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1 **New contributions to the drug profile of TNF α inhibitor SPD304:**
2 **affinity, selectivity and ADMET considerations**

3
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24

25 **Abstract**

26 **Tumor necrosis factor alpha** (TNF α) is a relevant clinical target for the treatment of chronic
27 inflammatory diseases. Currently, only few small molecules are known as direct inhibitors of
28 TNF α . To date, none of these molecules has shown both an efficient activity and a low
29 toxicity to be considered for clinical trials. The SPD304 is considered as a reference of direct
30 inhibitors of TNF α because of its well demonstrated mechanism (He et al., 2005).
31 Herein, we provide new insights regarding the drug profile, selectivity and absorption,
32 distribution, metabolism, excretion **and** toxicity (ADMET) considerations of SPD304 to
33 evaluate its potential as a hit for the structure-based design of novel TNF α inhibitors. ELISA
34 experiments confirmed the inhibition of TNF α /**TNF receptor 1** binding ($IC_{50} = 12 \mu M$).
35 Cellular-based assays highlighted the cytotoxicity of SPD304, as well as its ability to inhibit
36 TNF α signaling pathways at non-cytotoxic concentrations. A **surface acoustic wave** (SAW)
37 experiment highlighted only one binding site with a dissociation constant of 6.1 ± 4.7 nM.
38 SPD304 inhibited the binding of the cytokines **like** interleukins (IL)-4 and IL-13 to their
39 receptors and showed no direct inhibition on proteins involved in the TNF α pathway. Finally,
40 the thermodynamic solubility and Caco-2 cells permeability of SPD304 were experimentally
41 evaluated and ADMET *in silico* predictions are also discussed. The physicochemical,
42 pharmacological and ADMET **studies** of SPD304 have shown that is not an ideal hit for a
43 drug optimization program based on its chemical structure.

44 **Keywords:** *SPD304, Surface Acoustic Wave biosensor, Thermodynamic solubility,*
45 *TNF α , ADMET*

46

47

48 **1. Introduction**

49 Cytokines are proteins with a molar mass of between 6-60 kDa, often glycosylated,
50 synthesized by many cell types and acting on multiple cell targets, via specific membrane
51 receptors. Beyond the innate and adaptive immunity, cytokines have a major role in diverse
52 functions including immune cell differentiation, inflammation, angiogenesis, tumorigenesis,
53 neurobiology, viral pathogenesis, atherosclerosis and cancer (Gulati et al., 2016).

54 Tumor necrosis factor alpha (TNF α) is a homotrimeric cytokine of the immune system
55 which overproduction has been associated with several chronic inflammatory diseases such as
56 rheumatoid arthritis, inflammatory bowel diseases and psoriasis (Aggarwal, 2003). These
57 diseases are a major public health issue since they affect about 5% of the human population.
58 The use of biologic therapies as an adjunct to disease modifying drugs for the treatment of
59 autoimmune and rheumatologic diseases has now shown its clinical efficacy in many
60 pathologies such as systemic inflammatory autoimmune diseases (Rosman et al., 2013; Her
61 and Kavanaugh, 2016)

62 Since 2000, TNF α inhibition has become a major strategy for the alleviation of
63 inflammatory diseases and molecules targeting the TNF α pathway have been a therapeutic
64 intervention for the treatment of affected patients (Ratsimandresy et al., 2009). Several
65 “biologics” have been developed for TNF α inhibition such as monoclonal antibodies, soluble
66 TNF α receptor fusion proteins and recombinant TNF α binding proteins (Alizadeh et al.,
67 2017; Richmond et al., 2015). These marketed biotherapies exhibit several drawbacks due to
68 limited tissue penetration, route of administration, high production cost and consequently high
69 prices, immunogenicity resulting in treatment cessation and limitation due to immune
70 reactions (Rubbert-Roth and Finckh, 2009), and side effects, in particular an increased risk of
71 opportunistic infections (Fleischmann et al., 2004).

72 To circumvent these obstacles, several research programs have been conducted to
73 identify novel small-molecule inhibitors of TNF α (Davis and Colangelo, 2013). Among these

74 works, SPD304 has been identified as a reference small molecule to inhibit TNF α activity by
75 disrupting the protein trimerization (He et al., 2005).

76 However, the presence of the 3-substituted indole moiety in the structure of SPD304
77 makes it metabolically unstable. This metabolic instability coupled to a low kinetic solubility
78 (Papaneophytou et al., 2013) issue might explain why no further *in vivo* studies with SPD304
79 have been reported. SPD304 analogs with the aim to prepare less toxic and equally potent
80 TNF α inhibitor have been described (Alexiou et al., 2014).

81 Hit identification from screening campaigns for drug discovery and optimization of
82 primary hits in terms of activity but also of ADMET properties in order to derive lead
83 compounds is an approach whose effectiveness has been demonstrated for the discovery of
84 many drugs (Hoffer et al., 2018). This drug design approach has very recently made it
85 possible to identify a small anti-TNF α molecule based on a 3-indolinone backbone that
86 displays *in vivo* efficacy comparable to an anti-TNF α antibody in a mouse arthritis model
87 (Dietrich et al., 2021). Consequently, in this work, we wanted to analyze and deepen the
88 potential of SPD304 as a hit to evaluate whether SPD304 is an appropriate choice for the
89 structure-based design of new TNF α inhibitors.

90

91 **2. Materials and methods**

92

93 *2.1 Materials*

94

95 Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), phosphate buffered saline
96 (PBS) pH 7.4, PBS/tween 0.05% solution, 2,3-bis-(2-méthoxy-4-nitro-5-sulfophényle)-2H-
97 tétrazolium-5-carboxanilide (XTT), thiazolyl blue tetrazolium bromide (MTT), sulfuric acid
98 (H₂SO₄), hydrogen peroxide (H₂O₂), 11-mercaptoundecanoic acid (MUDA) and 6-mercapto-
99 1-hexanol (MHOH) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). 1-

100 ethyl-3-[3-dimethylaminopropyl] carbodiimidehydrochloride (EDC), *N*-hydroxysuccinimide
101 (NHS), [tetramethylbenzidine](#) (TMB) and [avidin-horseradish peroxidase](#) ([avidin-HRP](#)) were
102 from Thermo Fisher (Courtaboeuf, France). TNF α was from Proteros (Planegg, Germany).

103 Cytokines, their receptors, and corresponding biotinylated antibodies were obtained
104 from Bio-Techne (Rennes, France) and Proteros. Dulbecco's [modified eagle medium](#)
105 (DMEM), [fetal bovine serum](#) (FBS), penicillin/streptomycin, *L*-glutamine and PBS were
106 obtained from Dutscher (Brumath, France). Actinomycin D was obtained from Fisher
107 (Illkirch, France). L929 cell line has been grown in the Peptinov [Laboratory](#) for years (Paris,
108 France). HEK-Blue™ TNF α , HEK-Blue™ IL-4/IL-13 cell lines and QUANTI-Blue™ were
109 obtained from InvivoGen (Toulouse, France). Solvents, HPLC gold quality grade, were
110 purchased from Carlo Erba (Val-de-Reuil, France).

111 [SPD304 in a salt form](#) ([6,7-dimethyl-3-\[\[methyl\[2-\[methyl\[\[1-\[3-](#)
112 [\(trifluoromethyl\)phenyl\]-1H-indol-3-yl\]methyl\]amino\]ethyl\]amino\]methyl\]-\(4H-1-](#)
113 [benzopyran-4-one dihydrochloride](#)) ([Fig. 1E](#)) was synthesized on a 200 mg scale following
114 experimental conditions modified compared to the synthesis initially proposed [by He et al.](#)
115 [\(2005\)](#). SPD304 was obtained with 98% of HPLC purity and the general synthetic strategy is
116 described in the supporting information of this article.

117

118 2.2 *TNF α /TNF receptor 1 binding enzyme-linked immunosorbent assay (ELISA)*

119

120 Microtiter plates were coated with 10 ng of TNF receptor 1 in 100 μ l of PBS per well
121 overnight at 4 °C. Serial dilutions of SPD304 (in 0.5% DMSO) were mixed with a fixed
122 quantity of TNF α in PBS/BSA 1% and incubated 2 h at 37 °C. After blocking with PBS/BSA
123 2%, wells were washed and 100 μ l of the mix were added to the wells and plates were
124 incubated overnight at 4 °C. After washing, 20 ng of anti-TNF α biotinylated antibodies were
125 added to the wells and plates were incubated for 2 h at 37 °C before addition of avidin-HRP.

126 After washing, TMB solution was added to wells and reaction was quenched with 1 M H₂SO₄
127 solution. Absorbance was measured at 450 nm with a spectrophotometer, providing the
128 optical density (OD) of each well. Percentages of neutralization of TNF α by SPD304 were
129 determined using the following formula:

$$130 \quad \% \text{ Neutralization} = 100 - \left(\frac{OD_{\text{compound}} - OD_{\text{negative control}}}{OD_{\text{TNF}\alpha} - OD_{\text{negative control}}} \times 100 \right)$$

131 An IC₅₀ could be computed from the percentages of neutralization.

132

133 2.3 TNF α /TNF receptor 1 equilibrium

134

135 Microtiter plates were coated with 10 ng of TNF receptor 1 in 100 μ l of PBS per well
136 overnight at 4 °C. After blocking with PBS/BSA 2%, fixed quantity of TNF α was added to
137 the wells and plates were incubated for 2 h à 37 °C. After washing, serial dilutions of SPD304
138 (in 0.5% DMSO) were added to the wells and plates were incubated overnight at 4 °C. After
139 washing, 20 ng of anti-TNF α biotinylated antibodies were added to the wells and plates were
140 incubated for 2 h at 37 °C before addition of avidin-HRP. After washing, TMB solution was
141 added to wells and reaction was quenched with 1 M H₂SO₄ solution. Absorbance was
142 measured at 450 nm with a spectrophotometer providing the OD of each well. Percentages of
143 TNF α neutralization by SPD304 were calculated as above.

