Cytotoxic Assessment of Hydrogenated CBD (H_4CBD) in Primary Human Cells

Hydrogenated Cannabidiol (H_4CBD) used in preclinical in *vitro* studies was synthesized at Colorado Chromatography Labs (Parker, CO). The study was conducted at Charles River Laboratories Cleveland, OH; on primary normal human lung fibroblasts (NHLFs), primary human neural progenitor cells (NPCs), and primary human hepatocytes were cultured and exposed to H_4CBD. In addition, (hERG) test was also conducted to determine its effect of cardiac arrhythmias.

Cytotoxicity

Cell viability assay using primary normal human lung fibroblasts: The cell viability assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. Hydrogenated Cannabidiol (H_4CBD) was compared to the negative control (1% DMSO) and positive control (1% SDS in media) were tested at a final concentration ranging from 1.56 to 50 µM. Hydrogenated Cannabidiol (H_4CBD) showed better cell viability results in all concentrations compared to the positive control in 24 hours. However, significant cytotoxicity was observed at concentrations above 1.56 µM and near total loss of cell viability with greater concentration and increased exposure, 48 and 72 hours, as shown in figure 1.

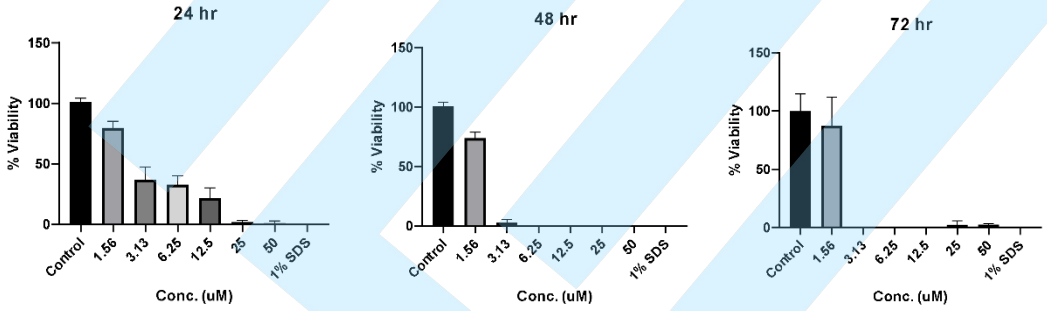

Figure 1: Cytotoxicity in NHLF

Cell viability assay using plated human hepatocytes: Similar to the lung fibroblasts, the high sensitive ATP CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the potential of *in vitro* liver toxicity of hydrogenated cannabidiol (H_4CBD). Hydrogenated Cannabidiol (H_4CBD) didn't not show significant drop in cell viability. The data showed H_4CBD had a better and favorable result than the positive control (1% SDS in media), as shown in figure 2. The results indicate there is no statistically significant drop in viability among hepatocytes.

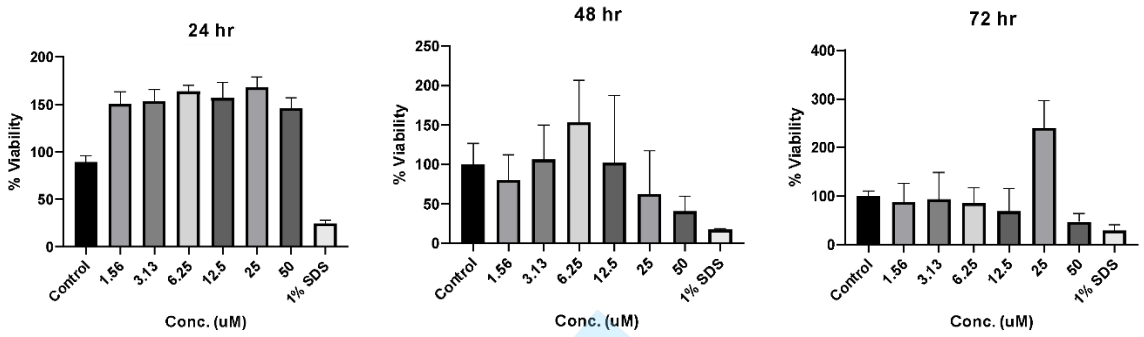

Figure 2: Cytotoxicity in Hepatocytes

Cell viability assay using primary human neural progenitor cells (NPCs): Similar to the lung fibroblasts and pated human hepatocytes, the high sensitive ATP CellTiter-Glo® Luminescent Cell Viability Assay was used to determine the potential of *in vitro* primary human neural progenitor cells (NPCs) of hydrogenated cannabidiol (H_4CBD). Hydrogenated Cannabidiol (H_4CBD) showed better results in all concentrations compared to the positive control ((1% SDS in media) in 24 hours. Loss in cell viability was observed in concentrations 6.25 µM and above at 24 hours. At 48 and 72 hours, severe loss in cell viability was observed.

