The ansamycins: hypolipidemic agents stimulating cholesterol removal by nonclassical mechanisms

Joyce C. Gibson,[1] Warren H. Lee, and Joseph R. Piccolo

Research Department, Pharmaceuticals Division, CIBA-GEIGY Corp., Summit, NJ 07901

Abstract The ansamycins CGP 43371 and CGS 24565 are derivatives of the antibiotic rifamycin that reduce plasma cholesterol levels in both primate and nonprimate species. In vivo, a striking accumulation of macrophage cholesteryl ester was seen in ansamycin-treated rats and hamsters, but carbon clearance studies and reticuloendothelial system blockade by gadolinium chloride indicated that phagocytosis was not involved. Simple addition of an ansamycin to macrophages or monocytes in vitro failed to stimulate radiolabeled lipoprotein cholesteryl ester association or mass accumulation. In contrast to mononuclear cells, however, the ansamycins did enhance radiolabeled lipoprotein cholesteryl ester association by liver cells in vitro. Primary hepatocyte cultures prepared from rats treated with radiolabeled CGP 43371 secreted CGP 43371 over an 18-h period in a fraction floating at d < 1.02 g/ml after density gradient ultracentrifugation that was relatively enriched in apoA-I. The medium containing this secreted [^{14}C]GP 43371-labeled lipoprotein was capable of enhancing the cholesteryl ester content of macrophages in vitro, suggesting that ansamycin-induced liver modification of lipoproteins might be involved. These drugs may serve as valuable tools for studying mechanisms of lipoprotein uptake.—**Gibson, J. C., W. H. Lee, and J. R. Piccolo.** The ansamycins: hypolipidemic agents stimulating cholesterol removal by nonclassical mechanisms. *J. Lipid Res.* 1994. **35:** 1524–1534.

Supplementary key words lipoproteins • mononuclear cells • hepatocytes

The development of pharmacological agents capable of reducing plasma cholesterol levels has been an important objective ever since the recognition that such a reduction can have beneficial effects on the incidence of coronary artery disease (1–3). The mechanism of action of currently available agents is diverse and, in many cases, not yet defined. Some reduce hepatic lipoprotein production, while others stimulate clearance by up-regulation of low density lipoprotein (LDL) (apoB-E) receptors. Others, however, may act through less well-defined receptors and/or lipid binding sites. Specifically, the scavenger receptor has been well characterized, though its endogenous role and regulation are not understood (4). In addition, a chylomicron remnant receptor has been hypothesized (5), though not characterized, as have several other putative lipoprotein receptors (6–8), all of which may contribute to non-LDL receptor-mediated clearance. The sum of these non-LDL receptor-mediated pathways may possibly account for one-third of the total lipoprotein clearance in humans (9).

The ansamycins are a class of compounds, derived from the antibiotic rifamycin, that have been shown to be effective in reducing plasma cholesterol levels in a variety of nonprimate (10) and primate (11) species. While the precise mechanism of action is not known, the ansamycins appear to act by facilitating lipoprotein cholesterol clearance as shown by enhanced catabolism of ^{125}I-labeled LDL in rats (12) and monkeys (11). As the ansamycins are lipophilic compounds that associate readily and completely with lipoproteins (J. C. Gibson, W. H. Lee, and Z. F. Stephan, unpublished results), it is our working hypothesis that the increased clearance is related to the capacity of the drugs to bind to lipoproteins and facilitate cell association. The present study has used two ansamycins, CGP 43371 and CGS 24565 (**Fig. 1**), to study how the interactions of these drugs with lipoproteins could enhance lipoprotein uptake by two types of cells, mononuclear cells and hepatocytes, both known to have the capacity to participate in LDL receptor-mediated and non-LDL receptor-mediated lipoprotein clearance. The data support a mechanism that uses a nonclassical clearance pathway and raise the possibility of using these drugs as tools to explore as yet undefined mechanisms by which lipoproteins interact with cells.

MATERIALS AND METHODS

Animals

For in vivo protocols, both rats and hamsters were used. Male Sprague-Dawley rats [Tac:N(SD)FBR] (150–200 g) were fed either a chow diet or a semi-purified diet containing 1.25% cholesterol and 0.5% cholic acid (13) prepared

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

[1]To whom correspondence should be addressed.

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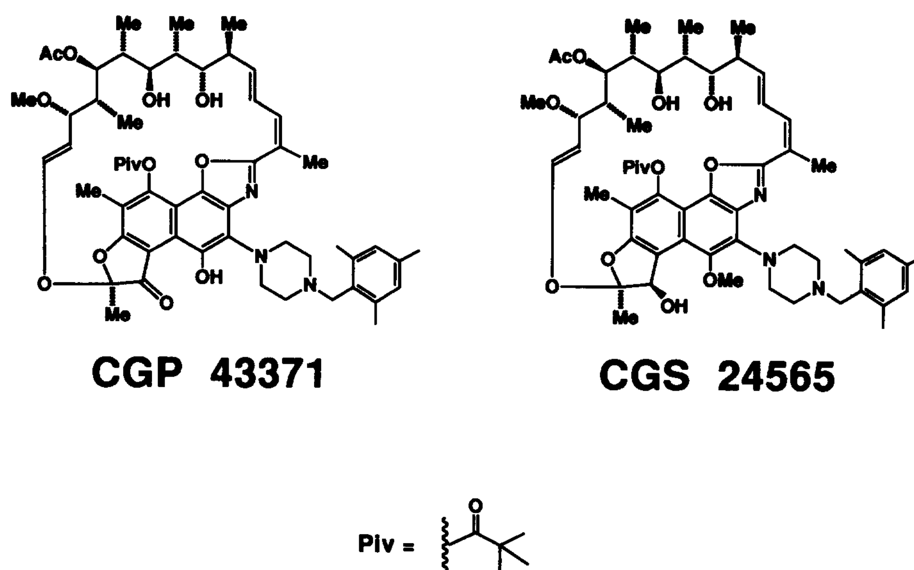