144

145 2.4 TNF α /SPD304 affinity using surface acoustic wave (SAW) biosensor

146

147 2.4.1 SAW biosensor design

148

149 The designed SAW sensor consists of dual delay lines fabricated on 36° rot lithium
150 tantalate piezoelectric substrate. The interdigital transducers, realized by evaporation of

151 (20/80) nm Cr/Au layers, were photolithographically patterned with a periodicity of $\lambda = 40$
152 μm which corresponds to an operating frequency of about 103 MHz. The measurement setup
153 consists of a TNF α grafted SAW sensor, a Kalrez[®] flow cell, a **poly methyl metacrylate**
154 (PMMA) cover including inlets and outlets connected to a Gilson[®] 3 peristaltic pump, and a
155 HP8214 network analyser (**Fig. 1**). This latter was used to monitor phase output versus time at
156 a fixed frequency.

157

158 *[Insert Fig. 1. here]*

159

160 *2.4.2 Biosensors functionalization strategy*

161

162 Several cleaning and activation steps were needed prior to TNF α grafting on the gold
163 sensing area of the SAW sensor: 50 μl drop of a piranha solution (98% H₂SO₄ / 30% H₂O₂ 2:1
164 v/v) were first deposited on the substrates, for 20 minutes, to favour the formation of OH
165 groups on gold surfaces. The SAW devices were after that rinsed copiously with ultrapure
166 water and then with ethanol, for 10 min, before being dried under ambient air.

167 Afterwards, an ethanol solution was injected on the SAW sensing area with a
168 peristaltic pump at a constant rate of 190 $\mu\text{l}/\text{min}$ before to the injection of a solution of 1 mM
169 MUDA/10 mM MHOH in ethanol. A subsequent rinsing with ethanol and then with PBS
170 permitted to remove the non-grafted molecules. Then, the obtained self-assembled
171 monolayers (SAMs) were activated with: **EDC/ NHS**: 75 mM/ 15 mM prior to the grafting of
172 0.01 mg/ml of TNF α dissolved in PBS at pH 7.4. All measurements were made at room
173 temperature.

174 2.5 *In cellulo* studies

175

176 2.5.1 Neutralization of cellular TNF α induced apoptosis in L929 cells

177

178 Eighty percent confluent L929 cells were plated in flat bottom plates at 4×10^5 cells
179 per well in 100 μ l of DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin -
180 100 μ g/ml streptomycin and incubated for one night at 37 °C, 5% CO₂. TNF α (150 pg/ml),
181 actinomycin D (4 μ g/ml) and serial dilutions of SPD304 (in 0.5% DMSO) were mixed in 150
182 μ l DMEM medium 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin - 100 μ g/ml
183 streptomycin in U-bottom plates. After 2 h incubation at 37 °C, 5% CO₂, 100 μ l of the mix
184 were added to the plated cells and incubated at 37 °C, 5% CO₂ for 24 h. Supernatants were
185 discarded and 100 μ l of MTT at 0.5 mg/ml were added to wells. After 2 h, supernatants were
186 discarded and 200 μ l of DMSO were then added. Plates were read at 570 nm with a
187 spectrophotometer providing the OD of each well. Percentages of neutralization of TNF α by
188 SPD304 were calculated using the following formula:

189
$$\% \text{ Neutralization} = \left(\frac{OD_{\text{compound}} - OD_{\text{TNF}\alpha}}{OD_{\text{negative control}} - OD_{\text{TNF}\alpha}} \times 100 \right)$$

190 An IC₅₀ could be computed from the percentages of neutralization.

191

192 2.5.2 Secreted embryonic alkaline phosphatase reporter assay.

193

194 Serial dilutions of SPD304 (in 0.5% DMSO) were mixed with 400 pg/ml of human
195 TNF α , 400 ng/ml of human IL-4 or 400 ng/ml of human IL-13 in DMEM containing 10% of
196 FBS, 2 mM L-glutamine, 100 U/ml penicillin - 100 μ g/ml streptomycin in flat-bottom plates.
197 After 2 h of incubation at 37 °C, 5% CO₂, 80% confluent HEK-Blue™ TNF α or IL-4/IL-13
198 cells were added to the mixes at 5×10^4 per well in DMEM containing 2 mM L-glutamine,

199 100 U/ml penicillin - 100 µg/ml streptomycin and incubated at 37 °C, 5% CO₂ for 24 h. 20 µl
200 of supernatants were incubated for 3 h with 180 µl of QUANTI-Blue™ to reveal secretion of
201 phosphatase alkaline and plates were read at 620 nm with a spectrophotometer, providing the
202 OD. XTT was added to wells containing cells and incubated for 3 h and plates were read at
203 450 nm with a spectrophotometer providing the OD. Percentages of neutralization of TNFα
204 by SPD304 were calculated using the following formula:

$$205 \quad \% \text{ Neutralization} = 100 - \left(\frac{OD_{\text{compound}} - OD_{\text{negative control}}}{OD_{\text{cytokine}} - OD_{\text{negative control}}} \times 100 \right)$$

206 An IC₅₀ could be computed from the percentages of neutralization.

207

208 *2.6 Selectivity against other targets*

209

210 *2.6.1 Inhibition activity of SPD304 on kinases and caspases related to TNFα pathway*

211

212 Kinases and caspases activities, implicated in TNFα pathway, were evaluated by
213 CEREP/Eurofins (Celle-Lévescault, France) (CEREP/Eurofins, 2019) in presence of 1 µM of
214 SPD304. The direct inhibition of SPD304 on the different kinases (IKKα, IKKβ, JNK1 &
215 p38α) and caspases (3 and 8) was compared to control values. Data are expressed as
216 percentages of inhibition of control values. For IKK proteins (IKKα and IKKβ), SPD304 was
217 mixed with either IKKα or IKKβ. Enzymatic reaction was initiated with ULight™-IkappaBα
218 substrate and ATP then incubated for 30 min (IKKα and IKKβ). SPD304 was also mixed
219 with either p38α or JNK1. The enzymatic reactions were initiated with ULight™-myelin
220 basic protein peptide substrate and ATP and incubated for 30 min (p38α) or 60 min (JNK1).
221 For each experience, reactions were stopped and a labeled-anti-phospho-myelin basic protein
222 antibody (for p38α and JNK1) or labelled-anti-phospho-IkappaBα (for IKKα and IKKβ) was

223 added for 60 min. After incubation, fluorescence intensity was measured using a microplate
224 reader at 620 nm and 665 nm.

225 For caspases 3 and 8, SPD304 was mixed with either caspase 3 or 8 and enzymatic
226 reaction was initiated with benzyloxycarbonyl-Ile-Glu-Thr-Asp-AFC then incubated for 30
227 min. Fluorescence intensity was measured using a microplate reader at 355 nm. The results
228 were expressed as percentage of inhibition of control enzyme activity.

229

230 2.6.2 IL-4/IL-4 receptor subunit α and IL-13/IL-13 receptor $\alpha 2$ binding ELISA

231

232 Microtiter plates were coated with 10 ng of IL-4 receptor subunit α or IL-13 receptor
233 $\alpha 2$ in 100 μ l of PBS per well overnight at 4 °C. Serial dilutions of SPD304 (in 0.5% DMSO)
234 were mixed with a fixed quantity of IL-4 or IL-13 in PBS/BSA 1% and incubated 2 h at 37
235 °C. After blocking with PBS/BSA 2%, wells were washed and 100 μ l of the mix were added
236 to the wells and plates were incubated overnight at 4 °C. After washing, 30 ng of anti-IL-4 or
237 anti-IL-13 biotinylated antibodies were added to the wells and plates were incubated for 2 h at
238 37 °C before addition of avidin-HRP. After washing, TMB solution was added to wells and
239 reaction was quenched with 1 M H₂SO₄ solution. Absorbance was measured at 450 nm with a
240 spectrophotometer providing the OD of each well. Percentages of neutralization of IL-4 or IL-
241 13 binding by SPD304 were calculated using the following formula:

$$242 \quad \% \text{ Neutralization} = 100 - \left(\frac{OD_{\text{compound}} - OD_{\text{negative control}}}{OD_{\text{TNF}\alpha} - OD_{\text{negative control}}} \times 100 \right)$$

243 An IC₅₀ could be computed from percentages of neutralization.

244

245 2.7 Evaluation of ADME parameters

246

247 *2.7.1 Thermodynamic solubility of SPD304*

248

249 Thermodynamic solubility was determined by measuring the concentration of a
250 saturated solution after equilibration. For this purpose, 1 mg of SPD304 was dissolved in 1 ml
251 of phosphate buffer pH 7.4 and samples were prepared in duplicate. Samples were
252 equilibrated in vertical agitator at 25 rpm, during 24 h at room temperature followed by 5 min
253 centrifugation at 20 °C at 12820 rpm, in order to separate undissolved material. Supernatants
254 were analyzed by ultra-performance liquid chromatography (injection of 8 µl). Solubility was
255 determined against a single point standard prepared from a 10 mM DMSO stock solution
256 diluted in acetonitrile/H₂O 1/1 (100 µM). Measurements were done using a Waters
257 ACQUITY H-class series, equipped with a photo diode array as detector (max plot was used
258 between 210 and 400 nm), controlled by Empower 3 software. The separation was performed
259 on stationary phase of Kinetex C18 column (50 mm × 2.1 mm × 1.7 µm, 100 Å) with a
260 mobile phase composed of water (0,1 % formic acid) and acetonitrile, in gradient elution (0-
261 0.25 min 95% H₂O/5% acetonitrile, 0.25-3.25 min 5% H₂O/95% acetonitrile, 3.25-4 min 5%
262 H₂O/95% acetonitrile, 4-7 min 95% H₂O/5% acetonitrile), at 0.5 ml/min as flow rate. The
263 samples were analysed at 210-400 nm.

