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From *In Vitro* to *In Cellulo*: Evaluation of Anti-TNFα Activity of a New Series of Small Molecules

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Abstract: The Tumor Necrosis Factor alpha (TNFα) is a relevant clinical target for the treatment of chronic inflammatory diseases as rheumatoid arthritis or Crohn's disease. Anti-TNFα biotherapies are used for the treatment of these diseases, improving considerably patient living conditions but they are not without drawbacks. Small molecules inhibitors of TNFα could present fewer disadvantages than existing biotherapies, with less side effects, no resistance, oral administration and would probably lead to less expensive costs. Today only few small molecules are known as direct inhibitors of TNFα, SPD304 was the first small molecule described by He *et al* in 2005. None of these molecules showed both an efficient activity and a low toxicity, necessary to yield them into clinical trial.
We have set-up a program aiming at finding new small molecules inhibitors of TNFα. A preview *in silico* docking study led to the identification of potential anti-TNFα molecules. Based on the docking results, new small molecules have been designed, synthetized and biologically evaluated. Herein we describe the biological evaluation of a series of thirty new synthetized compounds for their capacity to inhibit the TNFα. These molecules were evaluated *in vitro* using ELISA and cellular tests and appear promising compared to previously described small molecules.

Keywords: TNFα, Small-Molecules, Chronic Inflammatory Diseases

1. Introduction

The Tumor Necrosis Factor alpha (TNFα) is a major cytokine of immunity. It is know that a dysregulation of TNFα expression is involved in many diseases as diabetes, cancer and especially in chronic inflammatory diseases such as Crohn's disease [1] or rheumatoid arthritis [2] in which TNFα is a prime target for treatment. The commercialization of anti-TNFα biotherapies, mainly monoclonal antibodies: infliximab (Remicade®), adalimumab (Humira®), certolizumab pegol (Cimzia®) and golimumab (Simponi®) and one soluble receptor: etanercept (Enbrel®) have significantly improved the living conditions of patients for more than 15 years. However, those biotherapies are not without drawbacks. These biomolecules can promote resistance to treatment or side effects such as weakening of the immune system [3][4][5]. In addition, they are expensive (approximately $ 15,000 per year per patient) and the administration route is restrictive (intravenous or subcutaneous injections). Taking to account all this, it would be useful to find an alternative to those biotherapies. The use of small synthetic molecules would have several advantages. Production and treatment costs are significantly lower than for biotherapies.

Oral administration will be easier to implement. A decrease of the undesirable effects is also possible as no immune response directed to the treatment is expected. Moreover, in case of appearance of serious side effects, the treatment can be stopped immediately as the half-life of a small molecule is shorter in comparison to biotherapies [3].

SPD304 was the first small molecule describe for the direct inhibition of the TNFα by He *et al* in 2005 [6]. The authors demonstrated that the SPD304 promotes the formation of the inactive dimeric form of the TNFα by displacing a subunit of active trimeric form. Today, only few small molecules are known as direct inhibitors of TNFα and SPD304 remains a reference as TNFα inhibitor despite its toxicity [7][8].

Recently, we identified a new scaffold of anti-TNFα small molecules though combined *in silico*/*in vitro* studies. Based on the proposed scaffold, 30 new compounds have been synthesized and then evaluated for their TNFα inhibitory capacity.

2. Results and Discussion

First of all, the 30 compounds are evaluated *via* two ELISA assays, using SPD304 as a reference. A binding test, allowed us to determine the inhibitory activity of compounds for the binding of the TNFα to its receptor TNFRI (IC_{50} values **table 1**). The shifting test, compares the activity of the evaluated molecules to the inhibitory activity of SPD304 (shifting values **table 1**).

According to the *in vitro* results, we can classify our compounds into three groups. The first one comprises the non-active molecules with $IC_{50} \geq 50$ µM (compounds **1**, **16**, **17** and **18**). The second one includes 15 compounds with low activity, 50 µM > IC_{50} > 12 µM (compounds **2-8**, **12**, **13**, **15**, **22**, and **27-30**). The third group concerns the active compounds with $IC_{50} \leq 12$ µM and Shifting ≥ 100 % (**9-11**, **14**, **19-21** and **23-26**). Taking into account these results, 11 compounds of our series are active, with a better or comparable activity to SPD304.

