and therefore very sensitive to side-effects such as diarrhea and emesis.

These effects were local, as demonstrated by an experiment with two isolated loops in the same animal, the tested compound being present in only one of the loops. Only the loop in contact with the compound displayed secretory and lesional effects. Moreover, the observed side-effects were not mediated by induction of prostaglandin secretion, as demonstrated by the lack of protection following an indomethacin pretreatment (data not shown).

Any substitution of the 3-hydroxy group of diosgenin or tigogenin by a hydrophilic group (F1–5 and Tiqueside) gave molecules, which increased water and ionic secretion with an alteration of jejunal and ileal morphology, while diosgenin by itself was inactive towards all determined parameters. The sugar derivatives F3 and Tiqueside were less deleterious and it is noteworthy that Tiqueside, which was the most potent cholesterol absorption inhibitor, was described as being well tolerated in rodents,

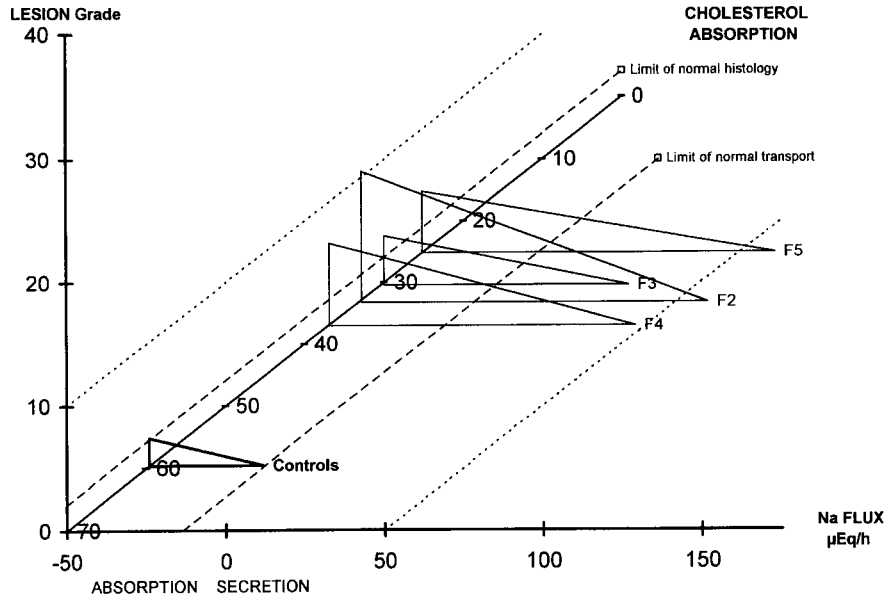

Fig 3. Three-dimensional representation of cholesterol absorption, Na movements and lesion grade in rat jejunal loop after 1 h incubation, for F2 to F5, and controls. Same legend as in figure 2.

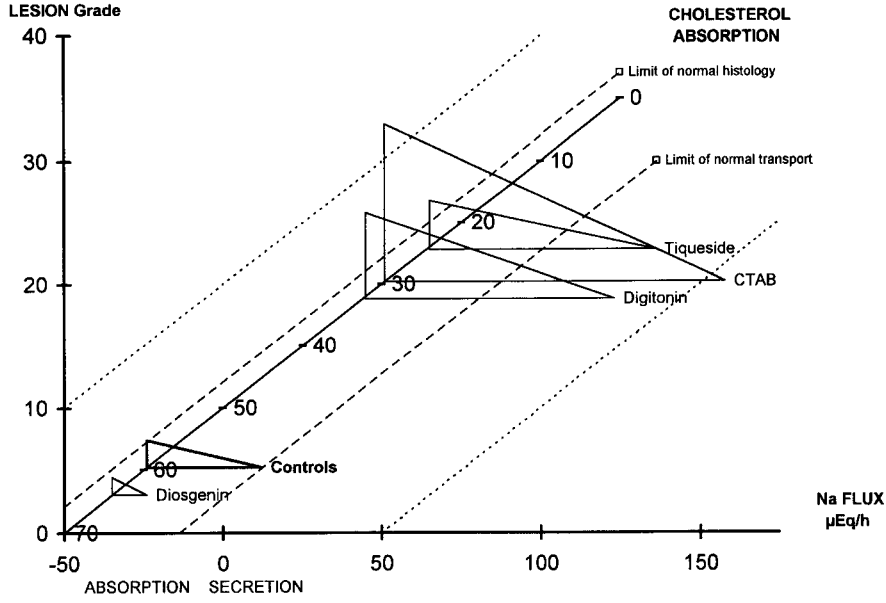

Fig 4. Three-dimensional representation of the three parameters for reference derivatives and controls. Same legend as in figure 2.

