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REDUCTION OF CARDIOVASCULAR AND THYROXINE-SUPPRESSING ACTIVITIES OF L-T_3 BY LIVER TARGETING WITH CHOLIC ACID

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Abstract—This study was designed to determine whether the conjugation product of L-T_3 with cholic acid would result in a liver-targeted compound (CGH 509A) with hypocholesterolemic (HC) activity significantly dissociable from cardiovascular (CV) and thyroxine-suppressing (TS) effects normally observed with thyroid hormone. Evaluation of HC activity in lipemic rats showed that CGH 509A was 6 times less potent than L-T_3 with ED_{25} values estimated at 150 and 25 nmol/kg, respectively. CV function measured as changes in atrial rate, atrial tension and heart weight was determined in euthyroid rats. CGH 509A was at least 64 times less cardio-stimulant than L-T_3 with minimum effective doses estimated at 2350 and 37 nmol/kg, respectively. TS activity was assessed in euthyroid rats as the potency of any compound to reduce plasma T_4 levels. CGH 509A was 50 times less potent than-L-T_3 with ED_{50} values estimated at 900 and 18 nmol/kg, respectively. From these results, it is clear that, while L-T_3 was equally potent on HC, CV and TS activities, the HC potency of CGH 509A was at least 15 and 6 times greater than its CV and TS potencies, respectively.

The hypocholesterolemic (HC†) activity of thyroid hormone (L-T_3) has been well known for more than three decades [1–7]. However, the use of L-T_3 as a safe lipid-lowering agent has not been possible because of the severe cardiovascular (CV) stimulation [8–11] and thyroxine-suppressing (TS) effects associated with this hormone.

Whereas the cholesterol-lowering activity of L-T_3 is thought to result from the interaction of the hormone with its receptor in liver cell nuclei [12] and its subsequent upregulatory action on the LDL receptor [13–16], the CV and TS activities result from interaction of the hormone with extrahepatic cells such as muscle, heart and pituitary cells [17]. Therefore it became apparent that, if the adverse activity of L-T_3 were to be limited by either minimizing its access to non-hepatic cells or targeting its uptake to the liver, then a cardiac and pituitary-sparing thyromimetic hypolipidemic could be identified. This concept was validated in part by Boyd and Oliver, Cuthbertson *et al.*, [18, 19] and Underwood *et al.* [20, 21] whose work with D-T4 and SKF L-9401 respectively demonstrated that these thyromimetics have much greater access to hepatic than to non-hepatic cells, and thus exhibit lipid-lowering activity significantly dissociable from cardiac stimulation *in vivo*.

In this report, we demonstrate that, by conjugating L-T_3 with a liver-targeted molecule such as cholic acid, it is possible to obtain a compound (CGH 509A; Fig. 1) with significantly less cardiac and TS activities than L-T_3 while retaining good cholesterol-lowering potency.

MATERIALS AND METHODS

Materials. CGH 509A, a compound formed by an amide linkage between the C_{25} carboxyl group of cholic acid and the α-amino group of the alanine side-chain of L-triiodothyronine, was synthesized in our laboratories at CIBA-GEIGY Ltd. (Horsham, England). ^{125}I-Labeled L-T_3 was obtained from New England Nuclear (Boston, MA). Unlabeled L-T_3 and all other materials were purchased from the Sigma Chemical Co. (St. Louis, MO). Rat chow supplemented with 1.5% cholesterol and 0.5% cholic acid was purchased from Dyets (Bethlehem, PA).

Binding to nuclear T_3 receptor. Rat liver nuclei

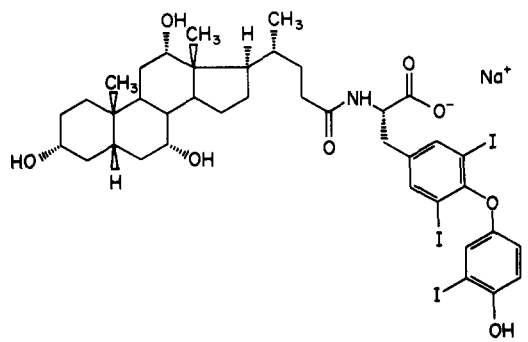

Fig. 1. Structure of CGH 509A.