Entry	Compound	IC_{50} (µM)	Shifting (%)
1	**1**	> 100	1
2	**2**	38.2	18
3	**3**	34.7	15
4	**4**	35.8	19
5	**5**	37.1	20
6	**6**	18.2	24
7	**7**	27.5	36
8	**8**	16.9	45
9	**9**	10	142
10	**10**	10.7	136
11	**11**	7.2	109
12	**12**	14.3	103
13	**13**	12.4	91
14	**14**	11.3	121
15	**15**	36.7	35
16	**16**	50.8	61
17	**17**	> 100	20
18	**18**	> 100	10
19	**19**	8.9	117
20	**20**	7.7	198
21	**21**	7.3	152
22	**22**	15.7	80
23	**23**	8.4	117
24	**24**	5.6	123
25	**25**	0.6	226
26	**26**	0.6	233
27	**27**	17.7	94
28	**28**	23.5	94
29	**29**	14.4	140
30	**30**	25.1	104
31	SPD304	12	100

Table 1 : ELISA data

Then, these best 11 compounds were evaluated *in cellulo* using the HEK-Blue™ TNFα cells. At 100 µM, the small molecules are cytotoxic, with survival percentages comprising between 0 and 18%. At lower concentrations, the cytotoxicity decreases. The results for 6.25 µM and 1.56 µM are presented in **Figure 1**. At 6.25 µM, 5 compounds (**10**, **11**, **14**, **19** and **23**) displayed a survival percentage higher than 60%. Those molecules have an inhibitory activity at this

concentration, with neutralization percentages comprising between 57 and 80%, except for compound **23** with 17% of neutralization. However, 5 compounds remain highly toxic at 6.25 µM (**20**, **21**, **24**, **25** and **26**), with survival percentages lower than 35%. At 1.56 µM, the 11 compounds are not cytotoxic with survival percentages higher than 80%. Nevertheless, the inhibitory activity decreases at this concentration. Only 3 compounds (**20**, **25** and **26**) are still active with 60% of neutralization on cells for the inhibition of the interaction of the TNFα with its receptor TNFRI, at this low concentration of 1.56 µM.

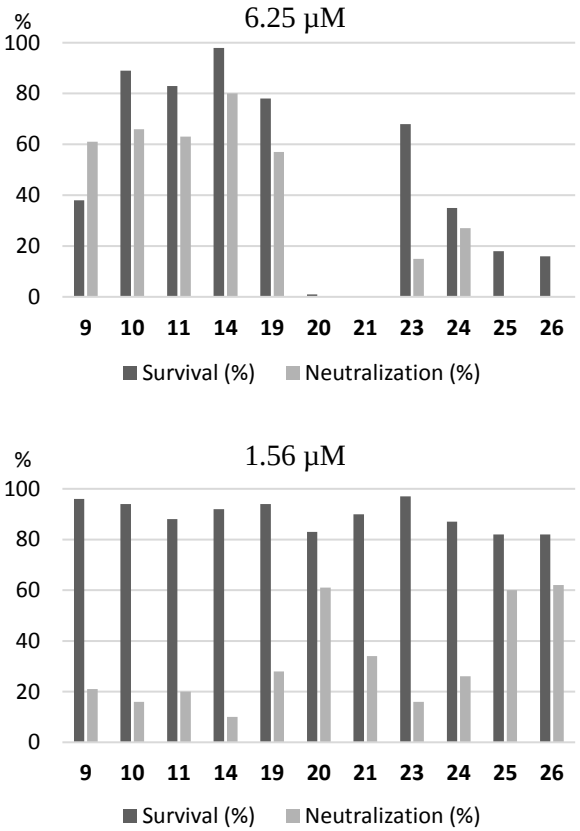

Figure 1: Cytotoxicity and anti-TNFα activity at 6.25 µM and 1.56 µM on HEK-Blue™ TNFα cells

3. Materials and Methods

Materials and cell line. Compounds were synthesized in molecular chemistry team of GBCM laboratory at Cnam. Dimethyl Sulfoxide (DMSO), TMB, XTT and SPD304 were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Human TNFα, human TNFRI and anti-TNFα antibody were obtained from R&D Systems (Lille, France). Avidin-HRP was obtained from eBioscience (ThermoFisher, Illkirch, France). HEK-Blue™ TNFα reporter cell line and QUANTI-Blue™ were obtained from InvivoGen (Toulouse, France). DMEM, L-Glutamine and Penicillin/Streptomycin were obtained from Dominique Dutcher (Issy-les-Moulineaux, France).

