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ORIGINAL ARTICLE

Cytotoxicity of the synthetic cannabinoids 5C-AKB48, 5F-MDMB-PINACA, ADB-CHMINACA, MDMB-CHMICA and NM-2201 in A549 and TR146 cell lines

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Abstract

Purpose The aim of the present study was to evaluate the cytotoxicity of five synthetic cannabinoids in two cell lines, for which both reference standards and herbal blends were available.

Methods An in-house smoking device was developed to produce smoke condensates. The cell viability of reference standards, herbal blend extracts and smoke condensates was measured using an MTT assay in the A549 lung carcinoma cell line and the TR146 buccal carcinoma cell line. Damiana extracts and damiana smoke condensates were also tested for cytotoxicity.

Results For the reference standards of 5F-MDMB-PINACA, ADB-CHMINACA and MDMB-CHMICA, a significant concentration-dependent decrease in cell viability was observed. ADB-CHMINACA and MDMB-CHMICA extracts were more potent than the damiana extract, whereas the NM-2201 blend extract abrogated the effects of the damiana leaves. Compared with damiana smoke condensate, MDMB-CHMICA smoke condensate had higher sensitivity, while ADB-CHMINACA smoke condensate was less potent.

Conclusions This is the first study to investigate the comparative cytotoxicity of reference standards, herbal blend extracts and smoke condensates of synthetic cannabinoids. The data showed that the presence of damiana plant contributed to enhancing the cytotoxicity of 5C-AKB48, 5F-MDMB-PINACA, and MDMB-CHMICA. The plant effects were generally more marked for smoke condensates. MDMB-CHMICA was the most potent substance. The NM-2201 reference standard and extract led to an increase in cell viability as compared to the control. This is of special interest, because the NM-2201 herbal blend extract seemed to abrogate the effects of the damiana leaf extracts. Further studies would be necessary to assess potential procarcinogenic or tumorigenic effects of NM-2201.

Keywords NPS · Synthetic cannabinoids · Damiana · A549 and TR146 cell lines · Cytotoxicity · Smoke condensates

Introduction

"Spice" drugs were the first new psychoactive substance (NPS) to receive global attention, in 2008. Although these herbal blends were labelled "not for human consumption", they were advertised as legal alternatives to cannabis and available on the Internet and in so-called head shops [1]. In 2009, Auwärter et al. [1] identified the synthetic cannabinoids JWH-018 and CP47,497 in "Spice" material, and noted that the psychoactive properties did not result from the plant itself. At that time, it was not possible to detect synthetic cannabinoids using commercial drug screening methods [2]. The European Union Early Warning System on New Psychoactive Substances (EWS) was established in 2005 and is operated by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Currently, more than 700 NPS have been reported to the EWS [3]. Synthetic cannabinoids, which were originally developed for pharmaceutical research, constitute the largest chemical class of monitored NPS [3, 4]. Law enforcement officials have confiscated large quantities of synthetic cannabinoids

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in powder form on their way into European countries [5]. However, the main form of consumption is smoking of plant material sprayed with synthetic cannabinoids or dipped in their solvent. One such plant material that is often used is the shrub *Turnera diffusa*, also known as damiana [6]. Although there have been reports of its aphrodisiac effects, no relation has been established between the distinct chemical constituents of damiana and its supposed psychoactive effects [6]. Synthetic cannabinoids target the cannabinoid receptors CB_1 and CB_2 [7], but in contrast to Δ^9-THC (the main psychoactive component of cannabis), synthetic cannabinoids are full agonists of both receptors [8]. CB_1 and CB_2 are both G protein-coupled receptors, which play an important role in physiological processes such as autoimmune diseases, pain relief, regulation of appetite and nausea suppression. CB_1 receptors are predominantly expressed in the central nervous system and mediate the psychoactive effects of cannabinoids [9]. CB_2 receptors can be found in cells from the immune system, such as in the tonsils and spleen [10, 11]. The consumption of synthetic cannabinoids can result in adverse effects including hallucinations, severe mood changes, tachycardia, cardiovascular toxicity, coma, respiratory depression, seizures, convulsions and hyperemesis [12–14]. Schoeder et al. [15] and Hess et al. [16] previously reported the binding affinities of four of the synthetic cannabinoids investigated in this study (see Fig. 1). For the fifth compound, 5C-AKB48 (also known as 5-chloro-AKB48), no such data are yet available, and only a study investigating its metabolism has been reported [17].

While the first synthetic cannabinoids available as "Spice" were derived from academic publications, preclinical research and older patents, the current products on the market were designed with the sole purpose of evading drug laws. Hence, pharmacological and toxicological data for these substances are scarce. To date, the toxicological profiles of a number of synthetic cannabinoids, synthetic cathinones and their metabolites have been reported, such as the reference standards of JWH-018, JWH-018 metabolite, JWH-073, JWH-122, JWH-210, AM-694, CP-47,497-C8, JWH-133, pyrovalerone, 3,4-MDPV, α-PVP, 3,4-MDPPP and 3,4-MDPBP [18–21]. These studies evaluated the cytotoxicity and genotoxicity of reference standards of synthetic cannabinoids.

The aim of the present study was to investigate the cytotoxicity of three synthetic cannabinoids with an indazole core structure [5C-AKB48, 5F-MDMB-PINACA (also known as 5-fluoro-ADB), ADB-CHMINACA (also known as MAB-CHMINACA)] and two with an indole core structure (MDMB-CHMICA, NM-2201) in two different cell lines (see Fig. 1). These substances were chosen because both the reference standards and authentic herbal blend samples obtained from the police were available for all of them. The Forensic Institute Zurich confirmed that each herbal blend contained only one synthetic cannabinoid. In order to consider the main route of consumption—smoking, an in-house smoking device was developed to produce smoking condensates of each studied compound. For the same reason, the human lung carcinoma cell line A549 and the human buccal carcinoma cell line TR146 were chosen to test for cytotoxicity. An MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to evaluate cell viability. Its mechanism is based on the metabolic properties of a cell to reduce the MTT when the mitochondrial functions are still intact. To the best of the authors' knowledge, this is the first study investigating the cytotoxicity of reference standards, herbal blend extracts and smoke condensates of synthetic cannabinoids in a single study.

