

Cannabinoids Impair the Formation of Cholesteryl Ester in Cultured Human Cells

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The ability of cultured human fibroblasts to form cholesteryl esters from ^{14}C -oleate is impaired by Δ' -tetrahydrocannabinol, cannabidiol, and cannabinol, a group of natural products isolated from *Cannabis sativa*. This inhibition is compound and dose-related; 30 μM cannabidiol reduced esterification to less than 20% of the control values. The esterification of endogenous and exogenous cholesterol was affected, since inhibition was seen with either low density lipoproteins (200 $\mu\text{g/ml}$) or 25-hydroxycholesterol (5 $\mu\text{g/ml}$) as esterification stimuli. Cells treated with these compounds at doses of from 1 to 30 μM showed no impairment of protein synthesis, triglyceride or phospholipid formation, or ability to metabolize ^{125}I -low density lipoproteins. An inhibition of cholesterol esterification was seen in human aortic medial cells. With increasing doses of these compounds, low density lipoproteins (25 $\mu\text{g/ml}$) became progressively less effective in suppressing HMG-CoA reductase in cultured human fibroblasts; with 30 μM cannabidiol the enzyme suppression was only 24% of that found in cells incubated with low density lipoproteins in the absence of drugs. Based on these data, we conclude that the cannabinoids "compartmentalize" cholesterol and, thus, make it unavailable for regulating cellular cholesterol metabolism. This may occur as a result of enhanced sterol efflux. (*Arteriosclerosis* 1:449-454, 1981)

Cannabinoids are a group of natural products derived from *Cannabis sativa*. These compounds are the subject of intense pharmacological investigation due to their wide, illegal consumption in the form of marijuana. They possess a variety of physiologic, as well as psychogenic, properties. Currently, Δ' -tetrahydrocannabinol is being considered as a therapeutic agent in the treatment of glaucoma¹ and as an adjunct in the chemotherapy of neoplastic diseases.²

Several investigations have shown that cannabinoids can affect lipid metabolism, including an enhancement of lipolysis in adipose tissue³ and a depression of steroid hormone production in steroidogenic tissue.⁴⁻⁶ These studies have demon-

strated the effects of these drugs on the metabolism of preformed lipid esters. We have examined the effects of Δ' -tetrahydrocannabinol, cannabidiol, and cannabinol on the formation of cholesteryl esters in cultured human fibroblasts and aortic smooth muscle cells.

Methods

Materials

$1\text{-}^{14}\text{C}$ -oleic acid (40-60 mCi/mmole), $1,2\text{-}^3\text{H(N)}$ -cholesterol (58 Ci/mmole), Na ^{125}I (carrier free in 0.1 N NaOH), D,L-3-hydroxy-3-methyl (3- ^{14}C) glutaryl coenzyme A (40-60 mCi/mmole), R,S-5- ^3H -mevalonolactone (2-10 Ci/mmole), and $^3\text{H-L}$ -amino acid mixture were purchased from New England Nuclear (Boston, Massachusetts). Cholesterol, sodium oleate, cholesteryl oleate, and 25-hydroxycholesterol were obtained from Applied Science Division (State College, Pennsylvania). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XV), nicotinamide adenine dinucleotide phosphate (sodium salt), dithiothreitol, D,L-mevalonolactone, D,L-3-

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hydroxy-3-methylglutaryl coenzyme A (sodium salt), and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, Missouri). Dextran sulfate was obtained from Calbiochem-Behring Corporation (San Diego, California). Kryo EOB, a synthetic nonionic detergent that solubilizes the plasma membranes of cultured cells but not the endoplasmic reticulum,⁷ was a generous gift from Robert Dowben of Procter and Gamble (Cincinnati, Ohio). Dulbecco's Modified Eagle Medium, fetal calf serum (FCS), penicillin (10,000 U/ml), streptomycin solution (10,000 µg/ml), and trypsin/EDTA solution were purchased from Grand Island Biological Company (Grand Island, New York). Silica gel G (without gypsum) plastic-backed, thin-layer chromatography sheets were obtained from Brinkman Industries, Incorporated (Westbury, New York). All tissue culture plastic ware was from Falcon Plastics (American Scientific Products, McGaw Park, Illinois). Δ^1 -tetrahydrocannabinol (2789-50-1), cannabidiol (2310-139), and cannabinol (1823-65-3) were supplied by the National Institute on Drug Abuse (Rockville, Maryland).