TNFα-TNFRI binding ELISA. Microtiter plates were coated with 10 ng of TNFRI per well and incubated one night at 37 °C. The wells were washed, blocked with PBS/BSA 2% for two hours and washed as before. Serial dilutions of compounds were mixed with a fixed quantity of TNFα in PBS/BSA 1% and incubated two hours at 37 °C. 100 µL of the mix were added to the wells and plates were incubated overnight at 4 °C. Wells were washed incubated with 30 ng of TNFα biotinylated antibody in 100 µL of PBS/BSA 1% for two hours at 37 °C. Wells were washed and incubated with avidin-HRP (1:500) in 100 µL of PBS/BSA 1% for 30 minutes at 37 °C. After washing, TMB solution was added to wells, quenched with 50 µL of 1 M H_2SO_4 solution. Absorbance was measured at 450 nm.

TNFα-TNFRI shifting ELISA. Microtiter plates were coated with 10 ng of TNFRI per well, incubated one night at 37 °C. The wells were washed, blocked with PBS/BSA 2% for two hours and washed as before. Serial dilutions of TNFα in PBS/BSA 1% were mixed with a fixed quantity of compounds and incubated two hours at 37 °C. 100 µL of the mix were added to the wells and plates were incubated overnight at 4 °C. Wells were washed incubated with 30 ng of TNFα biotinylated antibody in 100 µL of PBS/BSA 1% for two hours at 37 °C. Wells were washed and incubated with avidin-HRP (1:500) in 100 µL of PBS/BSA 1% for 30 minutes at 37 °C. After washing, TMB solution was added to wells, quenched with 50 µL of 1 M H_2SO_4 solution. Absorbance was measured at 450 nm.

TNFα neutralization on HEK-Blue™ TNFα cells. Serial dilutions of compounds (ranging from 100 µM to 0.8 µM) were mixed with 400 pg/mL of human TNFα in DMEM containing 10% of fetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/mL Penicillin−100 µg/mL Streptomycin in Flat-bottom plates. After two hours of incubation at 37 °C, 5% CO_2, 80% confluent HEK-Blue™ TNFα were added 5×10^4 per well in 40 µL of DMEM containing 10% FBS, 2 mM *L*-Glutamine, 100 U/mL Penicillin, 100

µg/mL Streptomycin and incubated at 24 h at 37 °C, 5% CO_2. 20 µL of supernatants were incubated for 3 hours with 180 µL of QUANTI-Blue™ to reveal secretion of phosphatase alkaline. 45 µL of XTT were added per well. Plates were read at 620 nm (QUANTI-Blue™) or 450 nm (XTT) with a spectrophotometer providing the optical density (OD).

Survival percentages were calculated using equation 1:

$$Suvival\ \% = \left(\frac{cells\ with\ compound\ DO - without\ cells\ DO}{cells\ alone\ DO - without\ cells\ DO}\right) \times 100$$

Neutralization percentages were calculated using equation 2:

$$Neutra\ \% = \left(\frac{cells\ with\ compound\ and\ TNF\alpha\ DO - cells\ with\ TNF\alpha\ DO}{cells\ alone\ DO\ - cells\ with\ TNF\alpha\ DO}\right) \times 100$$

4. Conclusions

We evaluated the TNFα inhibitory activity of 30 new small molecules. Eleven compounds displayed a better or comparable activity to SPD304 used as reference.

A test using HEK-Blue™ TNFα cells was used to confirm this inhibition ability. At low concentration (1.56 µM), 3 compounds are non cytotoxic and still active for the inhibition of the interaction of the TNFα with its receptor TNFRI.

Optimization of these 3 compounds is currently investigated in our laboratory and will be reported in due course.

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Author Contributions

All authors contribute extensively to the work presented in this paper. AM and HM carried out the biological studies and participates in the drafting of the article. AM, DC et CR carried out the synthesis of compounds. M S-IV, MP and JFZ are responsible of the data analysis and the revision of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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