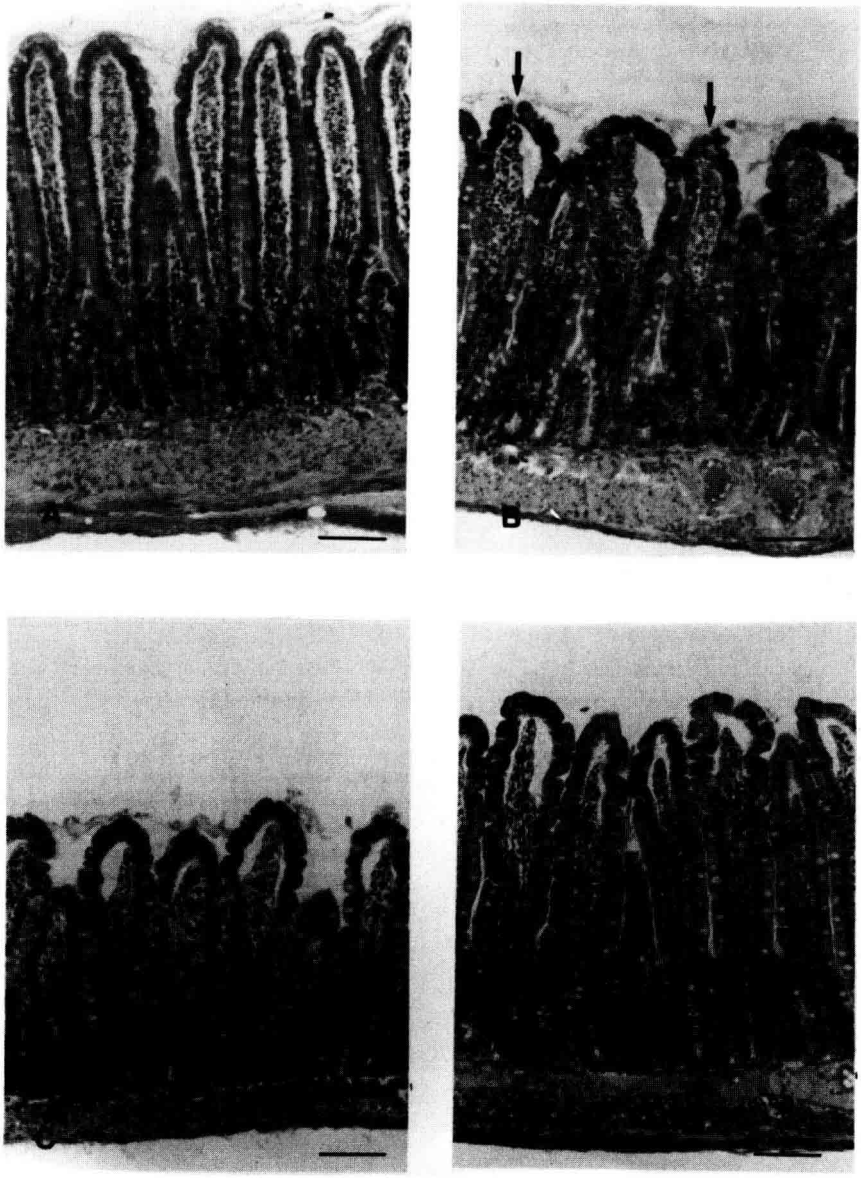

Fig 5. Histological aspect of the jejunal tissue after 60 min contact with the test solution containing hypocholesterolemic agents. Bouin fixation, hematoxylin, eosin, saffron staining. Bar = 100 µm. **A:** Control loop; **B:** Tiqueside; **C:** F1; **D:** F8. Compared to the control (A, grade 2), Tiqueside (B, grade 4) induced a decrease in the height with desquamation (arrow) and mucus secretion. F1 (C, grade 6) induced a decrease in the villus height with desquamation and vasodilatation (arrow). F8 (D, grade 1) did not induced any change in the histological structure of the intestinal wall. The sub-epithelial lacunae, present during absorption (A and D) or reabsorption of the induced secretion (B and C) were not taken into account for the estimation of the injury.