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† Abbreviations: HC, hypocholesterolemic; CV, cardiovascular; TS, thyroxine-suppressing; bpm, beats per minute; BMR, basal metabolic rate; MED, minimum effective dose; and TSH, thyroid-stimulating hormone.

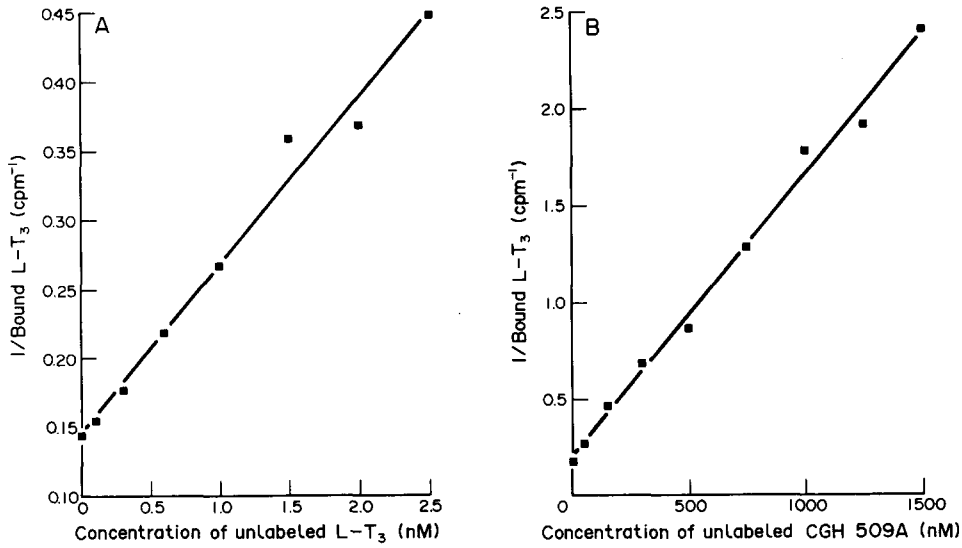

Fig. 2. Reciprocal plot of specifically bound L-T_3 (A) or CGH 509A (B) to the nuclear T_3 receptor. Specific binding of test compounds to the nuclear T_3 receptor was determined as the difference in ^{125}I-labeled L-T_3 displaced from rat liver nuclei in the absence (total binding) and presence (nonspecific binding) of excess unlabeled L-T_3. Each point is the mean of two determinations.

preparations were obtained from male Sprague–Dawley rats [Tac:N(SD)fBR] by differential ultracentrifugation as described by Emmelot *et al.* [22]. The nuclear fraction obtained from the 275 *g* pellet was further purified, and binding of test compounds to this fraction was determined according to the method of Spindler *et al.* [23] with minor modifications. To measure total binding, nuclei (300 μg of nuclear protein) were incubated with 0.3 nM ^{125}I-L-T_3 (1080 μCi/μg) for 50 min at 22° in a final volume of 1.0 mL of buffer A consisting of 20 mM Tris–HCl, 0.25 M sucrose, 1 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl and 5% glycerol (pH 7.2). Parallel incubations were conducted with tubes containing, in addition to the nuclear suspensions and radioactive L-T_3, either various concentrations of the test compounds or excess of unlabeled L-T_3 (3 μM). The latter served as a measure of nonspecific binding. Following incubation, the samples were chilled in an ice bath and centrifuged at 800 *g* for 7 min at 4°. The pellet was washed by suspending in 2 mL of buffer B (buffer A with 0.5% Triton X-100; pH 7.2) and mixing for 5 sec. Tubes were then centrifuged at 800 *g* for 7 min at 4°. The supernatant was aspirated off and the pellet was washed again and recentrifuged as described above. Radioactivity in the pellet was determined in an LKB 1282 gamma counter. Specific binding was calculated as the difference between total binding (incubation without excess unlabeled L-T_3) and nonspecific binding (incubation in the presence of excess unlabeled L-T_3). The concentration of test compounds corresponding to half-maximal inhibition (IC_{50}) of specific binding of ^{125}I-L-T_3 was determined from the reciprocal plot of specific binding versus concentration of test compound.