Fig. 1. Chemical structures of the ansamycin derivatives CGP 43371 and CGS 24565.

by Dyets (Bethlehem, PA). Male, hybrid Golden Syrian hamsters [Lak:LVG(SYR)] (100–150 g) were obtained from Bio Breeders, Inc. (Watertown, MA) and fed a semipurified hypercholesterolemic diet containing 15% coconut oil and 0.05% cholesterol, also prepared by Dyets. CGP 43371 and CGS 24565 were administered either by oral or intravenous routes. For oral dosing, ansamycins were suspended in 3% cornstarch in water with 0.33% Tween 80 and 5% polyethylene glycol (PEG) 400. Doses were administered by intragastric bolus once a day at doses and times indicated. The intravenous dosing vehicle for the ansamycins was PEG–dimethylacetamide (DMA) 75:25 and doses were administered via the tail vein of the rat.

Cell preparations

Rat peritoneal macrophages. All studies but one were performed with resident peritoneal macrophages. In this exception, peritoneal macrophages were elicited with sodium caseinate (8% via intraperitoneal injection) and harvested at indicated time points. For all macrophage studies, peritoneal macrophages were harvested by the following procedure. Fasted rats or hamsters were anesthetized with carbon dioxide and 20 ml phosphate buffer was infused into the peritoneal cavity. The abdomen was gently massaged and 15–17 ml of fluid was aspirated from the cavity by syringe. Cells were pelleted by centrifugation at 800 *g* for 10 min at 4°C. In the studies using elicited macrophages, the monocyte/macrophage population was isolated from the total peritoneal cell population by subjecting the total cell preparation to density gradient centrifugation on Ficoll-Hypaque at a density of 1.077 g/ml. For all other protocols, peritoneal cells were resuspended in RPMI 1640 and plated in plastic dishes (approximately 1×10^7 cells/ 6-well dish). After a 2-h incubation in a 5% CO_2/95% air incubator at 37°C, nonadherent cells were removed by washing with three sequential additions of phosphate-buffered saline, leaving the adherent macrophage population intact. For protocols in which the cholesteryl ester content of peritoneal macrophages from control and ansamycin-treated animals was determined directly, adherent cells were immediately extracted with hexane–isopropanol 2:1, resuspended in isopropanol, and analyzed for cholesteryl ester by a fluorometric procedure (14). For all other protocols, fresh RPMI 1640 containing antibiotics and 2% BSA was added to the adherent cells. For study, rat very low density lipoprotein (VLDL), LDL, or high density lipoprotein (HDL) was preincubated in the presence or absence of CGP 43371 at 37°C for 30 min prior to addition to the cells. Final concentrations of lipoprotein protein and of CGP 43371 were as indicated in the text or figures. Incubation of cells with test medium was continued for 18 h at 37°C prior to removal of medium, washing and extraction of cells for analysis of radioactivity and/or cholesteryl ester mass (14). Other studies examined the effect of medium harvested from hepatocyte incubations on peritoneal cell cholesterol content. In these experiments, Williams E medium was removed from hepatocyte cultures after 18 h of incubation and was transferred to adherent macrophages as prepared above.

Human monocytes. Human monocytes were isolated from whole blood by counterflow elutriation (15). Mononuclear cells were obtained by Ficoll-Hypaque centrifugation of whole blood from normal donors, and monocytes were isolated by elutriation using a Beckman JE-6B elutriator rotor (Beckman Instruments, Fullerton, CA). The resulting cell population was greater than 90% monocytes. Monocytes were then suspended in RPMI and plated in

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plastic dishes at a cell density of approximately 1×10^7 cells/6-well dish. Studies were performed exactly as described for resident peritoneal macrophages.

Rat hepatocyte cultures. Rats were anesthetized and hepatocytes were prepared under sterile conditions by perfusion of collagenase solution (100 U collagenase /ml) at a rate of 20 ml/min. Liver cells were suspended in Williams E medium, filtered, and resuspended in fresh Williams E medium for evaluation of cell viability by Trypan Blue exclusion prior to plating. For culture, cells were suspended in supplemented Williams E medium containing glutamine (292 mg/l), insulin (270 units/l), hydrocortisone (5 mg/l), and fetal bovine serum (100 ml/l) and plated into culture dishes (3 ml/dish) at a density of 1×10^6 cells per plate. At least 4 h after attachment, the medium was removed and fresh culture medium was added. Twenty-four hours after plating, fresh Williams E medium containing 8% fetal bovine serum was added to cells. For study of the effect of CGP 43371 on lipoprotein uptake, rat lipoproteins were preincubated with CGP 43371 for 30 min at 37°C prior to addition to cells for an additional 18 h. After 18 h, medium was removed and the cells were washed two times prior to extraction and analysis of radioactivity and/or cholesteryl ester mass (14).

In vivo assessment of phagocytic function

Gadolinium chloride is a rare earth metal that is known to block Kupffer cell phagocytic activity (16). To study the effects of this compound on ansamycin-induced hypolipidemia, rats were injected intravenously with saline vehicle (n = 12) or with $GdCl_2$ (n = 12) (10 mg/kg), 24 h prior to injection with CGP 43371 (3 mg/kg) or PEG/DMA vehicle (n = 6 for each subgroup). Four h after ansamycin injection, rats were bled for determination of plasma lipid levels.