Cells and Media

Normal human dermal fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey) and the American Type Culture Collection (Rockville, Maryland). The aortic medial cells were obtained from a kidney cadaver donor and were established from explants from the lower abdominal aorta as described.⁸ The cells were grown as monolayers on tissue culture plates and maintained in a humidified incubator at 37°C with an atmosphere of 92.5% air/7.5% CO₂. The stock culture medium consisted of Dulbecco's Modified Eagle Medium, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 44 mM NaHCO₃, and 10% (v/v) FCS.

All experiments were carried out using a standard format unless otherwise noted. Cells were dissociated from confluent stock cultures with 0.05% trypsin/0.02% EDTA and then seeded into 60 mm petri dishes, in fresh stock culture medium, at either 7.5×10^4 or 1×10^5 cells/plate. On Day 3 the medium was replaced with fresh stock culture medium. On Day 5 the medium was removed, the cells were washed once with Dulbecco's Modified Eagle Medium, and new media containing 10% human lipoprotein deficient serum (LPDS) (5 mg/ml) was added as the serum source. The cells were maintained in this medium for 48 hours before the start of each experiment.

Lipoproteins and Lipoprotein-Deficient Serum

Human low density lipoproteins (LDL) ($d = 1.019-1.063$ g/cc) were obtained from the plasma

of healthy volunteers by ultracentrifugation as described.⁹ ¹²⁵I-labeled LDL (¹²⁵I-LDL) was prepared using the iodine monochloride method of MacFarlane¹⁰ as modified by others.¹¹

Assays

The incorporation of ¹⁴C-oleate into labeled cholesteryl oleate was determined as described by Goldstein et al.¹² HMG-CoA reductase was measured by the method of Brown et al.¹³ Protein synthesis was assessed by examining the incorporation of a mixture of ³H-L-amino acids into trichloroacetic acid precipitable protein as outlined by Liberti and Miller.¹⁴ The metabolism of ¹²⁵I-LDL in cultured human fibroblasts at 4° and 37°C was measured by methods outlined by Goldstein et al.^{15, 16} Cell protein was determined by the method of Lowry et al.¹⁷ using a protein standard solution obtained from Sigma Chemical Company (St. Louis, Missouri).

Results

Figure 1 illustrates the effects of Δ^1 -tetrahydrocannabinol, cannabidiol, and cannabinol on LDL-induced cholesteryl ester formation in cultured human fibroblasts. When the cells were exposed to these cannabinoids before and during exposure to LDL, a marked, dose-related decrease in the incorporation of ¹⁴C-oleate into cellular cholesteryl esters was observed. There was no effect, however, on the incorporation of labelled compound into cellular phospholipid or triglyceride. The compounds also did not alter the incorporation of a mixture of ³H-amino acids into cellular trichloroacetic acid precipitable protein (data not shown). The inhibition of cholesteryl ester formation was also observed in a second normal fibroblast line as well as in human aortic medial cells (table 1). While all three drugs show inhibitory activity, a structural relationship seems to exist; the order of potency is cannabidiol > cannabinol > Δ^1 -tetrahydrocannabinol. These observations demonstrate that cultured human cells exposed to relatively low doses of cannabinoids are impaired in their ability to form steryl esters from LDL-derived cholesterol. The effect does not seem to be a generalized depression of lipid synthesis since phospholipid and triglyceride formation are not affected. We observed no changes in cell attachment or morphology throughout the course of the experiments and there were no significant differences in total cellular protein content between treatments. The cells also synthesized protein as well as control cells. These findings show that the experimental cells were viable and that the effect was specific only for cholesteryl ester formation.