264

265 *2.7.2 Metabolic stability of SPD304*

266

267 Metabolic stability was evaluated by the TechMed^{III}/platform of integrative chemical
268 biology of Strasbourg (Illkirch, France). Metabolic stability of SPD304 was assessed on
269 mouse liver microsomes to determine if SPD304 was a substrate of cytochromes P450 in
270 presence of NADPH as cofactor. Briefly, 1 µM of SPD304 (final concentration) was mixed
271 with a 0.5 mg/ml solution of mouse liver microsomes in presence of 1 mM of NADPH and 3
272 mM of MgCl₂. After 1 h of incubation, reaction was stopped with acetonitrile and samples

273 were frozen. After thawing, metabolic stability of SPD304 was measured by analyzing the
274 presence of the remaining compound (non-metabolized) in the samples by liquid
275 chromatography coupled to tandem mass spectrometry (LC-MS/MS) using Shimadzu
276 equipment, equipped with a triple quadrupole as detector (LC-MS 8030). The LC-MS
277 analysis was performed on stationary phase of Kinetex C18 column (50 mm × 2.1 mm × 2.6
278 μm, 100 Å) and mobile phase composed of water with 0.5 % formic acid (solvent A) and
279 acetonitrile (solvent B), in a gradient elution system (0 min: 95 % A, 5 % B; 1.20 - 1.4 min: 5
280 % B, 95 % B; 1.42 - 2.8 min: 95 % A, 5 % B), at 0.5 ml/min as flow rate. Multiple reaction
281 monitoring mode was used for quantitation and transition was 548.1 → 274.2, 287.3 for
282 SPD304.

283

284 2.7.3 Caco-2 permeability assay

285

286 The permeability assay on Caco-2 cells was carried out by TechMed^{ill}/ the platform of
287 integrative chemical biology of Strasbourg (Hikreh, France). Caco-2 cells were used as
288 predictive *in vitro* model in order to evaluate the absorption of SPD304 by the intestine
289 mucosa.

290 Caco-2 cells (ATCC HTB-37TM) were plated in the upper chambers (apical) of a 24-well plate
291 at 1.5x10⁵ cells per well as lower chambers (basolateral) of the plate were filled with Hanks
292 balanced salt solution. The chambers were separated with a permeable membrane. Cells were
293 cultured at 37 °C, 5% CO₂ until transepithelial electric resistance reached 700 Ω. Then,
294 SPD304 was placed in the upper chamber and incubated for 2 h at 37 °C, 5% CO₂. Apparent
295 permeability of SPD304 was measured by analyzing the presence of the compound in the
296 basolateral chamber by LC-MS/MS using Shimadzu equipment. Quantitation was performed
297 as described in section 2.7.2.

298

299 2.7.4 ADMET predictions

300

301 In order to complete the druglikeness study, we sought to predict several ADMET
302 features for SPD304 using the web platform ADMETlab (<http://admet.scbdd.com/>). (Dong et
303 al., 2018; “Home-ADMElab: ADMET Prediction|ADMET Predictor|QSAR|ADMET
304 Database,” n.d.) This web platform is based on a vast database which is implemented with
305 experimental ADMET data training sets collected from peer-reviewed publications, ChEMBL
306 (Gaulton et al., 2017), EPA (“US EPA,” n.d.) and DrugBank (Wishart et al., 2006) databases.
307 Thus, it provides after processing, 288,967 unique entries to be used in the molecular
308 similarity models.

309 2.7.5 Statistical analysis

310 All quantitative results are expressed as the mean \pm standard deviation (SD).

311

312 3. Results

313

314 Initial results obtained in biological assessments using SPD304 from a commercial
315 source (Sigma-Aldrich) were found to be non-reproducible. In order to guarantee the
316 reproducibility of our biological studies, SPD304 ~~used~~ was synthesized in our laboratory with
317 98% purity by HPLC (see supplementary data).

318

319 3.1 TNF α /TNF receptor 1 binding study and TNF α /TNF receptor 1 equilibrium

320

321 Our experimental ELISA set-up confirmed that SPD304 was able to disrupt
322 TNF α /TNF receptor 1 equilibrium. For that purpose, we incubated TNF α with serially diluted
323 SPD304 before addition on TNF receptor 1. As shown in Fig. 2, incubation of SPD304 with
324 TNF α prior addition on TNF receptor 1 inhibited TNF α /TNF receptor 1 binding with an IC₅₀

325 of $12 \pm 4.5 \mu\text{M}$. This value is of the same order of magnitude to the one published by He *et al.*
326 ($22 \mu\text{M}$) (He *et al.*, 2005), Alexiou *et al* and Melagraki *et al* ($5 \mu\text{M}$) using other ELISA assay
327 (Alexiou *et al.*, 2014; Melagraki *et al.*, 2017).

328 However, when $\text{TNF}\alpha$ was pre-incubated with **TNF** receptor 1, SPD304 could also
329 disrupt the association between $\text{TNF}\alpha$ and its receptor but with a milder activity. Indeed,
330 when $\text{TNF}\alpha$ was prior incubated with its receptor, SPD304 disrupted the $\text{TNF}\alpha$ /**TNF** receptor
331 1 binding with a neutralization percentage of 26%, at $100 \mu\text{M}$, compared to 90% when $\text{TNF}\alpha$
332 was firstly exposed to SPD304, corresponding to a decrease in the inhibitory activity of a 3.5
333 factor. The same results were obtained for other concentrations, SPD304 inhibitory activity is
334 inferior when the $\text{TNF}\alpha$ is pre-incubated with **TNF** receptor 1: at $50 \mu\text{M}$, 18% of
335 neutralization versus 90%, and at $25 \mu\text{M}$, 6% versus 76%. This is consistent with He *et al.*
336 (2005) results, who observed that the IC_{50} of SPD304 was lower by a factor 2 in the presence
337 of **TNF** receptor 1 and attributed these differences to the fact that **TNF** receptor 1 binding
338 could stabilize $\text{TNF}\alpha$ trimer (Fig. 2).

339 [Insert Fig. 2. here]

340

341 3.2 $\text{TNF}\alpha$ /SPD304 affinity using SAW biosensor

342

343 Here we have designed a surface acoustic wave biosensor to estimate the dissociation
344 constant of the $\text{TNF}\alpha$ /SPD304 complex. Gravimetry was investigated to determine
345 $\text{TNF}\alpha$ /SPD304 affinity. This technique allows the follow-up of the kinetic of recognition
346 between a given analyte and the corresponding recognition layer and to estimate the
347 corresponding dissociation constants (K_d). The basic principle of gravimetric sensors consists
348 in the measurement of changes of the velocities of surface acoustic waves, which travel along
349 the piezoelectric substrate. Analytes detection by a given recognition layer, deposited on the

350 sensing area, reduces surface acoustic wave velocity, and induces thus a phase decrease of the
351 output signal.

352 SAW biosensors' functionalization strategy was presented in paragraph 2.4.2. TNF α
353 grafting on the sensing area was followed by PBS rinsing, to eliminate the non-adsorbed
354 proteins, and once the signal stability was reached, increasing concentrations of SPD304
355 dissolved in PBS solution were injected. A typical gravimetric response after the injection of
356 10⁻⁵ M of SPD304 is represented in Fig. 3A. The corresponding time constant was of order of
357 176 \pm 7 s.

358 [Insert Fig. 3. here]

359

360 The calibration curve, i.e. cumulative phase-shift variations versus cumulative
361 SPD304 concentrations, is presented in Fig. 3B. This curve was obtained by averaging three
362 experimental ones. Fig. 3B shows that the saturation value was of order of 2x10⁻⁵ M. The
363 limit of detection of the designed SAW sensor, i.e the lowest detected concentration of
364 SPD304, was equal to 1 nM. Several interaction models were then tested to estimate the
365 dissociation constant value (K_d) between SPD304 and TNF α . The most appropriate one was a
366 combined one site binding / Hill model:

367
$$\Delta\Phi(C) = \frac{A_1 \times C}{K_{d1} + C} + \frac{A_2 \times C^\alpha}{K_{d2}^\alpha + C^\alpha}$$

368 $\Delta\Phi$ corresponds to phase variations for a given concentration C of SPD304, K_{d1} and K_{d2} are
369 the first and second dissociation constants. K_{d1} is related to the TNF α /SPD304 affinity, while
370 K_{d2} provides information on the spatial rearrangement of the protein after detection of the
371 analytes and/or possible non-specific adsorption. A_1 and A_2 are empiric constants (their sum is
372 equal to the saturation value of the sensor), and α is the Hill coefficient.

373 Dissociation constants were estimated at: $K_{d1} = 6.1 \pm 4.7$ nM and $K_{d2} = 4.2 \pm 1.7$ μ M.

374 The effect of DMSO concentration, as co-solvent, on the kinetic of interaction
375 between TNF α and SPD304 is presented in the Data in brief of this article.