A direct test of monocyte/macrophage system phagocytic activity in rats treated with CGS 24565 was performed in vivo using colloidal carbon (17). Rats were treated per os for 4 days with CGS 24565 (0.3 mg/kg). On day 4 of treatment, 2 h after the last dose of CGS 24565, fasted rats were injected intravenously via tail vein with India Ink (20 mg/kg) prepared by centrifugation of a commercial preparation (17). Blood samples were taken from the orbital sinus of rats at 1, 5, 10, and 15 min after injection for determination of blood carbon concentration and calculation of carbon clearance rates. The clearance rate, k, and the $t_{1/2}$ were calculated between 1 and 15 min as follows:

$$k = (\ln C^1 - \ln C_2)/(t_2 - t_1)$$
$$t_{1/2} = \ln 2/k$$

Lipoprotein preparation

Lipoproteins for radiolabeling and for hepatocyte and monocyte/macrophage incubations were prepared from rat plasma anticoagulated with EDTA. VLDL, LDL, and HDL were prepared by ultracentrifugation (18) at densities of 1.006 g/ml (VLDL), 1.02-1.04 g/ml (LDL), and 1.08-1.19 g/ml (HDL) and used after washing at appropriate densities and after dialysis. Where indicated, lipoproteins were labeled with [4-^{14}C]cholesteryl oleate by exchange using human or cynomolgus monkey lipoprotein-deficient serum as a source of cholesteryl ester transfer protein (19). For studies with human monocytes, human plasma lipoproteins were prepared from human blood as described for rat lipoproteins, but with density intervals changed to reflect the densities of human LDL (1.02-1.06 g/ml) and HDL (1.09-1.19 g/ml). In other studies, lipoproteins secreted by hepatocytes were submitted to single-spin density ultracentrifugation (20) or preparative sequential ultracentrifugation.

Analytical

Plasma and lipoprotein fractions were analyzed enzymatically for cholesterol and triglycerides on a Beckman Biomek automated work station using reagent kits (Sigma

TABLE 1. Effect of ansamycins on peritoneal macrophage cholesteryl ester content in rats and hamsters

Animals	Plasma		Macrophage	
	Control	Treatment[a]	Control	Treatment[a]
	mg/dl		*µg/10⁻⁷ cells*	
Rats				
Chow	65 ± 6.8	29 ± 4.8[b]	1.76 ± 0.38	6.70 ± 0.82[b]
Chol-fed	277 ± 37.2	168 ± 40.7[b]	14.6 ± 3.93	30.9 ± 6.84[b]
Hamsters				
Chol-fed-1	365 ± 30.8	196 ± 19.4[b]	3.1 ± 0.30	41.2 ± 6.5[b]
Chol-fed-2	692 ± 93.9	665 ± 109.6	27.0 ± 13.0	26.5 ± 8.0

Values represent mean ± SEM of 6 animals per group.
[a]Treatment regimens represent oral dosing of CGP 43371 at 10 mg/kg (chow-fed rats) or 30 mg/kg (cholesterol-fed rat and hamster study 2 or CGS 24565 at 10 mg/kg (hamster study 1) for times indicated in text.
[b]$P < 0.001$.

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Chemical Co., St. Louis, MO). Cellular cholesteryl ester was determined according to the fluorometric method of Heider and Boyett (14). Rat apolipoproteins A-I and B were quantified by specific enzyme-linked immunoadsorbent assays as previously described (10). Protein was determined by the method of Lowry et al. (21). Statistical evaluation of group differences was by Student's *t*-test.

RESULTS

Peritoneal macrophage cholesteryl ester accumulation in vivo

Table 1 summarizes the results of two rat and two hamster studies in which the association between plasma cholesterol reduction and peritoneal macrophage cholesteryl ester accumulation was studied. In chow-fed rats, the 55% reduction in total plasma cholesterol (including both LDL and HDL fractions) resulting from CGP 43371 treatment was accompanied by a 2.8-fold increase in peritoneal macrophage cholesteryl ester content. Feeding a cholesterol/cholic acid diet to rats for 10 days resulted in both enhanced plasma and peritoneal macrophage cholesterol levels in control rats. As with chow-fed rats, treatment with CGP 43371 significantly reduced plasma cholesterol levels and doubled peritoneal cell cholesteryl ester content. In hamsters fed a semi-purified 0.05% cholesterol diet for either 15 days (study 1) or 12 weeks (study 2), plasma cholesterol levels were also elevated. As with rats treated with CGP 43371, treatment with the more potent ansamycin, CGS 24565 (10 mg/kg), per os for 5 consecutive days resulted in a significant 46% reduction in plasma cholesterol levels and a level of peritoneal macrophage cholesteryl ester content that was 13× greater than that of control hamsters. In the second study, however, in which CGP 43371 was admixed in diet to give a dose of 30 mg/kg per day and administered for 12 weeks, no plasma cholesterol reduction or enhancement of peritoneal macrophage cholesteryl ester accumulation was seen.

In order to further investigate the mechanism(s) by which macrophage cholesteryl ester accumulates, a time course study was undertaken in cholesterol/cholic acid-fed rats. In this study an influx of blood-borne monocytes and lymphocytes into the peritoneal cavity was elicited by intraperitoneal sodium caseinate injection. After sodium caseinate injection, a striking change in the profile of peritoneal cells occurred. As shown in **Fig. 2A,** 24 h after injection, elicited cells represented largely lymphocytes and polymorphonuclear cells. By 72 h, however, the peritoneal cells reflected a striking increase in blood monocytes, with a proportionate fall in the leukocyte population. No change in plasma cholesterol levels was seen. Two intravenous doses of ^{14}C-CGP 43371 (10 mg/kg) were then superimposed upon this regimen in order *1)* to