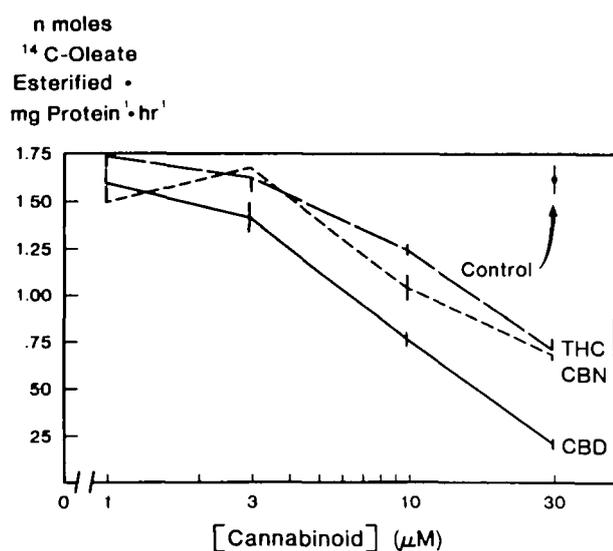


Figure 1. The effects of cannabinoids on low density lipoprotein (LDL)-induced cholesteryl ester formation. On Day 7 of cell growth, each plate received 2 ml of fresh media containing 10% lipoprotein deficient serum (LPDS) and the indicated concentration of Δ' -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabinol (CBN) dissolved in ethanol and diluted with 10% LPDS. Control cultures received an amount of ethanol equivalent to that contained in the highest drug dose, 0.14% final concentration. After incubation for 2 hours at 37°C, each plate received 200 μ g LDL protein/ml and was further incubated at 37°C for 4 hours. The plates were pulsed with 0.1 mM 1- 14 C-oleate (3146 DPM/nmole) and replaced in the incubator at 37°C for 2 hours. The media was removed, and the cells were washed and processed as described in the text. Each value is the mean of triplicate determinations \pm SEM.

To determine if the cannabinoids might be affecting the interaction of LDL with cell surface receptors, we carried out 125 I-LDL binding studies at 4°C. The results of these studies are presented in figure 2. No alteration of 125 I-LDL binding to its cell surface receptor was observed, although a less than 20% reduction was seen with 30 μ M cannabidiol. However, this percentage of perturbation was small when compared to the degree of inhibition of ester formation at that dose. Studies were also conducted with 125 I-LDL at 37°C to determine whether or not alterations in the internalization or degradation of the lipoprotein took place. Figure 3 displays the results of these experiments. Again, small decreases in LDL binding, internalization, and degradation were detected. These perturbations were of insufficient magnitude to account for the 80% decrease observed in cholesteryl ester formation. It does not appear, therefore, that the cannabinoids interacted with either the LDL particle or the LDL receptor to cause a significant decrease in lipoprotein binding, internalization, or degradation.

Several laboratories have shown that certain oxygenated sterols, such as 7-ketocholesterol and 25-hydroxycholesterol (25-OH), are freely taken up by cells independently of the LDL receptor. When presented to cells grown in LPDS, these compounds caused an immediate esterification of newly synthesized cholesterol by directly activating acyl-cholesterol acyltransferase (A-CAT). Therefore, all cells treated with 25-OH maximally esterified cholesterol regardless of LDL receptor status so long as the A-CAT system functioned effectively.¹⁸ Fibroblasts incu-

Table 1. Cannabinoid Inhibition of LDL-Induced Cholesteryl Ester Formation in Cultured Human Aortic Medial Cells

Concentration (μ M)	nmoles 14 C-oleate esterified/mg/hr†		
	THC	CBD	CBN
Control*	8.46 \pm 0.09		
1	8.02 \pm 0.31	7.97 \pm 0.19	8.07 \pm 0.85
3	7.81 \pm 0.52	5.86 \pm 0.84	5.18 \pm 0.32
10	6.22 \pm 0.26	2.54 \pm 0.13	4.17 \pm 0.12
30	3.00 \pm 0.36	0.66 \pm 0.05	1.62 \pm 0.34

*Control incubations carried out with vehicle (ethanol) only.

†Values are the mean of triplicate determinations \pm SEM.