376

377 *3.3 In cellulo studies*

378

379 In order to evaluate the inhibitory activity of SPD304 on the TNF α /TNF receptor 1
380 signaling pathway, we then used two cell-based assays. First, we tested SPD304 on the L929
381 cell line in which apoptosis is induced by TNF α . Consequently, cell survival is directly
382 correlated with SPD304 inhibitory activity. The obtained results showed a high cytotoxicity of
383 SPD304 from 100 μ M to 25 μ M (Fig. 4A). At 6.25 μ M, the percentage of neutralization of
384 TNF α was 52 % which was like the results obtained by Alexiou et al. (Alexiou et al., 2014)
385 who described an IC₅₀ of 5 μ M for SPD304 in the L929 cells.

386 We also evaluated SPD304 in the HEK-Blue™ TNF α cell-based reporter gene assay
387 where TNF α activity is linked to SEAP production regulated by a transcription factor, NF κ B
388 (Fig. 4B). Similar to L929 cells, SPD304 displayed high toxicity from 100 μ M to 12.5 μ M. At
389 6.25 μ M, the percentage of neutralization of TNF α was 32%. Due to SPD304 cytotoxicity,
390 we were not able to determine an accurate IC₅₀ for SPD304 but the value of this IC₅₀ should
391 be between 12.5 and 6.25 μ M.

392 [Insert Fig. 4. here]

393

394 *3.4 Selectivity against other targets*

395

396 We tested the selectivity of SPD304 for different proteins involved in the TNF α
397 pathway. As shown in Fig. 5, 1 μ M of SPD304 displayed no direct inhibition on IKK α , IKK β

398 (I κ B kinases), JNK1, p38 α and caspases 3 and 8 as activities were below a cut-off value of
399 50%.

400 [Insert Fig. 5. here]

401

402 We then evaluated the selectivity of SPD304 for IL-4 and IL-13 (Fig. 6). First, we
403 evaluated the inhibitory activity of SPD304 for IL-4/IL-4 receptor subunit α and IL-13/IL-13
404 receptor α 2 equilibria using an ELISA-based assay. Unexpectedly, we highlighted the non-
405 selectivity of the SPD304 for the inhibition of the interaction of TNF α /TNF receptor 1 as
406 SPD304 also inhibited the IL-4/IL-4 receptor subunit α and IL-13/IL-13 receptor α 2
407 interactions with an IC₅₀ of 6 μ M and 79 μ M respectively.

408 [Insert Fig. 6. here]

409

410 To confirm the IL-4/IL-4 receptor subunit α and IL-13/IL-13 receptor α 2 inhibition,
411 SPD304 was then tested on a cell-based reporter gene assay, the HEK-Blue™ IL-4/IL-13
412 assay. SPD304 was toxic at high doses ranging from 100 μ M to 12.5 μ M (data not shown). At
413 6.25 μ M (non-toxic dose), IL-4 and IL-13 were neutralized by SPD304 with a percentage of
414 62% and 77% respectively (calculated from QUANTI-Blue™ OD_{450 nm} using the formula
415 described in section 2.5.2) but neutralization was absent with lower doses (3.125 to 0.78 μ M)
416 (Fig. 7). Due to its cytotoxicity, we were not able to determine an accurate IC₅₀ for SPD304
417 but the value of this IC₅₀ was between 6.25 and 3.125 μ M for both cytokines.

418 [Insert Fig. 7. here]

419

420 3.5. ADMET considerations of SPD304

421

422 3.5.1 Thermodynamic solubility of SPD304

423

424 To assess the potential of SPD304 as a hit for an oral drug, we first evaluated the
425 thermodynamic solubility of SPD304 in PBS, pH 7.4, insofar as this medium is representative
426 of the extracellular fluid. SPD304 was characterized by a quite poor thermodynamic solubility
427 of $23 \pm 1 \mu\text{g/ml}$ ($37 \mu\text{M}$), which was one hundred times lower than the ideal solubility for an
428 oral drug as optimal target for solubility would be between 500 and 3000 $\mu\text{g/ml}$ for oral
429 administration (Di et al., 2012; Sun, 2004) .

430

431 3.5.2 Metabolic stability and Permeability on Caco-2 cells

432

433 Metabolic stability of SPD304 was investigated in mouse liver microsomes in order to
434 determine if SPD304 was a substrate of P450 with NADPH as a cofactor. SPD304 appeared
435 to be poorly stable as more than 94% of the compound is metabolized after 1 h.

436 We investigated the permeability of SPD304 using an *in vitro* epithelial Caco-2 cell
437 culture model. The apparent permeability of SPD304 was $0.3 \times 10^{-6} \text{ cm.s}^{-1}$ corresponding to
438 0.6% of SPD304 able to cross Caco-2 cells monolayer in 16 h, which was a low permeability
439 as Kerns and Di (2008). These elements showed that SPD304 is a FDA class IV compound
440 (Center for Drug Evaluation and Research, FDA, 2017), namely poorly soluble, poorly
441 permeable, and therefore not suitable for oral administration.

442

443 3.5.3 ADMET in silico prediction

444 Twenty-six ADMET endpoint predictions were computed and furnished several
445 insights into SPD304 characteristics. The results are summarized in Table 1.

446 Overall, SPD304 fails to fully comply with the canonical rule of five (Lipinski et al.,
447 1997) (i.e. Ro5), especially in terms of lipophilicity with relatively high LogP and LogD_{7.4}

448 (6.93 and 3.10, respectively), predicting poor oral adsorption. The calculated solubility is only
449 of 0.8 $\mu\text{g/ml}$. In comparison, the experimental thermodynamic aqueous solubility value is 23
450 $\mu\text{g/ml}$. Furthermore, the data indicated a moderate percentage of human intestinal absorption
451 of 80% (i.e. HIA) as well as a modest Caco-2 cells permeability of $14.9 \times 10^{-6} \text{ cm.s}^{-1}$. This
452 latter result compares unfavorably with the experimental Caco-2 cells permeability of 0.3×10^{-6}
453 cm.s^{-1} . Lastly, SPD304 has a probability of 68% and 53% for bioavailabilities of 20% and
454 30%, respectively.

455 Projected distribution parameters showed that SPD304 is highly bound to the plasma
456 proteins (% plasma protein binding (PPB) of 94.8%) and displays a theoretical volume of
457 distribution of 0.422 l/kg, in line with an uniform distribution across the body. In terms of
458 metabolism, SPD304 is predicted to inhibit all the cytochromes P450 isoforms, predominantly
459 the 3A4 and the 2C19 isoforms. SPD304 serves also as a substrate for the 1A2, 3A4 and 2C19
460 isoforms. Furthermore, SPD304 showed a projected half-life of 2.24 h and a low clearance of
461 $1.64 \text{ ml.min}^{-1}.\text{kg}^{-1}$. Finally, SPD304 is predicted to be a hERG channel blocker as well as non-
462 mutagenic (Ames test) and presented a high probability of drug induced liver injury (DILI).

463 *[Insert Table1. here]*

464 **4. Discussion**

465 In this work, we provide new elements regarding the biological activity and physico-
466 chemical characteristics of SPD304. Indeed, we recently started a drug discovery program
467 (*Theranalpha*, n.d.) aiming to discover new small molecules inhibiting $\text{TNF}\alpha$ and the use of
468 SPD304 as reference was essential to compare the activity of the new molecules. In this
469 perspective, it seems important to complete the information concerning the drug profile and
470 the drug likeness character of SPD304 in order to clarify if the chemical structure of SPD304
471 can be considered as a hit for a drug optimization program. ELISA experiments confirmed
472 that SPD304 inhibited $\text{TNF}\alpha/\text{TNF}$ receptor 1 binding with an IC_{50} of 12 μM . Cellular-based
473 assays results on two cell lines, L929 (apoptosis) and HEK-Blue™ $\text{TNF}\alpha$ (inflammation),

474 emphasized SPD304 cytotoxicity and also showed SPD304 ability to inhibit TNF α signaling
475 pathways at non-cytotoxic concentrations.

476 In a previous work (Moreau et al., 2016), we investigated a gravimetric SAW
477 biosensor to quantify the TNF α /SPD304 affinity and to estimate the further dissociation
478 constants (K_d). In this preliminary study, TNF α was deposited directly on the gold sensing
479 area of the SAW device, prior to the injection of increasing concentrations of SPD304. A two
480 sites model was used to fit the obtained calibration curves. The dissociation constant related to
481 TNF α /SPD304 affinity was of order of $K_{d1} = (0.6 \pm 0.3) \mu\text{M}$. However, while trying to use
482 this test to systematically assess the affinity of a new library of TNF α inhibitors, we recently
483 highlighted a problem of reproducibility. We have attributed that to poorly controlled
484 adsorption of TNF α on the gold surface of the biosensor. Consequently, in the present work,
485 we investigated a novel surface functionalization strategy to guarantee a reproducible
486 anchoring of TNF α . Here, we chosen to functionalize the sensing area of the SAW sensors
487 with mixed self-assembled monolayers of 1/9 (v/v) ratio of MUDA and MHOH. This latter
488 was used as a spacer to minimize the non-specific binding of TNF α , whereas MUDA permits
489 to immobilize the protein on SAW gold surfaces (Park et al., 2011; Patel et al., 1997).
490 Thereafter, TNF α protein was grafted on the SAMs via a carbodiimide coupling reaction.
491 Details concerning the functionalization and activation steps and the corresponding
492 gravimetric results are presented in the Data in brief of this article.