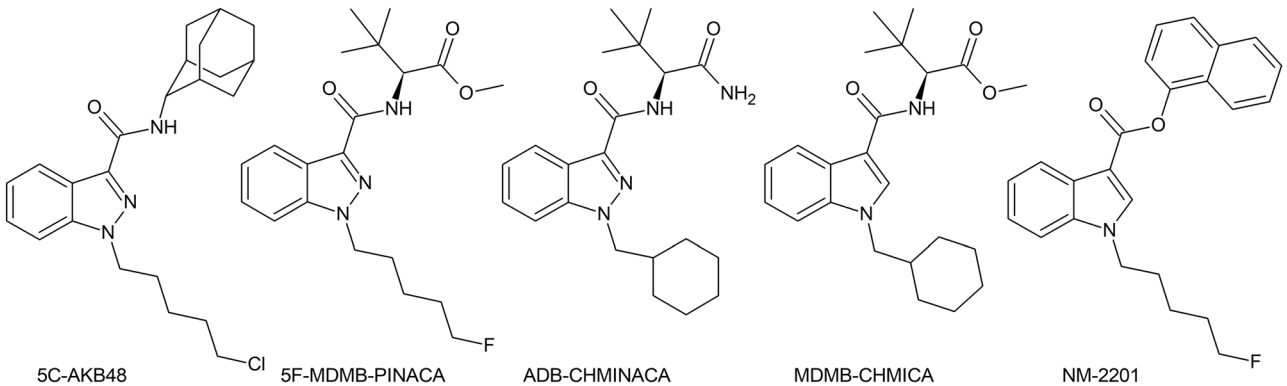

Fig. 1 Structures of the five investigated synthetic cannabinoids. The data on binding affinities for CB_1 and CB_2 were taken from Schoeder et al. [15] and Hess et al. [16]

Materials and methods

Chemicals and reagents

The TR146 carcinoma buccal cell line, RPMI 1640 medium, Ham's F12 medium, glutamine, foetal bovine serum, Triton X-100, thiazolyl blue tetrazolium bromide, sodium chloride, potassium chloride, sodium phosphate and potassium phosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). The A549 lung carcinoma cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Ethyl acetate, glass wool and silanized glass wool were obtained from Grogg Chemie AG (Stettlen, Switzerland). Dimethyl sulfoxide (DMSO) was obtained from Carl-Roth (Arlesheim, Switzerland). The reference standards of 5C-AKB48 (5-chloro-AKB48), 5F-MDMB-PINACA (5-fluoro-ADB), ADB-CHMINACA (MAB-CHMINACA) and MDMB-CHMICA, NM-2201 (> 98%), and the dried damiana leaves (*Turnera diffusa*) were kindly provided by the Institute of Forensic Medicine Freiburg, Germany. The five authentic herbal blends, containing each one of the synthetic cannabinoids, were generously provided by the Forensic Institute Zurich, Switzerland.

Quantification of synthetic cannabinoids in herbal blends using gas chromatography–mass spectrometry (GC–MS)

The herbal blends were weighed into an Eppendorf tube and mixed with ethyl acetate to obtain a concentration of 100 mg/mL of the resulting plant extract. Samples were then ultrasonicated for 15 min, followed by centrifugation at 8 °C and 17,000 *g* for 10 min. The supernatant was then filtered and transferred to a new vial. For each synthetic cannabinoid, a five-point calibration was prepared at concentrations of 5, 10, 25, 50 and 100 µg/mL.

Herbal blend extracts and calibrators were measured using a Clarus 500 gas chromatograph coupled to a Clarus 560S single quadrupole mass spectrometer with TurboChrom software (Perkin Elmer, Waltham, MA, USA). A 5% phenyl methyl polysiloxane capillary column (30 m, 0.25 mm i.d., film thickness 0.25 µm; Perkin Elmer) was used for chromatographic separation. The following gas chromatography temperature gradient was applied, with a total run time of 30.5 min: 0–3 min at 80 °C, 3–7 min at 20 °C/min to 150 °C, 7–22 min at 10 °C/min to 300 °C, and held for 8.5 min. Other conditions included carrier gas helium at a flow rate of 1 mL/min, injection volume (splitless) 1 µL, a solvent delay of 3 min, scan time from 3.1 to 30 min, and a mass spectrometry scan range from *m/z* 25 to 700.

Accuracy and precision were determined by sixfold analysis of damiana spiked with an NPS mixture containing all five synthetic cannabinoids, and the analysis of this pure NPS mixture in extraction solvent was carried out, respectively. The quantification results were used to prepare stock concentrations, with the molarity of the herbal blend extracts and smoke condensates dependent on the concentration of synthetic cannabinoid in the blend material. In order to compare results with the damiana leaf extracts and damiana leaf smoke condensates, the concentrations were also given in weight per volume.

Pyrolysis of the herbal blend and damiana leaf material

An in-house smoking device was used for the pyrolysis of the herbal blends and the damiana leaves (see Fig. 2). The samples were mounted on a single-use 6 mL Chromabond solid-phase extraction glass cartridge (Macherey-Nagel, Düren, Germany), filled with 0.5 cm of silanized glass wool. Each concentration stated for the smoke condensate corresponded to the initial concentration of synthetic cannabinoid on each herbal blend sample before burning. This is because the herbal blend extracts and smoke condensates were derived from the same sample sources. Hence, the comparability of the results was better using this approach. The cartridge was fixed on a glass adapter with a 90° angle and connected to two gas washing flasks, filled with 5 mg glass wool. The two gas washing flasks were connected by a glass tube. All connections were either with ball joints or with inert tubes. Suction was simulated using a PC 3000 pump (VacuuBrand, Wertheim, Germany) and set to constant 50 mbar. Samples were burned for 10 s using a commercial lighter, and the total pump running time was 60 s. The commercial lighter had a temperature of 550–600 °C, which was measured using a Testo 925 thermocouple (Testo SE & Co. KGaA, Lenzkirch, Germany).

Fig. 2 Experimental setup of the smoking device, from left to right: an SPE glass cartridge for mounting sample; two gas washing flasks filled with glass wool to trap smoke; a pump for suction

The glass wool in the gas washing flasks was extracted using ethyl acetate and sonicated for 15 min, after which the ethyl acetate solution was transferred to a round-bottom flask. The samples were evaporated on a Büchi RE-111 rotary evaporator coupled to a Büchi 461 water bath (Büchi, Flawil, Switzerland), under vacuum at 50 °C. The samples were then redissolved in ethyl acetate to obtain a final concentration of either 500 µM (5F-MDMB-PINACA 19.5 mg/mL, ADB-CHMINACA 17.1 mg/mL, MDMB-CHMICA 11.3 mg/mL), 1000 µM (5C-AKB-48 20.3 mg/mL, NM-2201 19.8 mg/mL) or 10.0 mg/mL (damiana). For the cytotoxicity experiments, samples were evaporated under nitrogen at 50 °C and redissolved in DMSO at concentrations of either 5000 µM (synthetic cannabinoids) or 8000 µg/mL (damiana) (see Table 1).

Cell culture and treatment conditions

The A549 lung carcinoma and TR146 buccal carcinoma cell lines were kept in culture in T75 cm^2 culture flasks containing RPMI 1640 medium with 10% foetal bovine serum, and Ham's F12 medium supplemented with 2 mM glutamine and 10% foetal bovine serum, respectively. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2 (Heracell 150; Thermo Fisher Scientific, Waltham, MA, USA). The cells were passaged every 2–3 days, and the experiments were performed using passages 6–10 for the A549 cell line and passages 3–8 for the TR146 cell line.

In vitro cytotoxicity assessment using MTT assay

The cytotoxicity experiments were carried out in 96-well plates. On experimental day 1, the cells were seeded at a concentration of 2500 cells per well for cell line A549 and 2000 cells per well for cell line TR146. On day 2, the cells were incubated for 72 h with eight different drug concentrations, as follows: 100, 50, 25, 12.5, 6.13, 3.13, 1.56 and 0.78 µM. For the TR146 cell line, the medium was changed before adding the drug substrates. On day 5, the old medium was discarded and new medium added to the cells including 10% MTT at a concentration of 5 mg/mL. After 4 h, the formed crystals were dissolved in DMSO and the optical density was measured at 550 nm using the Molecular Devices VMax Kinetic ELISA microplate reader (Molecular Devices, San Jose, CA, USA). Synthetic cannabinoid reference standards, synthetic cannabinoid herbal blend extracts, synthetic cannabinoid smoke condensates, damiana leaf extracts and damiana smoke condensates were tested at least three times in each independent experiment. In each independent experiment, a control culture [cells, medium and an equivalent amount of vehicle (DMSO), $n = 6$)], a negative control (cells and medium, $n = 4$) and a positive control (cells, medium, 10% Triton X-100, $n = 4$) were run, respectively. At least four independent experiments were performed for each compound.