On Day 7 of cell growth, each monolayer received 2 ml of fresh media containing 10% lipoprotein deficient serum (LPDS) and the indicated concentration of Δ' -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabinol (CBN) dissolved in ethanol and diluted with 10% LPDS. Control cultures received an amount of ethanol equivalent to that contained in the highest drug treatment, 0.14% final concentration. After 2 hours of incubation at 37°C, each plate received 200 μ g of low density lipoproteins (LDL) protein/ml and was further incubated at 37°C for 4 hours. The plates were pulsed with 0.1 mM 1- 14 C-oleate (2344 DPM/nmole) and replaced in the incubator for 2 hours. The media was removed and the cells rinsed and processed as described.

bated with increasing concentrations of cannabinoids for 2 hours before and during exposure to 25-OH showed a dose-related inhibition of cholesterol esterification similar to that observed with LDL (figure 4). Since the experiment was performed in the absence of LDL, and the 25-OH stimulus bypasses the LDL receptor, the data suggest that Δ' -tetrahydrocannabinol, cannabidiol, and cannabinol might act to inhibit cholesteryl ester formation by inhibiting A-CAT activity.

If the above hypothesis were true, an accumulation of free cholesterol within the cells would occur. This free cholesterol might inhibit the activity of HMG-CoA reductase. Thus, with increasing inhibition of A-CAT, and the accompanying increase in cellular free cholesterol content, we should observe increasing inhibition of HMG-CoA reductase activity. To test this hypothesis, we measured reductase activity in cultured human fibroblasts treated with various concentrations of Δ' -tetrahydrocannabinol, cannabidiol, and cannabinol followed by incubation with LDL.

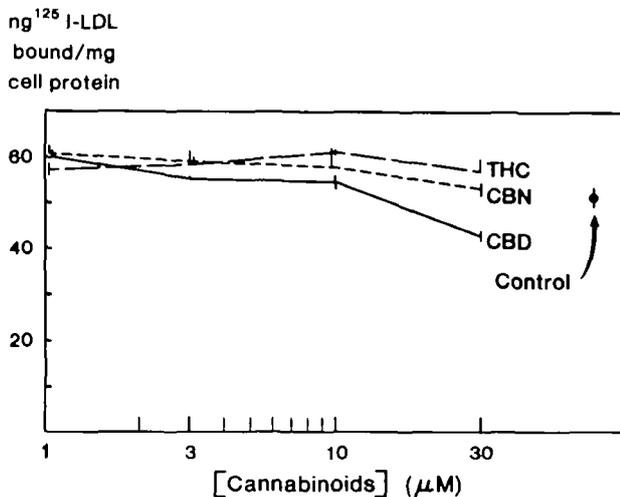


Figure 2. Absence of a cannabinoid effect on ^{125}I -labeled low density lipoproteins (^{125}I -LDL) binding to human fibroblasts at 4°C . On Day 7 of cell growth, each monolayer received 2 ml of fresh media containing 10% lipoprotein deficient serum (LPDS), and the indicated concentration of Δ' -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabinol (CBN) dissolved in ethanol and diluted with 10% LPDS. Control cultures received an amount of ethanol equivalent to that contained in the highest drug dose, 0.14% final concentration. After 2 hours incubation at 37°C , each plate was chilled to 4°C and presented with $5\ \mu\text{g}\ ^{125}\text{I}$ -LDL/ml (168 cpm/ng). After incubation for 2 hours at 4°C , the cells were washed extensively,¹⁵ and the amount of dextran sulfate-releasable ^{125}I -LDL was determined. Values plotted are the average of triplicate determinations \pm SEM.

The results of these studies are presented in table 2. Instead of observing a dose-related decrease in enzyme activity, we found a rise in reductase activity with increasing cannabinoid concentration. Apparently, the cannabinoids do not act in a manner that appreciably increases the regulatory pool of intracellular free cholesterol.