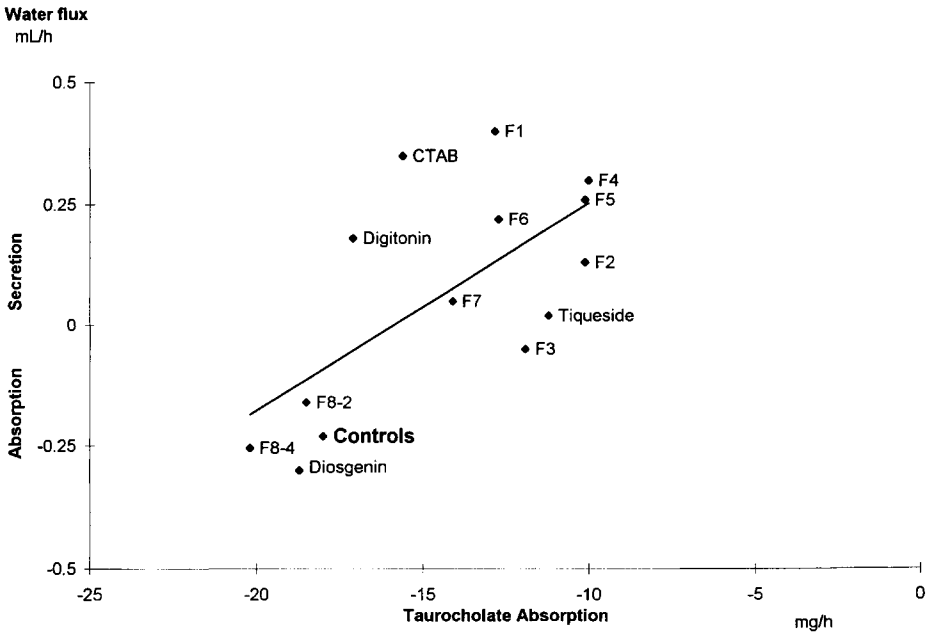

Fig 6. Correlation between taurocholate absorption and water secretion in ileum, after 1 h of incubation for all derivatives and control (negative values are absorption, positive ones secretion).

dogs and monkeys (Harwood et al, 1993). As the deleterious effects seemed to be poorly specific on the hydrophilic part of the molecules and were not observed with diosgenin alone, we hypothesized that the lysis of the mucosal cells was the consequence of the surfactant properties of the molecules, and was due to the addition of a very hydrophilic group to a very hydrophobic one (Nagawa and Regen, 1991). Similar effects were described with unconjugated bile acids (Low-Beer et al, 1970; Teem and Phillips, 1972). In order to verify this hypothesis, we tested two amphiphilic structures, F6 and CTAB. We also tested digitonin, known to increase the permeability of intestinal mucosal cells, via the formation of saponin-membrane cholesterol complexes (Milgate and Roberts, 1995). F6 is a F1 derivative in which the tigogenin moiety was substituted by cyclododecane, a nonsteroidic hydrophobic moiety. CTAB is a standard detergent composed of a hydrophilic alkyl chain with a quaternary ammonium. The three components displayed the same deleterious effects as the previous diosgenin and tigogenin derivatives in the in-situ isolated loop, and inhibited cholesterol and taurocholate absorption. Nevertheless F6 and CTAB were unable to inhibit cholesterol absorption in the intact rat as estimated by the Zilversmit method. In contrast, digitonin was very potent in this test (–64% at 5 mg/kg), as is expected because it is capable of precipitating cholesterol (Akiyama et al, 1980). These data led to the conclusion that, in the in-situ isolated loop model, the amphiphilic properties of the molecules, independently of the nature of the hydrophobic moiety, were responsible for the mucosal cell damages and the inhibition of cholesterol and