Cholesterol-lowering activity. Euthyroid male Sprague–Dawley rats (230–250 g) were maintained *al lib.* on water and rat chow diet containing 1.5% cholesterol and 0.5% cholic acid for 2 weeks prior to and during the 7-day treatment period. Groups of six animals were treated orally once a day with vehicle (water) alone or with test compounds for 7 consecutive days. After the last dose, animals were fasted for 18 hr and blood was collected by cardiac puncture under CO_2 anesthesia. Blood was withdrawn in 5% EDTA (50 μL/mL blood) and plasma was prepared by centrifugation at 2500 rpm for 10 min at 4°. Samples were analyzed enzymatically for total cholesterol on a Bio-Mek automated work station (Beckman Instruments) using a diagnostic reagent kit (Sigma Chemical Co.).

Cardiovascular activity. Euthyroid male Sprague–Dawley rats (235–265 g) were maintained *ad lib.* on standard chow and water. Groups of six animals were treated orally as described above. On day 8, animals were killed by decapitation. Hearts were quickly removed and rinsed in 20 mL of tissue bath containing Krebs buffer oxygenated with 95% O_2, 5% CO_2 at 28°. Right atria were dissected, hung with minimum applied tension and allowed to equilibrate for 30 min. The spontaneous atrial rate was then determined. Left atria were dissected from the heart and mounted on platinum electrodes positioned in tissue baths containing Krebs buffer. The resting tension was set at 2 g. Left atria were stimulated at 10 beats/min (bpm) with a stimulus duration of 2.5 msec. Atria were then allowed to equilibrate for 30 min. Tension–force curves were generated by altering tension and measuring the change in developed contractile force as described by Murayama and Goodkind [24]. The maximum developed contractile force was measured for each

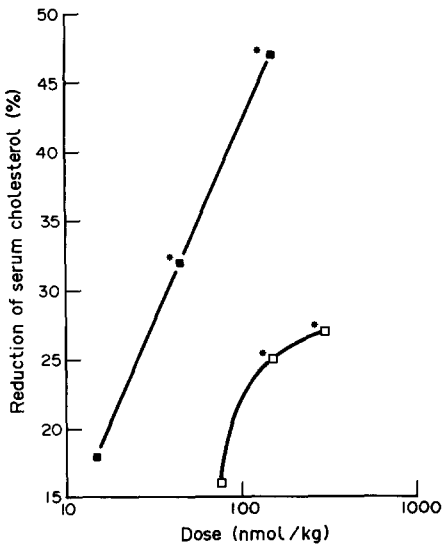

Fig. 3. Dose-dependent hypocholesterolemic activity of L-T_3 (■) vs CGH 509A (□). Cholesterol-lowering activity was determined after 7-day oral dosing of hyperlipemic rats pre-fed a diet containing 1.5% cholesterol and 0.5% cholic acid for 2 weeks. Each point on the curve is the mean of serum cholesterol reduction in six rats. Control rats had a cholesterol concentration of 178 ± 17 mg/dL. Key: (*) statistically significant change from the control group determined by Student's *t*-test ($P < 0.05$).

atrium. Isometric contractile forces were measured at frequencies of 10, 20, 40 and 80 bpm. Rat total body and heart weights were recorded to calculate relative heart weight. Plasma was collected and frozen for future biochemical analyses.

Thyroxine-suppressing activity. Plasma samples obtained from the above lipid and cardiovascular studies were analyzed for T_4 concentrations with a specific radioimmunoassay kit supplied by Amersham (Arlington Heights, IL).

Statistical analysis. Differences between experimental and control groups were tested for statistical significance by Student's *t*-test for independent samples of equal size [25].

RESULTS

Binding affinity of L-T_3 vs its cholic acid conjugate (CGH 509A). The reciprocal of the specifically bound radioactivity (1/B) was linearly regressed against the concentration of each compound, and binding affinity was expressed as the concentration required for half-maximal displacement (IC_{50}) of specifically bound L-T_3 to its nuclear receptor (Fig. 2, A and B). As shown in Fig. 2, the binding affinity of CGH 509A (IC_{50} = 130 nM) was at least 100-fold weaker than that of L-T_3 (IC_{50} = 1.25 nM) to the nuclear receptor.