Table 1 Concentrations of synthetic cannabinoid and damiana stock solutions expressed in molarity and weight per volume

Substance	Concentration stock [µM]	Concentration stock [µg/mL]
5C-AKB-48	5000	6846
5F-MDMB-PINACA	5000	7800
ADB-CHMINACA	5000	4184
MDMB-CHMICA	5000	4520
NM-2201	5000	3970
Damiana	–	8000

The molarity relates to the concentration of the synthetic cannabinoid on the plant material. The concentration weight per volume is dependent on the plant material

Statistical analysis

Raw data were processed using Microsoft Excel 2010 for Windows, and the mean, standard deviation and percentage of error were calculated. Dixon's Q test was used to detect outliers at a 95% confidence interval. The control culture was set to 100% cell viability and the data were normalized to this. Data are presented as mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics version 25 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 5 (version 5.03; GraphPad Software Inc., La Jolla, CA, USA) software programs. The normality of continuous variables was assessed using the Kolmogorov-Smirnov test. The significance was tested using one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Values of $p \leq 0.05$ were considered statistically significant. To calculate the half-maximal inhibitory concentration (IC_{50}), the data were normalized and the cell viability (%) [22] plotted against the log concentration (µg/mL or µM).

Results and discussion

Quantification of synthetic cannabinoids in herbal blends

Quantification of the respective synthetic cannabinoids in herbal blends was performed for all five substances with GC–MS using a five-point calibration. The mass spectra of each synthetic cannabinoid are presented in Fig. 3. Concentrations were determined in 1 mg/mL herbal blend, with results as follows: 5C-AKB48 19.7 µg/mL ($R = 0.981$), 5F-MDMB-PINACA 9.7 µg/mL ($R = 0.992$), ADB-CHMINACA 10.8 µg/mL ($R = 0.995$), MDMB-CHMICA 17.3 µg/mL ($R = 0.988$) and NM-2201 19.0 µg/mL ($R = 0.980$). The

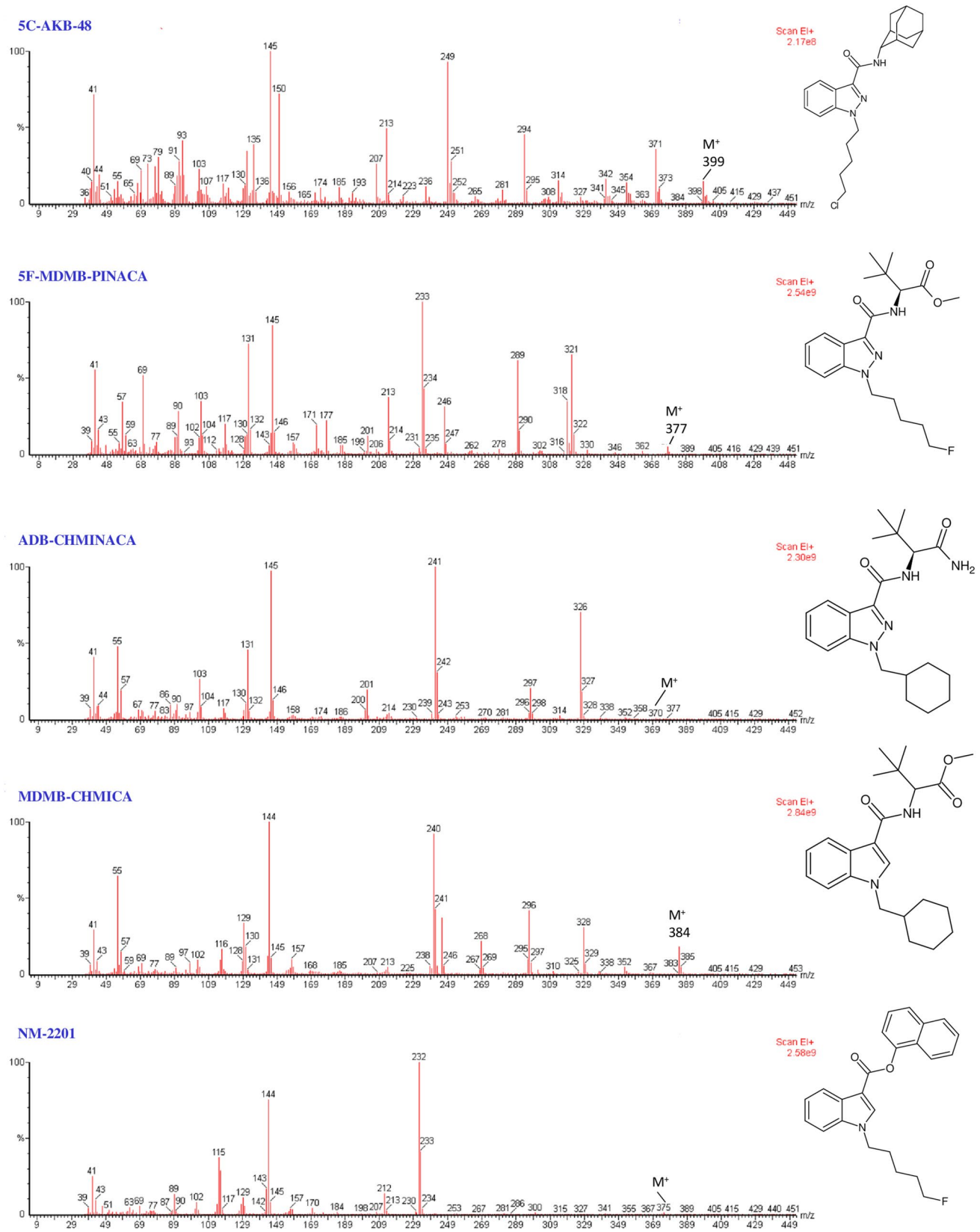

Fig. 3 Mass spectra of the synthetic cannabinoids investigated

smoking device was optimized and different parameters were tested as trapping components. Glass wool yielded 100% quantitative recovery and was therefore used as trapping component.

The precision of the NPS mixture in extraction solvent was 2.7–3.8% for the five synthetic cannabinoids. Additionally, the precision after sixfold extraction of damiana with the NPS mixture was 1.8–5.6%. The accuracy (expressed as % bias) determined by sixfold extraction versus sixfold injection of pure NPS mixture was 10.0% for 5F-MDMB-PINACA, −4.0% for ADB-CHMINACA, −0.4% for MDMB-CHMICA, 14.0% for NM-2201 and 15.6% for 5C-AKB-48. The extraction recoveries were 84 to 104%.