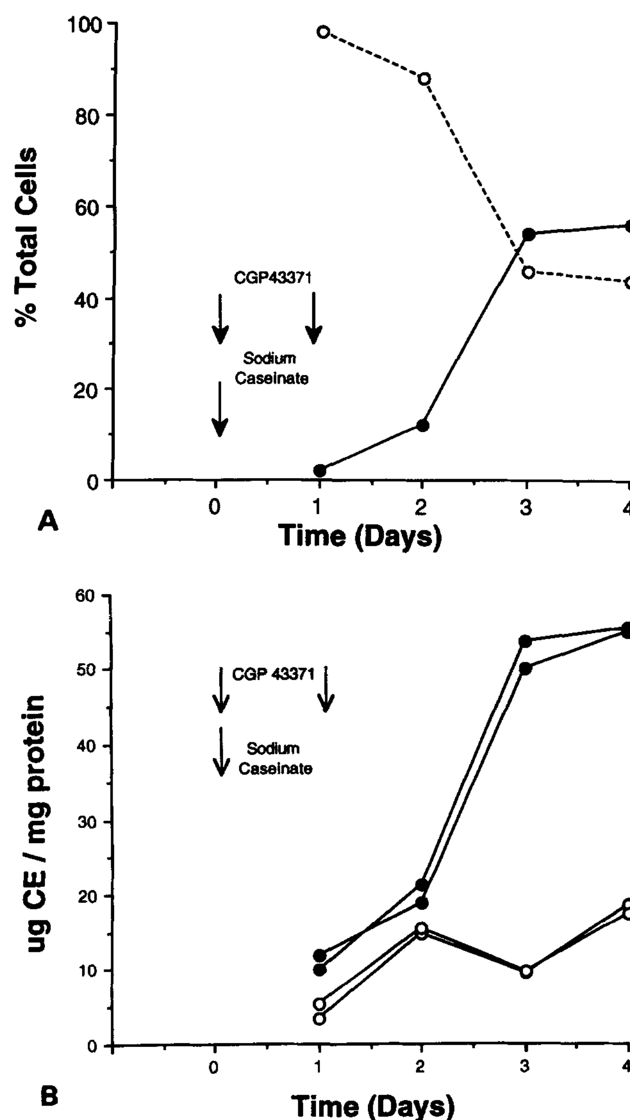

Fig. 2. A) Percent distribution of lymphocytes (○—○) and mononuclear cells (●—●) in peritoneal fluid after injection of sodium caseinate. B) Cholesteryl ester content of peritoneal macrophages harvested over time after injection of sodium caseinate and vehicle (○—○) or CGP 43371 (●—●).

follow the macrophage cholesteryl ester content over a time period in which plasma cholesterol levels were falling; and *2)* to determine whether the macrophage cholesteryl ester accumulation paralleled the appearance of radiolabeled drug. It has been shown previously (10) that intravenous administration of this dose of CGP 43371 resulted in a decrease in the cholesterol content of all lipoprotein fractions that was significant as early as 2 h after a single dose, maximal by 6 h, and sustained for at least 72 h. As shown in Fig. 2B, an increase in the peritoneal macrophage cholesteryl ester content seen in the two rats treated with CGP 43371 followed the influx of blood monocytes on day 3 (Fig. 2A). Furthermore, no radiolabeled CGP 43371 appeared in the peritoneal

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TABLE 2. Tissue distribution (%) of ^{14}C over time after injection of sodium caseinate and [^{14}C]CGS 43371

	Days of Study[a]			
	1	2	3	4
	% distribution of radiolabel per g tissue			
Tissue				
Liver	46.0	35.5	45.7	21.0
Adrenal	40.8	53.0	44.0	72.2
Kidney	2.0	2.7	2.2	1.6
Spleen	9.2	6.1	6.0	3.5
Peritoneal cells	N.D.	N.D.	N.D.	N.D.
	Plasma Cholesterol (mg/dl)			
Group				
Control	443	653	486	398
CGP 43371	235	288	287	233

[a] [^{14}C]CGP 43371 (10 mg/kg) was administered intravenously on days 0 and 1 of study to rats fed a cholic acid/cholesterol diet and injected intraperitoneally with sodium caseinate on day 0. N.D., not detectable.

cavity. **Table 2** represents the distribution of [^{14}C]CGP 43371 among four tissues from one of the test rats as a function of days after sodium caseinate treatment. Liver and adrenal glands represented the major areas of concentration of radiolabeled drug at all time points. Spleen and kidney showed low levels of drug, which could represent blood contamination of tissue. The liver clearly showed the greatest organ accumulation, but on a tissue weight basis, the adrenal was as high initially and three times greater by day 4. Radioactivity is likely to reflect CGP 43371 as it has been shown not to undergo significant metabolic transformation in vivo or in vitro (D. J. Moore and P. J. Robertson, unpublished data). This phenomenon of adrenal concentration has been noted previously in ansamycin-treated rats using whole body autoradiography (22) and may reflect the high rate of utilization of HDL cholesterol (in this case bound to CGP 43371) for adrenal steroidogenesis in the rat (23).

In vitro studies with mononuclear cells

In order to study in vitro the mechanism by which mononuclear cells accumulate cholesteryl ester, studies were performed with both rat peritoneal macrophages (**Table 3**) and with human blood monocytes (**Table 4**) under culture conditions. Table 3 summarizes studies in which the [^{14}C]-cholesteryl ester uptake by elicited mononuclear cells harvested on day 1, 2, or 4 after sodium caseinate injection of control rats fed cholesterol/cholic acid was measured after incubation with rat [^{14}C]-cholesteryl ester-labeled VLDL, LDL, or HDL in the presence or absence of CGP 43371 added in vitro. On each day, the cells represent those isolated from total peritoneal cells by Ficoll/Hypaque centrifugation. This study was designed to study effects in populations of mononuclear cells that were increasingly enriched in monocytes (Fig. 2A). The effect of the elicitation per se on mononuclear cell cholesteryl ester uptake from radiolabeled lipoproteins was examined by comparing cellular cholesterol radioactivity on days 1, 2, and 4 after sodium caseinate. As seen in Table 3, whether exposed to vehicle (control) or to CGP 43371 in vitro, there was a highly significant enhancement of cholesteryl ester uptake in cells exposed to VLDL on day 4 after elicitation relative to day 1. This was less pronounced with LDL and HDL