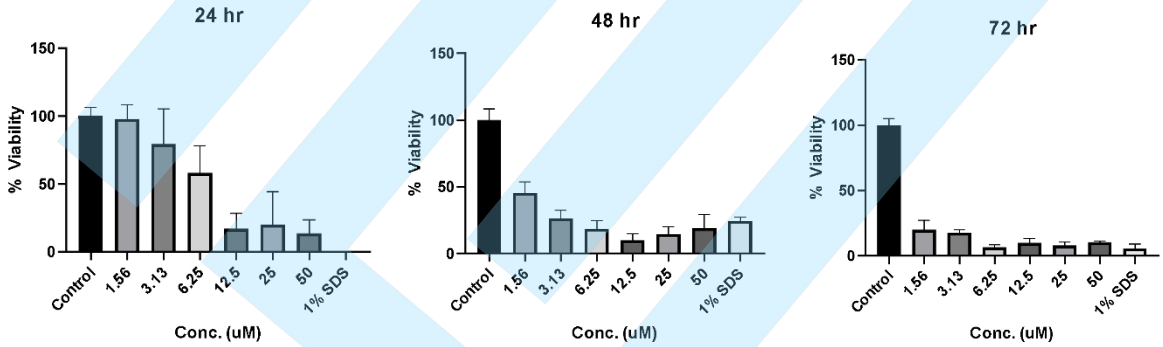

Figure 3: Cytotoxicity in NPCs

In vitro Cardiac Safety test

Evaluation of hERG current using patch clamp analysis. All along the drug development process, one of the most frequent adverse side effects, leading to the failure of drugs, is the cardiac arrhythmias. Such failure is mostly related to the capacity of the drug to inhibit the human ether-à-go-go-related gene (hERG) cardiac potassium channel. Inhibition of the hERG current causes QT interval prolongation resulting in potentially fatal ventricular tachyarrhythmia called Torsade de Pointes. To evaluate anticipated cardiovascular effects, early evaluation of hERG toxicity has been strongly recommended for instance by the regulatory agencies such as U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA).

The effect of hydrogenated cannabidiol (H_4CBD) on cloned hERG potassium channels (encoded by the KCNH2 gene and expressed in HEK293 cells) was examined using the QPatch II® (Sophion Bioscience A/S, Denmark), an automatic parallel patch clamp system. Hydrogenated cannabidiol (H_4CBD) was exposed to hERG at 0.0625, 0.125, 0.625, 1.25, 6.25, 12.5, 25, and 50 µM ($n \geq 3$). The duration of exposure to each test article concentration was a minimum of three (3) minutes. The positive control data confirmed the sensitivity of the test systems to ion channel inhibition.

The results suggest that hydrogenated cannabidiol (H_4CBD) does not block the HERG-encoding channels that expressed the HEK293 cells until above 1.25 µM. In order to determine if H_4CBD effects other channels, Cav1.2 and Nav1.5 assays were conducted. The results showed H_4CBD inhibited Cav1.2 calcium and Nav1.5 sodium channels with the same effect as the hERG potassium channels, as shown in tables 1-3. This effect of the depolarization and repolarization by these channels results in a net zero inhibition.

Table 1: Effects of H4CBD on hERG Ion Channel Current

Test Article ID	IC50 (µM)	Conc (µM)	Mean % hERG Inhibition	Standard Deviation	Standard Error	n
H4CBD A	<6.25	0.0625	7.1	4.3	1.6	7
		0.125	15.5	7.0	4.0	3
		0.625	38.0	5.9	3.4	3
		1.25	64.7	9.7	3.4	8
		6.25	100.0	0.3	0.1	9
		12.5	99.6	1.0	0.3	9
		25	100.0	0.2	0.1	5
		50	98.1	3.7	1.6	5
Cisapride (Positive control)		0.05	66.5	2.2	1.1	4

Table 2: Effects of H4CBD on Cav1.2 Ion Channel Current

Test Article ID	IC50 (µM)	Conc (µM)	Mean % hCav1.2 Inhibition	Standard Deviation	Standard Error	n
H4CBD A	<6.25	0.0625	3.0	3.9	2.2	3
		0.125	8.4	5.8	3.4	3
		0.625	24.4	3.5	2.0	3
		1.25	36.7	7.3	3.0	6
		6.25	97.1	2.8	1.4	4
		12.5	97.8	3.1	1.3	6

Table 3: Effects of H4CBD on Nav1.5 Ion Channel Current

Test Article ID	IC50 (µM)	Conc (µM)	Mean % Late Nav1.5 Inhibition	Standard Deviation	Standard Error	n
H4CBD A	<6.25	0.0625	5.0	3.7	2.1	3
		0.125	1.7	1.9	1.1	3
		0.625	20.7	6.9	2.4	8
		1.25	27.8	7.5	3.3	5
		6.25	70.0	8.8	3.6	6

Conclusion

Hydrogenated cannabidiol (H_4CBD) synthesized by Colorado Chromatography Labs, was shown to be non-cytotoxin in human hepatocytes cell viability assy. It showed better results in cell viability in NHFL, and NPCs compared to the positive control. Prolonged exposure and increase concentration led to significant and in some total loss of cell viability. The hERG, Cav1.2, and Nav1.5 assays showed H_4CBD had an equal inhibition of all three ion channels. Having equal inhibition, depolarization and repolarization, gives a net zero potential. This indicates H_4CBD having no cardiac safety issues.

