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MOLECULAR TOXICOLOGY

Impact of a synthetic cannabinoid (CP-47,497-C8) on protein expression in human cells: evidence for induction of inflammation and DNA damage

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Abstract Synthetic cannabinoids (SCs) are marketed worldwide as legal surrogates for marihuana. In order to predict potential health effects in consumers and to elucidate the underlying mechanisms of action, we investigated the impact of a representative of the cyclohexylphenols, CP47,497-C8, which binds to both cannabinoid receptors, on protein expression patterns, genomic stability and on induction of inflammatory cytokines in human lymphocytes. After treatment of the cells with the drug, we found pronounced up-regulation of a variety of enzymes in nuclear extracts which are involved in lipid metabolism and inflammatory signaling; some of the identified proteins are also involved in the endogenous synthesis of endocannabinoids. The assumption that the drug causes inflammation is further supported by results obtained in additional experiments with cytosols of LPS-stimulated lymphocytes which showed that the SC induces pro-inflammatory cytokines (IL12p40 and IL-6) as well as TNF-α. Furthermore, the proteome analyses revealed that the drug causes down-regulation of proteins which are involved in DNA repair. This observation provides an explanation for the formation of comets which was seen in single-cell gel electrophoresis assays and for the induction of micronuclei (which reflect structural and numerical chromosomal aberrations) by the drug. These effects were seen in experiments with human lymphocytes which were conducted under identical conditions as the proteome analysis. Taken together, the present findings indicate that the drug (and possibly other structurally related SCs) may cause DNA damage and inflammation in directly exposed cells of consumers.

Keywords Synthetic cannabinoid · Comet assay · Lymphocytes · Proteomics

Abbreviations

B(a)P	Benzo(*a*)pyrene
BSA	Bovine serum albumin
CBs	Cannabinoid receptors
DDT	Dithiothreitol
IAA	Iodacetamide
FDR	False discovery rate
GC–MS	Gas chromatography–mass spectrometry
^{1}H NMR	Proton nuclear magnetic resonance
LC–MS	Liquid chromatography–mass spectrometry
LFQ	Label-free quantification
MNi	Micronuclei
Nbuds	Nuclear buds
NDI	Nuclear division indices
NPBs	Nucleoplasmatic bridges
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
SCs	Synthetic cannabinoids
SCGE	Single-cell gel electrophoresis

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SDS Sodium dodecyl sulfate
THC Tetrahydrocannabinol

Introduction

Synthetic cannabinoids (SCs) are marketed worldwide as substitutes for cannabis. These drugs bind to cannabinoid receptors (CBs) and cause psychotropic effects which are similar to those of natural phytocannabinoids (Uchiyama et al. 2009). The chemical structures of SCs are heterogeneous (Presley et al. 2013). "Classical" representatives are structurally related to tetrahydrocannabinol (THC), while "non-classical" compounds which entered the market later belong to various chemical groups. Cannabicyclohexanol (CP-47,497-C8) was used in the present study as a model for SCs which bind to both CB receptors. It is a representative of the cyclohexylphenols and was detected for the first time in a herbal mix termed "Spice" in Germany and Japan in 2008 (Auwärter et al. 2009; Uchiyama et al. 2009). In the following years, several structurally related compounds (e.g., CP-47,497 and CP-55,940) were detected in smoking mixtures (Presley et al. 2013). Figure S1 depicts the chemical structure of different SCs.

Only few investigations have been conducted which concern the toxicological properties of SCs apart from their neurological effects. Recent findings indicate that some of these drugs, including CP-47,497-C8, cause damage of the genetic material (Koller et al. 2014). Furthermore, also acute toxic effects were observed in human-derived cells which were caused by interference with protein synthesis and damage of the cell membranes (Koller et al. 2013, 2014).

In order to elucidate the molecular mechanisms which cause these effects and other biological processes, which may have a negative impact on the health of drug users, we applied in the present investigation a proteomic-based screening approach which focused on a broad spectrum of cellular proteins. This method was successfully used in previous studies to improve the understanding of the toxicological properties of pharmaceuticals (Bileck et al. 2014; Klepeisz et al. 2013; Lorenz et al. 2009). Apart from damage of the genetic material, also inflammatory processes are associated with long-term effects of drugs including cancer (Okada 2014). Therefore, we focused in the present study particularly on expression patterns of proteins which are involved in DNA damage and repair and on changes of proteins which reflect inflammatory reactions.

For the proteome analysis, peripheral blood mononuclear cells (PBMCs) from different donors were exposed to CP-47,497-C8. Subsequently, nuclear proteins were isolated, and protein patterns were determined by means of a QExactive orbitrap mass spectrometer. The data analyses were supported by label-free quantification with MaxQuant software (Cox and Mann 2008). Furthermore, the altered expression of individual cytokines under identical experimental conditions was quantitatively determined with an additional approach, i.e., multiple reaction monitoring.

To find out whether alterations of the signatures of proteins which are involved in the maintenance of DNA stability are associated with damage of the genetic material, single-cell gel electrophoresis assays (SCGE) were performed which are based on the measurement of DNA migration in an electric field and enable the detection of single-, double-strand breaks and apurinic sites (Tice et al. 2000). Furthermore, also cytome experiments were conducted with the same indicator cells under identical conditions in which we monitored the impact of the drug on the formation of micronuclei (MNi) which reflects chromosome breakage and/or aneuploidy (Norppa and Falck 2003). In addition, also other nuclear anomalies which reflect genomic instability were scored in the cytome experiments, namely nuclear buds (Nbuds) which are caused by gene amplification and nucleoplasmatic bridges (NPBs), which are formed as a consequence of dicentric chromosomes (Fenech et al. 2003).

The observed alterations of the protein pattern indicated also the involvement of immune functions that are associated with inflammation. Therefore, additional experiments were conducted which concerned the impact of the drug on the immune status. The nuclear division indices (NDI), which were determined in the cytome experiments, provide information about the mitotic activity of lymphocytes. Furthermore, changes of the levels of important pro- and anti-inflammatory cytokines (IL-10, IL-6, IL12p40 and TNF-α) were measured under identical conditions as those used in the proteome experiments and in the genotoxicity assays.

Materials and methods

Test compound

Pure CP-47,497-C8 (CAS 70434-92-3) was obtained by extraction from commercial "Spice" products and subsequent purification by flash chromatography (Moosmann et al. 2012). The purity of the drug was assessed by GC-MS and ^{1}H NMR analysis and was >99 %. Stock solutions were prepared in DMSO and stored at −20 °C.

