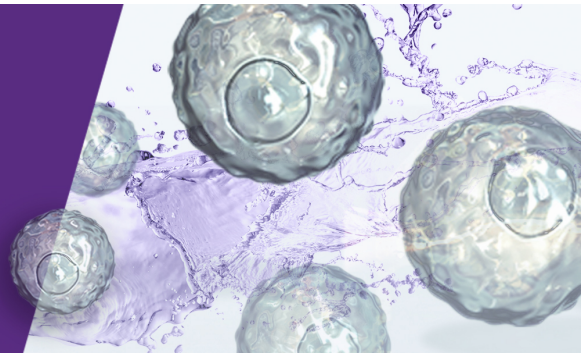


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CC-3052: A Water-Soluble Analog of Thalidomide and Potent Inhibitor of Activation-Induced TNF- α Production¹

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The immunomodulatory drug thalidomide has been shown to be clinically useful in a number of situations due to its ability to inhibit TNF- α synthesis. However, its use is restricted by potentially serious side effects, including teratogenicity and neurotoxicity; furthermore, insolubility may present problems in terms of systemic bioavailability. Recently, structural modifications of thalidomide have been designed enabling greatly enhanced anti-TNF- α activity in LPS-treated mice. In contrast to thalidomide (LPS-induced TNF- α IC₅₀ ~200 μ M in DMSO) and other analogs tested, one of these compounds, CC-3052 (IC₅₀ ~1 μ M in water), is water soluble. Furthermore, this analog exhibits increased stability in human plasma ($t_{1/2}$ ~17.5 vs 1.5 h for thalidomide) and appears to be nontoxic, nonmutagenic, and nonteratogenic. At pharmacologically active levels, cellular proliferation and LPS-induced IL-6 mRNA and IL-12p40 mRNA (as well as IL-1 β and IL-6 protein levels) in whole blood cultures were not affected; apparent inhibition of NK activity by CC-3052 was reversed upon addition of exogenous rTNF- α . In addition, IL-10 mRNA and protein levels were increased. These properties are consistent with results indicating inhibition of phosphodiesterase type IV activity by CC-3052. Furthermore, CC-3052 did not increase the degradation rate of macrophage TNF- α transcripts nor inhibit LPS-induced primary macrophage NF- κ B activation. Taken together, the potency of selective TNF- α inhibition, water solubility, and increased plasma stability make CC-3052 an excellent candidate for further development and clinical evaluation for the treatment of TNF- α -mediated disease. *The Journal of Immunology*, 1998, 161: 4236–4243.

Overproduction of TNF- α is associated with a wide range of pathologic conditions and has therefore led to much recent effort to find ways to down-regulate its production or inhibit its effects in vivo (reviewed in Ref. 1). Many drugs that are commonly used as immunosuppressants, such as cyclosporin A and dexamethasone, do show TNF- α inhibitory properties, although their effects are broad and associated with considerable toxicity. More specific TNF- α down-regulation, using drugs such as pentoxifylline (2) and thalidomide (3), has also been tried in clinical conditions with mixed results. Unfortunately, both pentoxifylline and thalidomide are limited by poor side effect profiles, in addition to limited efficacy. Treatment with humanized mouse anti-TNF- α mAbs has been reported to elicit marked clinical improvements in patients with rheumatoid arthritis (4) and Crohn's disease (5); however, the effect is transitory, and multiple administrations are likely to be associated with reduced efficacy, due to the generation of immune responses against the infused Ab itself. Therefore, current objectives involve the design of new anti-inflammatory drugs that are less toxic, more potent, and potentially more specific (1).

Thalidomide (α -N-phthalimidoglutarimide) is an immunomodulatory and anti-inflammatory drug that was originally used as a sedative, although it is now widely associated with its teratogenic and neurotoxic properties. This drug is now being reassessed because it has been shown to be clinically useful in a number of situations through its ability to selectively inhibit TNF- α synthesis (6). Indeed, thalidomide is the drug of choice in the treatment of erythema nodosum leprosum, an acute inflammatory complication often seen in patients with lepromatous leprosy (3), and it has also been used to treat patients with rheumatoid arthritis (7), HIV-associated aphthous ulceration (8, 9), chronic tuberculosis (10), and chronic graft-vs-host disease (11). Also, a number of double blind, placebo-controlled trials have indicated that thalidomide may be effective in the treatment of chronic diarrhea and wasting associated with HIV disease (10, 12, 13). However, reliable birth control methods must be used by women taking thalidomide, and monitoring for neurologic effects is required in all patients.

In this study, we have looked at a number of compounds synthesized by structural modifications of thalidomide in the hope of finding more potent and specific drugs (14). This strategy aims to take advantage of the beneficial moieties of the thalidomide structure and to enable potential therapy without the side effects associated with the use of the parent compound in vivo. We found that, in contrast to thalidomide and other analogs tested, one of these compounds, CC-3052, is water soluble: this compound was therefore studied further. Solubility is an important consideration in terms of systemic drug bioavailability, since insolubility further limits drug efficacy and the subsequent need for increased dosage compromises patient tolerance.

Materials and Methods

Whole blood and PBMC culture

Thalidomide analogs (Celgene, Warren, NJ) were dissolved in DMSO (0.2% v/v final concentration) or sterile water at 37°C for 1 h. Heparinized

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venous blood was diluted (1:4 for ELISA; 1:1 for PCR) in RPMI 1640 and stimulated at 1 ml/well in 24-well plates with LPS (1 $\mu\text{g/ml}$; *Escherichia coli* serotype 0127:B8; Sigma, St. Louis, MO) or TNF- α (5 ng/ml; R&D Systems, Minneapolis, MN); by incubation at 37°C in 5% CO₂ for 4 to 24 h \pm analog (0.1–100 μM). Cell-free supernatants were collected by microcentrifugation and stored in aliquots at –70°C until assayed by ELISA. For cultures in which PBMC were used, cells were first prepared by density centrifugation on Ficoll-Hypaque (Sigma). PBMC, resuspended at $1 \times 10^6/\text{ml}$ in complete RPMI medium/10% FCS, were stimulated in 24-well plates, and cell-free supernatants were collected and stored as above.

