A novel injectable nanotherapeutic platform increasing the bioavailability and antitumor efficacy of Arachidonylcyclopropylamide on an ectopic non-small cell lung cancer xenograft model: a randomized controlled trial

Running title: NanoACPA leads to non-small lung cancer regression in vivo

Ozge Boyacioglu[1,2], Cem Varan[3], Erem Bilensoy[4], Zaliha Gamze Aykut[5], Tuba Recber[6], Emirhan Nemutlu[6], Nedret Kilic[2], Petek Korkusuz[7,8(*)]

[1] Hacettepe University, Graduate School of Science and Engineering, Department of Bioengineering, 06800, Beytepe, Ankara, Turkey

[2] Atılım University, Faculty of Medicine, Department of Medical Biochemistry, 06830, Gölbaşı, Ankara, Turkey

[3] Hacettepe University, Graduate School of Science and Engineering, Department of Nanotechnology and Nanomedicine, 06800, Beytepe, Ankara, Turkey

[4] Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06100, Sıhhiye, Ankara, Turkey

[5] Bilkent University, Faculty of Science, Department of Molecular Biology and Genetics, 06800, Cankaya, Ankara, Turkey

[6] Hacettepe University, Faculty of Pharmacy, Department of Analytical Chemistry, 06100, Sıhhiye, Ankara, Turkey

[7] Hacettepe University, Faculty of Medicine, Department of Histology and Embryology, 06100, Sıhhiye, Ankara, Turkey

[8] METU MEMS Center, 06530, Ankara, Turkey

Corresponding author

Petek Korkusuz, M.D. Ph.D., Professor of Histology and Embryology

Hacettepe University, Faculty of Medicine, Department of Histology and Embryology, 06100, Sıhhiye, Ankara, Turkey

petek@hacettepe.edu.tr

[1] Özge Boyacıoğlu ORCID: 0000-0001-5240-8209

[2] Cem Varan ORCID: 0000-0002-9391-8691

[3] Erem Bilensoy ORCID: 0000-0003-3911-6388

[4] Zaliha Gamze Aykut ORCID: 0000-0003-2184-8628

[5] Tuba Reçber ORCID: 0000-0001-8257-7628

[6] Emirhan Nemutlu ORCID: 0000-0002-7337-6215

[7] Nedret Kilic ORCID: 0000-0002-5747-9433

[8] Petek Korkusuz ORCID: 0000-0002-7553-3915

Journal homepage: www.elsevier.com/locate/ijpharm

https://doi.org/10.1016/j.ijpharm.2024.125153

Received 22 October 2024; Received in revised form 29 December 2024; Accepted 29 December 2024

International Journal of Pharmaceutics 670 (2025) 125153

Available online 31 December 2024

Abstract

Rapid progressing non-small cell lung adenocarcinoma (NSCLC) decreases treatment success. Cannabinoids emerge as drug candidates for NSCLC due to their anti-tumoral capabilities. We previously reported the controlled release of Arachidonoylcyclopropylamide (ACPA) selectively targeting cannabinoid 1 (CB1) receptor in NSCLC cells in vitro. Hydrophobic polymers like polycaprolactone (PCL) offer prolonged circulation time and slower drug clearance which is suitable for hydrophobic molecules like ACPA. Thus, the extended circulation time with enhanced bioavailability and half-life of nanoparticular ACPA is crucial for its therapeutic performance in the tumor area. We assumed that a novel high technology-controlled release system increasing the bioavailability of ACPA compared to free ACPA could be transferred to the clinic when validated in vivo. Plasma profile of ACPA and ACPA-loaded PCL-based nanomedicine by LC-MS/MS and complete blood count (CBC) was assessed in wild-type Balb/c mice. Tumor growth in nanomedicine-applied NSCLC-induced athymic nude mice was assessed using bioluminescence imaging (BLI) and caliper measurements, histomorphometry, immunohistochemistry, TUNEL assay, and Western blot on days 7-21. Injectable NanoACPA increased its systemic exposure to tissues 5.5 times and maximum plasma concentration 6 times higher than free ACPA by substantially improving bioavailability. The potent effect of NanoACPA lasted for at least two days on ectopic NSCLC model through Akt/PI3K, Ras/MEK/Erk, and JNK pathways that diminished Ki-67 proliferative and promoted TUNEL apoptotic cell scores on days 7-21. The output reveals that NanoACPA platform could be a chemotherapeutic for NSCLC in the clinic following scale-up GLP/GMP-based phase trials, owing to therapeutic efficacy at a safe low dose window.

Keywords: Non-small cell lung cancer, Arachidonoylcyclopropylamide (ACPA), polycaprolactone (PCL), cannabinoids, nanomedicine, nanoparticle-based drug delivery system

1. Introduction

Lung cancer is responsible for the majority of cancer-related mortality worldwide due to its high invasive and metastatic features (Lahiri et al., 2023) with crude and age-standardized incidence rates of 28.3 and 23.0 per 100,000 according to a global burden report published in 2023 (Li et al., 2023). More than 85% of cases are defined as non-small cell lung cancer (NSCLC) (Padinharayil et al., 2023). FDA-approved or currently tested targeted agents are known to inhibit PI3K/Akt, Ras/MEK/Erk and JNK pathways through epidermal growth factor (EGF), ROS proto-oncogene 1 (ROS1), anaplastic lymphoma kinase (ALK), mesenchymal-epithelial transition (MET) or rearranged during transfection (RET) receptors but with low success and systemic side-effects complicating the treatment process (Hsu et al., 2023; Liu et al., 2019; Mausey and Halford, 2023).

Widely distributed endocannabinoid system components within the respiratory epithelia and the immune cells of the stroma act on major immunophysiological functions of the lung (Corrado et al., 2018; Fantauzzi et al., 2020; Gkoumassi et al., 2007; Wiese et al., 2023) and cannabinoid 1 (CB1) receptor presents higher expression than that of cannabinoid 2 (CB2) receptor in the parenchyme of the respiratory system (Turcotte et al., 2016). Cannabinoid ligands emerge as drug candidates for NSCLC treatment due to their anti-proliferative, apoptotic, anti-metastatic, and anti-angiogenic capacities (Boyacıoğlu and Korkusuz, 2023). Pan cannabinoid agonists Tetrahydrocannabinol (Δ^9-THC) (Preet et al., 2008; Sarafian et al., 2008), Cannabidiol (CBD) (Haustein et al., 2014; Milian et al., 2020; Ramer et al., 2012; Ramer et al., 2014; Ramer et al., 2010a; Ramer et al., 2010b; Vidinsky et al., 2012) and WIN55,212-2 (when applied with CB2 agonist JWH-015 (Preet et al., 2011) or Met-AEA (Ravi et al., 2014)) at a range between 10^{-8}-10^{-4} M dose inhibited proliferation, chemotaxis, migration and/or invasion and promoted apoptosis of NSCLC cells *in vitro*. The *in vivo* validation of the anti-tumoral effect for Δ^9-THC, CBD, and WIN55,212-2 revealed a limited performance at a high dose range. Two clinical trials revealed the anti-tumoral effects of Δ^9-THC in two over nine patients (Guzmán et al., 2006) and of Nabiximols (Δ^9-THC and CBD) (Twelves et al., 2021) in recurrent glioblastoma with serious side effects. Though presenting as the most potent candidate, Δ^9-THC is the major psychoactive cannabis constituent, can quickly cross the blood-brain-barrier, currently can be used for very

restricted indications, and is less likely to be prospective therapeutics for cancer in the clinical practice (Abuhasira et al., 2018; Calapai et al., 2020; Cristino et al., 2020). Our group previously reported a very low real-time antiproliferative and apoptotic dose window ($1.39x10^{-12}$ M) for a synthetic specific CB1 receptor agonist Arachidonylcyclopropylamide (ACPA) and generated a novel ACPA-loaded polycaprolactone (PCL) nanoparticulate delivery system (PCT/TR2020/050618) (Boyacıoğlu et al., 2021; Boyacioglu et al., 2022) in conjunction with its metabolic pathway in NSCLC *in vitro* comparing to other epithelial cancers (Endometrial cancer; 10^{-6} M (Bilgic et al., 2017), pancreatic cancer; 10^{-4} M (Brandi et al., 2013; Dando et al., 2013; Donadelli et al., 2011)). PCL is a more hydrophobic polymer with a slow degradation rate providing a prolonged circulation time in the bloodstream and thus, gradual release and slower clearance of the encapsulated drug compared to PLA or PLGA (Assani et al., 2022; Sadeghi et al., 2024) which is the best choice for hydrophobic active molecules, such as ACPA. Although, PCL provided a controlled release of ACPA, as a low-soluble cannabinoid with a shorter half-life and poor stability (Boyacıoğlu et al., 2021; Palrasu et al., 2022), an improvement in the plasma profile with a prolonged circulation of the active molecule including enhanced bioavailability and half-life (Elmowafy et al., 2023) is crucial for therapeutic performance at the tumor site (Bhardwaj and Jangde, 2023; Liu et al., 2024; Öztürk et al., 2024).

Herein, we hypothesized that the novel ACPA-loaded PCL-based drug delivery platform (NanoACPA), could exhibit superior bioavailability and systemic exposure compared to free ACPA and inhibit tumor growth by decreasing the proliferation rate, promoting the apoptosis of the tumor cells through Akt/PI3K, Ras/MEK/Erk and JNK pathways in vivo NSCLC ectopic xenograft nude mouse model by selectively targeting CB1 receptor. First, the plasma profile of ACPA and NanoACPA system and the complete blood count (CBC) parameters of a wild-type Balb/c mouse model were evaluated. The antitumoral effect of the intraperitoneally administered ACPA and NanoACPA system on tumor size was eventually assessed by bioluminescence imaging (BLI) and caliper measurement. Tumor nodules and potential metastatic loci (lung, costal bone, brain, testis, spleen, kidney, and liver) were evaluated by quantitative histomorphometric analysis. Antiproliferative and apoptotic mechanisms of action of the ACPA and NanoACPA

nanomedicine on tumor nodules were assessed by Ki-67 immunohistochemical analysis, TUNEL, and Western blot for caspases, PI3K/Akt, Ras/MEK/Erk, and JNK pathways.

2. Materials and methods

2.1. Study design

An in vivo randomized controlled trial, predetermined by 80% test power and 95% confidence interval (G-Power v3.1), was conducted under ARRIVE guidelines (Voehringer and Nicholson, 2020) following ethics approval by the Institutional Animal Care and Use Committee (BILHADYEK) of Bilkent University (24.11.2021/Approval no:2021/15).

