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Chlorpromazine inhibits tumour necrosis factor synthesis and cytotoxicity *in vitro*

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SUMMARY

Chlorpromazine (CPZ) has been previously shown to protect against endotoxin [lipopolysaccharide (LPS)] lethality and inhibit the release of tumour necrosis factor *in vivo*. We investigated at the cellular level whether this was due to direct inhibition of tumour necrosis factor-α (TNF-α) synthesis, using LPS-stimulated THP-1 human monocytic leukemia cells. We also studied the effect of CPZ on human TNF-α action by assessing TNF-α cytotoxicity on mouse fibrosarcoma L929 cells. CPZ (1–100 μM) inhibited TNF-α production in THP-1 cells in a dose dependent manner by a maximum of 80%. This effect was comparable to that of two well-known inhibitory drugs, dexamethasone and cyclicAMP. Inhibition was also evident at the mRNA level. On the other hand CPZ (10–25 μM) also inhibited TNF-α activity: in fact it reduced the cytotoxicity of TNF-α on L929 cells (EC_{50} was increased four times) and could provide protection even as a post-treatment. CPZ inhibited TNF-induced apoptosis in L929 cells, as detected by analysis of nuclear morphology. However, since we showed that apoptosis was very limited, and was not the main mode of cell death in our conditions, this could not explain the overall protection. Since CPZ did not interfere with either the oligomerization state of TNF-α or its receptor binding, our data suggest that it reduced cytotoxicity by inhibiting some steps in the TNF-α signalling pathways.

INTRODUCTION

Chlorpromazine (CPZ) is well known as an antipsychotic drug, and in addition has several peripheral actions too.[1–3]

Our group[4] and others [5] have shown that CPZ protects mice and guinea-pigs against the lethal effect of endotoxin (lipopolysaccharide; LPS). We have also shown that this protection could be explained by a strong inhibition of tumour necrosis factor-α (TNF-α) release *in vivo*, in mice.[6] TNF-α is in fact a key mediator in the toxic effect of LPS and is involved in the pathogenesis of septic shock; serum levels of TNF-α are elevated after administration of LPS to animals and human volunteers, or in septic patients,[7,8] and anti-TNF-α antibodies protect against the lethal effect of LPS and live bacteria in a variety of animal models.[9,10]

We investigated at the cellular level whether the results obtained with CPZ *in vivo* were due to a direct inhibition of TNF-α synthesis, using LPS-stimulated THP-1 human monocytic leukemia cells. We also studied the effect of CPZ on TNF-α action by assessing the TNF-α cytotoxicity on mouse fibrosarcoma L929 cells.

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Abbreviations: CPZ, chlorpromazine; TNF, human tumour necrosis factor; LPS, lipopolysaccharide; L-NMMA, L-*N*-monomethyl-arginine.

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Our findings, excluding an interaction with the active TNF-α trimer (by fast protein liquid chromatography gel filtration chromatography) or with the TNF-α receptor (by radioligand binding), indicate that CPZ reduced cytotoxicity by directly inhibiting TNF-α-stimulated signalling pathways.

MATERIALS AND METHODS

Cell culture

THP-1 and L929 cells (from American Type Culture Collection; Rockville, MD) were maintained in a humidified incubator under an atmosphere of 5% CO_2 in air, in RPMI-1640 (Seromed, Berlin, Germany) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate (all from Gibco, Paisley, UK), 10% fetal bovine serum (FBS, Hyclone Lab. Inc., Logan, UT), and 1·5 g/l $NaHCO_3$, and 12 mM HEPES buffer (Carlo Erba, Milan, Italy). THP-1 cells were used for the TNF-α synthesis experiments at a density of 10^6/ml in fresh medium in 24-well plates, 1 ml/well (Falcon Becton Dickinson, Lincoln Park, NJ) and incubated for 4 hr.

TNF-α protein

Cell suspension after incubation was subjected to three cycles of freeze-thawing. TNF-α present in this cell lysate (i.e. cell-associated plus secreted) was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) method performed as

described.[11] Anti-TNF-α monoclonal antibodies were from Pharmacia-Farmitalia-Carlo Erba (Nerviano, Italy) and rabbit polyclonal antibodies anti-TNF-α were a gift from Sclavo, Siena, Italy. Recombinant TNF-α (specific activity $6{\cdot}6 \times 10^6$ U/mg, kindly provided by BASF-Knoll, Ludwigshafen, Germany) was used as a standard. The sensitivity of the ELISA was 30 pg/ml.