TABLE 3. Effect of CGP 43371 (8 μM) on in vitro uptake of radiolabeled cholesterol from VLDL, LDL, and HDL by rat peritoneal macrophages elicited by treatment with sodium caseinate on day 0

Days after Na Caseinate	VLDL		LDL		HDL	
	Control	CGP 43371	Control	CGP 43371	Control	CGP 43371
	nmol CE/mg protein					
Day 1	44.2 ± 2.26	35.1 ± 2.02	39.5 ± 4.15	33.3 ± 1.92	10.2 ± 0.30	13.2 ± 0.52[c]
Day 2	71.8 ± 3.01[a]	44.2 ± 3.88[c]	30.5 ± 1.40	21.3 ± 0.87[c]	7.3 ± 0.70	7.0 ± 0.40
Day 4	200.4 ± 11.7[b]	76.6 ± 1.51[b, d]	51.2 ± 2.79	40.1 ± 0.17[a, c]	20.8 ± 1.98[a]	18.2 ± 0.59[a]

Significantly different from day 1: [a] $P < 0.05$; [b] $P < 0.001$.
Significantly different from control on same day: [c] $P < 0.05$; [d] $P < 0.001$.

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TABLE 4. Effect of CGP 43371 on cholesteryl ester content of human blood monocytes incubated in vitro with and without human lipoproteins

	Total Cholesterol		Free Cholesterol		Cholesteryl Ester	
	Control	CGP 43371	Control	CGP 43371	Control	CGP 43371
			$\mu g/mg$			
None	15.8 ± 0.31	16.5 ± 0.16	15.2 ± 0.31	15.9 ± 0.18	0.63 ± 0.01	0.60 ± 0.01
VLDL	22.7 ± 1.38	23.8 ± 3.93	15.7 ± 1.16	18.1 ± 3.62	7.1 ± 0.40	5.8 ± 0.38
LDL	21.2 ± 1.02	18.6 ± 0.42	16.7 ± 0.81	16.8 ± 0.18	4.4 ± 0.39	1.8 ± 0.34
HDL	18.4 ± 0.30	18.6 ± 0.66	15.8 ± 0.62	16.3 ± 0.49	2.6 ± 0.32	2.5 ± 0.32

and could reflect stimulation of receptors specific for VLDL in this elicited macrophage population. Examining the effect of CGP 43371 in vitro on mononuclear cell [^{14}C]-cholesteryl ester uptake after exposure to VLDL or LDL, it is noteworthy that cholesteryl ester uptake was actually significantly less by cells exposed to CGP 43371 on days 2 and 4 after elicitation than by cells incubated in the absence of CGP 43371. Thus, the accumulation seen in vivo was not reproduced in vitro, as reflected by [^{14}C]-cholesteryl ester uptake after simple exposure of lipoproteins and cells to CGP 43371. Other in vitro studies examined the effect of CGP 43371 on the uptake of [^{14}C]-cholesteryl oleate-labeled HDL and LDL by resident peritoneal macrophages (data not shown). These studies, too, failed to reproduce the effect documented in vivo in rats and hamsters.

Table 4 summarizes the effect of CGP 43371 in vitro on the cholesteryl ester content of human blood monocytes incubated with or without human lipoproteins. Monocytes incubated with or without 5 μM CGP 43371 in the absence of lipoproteins had very low levels of cholesteryl ester and no effect of CGP 43371 was seen. When VLDL, LDL, or HDL (50 μg each) were added, however, the free cholesterol remained constant, but a significant increase in cell esterified cholesterol occurred. As shown by [^{14}C]-cholesterol uptake by rat peritoneal macrophages, however, addition of CGP 43371 in vitro did not result in significant enhancement of cellular cholesterol relative to control. Despite the apparent capacity of CGP 43371 in vivo to induce macrophage and monocyte cholesteryl ester accumulation, neither cell type could be shown to reproduce this phenomenon in vitro.

In vivo studies on phagocytic function

As one mechanism by which mononuclear cells can take up modified lipoproteins is via phagocytosis, two in vivo studies were performed to assess whether phagocytosis might be involved in the lipid-lowering induced by ansamycin treatment. In the first of these, the effect of blockade of phagocytosis by gadolinium chloride ($GdCl_2$) on the plasma cholesterol-lowering effect by CGP 43371 was examined. In the second, reticulo-endothelial system function was directly evaluated by measuring the rate of colloidal carbon clearance from blood. In preliminary experiments in control rats, $GdCl_2$ (20 mg/kg, i.v.) was shown to increase the time of elimination of colloidal carbon 2.2-fold 24 h after administration, thus confirming that blockade of phagocytic activity had occurred. The effect of $GdCl_2$ on plasma cholesterol levels was then examined in the presence or absence of intravenous CGP 43371 (10 mg/kg)(**Table 5**). $GdCl_2$ alone was associated with a slight, nonsignificant increase in plasma total and HDL cholesterol levels. When CGP 43371 was administered, however, the same degree of reduction in total and HDL cholesterol was seen independent of the pretreatment with $GdCl_2$. For direct measurement of phagocytic function, the rate of elimination from plasma of an intravenous dose of colloidal carbon was determined in rats treated p.o. for 4 days with CGS 24565 (0.3 mg/kg). As shown in **Fig. 3,** the rate of elimination in treated rats was identical to that in control rats, indicating no stimulation of phagocytic activity.