Proteomics

Isolation and treatment of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were collected from four male healthy non-smoking volunteers (age

25–32 years) with their written consent. None had a history of a recent disease and exposure to toxic chemicals and drugs. A total of 30.0 mL of whole blood were collected in 6.0-mL CPDA tubes (Greiner Bio-One GmbH, Austria) and subsequently diluted 1:2 with RPMI 1640 medium (Gibco, Life Technologies, Austria) supplemented with 100 U/mL penicillin and 100.0 µg/mL streptomycin (ATCC, LGC Standards GmbH, Germany). Subsequently, the suspensions were carefully overlaid on Ficoll Paque (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 500×*g* for 20 min at 24 °C. PBMCs were collected from the interphase and washed with PBS. Afterward, the cell pellets were re-suspended in RPMI 1640 medium and transferred to Petri dishes (Greiner Bio-One GmbH, Austria). The cell suspensions of the individual donors were treated separately either with a solvent control or with a solution of the test compound (final concentration 10.0 µM). We used in all experiments a 3-h exposure time since we aimed to find changes in protein pattern which may provide an explanation for the results of the SCGE experiments which were also conducted with a 3-h treatment protocol. The subsequent proteome analyses were conducted separately with samples from the different donors.

Subcellular fractionation

After removal of the supernatants, the cells were lysed by addition of isotonic lysis buffer (10 mM HEPES/NaOH, pH 7.4, 0.25 M sucrose, 10.0 mM NaCl, 3 mM $MgCl_2$, 0.5 % Triton X-100) containing protease inhibitors (pepstatin, leupeptin and aprotinin, each at 1.0 µg/mL; 1.0 mM PMSF) and by mechanical shear stress as described previously (Slany et al. 2014). After centrifugation at 2300×*g* (4 °C for 5 min), the cytoplasmic proteins were separated from the nuclei and subsequently precipitated overnight with ice-cold ethanol at −20 °C. The nuclear pellets were treated with extraction buffer (500.0 mM NaCl) for 10 min and diluted 1:10 with NP-40 buffer (Nonidet P40 Substitute, BioXtra, mixture of 15 homologs, Sigma-Aldrich). After 15-min incubation, the samples were again centrifuged, and the supernatants were precipitated by addition of ethanol.

MS sample preparation

Subsequently, the samples were solubilized in buffer (7.5 M urea, 1.5 M thiourea, 4 % CHAPS, 0.05 % SDS, 100 mM DDT) and the protein concentrations were determined with the Bradford assay (Bio-Rad-Laboratories, Germany). As described previously, 20.0-µg protein of each sample was loaded on SDS-PAGE and stained with MS-compatible silver stain (Mortz et al. 2001; Slany et al. 2009). Before enzymatic digestion of the proteins at 37 °C by using trypsin (Roche Diagnostics, Germany) overnight, each protein band was cut into four slices, reduced with DTT and alkylated with IAA. The peptides were then eluted, dried and kept at −20 °C until further analysis.

LC–MS analysis

For LC–MS analysis, samples were dissolved in 5.0 µL 30 % formic acid (FA) containing four synthetic standard peptides (each 10 fmol) and diluted with 40.0 µL mobile phase A (98 % H_2O, 2 % ACN, 0.1 % FA). A total of 10.0 µL of the solutions were injected into a Dionex Ultimate 3000 nano-LC-system combined with a QExactive orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA). A 2 cm × 75 µm C18 Pepmap100 pre-column (Thermo Fisher Scientific, Waltham, MA USA) was used for pre-concentration, on which peptides were loaded at a flow rate of 10.0 µL/min using mobile phase A. A 50 cm × 75 µm Pepmap 100 (Thermo Fisher Scientific, Waltham, MA USA) was used as an analytical column. Peptides were eluted from the pre-column and separated on the analytical column using a gradient of 8–40 % mobile phase B (80 % ACN, 20 % H2O, 0.1 % FA) over 235 min at a flow rate of 300 nL/min. Mass spectrometric analyses were performed with a nanospray ion source. MS scans were achieved in the range from *m*/*z* 400–1400 at a resolution of 70,000 (at $m/z = 200$). HCD fragmentation at 30 % normalized collision energy and an isolation window of 2.0 *m*/*z* were used to generate MS/MS scans of the 12 most abundant ions, which were further analyzed in the orbitrap at a resolution of 17,500 (at $m/z = 200$).

Multiple reaction monitoring (MRM)

For targeted analyses, supernatants of CP 47,497-C8-treated PBMCs from further three male healthy volunteers were obtained after 3-h incubation and digested in-solution using trypsin as described previously (Bileck et al. 2014). Briefly, protein samples were concentrated and washed on 3kD MWCO filters (Pall Austria Filter GmbH) by centrifugation at 15,000*g* for 15 min. Again, DTT (5 mg/mL dissolved in 8 M guanidinium hydrochloride in 50 mM ammonium bicarbonate buffer (ABC buffer), pH 8) and IAA (10 mg/mL in 8 M guanidinium hydrochloride in 50 mM ABC buffer) were used for reduction and alkylation, respectively. Afterward, trypsin (0.1 µg/µL) was added and incubated at 37 °C for 18 h. Peptides were eluted using 25 mM ABC buffer and dried and stored at −20 °C until further MS analyses. For targeted LC–MS/MS analyses, dried peptide samples were reconstituted in 30 µL of 30 % FA containing 10 fmol each of the four synthetic standard

peptides. MRM analysis was performed as described in detail recently (Muqaku et al. 2015): 1 µL of this solution was then injected to a nano-flow LC-system (1260 Infinity Series, Agilent, Palo Alto, CA) using the HPLC-Chip cube (Agilent) coupled to the 6490 triple quadrupole mass spectrometer (Agilent). Peptides were separated by large-capacity protein chip (G4240-62010) with a 160-nl enrichment column and a 150mm × 75 µm separation column (5-µm ZORBAX 300SB-C18, 30-Å pore size) at a flow rate of 400 nl/min using a gradient from 8 % mobile phase A (99.8 % H2O, 0.2 % FA) to 30 % mobile phase B (99.8 % ACN, 0.2 % FA) over 20 min. All samples were analyzed in triplicates.

Genotoxicity experiments (SCGE and MN assay)

Isolation of human lymphocytes

Peripheral blood samples were collected from four non-smoking healthy male volunteers (aged between 25 and 32 years) without any history of recent disease or exposure to chemical toxins. The samples were collected by venipuncture in heparinized tubes (Vacutainer Systems, Becton, Dickinson, Erembodegem, Belgium). Venous heparinized blood was collected for comet and MN assays on separate days.

For comet assays, the blood samples were centrifuged (650×*g*, 10 min at 4 °C) immediately after collection. Plasma was removed, and lymphocytes were isolated by gradient centrifugation (800×*g*, 16 °C min, 16 °C) with Histopaque (Sigma-Aldrich, Steinheim, Germany) in Accupin tubes (Sigma-Aldrich, Steinheim, Germany). Lymphocytes were collected and washed twice in RPMI 1640 (332×*g*, 10 min 16 °C). Subsequently, aliquots were dispersed in serum-free freezing medium (1:10 dilution in Biofreeze, Biochrom AG, Berlin, Germany), frozen and stored at −80 °C. The SCGE experiments were performed within 2 weeks after sampling.

The lymphocytes for CBMN assays were isolated separately under sterile conditions from the same donors.

Single-cell gel electrophoresis (SCGE) assays

SCGE assays which reflect the formation of single- and double-strand breaks and apurinic sites were conducted under standard alkaline conditions as described by Tice et al. (2000).