Enzyme-linked immunosorbent assay

Supernatants were assayed for TNF- α and IL-10 using an assay procedure and reagents (anti-cytokine capture mAb, biotinylated anti-cytokine-detecting Ab, and recombinant cytokine) provided by PharMingen (Cambridge Bioscience, Cambridge, U.K.). Assays for IL-1 β and IL-6 were performed using kits provided by R&D Systems. In each case, the manufacturer's instructions were followed exactly.

Competitive PCR

PBMC were prepared from prestimulated whole blood cultures as described above and frozen at –70°C in an RNA isolator (Genosys, Cambridge, U.K.) until RNA preparation. Total RNA was prepared from PBMC incorporating treatment with RNase-free DNase (Promega, Madison, WI) at 37°C for 20 min. RNA was reverse transcribed at 42°C for 1 h using Reverse Transcriptase (R&D Systems). Each sample was assayed for β -actin and cytokine mRNAs by competitive PCR. For each PCR amplification, cDNA was coamplified with a known amount of specific competitor "mimic" fragment (Clontech, Basingstoke, U.K.) designed to give a product that differed in size from the target product by 150 base pairs. For each sample cDNA was coamplified in a series of five or six reactions with a fivefold dilution series of mimic concentrations. The products were separated on 1.5% agarose gels, and the relative amount of target and mimic DNA in each reaction was determined by densitometry (UV DNA Products, Cambridge, U.K.) and plotted to enable the determination of equimolarity and thus the amount of target in the sample. The conditions for amplification were as follows: β -actin and IL-6, 5 min at 96°C, then 40 cycles of 96°C for 45 s, 60°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 5 min; TNF- α , 34 cycles of 94°C for 45 s, 65°C for 45 s, 72°C for 90 s, and a final extension at 72°C for 5 min; for TNFR-1, 40 cycles of 94°C for 45 s, 65°C for 45 s, 72°C for 2 min, and finally 72°C for 5 min; for IL-10, 5 min at 94°C, then 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 5 min; for IL-12p40, 5 min at 94°C, then 40 cycles of 94°C for 60 s, 58°C for 60 s, 72°C for 60 s, and a final extension at 72°C for 5 min.

Flow cytometric analysis

PBMC were prepared and washed in ice-cold PBS/E/G solution (PBS/3 mM EDTA/10 mM glucose). Monocytes were purified by adherence to gelatin (15). Adherent cells were detached, resuspended in complete T cell medium, and stimulated (at $2.5 \times 10^6/\text{ml}$) in 25-cm² flasks for 6 h with LPS (10 $\mu\text{g/ml}$) at 37°C in 5% CO₂. Brefeldin A (10 $\mu\text{g/ml}$; Sigma) was added for the final 4 h of stimulation to prevent extracellular secretion of TNF- α . Cells were then detached by scraping and washed and resuspended in wash buffer (PBS/0.5% BSA/0.1% azide). Cells (2×10^5) were first stained extracellularly for the monocyte-specific marker CD14 (Dako, High Wycombe, U.K.), before fixation and permeabilization with 1 \times FACS lysis and 1 \times permeabilization solutions (Becton Dickinson, San Diego, CA) according to the manufacturer's instructions. Cells were then stained using fluorescence-conjugated α -TNF mAb (R&D Systems) for 30 min before washing once in 2 ml of wash buffer followed by a final resuspension in 1% paraformaldehyde/PBS. Stained samples were kept in the dark at 4°C before cytofluorometric analysis on a FACScan (Becton Dickinson).

Proliferation assay

PBMC (2×10^5) were resuspended in complete RPMI/10% FCS with CC-3052 added at the indicated concentrations into 96-well round-bottom plates (Falcon, Becton Dickinson) to a final volume of 200 μl per well. Cells were cultured with PHA (5 $\mu\text{g/ml}$) at 37°C in 5% CO₂. Proliferation was assessed by [³H]thymidine incorporation (2 mCi/well) for the final 8 h of a 72-h culture. Cells were harvested onto glass fiber filter paper and counted in a Matrix 96 direct beta counter (Canberra Packard, Pangbourne, U.K.). Uptake of [³H]thymidine was expressed as mean cpm of triplicate culture wells.

NK cell activity assay

Cytotoxic activity was measured by a standard chromium-release assay using the NK-sensitive K562 erythroleukemic cell line as target cells. K562 cells (1×10^6) were labeled with 100 μCi ⁵¹Cr (ICN, Thame, U.K.) for 4 h and washed three times before use. Labeled cells (5×10^4) were added to dilutions of PBMC and CC-3052 in 96-well microtiter plates in a total volume of 200 μl . The plates were centrifuged at 300 \times g for 3 min and incubated for 18 h at 37°C. One hundred microliters of supernatant was removed and ⁵¹Cr release was estimated by gamma counting. Maximum ⁵¹Cr release was determined in target cell cultures treated with 10% SDS. Spontaneous ⁵¹Cr release from K562 cells was always <12%. All E:T cell ratios (40:1, 20:1, 10:1, and 5:1) were performed in triplicate, and NK cell cytotoxicity was expressed as percentage of specific cell lysis.

Electrophoretic mobility shift assay (EMSA)

Primary monocytes were purified from PBMC by negative selection using anti-CD2 and anti-CD19 magnetic beads (Dyna, Wirral, U.K.) and were >90% pure as identified by CD14 staining and FACS analysis. Two million monocytes were stimulated for 90 min with LPS (1 $\mu\text{g/ml}$) with or without inhibitors, at 37°C. Nuclear extracts were prepared as described (16). EMSA⁴ was performed using a labeled PRDII probe as previously described (17).

