Percent Composition Testing Should Not Be Confused with Potency Testing:

Why the Expanding Cannabis Market Needs to Move Beyond Old Metrics

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We assert that the interaction between plant cannabinoids and the human endocannabinoid system, which produces the qualitative experience, cannot be predicted from percent cannabinoid composition alone. The addition of biologic potency testing will provide the cannabis industry with a means of evaluating plant grows, plant extracts, and synthetic blends before bringing products to market. This will allow for the formulation of products with known dose-dependent outcomes and result in informed consumer choice and product loyalty. In this article we examine the unmet industry need for commercial cannabis testing that anticipates the dose-dependent biologic potency of cannabinoids.

Introduction

Percent Composition as the Status Quo

The measurement and subsequent regulation of cannabis and hemp products based on Δ^9-tetrahydrocannabinol (THC) percent composition has been a major focus of the industry. Boutique cannabis products continue to enter the medicinal and adult-use markets, bringing with them an ever-increasing diversity of cannabinoid content. However, the industry has not responded in kind with novel or more targeted means of determining product potency. Instead, consumers are left to infer product potency from the amount of a handful of measured cannabinoids in these products. A typical cannabinoid panel will report the percent composition of cannabidiol (CBD), Δ^9-THC, Δ^8-THC, cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabidivarin (CBDV), tetrahydrocannabivarin (THCV), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), and tetrahydrocannabinolic acid (THCA). For some of these cannabinoids, most notably Δ^9-THC and more recently CBD, their ability to alter human physiology is common knowledge. However, reports in scientific literature reveal the ability of numerous minor cannabinoids to alter human physiology (1). Furthermore, combinations of major and minor cannabinoids and even terpenes interact with the human endocannabinoid system (ECS) to produce responses distinct from each cannabinoid in isolation (2-4). Thus, while major and minor cannabinoids are frequently thought of as independently acting agents, it should not be surprising that consumers of cannabis products report experiencing greater benefits from full and broad-spectrum products (containing a diverse set of cannabinoids and terpenes) than from isolates (containing as few as a single cannabinoid) (2,5).

Given that potency is a measure of the human biologic response to a given drug, what does percent cannabinoid composition really predict about

a product? The current cannabis market is broadly consumer-driven, which forces producers to be reactionary (6). Rather than relying on quantitative methods, which provide companies with predictive power at the level of individual cannabinoids and blends, the industry relies on data trends in consumer purchases to inform the next round of product formulation (6). Without reconciling percent composition with biologic potency, the industry is walking on thin ice as consumers are faced with an ever-increasing diversity of products with little basis for consumer choice. This is in addition to the problem reported in studies conducted by the Food and Drug Administration (FDA), National Institutes of Health (NIH), the *Journal of the American Medical Association* (JAMA) and Johns Hopkins research institutes which each demonstrated that Δ^9-THC and CBD content in products can be vastly different from the product label (7). While some of this chemical composition inconsistency can be attributed to the lack of universally adopted standards and methods for determining chemical composition (8), we assert that the true risk to the industry is an absence of standards and methods that rely on medically relevant biologic endpoints for validating potency and consistency. Biologic potency testing will also reveal grow-to-grow consistency, batch-to-batch extraction consistency and product shelf life.

How did the cannabis industry and government regulation get so far off track? It is important to acknowledge the history of cannabis regulation and Δ^9-THC's standing as an illicit substance that affected all cannabinoids and impeded scientific understanding of the endocannabinoid system. As cannabis and cannabis research become more mainstream, it has become clear to the research community that the combinations of major and minor cannabinoids affect the endocannabinoid system with significantly more complexity than can be inferred from how much of each is in a product. This includes the observations that: 1) higher purity formulations of cannabis don't guarantee higher therapeutic efficacy, 2) source material containing contamination of pure cannabinoid isolates with stereoisomers or chemically similar cannabinoids can result in inconsistent reports, and 3) the capacity of cannabinoids to act synergistically (or antagonistically) has not been adequately understood for its impact on potency (9).

Why Does Method Matter?