2.2. Plasma profile of ACPA and NanoACPA

NanoACPA was prepared and characterized as previously described (Boyacıoğlu et al., 2021). Briefly, NanoACPA formulation was prepared by the nanoprecipitation method using PCL MW:80,000 Da (#440744-250 G, Sigma-Aldrich). A mixture of acetonitrile:ethanol (9:1; v/v), containing 0.1% (w/v) PCL and 0.01% (w/v) ACPA (#91053, Batch no: 0435943-15, Cayman Chemical, C23H37NO, MW: 343.6 g/mol, purity: ≥98%, colorless liquid, 5 mg/100 ul absolute ethanol, solubility in ethanol: 25 mg/ml, organic solvent content: 95%, solid content: 5%), was used to form the organic phase. This phase was then slowly added to ultra-pure water (1:2 v/v) with 0.05% (w/v) Pluronic F68 at room temperature. After stirring the colloidal mixture, the organic solvent was removed and nanoparticle dispersion was obtained (Boyacıoğlu et al., 2021; Boyacioglu et al., 2022). As ACPA encapsulation efficiency and 7-day release profile assessments were previously provided (Boyacıoğlu et al., 2021), to observe the long-term physicochemical stability of NanoACPA, *in vitro* characterization by particle size distribution, polydispersity index (PDI) and zeta potential was performed using Zeta Sizer Nano ZS (United Kingdom) (n=3) (Boyacıoğlu et al., 2021) on the day the formulation was prepared and after 3, 5 and 7 days of storage at +4^{o}C, since the maximum time interval between two consecutive administrations was 7 days. As a preliminary step, NanoACPA formulation at 0.5 mg/kg dose was applied to wild-type Balb/c mice, however, no peak was identified in the plasma after 60 minutes of i.v. administration by LC-MS/MS (Supplementary Table 1). Thus, ACPA or NanoACPA

formulation at 1.0 mg/kg dose, also determined according to previous studies (Ebrahimi-Ghiri et al., 2019; Kumar et al., 2016; Shafaroodi et al., 2004), in a vehicle of 100 µl of ethanol:PBS (35:65, v/v, EtOH:PBS), was intravenously (i.v.) administered through the tail vein of 6–8-week-old wild type male BALB/c mice (n=6). Blood samples were collected at 5, 30, 60, 120, 240, and 480 min into K_2-EDTA blood collection tubes and centrifuged at 12000 rpm for 15 min at +4°C to obtain plasma. Previously validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) evaluated the amount of ACPA (Boyacıoğlu et al., 2023). Briefly, 50 µl plasma was spiked with 50 µl acetonitrile containing internal standard anandamide (10 µg/ml). Following centrifugation at 10,000 rpm for 10 min, the supernatant was analyzed. Plasma profile (Dissociation rate constant (k_d), half-life ($t_{1/2}$), volume of distribution (V_d) and clearance (Cl)) and bioavailability (Area under the curve (AUC), maximum plasma concentration (C_{max}) and time to reach the maximum concentration (T_{max})) parameters were calculated by WinNonlin® pharmacokinetic software.

2.3. Complete Blood Count

The hemogram was evaluated in i.v. ACPA (n=6) or NanoACPA platform (n=6)-injected 6–8-week-old wild-type Balb/c mice. Briefly, peripheral venous blood samples collected at t_0 and t_f (480 min after drug application) were transferred into K_2-EDTA tubes and CBC parameters including hemoglobin (HGB), hematocrit (HCT), red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width-coefficient of variability (RDW-CV), white blood cell (WBC), absolute lymphocyte count (ALC), lymphocyte (LYMPH), absolute monocyte count (AMC), monocyte (MONO), absolute eosinophil count (AEC), eosinophil (EOS), absolute basophil count (ABC), basophil (BASO) and platelet count (PLT) were analyzed by fully automated blood counting device (XN-1000, Sysmex, Turkiye).

Peripheral smears of blood samples were stained with the routine Giemsa staining method by Beckman Coulter Unicel DxH smear preparation staining device (DXH 800 SMS). The slides were treated with Giemsa stain and washed with distilled water for 5 min each and evaluated on an automated microscope attached digital camera by image analysis program (Leica DMB6B, DFC7000T, LASV3 Wetzlar, Germany).

2.4. Tumor growth assessment of NSCLC xenografts

The highest CB1 agonistic antitumoral effect of ACPA and NanoACPA on A549 NSCLC cells in vitro, in our previous study (Boyacıoğlu et al., 2021), constituted the main rationale for the use of this cell line for tumor induction in vivo in this study. $1x10^6$ A549-Luc2 (CCL-185-LUC2, ATCC, Virginia, USA) cells, expanded with F12-K medium (Gibco, New York, USA) enhanced by 10% FBS (Capricorn, Germany) and 8 µg/ml blasticidin (Invivogen, France), were injected in 100 µl PBS subcutaneously into the right flank of 6–8-week-old male athymic BALB/c nude mice for tumor induction (Dothager et al., 2009; Preet et al., 2008). When the tumor nodules reached palpable size (average of 100-150 mm^3) (K and Choppala, 2022; Liu et al., 2023) by caliper measurement (day 35, T35), mice were divided into control (0.9% NaCl/2 days, G1), vehicle-treated (EtOH:PBS, 35:65, v/v per 2 days, G2), NanoACPA (1 mg/kg/week, G3, or 1 mg/kg/2 days, G4) or ACPA-treated (1 mg/kg/2 days, G5) groups to be injected in 100 µl intraperitoneally (i.p.) for 7, 14 or 21 days (n=6, N=90). Comparison of vehicle- or drug-treated groups and negative control healthy nude mice (n=6, N=18) were done for further analyses following sacrification on days 7 (SET 1), 14 (SET 2), or 21 (SET 3). Evaluation of the nano system-alone on the NSCLC xenograft model was not performed, since the blank PCL nanoparticles have been already tested inert on NSCLC cells by RTCA in our previous work (Boyacıoğlu et al., 2021).

BLI for tumor growth assessment was performed under isoflurane anesthesia after i.p. D-luciferin (150 mg/kg) injection on days 0, 14, and 28 of tumor induction and days 7 (day 42), 14 (day 49), and 21 (day 56) of drug administration (Puri et al., 2022). Images were acquired with IVIS Spectrum in vivo Imaging System (PerkinElmer, Massachusetts, USA) and the measurement of light intensity (bioluminescence unit) observed for luciferase-expressing A549 cell-made tumor nodules was presented in terms of total flux radiance as a calibrated measurement based on the photon emission (Lu et al., 2021).

Tumor size was also measured with caliper on days 35 (baseline), 42, 49, and/or 56, and tumor volumes were calculated as $½length x (width)^2$ (Preet et al., 2008; Ravi et al., 2014). After 7, 14, or 21 days of drug application, animals were sacrificed, tumor nodules were excised, and weights

were measured by a high-precision scale and quick-frozen in liquid nitrogen for further analyses (Supplementary Fig. 1).

2.5. Histomorphometric analysis

After fixing a small piece of tumor nodules in 10% phosphate-buffered formalin (pH 7.0), they were dehydrated in a graded series of ethanol, cleared with xylol, and embedded in paraffin. 3-5 µm-thickened sections, taken by rotary microtome (Leica, Germany), were deparaffinized and stained with Hematoxylin-Eosin. The tumor area was calculated on each section under a light microscope and digital camera attachment (Akkın et al., 2023). Total lung samples were kept in Bouin's fixative at room temperature (RT) for 1 day for macroscopic superficial metastatic nodules. For micrometastasis evaluation, lung, liver, costal bone, kidney, spleen, testicle, and brain tissues were processed for Hematoxylin Eosin staining. Metastatic tumor, inflammation (edema, congestion, cell infiltration), fibrotic and necrosis/epithelial parenchymal disruption (metaplasia, cell damage) areas were evaluated within a range of 0-4; scored as 0 for no inflammation, fibrosis, metastasis or damage in any area, 1 for granulation tissue and inflammatory cell infiltration, fibroblasts, collagen, metastatic and necrotic cell accumulation, blebbing on the apical surfaces of epithelial cells, loss of epithelial cell interface connections and cellular debris in <25% area, 2 for 25-50%, 3 for 50-75% and 4 for 75-100%. The score criterion of the quantitative histomorphometric analysis is summarized (Supplementary Table 2) (Knoblaugh and Himmel, 2019).

2.6. Immunohistochemistry

Tumor nodule sections were passed through xylene and alcohols, treated with 0.1 M citrate buffer and heated for antigen retrieval, then treated with 3% hydrogen peroxide and incubated with Ki-67 primary antibody (#ab15580, Abcam, UK) for 1h at RT. Sections were labeled with HRP-conjugated secondary antibody (#ab236466, Abcam, UK) for 2h at RT, dehydrated in graded series of ethanol. The sections were examined under a light microscope and digital camera attachment. Ki-67 positive tumor cells were counted and divided by the total number of cells in the whole tumor area by Image J software (v1.54h) and the percentage was scored as 1 for <15%, 2 for 16-45%, 3 for 46%-75%, and 4 for >75% (Wang et al., 2021).

2.7. TUNEL assay

Tumor nodule sections were evaluated by *ApopTag Peroxidase In Situ Apoptosis* Kit (#S7101, Merck Millipore, Germany) (Bilgic et al., 2017). Briefly, cleared and dehydrated sections were subjected to Proteinase K (#21627, Merck Millipore, Germany) for 15 min at RT. Sections were kept in a 3% hydrogen peroxide, equilibration buffer, TdT solution, and anti-digoxigenin conjugate, respectively. 3,3′-Diaminobenzidine (DAB) for apoptotic cell nuclei staining and methyl green for background staining were performed and evaluated under a light microscope. Quantitative apoptotic cell indices were determined by dividing the number of apoptotic cells in the whole tumor area by the total cell number by Image J software (v1.54h) (Bilgic et al., 2017).

2.8. Western blot

Phospho-Akt^{S473} (1:2500, #MAB887, R&D, Minnesota, USA), phospho-PI3K$^{Y467/Y199}$ (1:1000, #ab278545, Abcam, UK), phospho-JNK$^{T183/Y185}$ (1:1000, #MAB1205, R&D, Minnesota, USA), pERK1$^{T202/Y204}$+ pERK2$^{T185/Y187}$ (1:1000, #P00104, Boster Bio, California, USA), total Akt (1:2500, #MAB2055, R&D, Minnesota, USA), PI3K (1:1000, #MAB6777, R&D, Minnesota, USA), JNK (1:2500, #MAB1387, R&D, Minnesota, USA), ERK1+ERK2 (1:10000, #ab184699, Abcam, UK), cyclin D1/D2 (1:2000, #AF4196, R&D, Minnesota, USA), caspase 3 (1:400, #634101, Biolegend, California, USA), caspase 9 (1:500, #E-AB-30760, Elabscience, China) and internal control GAPDH (1:500, #bs-2188R, Bioss, Massachusetts, USA) protein levels in tumor tissues were determined by Western blot as previously done (Kılıç et al., 2022). Following homogenization and centrifugation of 50 mg of tumor tissue, total protein concentration was measured by bicinchoninic acid (BCA) assay (#23227, ThermoScientific, Massachusetts, USA) at 562 nm. After boiling 30 µg protein samples with sample buffer for 5 min at 95 ^{o}C, all proteins were separated on 8-12% SDS-PAGE (#1658004, Biorad, California, USA) in 1x running buffer and transferred to PVDF membrane with Transfer blot system (#1704150, Biorad, California, USA). After 2h of the blocking step with 5% non-fat milk or BSA/TBS-t, membranes were kept overnight with primary antibodies +4^{o}C on a shaker. After incubation, the membranes were washed with TBS-t, incubated with a secondary antibody for 1h, and washed again with TBS-t. Due to the limited amount of samples to be used for the analysis of many proteins, mild stripping was performed according to the

literature (Litovchick, 2020). Enhanced chemiluminescence was performed for the visualization of bands (ChemiDoc Imaging System, #12003153, Biorad, California, USA). Bands were analyzed against GAPDH by Image Lab version 6.0 (Biorad, California, USA).

2.9. Statistical Analysis

Plasma profile data were distributed non-normally by Shapiro-Wilk test, thus all parameters were evaluated by the Mann-Whitney U test (Xu, 2023). Since raw data regarding CBC parameters showed normal distribution by the Shapiro-Wilk test, significance between time points and groups was separately determined by Student's t-test. Raw data of BLI, histomorphometric analysis, TUNEL and Ki-67 labeling, and Western blot were normally distributed by the Shapiro-Wilk test. Multiple comparisons between groups were evaluated by one-way analysis of variance (ANOVA), and pairwise comparisons were evaluated by the post-hoc Duncan test. Spearman's correlation test assessed the correlation between Ki-67 immunostaining–TUNEL and tumor area by histomorphometric analysis–BLI measurements and tumor volume by caliper–BLI measurements. All data were evaluated within the 95% confidence interval (SPSS v25.0). Graphs were drawn with Graphpad Prism 8.