Phosphodiesterase (PDE) type IV assay

PDE purification from U937 cells was conducted using the method of Hill and Mitchell (18). Cells (1×10^9) were washed in PBS and lysed in cold homogenization buffer (20 mM Tris-HCl, pH 7.1, 3 mM 2-ME, 1 mM MgCl₂, 0.1 mM EGTA, 1 μM PMSF, 1 $\mu\text{g/ml}$ leupeptin). Following homogenization by 20 strokes in a Dounce homogenizer, the supernatant was collected by centrifugation and loaded onto a Sephacryl S-200 column equilibrated in homogenization buffer. PDE was eluted in homogenization buffer and rolipram-sensitive fractions pooled and stored in aliquots. PDE activity was assayed by a procedure described by Di Santo and Heaslip (19) and in the presence of varying concentrations of CC-3052, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 1 μM cAMP (of which 1% was [³H] cAMP). The amount of extract used was predetermined to ensure that reactions were within the linear range and consumed <15% of the total substrate. Reactions were performed at 30°C for 30 min and terminated by boiling for 2 min. The samples were then chilled and treated with snake venom (1 mg/ml) at 30°C for 15 min. Unused substrate was removed by addition of 200 μl AG1-X8 resin (Bio-Rad, Richmond, CA) for 15 min. Samples were then spun at 3000 rpm for 5 min, and 50 μl of the aqueous phase was taken for counting. Each data point was conducted in duplicate with activity expressed as percentage of control. IC₅₀ was determined from dose-response curves derived from three independent experiments.

Teratogenicity, viability, and mutagenicity assays

CC-3052 (up to 1407 $\mu\text{M}/500 \mu\text{g/ml}$) was assessed for teratogenic potential by its effects on rat embryo limb bud cells cultured as micromass as previously described (20). After 5 days of culture with the appropriate CC-3052 concentration, cells were fixed and then stained with 1% alcian blue solution. In culture, mesoderm cells may differentiate into chondrocytes, and the associated glycosaminoglycans can be specifically stained with alcian blue. Hence, the number of chondrocyte foci in a micromass can be measured. A teratogen may inhibit the process of embryonic cell differentiation and hence the formation of chondrocyte foci.

Cell toxicity/viability (survival) was assessed by using the neutral red cytotoxicity test (21). Cells were cultured as above and fixed, followed by staining with 0.1% neutral red solution. Cell uptake of neutral red was then eluted with acid alcohol and measured spectrophotometrically. Measurement of the toxicity of CC-3052 indicates whether potential teratogenic activity is real or simply due to cytotoxic effects. PBMC viability after culture in the presence of CC-3052 (up to 100 μM) was also assessed by trypan blue exclusion analysis.

Mutagenicity was assessed using a six-strain bacterial mutation assay. CC-3052 was tested (up to 5000 $\mu\text{g}/\text{plate}$), in the presence and absence of a rat liver metabolic activation system (S-9), with four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) or two strains of *E. coli* (WP2 pKM101 and WP2 uvrA pKM101). Results are expressed as the mean number of revertant colonies at each analog concentration.

⁴ Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; PDE IV, phosphodiesterase type IV; IC₅₀, median inhibitory concentration.

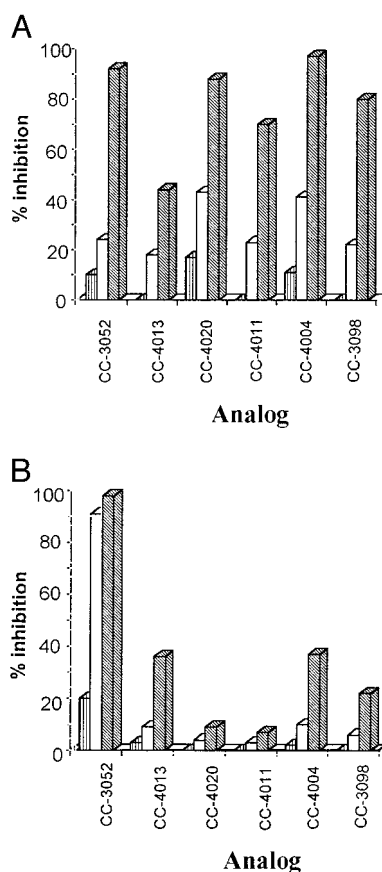


FIGURE 1. The effect of six thalidomide analogs, dissolved in DMSO (0.2% v/v final concentration) (A) and distilled water (B), on TNF- α production by LPS-stimulated whole blood cultures. The inhibitory effect of CC-3052 at 1 μ M (vertical shading), 10 μ M (no shading), and 100 μ M (diagonal shading) was assessed by ELISA. The concentration of each test sample (tested in duplicate) was determined from a standard curve of known concentrations of TNF- α and expressed as the percentage inhibition of cytokine production compared with control. The baseline (100% inhibition) was represented by the unstimulated culture without analog. The data shown are representative of two separate experiments.

Results

We initially tested six analogs of thalidomide for the ability to down-regulate LPS-induced TNF- α production in vitro (Fig. 1). All showed potent anti-TNF- α effects in LPS-stimulated whole blood cultures, when dissolved in DMSO (Fig. 1A). However, in our assay DMSO (0.1% v/v) on its own also exhibited a considerable number of immunomodulatory effects, affecting both TNF- α and IL-10 production (Fig. 2). Only one analog, CC-3052, completely retained its anti-TNF- α properties when water was used as solvent (Fig. 1B). Solubility studies show that this analog is soluble up to at least 0.2 mg/ml. The structure of CC-3052 is shown in Figure 3. We therefore assessed the immunomodulatory properties of CC-3052 (dissolved in water) in terms of cytokine production in vitro, lymphoproliferative response, and NK cell activity. We also determined its effect on cell viability and its capacity as a mutagenic and teratogenic agent.

Inhibition of TNF- α mRNA and protein levels by CC-3052

As shown in Figures 1B and 6, LPS-induced synthesis of TNF- α protein by whole blood was inhibited strongly by CC-3052 in a dose-dependent manner (from Fig. 6: mean IC₅₀ of 1.22 μ M; range, 0.052–2.73 μ M). These data represent numerous separate

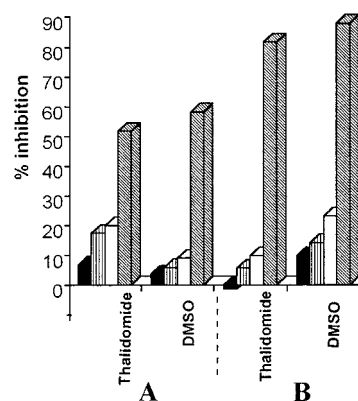


FIGURE 2. The effect of thalidomide dissolved in DMSO or of DMSO alone on TNF- α production (A) and IL-10 production (B) by LPS-stimulated whole blood cultures. The inhibitory effect of thalidomide/DMSO at 1 μ M/0.01% (black), 10 μ M/0.1% (vertical shading), 100 μ M/1% (no shading), and 1000 μ M/10% (diagonal shading) was assessed by ELISA and expressed as described in the legend to Figure 1. The data shown are representative of two separate experiments.

experiments conducted during our studies in which repeated samples from at least 10 apparently healthy laboratory workers were used. Flow cytometric analysis of isolated primary monocytes also showed inhibition of intracellular TNF- α protein production (Fig. 4). Furthermore, inhibition of TNF- α mRNA was observed using competitive PCR, indicating that CC-3052 affects gene expression (Fig. 5A).