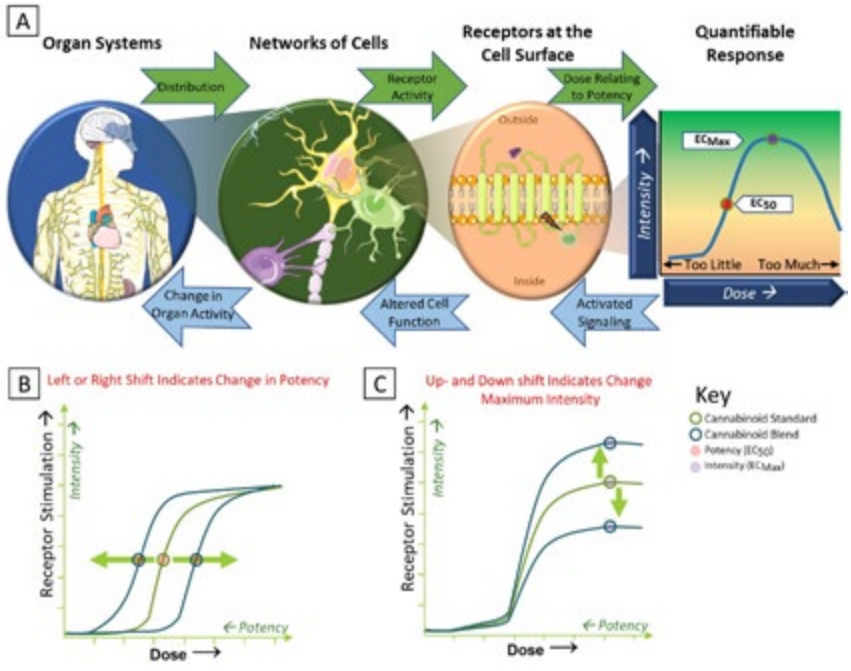
The percentage of Δ^9-THC by weight in a product is often colloquially referred to as its potency which is used to roughly differentiate between cannabis products with psychoactive properties and those without. In this context, potency in units of % total mass become the preferred measurement following the 2018 Farm Bill, which sets the legal limit for Δ^9-THC in a hemp product at 0.3% of total mass (10). However, this limit is a carryover from a 1976 research publication which "arbitrarily" assigned 0.3% THC as the means to distinguish between northern grown "low-intoxicant" type plants, and southern grown "high-intoxicant" type plants (11). The publication, *Taxon*, was quick to point out that factors including laboratory methodology, plant maturity, storage conditions, and which parts of a plant were used in the measurements can all drastically change the percent composition of Δ^9-THC reported (11). As a benchmark for intoxication, percent composition for Δ^9-THC alone follows a simple "more is more" association with potency and defines 0.3% as being sufficiently below the psychoactive threshold of Δ^9-THC (generally set at 0.9%) (12,13). In this respect percent composition is similar in its regulatory capacity to the 0.5% threshold for alcohol by volume (ABV).

Biologic potency, however, is a rigorously defined term which provides two quantitative measures: 1) a chemical/drug/medicine's dose, and 2) the intensity of the cell/tissue/body's response (14,15). The potency of cannabinoids arises from the strength of an interaction between a chemical and the receptor for that chemical found on the surface of cells throughout the body and from the outcome of that interaction. The relationship between dose and response is measured by the healthcare industry by quantifying the magnitude of the response from very low doses of a chemical which may trigger little or no response, and repeating the measurement with increasing amounts of the same compound until a maximum response is recorded (Such as, a dose-response study). Comparatively speaking, products that produce stronger biologic responses at a lower dose would be considered more potent and thus follow a "less-is-more" relationship with potency and safety. The potency of a chemical acting on a given target or of multiple chemicals acting concertedly on multiple targets can be calculated and reported as a single value, the 'EC50', the dose at which half of the maximum possible response can be observed. EC50 is used across the pharmaceutical industry as a standard for a chemical or drug's potency. A correlation in the dose response is that biologically relevant drug targets will not have unlimited ability to respond to an ever-increasing dose and will eventually stop responding to higher doses, known as 'saturation', also known as the Effective Concentration Maximum (ECmax). Chemical composition is important as it provides information on the chemical purity of the preparation in establishing the doses used to determine EC50 and ECmax.