Effect of L-T_3 vs CGH 509A on plasma cholesterol. Because of the large difference in molecular weight between the two compounds (L-T_3 = 673; CGH 509A = 1064), and since CGH 509A is considered the prodrug for L-T_3, the doses for all the animal studies reported herein will be expressed as nmol/kg instead of μg/kg for more accurate comparison.

In lipemic rats, plasma cholesterol (178 ± 17 mg/dL) was reduced dose dependently by both compounds (Fig. 3). However, the HC potency of CGH 509A measured by ED_{25} (the dose required to reduce serum cholesterol significantly by 25%) was 150 nmol/kg which was about 6 times weaker than that for L-T_3 (ED_{25} = 25 nmol/kg). Furthermore, plasma cholesterol reduction of as much as 47% was possible with L-T_3, while an efficacy of 27% reduction could not be exceeded by CGH 509A.

Effect of L-T_3 and CGH 509A on cardiovascular function. Three parameters were measured to assess cardiovascular function: heart weight, right atrial rate, and left atrial tension. As shown in Fig. 4, A, B and C, all three parameters were stimulated by L-T_3 in a dose-dependent manner. The minimum effective dose of L-T_3 on any one of these parameters was estimated at about 37 nmol/kg, and stimulation of up to 60% of all three parameters was obtained at 1500 nmol/kg. In contrast to L-T_3, treatment with CGH 509A had to be increased up to 2350 nmol/kg in order to obtain a moderate but statistically significant stimulation of atrial rate and atrial tension of 26 and 13%, respectively (Fig. 4, B and C). However, the latter dose of CGH 509A had no significant effect (5%) on cardiac hypertrophy (Fig. 4A).

Effect of L-T_3 and CGH 509A on plasma T_4. As shown in Fig. 5, plasma T_4 was reduced dose dependently by both compounds but to a greatly different degree. For example, plasma T_4 was reduced significantly by about 40% even at the lowest dose of L-T_3 (15 nmol/kg), while no detectable effect was observed with CGH 509A up to 300 nmol/kg. The ED_{50} values for L-T_3 and CGH 509A were estimated from dose–response curves at 18 and 900 nmol/kg, respectively.

Separation of HC from CV and TS activities of L-T_3 and CGH 509A. Combining all the results reported above, it is possible to estimate the relative safety of each compound with respect to CV and TS activities. The data in Table 1 show that the effective doses of L-T_3 on desirable (HC) versus undesirable (CV and TS) effects were essentially similar, thus yielding a relative safety ratio close to unity (1.48 and 0.72). In contrast to L-T_3, CGH 509A had a significantly greater HC effect compared to its effects on cardiovascular function and plasma T_4, thus resulting in relative safety ratios of 15.7 and 6.0, respectively.

DISCUSSION

The use of thyromimetics as safe and efficacious HC agents is largely dependent on the ability to dissociate the cholesterol-lowering activity of these compounds from their CV and TS activities. This has been attempted, with limited success, by several groups of investigators [18–21] who searched for compounds with poor accessibility to non-hepatic tissues. The work described in this paper attempts to identify a novel way to increase the hepatoselectivity of thyromimetics, thereby reducing their