Effect of CC-3052 on cytokine production

We next assessed the effect of CC-3052 on the expression of transcripts for IL-10, IL-6, and IL-12-p40 (Fig. 5) and on the secretion of IL-1 β , IL-6, and IL-10 protein (Fig. 6). In our system, the levels of IL-10 mRNA were increased by more than twofold at 1 μ M CC-3052, although at higher doses IL-10 mRNA (Fig. 5B) was inhibited. A similar pattern was seen with IL-10 protein, although its augmentation was not as marked (~15%). However, expression of IL-6 mRNA (Fig. 5C) was unchanged even at 100 μ M. Inhibition of IL-12p40 mRNA (Fig. 5D) was seen only at 100 μ M CC-3052. Secretion of IL-1 β and IL-6 was inhibited to a small extent at 10 μ M, becoming more pronounced at 100 μ M.

Negligible antiproliferative effect of CC-3052 at pharmacologically active levels

Proliferation of PHA-treated PBMC was reduced in a dose-dependent manner on addition of CC-3052 down to 20% of control at 100 μ M (Fig. 7A), although this effect was negligible at lower, but pharmacologically active doses (1–10 μ M). Addition of exogenous TNF- α had no effect on proliferation in the presence of analog (100 μ M), indicating that the antiproliferative effect is not due to inhibition of TNF- α production per se.

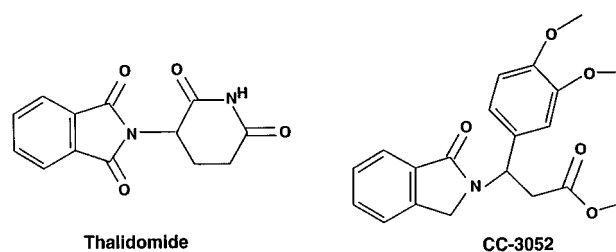


FIGURE 3. Structure of thalidomide and CC-3052.

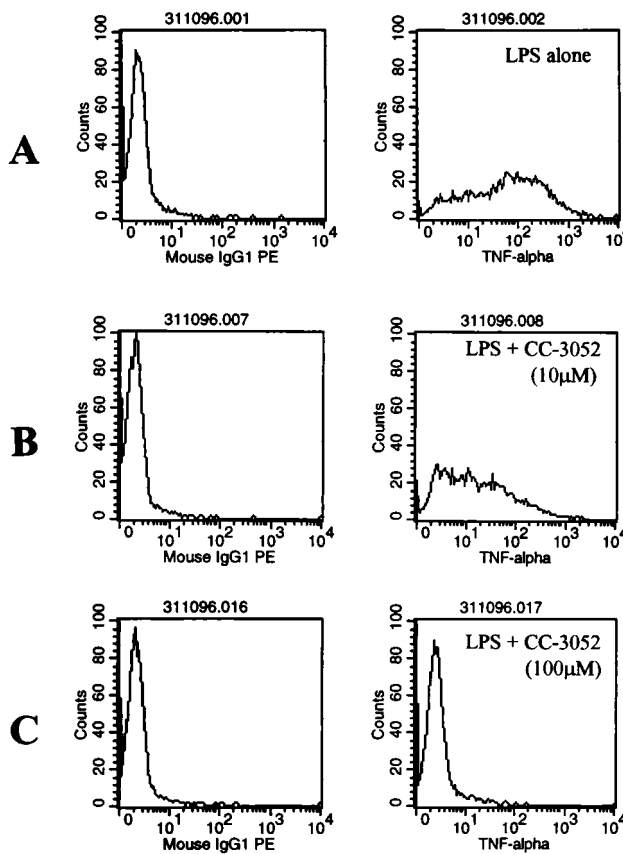


FIGURE 4. FACS analysis of the effect of CC-3052 on the production of intracellular TNF- α by purified monocytes. Cells were prepared as described and stained for CD14 (Dako) by a 15-min incubation at room temperature before fixation and permeabilization with 1 \times FACS lysis and 1 \times permeabilization solutions (Becton Dickinson). Cells were stained intracellularly using fluorescence-conjugated α -TNF mAb (R&D Systems) for 30 min at room temperature before washing once in wash buffer and resuspension in 1% paraformaldehyde/PBS. Samples were analyzed on a FACSscan using CellQuest software. Results are expressed as the percentage of CD14⁺ cells producing intracellular TNF- α and mean fluorescence intensity (MFI). *Left-hand plots* are of cells stained with irrelevant isotype-matched Ab; *right-hand plots* are of cells stained with anti TNF- α Ab. *A*, medium alone, MFI = 49.7; *B*, CC-3052 (10 μ M), MFI = 13.1; *C*, CC-3052 (100 μ M), MFI = 2.5. Plots shown are representative of two separate experiments.

CC-3052-induced TNF- α inhibition leads to partial reduction in NK cell activity

The addition of CC-3052 reduced NK activity in 18-h cultures (Fig. 7*B* shows results at E:T ratio of 20:1). Normalized lysis in NK assays supplemented with CC-3052 (at 10 μ M and 100 μ M, but not 1 μ M) was reduced compared with control samples at all E:T ratios (40:1, 20:1, 10:1, 5:1). The reduction in lysis was dose-dependent with the addition of rTNF- α augmenting or partly rescuing NK activity at all analog concentrations, in agreement with the known up-regulatory role of TNF- α in NK activity.

