radiosensitization studies *in vitro* and *in vivo*. *Int J Radiat Biol* **46**: 731–745, 1984.
9. Rybczynska M, Chmiel J and Philips H, The influence of nitroheterocyclic radiosensitizers on the membrane of red blood cells. *Pol J Pharmacol Pharm* **36**: 307–312, 1984.
10. Saneyoshi M, Inomata M, Sekine T, Hoshi A and Fukuoka F, Synthetic nucleosides and nucleotides. X. Synthesis and biological activities of several purin-6-yl benzyl disulfides and their ribonucleosides. *J Pharmacobiodyn* **1**: 168–174, 1978.
11. Sirakawa K, Aki O, Tsujikawa T and Tsuda T, *S*-Alkylthioisothioureas. I. *Chem Pharm Bull (Tokyo)* **18**: 235–242, 1970.
12. Bond GH and Green JW, Effects of monovalent cations on the (Mg^{2+} and Ca^{2+})-dependent ATPase of the red cell membrane. *Biochim Biophys Acta* **241**: 393–398, 1971.
13. Warren L, Isolation of plasma membrane from tissue culture L cells. *Methods Enzymol* **31**: 159–162, 1974.
14. Jorgensen P, Isolation of (Na^+ and K^+)-ATPase. *Methods Enzymol* **32**: 277–290, 1974.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Tsai C, Chen K and Canellakis E, Isolation and characterization of the plasma membrane of L-1210 cells. Iodination as a marker for the plasma membrane. *Biochim Biophys Acta* **401**: 196–212, 1975.
17. Fiske C and Subbarow Y, The colorimetric determination of phosphorus. *J Biol Chem* **66**: 375–400, 1925.
18. Robinson JD, Nucleotide and divalent cation interactions with the ($Na^+ + K^+$)-dependent ATPase. *Biochim Biophys Acta* **341**: 232–247, 1974.
19. Patzelt-Wenczler R and Schoner W, Evidence for two different reactive sulfhydryl groups in the ATP-binding sites of ($Na^+ + K^+$)-ATPase. *Eur J Biochem* **114**: 79–87, 1981.
20. Patzelt-Wenczler R and Mertens W, Effects of cations on high-affinity and low-affinity ATP-binding sites of (Na^+,K^+)-ATPase as studies by disulfides of thioinosine triphosphate and its analogue. *Eur J Biochem* **121**: 197–202, 1981.
21. Dixon M and Webb EC, *Enzymes*, 3rd Edn. Academic Press, New York, 1979.
22. Walter C, *Steady-State Applications in Enzyme Kinetics*. Ronald Press, New York, 1965.
23. Jardetsky O, Simple allosteric model for membrane pumps. *Nature* **211**: 969–970, 1966.

Biochemical Pharmacology, Vol. 39, No. 9, pp. 1487–1489, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Effects of octimibate, an inhibitor of acyl coenzyme A: cholesterol acyltransferase, on cholesterol metabolism in the hamster and rat

(*Received* 19 *June* 1989; *accepted* 22 *December* 1989)

Inhibition of esterification of cholesterol in cells has been considered by many to be a desirable goal. In the intestine this would lead to reduced absorption of cholesterol and in arteries would result in reduced deposition of cholesterol and hence a reduction of growing atherosclerotic plaques [1, 2]. A number of compounds have been reported that inhibit the intracellular enzyme that catalyses cholesterol esterification, acyl-CoA:cholesterol acyltransferase (ACAT), but to date none has been successful in the clinic [1–4]. Octimibate (Nattermann) is one of a number of compounds of this type in development and it appears to have a number of advantages over previous compounds through its solubility and absorbability [5]. We have used inhibitors of ACAT to characterize the role of hepatic pools of cholesterol in primary cultures of rat hepatocytes and in bovine adrenal cortical cells [6, 7]. Since little information is available on octimibate we carried out a short study to characterize its effects on cholesterol metabolism in the hamster, an animal that offers a number of advantages in studies of cholesterol metabolism over the rat. We compared these results with parallel studies in the rat.

Materials and methods

Male Syrian hamsters were obtained from Shamrock Farm or Belgrave Trading Ltd and had a body weight of 104–136 g at the time of the experiments. Male Wistar strain rats were obtained from Charles River and had a body weight of 319–366 g at the time of the experiments. A week prior to dosing, the animals were housed individually in wire-bottomed cages. The rats were allowed to feed on powdered PRD chow *ad lib.* from rat food hoppers and hamsters were allowed to feed on powered PRD chow *ad lib.* from pots clipped to the grid floor. During this acclimatization period food consumption was measured. The animals were weighed on alternate days and the expected mean body weight of the animals at the time of the experiment calculated.