Briefly, frozen lymphocytes were thawed in a water bath (37 °C) and centrifuged (200×*g*, 5 min at 16 °C). Subsequently, the pellets were dissolved in RPMI and washed twice under identical conditions. Thereafter, the vitality and number of cells were determined with the trypan blue dye exclusion test with a Neubauer chamber (LO-Laboroptik GmbH, Germany).

Aliquots of the lymphocytes were treated with different concentrations (5.0, 7.5, 10.0, 15.0 and 20.0 µM, 3 h) of the synthetic cannabinoid with and without metabolic activation with S9 mix (10 %). Aroclor™ 1254-induced rat liver S9 was purchased from Trinova Biochem GmbH (Giessen, Germany), and the S9-activation mix was freshly prepared before the experiments according to Maron and Ames (1983) prior to the experiments.

The indicator cells were incubated with serum-free medium containing 1 % DMSO (negative control) or with H_2O_2 (50 µM, 10 min) in experiments without S9 mix or with benzo(a)pyrene (B[a]P, 4 µM, 4 h, CAS no. 50-32-8, Sigma-Aldrich, Steinheim, Germany) in assays with S9-activation mix as a positive control. We used rat-derived liver homogenate as it is the most widely used activation mix for routine testing in genetic toxicology. In contrast to expensive, standardized liver preparations from humans, it is easily available, and a validated procedure for the composition of the homogenate has been published (for details see Maron and Ames 1983). Stock solutions of the drug were freshly made and further dissolved in serum-free medium. After incubation in the dark (37 °C; shaking 250 rpm) in presence and absence of S9-activation mix for 3 h, the cells were washed twice with RPMI (containing 10 % FCS), centrifugated (200×*g*, 8 min, 16 °C). The incubation time was chosen for reasons of comparison with earlier SCs studies in which positive results were obtained with various representatives (Koller et al. 2015). Subsequently, the pellets were resuspended in low-melting agarose (LMA, 0.5 %), spread on pre-coated agarose slides (1.5 % normal melting agarose) and lysed in the dark at 4 °C overnight. After 30-min unwinding under alkaline conditions (pH > 13), electrophoresis was carried out for 30 min (300 mA, 25 V). Subsequently, neutralization was performed twice for 8 min. Air-dried slides were stained with propidium iodide (10.0 µg/mL, Sigma-Aldrich, Steinheim, Germany).

The percentage of DNA in tail was measured by use of a computer-aided image analysis system (Comet IV, Perceptive Instruments Ltd., Haverhill, UK). For each experimental point, three slides were prepared from each person, and 50 randomly distributed cells were evaluated per slide.

Cytokinesis-blocked micronucleus (CBMN) assay with mitogen-stimulated human lymphocytes

The experiments were conducted according to OECD guideline # 487 (OECD 2012). Human lymphocytes are more sensitive toward genotoxic effects during S, G2 and M phase; therefore, we used cells which were stimulated

with phytohemagglutinin (PHA, Remel Inc., USA) before treatment with the test compound.

Briefly, 1 × 10^6 lymphocytes were added to 750.0 µL culture medium (RPMI 1640 containing 10 % FCS, 2.0 mM L-glutamine and 1.0 mM sodium pyruvate) and 10 µL PHA solution (30.0 µg/mL, Sigma-Aldrich, Steinheim, Germany). Four cultures were set up per concentration for each experimental point. The cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 44 h. After PHA stimulation, the medium was replaced by fresh medium without FCS (Sigma-Aldrich, Steinheim, Germany) containing different concentrations of CP-47,497-C8 (1.0, 2.5, 5.0, 7.5 and 10.0 µM). Treatment was performed for 3 h (37 °C, 250-rpm shaking in the dark). Mitomycin C (1.0 µg/mL, Sigma-Aldrich Chemical Co., USA) was used as a positive control. The solvent (DMSO, 1.0 %) was added to untreated cultures as a negative control. After treatment, the cells were washed twice in RPMI (332×*g*, 10 min, 16 °C), and the pellets were re-suspended in culture medium. Cytochalasin B (Sigma-Aldrich Chemical Co., USA) was added to the cultures at a final concentration of 4.5 µg/mL to block cytokinesis. The cells were harvested 72–73 h after stimulation with PHA. Slides were made by use of cytocentrifugation (Fenech 2007) and were air-dried, fixed and stained with Diff-Quick (Dade Behring, Deerfield, IL, USA).

From each culture, 2000 cells were evaluated. Different endpoints were scored, namely the number of binucleated (BN) cells with micronuclei (BN-MN), the total number of micronuclei in binucleated cells (MNi), nuclear buds (Nbuds) and nucleoplasmic bridges (NPBs). Furthermore, the cytokinesis-blocked proliferation indices (CBPI) were calculated according to the formula CBPI = (M1 + 2M2 + 3(M3 + M4))/N (N is the total number of scored cells, and M1–M4 refers to the number of cells with one to four nuclei). The toxicity of the compound was indirectly assessed by the assumption that a CBPI of 1.0 corresponds to 100 % cytotoxicity (OECD Guideline 2012). Five concentrations of each drug were used to determine the CBPI values. In agreement with OECD guideline 487 (OECD), only doses were analyzed in regard to formation of MN which caused less than 60 % cytotoxicity.

Cell isolation and measurement of cytokine induction

The experiments were performed as described by Saemann et al. (2000) with slight modifications. Briefly, heparinized blood was collected from four healthy donors, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway). To evaluate the impact of the drugs on the production of cytokines, the cells were cultivated in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 2.0 mM L-glutamine, 100.0 µg/mL streptomycin, 100 U/mL penicillin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 10 % FCS (Hyclone, Logan, UT, USA) and stimulated with LPS (100 ng/mL, *Escherichia coli* 0111:B4; Sigma-Aldrich Chemical Co., St. Louis, MO) in the presence and absence of increasing concentrations of the drug (0.1, 0.3, 1.0, 3.0 and 10.0 µM). The solvent (DMSO, 1.0 %) was added to untreated cultures as a negative control.

All experiments were conducted in 96-well plates in humidified atmosphere with 5 % CO_2. After exposure for 24 h, the supernatants were collected and stored at −80 °C after centrifugation. The treatment period was chosen on the basis of the experimental design of earlier investigations with SCs (Berdyshev et al. 1997). Cytokines (TNF-α, IL12/23p40, IL-6 and IL10) were determined in the samples by Luminex using matched-pair antibodies specific for the respective cytokines and recombinant cytokines as standards. Coating and detection antibodies for human IL-6, IL-10 and IL-12/23p40 were obtained from R&D (Minneapolis, MIN), and antibodies to human TNF-α were from PharMingen (San Diego, CA, USA).

Statistical analysis

Cytokines, comet formation and nuclear anomalies

Determination of cytokine levels was performed by use of the software package SoftMax Pro (Molecular Devices, Sunnyvale CA). The statistical evaluation of results of the SCGE experiments, and evaluation of the CBMN assays was performed with the GraphPad Prism 5 Project software system (La Jolla, CA, USA). Results are reported as means ± standard deviations (SD). Results of the comet assay and cytokines were analyzed with one-way ANOVA and Dunnett's multiple comparisons Test. The results of cytome assays were analyzed with the Kruskal–Wallis test followed by the Dunn's test; p values ≤ 0.05 were considered as statistically significant.