CC-3052 does not affect the stability of TNF- α mRNA

We then set out to determine whether CC-3052 was able to inhibit TNF- α production by specifically destabilizing TNF- α mRNA. We used LPS-stimulated adherent monocytes and added actinomycin D at the zero time point to inhibit transcription. By measuring the breakdown of TNF- α message over time, in the presence or absence of CC-3052 (10 μ M), we were able to show that

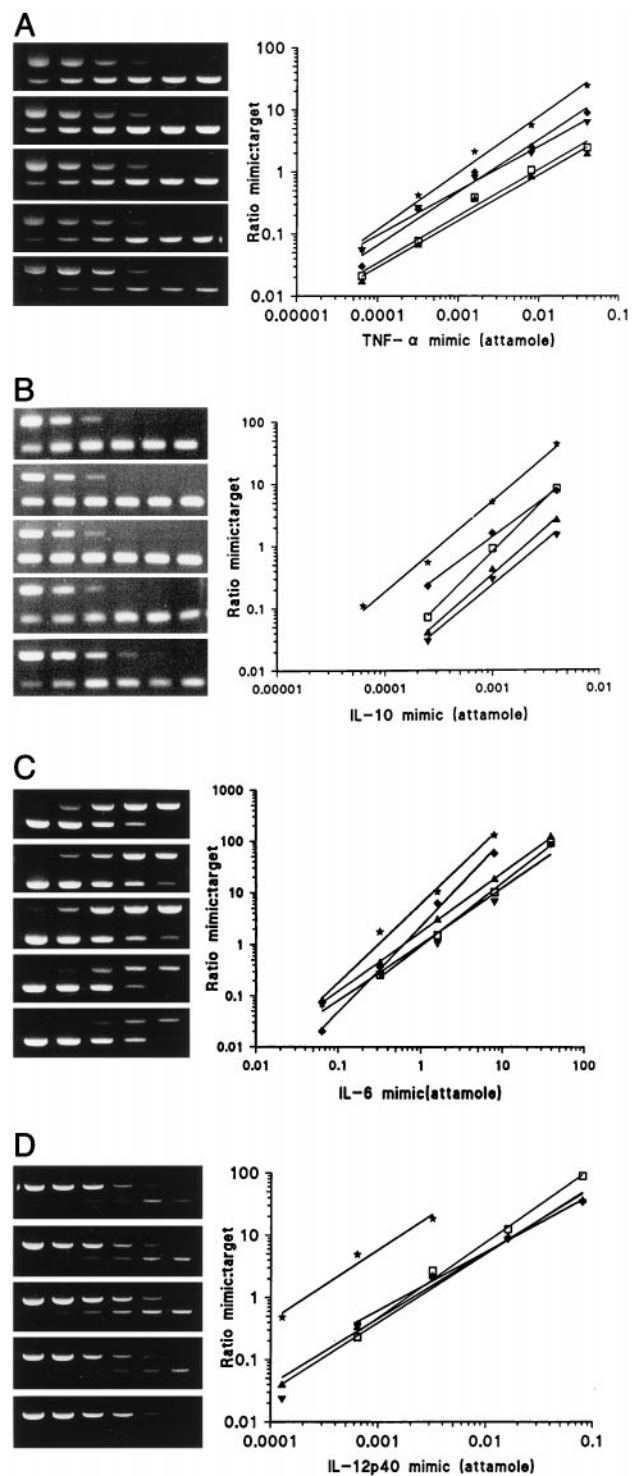


FIGURE 5. The effect of CC-3052 on LPS-induced expression of *A*, TNF- α , *B*, IL-10, *C*, IL-6, and *D*, IL-12p40 mRNA expression by whole blood cultures determined by competitive PCR. A series of PCR reactions plots is obtained from ethidium bromide-stained gels showing the ratio of competitor fragment to target vs competitor fragment concentration. Each gel (and graph line) represents one CC-3052 concentration: at the *top*, 0 μ M (open box), 0.1 μ M (up triangle), 1 μ M (down triangle), and 10 μ M (diamond); and at the *bottom*, 100 μ M (star). Each gel lane represents one PCR fraction with the highest competitor “mimic” concentration at the *left* (cDNA is constant) descending in a fivefold reaction series (fourfold for IL-10) to the *right*. Target band is the *lower band*, except for IL-6. For each cytokine, cDNA concentration (in attamoles) corresponds to the *x*-value where *y* = 1. *A* and *B* are representative of four, and *C* and *D* of two, separate experiments.

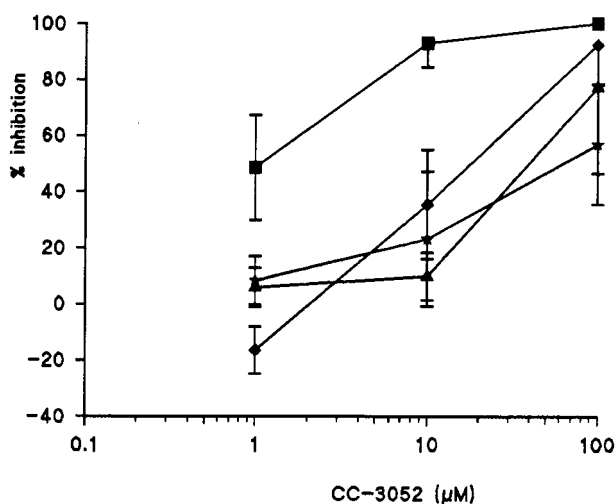


FIGURE 6. The effect of CC-3052 on LPS-induced production of TNF- α (square), IL-6 (triangle), IL-1 β (star), and IL-10 (diamond) by whole blood cultures determined by ELISA. Results are expressed as the mean (\pm SEs) inhibition of 10 blood samples derived from three separate experiments. The concentration of each test sample (tested in duplicate) was determined from a standard curve of known concentrations of TNF- α and expressed as the percentage inhibition of cytokine production compared with control. The baseline (100% inhibition) was represented by the unstimulated culture without analog.

the analog had no effect (Fig. 8). Therefore, our data indicates that CC-3052 inhibits TNF- α mRNA by a different mechanism than that proposed for the parent compound.

CC-3052 does not inhibit LPS-induced NF- κ B nuclear translocation

We then determined whether CC-3052 is able to effect the LPS-induced activation of NF- κ B in adherent monocytes. Activation of NF- κ B occurs via the dissociation of I κ B and subsequent nuclear translocation. By using EMSA, we were able to show that treatment with CC-3052 (10 μ M) did not result in reduction of NF- κ B in the nucleus (Fig. 9).