TNF-α mRNA

Total RNA was extracted from 10^7 cells by centrifugation through 5·7 M CsCl as previously described.[12] Ten micrograms of each sample were electrophoresed through denaturing gels (containing 1·7% formaldehyde) in MOPS buffer (MOPS 40 mM, sodium acetate 10 mM, EDTA 1 mM, pH 8) for 5 hr at 70 V. Gels were blotted on nylon membranes (Gene Screen™ Plus, DuPONT, Dreieich, Germany) in 10 × SSC (NaCl 1·5 M, sodium citrate trisodium salt dihydrate 150 mM, pH 7). A cDNA fragment (kind gift of Dr Emanuela Palla, Siena, Italy) corresponding to nt 220–1003 of human TNF-α sequence,[13] was used as a probe. Membrane hybridization was carried out with 10^6 c.p.m./ml in 50% formamide, 10% dextran sulphate, 1% sodium dodecyl sulphate (SDS), and 250 μg/ml salmon sperm DNA at 45° for about 15 hr. Membranes were washed to a final stringency of 0·1 × SSC, 0·1% SDS at 65° and exposed to Kodak X-OMAT AR-5 film (Eastman Kodak, Rochester, NY) with an intensifying screen at −70° for 24 hr.

TNF cytotoxicity

When indicated, total TNF bioactivity (in the cell lysate) was measured by a cytotoxicity assay on L929 cells.[14] Briefly, L929 cells were plated at the density of $1{\cdot}5 \times 10^5$/ml in 96-well plates, 100 μl/well, and used 24 hr later. Samples, drugs and/or recombinant TNF-α (as the standard) were incubated for a further 24 hr, in the presence of 1 μg/ml actinomycin D. Cell survival was determined by measuring optical density at 540 nm after crystal violet staining. The detection limit of the bioassay was 80 pg/ml.

Nuclear morphology

L929 cells were plated at the density of $1{\cdot}5 \times 10^5$/ml in six-well plates, 3 ml/well; 24 hr later 1 μg/ml actinomycin D, 0·5 ng/ml TNF-α and the indicated drugs were added. Nuclear morphology was examined after 8 and 24 hr of incubation as previously described.[15] Briefly, cells (detached with trypsin treatment, when substrate-adhering, and resuspended at the density of 5×10^5/ml) were seeded on microscope slides (100 μl) by cytospin centrifugation (Heraeus, Handu, Germany) at 50 *g* for 10 min, fixed in Carnoy solution (methanol: acetic acid, 3:1) for 1–20 hr, stained with Hoechst 33258 [0·1 μg/ml in phosphate-buffered saline (PBS)] for 1 hr at 37°, washed with tap water for 1 hr, air-dried and mounted. Slides were observed with a fluorescence microscope (Zeiss, Oberkochen, Germany) at an excitation wavelength of 365 nm. The number of nuclei with apoptotic morphology was counted over a total of at least 2000 cells, from coded triplicate slides. When cell density was judged to be uniform in the different regions of the preparation, the total number of nuclei in a single microscope field was counted only once for each slide.

Fast protein liquid chromatography–gel filtration chromatography of TNF-α

Gel filtration chromatography was done as previously described.[16] Briefly, TNF-α spiked with ^{125}I-labelled TNF-α was chromatographed on a Superose 12 column equilibrated and eluted in PBS containing 0·05% bovine serum albumin and 0·02% NaN_3 at a flow rate of 0·05 ml/min. Fractions of 0·25 ml were collected and tested for radioactivity in a gamma-counter.

Statistical analysis and computer-assisted curve fitting

Data are presented either as means ± SEM of the results from independent experiments or as means ± SD of triplicate determinations from a single experiment. Statistical comparisons were made by a paired Student's *t*-test, by Dunnett's test (following one-way analysis of variance) or by Tukey's test (following two-way analysis of variance). EC_{50} were calculated and compared by the Allfit non-linear fitting computer program.[17] Parameters of individual curves are presented with standard error, derived from the fitting.