LC–MS data analysis

MaxQuant 1.3.0.5 comprising the Andromeda search engine, and the Perseus statistical analysis package (Cox and Mann 2008, 2012) was employed for label-free quantitative data analysis. For protein identification, the SwissProt database (version 012013 with 20 264 entries) was used, allowing a 5-ppm mass tolerance for peptide m/z values as well as a maximum of two missed cleavages. Additionally, carbamidomethylation on cysteines was set as fixed modification and methionine oxidation as well as N-terminal protein acetylation as variable modifications. Search criteria further included a minimum of two peptide identifications per protein, at least one of them unique and a FDR

calculation based on q values less than 0.01 for both peptide and protein identification. Further data analysis was performed using Perseus (version 1.3.0.4). The proteins were filtered for reversed sequences and contaminants as well as a minimum of three independent identifications. Applying a two-sided t test with $p < 0.05$, significantly up- and down-regulated proteins were determined. Additionally, a 2D annotation enrichment analysis based on GO terms for molecular functions (GOMF) and biological processes (GOBP) was performed according to (Geiger et al. 2010).

Analysis of LC-MRM data

Data analysis was accomplished using Skyline version 1.5 (MacLean et al. 2010). The total peak area of each peptide was normalized to the internal standard peptides. Afterward, means and SDs of normalized peak areas were calculated for each protein separately for the different donors and conditions. For graphical representation, the highest determined peak area was set to 100 %, and the other peak areas were calculated relative to it.

Results

Alterations in protein regulation in PBMC by CP-47,497-C8

In total 6883 proteins were identified with at least two distinct peptides per protein. Comprehensive profiling of the status of untreated and CP-47,497-C8 treated cells showed that in total 249 proteins were significantly up- or down-regulated with a minimum of a twofold change of the LFQ values. Figure 1 depicts the results of a volcano plot analysis and shows the pattern of up- and down-regulation of individual proteins; Table S1 contains a list of all proteins which were found significantly up- or down-regulated in our study.

The results of a cluster analysis using gene ontology terms performed with Perseus (Geiger et al.) show that most up-regulated genes fall in three categories, namely, regulation of metabolic processes, signal transduction involved in mitosis and antigen processing and presentation. Most down-regulated proteins are involved in the metabolism of macromolecules, RNA splicing and in the activation of transcription cofactor activities (Fig. S2A–B—supplementary material).

Since the formation of endocannabinoids is linked to interactions with lipid metabolism (see "Discussion" section), we anticipated that CP-47,497-C8 may interact with these pathways. Indeed, significant up-regulation of a number of proteins which are involved in lipid metabolism was observed after treatment of the cells with the drug. It can be seen in Fig. 2, that the level of arachidonate 5-lipoxygenase was induced more than 26-fold, and fatty acid synthase was induced 2.7-fold upon treatment of the cells. Interestingly, also monoacylglycerol lipase ABHD12, a regulator of the endocannabinoid signaling pathway, was clearly increased after treatment of the cells with the drug.

Some of these proteins are important regulators of inflammatory processes. Therefore, we looked on further interactions of the drug with inflammatory signaling. The results are summarized in Fig. 3. It can be seen that a

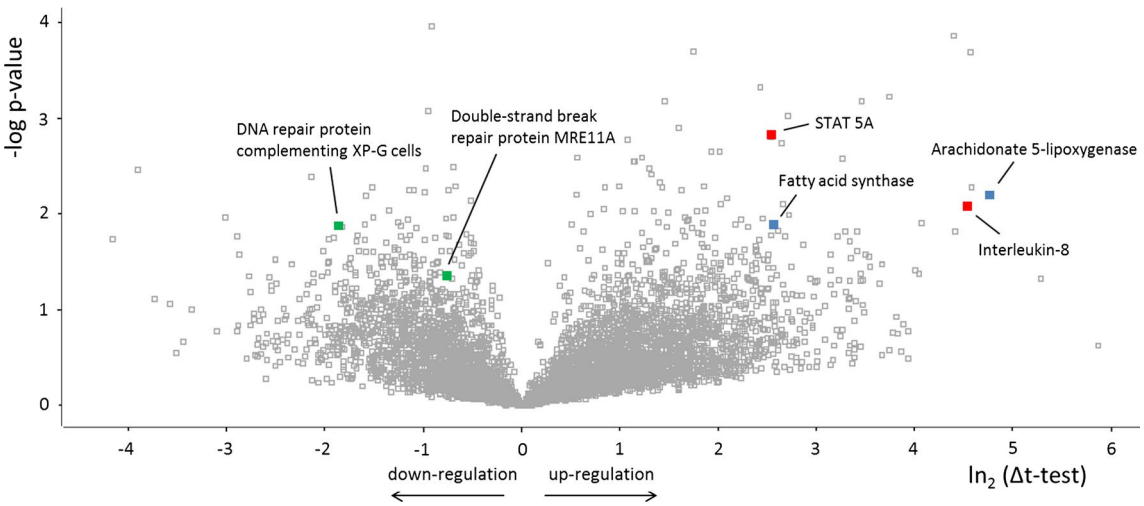

Fig. 1 Protein regulation in lymphocytes upon treatment with CP-47,497-C8 (exposure concentration 10 µM; treatment time 3 h). The distribution of up- and down-regulated nuclear proteins upon treatment with CP-47,497-C8 is displayed by a volcano plot. For each identified protein the fold-change in a logarithmic scale to the basis of 2 ($\ln_2 \Delta t$ test) and the corresponding p value ($-\log p$) are indicated