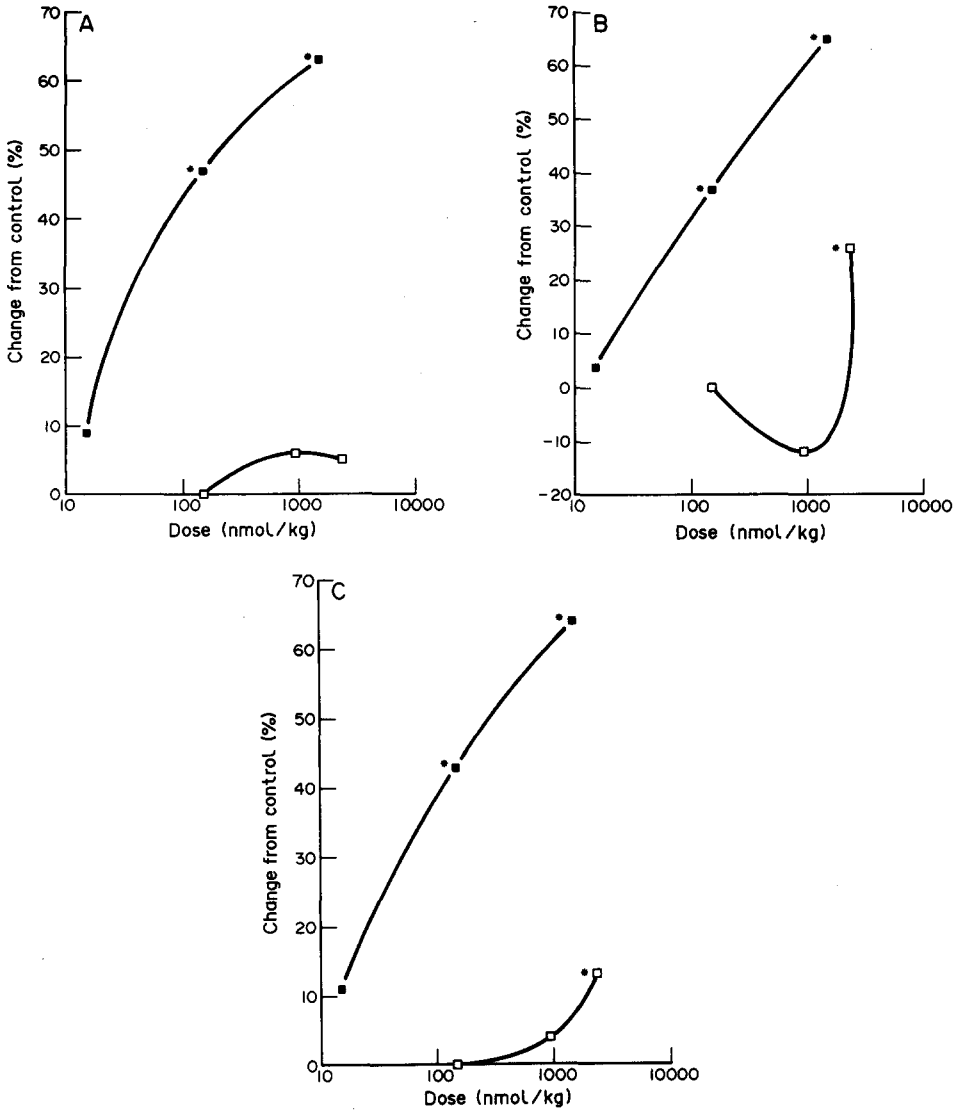

Fig. 4. Dose-dependent effect of L-T_3 (■) vs CGH 509A (□) on heart weight (A), right atrial rate (B) and left atrial tension (C). All cardiovascular parameters were measured in euthyroid rats. Relative heart weight was calculated as the ratio of heart to body weight. Atrial rate was determined in the spontaneously beating isolated right atria after appropriate equilibration time in oxygenated Krebs buffer at 28°. Atrial tension was expressed as the percent of maximal developed contractile force by the left atria when the tissue was electrically paced at 40 bpm. Each point on the curve is the mean of percent change from control of six rats. Control values for relative heart weight, atrial rate and atrial tension were 0.37% of total body weight, 191 bpm and 87% of maximum tension, respectively. Key: (*) statistically significant change from the control group determined by Student's *t*-test ($P < 0.05$).

chances of producing cardiac and pituitary-related side effects. CGH 509A (see Fig. 1), a cholic acid conjugate of L-T_3, was used to demonstrate this concept of improved hepatoselectivity.

The poor binding affinity of CGH 509A (Fig. 2) to the nuclear receptor (100-fold weaker than L-T_3) was not surprising since the addition of such a bulky molecule as cholic acid would be expected to drastically change the molecular interaction between the ligand (L-T_3) and its receptor. Usually, thyromimetics with poor binding affinity similar to that of CGH 509A (IC_{50} = 130 nM) would not be active *in vivo* [26–28]. However, the retention of significant *in vivo* activity by this compound strongly suggests that hydrolysis and subsequent release of the active L-T_3 molecule must have occurred. Furthermore, the reduced potency of CGH 509A on HC (Fig. 3) can be partially explained by either poor intestinal absorption and/or inefficient hydrolysis of the amide bond by hepatic enzymes. Based on the ED_{25} values (Table 1), the activity of CGH 509A on HC was reduced only by a factor of 6 compared to L-T_3.