3. Results

3.1. Nanoencapsulation improves the bioavailability of ACPA with no WBC but slight RBC alteration in wild-type mice

NanoACPA showed a mean particle size of 129.4 ± 0.3 nm, PDI of 0.081 ± 0.006, and zeta potential of -16.11 ± 0.83 mV on the day the formulation. Average nanoparticle size, PDI and zeta potential of NanoACPA were 135.6 ± 0.8 nm, 0.062 ± 0.024 and -20.16 ± 0.38 mV, respectively, on day 3; they were 145.0 ± 3.7 nm, 0.048 ± 0.010 and -21.14 ± 0.73 mV, respectively, on day 5 and 143.2 ± 3.9 nm, 0.065 ± 0.019 and -16.22 ± 0.47 mV on day 7 (Fig 1a, b) indicating that NanoACPA is physicochemically stable when stored at +4 ^{o}C for 7 days.

NanoACPA ensured a higher and sustainable plasma concentration with a relatively rapid increase and a significantly higher C_{max}, AUC, and $t_{1/2}$ and lower T_{max}, k_d, V_d, and Cl from 5 min to 2h compared to the ACPA in wild-type Balb/c mice (Fig. 1c, d). Both ACPA and NanoACPA

caused a slight depression in HGB (Fig. 1f) and red blood cell-related counts (RBC, MCH, MCHC, RDW-CV) (Fig. 1g, i-k) excluding the MCV (Fig. 1h) which was also in line with the peripheral blood smear examinations (Fig. 1v-y). The WBC (LYMPH, ALC, MONO, AMC, EOS, AEC, BASO, ABC) (Fig. 1l-t) and PLT (Fig. 1u) counts remained unchanged following NanoACPA injection. WBC parameters, however, presented a suppression that was assisted by a decrease in HCT count (Fig. 1e) following ACPA application at 8h.

3.2. NanoACPA prominently inhibits NSCLC growth in ectopic nude mouse xenograft model on days 7-21

Tumor nodules reached a homogenous size by caliper measurement and BLI in all groups on day 35 (T35, M0) following s.c. tumor cell injection in all sets (SET 1, 2 & 3) (Fig. 2a-e, 3a-e, 4a-e). All sets presented an acceleration in tumor growth from day 28 (T28) to 42 (T42, M7) excluding the weekly-applied NanoACPA group (Fig. 2f, 3f, 4f) that provided 67% and 48% of tumor growth reduction compared to control and vehicle, respectively. NanoACPA similarly reduced the tumor weight (Fig. 5a-f), however, weekly-applied NanoACPA presented a maximum efficiency in reducing the tumor growth by BLI and by caliper measurement on day 7 (T42, M7) compared to all other groups including every 2-day application (Fig. 2f-g, 3f-g, 4f-g). Weekly-applied NanoACPA diminished the tumor growth by 42% and 65% on average on day 14 (M14) and 21 (M21) compared to control, respectively. Every 2-day-applied NanoACPA exhibited superior performance on tumor growth in terms of weight and volume compared to weekly-applied NanoACPA and all the other groups from day 14 (T49, M14) to 21 (T56, M21) in all sets of experiment (Fig. 3f-g, 4f-g, 5) that exhibited 50% and 96% decrease in tumor growth comparing to control, respectively. Free ACPA application provided a decrease in tumor volume by BLI and caliper measurement but not in tumor weight from day 14 to 21 compared to controls (Fig. 3f-g, 4f-g, Fig. 5f). Free ACPA exhibited 77% and 11% decrease in tumor growth on days 14 and 21 comparing to control, respectively (Fig. 3f-g, 4f-g). A dramatic alteration in tumor volume was observed by every 2 day-applied NanoACPA presenting 48% and 56% reduction on day 14 (Fig. 3g) and 78% and 43% reduction on day 21 (Fig. 4g) compared to control and vehicle by calliper measurement. Free ACPA presented 53% (fig. 3g) and 68% (Fig. 4g) decrease in tumor volume

on days 14 and 21 compared to control by calliper measurement. Metastatic foci were not noted in the lungs of the drug-applied and control groups from day 7 to 21 (Fig. 5g-l).

3.3. NanoACPA exhibit anti-tumoral efficacy by Akt/PI3K, Ras/MEK/Erk and JNK pathways

NanoACPA ensured poorer infiltrative cell and vessel density patterns within tumor parenchyma when applied weekly or every 2 days compared to all groups from day 7 to 21 by quantitative histomorphometry (Fig. 6a, c, f).

NanoACPA presented maximum efficiency in reducing the tumor area and inflammation on day 7 when applied weekly compared to all other groups (Fig. 6c, f). NanoACPA groups exhibited similar better performance on tumor area from day 14 to 21 but a superior effect of every 2-day injection on day 21 on inflammation rate compared to controls (Fig. 6c, f). Free ACPA did not change the tumor area and inflammation rate on day 7 but on days 14 and 21 (Fig. 6c, f). Tumor area measurements by histomorphometry (Fig. 6d, R^2=0.7787, p=0.001) and tumor volume measurements by caliper (Fig. 6e, R^2=0.8484, p=0.001) presented a significantly strong positive correlation with BLI measurements. Fibrosis (Fig. 6g) and necrosis (Fig. 6h) scores slightly increased in time at variable levels in drug-applied and control groups.

Lung (Fig. 6b), costal bone (Supplementary Fig. 2a), brain (Supplementary Fig. 2b), testis (Supplementary Fig. 3a), spleen (Supplementary Fig. 3b), kidney (Supplementary Fig. 4a) and liver (Supplementary Fig. 4b) samples presented a nonspecific mild to moderate stromal vascular congestion and inflammation with mononuclear cell infiltration and mild edema. Fibrotic alterations or necrotic areas were not observed in these organs (Supplementary Table 3). Foreign body reaction or granulomatous focus was furthermore not detected in the lung (Fig. 6b), spleen (Supplementary Fig. 3b), and liver (Supplementary Fig. 4b). NanoACPA (1 mg/kg/week) and free ACPA reduced inflammation in the lungs compared to vehicle or control on day 7 (Fig. 6i). All drug-applied groups decreased the inflammation level in the lungs compared to vehicle-applied and healthy negative control groups on days 7-14; whereas control and vehicle-applied groups had higher inflammation scores than that of negative control on day 21 (Fig. 6i). NanoACPA (1 mg/kg/week) and free ACPA caused a higher score of inflammation in the lungs than that of the negative control on day 21 (Fig. 6i). NanoACPA (1 mg/kg/2 days) lowered the inflammation scores

in the lungs compared to free ACPA and vehicle on day 21 (Fig. 6i). NSCLC control group presented a significant increase in inflammation scores, whereas negative control group exhibited a significant decrease over time (Fig. 6i).

TUNEL (+) apoptotic tumor cells were abundant collectively in the center and scarce in the periphery of the tumor in all control and experimental groups (Fig. 7b) in accordance with the labelling of positive (Fig. 7d) and negative (Fig. 7e) controls, vice versa for Ki-67 (+) proliferative tumor cells (Fig. 7a). All-drug applied groups increased TUNEL scores (Fig. 7b, f) and reduced proliferation rate of tumor cells by Ki-67 labelling (Fig. 7a, c) comparing control and vehicle-applied groups on days 7-21. Free ACPA presented a higher apoptosis rate than that of every 2-day-applied NanoACPA on days 14-21 (Fig. 7f). Every 2-day NanoACPA application caused a sharp decrease in proliferation index over time (Fig. 7c). A strong negative correlation was detected between the apoptotic index by TUNEL and proliferative cell index by Ki-67 labelling (Fig. 7g, R^2=0.9009, p=0.001).

Both weekly- and every 2-day-applied NanoACPA inhibited p-Akt, p-JNK, p-ERK1/2, CycD1/D2 and promoted Casp-9 and cleaved Casp-9 expression compared to vehicle and/or control groups on days 7-21 by Western blot (Fig. 7h-i, k-l, n-o). Free ACPA suppressed p-Akt and CycD1/D2 protein expression compared to the vehicle-applied group only on day 21 (Fig. 7h-i, n). Every 2-day-applied NanoACPA and free ACPA suppressed p-PI3K and elevated Casp-3 protein expression compared to control on days 7-21 (Fig. 7h, j, m). Weekly-applied NanoACPA reduced p-PI3K on day 7 and elevated Casp-3 expression on day 21 compared to control by Western blot (Fig. 7h, j, m). Free ACPA inhibited p-JNK and induced Casp-9 and cleaved Casp-9 expressions on days 7-21 compared to control and/or vehicle (Fig. 7k, o, Fig. 8).

4. Discussion

In this research, we report the in vivo biodistribution and anti-tumor efficacy performance of a novel nanotherapeutic for NSCLC. We revealed, for the first time, the plasma profile of ACPA and novel PCL-based drug delivery formulation that establishes an improved bioavailability of ACPA with higher peak concentration of plasma, C_{max}, overall exposure AUC and $t_{1/2}$ and lower k_d, V_d and Cl values comparing to free ACPA with a slight depression in RBC and WBC-related counts

in a wild-type mouse model directly validating nude mice heterotypic tumor model (Li et al., 2016). All of the WBC counts and the majority of the erythroid cell parameters (MCV, MCH, MCHC, RDW-CV) remaining intact confirmed the biosafety of the new formulation in terms of systemic tolerability. However, the slight decrease in RBC count with no abnormality of other erythroid parameters should be taken into consideration as a potential adverse event in scale-up animal studies. Here, we report that 1.0 mg/kg free ACPA and NanoACPA presented C_{max} and AUC values of 2.443 and 14.173 ng/ml, and 74.918 and 414.982 min.ng/ml, respectively, at 8 h. The systemic exposure expressed as AUC increased 5.5 times and the peak concentration C_{max} increased 6 times in the plasma profile of nanoparticulate ACPA compared to free ACPA, in this study. Two studies assessed the plasma profiles of the potent psychoactive pan cannabinoid agonist Δ^9-THC (Torrens et al., 2020) and non-psychoactive pan agonist CBD (Xu et al., 2019) in wild-type mice. Δ^9-THC at 1.6 and 0.5 mg/kg exhibited mean C_{max} and AUC values of 122 and 22 ng/ml and 10054 and 2838 min.ng/ml, respectively, when administered to C57BL/6 mice (Torrens et al., 2020). C_{max} and AUC values of ACPA were lower than that of Δ^9-THC which was significantly improved by nanoencapsulation. Herein, CB1 agonist ACPA at 1 mg/kg presented also a lower C_{max} value than 10 mg/kg i.v.-administered CBD (C_{max} values are 1350 and 48.7 ng/ml at 10 min and 8 h, respectively) to ICR mice (Xu et al., 2019) which was substantially enhanced by entrapping ACPA. Δ^9-THC presented mean C_{max} and AUC values of 198 ng/ml and 28763 min.ng/ml, respectively, at 5 mg/kg (Torrens et al., 2020) showing long-lasting anti-depressant-like (Xu et al., 2019) or psychotropic (Cristino et al., 2020) effects when i.v. administered at high doses. The present study established the lowest dose with a potentially adequate yet-to-be-demonstrated antitumor therapeutic efficacy in vivo for the non-psychoactive ACPA and NanoACPA, through CB1 receptor agonism only, in the frame of other cannabinoid therapeutics for NSCLC (Preet et al., 2011; Shafaroodi et al., 2004). The C_{max}, AUC, and half-life of the drug candidate depend on the dose and/or elimination rate of the drug from the body (Hallare and Gerriets, 2024), which requires further plasma profile analysis of ACPA at higher dose levels to compare with other cannabinoids such as CBD. Still, the improved bioavailability of ACPA with a lower dose via a selective CB1 agonism might enable maximizing the clinical benefit by eradicating potential systemic involvement and the side effects of pan agonists (Lazic

and Williams, 2020). Herein, the Cl, $t_{1/2}$, and V_d values of ACPA and nanoparticle-bound ACPA were 209.568 and 47.265 ml/min, 36 and 71 min, and 9323.957 and 4866.329 ml, respectively, at 8 h indicating the improvement of bioavailability of ACPA when applied in PCL-based drug delivery formulation. Within this perspective, the successfully developed NanoACPA system improves the tolerability, safety, and efficacy of ACPA in plasma. The V_d and Cl values of 5 mg/kg i.v.-administered Δ^9-THC to C57BL/6 mice was calculated as 808 ml and 4.4 ml/min, respectively (Torrens et al., 2020). The same research also revealed the plasma half-lives of 5.0, 1.6, and 0.5 mg/kg doses of Δ^9-THC as 109, 108, and 92 min, respectively (Torrens et al., 2020). Intravenously administered CBD at 10 mg/kg exhibited $t_{1/2}$, Cl, and V_d values of 234 min, 1.983 ml/min, and 682.5 ml, respectively, in 35 g-weighed ICR mice (Xu et al., 2019). The half-lives of ACPA and NanoACPA system, in our study, were found to be lower than that of CBD and Δ^9-THC. On the other hand, ACPA and NanoACPA showed higher Cl and V_d compared to those of CBD and Δ^9-THC. Free ACPA and NanoACPA representing shorter half-life and higher Cl and V_d could be associated with the lower dose application compared to that of CBD or Δ^9-THC. Furthermore, high V_d observed by ACPA might reveal that the drug leaves the plasma faster followed by passing into the extravascular areas of the body which may require testing higher doses (Mansoor and Mahabadi, 2023). Overall, the superiority of Cl and V_d values of nanoencapsulation maintained the level of ACPA in the blood for a long time in a high, reliable, and permissible dose compared to free ACPA.