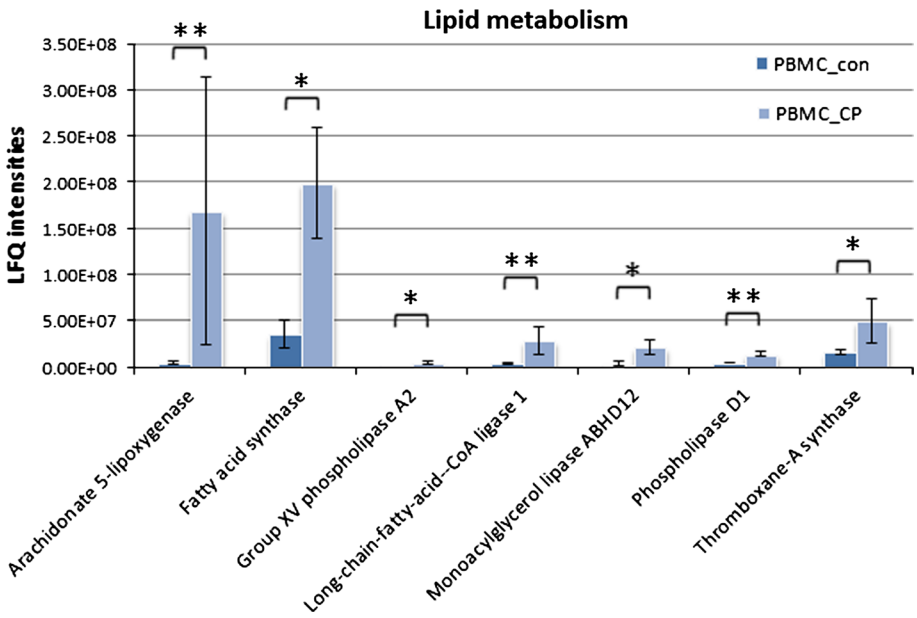

Fig. 2 Regulation of proteins related to lipid metabolism (exposure concentration 10 µM; treatment time 3 h). Label-free quantification (LFQ) intensities of proteins are shown. *Bars* represent mean ± SD of results obtained with samples from four donors. The samples were analyzed separately. These proteins were significantly (*t* test, $*p \leq 0.05$; $**p \leq 0.01$) up-regulated in PBMC upon treatment with CP-47,497-C8 (PBMC_CP; *light colored*) in comparison with control PBMC (PBMC_con; *dark colored*) (color figure online)

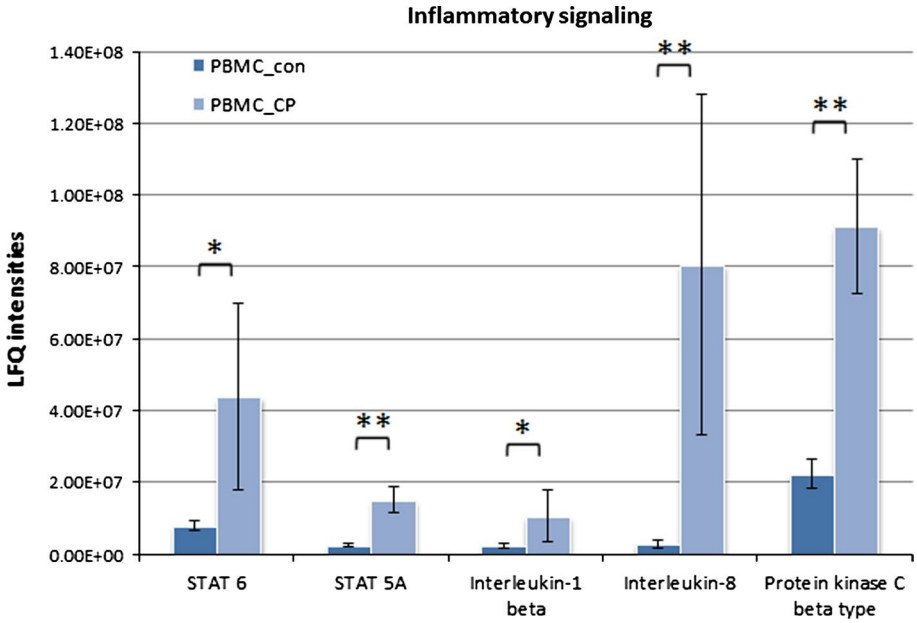

Fig. 3 Regulation of proteins related to inflammatory signaling (exposure concentration 10 µM; treatment time 3 h). Label-free quantification (LFQ) intensities of proteins are shown. *Bars* represent mean ± SD of results obtained with samples from four donors. The samples were analyzed separately. These proteins were significantly (*t* test, $*p \leq 0.05$; $**p \leq 0.01$) up-regulated in PBMC after treatment with CP-47,497-C8 (PBMC_CP; *light colored*) in comparison with control cells (PBMC_con; *dark colored*) (color figure online)

number of cytokines and also STAT proteins were up-regulated by CP-47,497-C8.

As mentioned in the "Introduction" section, we found in earlier investigations that several SCs including CP-47,497-C8 cause DNA damage in human-derived cells. The molecular mechanisms by which SCs cause instability of the genetic material are not known at present. The results of the present proteome analysis can be seen in Fig. 4. It was found that proteins which play a role in DNA excision repair and in double-strand break repair were down-regulated after treatment of the cells.

Cytokine analysis with multiple reaction monitoring (MRM)

An independent targeted MS analysis was used in order to verify data obtained from the shotgun proteomics approach. Therefore, two important pro-inflammatory mediators were selected, namely IL-8 and IL-1α. As demonstrated in Fig. 5, these cytokines were significantly up-regulated in the PBMCs of two (out of three) donors.

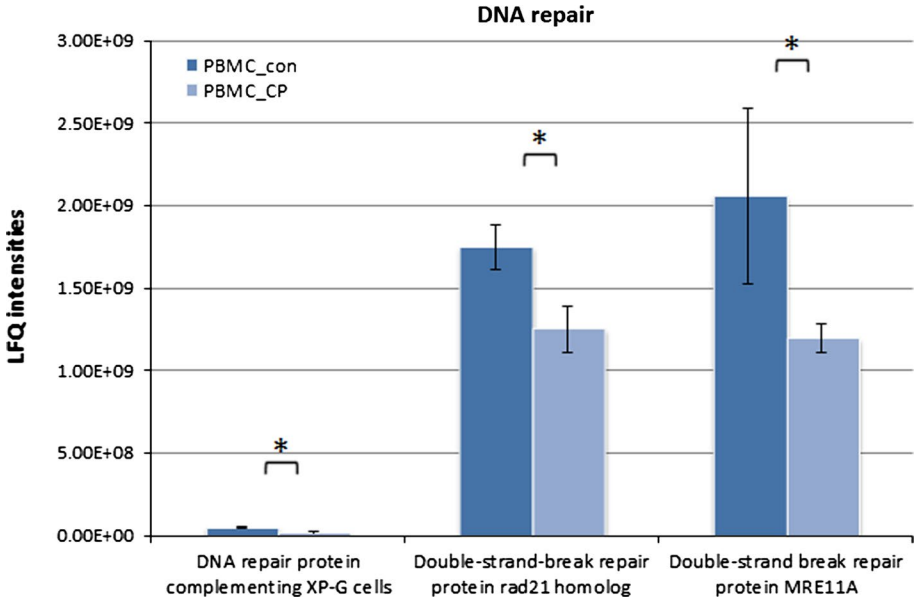

Fig. 4 Regulation of proteins related to DNA repair (exposure concentration 10 µM; treatment time 3 h). Label-free quantification (LFQ) intensities of proteins are shown. *Bars* represent mean ± SD of results obtained with samples from four donors. The samples were analyzed separately. These proteins were significantly (*t* test, $*p \leq 0.05$) up-regulated in PBMC upon treatment with 10 µM CP-47,497-C8 (PBMC_CP; *light colored*) in comparison with control cells (PBMC_con; *dark colored*) (color figure online)