Qualitative analysis of smoke condensate

The same GC–MS setup was used to qualitatively analyse the smoke condensates of the herbal blends. Figure 4 depicts the total ion chromatograms obtained. In all five samples, the corresponding synthetic cannabinoid was still present. For 5C-AKB48 and NM-2201, parts of their structures, such as the linker head group, the side chain or the basic structure, were found in each smoke condensate. For example, for 5C-AKB48, the AKB48 basic structure was found, which resulted from the loss of the 5C side chain and the linker head group 1-isocyanato-adamantane. For NM-2201, we identified the 5F-pentylindole corresponding to the basic structure, together with the side chain and 1-naphthol corresponding to the head group. We found phenols and alkanes in all samples. Alkanes were also detected in the herbal blend extracts. Various studies have investigated the smoke composition of a range of synthetic cannabinoids. Kevin et al. [22] analysed the thermolysis products of reference standards of CUMYL-PICA, 5F-CUMYL-PICA, AMB-FUBINACA, MDMB-FUBINACA, NNEI and MN-18 formed by exposure to 200, 400, 600 and 800 °C. They found that, depending on the temperature and the constitution of the synthetic cannabinoid, degraded compounds were formed corresponding to parts of the original structure. For 400 and 600 °C, the parent compound was always identified.

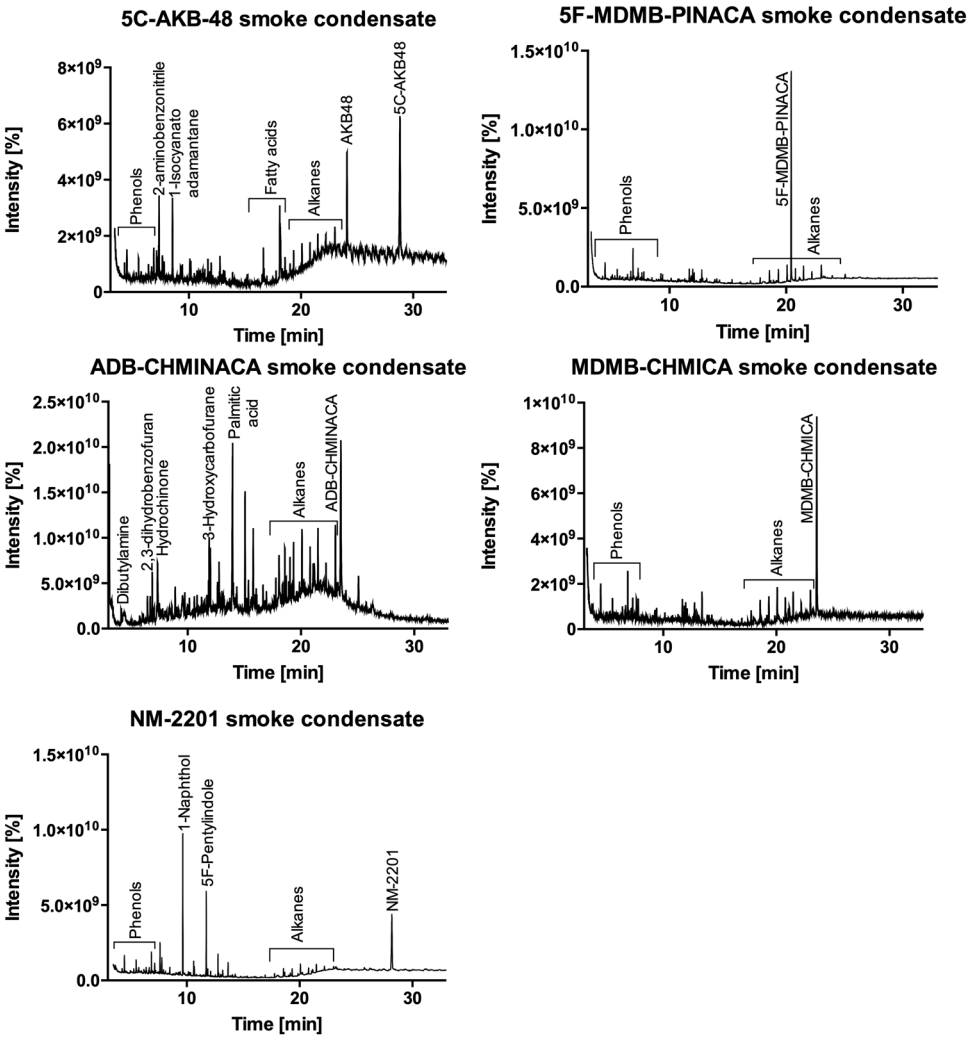

Fig. 4 Total ion chromatograms of the smoke condensates of each herbal blend. The chromatograms were reproduced using GraphPad Prism 5

However, for the higher temperature of 800 °C, the parent compound was observed for only two of the five compounds tested. Regarding our samples, which were burned with a flame at 550–600 °C, glowing of the leaves was observed. However, this lasted for a short time due to the air suction by the pump. Hädener et al. [23] reported that the actual temperature of the vapour phase of marihuana in a similar smoking device was in the range of 60–92 °C. Our results showed that the formation of degradation compounds was largely dependent on the constitution of the synthetic cannabinoid, in line with the results by Kevin et al. [22].

Cytotoxicity assay

The results of the MTT assay are presented in Figs. 5 and 6, with cell viability (%) plotted against the concentration in