493 A 103 MHz surface acoustic wave biosensor, functionalized with mixed self-
494 assembled monolayers (MUDA/MHOH, 1:9 v/v), was designed to quantify the affinity
495 between TNF α protein and SPD304 ligand. Adequate modelling of the gravimetric calibration
496 curve, with a combined one site binding and Hill mode, allowed to estimate two dissociation
497 constants at: $K_{d1} = 6.1 \pm 4.7 \text{ nM}$ and $K_{d2} = 4.2 \pm 1.7 \mu\text{M}$. The first dissociation constant K_{d1} ,
498 which is related to TNF α /SPD304 affinity, is inferior by about three orders of magnitude to

499 that of Papanephytous et al, (Papanephytous et al., 2013) and by about two orders of
500 magnitude to our previous work (Moreau et al., 2016). This is probably related to our
501 functionalization strategy, which allows a better grafting of TNF α protein to the SAW sensing
502 area due the use of mixed SAMs. K_{d2} value can be related to nonspecific adsorption between
503 the ligand and the protein. Hill coefficient α was of order of 0.2, indicating negative
504 cooperative interactions between SPD304 and the binding sites (Kurganov et al., 2001).

505 In terms of mechanism of action, co-crystallization showed that SPD304 displaced a
506 subunit from the trimer and bound to the resulting dimer in a folded conformation. SPD304
507 stabilized the dimeric form of TNF α by forming 16 hydrophobic contacts in particular with
508 Tyr59 and Tyr119 (protein data bank ID: 2AZ5) that are crucial to the trimer association
509 (Mukai et al., 2010). In this pioneering work (He et al., 2005), mass spectrometry
510 experiments confirmed this dissociation mechanism as 20% of the dimer was detected in
511 complex with one SPD304 molecule. He et al. in 2005, using a fluorescence homoquenching-
512 based assay, postulated that the mechanism was described by a predissociation-independent
513 model where SPD304 interacts with the TNF α trimer to form an intermediate complex which
514 promotes the TNF α monomer dissociation. This mechanism of action was further confirmed
515 by other mass spectrometry experiments (Cubrillovic et al., 2014). Our different results do not
516 call into question this predissociation-independent model mechanism. However, more
517 surprisingly, we showed in this work that SPD304 was not selective for TNF α or proteins
518 members of the TNF superfamily, such as RANKL, (Douni et al., 2011). Even if we showed
519 that SPD304 displayed no direct inhibition on proteins involved in the TNF α pathway (I κ B
520 kinases, JNK1, p38 α and caspases 3 et 8), we also demonstrated using biochemical (ELISA
521 tests) and cell-based assays (HEK-Blue™ cell line) that SPD304 inhibited two other
522 cytokines, IL-4 and IL-13, which are not part of the TNF superfamily. All these results taken
523 together clearly showed that the small molecule SPD304 was not selective for the inhibition

524 of the TNF α /TNF receptor 1 interaction. The biopharmaceutics classification system (Center
525 for drug evaluation and research, FDA, 2017) allows differentiating drugs based on their
526 solubility and permeability. The solubility classification is based on the highest dose strength
527 in an immediate-release product. A drug is considered as highly soluble when the highest
528 strength is soluble in 250 ml or less of aqueous media over the pH range of 1.0-7.5; otherwise,
529 the drug substance is considered poorly soluble. On the other hand, a drug is considered
530 highly permeable when its level of intestinal absorption is higher or equal to 90%, if not the
531 drug substance is considered to be poorly permeable (Navnit H. Shah et al., 2008).

532 Previously, the kinetic solubility in 10 mM citrate-phosphate buffer (pH 6.5) of
533 SPD304 had been evaluated in the literature at 10 μ M (Papanephytous et al., 2013). Even if
534 the authors mentioned that this solubility value is not precise taking into account the
535 experimental device used, it was significantly different from that which we measured. This
536 difference could be explained by the different pH of measurement and by the methods of
537 measurement (thermodynamic versus kinetic).

538 The thermodynamic solubility of $23 \pm 1 \mu\text{g/ml}$ ($37 \mu\text{M}$), highlighted the need to
539 improve the molecular structure of SPD304 to obtain a more soluble molecule, as poorly
540 soluble compounds carry a higher risk of failure during development (Di et al., 2012). These
541 elements showed that SPD304 is poorly soluble and therefore, very poorly suitable for oral
542 administration. We also confirmed that SPD304 was a very rapidly metabolized molecule
543 (poorly stable as more than 94% of the compound is metabolized after 1 h) and showed a low
544 permeability on Caco-2 cells. The metabolic instability of SPD304 had been already identified
545 by Sun and Yost, 2008. Indeed, the 3-substituted indole moiety of SPD304 is dehydrogenated
546 by the P450 isoform, CYP3A4, producing reactive and toxic electrophilic iminium. SPD304
547 is also metabolized through hydroxylation, N-dealkylation, and epoxidation pathways (Sun
548 and Yost, 2008). According to these authors, the 3-alkylindole moiety should be considered as

549 a toxicophoric as it is metabolically transformed into electrophilic unsaturated iminium
550 species that could react with protein and/or DNA nucleophilic residues and cause toxicities.

551 The evaluated physicochemical and pharmacological profile of SPD304 completed by
552 the ADMET *in silico* prediction makes it possible to conclude that this hit is of rather low
553 quality for a drug optimization program based on its chemical structure. In particular, the non-
554 selectivity of SPD304 constitutes a major drawback in terms of drug design of SPD304
555 analogs since non-selectivity (or promiscuity) is very often a source of adverse drug reactions
556 in animal models or clinical studies due to off-target pharmacological interactions.

557

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563

564 **CRedit authorship contribution statement**

565 All authors contribute to the work presented in this paper. AM, HM, GA carried out
566 the biological and physicochemical studies and participates in the drafting of the article. DC
567 performed the synthesis of SPD304, PG was in charge of metabolic stability and permeability
568 studies and MB carried out the ADMET predictions. NF, CZ, J-FZ, MS-IV and MP are
569 responsible of the data analysis and the revision of the manuscript. All authors have given
570 approval to the final version of the manuscript.

571

572 **Declaration of competing interest**

573 There is no conflict of interest on this study

574

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Figures and Captions

New contributions to the drug profile of TNF α inhibitor SPD304: affinity, selectivity and ADMET considerations

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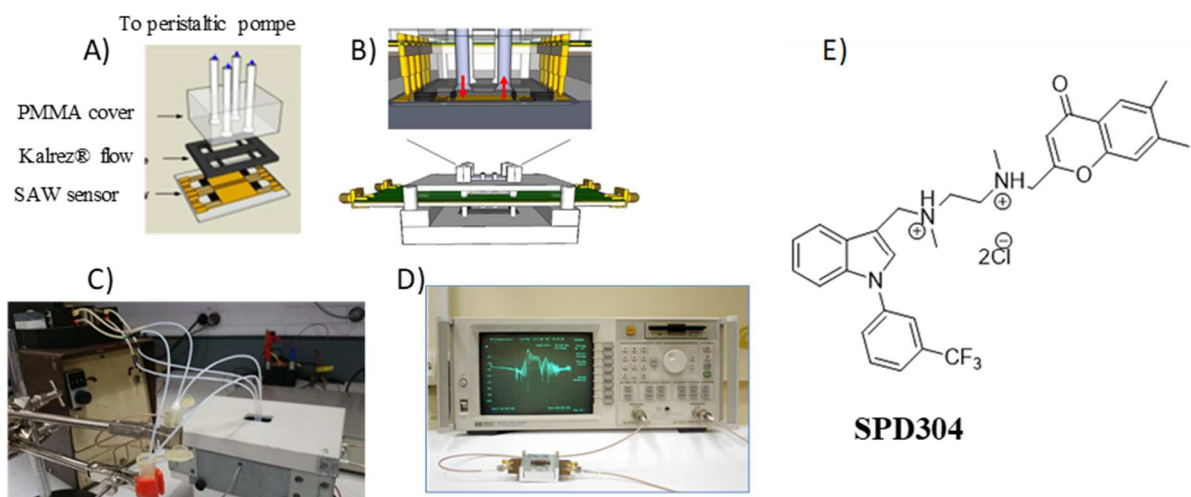


Fig. 1. Schematic representation of a part of the SAW sensor's setup. (A). The various parts of the sensing system. (B). Complete assembly including the printed circuit board. (C). Overview of the system assembly and the fluidic circuit with peristaltic pump. (D). HP8214 network analyser. (E). Structure of SPD304 in dihydrochloride salt form.