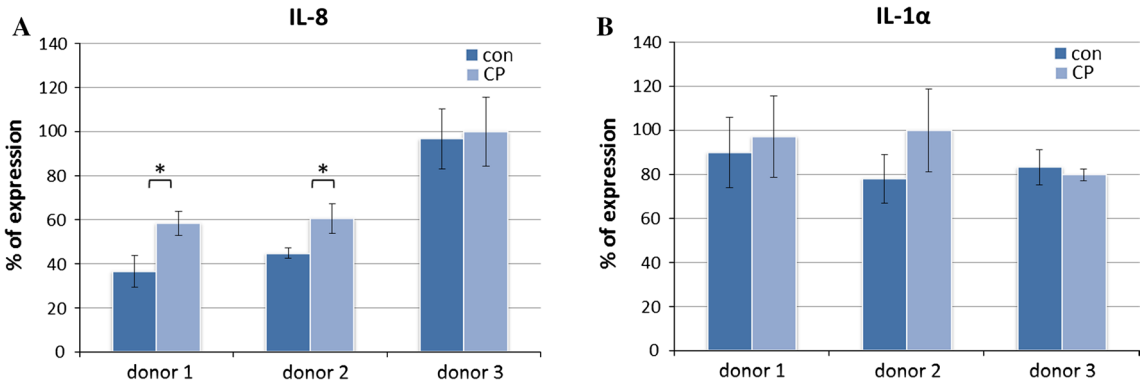

Fig. 5 Impact of exposure on PBMCs from three different donors on the levels of IL-1α and IL-8. The proteins were quantified with MRM as described in the "Materials and methods" section. *Bars* represent results obtained with cells from individual participants. The highest peak area determined for a protein was set to 100 %, and the other peak areas were calculated relative to it. *Stars* indicate statistical significance (*t* test, $p \leq 0.05$)

Induction of DNA damage in lymphocytes by CP-47,497-C8

To find out whether the drug causes DNA damage in human lymphocyte under identical experimental conditions as those used in the protein analyses, SCGE experiments were carried out in which the cells were treated with CP-47,497-C8 in the presence and absence of liver-derived metabolic activation mix (S-9). The results are summarized in Fig. 6a–f. Exposure of the indicator cells to the drugs had no impact on their vitality (see Fig. 6a, c and e), but significant induction of comets was seen which reflect single- and double-strand breaks and apurinic sites (Fig. 6b, d and f).This effect was less pronounced in presence of liver S9 mix. This observation indicates that the compound may be detoxified via protein binding. In order to verify this assumption, additional experiments were conducted in which the drug was incubated with BSA and heat-inactivated S9. Both suspensions contained identical protein concentrations (final protein concentration 0.3 mg/mL). It can be seen in Fig. 6f, that addition of the proteins and of the enzyme mix to the incubation mix reduced the genotoxic activity of the drug.

To find out whether the interaction of the drug with DNA leads to persisting chromosomal aberrations, cytome experiments were conducted in which MN were scored which reflect structural and numerical chromosomal aberrations. Furthermore, also other nuclear anomalies were evaluated, namely Nbuds and NPBs.

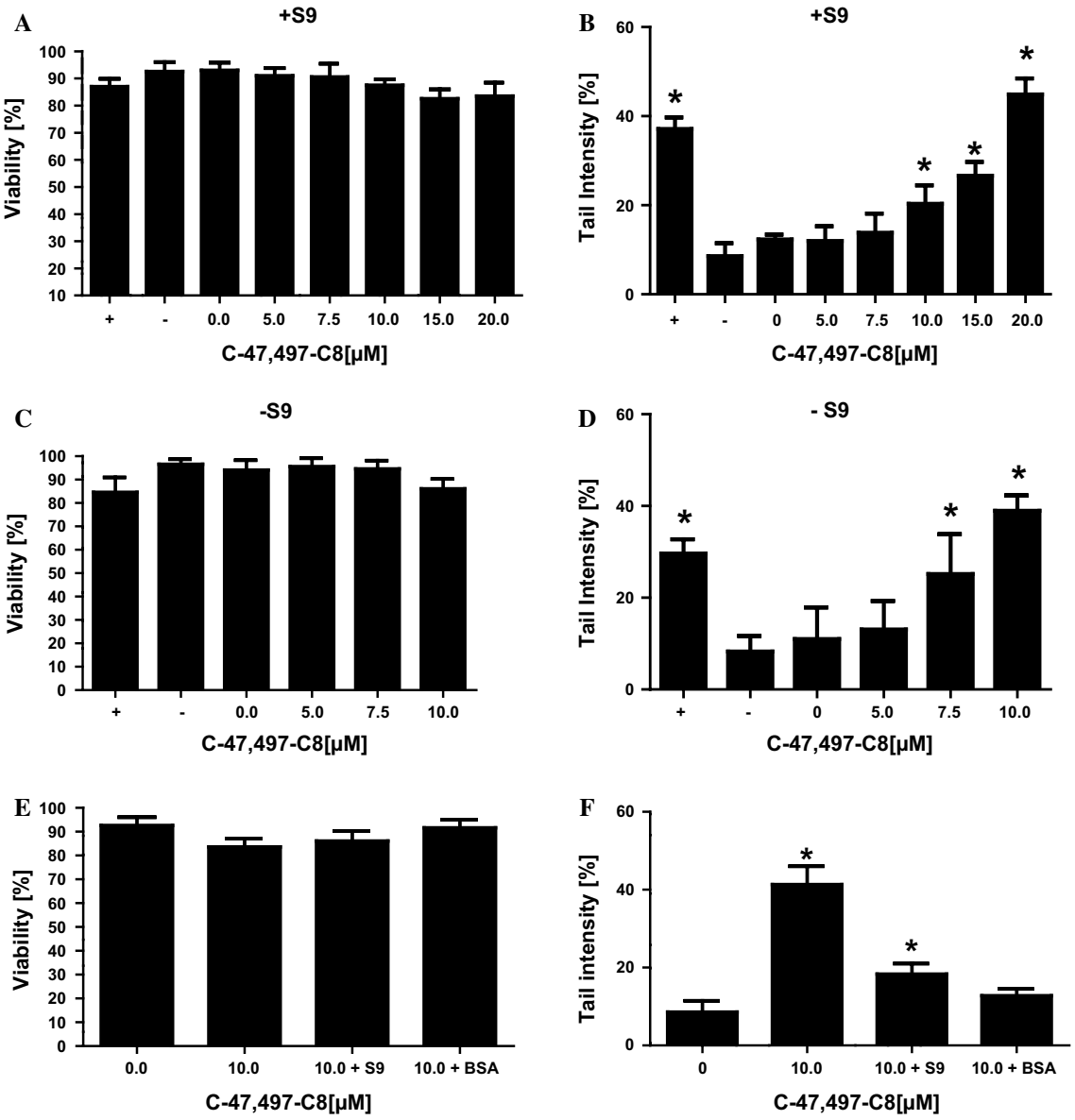

Fig. 6 Results of SCGE experiments with CP-47,497-C8. Human lymphocytes were treated in the presence and absence of rat-derived liver S9 mix with CP-47,497-C8 (treatment time 3 h; 5.0–20 µM). Furthermore, experiments were conducted in which the cells were incubated under identical conditions with S9 activation mix and heat-inactivated BSA (final protein concentration 0.4 mg/mL). **a**, **c** and **e** depict the impact of the drugs on the vitality of the indicator cells (which was monitored with *trypan blue*). **b**, **d** and **f** shows results which were conducted in SCGE assay which were conducted under identical experimental conditions. Cells were exposed in experiments without metabolic activation to the drug for 3 h or to 50 µM H_2O_2 as positive control for 10 min. In the positive control experiments with S9 mix, cells were treated with 4 µM B(a)P for 4 h. Subsequently, the nuclei were isolated and the percentage of DNA in comet determined as described in "Materials and methods." *Bars* represent mean ± SD of results obtained with three cultures per experimental point. From each culture, three slides were made, and 50 cells from each slide were analyzed for comet formation. The viability of the cells was determined after treatment with trypan blue and was in all experiments ≥95 % (data not shown). *Stars* indicate statistical significance (ANOVA, $p \leq 0.05$)