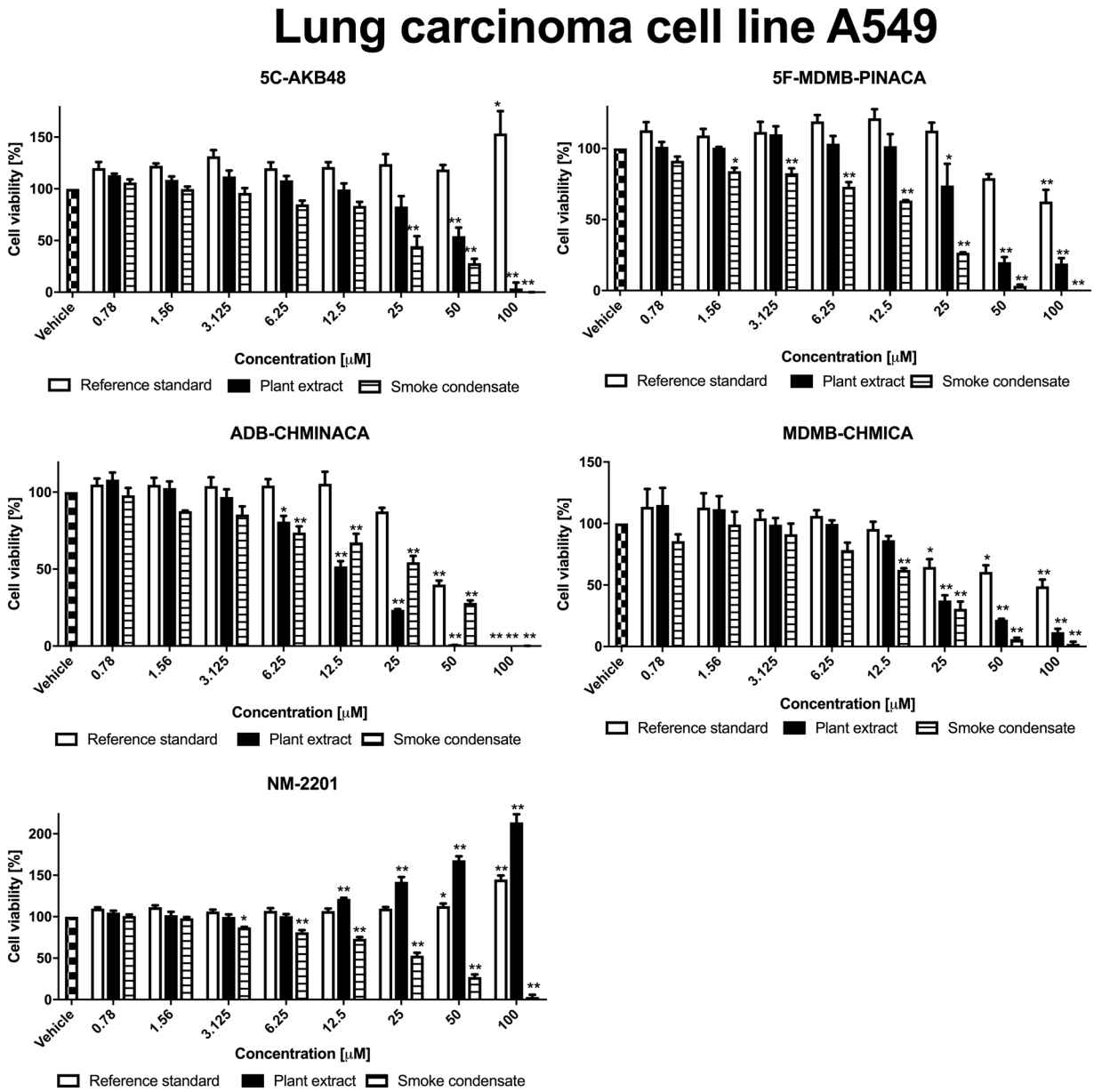

Fig. 5 Percentages of cell viability in A549 treated for 72 h with reference standards, herbal blend extracts and smoke condensates of 5C-AKB48, 5F-MDM-PINACA, ADB-CHMINACA, MDMB-CHMICA and NM-2201. An MTT assay was used to evaluate cytotoxicity. The concentration is expressed in molarity and is dependent on the concentration of synthetic cannabinoid in the plant material. Typically, different concentrations of synthetic cannabinoids were found among charges of the same herbal blend. Mean percentage change ± standard error of the mean (SEM) are shown. One-way analysis of variance was used to evaluate statistical significance. Bonferroni post hoc analysis was used to determine the dose response at which the percentage of cell viability was significantly different from the vehicle (*$p < 0.05$ and **$p < 0.001$)

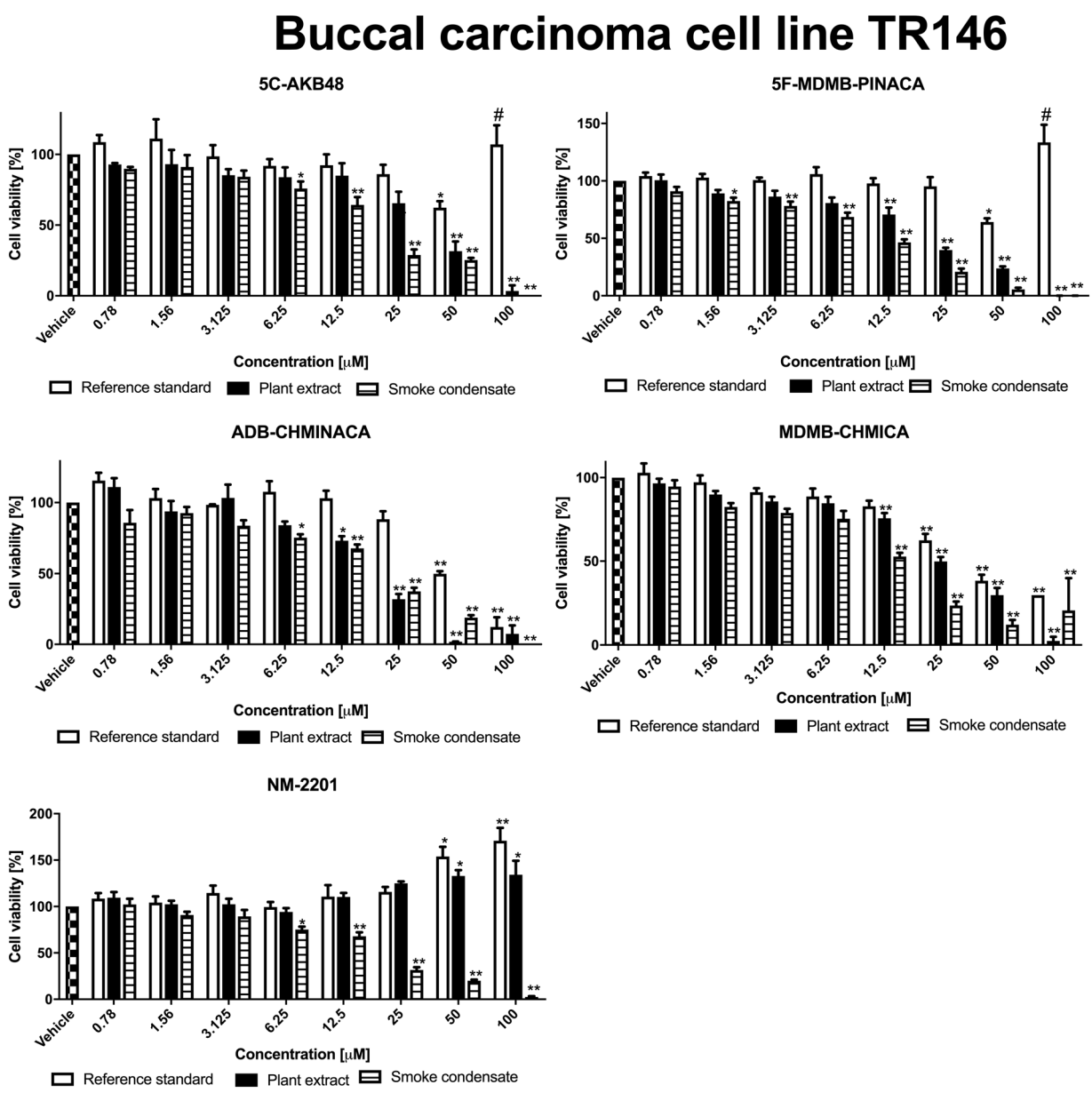

Fig. 6 Percentages of cell viability in TR146 treated with reference standards, herbal blend extracts and smoke condensates of 5C-AKB48, 5F-MDM-PINACA, ADB-CHMINACA, MDMB-CHMICA and NM-2201 for 72 h. Other details are the same as described in Fig. 5. # indicates that an anomaly was identified, possibly resulting from solubility issues such as aggregation

μM. Eight different concentrations from 100 μM to 0.78 μM were tested. The results from the two cell lines are in agreement with each other, with the exception of the reference standard of 5C-AKB48. For the reference standards, a concentration-dependent decrease in cell viability was observed for 5F-MDMB-PINACA, ADB-CHMINACA and MDMB-CHMICA. Interestingly, a concentration-dependent increase in cell viability was observed for NM-2201 at concentrations of ≥ 50 μM in both cell lines ($p < 0.05$ vs. vehicle). The data for the reference standard of 5C-AKB48 showed no difference in cell viability at ≤ 25 μM for either cell line, but a significant increase in cell viability was observed for A549 at 100 μM. In the cell line TR146, however, a significant decrease in cell viability was observed at 50 μM. In addition, an anomaly was identified for the reference standard at 100 μM, as this showed a significant increase, which may be due to solubility issues such as aggregation. A similar solubility issue was observed for 5F-MDMB-PINACA at 100 μM in TR146.