In vitro studies with isolated hepatocytes

As in vitro as well as in vivo studies (22) clearly documented a rapid association of both drug and lipoprotein cholesterol with hepatic tissue, additional in vitro studies were undertaken to examine whether ansamycins could directly stimulate the uptake of lipoprotein cholesterol by isolated hepatocytes. Rat LDL and HDL were exchange labeled with [^{14}C]-cholesteryl oleate and incubated in the presence or absence of CGP

TABLE 5. Effect of gadolinium chloride on plasma lipid-lowering activity of CGP 43371

	Control		CGP 43371	
	Vehicle	$GdCl_2$	Vehicle	$GdCl_2$
		mg/dl		
Total cholesterol	62 ± 4.2	72 ± 4.1	42 ± 1.9	46 ± 2.7
HDL cholesterol	45 ± 6.3	52 ± 3.9	30 ± 2.0	39 ± 2.3

Values represent mean ± SEM of six chow-fed rats treated with gadolinium chloride (10 mg/kg) 24 h prior to intravenous administration of CGP 43371 (3 mg/kg). Blood for analysis was collected 4 h after CGP 43371 dosing.

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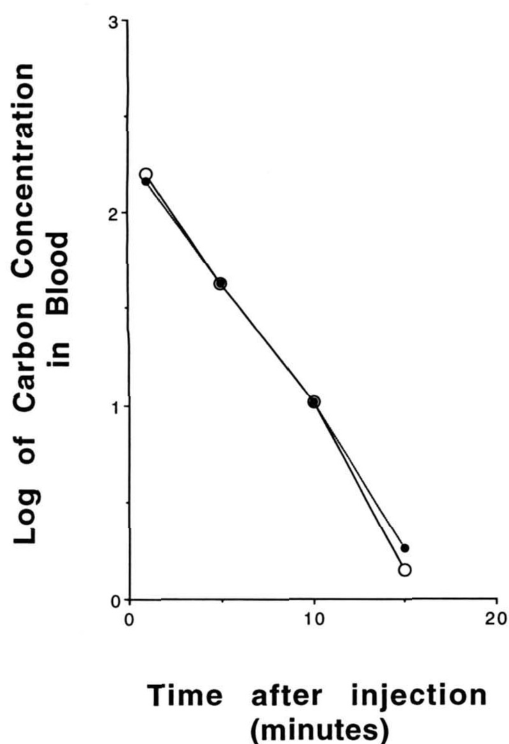

Fig. 3. Clearance of colloidal carbon from plasma of rats treated with vehicle (●) or CGP 43371 (○).

43371 (4 μM) and increasing concentrations of HDL or LDL for 30 min at 3°C. These preincubated lipoproteins, with or without CGP 43371, were then added to hepatocyte cultures for an additional 18 h. As shown in **Fig. 4,** CGP 43371 significantly increased the mass of radiolabeled cholesteryl ester that associated with the cells at each concentration of LDL. CGP 43371 also increased the association of radiolabeled HDL cholesteryl ester relative to control incubations, but the increase was only significant at the highest concentration of HDL (Fig. 4). Thus, unlike in vitro studies with mononuclear cells, hepatocytes in vitro did show enhanced lipoprotein cholesterol association in the presence of CGP 43371.

In vitro studies with ansamycin-labeled lipoproteins

The previous in vivo and in vitro studies documented an ability of ansamycins to enhance mononuclear cell cholesteryl ester accumulation in vivo that could not be reproduced in vitro. In addition, studies also showed that the ansamycins themselves readily associate with the liver in vivo (Table 2) (22) and enhance the uptake by hepatocytes of lipoprotein cholesterol (Fig. 4). Studies were therefore undertaken to determine whether the ansamycins might be taken up by hepatocytes, incorporated into lipoproteins, and secreted in a form capable of enhancing mononuclear cell cholesteryl ester in vitro. To do this, an in vivo protocol was used to maximize the potential incorporation of CGP 43371 into lipoproteins by the liver. Radiolabeled CGP 43371 (sp act = 10.8 in μCi/mg, dose = 10 mg/kg) or vehicle was injected intravenously into chow-fed rats. Six hours later, the livers from control and treated rats were excised and hepatocytes were isolated as described previously. The hepatocytes were then incubated in fresh medium for 18 h at 37°C. After incubation, the medium was removed and divided into two aliquots. The cells were washed and analyzed for radiolabeled ansamycin. One fraction of the medium was submitted to single-spin density ultracentrifugation in order to identify whether radiolabeled ansamycin was incorporated into secreted lipoproteins. The other fraction was transferred to resident peritoneal macrophages for an additional 18 h to determine whether the secreted lipoproteins had the capacity to enhance macrophage cholesterol ester content in vitro. **Table 6** shows that the isolated hepatocytes contained high levels of radiolabeled CGP 43371 and that approximately 31% of the drug was secreted into the medium over the 18-h incubation period. **Fig. 5** demonstrates flotation of secreted radiolabeled

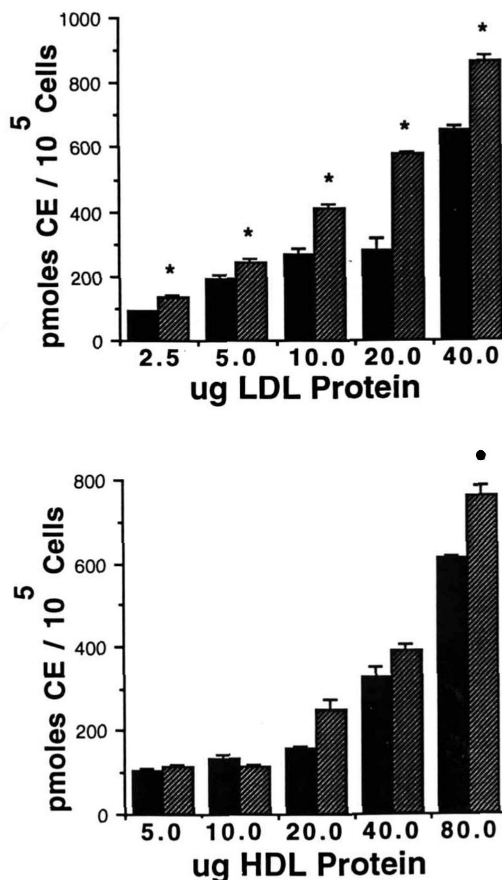

Fig. 4. Cell association of [^{14}C]cholesteryl oleate labeled LDL (top) or HDL (bottom) after incubation in the presence (striped bars) or absence (solid bars) of CGP 43371 (4 μM).