The appropriate amount of octimibate, calculated from the food consumption and expected body weight of the animals, was dissolved in a large volume of 99% ethanol, typically 1.2 L for a 4 kg batch of diet. This solution was then thoroughly mixed into the powdered PRD chow to give a homogeneous mixture. The wet diet was then transferred to a large photographic developing tray and placed in a fume cupboard to dry. During the experiment the daily dose of octimibate received by each animal was calculated from its food consumption and body weight. After the animals had been fed on the appropriate diet for 7 days, they were anaesthetized with diethyl ether and a blood sample (2–3 mL) was taken from the superior vena cava. The liver was perfused with cold saline and then weighed. A portion of liver (~ 1 g) was removed, weighed and homogenized in chloroform/methanol (2:1 v/v) with a Polytron homogenizer. [^{14}C]Cholesterol was added as a recovery marker to the resulting homogenate, which was then filtered through Whatman number 1 filter paper and blown down to dryness under nitrogen. The residue was taken up in 1 mL of propan-2-ol and centrifuged at 2900 *g* for 10 min in a microcentrifuge. The supernatant was assayed for free and total cholesterol using the AMES enzymatic colorimetric method. Octimibate was synthesized by the Department of Medicinal Chemistry at Smith Kline and French Laboratories in Welwyn.

Table 1. The effect in the hamster of octimibate on liver cholesterol

Octimibate dose (mg/kg/day)	N	Liver weight (g)	Total cholesterol (mg/g)	Free cholesterol (mg/g)	% Free cholesterol
Control	12	4.54 ± 0.46	2.44 ± 0.26	2.34 ± 0.27	95.9 ± 6.3
21 ± 3	6	5.11 ± 0.48	2.71 ± 0.25	2.29 ± 0.31	84.8 ± 8.9*
70 ± 25	6	4.63 ± 0.44	3.21 ± 0.35	2.64 ± 0.39	82.4 ± 11.3*
130 ± 20	6	5.18 ± 0.45	4.06 ± 1.16	2.56 ± 0.27	66.1 ± 12.7*

Hamsters were dosed with octimibate in the diet for 7 days. All values expressed as mean ± SD.

* Different from control $P < 0.05$ (Student's *t*-test).

Table 2. The effect in the rat of octimibate on liver cholesterol

Octimibate dose (mg/kg/day)	N	Liver weight (g)	Total cholesterol (mg/g)	Free cholesterol (mg/g)	% Free cholesterol
Control	12	13.89 ± 2.11	2.46 ± 0.30	2.30 ± 0.35	93.3 ± 4.7
18 ± 1	6	13.30 ± 1.31	2.34 ± 0.15	2.11 ± 0.12	90.6 ± 1.5
63 + 4	6	13.08 ± 1.26	2.38 ± 0.06	2.17 ± 0.05	90.0 ± 1.8
130 ± 14	6	14.17 ± 0.86	2.45 ± 0.11	2.43 ± 0.14	99.1 ± 1.3

Rats were dosed with octimibate in the diet for 7 days. All values expressed as mean ± SD.

Results and discussion

Table 1 shows the results obtained after 1 week of dosing with octimibate at several doses in the hamster. There was a small but significant reduction in the level of plasma cholesterol (control 126.2 ± 14.7 mg/dL, N = 5, 100 mg/kg octimibate for 1 week, 118.0 ± 6.6 mg/dL, N = 6). Analysis of the lipoprotein fractions showed small reductions in all the fractions, with perhaps a larger effect in the high density fraction (data not shown). The concentration of cholesterol and cholesteryl ester found in the liver after dosing was paradoxical. The octimibate-treated animals showed a small increase in free cholesterol (32% at 90 mg/kg/day) but a substantial increase in cholesteryl ester was observed (260-fold). The accumulation of cholesteryl ester in the liver was dose-dependent (Table 1). The cholesteryl ester was identified by HPLC as mainly cholesteryl oleate.