RESULTS

CPZ inhibits LPS-induced TNF protein and mRNA synthesis in THP-1 cells

THP-1 cells produced TNF in response to LPS stimulation, with maximal effect at the dose of 1 μg/ml, during a 4-hr incubation.[18]

CPZ was not toxic to THP-1 cells in these conditions. In fact cell viability at the end of this incubation was always more than 90%, whether in the presence of CPZ or not. Therefore this model was used to study how the drug interfered with TNF-α synthesis.

As shown in Fig. 1a, 100 μM CPZ, incubated with THP-1 cells from 30 min before the addition of 1 μg/ml LPS, inhibited TNF-α protein production by about 80%. This was comparable to the effect of 2 μM dexamethasone, a well-known inhibitor of TNF-α synthesis *in vivo*[19] and *in vitro*;[20] another known inhibitor (dibutyryl cAMP, 1 mM)[21–23] completely abolished TNF-α synthesis.

Northern blot analysis of total RNA from THP-1 cells (Fig. 1b) revealed a single band of the expected molecular weight. CPZ markedly inhibited the increase in TNF-α mRNA levels induced by LPS, almost as effectively as dibutyryl cAMP.

CPZ inhibits TNF bioactivity induced by LPS in THP-1 cells

The reduction in TNF-α mRNA and protein synthesis was paralleled by an even stronger reduction in TNF bioactivity. Figure 2 shows a quantification of TNF bioactivity recovered in THP-1 cell lysates, obtained with a cytotoxicity assay on L929 cells. CPZ reduced the production of TNF bioactivity in a dose-dependent manner, reaching maximal inhibition (100%) at 100 μM. The same CPZ concentration never completely blocked TNF-α protein synthesis (see Fig. 1a).

CPZ inhibits TNF-α cytotoxicity on L929 cells

Since bioactivity was inhibited more than protein synthesis, we checked whether CPZ interfered in the TNF-α cytotoxicity assay. As shown in Fig. 3, 10 μM CPZ, coincubated with various concentrations of TNF-α for 24 hr, did in fact reduce TNF-α cytotoxicity on L929 cells (EC_{50} was increased from 0·4 ± 0·05 to 1·63 ± 0·36 ng/ml). A similar protection was

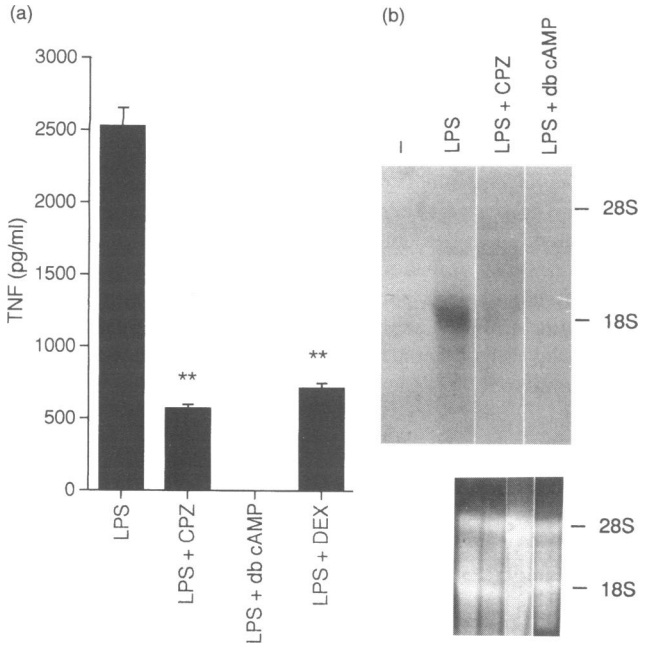

Figure 1. Effect of CPZ on LPS-induced TNF-α synthesis (a) and on the induction of TNF-α mRNA (b) in THP-1 cells. (a) THP-1 cells (1×10^6 cells/ml) were incubated with 100 μM CPZ, 2 μM dexamethasone (DEX), 1 mM dibutyryl cAMP (db cAMP) or medium for 30 min, then with 1 μg/ml LPS for a further 4 hr at 37°. TNF-α protein, measured with an ELISA assay, was undetectable (below the sensitivity of the assay—30 pg/ml) in the absence of LPS, whether or not other drugs were present. The results shown are the mean ± SD of triplicate determinations (**$P < 0.01$, Dunnett's test) of a single experiment, representative of four. (b) THP-1 cells (1×10^6 cells/ml) were incubated with 50 μM CPZ, 1 mM dibutyryl cAMP (db cAMP) or saline for 30 min, then with 1 μg/ml LPS for a further 4 hr at 37°. The upper panel shows the autoradiography of the membrane and the lower panel shows the ethydium bromide staining of RNA loaded on the gel. This experiment is representative of two that gave similar results.