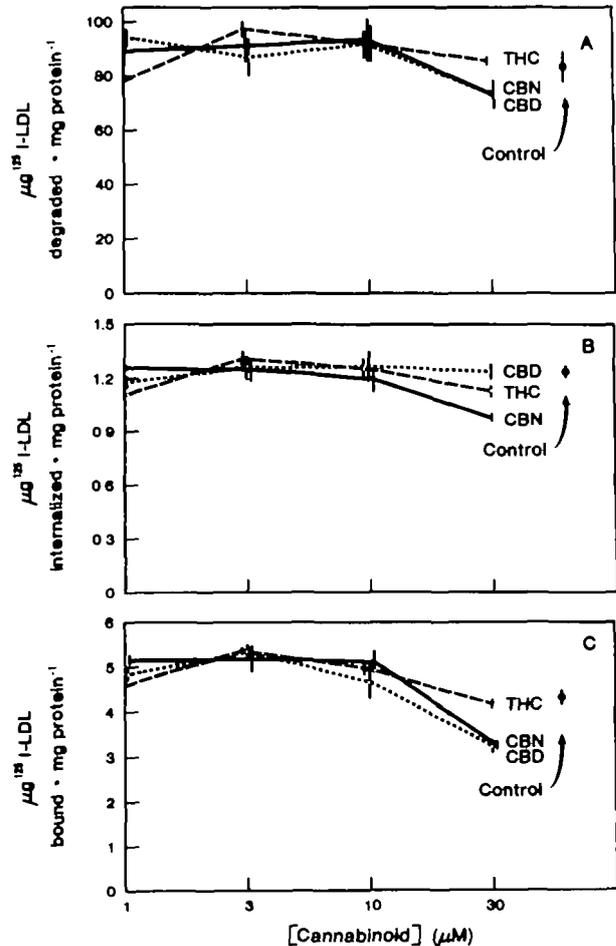


Figure 3. The effect of cannabinoids on ^{125}I -labeled low density lipoproteins (^{125}I -LDL) binding, internalization and degradation at 37°C . On Day 7 of cell growth, each monolayer received 2 ml of fresh media containing 10% lipoprotein deficient serum (LPDS), and the indicated concentration of Δ' -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabinol (CBN) dissolved in ethanol and diluted with 10% LPDS. Control cultures received an amount of ethanol equivalent to that contained in the highest drug dose, 0.14% final concentration. After 2 hours of incubation at 37°C , each plate received $25\ \mu\text{g}/\text{ml}\ ^{125}\text{I}$ -LDL (93.2 cpm/ng). The cells were further incubated at 37°C for 5 hours and then processed accordingly.^{15,16} Panels A, B, and C show the amount of ^{125}I -LDL degraded, and bound, respectively, as a function of cannabinoid concentration.

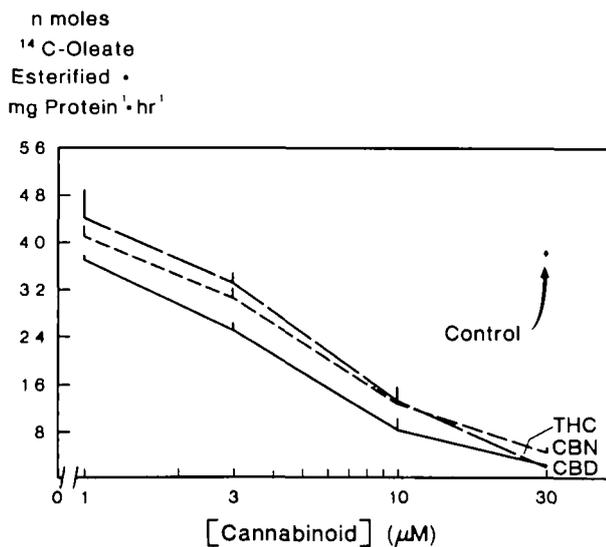


Figure 4. Cannabinoid inhibition of 25-hydroxycholesterol induced cholesteryl ester formation. On Day 7 of cell growth, each monolayer received 2 ml of fresh media containing 10% lipoprotein deficient serum (LPDS) and the indicated concentration of Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabinol (CBN) dissolved in ethanol and diluted with 10% LPDS. Control cultures received an amount of ethanol equivalent to that contained in the highest drug dose, 0.14% final concentration. After incubation at 37°C for 2 hours, each plate received 5 $\mu\text{g}/\text{ml}$ of 25-hydroxycholesterol and 0.1 mM 1- ^{14}C -oleate (1845 DPM/nmole). The monolayers were incubated at 37°C for 6 hours, after which the media was removed, the monolayers rinsed and processed as described in the text. Values are the mean of triplicate determinations \pm SEM.