Biologic Potency and the Endocannabinoid System

Cannabis receptors are components of the endocannabinoid system which

Legend for Figure 1: CannaMetrix Receptor Technology at A Glance. (A) *From left to right* (green arrows): the fundamental basis of biologic potency testing is that cannabinoids are absorbed and distributed throughout the body to interact with protein receptors exposed on the external surfaces of our cells. When an interaction induces a response by the receptor, a change in biology is initiated. The ability of a compound to initiate this change is the biological potency of that compound. *From right to left* (blue arrows): The receptor response on the cell surface sends a signal to the inner workings of the cell that triggers a new set of functions in the cell. The altered cellular functions can cause major changes in how cell networks or organs function, leading to a change in physiology. CannaMetrix uses cells engineered to intercept the receptor signal and produce a quantifiable response. (B, C) Hypothetical dose of a CB1 receptor agonist (○) increases from left to right on the *x*-axis and intensity of the receptor response increasing from bottom to top along the *y*-axis. (B) A cannabinoid in a blend (○) that increases potency (●) can be observed as a leftward shift whereas a decrease can be observed as a rightward shift. (C) A cannabinoid in a blend that increases intensity (●) can be observed as upward shift whereas a decrease can be observed as a downward shift. Parts of the figure were drawn by using open-source images from Servier Medical Art (24).



trigger changes in the biochemistry inside the cell leading to altered cellular function. Typically, these receptors are triggered by endocannabinoids made within our bodies and are believed to play an important role in keeping body functions such as mood, metabolism, pain sensation, and immune response, within their acceptable ranges (16). When stimulated by plant-based cannabinoids, these receptors can initiate stronger and more prolonged changes in cellular biochemistry. Multiple cells triggered in this way at the same time results in new functions at the tissue and organ levels, known as the entourage effect, which ultimately determines the consumer's physiologic and psychologic experience (see **Figure 1A**). This is particularly important for cannabis products which may contain several of the over one hundred different cannabinoid chemistries produced by the plant whose net potency can only be fully appreciated by measuring cellular responses. While current understanding is limited, studies examining the potency of at least five psychoactive (17) and nine non-psychoactive (18) cannabinoids against the canonical cannabinoid receptors, demonstrate a broad dose range at which ECmax values are observed, and implicate up to 22 other biologic targets for these cannabinoids. Different cannabinoids will have different EC50s; some act potently at low doses. Hence, dose curves will reveal a product's potential activity in human physiology.

To be clear, potency (EC50) and efficacy (ECmax) are distinct characteristics of each cannabinoid, but can change when combinations of cannabinoids are consumed. Therefore, it is inappropriate to rule out the contribution of so-called minor cannabinoids in a product as not being potent simply due to their smaller percent composition. In addition, numerous cannabinoids are converted through human tissue metabolism to their biologically active form before they can exert an effect on their endocannabinoid targets (19). Many of the cannabinoid conversions are carried out by enzymes that have their inherent efficiencies. The rate of cannabinoid conversion and removal by these enzymes further contributes to the actual or perceived potency of products. Taken together, the percent composition of each cannabinoid in an extract or product is more of a chemical starting point that may or may not have much to do with the actual or perceived potency of the product which needs to be measured empirically. The need to regulate Δ^9-THC as a psychoactive drug has biased the entire industry into erroneously referring to cannabinoid composition as potency.

Herein we describe the CannaMetrix EC50 Array™ as a high throughput method for quantifying the biologic potency of products developed in the cannabis and hemp industry for human consumption. The basis for this critical approach is shown as examples of dose response curves (see **Figure 1B** and **1C**). The dose response curve is produced by plotting how much response from the cell surface receptor can be measured (vertical or *y*-axis) relative

to an escalating amount of dose (horizontal or *x*-axis). When measuring biologic potency of Δ^9-THC alone, the activation of cannabinoid receptor one (CB1) promotes cellular responses. Different combinations of cannabinoids will cause the curve to shift relative to a pure cannabinoid standard which provides the means of quantifying changes in the EC50 and ECmax values due to cannabinoid combinations (see **Figure 1B** and **2C**, respectively).