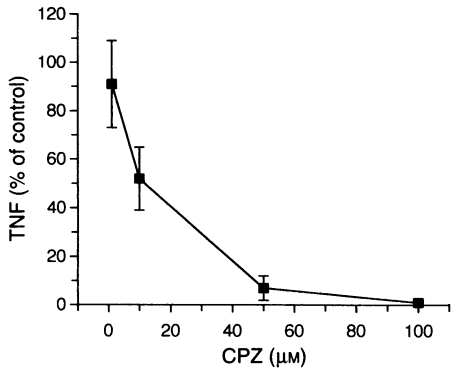

Figure 2. Dose–response curve of CPZ on LPS-induced TNF bioactivity in THP-1 cells. Cells were incubated with various concentrations of CPZ for 30 min, then with 1 μg/ml LPS for a further 4 hr at 37°. TNF bioactivity was assessed by a standard cytotoxicity test on L929 cells. The results shown are the mean ± SEM of the data expressed as percentage of LPS stimulation (absolute values: range 500–1900 pg/ml) from four independent experiments.

obtained when CPZ was incubated 30 min before or after addition of TNF-α.

In a second set of experiments, L929 cells were incubated with TNF-α (6 ng/ml) for only a short period (10 min), to trigger a cytotoxic response. Then they were washed and left for another 24 hr either in control medium or with 25 μM CPZ. At the end of this period cell survival in controls was less than 50% (OD reduction: 58·7 ± 8·6% of untreated cells, mean ± SD of four independent experiments), and was significantly improved (24% increase) by CPZ (OD reduction: 44·8 ± 13·7%, mean ± SD, $n = 4$, $p < 0.05$ paired Student's t-test).

TNF-α-induced apoptosis/necrosis in L929 cells

To investigate the mechanism by which CPZ is protective against TNF-α cytotoxicity, we measured the proportion of nuclei with apoptotic morphology in L929 cells 8 and 24 hr after 0·5 ng/ml TNF-α challenge. Some apoptosis could be detected 8 hr after TNF-α, when all cells were still adhering and trypan blue negative (Fig. 4a). However, nuclear morphology after 24 hr, both in the adhering and non-adhering cells, was more of the necrotic type, showing sparse chromatin in swollen nuclei (not shown). When measured at 8 hr, the proportion of apoptotic nuclei was significantly reduced by 25 μM CPZ, but also by the nitric oxide synthase inhibitor L-*N*-monomethyl-arginine (L-NMMA, 200 μM) (Fig. 4b). However, L-NMMA did not inhibit TNF-α cytotoxicity on L929 cells (Fig. 4c), indicating that apoptosis is not the main cause of cell death in our conditions.

CPZ does not interfere with TNF-α oligomerization or receptor binding

The demonstration of a protective effect of CPZ even after TNF-α removal, suggested that the drug's action was not directed at TNF-α or TNF–receptor interaction. To support this hypothesis we tested whether CPZ interfered with the

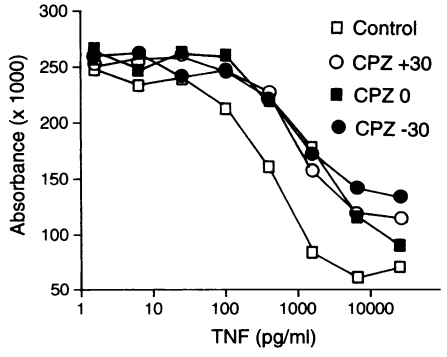

Figure 3. CPZ inhibition of TNF-α cytotoxicity on L929 cells. L929 cells were plated at a density of $1·5 \times 10^5$/ml (100 μl/well) and incubated 24 hr later, for a further 24 hr, with various concentrations of TNF-α either with vehicle (control) or with 10 μM CPZ added at the same time of TNF-α (CPZ 0) or 30 min before or after TNF-α (CPZ −30 and CPZ +30, respectively). EC_{50} were 0·41 ± 0·05 ng/ml for control and 1·63 ± 0·36, 0·89 ± 0·22, 0·96 ± 0·15 ng/ml for CPZ 0, −30 and +30, respectively. The values for CPZ curves were all different from control ($P < 0.01$) and not different between themselves (Allfit). The data are from a single experiment representative of three.