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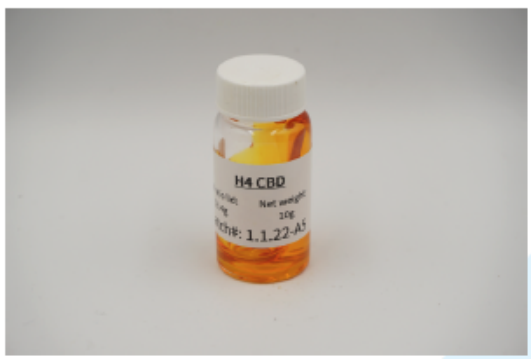
Certificate of Analysis

H4-CBD

Sample ID: SA-220427-8842
Batch: 1.1.22-A5
Type: In-Process Materials
Matrix: Concentrate - Distillate
Unit Mass (g):

Received: 04/27/2022
Completed: 05/06/2022

Client
Colorado Chromatography
1050 S Progress Way, Unit 105
Parker, CO 80134
USA



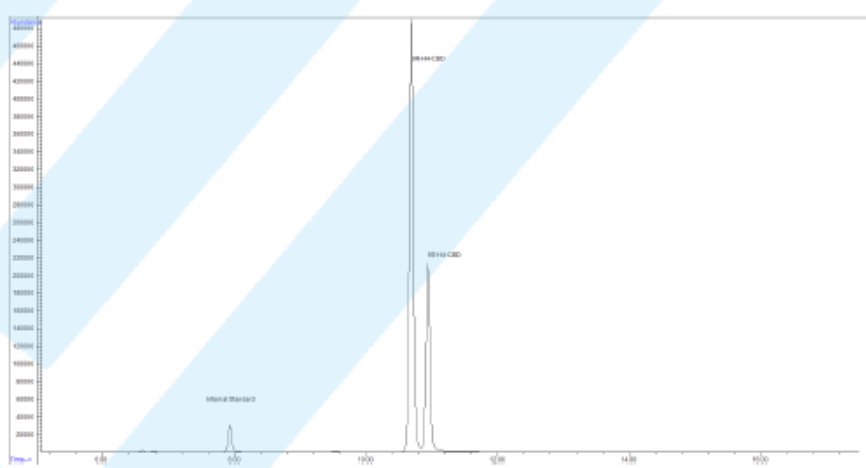
Summary

Test	Date Tested	Status
Cannabinoids	05/06/2022	Tested

ND	69.1%	99.5%	Not Tested	Not Tested	Yes
Total Δ9-THC	9R-H4-CBD	Total Cannabinoids	Moisture Content	Foreign Matter	Internal Standard Normalization

Cannabinoids by HPLC-PDA, LC-MS/MS, and/or GC-MS/MS

Analyte	LOD (%)	LOQ (%)	Result (%)	Result (mg/g)
CBC	0.0095	0.0284	ND	ND
CBCA	0.0181	0.0543	ND	ND
CBCV	0.006	0.018	ND	ND
CBD	0.0081	0.0242	ND	ND
CBDA	0.0043	0.013	ND	ND
CBDV	0.0061	0.0182	ND	ND
CBDVA	0.0021	0.0063	ND	ND
CBG	0.0057	0.0172	ND	ND
CBGA	0.0049	0.0147	ND	ND
CBL	0.0112	0.0335	ND	ND
CBLA	0.0124	0.0371	ND	ND
CBN	0.0056	0.0169	ND	ND
CBNA	0.006	0.0181	ND	ND
CBT	0.0181	0.0543	ND	ND
Δ8-THC	0.0104	0.0312	ND	ND
Δ9-THC	0.0076	0.0227	ND	ND
Δ9-THCA	0.0084	0.0251	ND	ND
Δ9-THCV	0.0069	0.0206	ND	ND
Δ9-THCVA	0.0062	0.0186	ND	ND
Δ9-cis-THC	0.0095	0.0284	ND	ND
9R-H4-CBD	0.1	0.3	69.1	691
9S-H4-CBD	0.1	0.3	30.3	303
Total Δ9-THC			**ND**	**ND**
Total CBD			**ND**	**ND**
Total			**99.5**	**995**



ND = Not Detected; NT = Not Tested; LOD = Limit of Detection; LOQ = Limit of Quantitation; RL = Reporting Limit; Δ = Delta; Total Δ9-THC = Δ9-THCA * 0.877 + Δ9-THC; Total CBD = CBDA * 0.877 + CBD;

Generated By: Ryan Bellone
Commercial Director
Date: 05/06/2022

Tested By: Scott Caudill
Senior Scientist
Date: 05/06/2022

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