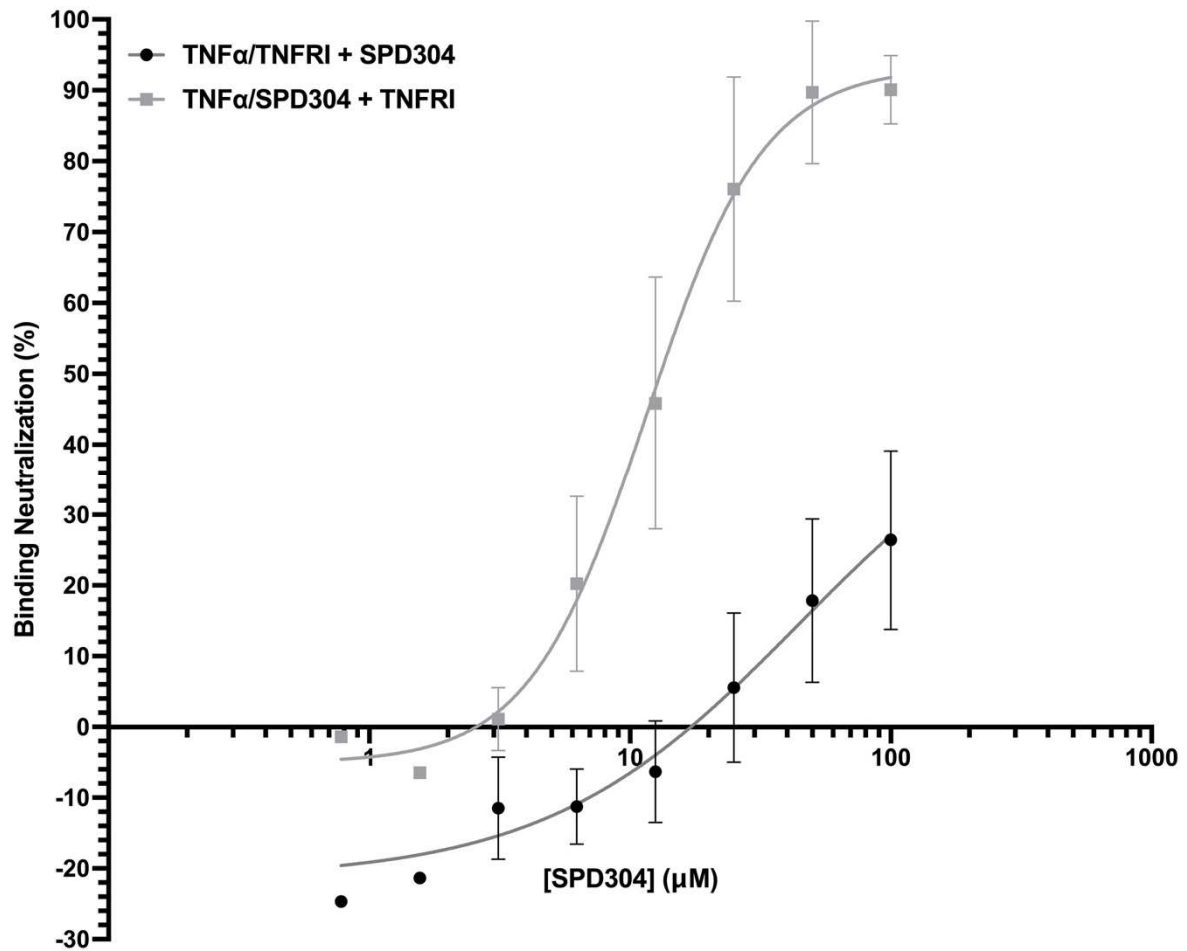


Fig. 2. Effect of SPD304 on the TNF α /TNF receptor 1 equilibrium. SPD304 inhibited TNF α /TNF receptor 1 interaction in a dose-dependent manner. TNF α /SPD304 + TNF receptor 1 $IC_{50} = 12 \pm 4.5 \mu M$. Data are representative of four experiments.

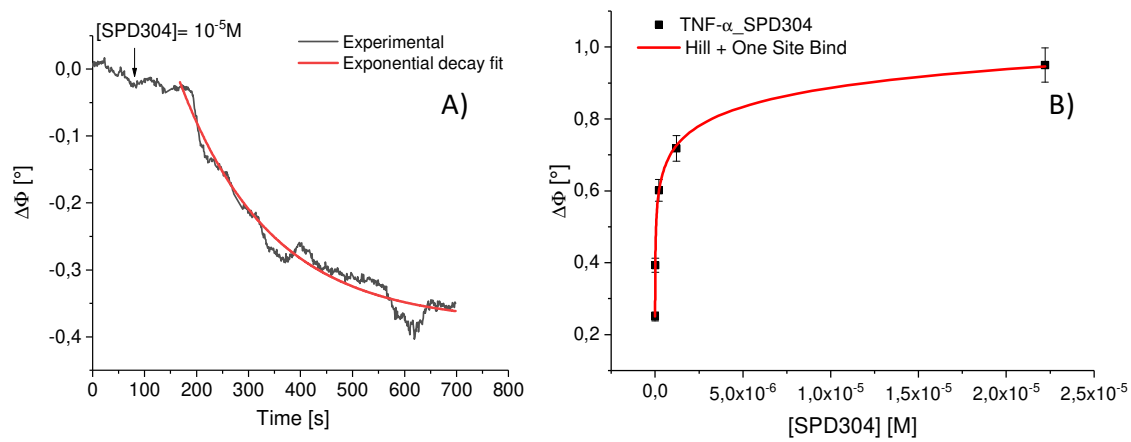


Fig. 3. (A). Phase variations versus time after the injection of 10^{-5} M of SPD304 on the sensing area. (B). Phase-shift variations cumulative ($\Delta\Phi$) versus SPD304 concentration.

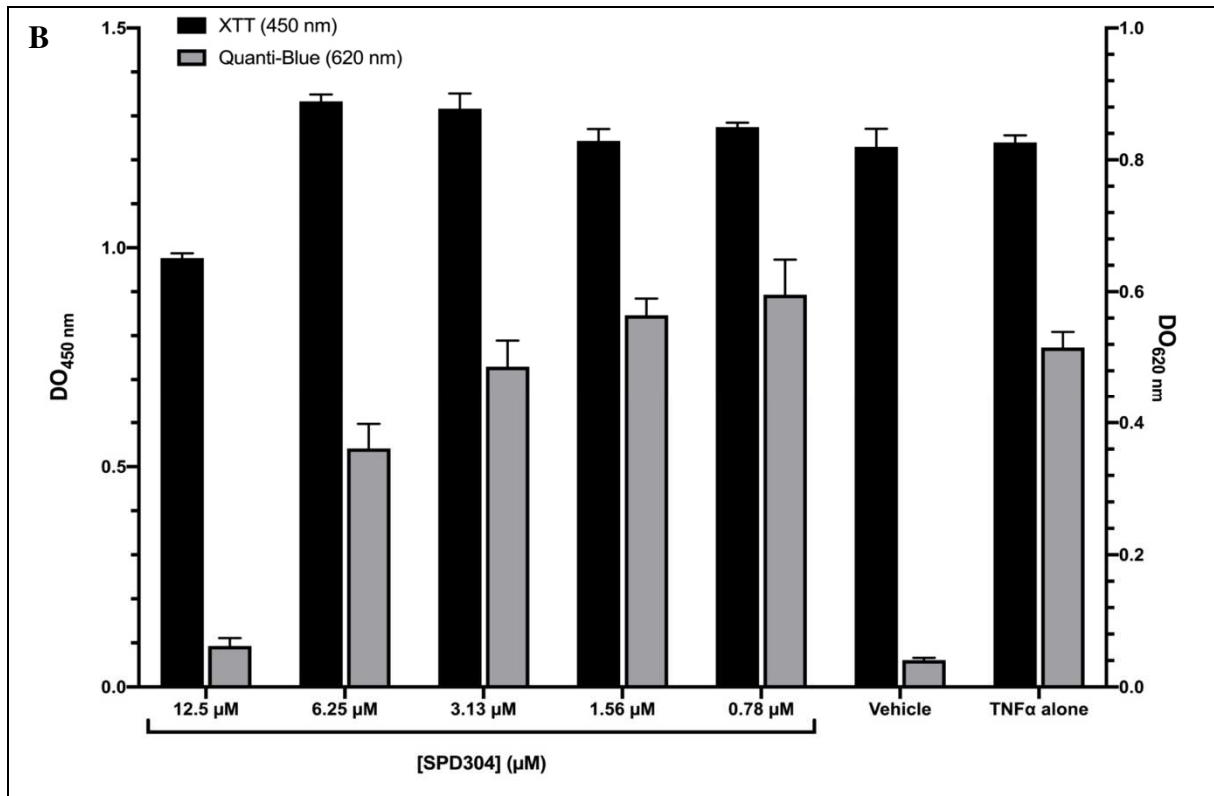
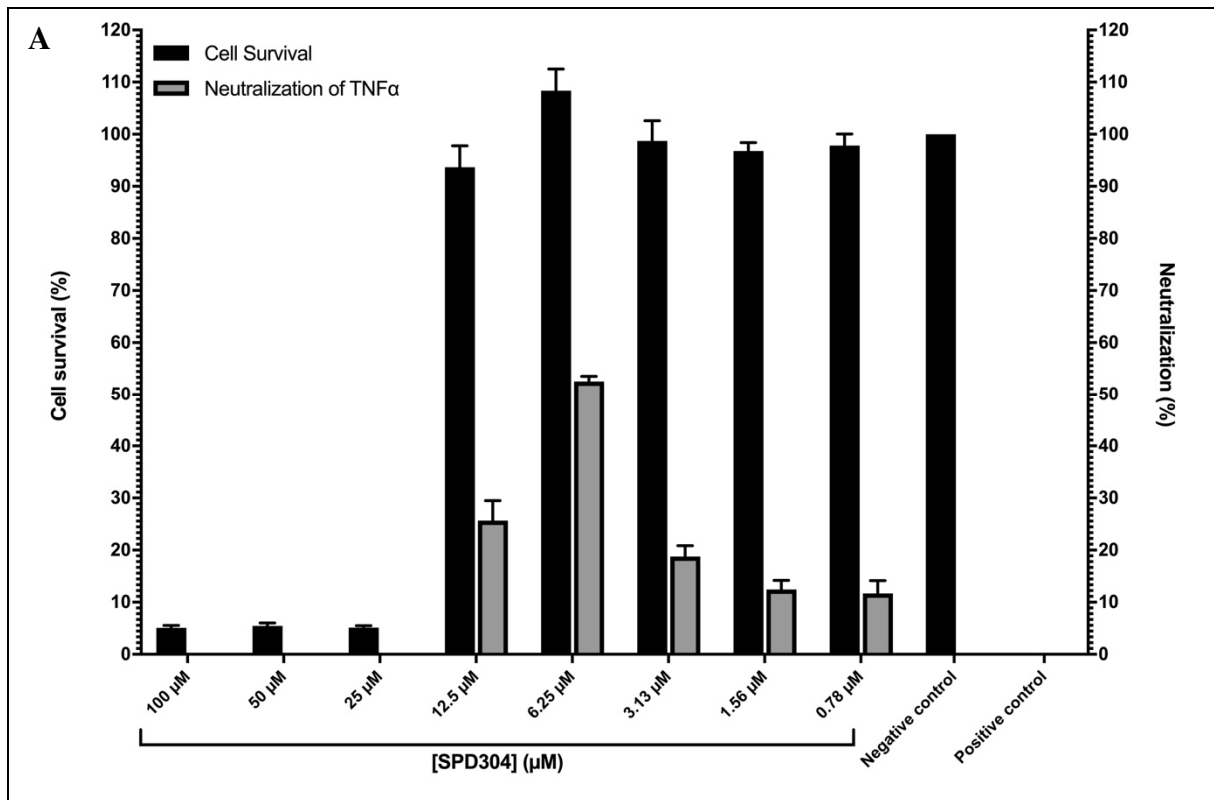