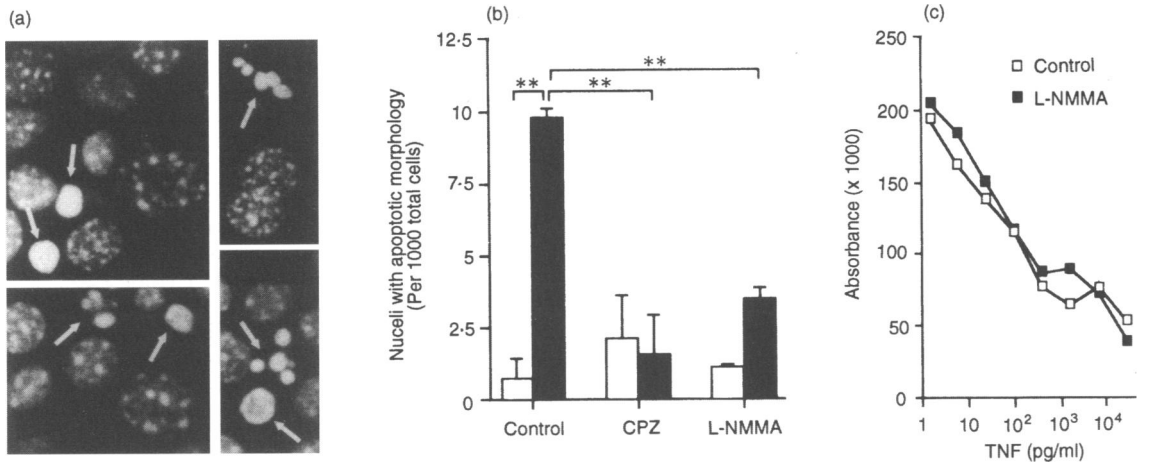

Figure 4. Characterization of TNF-induced apoptosis on L929 cells. Cells were plated at a density of $1{\cdot}5 \times 10^5$/ml (3 ml/well in a and b, 100 μl/well in c) and incubated 24 hr later with TNF-α (0·5 ng/ml in a and b, 1·5 pg/ml–25 ng/ml in c) either alone or with 25 μM CPZ or 200 μM L-NMMA, for 8 hr (in a and b), or 24 hr (in c). (a) Nuclear morphology of L929 cells stained with Hoechst 33258. Arrows indicate nuclei presenting chromatin condensation and fragmentation characteristic of apoptosis. (b) Proportion of nuclei with apoptotic morphology in the presence of TNF-α (black bars) or in its absence (white bars, average ± SD of triplicate samples). Data were analysed by two-way analysis of variance followed by Tukey's test (**$P < 0{\cdot}01$). This single experiment is representative of three that gave similar results. (c) Dose–response curve of TNF-α cytotoxicity with or without 200 μM L-NMMA. The data are from a single experiment representative of three.

TNF-α tridimensional structure. Since this cytokine exists in solution as a trimer, we studied TNF-α de-oligomerization in gel filtration chromatography.[16] When TNF-α was chromatographed after a 24-hr incubation with 1 mM CPZ, a single peak was detected (Fig. 5), with an apparent molecular weight of 31 000 MW, corresponding to trimeric TNF-α, thus demonstrating that TNF-α conserved its trimeric structure even in the presence of very high concentrations of CPZ.