The data from the herbal blend extract samples, with the exception of NM-2201, showed a significant concentration-dependent decrease in cell viability in both cell lines. For

5C-AKB48, a decrease was observed in a 99% confidence interval for concentrations of ≥ 50 µM in A549 and ≥ 25 µM in TR146. However, for 5F-MDMB-PINACA and MDMB-CHMICA, a decrease was already seen at concentrations of ≥ 25 µM in A549 ($p < 0.05$ vs. vehicle) and ≥ 12.5 µM in TR146 ($p < 0.001$ vs. vehicle). ADB-CHMINACA displayed the highest decrease in cell viability at concentrations of ≥ 6.25 µM in A549 and ≥ 12.5 µM in TR146 ($p < 0.05$ vs. vehicle).

For all five synthetic cannabinoid smoke condensates investigated, a dose-dependent decrease in cell viability was observed in both cell lines. For 5F-MDMB-PINACA, ADB-CHMINACA and MDMB-CHMICA smoke condensates, we observed a decrease in cell viability in both cell lines at ≥ 1.56 µM ($p < 0.05$ vs. vehicle), ≥ 6.25 µM ($p < 0.05$ vs. vehicle) and ≥ 12.5 µM ($p < 0.001$ vs. vehicle), respectively. Cell viability was significantly decreased for the 5C-AKB48 smoke condensate at concentrations of ≥ 25 µM ($p < 0.05$ vs. vehicle) in A549 and ≥ 6.25 µM ($p < 0.001$ vs. vehicle) in TR146. For the NM-2201 smoke condensate, a reduction in cell viability was observed at ≥ 3.125 µM in A549 and ≥ 6.25 µM in TR146 ($p < 0.05$ vs. vehicle).

In order to take into consideration the contribution of the damiana leaves with respect to cell viability, the damiana leaf extracts and damiana smoke condensates were further investigated in A549 and TR146. From all obtained data, the IC_{50} values were calculated as depicted in Table 2. The corresponding IC_{50} graphs plot the cell viability against log concentration as presented in Figs. 7 and 8. The concentrations of the synthetic cannabinoids were stated in two different units: weight per volume and molarity. This was done on the one hand to be able to compare the results obtained from the synthetic cannabinoids to samples from the damiana leaves, and on the other hand because the concentrations of synthetic cannabinoids can vary between packages. The concentration of weight per volume is dependent on the weight of herbal blends per volume, whereas the molarity is dependent on the concentration of synthetic cannabinoids on damiana leaves.

For the reference standards 5C-AKB48 and NM-2201, proliferation occurred in both cell lines (Figs. 5–8). The MDMB-CHMICA reference standard was the most potent compound as compared with ADB-CHMINACA and 5F-MDMB-PINACA reference standards, with IC_{50} values of 17 µM (12.0–23.9 µM) in A549 and 19.3 µM (15.9–23.4 µM) in TR146 (Table 2).

For the damiana leaf extracts, IC_{50} values of 1222 µg/mL (1088–1373 µg/mL) in A549 and 784.5 µg/mL (592.7–1038.9 µg/mL) in TR 146 were calculated. The IC_{50} values for 5C-AKB48 and 5F-MDMB-PINACA herbal blend extracts were in similar ranges. It can be seen that the ADB-CHMINACA and MDMB-CHMICA herbal blend extracts are more potent than the damiana leaf extracts, with IC_{50} values of 413.4 µg/mL (375.1–455.7 µg/mL) in A549 and 496.7 µg/mL (416.7–592.1 µg/mL) in TR146, and 373.4 µg/mL (311.6–447.5 µg/mL) in A549 and 527.2 µg/mL (461.0–601.7 µg/mL) in TR146, respectively. As mentioned previously, the NM-2201 herbal blend extract clearly

Table 2 Assessment of IC_{50} values in µg/mL and µM using GraphPad Prism 5

Sample type	Substrate	A549		TR146	
		IC50 (µg/mL) ($p < 0.05$)	IC50 (µM) ($p < 0.05$)	IC50 (µg/mL) ($p < 0.05$)	IC50 (µM) ($p < 0.05$)
Plant extract	5C-AKB-48	828.2 (**703.3–975.2**)	39.6 (**33.6–46.6**)	629.0 (**503.4–785.8**)	30.1 (**24.1–37.6**)
	5F-MDMB-PINACA	1047.0 (**913.0–1202.0**)	26.9 (**23.4–30.8**)	818.1 (**715.0–936.1**)	21.0 (**18.3–24.0**)
	ADB-CHMINACA	413.4 (**375.1–455.7**)	12.0 (**10.9–13.2**)	496.7 (**416.7–592.1**)	14.4 (**12.1–17.2**)
	MDMB-CHMICA	373.4 (**311.6–447.5**)	16.5 (**13.7–19.8**)	527.2 (**461.0–601.7**)	23.3 (**20.5–25.6**)
	NM-2201	> 1985.0	> 100.0	> 1985.0	> 100.0
	Damiana	1222 (**1088–1373**)		784.5 (**592.7–1038.9**)	
Smoke condensate	5C-AKB-48	448.4 (**380.3–528.8**)	22.3 (**19.0–26.3**)	351.3 (**292.3–422.1**)	16.8 (**14.0–20.2**)
	5F-MDMB-PINACA	491.7 (**418.5–577.7**)	13.1 (**11.2–15.3**)	374.9 (**326–430.3**)	9.8 (**8.5–11.2**)
	ADB-CHMINACA	747.9 (**618.4–904.5**)	22.0 (**18.2–26.5**)	603.4 (**508.9–715.3**)	17.7 (**14.9–21.0**)
	MDMB-CHMICA	328.4 (**272.2–396.1**)	14.5 (**12.1–17.5**)	208.4 (**156.1–278.2**)	9.4 (**7.0–12.4**)
	NM-2201	432.7 (**383.8–487.9**)	21.8 (**19.3–24.6**)	300.8 (**258.1–350.7**)	15.2 (**13.0–17.7**)
	Damiana	532.6 (**437.8–648.0**)		422.6 (**382.2–467.3**)	
Reference standard	5C-AKB-48		> 100.0		> 100.0
	5F-MDMB-PINACA		39.2 (**29.6–52.0**)		29.1 (**21.3–39.8**)
	ADB-CHMINACA		43.3 (**39.9–47.1**)		36.9 (**31.2–43.7**)
	MDMB-CHMICA		17.0 (**12.0–23.9**)		19.3 (**15.9–23.4**)
	NM-2201		> 100.0		> 100.0