The results of a representative experiment are summarized in Table 1. It can be seen, that exposure of the cells to CP-47,497-C8 at concentrations ≥7.5 µM caused a significant increase in the frequencies of two endpoints (MN and BN-MN), and also the number of NPBs was elevated, but this latter effect did not reach significance. The impact of the drug on the nuclear division indices (NDI) of the cells is listed in the first column. It can be seen that no significant effects were detected under any experimental conditions.

Table 1 Impact of CP-47,497-C8 on human lymphocytes on the nuclear division index and various chromosomal anomalies

Compounds	Concentration µM	NDI Mean ± SD	BN-MN Mean (‰) ± SD	MNi Mean (‰) ± SD	Nbuds Mean (‰) ± SD	NPBs Mean (‰) ± SD
Neg. Cont.	0.0	2.04 ± 0.12	4.14 ± 0.52	4.26 ± 0.50	2.96 ± 1.56	1.67 ± 0.76
CP-47,497-C8	1.0	1.99 ± 0.14	4.40 ± 0.79	4.40 ± 0.79	3.91 ± 2.08	2.10 ± 1.67
	2.5	2.02 ± 0.12	4.89 ± 1.01	4.89 ± 1.01	4.33 ± 1.61	2.05 ± 0.79
	5.0	2.01 ± 0.14	5.26 ± 0.98	5.37 ± 1.20	2.57 ± 1.90	2.24 ± 2.79
	7.5	2.01 ± 0.15	**6.03 ± 1.28**	**6.03 ± 1.28**	3.97 ± 1.45	2.45 ± 2.41
	10.0	1.93 ± 0.19	**6.37 ± 1.83**	**6.58 ± 2.03**	4.48 ± 2.24	1.89 ± 0.96
Pos. Cont.	1.0 µg/mL	1.74 ± 0.16	**48.60 ± 9.35**	**50.62 ± 10.10**	**12.44 ± 5.92**	2.68 ± 1.16

Human mitogen-stimulated lymphocytes from four individuals were treated with different concentrations of the test compound for 3 h. Subsequently, the nuclear division indices (NDI) and nuclear aberrations were analyzed after incubation of the cells with cytochalasin B as described in "Materials and methods." Each experimental point represents results (mean ± SD) obtained from duplicate cultures from four donors

NDI Nuclear Division Indices, *BN-MN* binucleated cells with micronuclei, *MN* micronuclei, *Nbuds* nuclear buds, *NPB* nucleoplasmic bridges, *Neg. Control* solvent control (DMSO 1 %), *Pos. Control* mitomycin C (1.0 µg/mL)

Bold values indicate significant differences from negative control values (Kuskal–Wallis test followed by Dunnett's test, $p \leq 0.05$)

Table 2 Impact of CP-47,497-C8 on the cytokine production in human PBMCs

LPS + CP (µM)	IL-10 (ng/mL) Mean ± SD	Δ[a] (%)	IL-6 (ng/mL) Mean ± SD	Δ[a] (%)	IL12p40 (ng/mL) Mean ± SD	Δ[a] (%)	TNFα (ng/mL) Mean ± SD	Δ[a] (%)
0.0	0.97 ± 0.22	–	12.30 ± 1.18	–	0.64 ± 0.30	–	1.01 ± 0.35	–
0.1	0.84 ± 0.20	−13	12.23 ± 4.67	−1	0.71 ± 0.26	+14	1.07 ± 0.41	+6
0.3	1.01 ± 0.28	+4	10.15 ± 1.31	−17	0.64 ± 0.22	0	0.91 ± 0.28	−10
1.0	0.95 ± 0.11	−2	10.64 ± 1.76	−13	0.56 ± 0.30	−12	0.93 ± 0.27	−8
3.0	0.92 ± 0.13	−5	14.45 ± 1.15	+17	0.69 ± 0.26	+8	1.04 ± 0.30	+3
10.0	**0.78 ± 0.22**	−20	**15.83 ± 2.10**	+29	**0.91 ± 0.26**	+42	**1.46 ± 0.39**	+45

Human PBMCs were exposed to the synthetic cannabinoid CP-47,497-C8 for 24 h and processed as described in the "Materials and methods" section. Values indicate mean ± SD from triplicate measurements conducted with samples from four different donors. Statistical analyses were performed by one-way ANOVA and Dunnett's multiple comparison test

Bold values are significantly different from those found in the respective controls ($p < 0.05$)

[a] Δ Alteration in %

Impact of CP-47,497-C8 on the production of inflammatory cytokines

Since the results of proteome profiling indicated that the drug induces the synthesis of proteins which are involved in inflammation, experiments were conducted in which we measured the levels of cytokines after treatment of LPS-stimulated lymphocytes with the drug. The results are summarized in Table 2. It can be seen that inductions of several cytokines (IL-10, IL-6 and IL12p40) and of TNF-α were detected. The strongest effect was seen with the tumor necrosis factor, and its concentration was increased by approximately 40 % after exposure of the cells to the drug. On the contrary, we found down-regulation of IL-10; its level was decreased by approximately 20 % after treatment of the cells. It is notable that all these effects were not dose-related; up to concentrations of 3.0 µM, no differences were seen in comparison with the background levels, whereas only at the highest dose (10.0 µM), significant alterations were detected.

Discussion

This is the first investigation using proteome profiling to assess the impact of a synthetic cannabinoid on primary human cells. In contrast, several studies have been published which describe cellular alterations by THC, the most relevant phytocannabinoid (for review see Wang et al. 2011). However, these earlier investigations concerned the impact of the drug on global and psychotropic effects and were conducted with cytosolic extracts of neuronal cells (Bindukumar et al. 2008; Colombo et al. 2009; Quinn et al. 2008; Rubino et al. 2009); therefore, their findings can hardly be compared with the present results.

As described in the "Results" section, we observed up-regulation of several proteins by the SC which are involved in the metabolism of amino acids as well as in signal transduction involved in mitotic cell cycle; most down-regulated proteins play a role in the biosynthesis of macromolecules (Fig. S2A–B—supplementary material).

Furthermore, we detected a number of specific alterations which concern lipid metabolism, inflammatory reactions and other functions of the immune system as well as inhibition of DNA repair processes. These effects are of particular interest in regard to possible adverse health effects in drug users.