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TABLE 6. Uptake and release of radiolabel by isolated hepatocyte cultures after intravenous injection of [^{14}C]CGP 43371

Dish	CPM		Medium as % Total
	Hepatocytes	Medium	
1	87440	39300	31.0
2	88233	38400	30.3
3	87020	41120	30.9
Average	87564	39607	30.8

Hepatocytes were incubated for 18 h with medium prior to study.

TABLE 7. Macrophage cholesterol content with and without incubation with medium exposed in vitro to hepatocytes

Conditions		Macrophage Cholesterol		
Pre-incubation	CGP 43371	Total	FC	CE
		μg/mg protein		
18 h		16.61	10.43	6.18
18 h	exog.	18.45	11.56	6.89
	exog.	18.38	10.46	7.93
18 h	endog	26.27	15.93	10.34

Values represent the mean of three separate incubations.; FC, free cholesterol; CE, cholesteryl ester.

CGP 43371 at d < 1.02 g/ml. A preliminary study documented a 72% binding of this secreted buoyant [^{14}C]CGP 43371 to an anti-apoB IgG affinity matrix, in contrast to an apparent 33% nonspecific binding of [^{14}C]CGP 43371 to the gel in the absence of lipoprotein. Thus, a portion of the secreted [^{14}C]CGP 43371 appears to have been associated with apoB-containing lipoproteins.

Table 7 summarizes the results of in vitro studies in which the cholesterol content of resident peritoneal macrophages was determined after incubation with medium that had been derived from *1)* hepatocytes not exposed to CGP 43371 for 18 h; *2)* hepatocytes to which [^{14}C]CGP 43371 was added exogenously; or *3)* hepatocytes prepared from rats pretreated with [^{14}C]CGP 43371 intravenously. The addition of [^{14}C]CGP 43371 to medium at the time of addition to macrophages was associated with only a minor increase in macrophage cholesteryl ester compared to macrophages not exposed to [^{14}C]CGP 43371. In the presence of medium containing [^{14}C]CGP 43371 secreted from hepatocytes, however, a 66% enhancement of cholesteryl ester content was seen relative to control incubations.

In a separate study, CGS 24565 (0.5 mg/kg) and CGP 43371 (2 and 10 mg/kg) or vehicle were administered intravenously and hepatocytes were prepared as above. After 18 h of incubation, the ansamycin-enriched fraction was isolated at a density of 1.02 g/ml and analyzed for cholesterol, triglyceride, apoB, and apoA-I. The data are summarized in **Table 8.** The d < 1.02 g/ml fraction of medium prepared from hepatocytes treated with the highest dose of CGP 43371 was enriched in both cholesterol and triglyceride relative to control medium, whereas CGS 24565 medium and low dose CGP 43371 were not. Medium apoB concentrations were not different from control in hepatocytes from any of the treatment groups. A striking change was seen in apoA-I levels, however. Secreted apoA-I was markedly increased in a dose-dependent manner after ansamycin treatment.

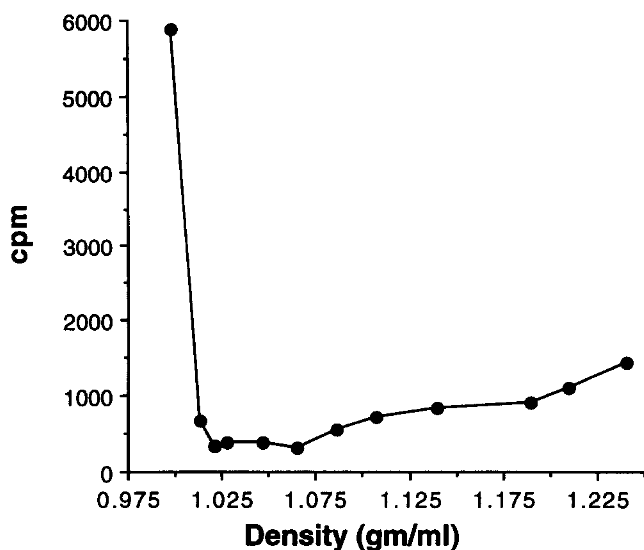

Fig. 5. [^{14}C]CGP 43371 distribution after single spin density ultracentrifugation of medium collected from hepatocytes isolated from rats treated with [^{14}C]CGP 43371.

DISCUSSION

The ansamycins are macrocyclic compounds that potently reduce VLDL, LDL, and HDL cholesterol levels in virtually every species tested (10, 11). Data presented here and elsewhere suggest that the mechanism involves a

TABLE 8. Lipid and apolipoprotein composition of the d < 1.02 g/ml fraction of rat hepatocyte medium after 18 h of incubation

	Total Chol	TG	ApoA-I	ApoB
	mg/dl		*ng/10^{-7} cells*	
Control	0.5	6.1	46	6770
CGP 43371				
2 mg/kg	0.4	7.7	498	4642
10 mg/kg	2.0	20.6	3041	5932
CGS 24565				
0.5 mg/kg	0.6	9.8	3644	7963

Chol, cholesterol; TG, triglyceride.

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physical association of the ansamycins with lipoproteins, facilitating hepatic uptake followed by resecretion of drug in a very low density form that is capable of enhancing cholesterol accumulation in mononuclear cells. Significant questions remain, however. *1)* Is the same recognition mechanism used by liver and mononuclear cells? *2)* Which tissue site is primarily responsible for the lipid-lowering effect? *3)* What is the nature of the binding site(s) mediating uptake?