Herein, we conducted the experiments regarding the evaluation of the plasma profile of Balb/c mice upon i.v. administration of ACPA and NanoACPA but i.p. administration for antitumoral efficacy of the drugs. Due to the rapid absorption of most substances from the peritoneal cavity, it is known that the systemic exposure (AUC and C_{max}) of a substance administered via the i.p. route is more similar to that of the i.v. route (Al Shoyaib et al., 2019). The absorption rate after i.p. administration has a typical rapidity of one-half to one-fourth compared to i.v. administration (Al Shoyaib et al., 2019) which might indicate similarity in overall exposure. Once absorbed from the peritoneal cavity, small molecules like ACPA (343.6 g/mol) are known to follow the absorption pathway to reach the systemic circulation, as the surface area of the membranes transporting the substances into the portal circulation is significantly larger. Another key factor is to consider

whether the i.p. route is appropriate for long-term or repeated treatments. Since ACPA and NanoACPA are applied every 2 days within the scope of this research, i.p. administration becomes more reproducible and easier to perform compared to other routes. Moreover, i.v. administration has technical challenges and is not suitable for large-volume administrations when needed. On the other hand, i.p. administration has various advantages including the administration of large volumes, higher absorption rates, and suitability for repeated administrations (Al Shoyaib et al., 2019). Thus, the antitumoral efficacy experiments were conducted by i.p. injecting ACPA and NanoACPA formulations to nude mice models. I.p. injection is a well-established and reliable protocol for antitumoral activity studies in tumor-induced animal models. The physicochemical properties of nanoparticles significantly influence their ability to traverse biological barriers. ACPA-PCL nanoparticles, with a size of approximately 130 nm and a negative surface charge, are considered relatively small. Nanoparticles within the 100-150 nm range have a prolonged circulation time and increased accumulation at the tumor site, as they effectively penetrate biological membranes and enter systemic circulation with rapid absorption upon i.p. administration resulting in higher bioavailability and lower clearance (Chenthamara et al., 2019). Moreover, the i.p. route can allow for prolonged drug release into the peritoneal space, potentially leading to extended therapeutic effect and ensuring systemic distribution. Therefore, we anticipate that our nanoparticles will not encounter significant obstacles in this regard as our research group has published several *in vivo* studies with PCL nanoparticle administration through the tail vein. The biodistribution and the fate of the nanoparticles are well established. Additionally, NanoACPA targeted higher CB1 receptor-expressing tumor cells *in vivo nude* mouse model without being captured, since the superior antitumoral efficacy of the drug candidate could be examined. I.p. administration, on the other hand, could elucidate the potential of nanoparticle-bound ACPA in the peritoneum. Nevertheless, an efficient and rapid diffusion through the biological membranes was expected from the nanoparticles.

Herein, we report for the first time that every 2-day 1 mg/kg dose-applied ACPA and NanoACPA inhibit NSCLC growth in ectopic nude Balb/c mice xenograft model on days 14-21 without causing systemic toxicity, increased infiltration, necrosis, fibrosis, and metastasis. ACPA and the novel NanoACPA system provided a 50% reduction in tumor weight and 60-80% in tumor volume,

induced apoptosis in more than 50% of the tumor cells and inhibited proliferation in less than 50% of the tumor cells through caspase, PI3K/Akt, Ras/MEK/Erk and JNK pathways, thus, ensured an efficacy with a 100% survival rate following the induction of NSCLC model on day 21. A very limited number of studies assessed the efficacy of synthetic cannabinoid ligands Δ^9-THC, WIN55,212-2, MET-F-AEA, JWH-133 and JWH-015 via CB1 and/or CB2 receptors to act on A549, H460 or ED1 cell-made NSCLC nude mouse models for 21-28 days (Preet et al., 2008; Preet et al., 2011; Ravi et al., 2014; Ravi et al., 2016). Daily peritumoral administration of CB1/2 pan agonist Δ^9-THC (Ki_{CB1R}=5.05-80.3nM; Ki_{CB2R}=3.13-75.03nM) at 5 mg/kg (Preet et al., 2008) and CB1/2 agonist WIN55,212-2 (Ki_{CB1R}=62.3nM; Ki_{CB2R}=3.3nM) at 0.1 mg/kg (Preet et al., 2011) exhibited a similar antitumoral efficacy by providing a 50% decrease in A549 cell-based tumor weight and volume, prevented 30-60% of tumor cell proliferation in SCID mice by Ki-67 immune labelling with an increased 40-50% of the apoptotic cell index by WIN55,212-2 and inhibition of p-FAK, p-Erk1/2, and p-Akt by Δ^9-THC compared to control on day 28. Every 3-day-applied anandamide analogue MET-F-AEA (CB1/2 agonist) at 5 mg/kg and fatty-acid amide hydrolase 1 (FAAH, AEA-degrading enzyme) inhibitor URB597 at 1 mg/kg reduced 30-40% of H460 cell-based tumor weight and volume by inhibiting p-EGFR, p-Erk, and p-Akt, diminished 60-70% rate in Ki-67 scores compared to control and vehicle and reduced the necrotic area thus biological aggressiveness in the ectopic NSCLC model on day 21 (Ravi et al., 2014). ACPA has been able to exhibit a comparable antitumoral capability by sole CB1 agonism on ectopic NSCLC nude mouse model at 1 mg/kg dose by i.p. injection that ensured systemic efficacy by maintaining sufficient blood C_{max} and AUC levels within 8h when loaded in the nanoparticular system. In this study, weekly-applied NanoACPA presented a greater reduction in tumor volume of NSCLC nude mice on day 7 compared to every 2-day application, however, every 2-day application exhibited a better performance in reducing tumor growth compared to weekly applied NanoACPA from days 14 to 21 which might make a contradiction in dose-response relationship. A possible explanation would be the internalization of G-protein-coupled receptors including CB1 receptors (Fletcher-Jones et al., 2020). Long-term cannabinoid treatment is known to cause desensitization and downregulation of the CB1 receptor corresponding to the removal and internalization of the receptor from the cell membrane followed by trafficking to lysosomes for degradation (Al-Zoubi et

al., 2019; Piscura et al., 2023). The frequency of CB1 receptor agonist administration, such as with ACPA, can dynamically influence CB1 receptors altering the endocytotic pattern and rate within the cells by agonist-induced desensitization (Fletcher-Jones et al., 2020). Furthermore, repeated cannabinoid exposure is known to display a reduction in desensitization along with adaptive receptor recovery followed by receptor upregulation (Augustin and Lovinger, 2022; Fletcher-Jones et al., 2020). Δ^9-THC at a dose of 10 mg/kg downregulated CB1 receptor expression in the brain after three days of administration, however, increased its expression on days 14-21 (Breivogel et al., 1999), reflecting the dose-, agonist- and region-dependent recovery of CB1 receptor downregulation around 3-7 days (Piscura et al., 2023). Similarly, tumor cells treated with NanoACPA weekly might recover their CB1 receptors in the cell membrane within one week, potentially leading to continuous desensitization with each subsequent dose. In contrast, tumor cells treated with NanoACPA every 2 days might develop a gradual adaptation to the drug, allowing the drug to exhibit higher efficacy on tumor cells. Additionally, the curative efficacy of a drug could be increased only at low doses following the reduction in the tolerance of the cells (Ozdemir, 2020) and CB1 receptor-specific ligands could provide the benefit of causing fewer severe side effects even with long-term use (Al-Zoubi et al., 2019). Those compensation mechanisms should be considered in scale-up studies while synthetic cannabinoid ligands are translated into oncology clinics as chemotherapeutic candidates.

Daily peritumoral administration of WIN55,212-2 with CB2 antagonist SR144528 presented a slight decrease in the antitumoral efficiency in terms of change in tumor volume and proliferation and apoptosis in A549 cell-made ectopic NSCLC nude mouse model (Preet et al., 2011). Daily peritumoral administration of CB2 agonist JWH-133 (Ki_{CB1R}=677nM; Ki_{CB2R}=3.4nM) at 1 mg/kg or WIN55,212-2 at 0.1 mg/kg with CB1 antagonist AM281 diminished 40-70% of A549 cell-based tumor weight and volume and 30-50% of tumor cell proliferation in SCID mice by Ki-67 immune labelling on day 28 (Preet et al., 2011). Every 3-day-applied CB2 agonist JWH-015 (Ki_{CB1R}=383nM; Ki_{CB2R}=13.8nM) at 7.5 mg/kg lowered approximately 30% of mouse ED1 lung tumor weight and volume and reduced more than 70% of cancer cell proliferation on day 21 (Ravi et al., 2016). Evaluated together, WIN55,212-2, JWH-133, and JWH-015 exhibit equivalent antitumoral potency when applied at different effective doses related to their different affinity

indices. On the other hand, ACPA (Ki_{CB1R}=2.2nM; Ki_{CB2R}=715nM) induced a higher and faster antitumoral effect on the similar ectopic mouse model compared to CB2-agonistic cannabinoids that might be attributed partly to its higher affinity to the receptor with lower Ki values but also to the abundance of CB1 receptors on NSCLC cells that would ensure a targeted chemotherapeutic potency. Previously many groups including ours reported a diffuse CB1R labelling, in healthy human bronchial and pulmonary cells (Turcotte et al., 2016) and in 90% of CB1R density of human NSCLC lines (A549, H1299, H358, and H838 NSCLC cells) in correlation with qRT-PCR data presenting 250 times higher mRNA expression comparing to CB2R (Boyacıoğlu et al., 2021). Taken together, CB1 agonistic chemotherapeutics acting on lung epithelial apoptotic pathways in vitro (Boyacıoğlu et al., 2021) have been validated in vivo in this study suggesting new nano strategies to prevent systemic toxicity but maintaining low anticancer potency.