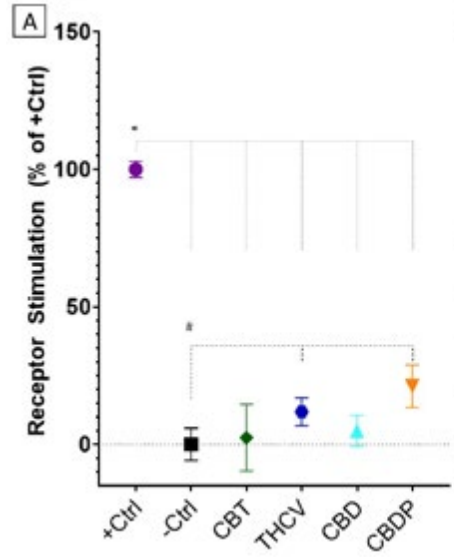
Results and Discussion

CannaMetrix Technology

The essential parameters of a dose response using the EC50 Array™ are: 1) a biologic target, 2) a compound that selectively interacts with the target, 3) a means of measuring the amount of response or change due to drug-target interaction, and 4) to conduct this measurement in a physiologically relevant setting, in this case the endocannabinoid system in human cells (20). Cells from non-human species or components of the endocannabinoid system from other species have no or different responses to cannabinoids than seen in humans; the use of an 'all human origin' testing method is essential (21).

The CannaMetrix EC50 Array™ uses cells of human origin which have or have been made to constitutively express human CB1 following integration of lentiviral transduction of the CNR1 open reading frame. As cannabinoid receptors are members of the G-protein coupled receptor (GPCR) family we also expressed the promiscuous human G-protein "Gα15" which interacts with a broad range of GPCRs to direct their activation to release of cellular calcium stores (22). This newly made cell line forms the cellular component of the EC50 Array™. We then incubated these cells with the cell permeable, ratio metric calcium dye Fura2-AM which allows for real time quantification of changes in cellular calcium levels. The response of these cells

Legend for Figure 2: Cannabinoid Activity on CB1 Receptor Measured Independently. (A) Real data collected showing direct effects of 0.3 µg/ml of either CB1 agonist (+ctrl ●), DMSO (-ctrl ▪), CBT (◆), THCV (●), CBD (▲), or CBDP (▼) on the CB1 receptor. CBT and CBD had no significant activity on the receptor when added alone (compare to –ctrl). THCV and CBDP had a small but significant stimulus of activity. All cannabinoids show significantly less activity than CB1 agonist. Data are n=5 "*" and "#" indicate significance compared to +ctrl and –ctrl respectively p<0.0017 Bonferroni correction. (B) Table shows mean value and 99% Confidence Interval (CI) for each cannabinoid.