Fig. 4. Effect of SPD304 on L929 and HEK-Blue™ TNF α cell survival and neutralization of TNF α . (A). Data represent cell survival of L929 cells in presence of various concentrations of

SPD304 (left axis, black bars) and neutralization of TNF α in presence of TNF α and various concentrations of SPD304 (right axis, grey bars). Due to high toxicity of SPD304, IC₅₀ was not calculated but was evaluated at 6.25 μ M. **(B)**. Data represent cell survival of HEK-Blue™ TNF α cells in presence of various concentrations of SPD304 (XTT, left axis, black bars) and neutralization of TNF α in presence of TNF α and various concentrations of SPD304 (QUANTI-Blue™, right axis, grey bars). Due to high toxicity of SPD304, IC₅₀ was not calculated but was evaluated between 12.5 μ M and 6.25 μ M. Data are representative of three replicates.

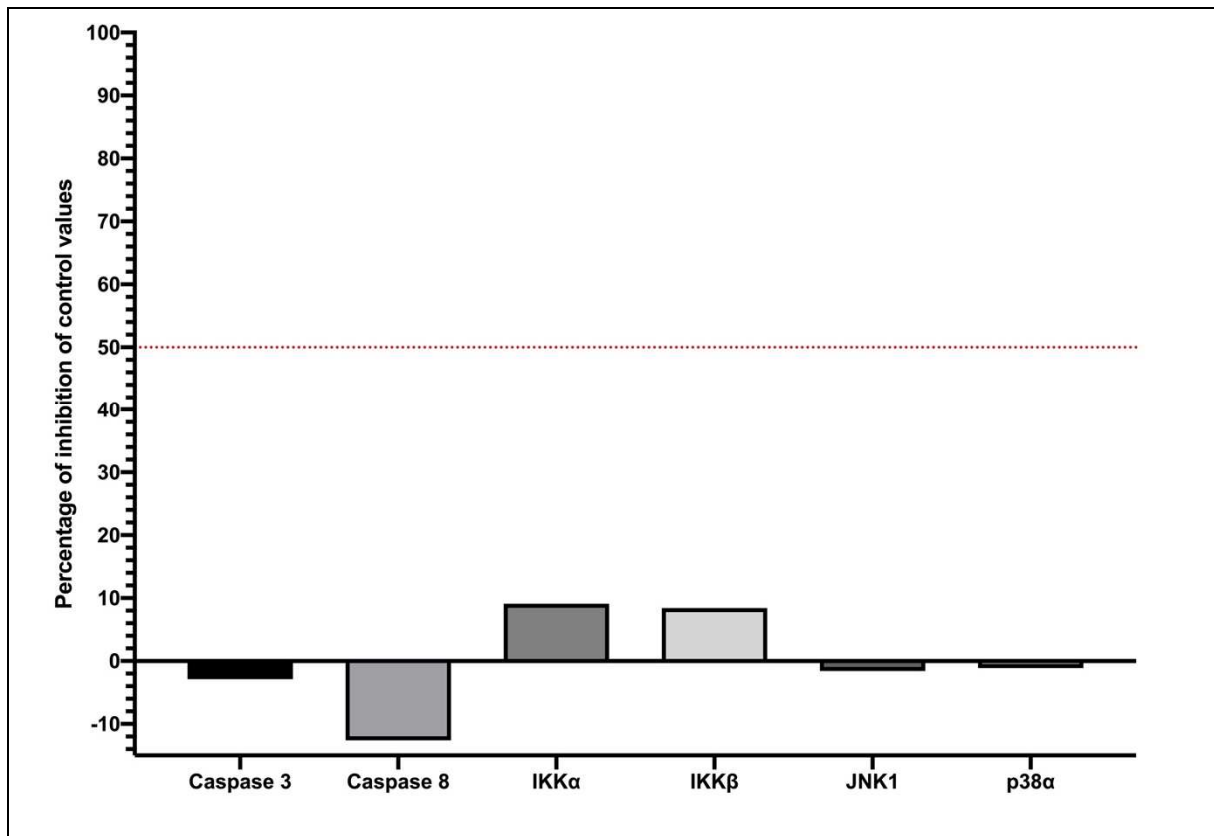


Fig. 5. Effect of direct inhibition of SPD304 on proteins related to TNF α signaling pathway:

Caspase 3, Caspase 8, IKK α , IKK β , JNK1 and p38 α . Red line is the cut-off value (50%).

Values under this cut-off are considered as negative.

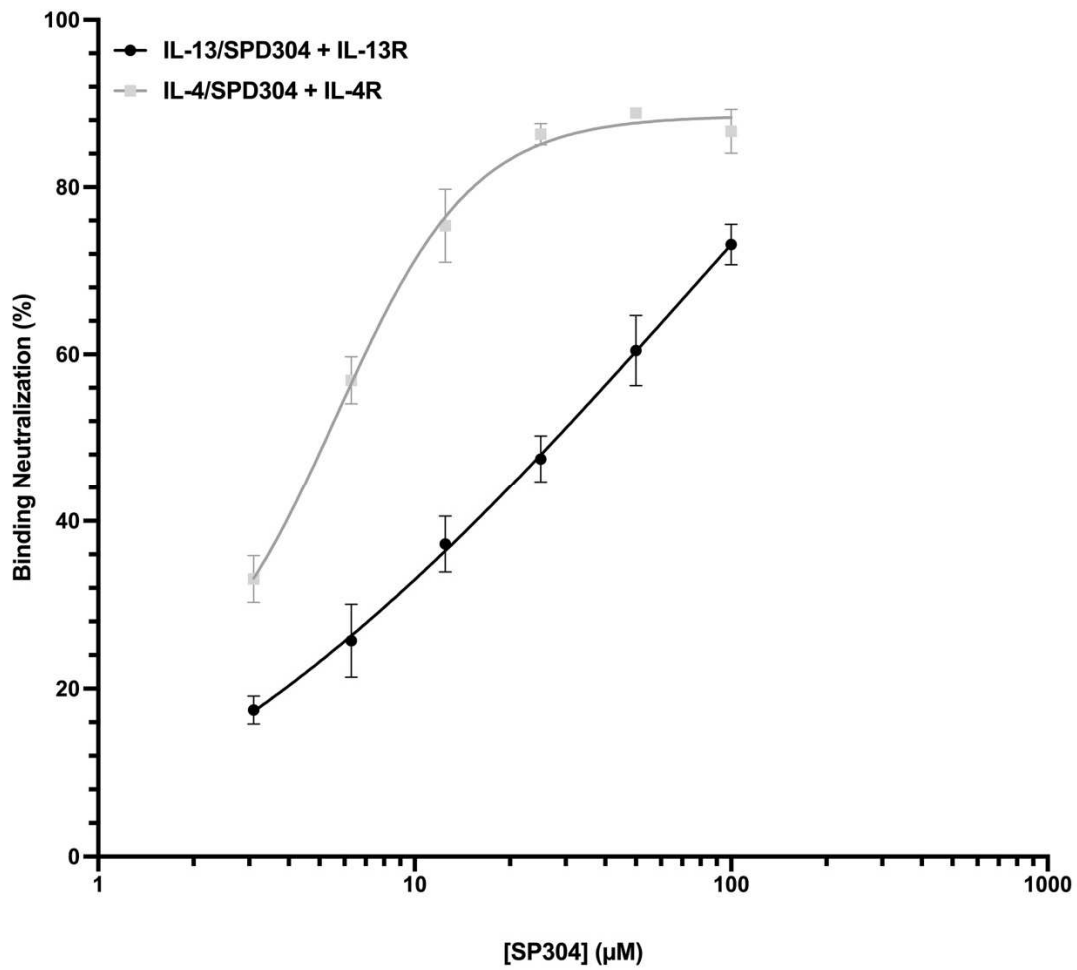


Fig. 6. Effect of SPD304 on the IL-4/IL-4 receptor subunit α and IL-13/IL-13 receptor $\alpha 2$ equilibria. SPD304 inhibited IL-4/IL-4 receptor subunit α and IL-13/IL-13 receptor $\alpha 2$ interaction in a dose-dependent manner. IL-4/IL-4 receptor subunit α $IC_{50} = 6 \mu M$, IL-13/IL-13 receptor $\alpha 2$ $IC_{50} = 79 \mu M$. Data are representative of three replicates.