We previously generated and in vitro tested a PCL-based targeted drug delivery system for NSCLC with a loading efficiency of 40% and a cumulative release of 63.9% in 7 days (Boyacıoğlu et al., 2021). Herein, NanoACPA improved all plasma profile parameters from 5 min to 2h with no WBC but slight RBC change, thus confirming a sustainable plasma concentration in wild-type mice and a lower infiltrative cell and vessel extent pattern within tumor parenchyme in nude mice. Every 2-day-applied NanoACPA exhibited superior antitumoral performance in terms of weight, area, and volume assisted by a sharp decrease in proliferative index over time compared to weekly applied- and other groups on day 21. Cannabinoid-loaded PCL-based drug delivery systems have been designed and tested for MDA-MB-231 breast cancer (Hernan Perez de la Ossa et al., 2012), U87MG human glioma (Hernán Pérez de la Ossa et al., 2013), and RBL2H3 rat basophil (Hernan Perez de la Ossa et al., 2013) cells in vitro. However, no other cannabinoid-loaded PCL chemotherapeutical formulation has been generated and assessed for NSCLC. Biodegradable CBD (Hernan Perez de la Ossa et al., 2012; Hernán Pérez de la Ossa et al., 2013) and Δ^9-THC (Castor and Purdum, 2014; Hernán Pérez de la Ossa et al., 2013)-loaded nano/microsystems provided 60–100% drug loading capacity and cumulative release profile which is in line with ours (Castor and Purdum, 2014; Hernan Perez de la Ossa et al., 2012). Taken together, NanoACPA has been validated for its low-dose antitumor capacity, bioavailability, biocompatibility, and low/no systemic toxicity within the frame of the animal model.

This study validated the in vivo antitumoral efficacy of the novel ACPA-loaded nanoparticular system through Akt/PI3K, Ras/MEK/Erk, and JNK pathways that correlate with our previous in vitro findings on the CB1 agonistic acting mechanism by inhibiting Akt/PI3K, glycolysis, pentose phosphate pathways; amino acid biosynthesis, urea, and TCA cycles pathways on NSCLC. This study further revealed that NanoACPA acts on caspase-mediated antiproliferative and apoptotic pathways including Ras/MEK/Erk within the tumor microenvironment. Late diagnosis and rapid and silent progression of lung cancer constitute the main challenge for the curative treatment of the disease. The FDA-approved targeted molecular treatment choices (Hsu et al., 2023; Liu et al., 2019; Mausey and Halford, 2023) are the unique option for advanced or recurrent NSCLC patients and considered upon the actionable oncogenic mutations including EGF, ROS1, ALK, MET and RET receptors that all act on common effector pathways of Akt/PI3K, Ras/MEK/Erk and JNK. ACPA's autophagy-inducing and proliferation-reducing effects have been reported in human pancreatic (Brandi et al., 2013; Dando et al., 2013; Donadelli et al., 2011) and lung (Boyacıoğlu et al., 2021) cancer cells via Akt/PI3K, Ras/MEK/Erk and JNK pathways. Here we demonstrate for the first time its in vivo performance within the NSCLC microenvironment. In contrast, the common in vitro performance of ACPA on epithelial cancers including endometrium (Bilgic et al., 2017), pancreas (Brandi et al., 2013; Dando et al., 2013; Donadelli et al., 2011), and lung (Boyacıoğlu et al., 2021) propose a potential as a chemotherapeutic with very low IC50. Thus, it is worth improving its bioavailability confirming safety for eventual reduction of potential adverse effects in further clinical trials by generating novel nano molecular formulations (Liu et al., 2025). Nabiximols cannabinoid oromucosal spray acting on recurrent glioblastoma in a phase 1 trial (Twelves et al., 2021) and a synthetic cannabinoid derivative Dexanabinol targeting advanced in cytologically confirmed, metastatic or progressive solid tumors (Juarez et al., 2021) have been published and an ongoing clinical tolerability study through escalation of Δ^9-THC and CBD on 20 child cancer patients (ClinicalTrials.gov ID NCT05754840) further suggest the use of cannabinoid-made mediators as potential cancer therapeutics in epithelial cancers such as NSCLC. On the other hand, Δ^9-THC-made pan agonistic cannabinoids pose major complications in patients including dizziness, fatigue, nausea, and headache due to their high dose therapeutic window (Twelves et al., 2021) despite their antitumoral efficacy. ACPA and NanoACPA,

distinctively, exhibited better antitumoral performance with a very low IC50 besides Δ^9-THC and CBD, both reported in preclinical and clinical studies, without a systemic adverse effect, which highlights its potential therapeutic benefits over other therapeutic options.

All data is limited to in vivo ectopic xenograft model performance at a one-month time interval. The plasma profile and pharmacodynamic modeling, in this study, were successfully achieved, however, the plasma profile and the antitumoral effect of i.p. ACPA and NanoACPA formulation were evaluated by single-dose administration, which might be a limitation of this study and requires dose and time adjustment. Moreover, to improve the plasma profile of free ACPA and NanoACPA, conducting experiments with multiple and relatively high doses and testing the therapeutic index by determining the maximum dose that mice can tolerate is crucial. On the other hand, the analysis of plasma profile parameters of ACPA and NanoACPA system at a dose set, based on the output of our previous study (Boyacıoğlu et al., 2021), also lower compared to previous studies (Torrens et al., 2020; Xu et al., 2019), might contribute to the literature and provided a strong basis for pharmacodynamic modeling of NanoACPA system on NSCLC regression. The ectopic subcutaneous tumor model evaluated the safety, tolerability, and antitumoral efficacy of the drug candidate (Stribbling et al., 2024), NanoACPA, on the NSCLC tumor model. On the other side, further researches including orthotopic and metastatic tumor models are required to evaluate the pharmacokinetics and pharmacodynamics of NanoACPA, since these models better simulate the tumor microenvironment and provide a more comprehensive assessment before translating preclinical outcomes to clinical practice (Stribbling et al., 2024). Finally, herein, we did not perform the specific antitumoral effect of ACPA and NanoACPA system on CB1R-knockout or any pathway inhibitor-administered NSCLC mouse model. However, we previously demonstrated the CB1R-mediated real-time effect of ACPA and NanoACPA in the presence of CB1-specific antagonist AM281 on NSCLC cells in vitro. We also did not consider a group receiving blank PCL nanoparticles alone. Since we have determined the blank PCL nanoparticles as inert on NSCLC cells by real-time proliferation analysis in our previous work (Boyacıoğlu et al., 2021), we designed our in vivo experiment model based on the minimum number to prevent unnecessary animals according to the 3R principle.

5. Conclusion

In conclusion, our group has successfully clarified nanoencapsulation, improved the plasma profile and bioavailability of ACPA, a specific CB1 receptor agonist, and increased its peak plasma concentration without causing systemic toxicity. The novel NanoACPA nanomedicine has been efficacious in inducing tumor regression through Akt/PI3K, Ras/MEK/Erk, and JNK pathways at a low dose within 21 days in the ectopic NSCLC nude mice model. The overall output validates the chemotherapeutic potency of the NanoACPA system being a candidate as an anticancer drug of a combined second or third-line treatment protocol of NSCLC and further suggests a targeted cannabinoid-based chemotherapeutic platform for solid tumors in oncology clinics following scale-up preclinical experiments of GLP-manufactured product and GMP-based phase trials encompassing the pharmacokinetics and pharmacodynamics of NanoACPA in orthotopic and metastatic CB1 receptor-knockout tumor models.

Abbreviations

Δ^9-THC	Tetrahydrocannabinol
ABC	Absolute basophil count
ACPA	Arachidonoylcyclopropylamide
AEC	Absolute eosinophil count
ALC	Absolute lymphocyte count
ALK	Anaplastic lymphoma kinase
AMC	Absolute monocyte count
AMPK	5'-adenosine monophosphate-activated protein kinase
AUC	Area under the curve
BASO	Basophil
BCA	Bicinchoninic acid
BLI	Bioluminescence imaging
CB1	Cannabinoid 1
CB2	Cannabinoid 2

CBC	Complete blood count
CBD	Cannabidiol
Cl	Clearance
C_{max}	Maximum plasma concentration
DAB	3,3′-Diaminobenzidine
EGF	Epidermal growth factor
EOS	Eosinophil
FAAH	Fatty-acid amide hydrolase 1
HCT	Hematocrit
HGB	Hemoglobin
ICAM-1	Intercellular adhesion molecule 1
k_d	Dissociation rate constant
LAK	Lymphokine-activated killer
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LYMPH	Lymphocyte
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MET	Mesenchymal-epithelial transition
MMP2	Matrix metalloproteinase-2
MONO	Monocyte
NanoACPA	ACPA-loaded PCL-based drug delivery system
NSCLC	Non-small cell lung cancer
PAI-1	plasminogen activator inhibitor-1
PCL	Polycaprolactone
PLT	Platelet
RBC	Red blood cell
RDW-CV	Red blood cell distribution width-coefficient of variability
RET	Rearranged during transfection

ROI	Region of interest
ROS1	ROS proto-oncogene 1
RT	Room temperature
$t_{1/2}$	Half-life
T_{max}	Time to reach the maximum concentration
TIMP-1	Tissue inhibitor of matrix metalloproteinases-1
TRPV	Transient receptor potential vanilloid receptor
V_d	Volume of distribution
WBC	White blood cell

Acknowledgements

We kindly acknowledge Beliz Taşkonak for her contribution to sectioning for histomorphometric analysis, Dr. Elif Karakoç for sharing her knowledge and experience in assessing antitumoral efficacy of ACPA and Prof. Dr. Zeynep Şafak Teksin for making the pharmaceutical analysis system available for use.

Conflict of Interest

Authors declare having no financial relationship with any commercial identity leading to a conflict of interest.

Author Contributions

OB designed and performed the experiments, analyzed and interpreted data and wrote the manuscript. CV and EB contributed to the design and preparation of PCL-based drug delivery system and assisted with the evaluation of plasma profile analysis and writing the manuscript. ZGA assisted with animal studies. EN and TR contributed to the evaluation of the amount of ACPA in the plasma by LC-MS-MS for plasma profile analysis. NK provided feedback and support for Western blot analyses. PK conceived the project, supervised the research and wrote and edited the paper. All authors wrote and reviewed the final version of the manuscript.

In figure 1, EN and TR generated the data, CV and EB analyzed the data and prepared panel A-D, whereas OB prepared the tables in figure 1, drew the graphs in figure 1E-U and prepared the panel V-Y with PK. In figure 2-4, OB and ZGA generated the data, OB assembled the figure and prepared the graphs in figure 2F-G, 3F-G and 4F-G under the guidance of PK. OB took the photos in figure 5A-E with the help of ZGA. OB prepared the graph in figure 5F and took the photos and prepared the panel G-L in figure 5 with PK. OB generated the histochemical and immunohistochemical data, drew the graphs and assembled the figures of 6-7 and supplementary figures of 2-4. OB and PK together evaluated the histochemical and immunohistochemical data and labelled the images. OB generated the western blot data with the help of NK, analyzed the data, and prepared the panel H-O in figure 7. OB prepared the illustrations in supplementary figure 1 and figure 8.

Ethics Statement

This study was approved by Institutional Animal Care and Use Committee (BILHADYEK) of Bilkent University (24.11.2021/Approval no:2021/15).

Funding

This study was supported by Scientific and Technological Research Council of Turkey (TUBITAK) (Grants #321S058, partially #122S459) and Hacettepe University Scientific Research Projects Coordination Unit (#THD-2023-20676, partially #TSA-2023-20427).

Availability of data and materials

All data supporting the findings of this study are available within the paper and Supplementary Information. Further information is available from the corresponding author upon reasonable request. No custom code was created within the context of this study.

References

Abuhasira, R., Shbiro, L., Landschaft, Y., 2018. Medical use of cannabis and cannabinoids containing products - Regulations in Europe and North America. Eur J Intern Med 49, 2-6.

Al Shoyaib, A., Archie, S.R., Karamyan, V.T., 2019. Intraperitoneal Route of Drug Administration: Should it Be Used in Experimental Animal Studies? Pharmaceutical Research 37, 12.

Al-Zoubi, R., Morales, P., Reggio, P.H., 2019. Structural Insights into CB1 Receptor Biased Signaling. International Journal of Molecular Sciences 20, 1837.

Akkın, S., Varan, G., Işık, A., Gökşen, S., Karakoç, E., Malanga, M., Esendağlı, G., Korkusuz, P., Bilensoy, E., 2023. Synergistic Antitumor Potency of a Self-Assembling Cyclodextrin Nanoplex for the Co-Delivery of 5-Fluorouracil and Interleukin-2 in the Treatment of Colorectal Cancer. Pharmaceutics 15, 314.