In contrast, in the rat over a similar dose range, no effect of octimibate was observed on the concentration of hepatic cholesteryl ester (Table 2). Another ACAT inhibitor, CL 277, 082 (2,4, American Cyanamid) was found to reduce the cholesteryl ester content of normochloesterolaemic hamster liver in experiments identical to those described for octimibate (113 mg/kg/day CL 277, 082 for 1 week had 97% free cholesterol in the total hepatic cholesterol: control animals contained 87% free cholesterol). It is known from the literature [5] and we have confirmed that octimibate is an effective inhibitor of ACAT, both in subcellular fractions and in cultured cells. If octimibate is acting systemically, as is thought to be the case, the accumulation of cholesteryl ester in the hamster liver is not consistent with this mechanism of action. It is possible that octimibate inhibits the hydrolysis of cholesteryl ester in the liver, an effect that may be expressed at the lysosomal or cytoplasmic cholesteryl ester hydrolase. Alternatively, it may prevent the secretion of cholesteryl ester in lipoproteins. Such an effect, if present in the intestine and liver, would help to account for the small hypocholesterolaemia observed with the drug in these experiments. These effects must be specific to the hamster. There are several significant differences in hepatic cholesterol metabolism between the rat and the hamster. In general, enzyme activities (e.g. ACAT, HMG-CoA reductase) in the hamster are lower than in the rat [8]. The hamster, unlike the rat, regulates its LDL-receptor activity to control the hepatic flux of cholesterol. The rat achieves this primarily through regulation of cholesterol synthesis [9]. The present experiments show that these two laboratory animals respond differently to a drug that is known to affect a specific enzyme of hepatic cholesterol metabolism.

Octimibate (Natterman) has been described as an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) and is in development as an anti-atherosclerotic and hypolipidaemic drug. We have studied its effect on the concentration of free and esterified cholesterol in the liver of the rat and hamster. In the rat octimibate caused no effect on the hepatic cholesterol concentration at doses up to 130 mg/kg for 7 days. In contrast a dose-dependent *increase* in cholesteryl ester concentration was found in the hamster livers. This paradoxical result is not consistent with the mode of action of octimibate being inhibition of cholesterol esterification.

Smith Kline & French Research Ltd
The Frythe
Welwyn
Herts AL6 9AR
U.K.

BRIAN JACKSON
ANDREW GEE
SUSAN BLACK
KEITH E. SUCKLING

REFERENCES

1. Heider JG, Agents which inhibit cholesterol esterification in the intestine and their potential value in the treatment of hypercholesterolaemia. In: *Pharmacological Control of Hyperlipidaemia* (Ed. Fears R), pp. 423–428. J. R. Prous, Barcelona, 1986.

2. Krause BR and Newton RS. ACAT Inhibitors: preclinical pharmacological profiles and implications for plasma lipid regulation and prevention of atherosclerosis. In: *Atherosclerosis VIII, Proceedings of the 8th International Symposium on Atherosclerosis,* (Eds. Gepaldi G, Gotto AM, Manzato E and Baggio G), pp. 707–710. Excerpta Medica, Amsterdam, 1989.
3. Ross CA, Go KJ, Heider JG and Rothblat GH, Selective inhibition of acyl-CoA:cholesterol acyltransferase by compound 58-035. *J Biol Chem* **259**: 815–819, 1984.
4. De Vries VG, Schaffer SA, Largis EE, Dutia MD, Wang CH, Bloom JD and Katocs AS, Potential antiatherosclerotic agents. 5. An acyl-CoA:cholesterol *O*-acyltransferase inhibitor with hypocholesterolemic activity. *J Med Chem* **29**: 1133–1134, 1986.
5. Rucker W, Prop G and Huther AM, Antiatherosclerotic and antihyperlipidemic effects of octimibate sodium in rabbits. *Atherosclerosis* **69**: 155–160, 1988.
6. Sampson WJ, Suffolk RA, Bowers P, Houghton JD, Botham KM and Suckling KE, The role of acyl-CoA:cholesterol acyltransferase in the metabolism of free cholesterol to cholesteryl esters or bile acids in primary cultures of rat hepatocytes. *Biochim Biophys Acta* **920**: 1–8, 1987.
7. Ochoa B and Suckling KE, Short-term metabolism of cholesteryl ester from low-density lipoprotein in primary monolayers of bovine adrenal cortical cells. *Biochim Biophys Acta* **918**: 159–167, 1987.
8. Suckling KE and Stange EF, Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* **26**: 647–671, 1985.
9. Dietschy JM, Spady DK and Meddings JB, A quantitative approach to low density lipoprotein metabolism in man and in various experimental animals. In: *Hyperlipidaemia and Atherosclerosis* (Eds. Suckling KE and Groot PHE), Chapter 2. Academic Press, London, 1988.