CC-3052, but not thalidomide, inhibits PDE IV

A possible mode of action of CC-3052 was indicated by direct analysis of its effect on PDE IV activity, the isoform that is expressed mainly in inflammatory cells. Inhibition of purified PDE IV by CC-3052 was observed with a mean IC₅₀ of 3 μ M (range, 0.7–5.32 μ M) correlating closely with its ability to inhibit TNF- α production (IC₅₀ = \sim 1 μ M) (Fig. 10). There was no PDE IV inhibitory effect seen with the addition of thalidomide (not shown).

CC-3052 appears to be nontoxic, nonmutagenic, and nonteratogenic

To address the safety aspects associated with the use of a drug derived from a class of compound such as thalidomide, we have undertaken standard drug assays to assess CC-3052 for any teratogenic, toxicologic, and mutagenic properties that would present problems in a clinical setting. The results of the rat limb bud assay (Table I) showed that CC-3052 did not fulfill the criteria to be considered potentially teratogenic (IC₅₀ teratogen, 576 μ M). Parallel cell viability studies indicate that toxicity is also not a problem at pharmacologically active doses (IC₅₀ survival, 797 μ M) (Table I). Furthermore, PBMC cell viability was not affected when cultured in the presence of CC-3052 up to 100 μ M (not shown). Initial analysis in standard pharmaceutical industry bacterial mu-

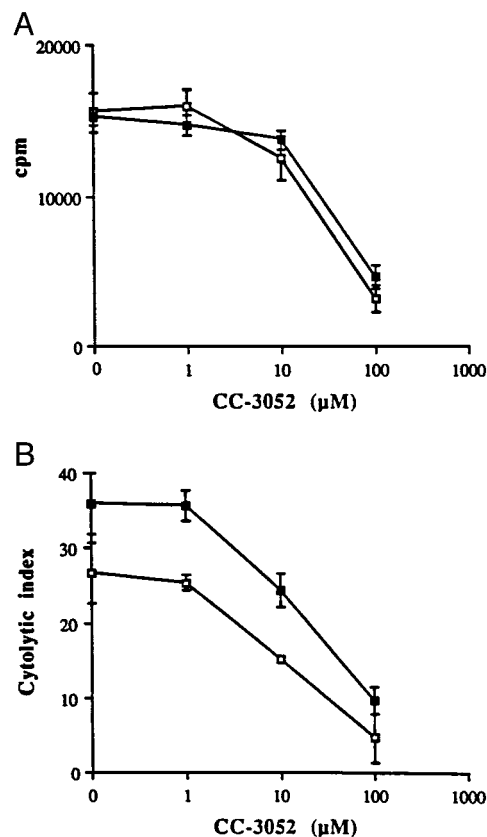


FIGURE 7. Effect of CC-3052 on cell proliferation and NK activity. *A*, Proliferation of PHA-stimulated PBMC was assessed by [³H]thymidine incorporation for the final 8 h of a 72-h culture. Uptake of [³H]thymidine was expressed as mean cpm (\pm SEs) of triplicate wells with CC-3052 alone (open box) and plus rTNF- α (20 ng/ml; filled box). Data are representative of five separate experiments. *B*, Cytotoxic activity was measured by chromium release assay using the NK-sensitive K562 cell line as target cells. Similar results were obtained at all E:T cell ratios; experiments were performed in triplicate, and NK cell cytotoxicity was calculated as described (\pm SEs). Data shown are at an E:T ratio of 1:20 and are representative of three separate experiments. Symbols are as above.

tation assays indicated that no strains showed any increases in bacterial revertant numbers in the presence of CC-3052 (Table II). Hence, there was no evidence that CC-3052 should be considered as potentially mutagenic.

Discussion

In contrast to other analogs tested and to thalidomide itself, we have shown that CC-3052 is highly water soluble at pharmacologically active concentrations. In our whole blood cultures, the use of DMSO at 0.1% (v/v) showed immunomodulatory effects when used alone; previous reports on the action of thalidomide *in vitro* have used DMSO at up to 0.5% (v/v) with apparently little effect (6, 28). Subsequently, we identified CC-3052 alone as water soluble. This is important in that it potentially allows the use of a smaller dosage to achieve systemic effects *in vivo*.

The poor solubility of thalidomide and subsequent poor absorption through the gut mucosa appears to provide a major barrier to good systemic bioavailability. Our experience of the use of oral thalidomide (in a phase II, placebo-controlled, double blind study) to treat asymptomatic HIV-positive patients, at similar doses (100 mg/day) shown to be effective on aphthous ulcers in the gut (8) and in the treatment of patients with microsporidium infection (12),

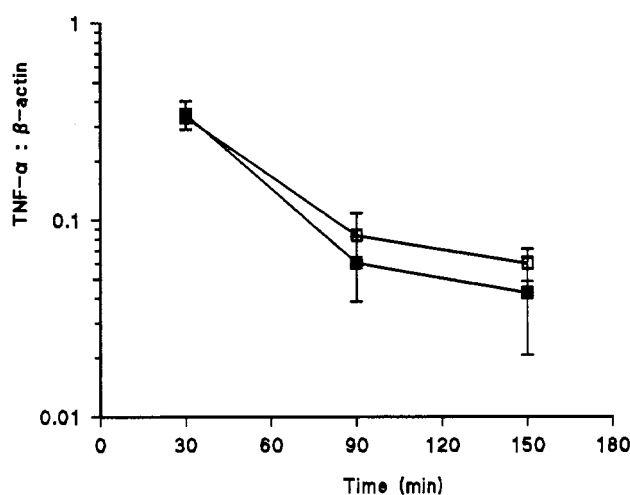


FIGURE 8. Effect of CC-3052 on the rate of TNF- α mRNA degradation in LPS-stimulated adherent cells. PBMC were stimulated with LPS (1 μ g/ml) for 2 to 3 h at 37°C. Actinomycin D (5 μ g/ml) was added to the cells, which were then transferred (at 4×10^6 /ml) to 25-cm² flasks. CC-3052 (10 μ M) or medium alone was added to flasks, and at various time points cells were collected for RNA preparation and analysis by competitive PCR as described. Results are expressed as TNF- α message (attamole) per attamole of β -actin with (open box) or without CC-3052 (filled box) and are mean levels (\pm SE) from two separate experiments.