Assani, K., Neidhard-Doll, A., Goswami, T., 2022. Mechanical properties of nanoparticles in the drug delivery kinetics. Journal of Pharmaceutical and Biopharmaceutical Research 4, 248-255.

Augustin, S.M., Lovinger, D.M., 2022. Synaptic changes induced by cannabinoid drugs and cannabis use disorder. Neurobiology of Disease 167, 105670.

Bhardwaj, H., Jangde, R.K., 2023. Current updated review on preparation of polymeric nanoparticles for drug delivery and biomedical applications. Next Nanotechnology 2, 100013.

Bilgic, E., Guzel, E., Kose, S., Aydin, M.C., Karaismailoglu, E., Akar, I., Usubutun, A., Korkusuz, P., 2017. Endocannabinoids modulate apoptosis in endometriosis and adenomyosis. Acta Histochem. 119, 523-532.

Boyacıoğlu, Ö., Bilgiç, E., Varan, C., Bilensoy, E., Nemutlu, E., Sevim, D., Kocaefe, Ç., Korkusuz, P., 2021. ACPA decreases non-small cell lung cancer line growth through Akt/PI3K and JNK pathways in vitro. Cell Death Dis 12, 56.

Boyacıoğlu, Ö., Korkusuz, P., 2023. Cannabinoids as Prospective Anti-Cancer Drugs: Mechanism of Action in Healthy and Cancer Cells, in: Turksen, K. (Ed.), Cell Biology and Translational Medicine, Volume 19: Perspectives in Diverse Human Diseases and Their Therapeutic Options. Springer Nature Switzerland, Cham, pp. 145-169.

Boyacıoğlu, Ö., Reçber, T., Kır, S., Korkusuz, P., Nemutlu, E., 2023. Development and validation of a sensitive assay for the quantification of arachidonoylcyclopropylamide (ACPA) in cell culture by LC–MS/MS. Journal of Analytical Science and Technology 14, 18.

Boyacioglu, O., Korkusuz, P., Bilgic, E., Bilensoy, E., Varan, C., Nemutlu, E., 2022. Use of cannabinoid 1 receptor agonist arachidonoyl cyclopropylamide (acpa) in non small cell lung cancer. Google Patents.

Brandi, J., Dando, I., Palmieri, M., Donadelli, M., Cecconi, D., 2013. Comparative proteomic and phosphoproteomic profiling of pancreatic adenocarcinoma cells treated with CB1 or CB2 agonists. Electrophoresis 34, 1359-1368.

Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E., Vogt, L.J., Sim-Selley, L.J., 1999. Chronic Δ9-tetrahydrocannabinol treatment produces a time-dependent loss of cannabinoid receptors and cannabinoid receptor-activated G proteins in rat brain. Journal of Neurochemistry 73, 2447-2459.

Brunetti, P., Lo Faro, A.F., Pirani, F., Berretta, P., Pacifici, R., Pichini, S., Busardò, F.P., 2020. Pharmacology and legal status of cannabidiol. Ann Ist Super Sanita 56, 285-291.

Calapai, F., Cardia, L., Sorbara, E.E., Navarra, M., Gangemi, S., Calapai, G., Mannucci, C., 2020. Cannabinoids, Blood–Brain Barrier, and Brain Disposition. Pharmaceutics 12, 265.

Castor, T.P., Purdum, G., 2014. Nanoencapsulated delta-9-tetrahydrocannabinol Aphios Corporation, Woburn, MA (US, United States.

Chenthamara, D., Subramaniam, S., Ramakrishnan, S.G., Krishnaswamy, S., Essa, M.M., Lin, F.-H., Qoronfleh, M.W., 2019. Therapeutic efficacy of nanoparticles and routes of administration. Biomaterials Research 23, 20.

Corrado, A., Battle, M., Wise, S.K., Lee, F.E., Guidot, D.M., DelGaudio, J.M., Molina, S.A., Levy, J.M., 2018. Endocannabinoid receptor CB2R is significantly expressed in aspirin-exacerbated respiratory disease: a pilot study. Int Forum Allergy Rhinol 8, 1184-1189.

Cristino, L., Bisogno, T., Di Marzo, V., 2020. Cannabinoids and the expanded endocannabinoid system in neurological disorders. Nat Rev Neurol 16, 9-29.

Dando, I., Donadelli, M., Costanzo, C., Dalla Pozza, E., D'Alessandro, A., Zolla, L., Palmieri, M., 2013. Cannabinoids inhibit energetic metabolism and induce AMPK-dependent autophagy in pancreatic cancer cells. Cell Death Dis. 4, e664.

Donadelli, M., Dando, I., Zaniboni, T., Costanzo, C., Dalla Pozza, E., Scupoli, M.T., Scarpa, A., Zappavigna, S., Marra, M., Abbruzzese, A., Bifulco, M., Caraglia, M., Palmieri, M., 2011.

Gemcitabine/cannabinoid combination triggers autophagy in pancreatic cancer cells through a ROS-mediated mechanism. Cell Death Dis. 2, e152.

Dothager, R.S., Flentie, K., Moss, B., Pan, M.-H., Kesarwala, A., Piwnica-Worms, D., 2009. Advances in bioluminescence imaging of live animal models. Current Opinion in Biotechnology 20, 45-53.

Ebrahimi-Ghiri, M., Nasehi, M., Zarrindast, M.R., 2019. Anxiolytic and antidepressant effects of ACPA and harmaline co-treatment. Behav Brain Res 364, 296-302.

Elmowafy, M., Shalaby, K., Elkomy, M.H., Alsaidan, O.A., Gomaa, H.A.M., Abdelgawad, M.A., Mostafa, E.M., 2023. Polymeric Nanoparticles for Delivery of Natural Bioactive Agents: Recent Advances and Challenges. Polymers 15, 1123.

Fantauzzi, M.F., Aguiar, J.A., Tremblay, B.J., Mansfield, M.J., Yanagihara, T., Chandiramohan, A., Revill, S., Ryu, M.H., Carlsten, C., Ask, K., Stämpfli, M., Doxey, A.C., Hirota, J.A., 2020. Expression of endocannabinoid system components in human airway epithelial cells: impact of sex and chronic respiratory disease status. ERJ Open Res 6.

Fletcher-Jones, A., Hildick, K.L., Evans, A.J., Nakamura, Y., Henley, J.M., Wilkinson, K.A., 2020. Protein Interactors and Trafficking Pathways That Regulate the Cannabinoid Type 1 Receptor (CB1R). Frontiers in Molecular Neuroscience 13.

Gado, F., Meini, S., Bertini, S., Digiacomo, M., Macchia, M., Manera, C., 2019. Allosteric modulators targeting cannabinoid cb1 and cb2 receptors: implications for drug discovery. Future Med Chem 11, 2019-2037.

Gkoumassi, E., Dekkers, B.G., Droge, M.J., Elzinga, C.R., Schmidt, M., Meurs, H., Zaagsma, J., Nelemans, S.A., 2007. Virodhamine and CP55,940 modulate cAMP production and IL-8 release in human bronchial epithelial cells. Br. J. Pharmacol. 151, 1041-1048.

Guzmán, M., Duarte, M.J., Blázquez, C., Ravina, J., Rosa, M.C., Galve-Roperh, I., Sánchez, C., Velasco, G., González-Feria, L., 2006. A pilot clinical study of Δ9-tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. British Journal of Cancer 95, 197-203.

Hallare, J., Gerriets, V., 2024. Half Life, StatPearls. StatPearls Publishing Copyright © 2024, StatPearls Publishing LLC., Treasure Island (FL).

Hernan Perez de la Ossa, D., Gil-Alegre, M.E., Ligresti, A., Aberturas Mdel, R., Molpeceres, J., Torres, A.I., Di Marzo, V., 2013. Preparation and characterization of Delta(9)-tetrahydrocannabinol-loaded biodegradable polymeric microparticles and their antitumoral efficacy on cancer cell lines. J. Drug Target 21, 710-718.

Hernan Perez de la Ossa, D., Ligresti, A., Gil-Alegre, M.E., Aberturas, M.R., Molpeceres, J., Di Marzo, V., Torres Suarez, A.I., 2012. Poly-epsilon-caprolactone microspheres as a drug delivery system for cannabinoid administration: development, characterization and in vitro evaluation of their antitumoral efficacy. Journal of controlled release : official journal of the Controlled Release Society 161, 927-932.

Hernán Pérez de la Ossa, D., Lorente, M., Gil-Alegre, M.E., Torres, S., García-Taboada, E., Aberturas Mdel, R., Molpeceres, J., Velasco, G., Torres-Suárez, A.I., 2013. Local delivery of cannabinoid-loaded microparticles inhibits tumor growth in a murine xenograft model of glioblastoma multiforme. PLoS One 8, e54795.

Hsu, R., Benjamin, D.J., Nagasaka, M., 2023. The Development and Role of Capmatinib in the Treatment of MET-Dysregulated Non-Small Cell Lung Cancer-A Narrative Review. Cancers (Basel) 15.

Juarez, T.M., Piccioni, D., Rose, L., Nguyen, A., Brown, B., Kesari, S., 2021. Phase I dose-escalation, safety, and CNS pharmacokinetic study of dexanabinol in patients with brain cancer. Neurooncol Adv 3, vdab006.

K, S.K., Choppala, A.d., 2022. Development and Optimization of Osimertinib-loaded Biodegradable Polymeric Nanoparticles Enhance In-vitro Cytotoxicity in Mutant EGFR NSCLC Cell Models and In-vivo Tumor Reduction in H1975 Xenograft Mice Models. AAPS PharmSciTech 23, 159.

Kılıç, N., Boyacıoğlu, Ö., Saltoğlu, G.T., Bulduk, E.B., Kurt, G., Korkusuz, P., 2022. Thioredoxin System and miR-21, miR-23a/b and let-7a as Potential Biomarkers for Brain Tumor Progression: Preliminary Case Data. World Neurosurgery.

Knoblaugh, S.E., Himmel, L.E., 2019. Keeping Score: Semiquantitative and Quantitative Scoring Approaches to Genetically Engineered and Xenograft Mouse Models of Cancer. Veterinary Pathology 56, 24-32.

784 Kumar, R., Prasoon, P., Gautam, M., Ray, S.B., 2016. Comparative antinociceptive effect of
785 arachidonylcyclopropylamide, a cannabinoid 1 receptor agonist & lignocaine, a local anaesthetic
786 agent, following direct intrawound administration in rats. Indian J Med Res 144, 730-740.
787 Lahiri, A., Maji, A., Potdar, P.D., Singh, N., Parikh, P., Bisht, B., Mukherjee, A., Paul, M.K., 2023.
788 Lung cancer immunotherapy: progress, pitfalls, and promises. Molecular Cancer 22, 40.
789 Lazic, S.E., Williams, D.P., 2020. Improving drug safety predictions by reducing poor analytical
790 practices. Toxicology Research and Application 4, 2397847320978633.
791 Li, C., Lei, S., Ding, L., Xu, Y., Wu, X., Wang, H., Zhang, Z., Gao, T., Zhang, Y., Li, L., 2023.
792 Global burden and trends of lung cancer incidence and mortality. Chin Med J (Engl) 136, 1583-
793 1590.
794 Li, J.Y., Ren, Y.P., Yuan, Y., Ji, S.M., Zhou, S.P., Wang, L.J., Mou, Z.Z., Li, L., Lu, W., Zhou, T.Y.,
795 2016. Preclinical PK/PD model for combined administration of erlotinib and sunitinib in the
796 treatment of A549 human NSCLC xenograft mice. Acta Pharmacol Sin 37, 930-940.
797 Litovchick, L., 2020. Stripping of the Immunoblot for Reprobing. Cold Spring Harb Protoc 2020,
798 098491.
799 Liu, G.F., Li, X.F., Yu, S.N., Miao, Y.Y., Zhang, S.H., 2019. Efficacy and adverse events of five
800 targeted agents in the treatment of advanced or metastatic non-small-cell lung cancer: A network
801 meta-analysis of nine eligible randomized controlled trials involving 5,059 patients. J. Cell Physiol.
802 234, 3445-3457.
803 Liu, J., Bai, Y., Li, Y., Li, X., Luo, K., 2024. Reprogramming the immunosuppressive tumor
804 microenvironment through nanomedicine: an immunometabolism perspective. eBioMedicine 107.
805 Liu, J., Li, X., Li, Y., Gong, Q., Luo, K., 2025. Metformin-based nanomedicines for reprogramming
806 tumor immune microenvironment. Theranostics 15, 993-1016.
807 Liu, P., Huang, Y., Zhan, C., Zhang, F., Deng, C., Jia, Y., Wan, T., Wang, S., Li, B., 2023. Tumor-
808 overexpressed enzyme responsive amphiphiles small molecular self-assembly nano-prodrug for
809 the chemo-phototherapy against non-small-cell lung cancer. Materials Today Bio 21, 100722.
810 Lu, F., Pan, X., Zhang, W., Su, X., Gu, Y., Qiu, H., Shen, S., Liu, C., Liu, W., Wang, X., Zhan, Z.,
811 Liu, Z., He, Z., 2021. A Three-Dimensional Imaging Method for the Quantification and Localization
812 of Dynamic Cell Tracking Posttransplantation. Frontiers in Cell and Developmental Biology 9.