taurocholate absorption. Our data show a relationship between ileal secretion and the decrease in taurocholate absorption. In contrast, in the intact rat, the presence of a sterol moiety was a prerequisite for cholesterol absorption inhibition. Our aim was therefore to modify the amphiphilic properties of the active diosgenin or tigogenin. Two new series of molecules were synthesized. In the first one, represented by F7, two hydrophilic parts were added at the opposite end of a sterol nucleus. These molecules, due to the loss of their amphiphilic properties, were effectively devoid of secretory and lesional effect in the in-situ loop model, but they were also unable to inhibit intestinal cholesterol absorption in both models. The second series was obtained by linking two diosgenin or tigogenin molecules with a spacer including at least one quaternary ammonium. F8, a representative of this series, confirmed the hypothesis. It inhibited cholesterol absorption in both models, without impairing taurocholate absorption in the ileum and without damaging the mucosal cells. The deleterious effects were not induced by increasing two-fold the F8 dose, to take into account the higher molecular weight. F8 is defined as a specific cholesterol absorption inhibitor, devoid of gastro-intestinal side-effects. The precise mechanism of its action is still unknown. We have previously reported (Guffroy et al, 1995) that this molecule, LF 13-0491c, was also a potent and unabsorbed hypocholesterolemic agent in different animal models such as the hamster and dog. In human, a similar hypocholesterolemic effect to 12 g/day of cholestyramine is achieved by 2–4 g/day of LF 13-0491c. In the dog, it is devoid of the gastro-intestinal secondary effects, which were clearly observed with molecules such as F1, F2, F3 and F5 when tested in the same model. The absence of lesional effects could be explained by the absence of surfactant properties, while these properties could be obtained by a possible hair-spin configuration (Menger and Littau, 1993), or to the impossibility for the molecule owing to its spacial configuration to incorporate into or eventually disrupt the cell membrane as demonstrated for saponins (Milgate and Roberts, 1995), sapogenins (Segal et al, 1970) and sterol-phosphorylcholine derivatives (Habiger et al, 1992).

The precise mechanism of cholesterol absorption is still poorly understood. The essential steps for absorption include: micellar solubilization, uptake by the brush border membrane, intracellular esterification and incorporation into chylomicrons. With respect to luminal events, inhibition of absorption by non-systemic agents could result from: 1) the direct precipitation of cholesterol; 2) displacement of cholesterol from micelles; 3) complexation with bile acids leading to a modification of the micellar structures required for efficient cholesterol absorption; 4) viscous inhibition of all transport phenomena; 5) exfoliation of intestinal mucosa with concomitant loss of endogenous cholesterol and decrease of the exchange surface between intestinal mucosa and the luminal content; 6) enzymatic inhibition of cholesterol esterases in the intestinal lumen or at the level of mucosal cell membrane. The poor structure–activity relationships observed with our dimeric phytosterol series are in favor of a non-catalytic inhibitory process. As a consequence, an hypocholestrolemic effect due to a specific pCEH inhibition can be excluded. Exfoliation of intestinal mucosal cells can also be excluded for LF 13-0491c, taking into account the present data. Finally, the only non-systemic mechanisms that could explain the inhibition of cholesterol absorption by LF 13-0491c are physicochemical interactions with cholesterol and/or bile salts or competition with these molecules into the micellar structures or modification of the intestinal content viscosity.

We have also previously reported (Guffroy et al, 1995) that LF 13-0491c was able to induce a reversible bile salt precipitation

in vitro and that bile salt intestinal reabsorption inhibition could be observed in vivo but at a higher dose than that required to demonstrate intestinal cholesterol reabsorption and hypocholesterolemic effects. Moreover, a total loss of bile salt precipitation in vitro and cholesterol absorption inhibition in vivo is observed for any substitution of the cationic part of LF 13-0491c by a non-cationic one. A reversible interaction between LF 13-0491c and bile salts (unpublished data), with, as a consequence, a modification of the micellar cholesterol solubilization in the intestinal lumen, could induce a cholesterol absorption inhibition without altering bile salt reabsorption. These unpublished data were in agreement with the lack of inhibition of taurocholate absorption observed with LF 13-0491c in the present report, but complementary studies are necessary to confirm this hypothesis.

In conclusion, these results suggested that the amphiphilic characteristics of the described phytosterol derivatives were responsible for their deleterious effects. Systematic modifications of these phytosterol derivatives and the use of the in-situ isolated loop technique in anaesthetized rats allowed the selection of new molecules such as LF 13-0491c, which were devoid of any deleterious effects and were effective non-systemic hypocholesterolemic agents.

Whether LF 13-0491c will lower plasma cholesterol levels in humans with the same efficacy that it showed in animals and whether it will also inhibit progression and induce regression of atherosclerosis in animals and humans, remain to be determined.

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