The IC_{50} range at a 95% confidence interval is shown in bold in parentheses

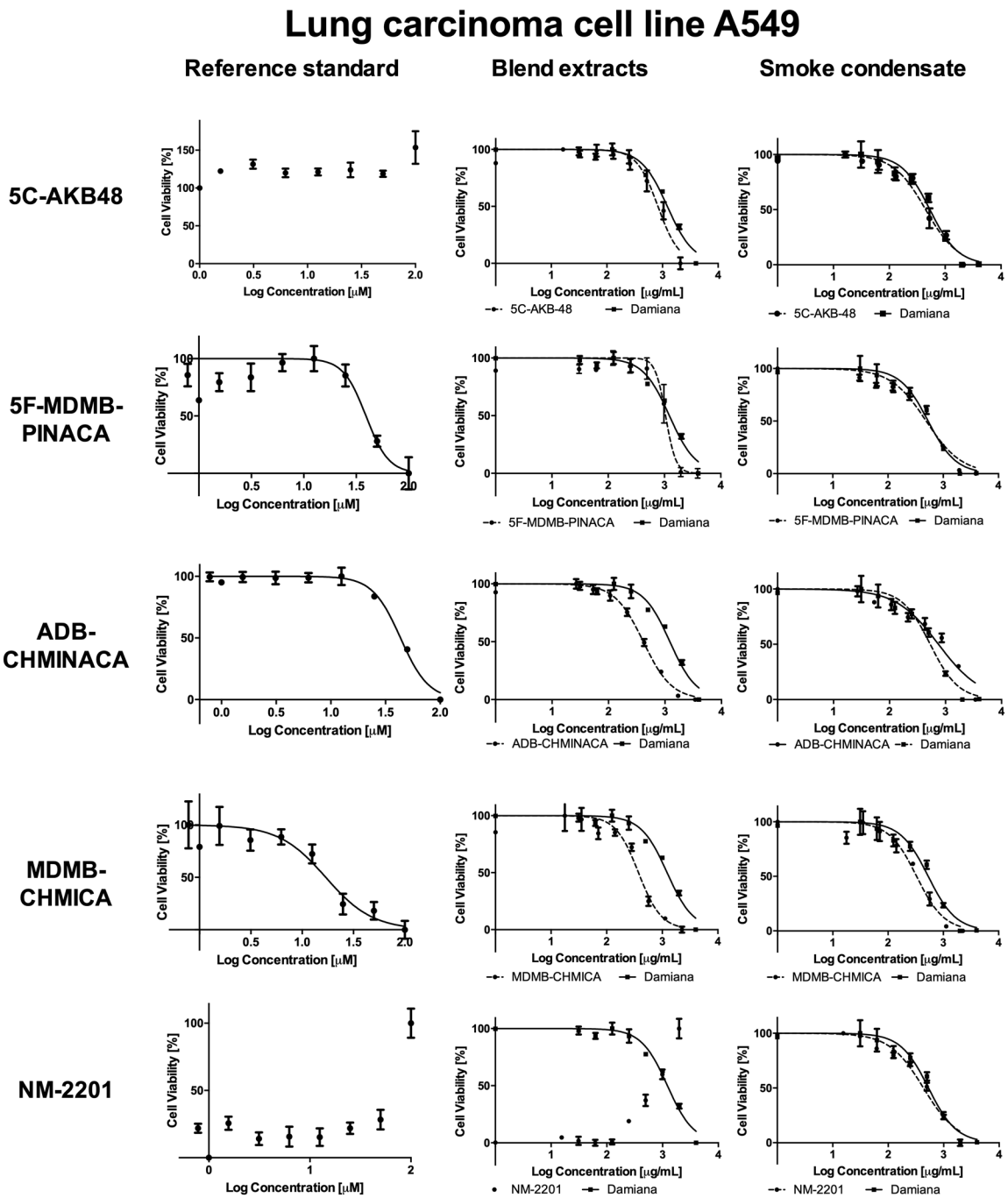

Fig. 7 Dose-effect curves for A549 after treatment with 5C-AKB48, 5F-MDMB-PINACA, ADB-CHMINACA, MDMB-CHMICA and NM-2201 for 72 h. Bars represent mean ± SEM of four to five independent experiments. The dashed lines represent the data obtained from the respective synthetic cannabinoid herbal blend extracts and smoke condensates; the solid lines represent the data obtained for the damiana leaves

has an effect on cell proliferation as compared to the damiana leaf extract. The effects of the NM-2201 herbal blend extract appeared to abrogate the effects of the damiana leaf extracts. Taking this into account, further experiments should be carried out using lung and buccal primary cells to determine whether cell proliferation also occurs in them. Both cell lines used in the current study are carcinoma cell lines; therefore, they are immortalized, which can result in differences as compared with primary cell lines [24]. Nevertheless, the use of cytotoxicity assays in cancer cell lines is a fair measure of overall cytotoxicity potential. As the degree of cytotoxicity may be different, the outcome of cytotoxicity assays may