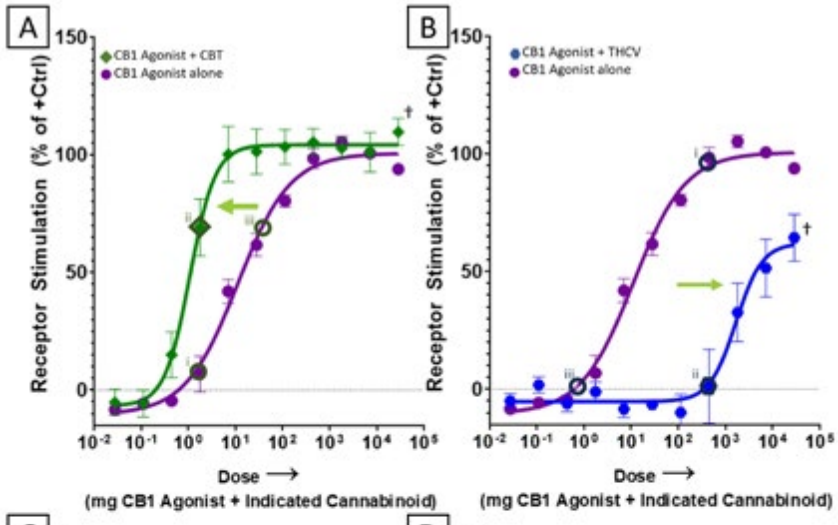
B

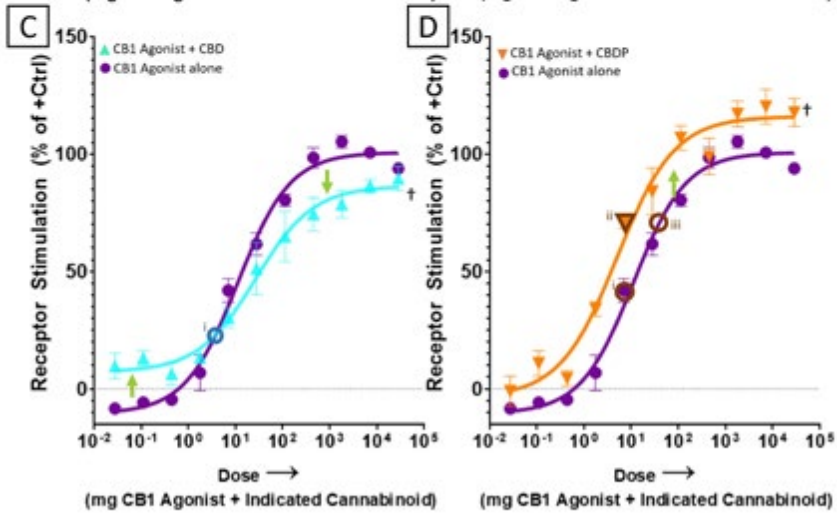
	Mean	+CI	-CI
+Ctrl	100.0	104.0	96.0
-Ctrl	0.0	8.1	-8.1
CBT	2.4	19.4	-14.7
THCV	11.8	19.0	4.7
CBD	4.8	12.7	-3.0
CBDP	21.2	32.1	10.3

to addition of cannabinoids and/or control compounds is determined kinetically by continuous detection of the change in fluorescence in Fura2. Cannabinoids and their metabolites have activities which span the range of pharmacologic mechanisms including agonists (turn on an activity), antagonists (turn off activated receptors), inverse agonists (decrease constitutive "basal" activity), and modulators (change the degree to which a target can be activated by other cannabinoids) (23). Critically, the potency of antagonists and modulators is dependent upon the simultaneous presence of agonists that activate the receptors (24). We therefore are determined to use a standard CB1 agonist as a control stimulus added alone and concurrently with cannabinoid blends, and to calculate the deviation from the control (agonist alone) that each cannabinoid or blend of cannabinoids produces. Our method of potency determination becomes extremely useful when measuring the simultaneous activities of multiple cannabinoids acting on the endocannabinoid targets. This comparative analysis constitutes the quantitative analytical component of the EC50 Array™.

Stated differently, the potency of some types of cannabinoids may not be evident by testing their activity separately but can be determined by quantifying the dose-dependent ability to alter the apparent potency of a plant-derived, endocannabinoid or synthetic CB1 agonist. Quantification of responses treated cells have following addition of cannabinoids will uniquely reveal the efficacy of blends or purified cannabinoids for modulating the endocannabinoid system and identify the range in which optimal dose-responses may be observed. In this way, the EC50 Array™ provides a method of directly measuring the potency that broad or full spectrum products or extracts from cannabis varietals

Legend for Figure 3: Practical Application of CannaMetrix Technology. Panels A–D. Examples of data collected with the EC50 Array™ potency test showing EC50 and ECmax effects on potency and intensity comparing a dose range of CB1 agonist equivalent to human doses of 0.03 mg to 30,000 mg added alone (+ctrl ●) or added concurrently with CBT (◆), THCV (●), CBD (▲), or CBDP (▼) equivalent to human doses of 15 mg (see "Methods" for conversion and exact concentrations). Green arrows highlight direction of curve shift. Specific points on curve emphasized with Roman numerals are described in the discussion. (A) CBT addition to the CB1 receptor agonist increased the potency of the agonist (leftward shift) while (B) THCV addition decreased the agonist's potency (rightward shift) and significantly decrease the ECmax. (C), CBD produced unique increases in receptor activation at low doses of CB1 receptor agonist but produced a decrease in receptor activation at higher doses of the CB1 receptor agonist. (D) CBDP enhanced receptor activity at all doses. Data are n=5, "†" indicate significance compared to +ctrl p<0.003 extra-sum-of-squares F-test correction. Points used for comparisons in the discussion section are highlighted with an unfilled shape and numbered with roman numerals "i," "ii," and "iii." (E) Table of calculated best fit dose-response curves indicating the mean ECmin, ECmax, and EC50 values with 99% Confidence Interval (99% CI) for the data shown in A–D.