There are several arguments against a mechanism of lipoprotein complex recognition common to both hepatic and mononuclear cells. In the first place, under identical in vitro conditions in which lipoproteins and ansamycin are preincubated to enhance physical association, hepatocyte uptake of lipoprotein cholesterol is stimulated, but neither monocytes nor macrophages demonstrate a direct ansamycin-induced enhancement of cellular cholesterol in vitro. Second, in vivo studies presented here and elsewhere (12, 22), as well as in vitro studies, have documented a striking uptake of ansamycins by liver tissue. The cholesterol enrichment of mononuclear cells in vivo demonstrated in the present study, however, occurred independently of ansamycin uptake into cells, as shown by the lack of radiolabeled CGP 43371 in peritoneal cells after injection of sodium caseinate. Thus, although the initial recognition site could be common, the data do not support a common response in terms of internalization.

Another important question is whether hepatic or mononuclear cells or both are responsible for the lipid-lowering effect of the ansamycins. From previous studies, it is known that the onset of hypocholesterolemia is very rapid after either intravenous or oral administration (10). As shown here, there is also rapid uptake of both drug and of lipoprotein cholesterol by the liver, suggesting that hepatic uptake may directly parallel and be responsible for the initial hypocholesterolemia. The involvement of mononuclear cells in the lipid lowering, however, appears more complex. While stimulation of macrophage function or number by pharmacological agents (24), growth factors (25), or pathophysiological state (26) is associated with profound lipid lowering, ansamycin treatment did not appear to activate macrophage phagocytic activity as shown by lack of effect on carbon clearance and the failure of blockade of Kupffer cell phagocytosis to prevent lipid lowering. Also, neither monocytes nor macrophages could be stimulated directly by the drug to accumulate cholesterol, suggesting that mononuclear cells may not play a major role in the initial stages of lipid lowering. Rather, hepatic interaction may be a prerequisite to a secondary involvement of the mononuclear cells in perhaps sustaining and/or augmenting the initial response.

The mechanism(s) by which hepatic and mononuclear cells are stimulated by ansamycins to take up lipoprotein cholesterol is also not known. Certain clearance mechanisms can, however, be eliminated on the basis of the studies reported here and elsewhere. In the first place, it is unlikely that the liver or mononuclear cell effect is mediated solely by the classical LDL receptor, as the ansamycins equivalently reduce cholesterol transported by HDL and by apoB-containing lipoproteins. Also, the ansamycins are potent lipid-lowering agents in homozygous WHHL rabbits, which have a genetic deficiency of LDL receptors (J. C. Gibson, W. K. Sawyer, and M. F. Prescott, unpublished studies). Phagocytosis can also be excluded on the basis of the gadolinium chloride blockade and carbon clearance studies reported here. Other mechanisms are not so easily eliminated, however. These include the well-characterized scavenger receptor (4), less well-defined receptors such as those for β-VLDL (6) or chylomicron remnants (5, 7, 8) or a mechanism involving a binding site or receptor not yet identified, such as that mediating selective cholesteryl ester uptake (27). Blood-borne monocytes, macrophages, and Kupffer cells all possess scavenger receptors. Thus, involvement of this receptor cannot be ruled out and is currently being investigated. Unlike all reported ligands for the scavenger receptor (28), however, the in vitro association of CGP 43371 with VLDL, LDL, or HDL does not change the lipoprotein charge, as shown by unchanged electrophoretic mobility (W. H. Lee, unpublished data). The β-VLDL receptor, present on macrophages, may be involved in the effect seen with mononuclear cells, although a mechanism involving stimulation of this receptor might be expected to be most effective in cholesterol-fed animal models where β-VLDL accumulate. The ansamycins, however, are less potent in lowering cholesterol of cholesterol-fed animals (10). Stimulation of one of the receptors hypothesized to mediate chylomicron remnant clearance is unlikely also as the clearance of an oral fat load is not enhanced in ansamycin-treated rats (J. R. Piccolo, unpublished data). The most intriguing potential mechanism is that mediating selective uptake of cholesteryl esters, described for both HDL (27, 29) and LDL (30). Involvement of this pathway is a particularly attractive possibility in view of the observation that the ansamycins reduce plasma HDL cholesterol levels to a much greater extent than they reduce plasma and HDL apoA-I (10). In addition, the data reported here on ansamycin-stimulated hepatocyte secretion of an apoA-I-enriched lipoprotein are compatible with a reversible association of apoA-I with the hepatocyte, such as has been described for the selective uptake process (31).

It should be considered whether the hepatic uptake and apparent resecretion described here is purely an in vitro phenomenon. The pharmacokinetics of this class of drug argue against this. Five minutes after intravenous administration of radiolabeled CGP 43371 to rats, > 95% of the compound was localized in the liver, as shown by

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whole body autoradiography (22). Over time, however, (up to 96 h), the drug was gradually released from the liver and became concentrated in adrenal and spleen (22), a result consistent with rapid hepatic targeting and resecretion in lipoprotein form. Although analysis of plasma lipoproteins isolated 6 h after i.v. injection of radiolabeled CGP 43371 failed to document a high specific activity VLDL subfraction (J. C. Gibson, W. H. Lee, and Z. F. Stephan, unpublished results), this secreted lipoprotein may be rapidly transformed to other density classes and/or be rapidly metabolized by mononuclear or other cells.

While many other questions remain to be answered, the ansamycins are clearly a class of potent hypolipidemic agent that appears to be acting via a clearance mechanism for lipoprotein cholesterol which, though unidentified, is effective in all animal models tested as well as in humans (J. Pincus and M. Gatlin, unpublished data). In this regard, this mechanism may represent a significant component of non-LDL receptor-mediated clearance and the ansamycins may provide valuable tools for investigating new pathways of lipoprotein modification and processing.

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