Biochemical Pharmacology, Vol. 39, No. 9, pp. 1489–1491, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Inhibition of tyrosine-3-monooxygenase by benserazide

(*Received* 12 *July* 1988; *accepted* 16 *November* 1989)

One procedure frequently used to estimate catecholamine synthesis *in vivo* is to measure the accumulation of 3,4-dihydroxyphenylalanine (DOPA) after inhibition of L-aromatic amino acid decarboxylase (3,4-dihydroxy-L-phenylalanine carboxy-lyase; EC 4.1.1.26; AAAD) [1]. In this procedure, the linear accumulation of DOPA in tissues, following a single, large dose of an inhibitor of AAAD, is considered to be a measure of the rate of tyrosine hydroxylation. The method makes at least three major assumptions: (1) AAAD is completely and immediately inhibited; (2) the DOPA formed is not metabolized further and does not diffuse out of the tissue; and (3) the inhibitor of AAAD does not change, directly or indirectly, the hydroxylation of tyrosine. The two most commonly used inhibitors of AAAD, for the *in vivo* estimation of catecholamine synthesis, are 3-hydroxybenzyl hydrazine (NSD-1015) and benserazide (Ro4-4602; DL-serine, 2-[(2,3,4-trihydroxyphenyl)methyl]hydrazide). The structures of these compounds are presented in Fig. 1. Examination of the structure of benserazide reveals a catechol (trihydroxyphenyl) structure. Since catechols have been known to inhibit tyrosine-3-monooxygenase (L-tryosine, tetrahydropteridine: oxygen oxidoreductase [3-hydroxylating]; EC 1.14.16.2; TH) [2], we examined NSD-1015 and benserazide as inhibitors of TH. We now report that benserazide, but not NSD-1015, is an inhibitor of TH, complicating its use for determining tyrosine hydroxylation *in vivo*.

Materials and methods

Benserazide was furnished by Hoffmann-LaRoche (Nutley, NJ), while 3-hydroxybenzyl hydrazine was purchased from the Aldrich Chemical Co. (Milwaukee, WI). The 6(*R*)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) was obtained from Dr B. Schircks (Jona, Switzerland). All other chemicals were obtained from the Sigma Chemical Co. (St Louis, MO).

The TH, used in these studies, was purified in its native form from bovine adrenal medulla by modifications to the method of Togari *et al.* [3]. The modifications were: (1) addition of 10 μg/mL of leupeptin and pepstatin-A to all of the buffers, except the homogenization buffer which additionally contained 0.1 mg/mL of aprotinin, soybean trypsin inhibitor and 1 mM diisopropylfluorophosphate; (2) replacement of the ion exchange column step with a 30–40% ammonium sulfate fractionation; (3) replacing the Biogel A-1.5 M column with a Sephacryl S-400 column (Pharmacia, Piscataway NJ); and (4) washing the heparin agarose affinity column at a flow rate of 3 mL/min. The enzyme, using BH_4 as cofactor, had a pterin K_m of 112 ± 11 μM, a tyrosine K_m of 6.4 ± 2.2 μM, a pH optimum of 6.55, and a specific activity of 37.45 units/mg protein. One unit is presently defined as the amount of enzyme which forms 1 nmol of product in 1 min.

The TH assay was performed using a recent modification of the ^3HOH release assay, where the unreacted isotopic substrate is adsorbed and precipitated with charcoal [4]. Each assay tube contained, in a final volume of 0.1 mL: $NaPO_4$, pH 6.55, 10 μmol; catalase, 30 μg; superoxide dis-

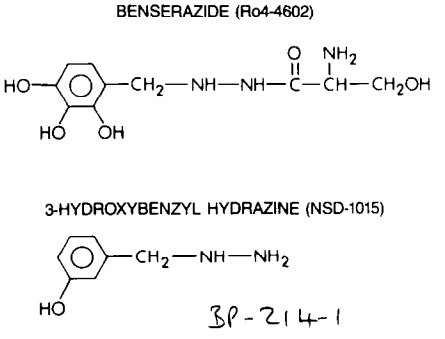

Fig. 1. Structures of benserazide and 3-hydroxybenzyl hydrazine.