indicate that efficacy in treating gut-localized pathogenesis is not indicative of systemic bioavailability (22). Furthermore, benefits in the treatment of HIV-associated cachexia using large oral doses (300/400 mg/day) have not been accompanied by alterations in markers of immune function (10, 13). There are also reports of adverse effects, including peripheral neuropathy, severe rash, and

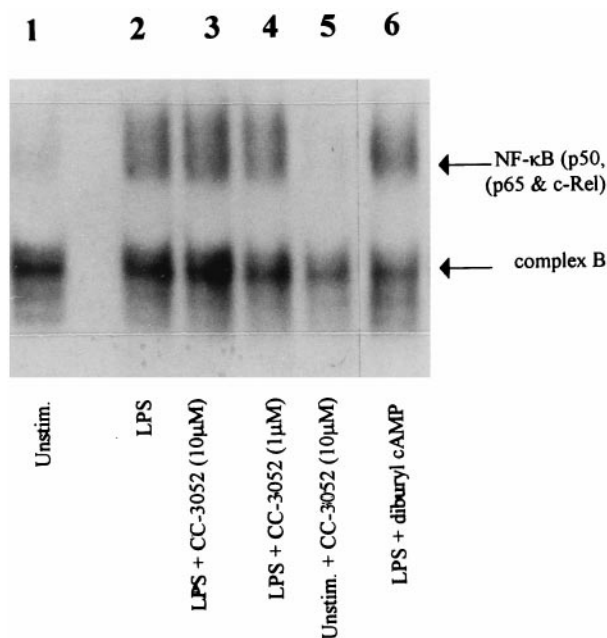


FIGURE 9. Effect of CC-3052 on activation of NF κ B in purified monocytes. Lane 1, unstimulated cells; lane 2, + LPS; lane 3, LPS + CC-3052 (10 μ M); lane 4, LPS + CC-3052 (1 μ M); lane 5, unstimulated + CC-3052 (10 μ M); lane 6, LPS + dibutyl cAMP (40 μ g/ml). Upper band contains NF- κ B, established using specific antisera against p50, p65, and c-Rel (data not shown). Lower band is complex B as described in Ref. 16. Results are representative of three separate experiments.

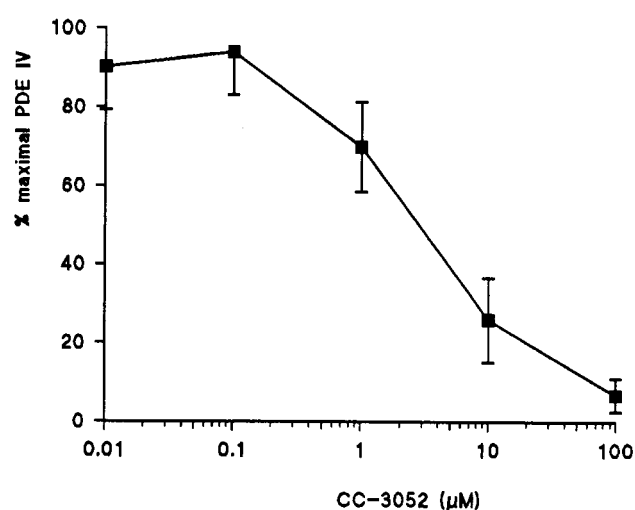


FIGURE 10. Effect of CC-3052 on purified PDE IV. Results are expressed as the percentage of maximal PDE IV activity. Each point represents the mean (\pm SE) of 10 assays.

somnolence. For example, results from a trial of thalidomide in patients with rheumatoid arthritis emphasized the limits imposed by the side effect profile of this drug (23); patients who were able to tolerate a daily dose of 350 mg showed clinical benefits, whereas those that could not tolerate >250 mg did not. Another study on patients with rheumatoid arthritis had to be stopped when it became clear that there were unacceptable side effects in the thalidomide group (24). It is apparent, therefore, that the blood thalidomide levels needed to achieve systemic efficacy may not be possible without considerable toxicity. In this regard, there is little pharmacokinetic data available.

Structural modification of thalidomide has enabled the generation of compounds that are far more potent in terms of TNF- α inhibition; our own studies, as well as those at Celgene, have established that CC-3052 is ~ 200 -fold more potent in inhibiting production of LPS-induced TNF- α . Similar inhibition is observed when net protein production (tested by ELISA), single-cell protein production (by FACScan), and mRNA (by PCR) is assessed, implying a transcriptional locus of control. Furthermore, similar inhibition was observed when whole blood, PBMC, or purified monocyte cultures were used (data submitted but not shown). Importantly, we have observed that CC-3052 is also able to inhibit PHA-induced and rTNF- α -induced TNF- α transcription in PBMC as well as TNF- α transcription due to adhesion of purified monocytes to tissue culture flasks (J.B.M., M.W., S.C., and A.G.D., unpublished observations). Hence, it appears that production of TNF- α due to multiple signaling pathways is affected. The effect on TNF- α appears to be specific, since other cytokines appear not to be inhibited at pharmacologically active doses (~ 1 μ M). Production of other monocyte/macrophage-derived cytokines (IL-1 β and IL-6) induced by LPS are affected to a much lesser extent. However, other cell types may contribute to cytokine production in these cultures, and the effect of CC-3052 on other cytokines, such as IFN- γ , in the presence of LPS and T cell mitogens remains to be resolved. Furthermore, inhibition of NK cell activity appears to be due to TNF- α inhibition itself, since this can be reversed by adding rTNF- α . General cytokine inhibition, observed at the highest dose of CC-3052 (100 μ M), may be due to an antiproliferative effect rather than to TNF- α inhibition per se.