The impact of the drug on lipid metabolism was not unexpected. The lipophilic character of phytocannabinoids led to the assumption that they exert part of their physiological effects via disturbance of the structures of the lipid part of the cell membranes (Leuschner et al. 1984; Paton 1975). THC and related compounds are incorporated in the lipid bilayers of cell membranes (Herbette et al. 1986) and cause their effects via interactions with microdomains of plasma membranes which are modulators of immune receptors and cannabinoid receptors (Fisar 2009). As described in the "Results" section (Fig. 2), we found in the present study significant alterations of a number of proteins which are involved in lipid metabolism. The most pronounced up-regulation concerned arachidonate 5-lipoxygenase. Furthermore, several other enzymes which were induced by CP-47,497-C8, for example fatty acid synthase and long-chain fatty acid CoA ligase 1, are also involved in inflammation (Kanter et al. 2012; Matsuo et al. 2014). The observation of increased levels of phospholipase D1 indicates that SCs may have an impact on the synthesis of endogenous cannabinoids. This enzyme is involved in the biosynthesis of anandamide (Fisar 2009) and is also required for TNF-α induced production of cytokines such as IL-1, IL-5, IL-6 and IL-13 (Sethu et al. 2008). Also monoacylglycerol lipase ABHD12, which was clearly activated by CP-47,497-C8, is involved in the synthesis of endocannabinoids (Chen et al. 2013).

It is notable that all lipid metabolism-associated proteins, which were up-regulated after exposure of the cells to CP-47,497-C8, are involved in inflammatory processes. Numerous studies have been published which concern the effects of pro- and anti-inflammatory properties of phyto- and endocannabinoids (Cabral and Griffin-Thomas 2009; Kaplan 2013; Suarez-Pinilla et al. 2014; Tanasescu and Constantinescu 2010). Also synthetic antagonists which bind selectively to both types of cannabinoid receptors (CB1 and CB2) were used in some of these investigations. In addition to the findings which are described in the previous chapter, we detected up-regulation of further inflammation-related proteins, for example induction of STAT5A and 6, which are involved in both, pro- and anti-inflammatory processes (Goenka and Kaplan 2011; Lu et al. 2007). Furthermore, we found also evidence for up-regulation of interleukin-1β, one of the most important pro-inflammatory cytokines in the human body, which is involved in the pathogenesis of several neurological disorders (Fogal and Hewett 2008). Notably, it is known that this protein regulates also the sensitivity of cannabinoid receptor CB1-controlled GABA transmission in the striatum (De Chiara et al. 2013). Other cytokines which are induced by the drug are interleukin-8 (its production is controlled by TNF-α and by IL-1 which are up-regulated by the drug) and tryptophan tRNA ligase, which has not only an enzymatic function, but is also induced upon inflammatory activation and acts also as a pro-inflammatory cytokine (Haudek-Prinz et al. 2012; Ivakhno and Kornelyuk 2004). Finally, we observed also significant induction of PKC-β, which is involved in the activation of NFκB1 and causes up-regulation of numerous inflammatory mediators (Kang et al. 2001; Kong et al. 2013).

Also in the cytoplasmic fractions, up-regulation of pro-inflammatory proteins was detected in LPS-stimulated cells. As shown in Table 2, we found that TNFα was significantly increased, and this protein activates NFkB1, the most important transcription factor regulating inflammatory responses. Furthermore, also the cytokines IL-6 and IL-12, which are involved in numerous inflammatory diseases (Mihara et al. 2012; Vignali and Kuchroo 2012), were induced by the SC. On the contrary, a reduction in IL-10 was observed under identical experimental conditions. IL-10 is an inhibitor of the synthesis of pro-inflammatory cytokines and controls the inducible form of COX (Sikka et al. 2013).

The former observations seem to contradict previous reports in which the effects of phytocannabinoids and SCs (JWH-015 and JWH133) on cytokine production were monitored in T cells (for review see Suarez-Pinilla et al. 2014). In these previous investigations, induction of IL-10 and suppression of IL-1β and IL-6 were observed in contrast to our findings. Also in experiments with LPS-stimulated human PBMCs, low concentrations of Δ9-THC (3 nM) were found to inhibit pro-inflammatory cytokine production (e.g., TNF-α, IL-8, IL-6), while a high dose (3 μM) which is similar to those we used caused stimulation of these proteins. These findings of Berdyshev et al. (1997) indicate that the responses of mammalian cells toward SCs depend strongly on the exposure concentrations and provide an explanation for the discrepancy between earlier findings and our results.

It is not known at present which molecular mechanisms trigger the up-regulation of inflammatory cytokines. One of the modes of action potentially involved is oxidative stress which may cause activation of a variety of transcription factors leading to expression of hundreds of genes

including those which encode for inflammatory cytokines (for details see (Reuter et al. 2010).

As mentioned in the introduction, recent investigations indicate that SCs of different chemical groups including CP-47,497-C8 cause chromosomal damage in human-derived cells (Koller et al. 2013). The molecular mechanisms which cause these effects are not known. The results of the present study provide a possible explanation; they indicate that CP-47,497-C8 inhibits several enzymes which are involved in DNA repair processes (see Fig. 4). ERCC5 is a synthetic endonuclease involved in excision repair (Kiyohara and Yoshimasu 2007), while MRE11A plays a causal role in double-strand break repair (Errico and Costanzo 2012). Both proteins were significantly down-regulated after exposure of the cells to CP-47,497-C8. These findings indicate that indirect effects may account for the induction of MNi (Table 1) and also for the formation of comets (Fig. 6b, d and f) in lymphocytes. This assumption is supported by results obtained with liver-S9-mix, which indicate that the drug is not converted by phase I enzymes to DNA-reactive metabolites. The reduction in the genotoxic activity of the compound after addition of BSA and also with heat-inactivated enzyme mix indicates that the drug is inactivated by proteins probably due to direct binding. Similar detoxification effects were seen in earlier studies with other direct acting genotoxic carcinogens for example with alkylating drugs and isothiocyanates (Kassie et al. 2003).

It can be seen in Figs. 2, 3 and 4 that the standard derivations in the proteome analyses are quite high indicating that strong inter-individual variations of the responses exist. Also the findings of the MRM experiments (Fig. 5) underline this assumption: As described above, only in two out of three participants induction of cytokines was detected. Nevertheless, it is obvious that the alterations of protein expression patterns which we described in the previous chapters were pronounced and highly significant.

In conclusion, the results of the present investigations indicate that the drug causes two main effects which may lead to adverse health effects in users, namely induction of inflammation via up-regulation of pro-inflammatory cytokines as well as induction of damage of the genetic material via inhibition of repair enzymes. It is notable that the concentrations which caused these effects in the present study in human cells are substantially (10^2–10^3 orders of magnitude) higher than the blood levels which were detected after consumption of selected SCs in humans (Kneisel and Auwärter 2012; Teske et al. 2010). However, it is likely that relatively high concentrations, which may cause inflammation as well as chromosomal damage in drug users, are reached in epithelia of the respiratory tract of drug users. Therefore, further investigations concerning possible adverse effects in consumers are warranted.

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Compliance with ethical standards

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

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