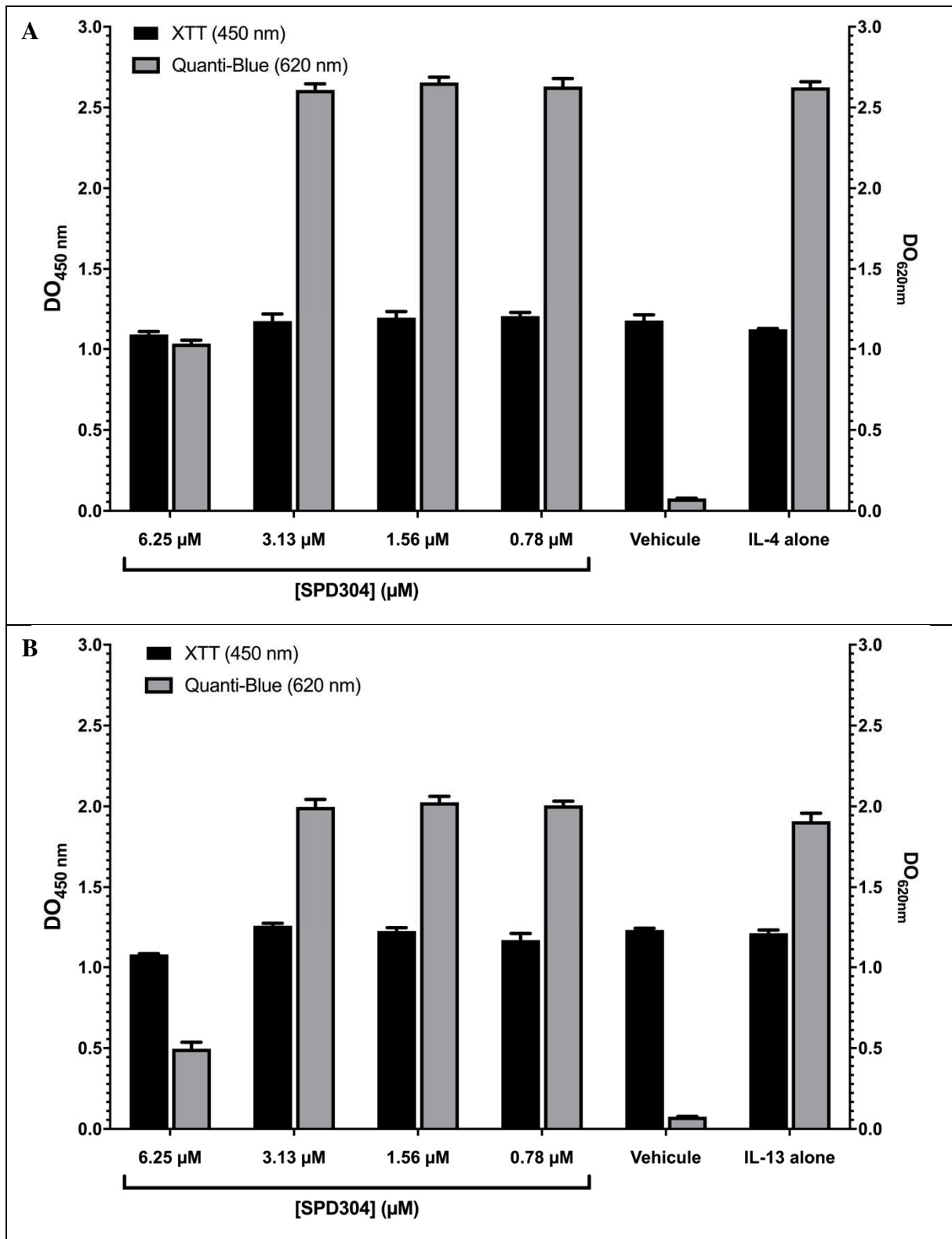


Fig. 7. Effect of SPD304 on HEK-Blue™ IL-4/IL-13 cell survival and neutralization of IL-4 and IL-13. (A). Data represent cell survival of HEK-Blue™ IL-4/IL-13 cells in presence of various concentrations of SPD304 (XTT, left axis, black bars) and neutralization of IL-4 in

presence of IL-4 and various concentrations of SPD304 (QUANTI-Blue™, right axis, grey bars). Due to high toxicity of SPD304, IC₅₀ was not calculated but was evaluated at between 6.25 et 3.125 μM. **(B)**. Data represent cell survival in presence of various concentrations of SPD304 (XTT, left axis, black bars) and neutralization of IL-13 in presence of IL-13 and various concentrations of SPD304 (QUANTI-Blue™, right axis, grey bars). Due to high toxicity of SPD304, IC₅₀ was not calculated but was evaluated at between 6.25 et 3.125 μM. Data are representative of three replicates.

Tables

New contributions to the drug profile of TNF α inhibitor SPD304: affinity, selectivity and ADMET considerations

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Table 1. ADMET parameters predicted for SPD304.

| Physicochemical properties | | | | | | | |
|--|--------------------------------------|--------------------------------------|-----------------------------|------------------------------------|-------------------------------|--------------------------------|-------------------|
| | MW ^a | cLogD ^b | cLogP ^c | Solubility ^d | HBA ^e | HBD ^f | TPSA ^g |
| | 547.61 | 3.1 | 6.9 | 0.8 | 5 | 0 | 41.62 |
| Absorption | | | | | | | |
| | Caco-2 P _{app} ^h | P-gp inhibition ⁱ | P-gp substrate ⁱ | % HIA ^k | | | |
| Category | Moderately permeable | Inhibitor | Non substrate | Moderately absorbed | | | |
| Probability | - | 91% | 61% | - | | | |
| Value | 14x10 ⁻⁶ | - | - | 81% | | | |
| Pharmacokinetics and distribution | | | | | | | |
| | F (20%) bioavailability ^l | F (30%) bioavailability ^m | %PBB ⁿ | V _d (L/Kg) ^o | Half-life (hour) ^p | CL _{int} ^q | |
| Category | F20+ | F30+ | Highly bound | Evenly distributed | - | - | |
| Probability | 68% | 53% | 94% | - | - | - | |
| Value | - | - | - | 0.42 | 2.23 | 1.64 | |
| Metabolism | | | | | | | |
| Oxydation ^r | CYP1A2 | CYP3A4 | CYP2C9 | CYP2C19 | CYP2D6 | | |
| Substrate ^s | Yes | Yes | No | Yes | No | | |
| Substrate probability | 58% | 74% | 49% | 70% | 48% | | |
| Inhibitor ^t | Yes | Yes | Yes | Yes | Yes | | |
| Inhibitor probability | 66% | 78% | 46% | 88% | 53% | | |
| Toxicity | | | | | | | |
| | hERG ^u | H-HT ^v | Mutagenicity ^w | DILI ^x | | | |
| Category | Blocker | Positive | Negative | Positive | | | |
| Probability | 91% | 71% | 63% | 67% | | | |

^a MW: molecular weight (g.mol⁻¹)

^b cLogD: calculated distribution coefficient at pH 7.4: < 1: solubility high; permeability low by passive transcellular diffusion; permeability possible via paracellular if MW < 200; metabolism low. 1 to 3: solubility moderate; permeability moderate; metabolism low. 3 to 5:

solubility low; permeability high; metabolism moderate to high. > 5: solubility low; permeability high; metabolism high.

^c cLogP: calculated octanol-water partition coefficient (lipophilicity)

^d solubility ($\mu\text{g/ml}$): < 10 $\mu\text{g/ml}$: low solubility; 10–60 $\mu\text{g/ml}$: moderate solubility; > 60 $\mu\text{g/ml}$: high solubility

^e HBA: hydrogen bond acceptor

^f HBD: hydrogen bond donor

^g TPSA: total polar surface area in \AA^2

^h Caco-2 permeability: $P_{\text{app}} < 2 \times 10^{-6} \text{ cm.s}^{-1}$: low permeability; $2 \times 10^{-6} < P_{\text{app}} < 20 \times 10^{-6} \text{ cm.s}^{-1}$: moderate permeability; $P_{\text{app}} > 20 \times 10^{-6} \text{ cm.s}^{-1}$: high permeability.

ⁱ P-gp inhibition: inhibition of P-gp has shown to be responsible for several clinical drug-drug interactions.

^j P-gp substrate: more likely to be a P-gp substrate: $N+O \geq 8$; $MW > 400$; acid with $\text{pKa} > 4$.

More likely to be a P-gp non-substrate: $N+O \leq 4$; $MW < 400$; acid with $\text{pKa} < 8$

^k %HIA: percentage of human intestinal absorption

^l F (20% bioavailability): $\geq 20\%$: F20+; $< 20\%$: F20-

^m F (30% bioavailability): $\geq 30\%$: F30+; $< 30\%$: F30-

ⁿ %PPB: percentage of plasma protein binding

^o V_d : volume of distribution. < 0.07 l/kg: confined to blood, bound to plasma protein or highly hydrophilic; 0.07-0.7 l/kg: evenly distributed; > 0.7 l/kg: bound to tissue components (e.g., protein, lipid), highly lipophilic.

^p Half-life ($t_{1/2}$, h): Range: > 8 hours: high; $3 < t_{1/2} < 8$ hours: moderate; < 3 hours: low

^q Clearance CL_{int} : Range: > 15 $\text{ml.min}^{-1}.\text{kg}^{-1}$: high; $5 < CL_{\text{int}} < 15 \text{ ml.min}^{-1}.\text{kg}^{-1}$: moderate; < 5 $\text{ml.min}^{-1}.\text{kg}^{-1}$: low

^r Most common chemical reaction of phase 1 metabolism (oxidation) by cytochromes P450 CYPxxx

^s Substrate: substrate classification models (yes/no) for human CYP_{xxx}

^t Inhibitor: inhibitor classification models (yes/no) for human CYP_{xxx}

^u hERG liability: Features may lead to hERG blocker: A basic amine (positively ionizable, pK_a > 7.3). Hydrophobic/lipophilic substructure(s) (clogP > 3.7). Absence of negatively ionizable groups or oxygen H-bond acceptors.

^v H-HT: Human hepatotoxicity

^w Ames mutagenicity test: Ames positive & negative: significantly induces revertant colony growth at least in one out of usually five strains, otherwise, negative.

^x DILI: Drug induced liver injury