NONCLINICAL *In Vitro* SAFETY ASSESMENT SUMMARY of HEMP DERIVED (R/S)-HEXAHYDROCANNABINOL ((R/S)-HHC)

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(R/S)-Hexahydrocannabinol ((R/S)-HHC) used in the preclinical *in vitro* studies was synthesized by Colorado Chromatography Labs (Parker, CO). The studies were conducted at Charles River Laboratories [Worcester, MA (cell viability); Cleveland, OH (hERG); and Skokie, IL (Ames)]. All studies were exploratory and carried out under a non-GLP environment. All procedures carried out in the laboratory were reviewed and approved by the Institutional Authorities.

Genotoxicity assay

Microbial mutagenesis. The Ames test is one of the most frequently applied tests in toxicology. Almost all new pharmaceutical substances and chemicals used in industry are tested by this assay. The (*Salmonella typhimurium* reverse mutation assay) is a bacterial short-term test for identification of carcinogens using mutagenicity in bacteria as an end point. The Ames test detects mutations in a gene of a histidine-requiring bacterial strain that produces a histidine-independent strain. Both direct and indirect (i.e., chemicals that require metabolic activation) mutagens can be identified using the Ames test.

Potential of (R/S)-HHC to induce reversion mutations in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA both with and without metabolic activation was evaluated. The plate incorporation screen for (R/S)-HHC was tested in duplicate with strains TA98, TA100, TA1535, TA1537, and WP2 uvrA in the presence and absence of a metabolic activation system (phenobarbital/5,6-benzoflavone-induced rat liver S9 microsomal fraction) at 1, 5, 10, 50, 100, 500, 1000, and 5000 µg/plate. The positive controls without metabolic activation were 2 nitrofluorene (TA98, 2.5 µg/plate), sodium azide (TA100 and TA1535, 1 µg/plate), ICR-191 Acridine (TA1537, 0.5 µg/plate), and 4 nitroquinoline N oxide (WP2 uvrA, 2.0 µg/plate). The positive control with metabolic activation for all strains was 2-

aminoanthracene (2.5 µg/plate for Salmonella strains and 10 µg/plate for the E. coli strain). DMSO was used as the vehicle control.

(R/S)-Hexahydrocannabinol was not mutagenic, up to concentrations at ≥ 100 µg/plate in strain TA1535 without metabolic activation, and at ≥ 500 µg/plate in strains TA98, TA100, TA1537, and WP2 uvrA both with and without metabolic activation and in strain TA1535 with metabolic activation. The data from Ames test suggests that (R/S)-HHC is a non-mutagenic compound.

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).

Table 1: Effects of (R/S)-HHC on hERG Ion Channel Current

Test Article	IC_{50} (µM)	Conc (µM)	Mean % hERG Inhibition	Standard Deviation	Standard Error	n
(R/S)-HHC	**>50**	6.25	4.8	3.0	1.0	9
		12.5	5.7	2.5	0.9	7
		25	2.3	3.8	2.2	3
		50	6.4	5.4	2.7	4
Cisapride (positive control)	**<0.05**	0.05	64.1	7.6	3.8	4

The effect of (R/S)-hexahydrocannabinol 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. (R/S)-Hexahydrocannabinol was exposed to hERG at 6.25, 12.5, 25 and 50 µM (n ≥ 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.

A summary of the results for (R/S)-hexahydrocannabinol and the positive controls are shown in Table 1 above. The data suggests that (R/S)-HHC does not block HERG-encoded channels expressed in HEK293 cells.

Cytotoxicity

Cell viability assay using 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. (R/S)-HHC and the control (chlorpromazine) were tested at a nominal concentration range of 0.156 to 50.0 µM for cell viability (cytotoxicity) in human lung fibroblasts. In lung fibroblasts, the IC50 for (R/S)-HHC was 14.4 µM, and the % cytotoxicity at 50 µM was 74.8. The control, chlorpromazine, showed an IC50 value of 14.3 µM and 86.4% cytotoxicity at 50 µM. The data is summerized in Table 2.

Table 2: Summary of cell viability (cytotoxicity) in fibroblasts following 24-hour incubation

Cell Line	Compound ID	IC_{50} (µM)	Cytotoxicity (at 50 µM)
Fibroblasts	(R/S)-HHC	**14.4**	74.8%
Fibroblasts	Chlorpromazine (control)	**14.3**	86.4%

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 determined the potential of *in vitro* liver toxicity of (R/S)-HHC. The test compound ((R/S)-HHC) and the control (Terfenadine) were tested at a concentration range of 0.05 to 50.0 μM for cell viability (cytotoxicity) in human hepatocytes. In human hepatocytes, (R/S)-HHC was non-cytotoxic and the IC_{50} for (R/S)-HHC was not measured, and the % cytotoxicity at 50 μM was also very low (8.9%) due to its weak response. Terfenadine showed an IC_{50} value of 15.8 μM and 99.9% cytotoxicity at 50 μM. The data is summerized in Table 3. The results indicated that there is no potential for hepatotoxicity, with a very weak results in this human hepatocytes cell viability assay.

Table 3: Summary of cell viability (cytotoxicity) in human hepatocytes following 48 hours incubation

Compound	Incubation Time	Cell Lines	IC_{50} (μM)	Cytotoxicity (at 50 μM)	Comments
(R/S)-HHC	48 hrs	Plated human hepatocytes	**Not determined**	8.9%	Nontoxic
Terfenadine	48 hrs	Plated human hepatocytes	**15.8**	99.9%	Showed toxicity as expected

Conclusion

(R/S)-HHC derived from Colorado Chromatography Labs, using their patent pending technology demonstated the following results. (R/S)-HHC was not mutagenic in *in vitro* genotoxic assay (Ames test). Preclinical assessments in the hERG patch clamp study did not indicate that (R/S)-HHC had any cardiac safety issues, which suggests that there does not appear to be an effect of (R/S)-HHC and QTc prolongation. The finding in the cell viability studies demonstrated potential cytotoxic effects of (R/S)-HHC in human lung fibroblasts at > 10 μM concentrations and it was not cytotoxic or damaging to human liver cells in *in vitro* human hepatocytes cell viability assay.