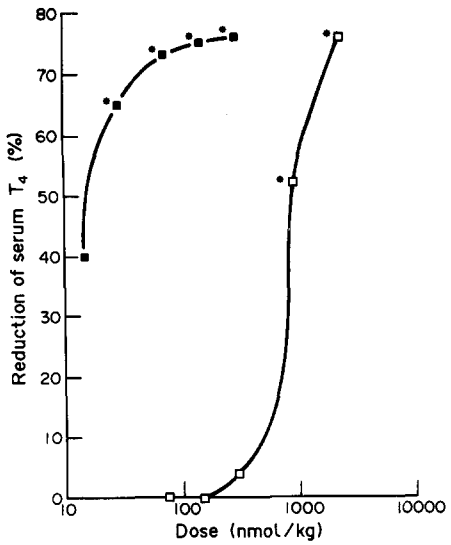

Fig. 5. Dose-dependent effect of L-T_3 (■) vs CGH 509A (□) on plasma T_4 concentrations. The thyroxine-suppressing activity of test compounds was evaluated in euthyroid rats after a 7-day oral treatment. Plasma T_4 was determined by a specific radioimmunoassay kit. Each point on the curve is the mean of plasma T_4 reduction in six rats. Control rats had a plasma T_4 concentration of 4.51 ± 0.56 μg/dL. Key: (*) statistically significant change from the control group determined by Student's *t*-test ($P < 0.05$).

Table 1. Hypocholesterolemic, cardiovascular stimulant and thyroxine-suppressing activities of L-T_3 and CGH 509A

Parameter	L-T_3	CGH 509A (nmol/kg)
[A] Serum cholesterol (ED_{25})	25	150
[B] CV functions (MED*)	37	2350
[C] Serum T_4 (ED_{50})	18	900
[B]/[A]	1.48	15.7
[C]/[A]	0.72	6.0

* MED = minimum effective dose on any cardiovascular parameter.

Relative to L-T_3, CGH 509A had a very weak effect on cardiovascular functions (Fig. 4). Both atrial rate and atrial tension, which normally increase in response to L-T_3 treatment in order to generate the cardiac output necessary to compensate for the enhanced needs of a stimulated basal metabolic rate (BMR), were barely affected by CGH 509A and only at a dose 64-fold higher than that of L-T_3 (Table 1). Interestingly, such stimulation of atrial function, which is normally accompanied by cardiac hypertrophy [10], was observed with L-T_3 but not with CGH 509A. The discrepancy between the two compounds cannot be attributed to insufficient stimulation at the minimum effective dose (MED) of CGH 509A, since cardiac hypertrophy occurred at the MED of L-T_3, but it possibly can be due to poor access of L-T_3 (released from CGH 509A) into the heart where cardiac hypertrophy is considered to be the result of an enhanced mechanical workload [29, 30] due to the stimulated BMR as well as a result of a direct growth factor-like [31–34] effect of L-T_3.

Finally, the reduction of plasma T_4 concentrations generally reflects the ability of a thyromimetic to access the pituitary gland and feed-back inhibit the formation of T_4 in the thyroid gland via thyroid-stimulating hormone (TSH). The data presented here (Table 1) indicate that CGH 509A is at least 50 times less potent a thyroxine-suppressant than L-T_3. Furthermore, CGH 509A was virtually without any pituitary-mediated effect even up to the highest effective HC dose tested (300 nmol/kg). In contrast, the cholesterol-lowering dose of L-T_3 was always accompanied by significant reduction of plasma T_4.

In conclusion, this paper demonstrates that conjugation of thyromimetics with a molecule having high affinity for the liver, such as cholic acid, may be a useful means to enhance the hepatoselectivity of thyromimetics, thereby reducing cardiac- and pituitary-associated side effects while retaining hypolipidemic activity.

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