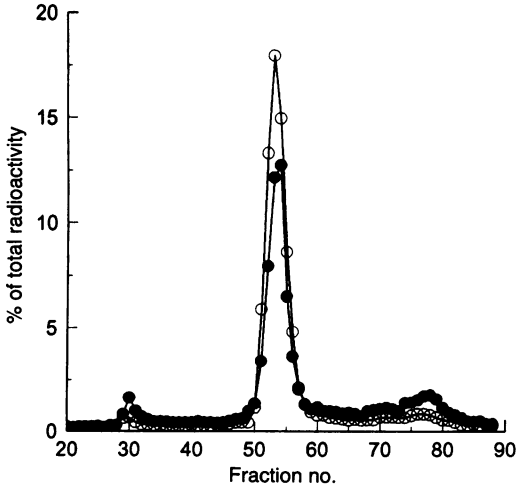

Figure 5. Lack of effect of CPZ on the oligomerization state of recombinant TNF-α. The effect of CPZ on TNF-α was assessed by fast protein liquid chromatography gel filtration chromatography. TNF-α spiked with ^{125}I-labelled TNF (100 : 1 for a total of 20 nM in 100 μl) was preincubated for 24 hr at 4° with buffer alone (open circles) or with buffer supplemented with 1 mM CPZ (filled circles) and then applied to a Superose 12 column. All fractions were tested for radioactivity and results expressed as a percentage of the total radioactivity used.

Additionally the drug, tested either on intact K562 human erythroleukemia cells (at the concentration of 15–30 μM) or on crude membrane preparations (60 nM–60 μM), did not affect the binding of 0·2 nM radioiodinated TNF-α (concentration which is close to K_d value—our unpublished observations) (data not shown).

DISCUSSION

We demonstrate here that CPZ inhibits TNF-α synthesis induced by LPS in THP-1 human monocytic leukemia cells. This observation confirms and extends our previous report of inhibition of serum TNF production in LPS-treated mice.[3] In fact, from the present results, we can conclude that the inhibition can be direct on the TNF-α producing cells, not mediated through any action on other organs. We also found that the inhibition was associated with a reduction in TNF-α mRNA. Several of the pharmacological properties of CPZ could be involved in this activity, including inhibition of phospholipase A_2[24] and of various Ca^{2+}-activated enzymes through binding to calmodulin.[25,26]

The inhibition of TNF-α synthesis is not the drug's only action in this system: it also inhibited TNF-α cytotoxicity. Moreover, the fact that CPZ provided protection even when added after TNF-α had been removed, suggests that it acts on the signalling pathways triggered by TNF-α.

One of the most common,[27,28] though not universal[29,30] observations in apoptosis is a sustained increase in intracellular Ca^{2+} which precedes cell death. Since Ca^{2+} chelation can, in some cases, protect cells (see ref. 31 for review) and one of the actions of CPZ is inhibition of calmodulin, we asked whether CPZ could protect L929 cells by inhibiting apoptosis. Actually, CPZ significantly reduced the proportion of nuclei with apoptotic morphology in TNF-α-treated cells, but this did not explain the overall protection. In fact we concluded that

apoptosis is not the main mode of cell death in our conditions, based on two observations. First, the proportion of apoptotic nuclei was low at short times, and gave place to high amounts of nuclei with necrotic morphology 24 hr after TNF-α challenge; and second, L-NMMA, which inhibits the TNF-α-induced increase in the proportion of apoptotic nuclei, does not change overall TNF-α toxicity.

TNF-α can induce either apoptotic or necrotic cell death depending on the target.[32] A recent paper demonstrated that 5000 U/ml TNF-α induce apoptosis (although with an atypical pattern) and not necrosis in L929 cells. However, our different incubation conditions, including the inhibitor of transcription, actinomycin D, and a low TNF-α concentration (3·3 U/ml) could account for the discrepancy in the results.

TNF-α, synthesized as a precursor protein of molecular weight 26 000 and transformed into a soluble form of 17 000 MW by proteolytic cleavage, is a trimeric structure in the mature form.[33] As a trimer, it binds two distinct receptors on the cell surface and activates several intracellular pathways, including phospholipase A2, phospholipase C and sphingomyelinase activation (see ref. 34 for review). In our hands CPZ did not interfere with the TNF-α tridimensional structure nor did it inhibit TNF-α receptor binding.

To conclude, it seems that CPZ inhibits the TNF-α pathway in two ways: on the one hand it inhibits synthesis and, on the other, it inhibits its cytotoxic activity, probably through an interaction with the signalling pathways elicited by TNF-α.

It remains to be established whether other biological actions of TNF-α are affected by CPZ and whether this activity downstream of TNF-α synthesis is relevant for the *in vivo* protection from LPS toxicity.

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