E

	Ctrl		CBT		THCV		CBD		CBDP	
	Mean	±99% CI	Mean	±99% CI	Mean	±99% CI	Mean	±99% CI	Mean	±99% CI
Min	-10.49	+7.7, -9.4	-6.78	+15.6, -20.5	-5.28	+8, -8.4	7.21	+10.2, -19.6	-3.47	+14.7, -30.5
Max	100.70	+7, -6.3	104.20	+8.4, -8.1	62.05	+212.8, -18.4	86.43	+24, -10.9	115.90	+14.6, -10
EC50	11.09	+6.5, -4.1	1.08	+0.7, -0.5	1665.00	+266842, -1040.8	26.66	+76.1, -17.4	5.08	+5.9, -3.2

(chemotypes) will have in a consumer. Similarly, the EC50 Array™ provides a potency validation method for establishing what are appropriate blends of synthetic cannabinoids for a desired end user experience. Furthermore, a unique advantage in cell-based potency testing is the ability to predict dose-dependent off-target or toxic effects that products may still have before they go on the dispensary shelf.

By way of example, we have included real data collected using the EC50 Array™ method to measure the contribution of cannabitriol (CBT), THCV, CBD, or cannabidiphorol (CBDP) to modulate the activity of the CB1 receptor agonist. These examples were chosen because they each represent one of the shifts described in **Figure 1**. CBD and CBT alone, do not significantly stimulate the CB1 receptor where CBDP and THCV stimulate a small but significant receptor response compared to the negative control (see **Figure 2A**). However, when the same mass percent of each cannabinoid is added to cells that have been exposed to a dose range of a CB1 receptor agonist, the ability of CBT (see **Figure 3A**, **3E**) and THCV (see **Figure 3B**, **3E**) to induce left and right shifts (respectively) becomes apparent. Similarly, when the same mass percent of each cannabinoid is added to cells that have also been dosed with a CB1 receptor agonist, CBD (see **Figure 3C**, **3E**) and CBDP (see **Figure 3D**, **3E**) induced downward and upward shifts (respectively) in receptor activation intensity. The data reported in **Figure 3** provides the basis for the following interpretations:

- The equivalent of dosing a human with 15 mg of pure CBT alone has an imperceptible stimulation of the CB1 receptor (see **Figure 2A**). The equivalent of ~0.5 mg dose of CB1 agonist has a 5% stimulation of the CB1 receptor (see **Figure 3A** highlighted shape "i"). When combined with CBT, the

CB1 receptor activity is stimulated to ~70% activity (see **Figure 3A** highlighted shape "ii"), a 50-fold stronger receptor activation equivalent to ~25 mg CB1 agonist alone (see **Figure 3A** highlighted shape "iii").

- The equivalent of dosing a human with 15mg of pure THCV alone has a small but significant stimulation of the CB1 receptor (see **Figure 2A**). The equivalent of ~475 mg dose of CB1 agonist has a 95% stimulation of the CB1 receptor (see **Figure 3B** highlighted shape "i"). When combined with THCV, the CB1 receptor activity is diminished to ~0% activity (see **Figure 3B** highlighted shape "ii"), equivalent to ~0.15 mg CB1 agonist alone (see **Figure 3B** highlighted shape "iii"), a roughly 4000-fold decrease in potency.
- The equivalent of dosing a human with 15 mg of pure CBD alone produces no stimulation of the CB1 receptor (see **Figure 2A**) however the combination of ~15 mg CBD with CB1 agonist above ~1.2 mg results in potency lower than anticipated by agonist alone, but higher potency when CBD is combined with CB1 agonist below ~1.2 mg (see **Figure 3C** highlighted shape "i").
- The equivalent of dosing a human with a 15 mg of pure CBDP alone can induce ~21% activation of the CB1 receptor (see **Figure 2A**). This same percent activation is achieved from ~3.4 mg dose of CB1 agonist alone (see **Figure 3D** highlighted shape "i"). If these effects were purely additive, a receptor activation of 42% would be predicted when the same mg amounts of CBDP and CB1 agonist are added concurrently (see **Figure 3D** highlighted shape "ii"). Our data show the combined response is close to 50% (see **Figure 3D** highlighted shape "iii"), representing a shift that has a small but significant synergistic relationship.