Mansoor, A., Mahabadi, N., 2023. Volume of Distribution, StatPearls. StatPearls Publishing Copyright © 2023, StatPearls Publishing LLC., Treasure Island (FL).

Mausey, N., Halford, Z., 2023. Targeted Therapies for Previously “Undruggable” KRAS-Mutated Non–Small Cell Lung Cancer: A Review of Sotorasib and Adagrasib. Annals of Pharmacotherapy 0, 10600280231197459.

Milian, L., Mata, M., Alcacer, J., Oliver, M., Sancho-Tello, M., Martín de Llano, J.J., Camps, C., Galbis, J., Carretero, J., Carda, C., 2020. Cannabinoid receptor expression in non-small cell lung cancer. Effectiveness of tetrahydrocannabinol and cannabidiol inhibiting cell proliferation and epithelial-mesenchymal transition in vitro. PLoS One 15, e0228909.

Ozdemir, E., 2020. The Role of the Cannabinoid System in Opioid Analgesia and Tolerance. Mini Rev Med Chem 20, 875-885.

Öztürk, K., Kaplan, M., Çalış, S., 2024. Effects of nanoparticle size, shape, and zeta potential on drug delivery. International Journal of Pharmaceutics 666, 124799.

Padinharayil, H., Varghese, J., John, M.C., Rajanikant, G.K., Wilson, C.M., Al-Yozbaki, M., Renu, K., Dewanjee, S., Sanyal, R., Dey, A., Mukherjee, A.G., Wanjari, U.R., Gopalakrishnan, A.V., George, A., 2023. Non-small cell lung carcinoma (NSCLC): Implications on molecular pathology and advances in early diagnostics and therapeutics. Genes & Diseases 10, 960-989.

Palrasu, M., Wright, L., Patel, M., Leech, L., Branch, S., Harrelson, S., Khan, S., 2022. Perspectives on Challenges in Cannabis Drug Delivery Systems: Where Are We? Medical Cannabis and Cannabinoids 5, 102-119.

Piscura, M.K., Henderson-Redmond, A.N., Barnes, R.C., Mitra, S., Guindon, J., Morgan, D.J., 2023. Mechanisms of cannabinoid tolerance. Biochemical Pharmacology 214, 115665.

Preet, A., Ganju, R.K., Groopman, J.E., 2008. Delta9-Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration in vitro as well as its growth and metastasis in vivo. Oncogene 27, 339-346.

Preet, A., Qamri, Z., Nasser, M.W., Prasad, A., Shilo, K., Zou, X.H., Groopman, J.E., Ganju, R.K., 2011. Cannabinoid Receptors, CB1 and CB2, as Novel Targets for Inhibition of Non-Small Cell Lung Cancer Growth and Metastasis. Cancer Prev. Res. (Phila.) 4, 65-75.

Puri, A., Ibrahim, F., O'Reilly Beringhs, A., Isemann, C., Zakrevsky, P., Whittenburg, A., Hargrove, D., Kanai, T., Dillard, R.S., de Val, N., Nantz, M.H., Lu, X., Shapiro, B.A., 2022. Stealth oxime ether lipid vesicles promote delivery of functional DsiRNA in human lung cancer A549 tumor bearing mouse xenografts. Nanomedicine: Nanotechnology, Biology and Medicine 44, 102572.

Ramer, R., Bublitz, K., Freimuth, N., Merkord, J., Rohde, H., Haustein, M., Borchert, P., Schmuhl, E., Linnebacher, M., Hinz, B., 2012. Cannabidiol inhibits lung cancer cell invasion and metastasis via intercellular adhesion molecule-1. FASEB J. 26, 1535-1548.

Ramer, R., Fischer, S., Haustein, M., Manda, K., Hinz, B., 2014. Cannabinoids inhibit angiogenic capacities of endothelial cells via release of tissue inhibitor of matrix metalloproteinases-1 from lung cancer cells. Biochem. Pharmacol. 91, 202-216.

Ramer, R., Merkord, J., Rohde, H., Hinz, B., 2010a. Cannabidiol inhibits cancer cell invasion via upregulation of tissue inhibitor of matrix metalloproteinases-1. Biochem Pharmacol 79, 955-966.

Ramer, R., Rohde, A., Merkord, J., Rohde, H., Hinz, B., 2010b. Decrease of Plasminogen Activator Inhibitor-1 May Contribute to the Anti-Invasive Action of Cannabidiol on Human Lung Cancer Cells. Pharmaceutical Research 27, 2162-2174.

Ravi, J., Elbaz, M., Wani, N.A., Nasser, M.W., Ganju, R.K., 2016. Cannabinoid receptor-2 agonist inhibits macrophage induced EMT in non-small cell lung cancer by downregulation of EGFR pathway. Molecular Carcinogenesis 55, 2063-2076.

Ravi, J., Sneh, A., Shilo, K., Nasser, M.W., Ganju, R.K., 2014. FAAH inhibition enhances anandamide mediated anti-tumorigenic effects in non-small cell lung cancer by downregulating the EGF/EGFR pathway. Oncotarget 5, 2475-2486.

Sadeghi, S., Mohammadnejad, J., Eidi, A., Jafary, H., 2024. Biocompatible PLGA-PCL nanobeads for efficient delivery of curcumin to lung cancer. Cancer Nanotechnology 15, 34.

Sarafian, T., Montes, C., Harui, A., Beedanagari, S.R., Kiertscher, S., Stripecke, R., Hossepian, D., Kitchen, C., Kern, R., Belperio, J., Roth, M.D., 2008. Clarifying CB2 receptor-dependent and independent effects of THC on human lung epithelial cells. Toxicology and Applied Pharmacology 231, 282-290.

Shafaroodi, H., Samini, M., Moezi, L., Homayoun, H., Sadeghipour, H., Tavakoli, S., Hajrasouliha, A.R., Dehpour, A.R., 2004. The interaction of cannabinoids and opioids on pentylenetetrazole-induced seizure threshold in mice. Neuropharmacology 47, 390-400.

Torrens, A., Vozella, V., Huff, H., McNeil, B., Ahmed, F., Ghidini, A., Mahler, S.V., Huestis, M.A., Das, A., Piomelli, D., 2020. Comparative Pharmacokinetics of Δ⁹-Tetrahydrocannabinol in Adolescent and Adult Male Mice. Journal of Pharmacology and Experimental Therapeutics 374, 151-160.

Turcotte, C., Blanchet, M.-R., Laviolette, M., Flamand, N., 2016. Impact of Cannabis, Cannabinoids, and Endocannabinoids in the Lungs. Front. Pharmacol. 7, 317.

Twelves, C., Sabel, M., Checketts, D., Miller, S., Tayo, B., Jove, M., Brazil, L., Short, S.C., 2021. A phase 1b randomised, placebo-controlled trial of nabiximols cannabinoid oromucosal spray with temozolomide in patients with recurrent glioblastoma. Br J Cancer 124, 1379-1387.

Vidinsky, B., Gal, P., Pilatova, M., Vidova, Z., Solar, P., Varinska, L., Ivanova, L., Mojzis, J., 2012. Anti-proliferative and Anti-angiogenic Effects of CB2R Agonist (JWH-133) in Non-small Lung Cancer Cells (A549) and Human Umbilical Vein Endothelial Cells: an in Vitro Investigation. Folia Biol. 58, 75-80.

Voehringer, P., Nicholson, J.R., 2020. Minimum Information in In Vivo Research, in: Bespalov, A., Michel, M.C., Steckler, T. (Eds.), Good Research Practice in Non-Clinical Pharmacology and Biomedicine. Springer International Publishing, Cham, pp. 197-222.

Wang, D., Ye, W., Shi, Q., 2021. Prognostic Value of Ki-67 Expression in Advanced Lung Squamous Cell Carcinoma Patients Treated with Chemotherapy. Cancer Manag Res 13, 6429-6436.

White, C.M., 2019. A Review of Human Studies Assessing Cannabidiol's (CBD) Therapeutic Actions and Potential. J Clin Pharmacol 59, 923-934.

Wiese, B.M., Alvarez Reyes, A., Vanderah, T.W., Largent-Milnes, T.M., 2023. The endocannabinoid system and breathing. Front Neurosci 17, 1126004.

Xu, C., Chang, T., Du, Y., Yu, C., Tan, X., Li, X., 2019. Pharmacokinetics of oral and intravenous cannabidiol and its antidepressant-like effects in chronic mild stress mouse model. Environmental Toxicology and Pharmacology 70, 103202.

Xu, J., 2023. Extensions of the Mann-Whitney U Test.

Figure Legends

Fig. 1. NanoACPA improves plasma profile properties and bioavailability of ACPA with no immune reaction in wild-type mice. A-B, Bar graphs representing the physicochemical stability of NanoACPA in terms of (**A**) particle size distribution and polydispersity index (PDI) and (**B**) zeta potential on the day the formulation was prepared and after 3, 5 and 7 days of storage at +4 ^{o}C. **C-D,** Graphs depicting ACPA concentration vs. time of i.v. administered (**C**) ACPA and (**D**) NanoACPA formulation demonstrating plasma profile data with p values (n=6). Significance between groups was assessed by nonparametric Mann-Whitney U test. **E-U,** Mean±SEM graphs of (**E**) hematocrit (HCT, %), (**F**) hemoglobin (HGB, g/dl), (**G**) red blood cell count (RBC) (M/mm^3), (**H**) mean corpuscular volume (MCV, fL), (**I**) mean corpuscular hemoglobin (MCH, pg), (**J**) mean corpuscular hemoglobin concentration (MCHC, g/dl), (**K**) red cell distribution width (RDW-CV, %), (**L**) white blood cell count (WBC, K/mm^3), (**M**) lymphocyte index (LYMPH, %), (**N**) absolute lymphocyte count (ALC, K/mm^3), (**O**) monocyte index (MONO, %), (**P**) absolute monocyte count (AMC, K/mm^3), (**Q**) eosinophil index (EOS, %), (**R**) absolute eosinophil count (AEC, K/mm^3), (**S**) basophil index (BASO, %), (**T**) absolute basophil count (ABC, K/mm^3) and (**U**) platelet count (PLT, K/mm^3) in the blood samples obtained before (t_0) and at 8h (t_{final}) following i.v. administration of ACPA and NanoACPA platform to wild-type Balb/c mice (n=6) were depicted. Significance between time points and groups was separately determined by Student's t-test indicating (*) for $p<0.05$ comparing to NanoACPA at t_0; (#) for $p<0.05$ comparing to ACPA at t_0; (**) for $p<0.05$ comparing to t_0 in ACPA-administered mice and (##) for $p<0.05$ compared to t_0 in NanoACPA-administered mice. **V-Y,** Micrographs of the peripheral smears of blood samples of i.v. administered ACPA (**V-W**) and NanoACPA formulation (**X-Y**) collected before (hour 0) and after (hour 8) administration show the erythrocytes with normal morphology, sparse distribution of lymphocytes, neutrophils, monocytes and platelets.