Previously published data have indicated that a number of inhibitors of TNF- α production, such as pentoxifylline, exert their

Table I. Assessment of teratogenicity and toxicity of CC-3052

Substance	Dosage Level (μ M)	Teratogenicity ^a	Toxicity ^b
		(foci per island)	(OD 540 nm)
		Mean \pm SD	Mean \pm SD
DMSO	0	195 \pm 10 (6) ^e	3.47 \pm 0.02 (4)
CC-3052 ^{c,d}	22 (7.8 μ g/ml)	210 \pm 26 (2)	3.00 \pm 0.18 (2)
	44	192 \pm 3 (3)	3.22 \pm 0.23 (2)
	88	201 \pm 11 (3)	3.37 \pm 0.05 (2)
	176	207 \pm 10 (3)	3.46 \pm 0.03 (2)
	352	126 \pm 6 (3)	3.24 \pm 0.11 (2)
	704	101 \pm 14 (3)	2.19 \pm 0.04 (2)
	1407	0 \pm 0 (3)	0.15 \pm 0.04 (2)
Positive control (5-FU)	0.1 (μ g/ml)	188 \pm 26 (3)	1.63 \pm 0.07 (2)
	0.2	37 \pm 3 (3)	1.80 \pm 0.09 (2)
	0.4	0 \pm 0 (3)	1.15 \pm 0.11 (2)

^a Teratogenicity was assessed by microscopic scoring of differentiated foci in each stained micromass and expressed as mean differentiated foci per island \pm SD.

^b Toxicity was assessed by spectrophotometric measurement of eluted neutral red at 540 nm (mean \pm SD).

^c IC₅₀ (teratogen) for CC-3052 was 576 μ M.

^d IC₅₀ (survival) for CC-3052 was 797 μ M.

^e Figures in parentheses are numbers of plates tested per condition. 5-FU, 5-fluorouracil.

action by increasing levels of cAMP via inhibition of PDE activity (25). Our data show that IL-10 mRNA expression is increased (approximately twofold) and IL-10 protein production is increased by \sim 15% at low concentrations of CC-3052. Increased IL-10 production is associated with cAMP-elevating drugs. However, Platzer et al. have showed that induction of IL-10 is inhibited by anti-TNF- α Ab. Furthermore, another report showed the importance of TNF- α in monocyte IL-10 production (26). Our data showing modest up-regulation of IL-10 mRNA and protein is therefore consistent with the idea that CC-3052 acts via inhibition of PDE IV; a net negative effect on IL-10 expression at high analog concentrations is probably due to TNF- α inhibition. It is likely that the TNF- α independent antiproliferative effect of a high concentration CC-3052 is due to elevation of cAMP. Furthermore, reduction of NK cell activity by CC-3052 appears to be partly dependent on TNF- α inhibition and partly on cAMP elevation.

Our results show that CC-3052 is not able to affect the stability of TNF- α mRNA. This is a well-documented method of controlling the level of specific gene transcripts (27). A previous report has indicated that this is the mechanism by which thalidomide itself exerts its effect (28), although we were unable to confirm this. Another report concerning a possible mode of action has indicated that thalidomide and the three other analogs that were tested, not including CC-3052, were able to inhibit activation of

NK- κ B in HIV-infected primary macrophages (29). We have used LPS-stimulated primary macrophages and shown thalidomide to partially inhibit activation of this transcription factor, although no effect has been seen with CC-3052. We are presently addressing the involvement of transcription factors in the action of thalidomide and its analogs. In addition to its solubility and increased potency of action, we have found that CC-3052 is far more stable in human plasma than thalidomide, with a $t_{1/2}$ in reconstituted human plasma of 17.5 h, compared with only 1.5 h for thalidomide due to hydrolysis of its glutarimide ring (Fig. 3). This finding has obvious implications when considering bioavailability and the use of multiple dosage regimens.

Of great importance when considering potential therapeutic applications of compounds based on a drug such as thalidomide are its potential for side effects when administered to patients. Initial results for CC-3052 are promising, as it appears not to exhibit teratogenic, toxic, or mutagenic properties. Furthermore, results obtained by Celgene with analogs similar to CC-3052 have shown these to be highly active in protecting mice from LPS-induced lethality and will soon be assessed in phase I clinical trials (30).

We have therefore identified a thalidomide analog that has far greater potential than its parent molecule for use as an anti TNF- α agent in vivo: CC-3052 is \sim 200-fold more potent than thalidomide

Table II. Assessment of mutagenicity of CC-3052 using a six-strain bacterial mutation assay^a

Substance	Dosage Level (μ g/plate)	TA98, Mean \pm SD	TA100, Mean \pm SD	TA1535, Mean \pm SD	TA1537, Mean \pm SD	WPpKM101, Mean \pm SD	WP2 uvrA pKM101, Mean \pm SD
DMSO	—	40 \pm 7 (5)	163 \pm 6 (4)	24 \pm 4 (5)	11 \pm 3 (5)	54 \pm 10 (5)	173 \pm 10 (5)
CC-3052	8	30 \pm 13 (3)	144 \pm 20 (3)	27 \pm 10 (3)	14 \pm 2 (3)	47 \pm 6 (3)	194 \pm 13 (3)
	40	37 \pm 7 (3)	142 \pm 8 (3)	28 \pm 4 (3)	13 \pm 3 (3)	46 \pm 10 (3)	177 \pm 2 (2)
	200	43 \pm 4 (3)	148 \pm 14 (3)	21 \pm 5 (3)	8 \pm 3 (3)	64 \pm 11 (3)	188 \pm 15 (3)
	1000	50 \pm 6 (3)	153 \pm 9 (3)	20 \pm 5 (3)	10 \pm 3 (3)	52 \pm 4 (3)	205 \pm 8 (3)
	5000	29 \pm 5 (3)	148 \pm 12 (3)	23 \pm 8 (3)	13 \pm 7 (3)	62 \pm 7 (3)	190 \pm 19 (3)
Positive control	Compound dose	AAN	AAN	ND	ND	ND	AAN
	Mean \pm SD	5 μ g 1021 \pm 54 (3)	5 μ g 1352 \pm 20 (3)				5 μ g 592 \pm 9 (3)

^a CC-3052 was tested in the presence of a rat liver metabolic activation system (S-9), with four strains of *S. typhimurium* (TA98, TA100, TA1535, and TA1537) and two strains of *E. coli* (WP2 pKM101 and WP2 uvrA pKM101). Data expressed as mean revertant colonies \pm SD. Figures in parentheses are numbers of plates tested per condition. AAN, 2-aminoanthracene; ND, not determined.

in vitro, is far more soluble in water, has greater stability in reconstituted human plasma, and appears to be nontoxic and non-teratogenic. These factors indicate that this compound is potentially an excellent candidate for further investigation in a clinical environment in which elevated TNF- α levels are associated with disease pathogenesis.

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