Therefore, CBT plus CB1 agonist can enhance the potency of the agonist whereas, THCV plus a CB1 agonist can diminish the potency of the agonist across a range of concentrations physiologically relevant to human usage. CBDP has no significant impact on the calculated potency, for example, EC50. However its ability to enhance the perceived intensity of a CB1 agonist potency is an important factor understanding human responses to blend compositions containing CBDP. CBD can modulate the potency of a CB1 agonist increasing or decreasing the perceived potency of the agonist depending on the specific ratio of the two compounds. In the data reported here a CBD:CB1 agonist ratio of 12.5:1 serves as the inflection point for this shift in perceived potency. Finally, addition of cannabinoids can induce a decrease in signal below resting levels in some conditions. One interpretation of these findings is that CB1 receptor activity is not "zero" in the unstimulated state but has a small basal activity which our assay has the sensitivity to detect.

Taken together we assert that chemical composition remains a critical component of product quality control (QC), however it fails to anticipate true biologic potency. This makes independent biologic potency testing essential to produce products with quantitative, reproducible activities.

Methods

Cells were cultured in DMEM (Gibco) with 10% FBS (Phoenix Biosciences), 1% PSF (HyClone), and grown in 37°C, 5% CO_2 incubator. EC50 Array™ cells were derived from HEK293A cells transduced with lentiviral particles containing human CNR1 (aka CB1 receptor), and human GNA15 (aka Gα15) and selected for using puromycin and blasticidin respectively (Origene Technologies). Cells were plated overnight in 96 well plates 100,000 cells/well, and assayed the following morning. Cells incubated with 4 µM Fura2-AM (ATT Bioquest) were washed once in HBSS containing 1 mM Calcium and 5 mM HEPES pH 7.4 (Corning). Cells were then assayed using a Flexstation III on "flexmode" to measure calcium bound (ex/em 340/510) and calcium free (ex/em 380/510) fluorescence. Following a 30 s baseline read compound was added to each well and the subsequent change in cytoplasmic calcium was recorded. All data reported are the 340/380 ratio normalized to baseline and scaled to the max signal obtained from the highest concentration of control CB1 agonist (HU210 at 3.3 µM). All compounds and cannabinoids were dissolved in DMSO and added to cell such that the final concentration of DMSO <0.1%.

Calculations: HU210 concentrations in µM are converted to human dose equivalent in units of mg using the following formula:

$$\text{Concentration (Mole/L)} * \text{MW (G/Mole)} * 52.8\ \text{L} * \text{HU210}/\Delta^9\text{THC potency ratio} \quad [1]$$

HU210 Dose curve consists of concentrations of $3.15*10^{-6}$ µM to 3.3 µM with a 4-fold increase between doses. Cannabinoid concentration used are 0.003 mg/ml and are converted to human dose equivalent in units of mg using the following formula:

$$\text{Concentration (G/L)} * 52.8\ \text{L} \quad [2]$$

52.8 L is the approximate accessible liquid volume for a drug in the body of an 88kg human male (25, 26). The HU210 to Δ^9-THC potency ratio used is the lower estimate (27). Cannabinoids tested

were obtained from Cayman Chemical (HU210), and Purysis, LLC (CBD, CBDP, CBT, THCV), all measured as 99.9% pure active pharmaceutical ingredient (API).

Statistics: Data are means and 99% confidence intervals, n=5. Stats for Figure 2: "*" indicating significance compared to +ctrl, and "#" indicating significance compared to –ctrl. ANOVA with p-value <0.01 followed by t-test using Bonferroni correction of p<0.0017). Stats for Figure 3: "†" indicating significance compared to +ctrl (Non-linear best fit p-value <0.01 with extra-sum-of-squares F test correction of p<0.003). Graphs and data analysis were performed using GraphPad Prism 10. Best fit curves were generated using a 4 parameter (agonist) vs. response variable slope model with the formula:

$$y=\text{Bottom}+(\text{Hillslope})*((\text{Top}-\text{Bottom})/(X^{\text{Hillslope}}+EC50^{\text{Hillslope}})) \quad [3]$$

Graphics are sourced from Servier Medical Art but independently modified for this publication (28).

Disclaimer/Conflict of Interest

The authors have financial interests in CannaMetrix, LLC and its patented method for testing the biologic potency of cannabis products based on the endocannabinoid system in human cells (US 11,782,050 B2 (29)). The Company seeks to transform the legal cannabis industry by differentiating products that have proven their potency to bring about dose-dependent responses from the human endocannabinoid system. In our opinion, the industry's reliance on percent of individual cannabinoids in a product as the sole metric to infer potency is misleading because combinations of major and minor cannabinoids bring about a combination or entourage effect that can be drastically different from the effect that chemical composition anticipates.

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