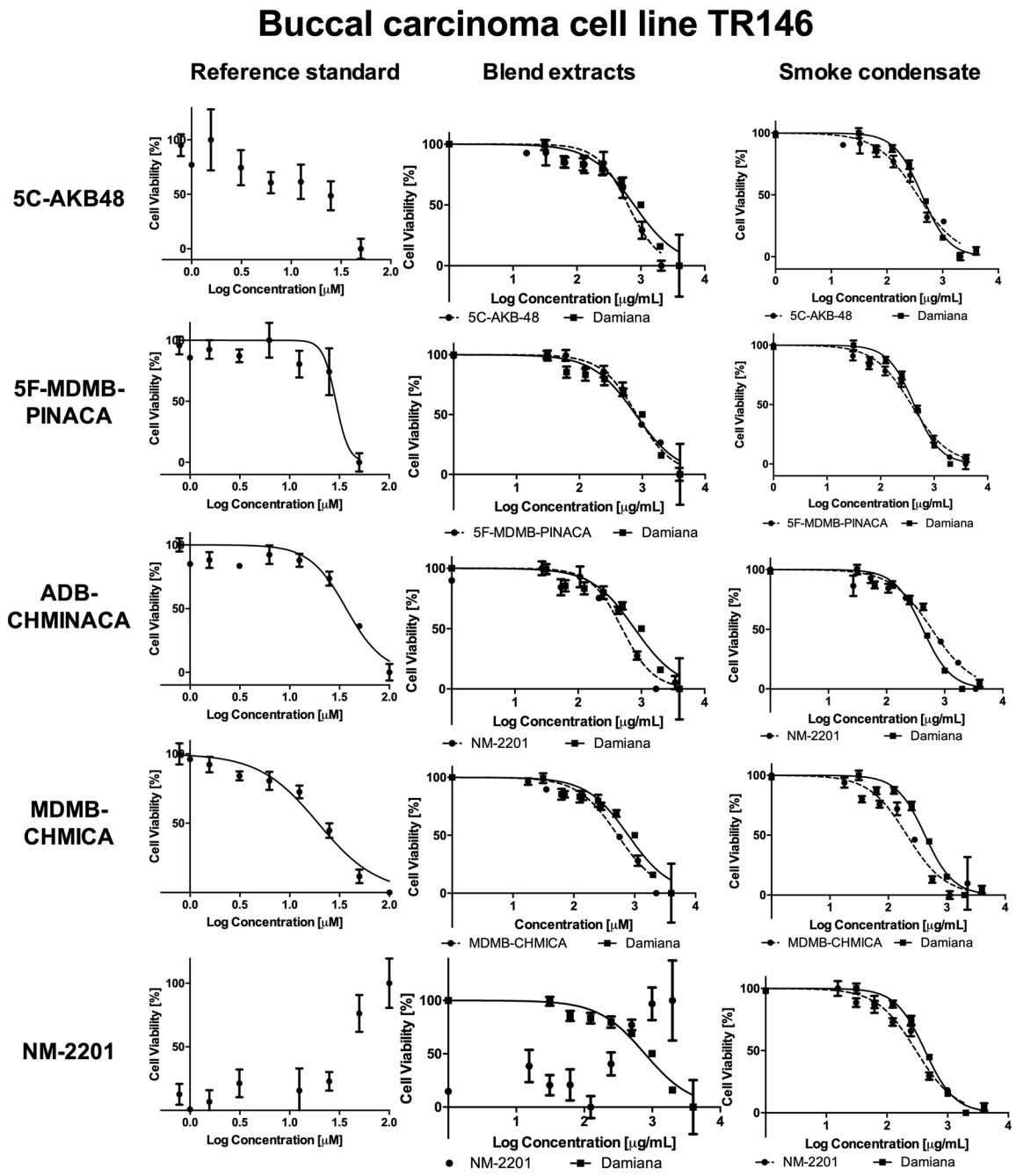

Fig. 8 Dose-effect curves for TR146 after treatment with 5C-AKB48, 5F-MDMB-PINACA, ADB-CHMINACA, MDMB-CHMICA and NM-2201 for 72 h. For other details, see Fig. 7

have only a comparative value. The case of NM-2201 is interesting, as a procarcinogenic or tumour-promoting effect cannot be excluded at this point.

As previously stated, the main route of consumption of synthetic cannabinoids is via smoking. Therefore, it is important to evaluate the herbal blend smoke condensates as compared to damiana smoke condensate. For damiana smoke condensate, IC_{50} values of 532.6 µg/mL in A549 and 422.6 µg/mL (382.2–467.3 µg/mL) in TR146 were calculated. No differences in IC_{50} values were observed between the damiana smoke condensate and 5C-AKB48, 5F-MDMB-PINACA and NM-2201 smoke condensates. However, higher sensitivity was found for the MDMB-CHMICA smoke condensate than the damiana smoke condensate in both cell lines. In addition, the ADB-CHMINACA smoke condensate was found to be less potent than the damiana smoke condensate.

This study is the first to report the cytotoxicity of herbal blend extracts and herbal blend smoke condensates. Previous studies have evaluated the cytotoxicity of reference standards of synthetic cannabinoids only. Koller et al. [19] reported that JWH-018, JWH-073, JWH-122, JWH-210 and AM-694 caused effects at concentrations of 100 µM in MCF-7 breast carcinoma and TR146 lines. These compounds have a high affinity for the CB_1 receptor. Couceiro et al. [18] investigated the cytotoxicity of the JWH-018 metabolite *N*-(3-hydroxypentyl) in SH-SY5Y neuroblastoma and human embryonic kidney HEK-293T cell lines. They reported that the JWH-018 metabolite was more potent than JWH-018 itself [18]. Tomiyama and Funada [25] demonstrated that CP-55,940, CP-47,497 and CP-47,497-C8 were cytotoxic towards the NG108-15 mouse neuroblastoma/rat glioma hybrid cell line in a concentration-dependent manner. Further studies by the same authors using the CB_1 agonists CP-55-940, CP-47,497, CP-47,497-C8, HU-210, JWH-018, JWH-210, AM-2201 and MAM-2201 and CB_2 agonists JWH-133 and CB-65 on primary neuronal mouse cells confirmed these results and suggested that the cytotoxicity towards this cell line was mediated by the CB_1 receptor and not by the CB_2 receptor [26]. However, Wojcieszak et al. [20] demonstrated that pretreatment of SH-SY5Y cells with AM-630, an inverse agonist of CB_2 receptors, did not affect the reduction in cell viability at concentrations of 20 µM JWH-133. This suggests that the JWH-133 cytotoxic action is not mediated by stimulation of the CB_2 receptor. Clearly, JWH-133 is a very pure CB_2 agonist that is specific for CB_2 receptors in the nanomolar range and starts to become unspecific at concentrations > 10 µM [20].

Severe and fatal intoxications have been reported for 5F-MDMB-PINACA, MDMB-CHMICA and ADB-CHMINACA [27–29]. Adverse effects observed include agitation, aggression, slurred speech, vomiting, muscle spasms, respiratory failure and loss of consciousness [12, 29]. Tachycardia, hypertension and acute kidney injury have also been reported [12]. To the best of our knowledge, no severe or fatal intoxications with 5C-AKB48 or NM-2201 have been reported in the literature. The results for 5C-AKB48 indicate that no severe cytotoxicity occurred in either cell line for any sample type. For both the herbal blend extracts and smoke condensates of 5C-AKB48, the concentration-dependent decrease in cell viability was likely the result of the damiana leaf extracts and smoke condensates themselves. The lack of reports of intoxication concerning this substrate would support this hypothesis. The results for the NM-2201 reference standard and herbal blend extract showed that this synthetic cannabinoid enhanced cell proliferation in A549 and TR146 (Table 2, Figs. 5–8). The smoke condensate of NM-2201 had influence on cell viability; such effect was rather the result of the damiana leaf extracts. Among the five synthetic cannabinoids investigated, MDMB-CHMICA was the most potent in both cell lines. This is supported by the high number of fatal intoxications reported from MDMB-CHMICA consumption [27, 30].

Conclusions

This study reports, for the first time, the comparative cytotoxicity of pure compounds, herbal blend extracts and smoke condensates of five synthetic cannabinoids found in "Spice" products and used as "legal highs". The main route of consumption of synthetic cannabinoids is smoking, thus rendering these results especially meaningful. In order to determine whether the effects on cell viability resulted from the synthetic cannabinoid or the damiana leaf extract itself, the data obtained were evaluated against the damiana leaf extracts and smoke condensates. The results showed that the presence of damiana plant extracts enhanced the cytotoxicity of 5C-AKB48, 5F-MDMB-PINACA and MDMB-CHMICA. The plant effects were generally more marked for smoke condensates. A comparison of the three sample types showed that, for 5C-AKB48, no cytotoxicity was observed as a result of the synthetic cannabinoid itself, but the cytotoxicity was stemmed from the damiana. Similar results were observed for 5F-MDMB-PINACA; however, its reference standard showed a concentration-dependent decrease in cell viability. The herbal blend extract and reference standard of ADB-CHMINACA both showed a concentration-dependent decrease in cell viability, independent of the carrier material. However, the decrease in cell viability of the smoke condensate resulted from the damiana itself. For MDMB-CHMICA, the IC_{50} values for all three sample types studied were in the same range, and the effects were independent of the damiana carrier material. Among the five tested substances, MDMB-CHMICA was the most potent, showing comparable cytotoxicity in both cell lines. Interestingly, the NM-2201 reference standard and herbal blend extract led to an increase in cell viability and pointed towards cell proliferation versus the control. This should be further investigated in studies involving primary cell lines. Additional studies would be necessary to assess potential procarcinogenic or tumorigenic effects of NM-2201. Given the potency of this compound at CB receptors, and the fact that the increased viability was observed only with this cannabinoid and only at micromolar concentrations (Figs. 5 and 6), we suggest that this effect is independent of CB receptors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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