Fig. 2. Weekly-applied NanoACPA inhibits NSCLC growth on day 7 by BLI and caliper measurements. A-E, Representative images of BLI assessed between days 0 (T0) and 42 (T42, M7) of (**A**) 0.9% NaCl (control, G1 from M0 to M7), (**B**) EtOH:PBS (vehicle, G2 from M0 to M7),

(**C**) NanoACPA (1 mg/kg/week from M0 to M7), (**D**) NanoACPA (1 mg/kg/2 days from M0 to M7) or (**E**) free ACPA (1 mg/kg/2 days from M0 to M7)-administered A549-luc2-injected nude Balb/c mice. (**F**) Quantification of bioluminescent intensity (10^8 photons/sec) of ROI (region of interest) vs. time (n=6). Note the differences in radiance values per group and time point in each scale. (*), (#) and (ǂ): $p<0.05$ for significance between NanoACPA (1mg/kg/week) and control, vehicle or free ACPA, respectively; (ǂǂǂ): $p<0.05$ for significance between NanoACPA (1mg/kg/2 days) and free ACPA by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (**G**) Curve indicates the tumor growth measured between the baseline (T28), where the tumor volume reaches an average of 100-150 mm^3, and day 7 (T42, M7) of drug application (n=6). (**), (##), (ǂ) and (ǂǂǂ): $p<0.05$ for significance between ACPA (1mg/kg/2 days) and control, vehicle, NanoACPA (1 mg/kg/week) or NanoACPA (1mg/kg/2 days), respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (T: Tumor injection; M: Medicine administration; G: Group)

Fig. 3. Repeated NanoACPA dosing inhibits NSCLC growth on day 14 by BLI and caliper measurements. A-E, Representative of BLI assessed between days 0 (T0) and 49 (T49, M14) of (**A**) 0.9% NaCl (control, G1 from M0 to M14), (**B**) EtOH:PBS (vehicle, G2 from M0 to M14), (**C**) NanoACPA (1 mg/kg/week from M0 to M14), (**D**) NanoACPA (1 mg/kg/2 days from M0 to M14) or (**E**) free ACPA (1 mg/kg/2 days from M0 to M14)-administered A549-luc2-injected nude Balb/c mice. (**F**) Quantification of bioluminescent intensity (10^8 photons/sec) of ROI (region of interest) vs. time (n=6). Note the differences in radiance values per group and time point in each scale. (#) and (ǂ): $p<0.05$ for significance between NanoACPA (1mg/kg/week) and vehicle or free ACPA, respectively; (ǂǂǂ): $p<0.05$ for significance between NanoACPA (1mg/kg/2 days) and free ACPA by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (**G**) Curve denotes the tumor growth measured between the baseline (T28), where the tumor volume reaches average of 100-150 mm^3, and day 14 (T49, M14) of drug therapy (n=6). (**) and (##): $p<0.05$ for significance between ACPA (1mg/kg/2 days) and control or vehicle, respectively; (***) and (###): $p<0.05$ for significance between NanoACPA (1mg/kg/2 days) and control or vehicle, respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (T: Tumor injection; M: Medicine administration; G: Group)

Fig. 4. High dosage frequency of NanoACPA preserves the reduction of NSCLC on day 21. A-E, Representative of BLI assessed between days 0 (T0) and 56 (T56, M21) of (**A**) 0.9% NaCl (control, G1 from M0 to M21), (**B**) EtOH:PBS (vehicle, G2 from M0 to M21), (**C**) NanoACPA (1 mg/kg/week from M0 to M21), (**D**) NanoACPA (1 mg/kg/2 days from M0 to M21) or (**E**) free ACPA (1 mg/kg/2 days from M0 to M21)-administered A549-luc2-injected nude Balb/c mice. (**F**) Quantification of bioluminescent intensity (10^8 photons/sec) of ROI (region of interest) vs. time (n=6). Note the differences in radiance values per group and time point in each scale. (*), (#) and (ǂ): $p<0.05$ for significance between NanoACPA (1mg/kg/week) and control, vehicle or free ACPA, respectively; (**) and (##): $p<0.05$ for significance between ACPA (1mg/kg/2 days) and control or vehicle, respectively; (***) and (###): $p<0.05$ for significance between NanoACPA (1mg/kg/2 days) and control or vehicle, respectively by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (**G**) Curve represents the tumor growth measured between the baseline (T28) and day 21 (T56, M21) of drug therapy (n=6). (*), (ǂ) and (ǂǂ): $p<0.05$ for significance between NanoACPA (1mg/kg/week) and control, free ACPA or NanoACPA (1mg/kg/2 days), respectively; (**) and (##): $p<0.05$ for significance between ACPA (1mg/kg/2 days) and control or vehicle, respectively; (***) and (###): $p<0.05$ for significance between NanoACPA (1mg/kg/2 days) and control or vehicle, respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (T: Tumor injection; M: Medicine administration; G: Group)

Fig. 5. Every 2-day-applied NanoACPA exhibits superior performance on tumor weight without metastatic foci in the lungs. A-E, Following sacrification, tumor nodules of (**A**) control, (**B**) vehicle (EtOH:PBS, 35%, v/v), (**C**) weekly-applied NanoACPA, (**D**) every 2-day-applied NanoACPA, and (**E**) every 2-day-applied free ACPA groups were photographed. **F,** Bar graphs represent the tumor weights (g) of each group after 21 days of treatment. **G-L,** Macro metastasis analysis demonstrated that no visible metastatic areas were noted total lung tissues of (**G**) control, (**H**) vehicle, (**I**) negative control, (**J**) weekly-applied NanoACPA and (**K**) every 2-day-applied NanoACPA groups. (*), (#) and (ǂ): $p<0.05$ for significance between NanoACPA (1 mg/kg/week) and control, vehicle or free ACPA respectively; (###) and (ǂǂǂ): $p<0.05$ for significance between NanoACPA (1 mg/kg/2 days) and vehicle or free ACPA, respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test.

Fig. 6. Every 2-day-applied NanoACPA presents maximum efficiency on reducing tumor area and inflammation on day 21. (**A**) Tile scan micrographs of the tumor nodules in the control, vehicle, weekly-applied NanoACPA, every 2-day applied NanoACPA and free ACPA groups exhibit infiltrative cells, vessels and glandular structures. 40x. N: Necrosis, Arrow: Collagen fibrils. (**B**) Note mild to moderate vascular congestion and mononuclear cell infiltration in lung sections. 100x. HE: Hematoxylin-Eosin. (*): Infiltration, C: Congestion. (**C**) Tumor area (μm^2) was higher in the control and vehicle than that of drug-applied groups by quantitative histomorphometry (n=6). **D-E,** Correlation graphs of tumor volume measurements by BLI and (**D**) tumor area measurements by histomorphometry (R^2=0.7787, p=0.001) or (**E**) tumor volume measurements by calliper (R^2=0.8484, p=0.001) are shown. Spearman's correlation test. **F-I,** Bar graphs represent the histomorphometric analysis for (**F**) inflammation, (**G**) fibrosis and (**H**) necrosis scores of tumor nodules and (**I**) inflammation scores of lung samples (n=6). (*) and (#): $p<0.05$ for significance between groups and time points, respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test.

Fig. 7. All drugs diminish tumor cell proliferation and enhance apoptosis through Akt/PI3K, Ras/MEK/Erk and JNK inhibition. A-B, Micrographs denote the tumor nodules on days 7-21 following drug application by (**A**) Ki-67 and (**B**) TUNEL indirect immunoperoxidase labelling. (**C)** Bar graph standing for the scores regarding Ki-67-labeled NSCLC cells. Chromogen: DAB, Nucleus stain: Hematoxylin. 400x (n=6). (**D**) Positive and (**E**) negative control groups by TUNEL indirect immunoperoxidase labelling. (**F**) Bar graph standing for the scores regarding TUNEL-labeled NSCLC cells. Chromogen: DAB, Nucleus stain: Hematoxylin. 400x. (n=6) (*) and (#): $p<0.05$ for significance between groups and time points, respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test. (**G**) Correlation graph demonstrates the relation between the apoptotic cell index by TUNEL and proliferative cell index by Ki-67 labelling (R^2=0.9009, p=0.001). Spearman's correlation test. (**H**) Western blot analysis indicates the bands for p-Akt (S473), p-PI3K (Y467/Y199), p-JNK (T183/Y185), pERK1 (T202/Y204) + pERK2 (T185/Y187), total Akt, PI3K, JNK, ERK1+ERK2, cyclin D1/D2, caspase 3, caspase 9 and internal control GAPDH immunolabeling of control and drug-applied groups on days 7-21. **I-R**, Heatmaps (left or above the bar graphs) and bar graphs demonstrate relative (**I**) p-Akt/Akt, (**J**) p-PI3K/PI3K,

(**K**) p-JNK/JNK, (**L**) pERK1+pERK2/ERK1+ERK2, (**M**) caspase 3, (**N**) cyclin D1/D2 and (**O**) caspase 9 normalized to GAPDH. (n=3) (*) and (#): $p<0.05$ for significance between groups and time points, respectively, by one-way analysis of variance (ANOVA) and post-hoc Duncan's test.

Fig. 8. ACPA and NanoACPA modulate the tumor microenvironment through key signalling pathways. Significant metabolites/proteins of caspase-mediated Akt/PI3K, Ras/MEK/Erk and JNK pathways affected in NSCLC microenvironment following i.p. applications of every 2-day ACPA or NanoACPA platform.

Supplementary Fig. 1. Schematic diagram for in vivo tumor model. A549-luc2 cells were s.c. injected to nude Balb/c mice on day 0 (T0). Tumor growth was assessed on days 14 (T14) and 28 (T28) by BLI and mice were treated every 2 days either with 0.9% NaCl (G1), vehicle (EtOH:PBS) (G2), free ACPA (1 mg/kg) (G5) or NanoACPA (1 mg/kg) (G4) or weekly with NanoACPA (1 mg/kg) (G3) on day 35 (T35, M0) for 7 (T42, M7, SET 1), 14 (T49, M14, SET 2) or 21 (T56, M21, SET 3) days and whole body scan was performed by BLI and tumor nodules were measured by calliper every week. Samples were collected on days 7, 14 or 21 including negative controls (n=6, N=108) for further molecular and microscopic analyses. (T: Tumor injection; M: Medicine administration; G: Group)

Supplementary Fig. 2. No brain and costal bone metastasis and inflammation are observed in treated and untreated groups. Micrographs of control and experimental groups show (**A**) costal bone and (**B**) brain tissue sections on days 7-21 following applications. E: Epiphyseal plate, Ca: Cartilage. HE: Hematoxylin-Eosin, 100x.

Supplementary Fig. 3. No testis and spleen metastasis and inflammation are observed in treated and untreated groups. Micrographs of control and experimental groups display (**A**) testis and (**B**) spleen tissue sections on days 7-21 following applications. Elongated spermatids were noted in the seminiferous tubules in the testicular samples. "{": Seminiferous tubule, (*): Elonge spermatids. WP: White pulp. HE: Hematoxylin-Eosin, 100x.

Supplementary Fig. 4. No kidney and liver metastasis and inflammation are observed in treated and untreated groups. Micrographs of control and experimental groups display (**A**)

kidney and (**B**) liver tissue sections on days 7-21 following applications. G: Glomerulus. HE: Hematoxylin-Eosin, 100x.