Discussion

These studies demonstrate that Δ^9 -tetrahydrocannabinol, cannabidiol, and cannabinol specifically decrease the esterification of cholesterol derived from either LDL or from new synthesis. This is the first time that these compounds have been shown to alter cholesteryl ester formation in cultured human fibroblasts. This effect was seen in the absence of an inhibition of ^{125}I -LDL binding at 4°C and of ^{125}I -LDL metabolism at 37°C. The cells were also shown to be competent to carry on protein, triglyceride, and phospholipid synthesis without significant cell loss when treated with the compounds at the doses used. Accordingly, any proposed mechanism for these effects must be consistent with these observations.

One possibility that might account for our findings would be a direct inhibition of A-CAT. This would account for the changes in cholesteryl ester formation in both LDL and 25-OH treated cells, without altering LDL metabolism. However, this does not appear to be consistent with the lack of suppression of HMG-CoA reductase activity in the cells exposed to LDL.

Alternatively, cellular cholesterol may be "compartmentalized" in some way by the drugs so as to be unavailable for regulating cellular cholesterol metabolism. This compartmentalization could be in the form of an intracellular cannabinoid-cholesterol complex. This proposed complex is quite feasible given the high solubility of these compounds in lipids. When incubated with plasma, Δ^9 -tetrahydrocannabinol binds primarily to lipoproteins,¹⁹ with most of the drug being bound to LDL.²⁰ This binding is related to the lipoprotein content of total or neutral lipid as opposed to that of phospholipid or protein. In

Table 2. Effect of Cannabinoid Treatment on LDL Suppression of HMG-CoA Reductase Activity

Concentration (μM)	pmoles MVA formed $\text{mg}^{-1} \text{min}^{-1}$ *		
	THC	CBD	CBN
LPDS	198.78 \pm 19.9		
LPDS + LDL	39.92 \pm 2.3		
1	37.06 \pm 3.4	37.55 \pm 0.2	31.46 \pm 2.7
3	36.16 \pm 4.4	30.49 \pm 2.7	27.77 \pm 0.5
10	45.29 \pm 1.9	49.24 \pm 10.0	63.98 \pm 12.1
30	81.26 \pm 3.9	168.30 \pm 14.1	143.06 \pm 9.1

*Values are the mean of triplicate determinations \pm SEM.

On Day 7 of cell growth, each monolayer received 2 ml of fresh media containing 10% lipoprotein deficient serum (LPDS) and the indicated concentration of Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabinol (CBN) dissolved in ethanol and diluted with 10% LPDS. Control cultures received an amount of ethanol equivalent to that contained in the highest drug dose, 0.14% final concentration. After incubation at 37°C for 2 hours, each plate received 25 μg low density lipoproteins (LDL) protein/ml. Half of the control cultures did not receive LDL. All plates were returned to the incubator for 5 hours, and then were processed for the determination of HMG-CoA reductase activity.¹³

our studies all cells were incubated with cannabinoids for 2 hours in media devoid of lipoproteins (10% LPDS) before LDL was introduced.

On the other hand, the drugs may act by promoting the efflux of cholesterol from the cells. This action would result in a decreased availability of cholesterol for esterification, whether it is derived from LDL or new synthesis. Since cellular stores of cholesterol would then be low, HMG-CoA reductase activity would be high even in the presence of LDL. Our data are consistent with this hypothesis. Additional studies of cellular balance are necessary to verify it.

If the cannabinoids do exert their effect by increasing cellular cholesterol efflux, they might provide a new range of therapeutic possibilities as well as experimental tools for the study of lipid metabolism derangements. One major problem in atherosclerosis treatment has been the mobilization of lipid already deposited in the arterial wall. Cannabinoids or cannabinoid-like compounds may provide the foundation for lipid-removal therapy. Cannabidiol proved to be the most potent compound tested. It is also the least active psychogenically. Using this compound and the efficacy of its structure, medicinal chemists may be able to develop more potent, related compounds. Further studies explaining the mechanism of cellular action in these substances will be needed before their therapeutic potential can be determined.

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