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Aminoalkylcarbamoylphosphonates reduce TNFα release from activated immune cells

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ABSTRACT

Some carbamoylphosphonates (CPOs) inhibit matrix metalloproteinases (MMPs). Although MMPs are involved in inflammatory processes, the anti-inflammatory activity of CPOs has not been reported. In this context we compared the biological activity of the three aminoCPOs, PYR-CPO, PIP-CPO and *cis*-ACCP. We were particularly interested in their capability to modulate the secretion of tumor necrosis factor alpha (TNFα). LPS-activated mouse peritoneal macrophages and LPS-activated mouse splenocytes were used to explore this question. It was found that the aminoCPOs were able to reduce TNFα secretion to a level equivalent to the reduction caused by the steroid drug budesonide (BUD). The reduction in TNFα levels was neither accompanied by cytotoxicity, nor did it inhibit cell proliferation. To explicate whether the aminoCPOs affect TNFα processing by TNFα-converting enzyme (TACE), TACE inhibitory properties of the three molecules was tested in vitro. Only PIP-CPO exerted TACE inhibitory activity at therapeutic (non-cytotoxic) concentrations, indicating on its potential to serve as an anti-inflammatory agent by reducing TNFα secretion.

Chronic inflammation involves a local, continuous release of pro-inflammatory cytokines.[1,2] The most abundant ones are tumor necrosis factor alpha (TNFα) and interleukin-1β.[3,4] The typical overproduction of TNFα in chronic inflammation such as rheumatoid arthritis and inflammatory bowel diseases,[3,5,6] has already led to the comprehension that blocking TNFα access to its receptor during inflammation can be used for the specific therapy of the two diseases.[4,7,8] However, the chronic use of anti-TNFα monoclonal antibodies (e.g., infliximab) is associated with severe adverse effects such as immunogenicity,[9,10] lymphoma risk and neuropathy.[4,11,12] Alternatively, the release of soluble TNFα from its membrane-bound precursor into the vicinity of the inflamed regions by TNFα-converting enzyme (TACE, ADAM17) could be blocked by small molecules.[13,14] Indeed, a variety of TACE inhibitors has already been tested clinically as anti-inflammatory agents.[4,15–17]

TACE is a member of the ADAM family. Together with matrix metalloproteinases (MMPs) they are included in a wider family of metalloproteinases, the metzincins. The structure similarity of these two groups, has led to the notion that some MMPs inhibitors could also serve as TACE inhibitors.[18–20] As yet, TACE inhibitors failed in the area of cancer therapy, apparently due to lack of selectivity toward transition metals, thus exerting hepatotoxicity.[4,18,21] Previously we reported that certain carbamoylphosphonates (CPOs) can act as selective MMP inhibitors, with mild zinc chelating properties.[21–23] In our studies we observed that the presence of a basic amino group near the CPO function increases zinc binding potency.[21] Thus, we suggest that some aminoCPOs could serve as TACE inhibitors. In the present study, aimed at biological evaluation of these compounds, we also included a recently reported MMP-2 specific CPO, namely, *cis*-2-aminocyclohexylcarbamoylphosphonic acid (*cis*-ACCP, Scheme 1), which was found to act as a non-toxic anti-metastatic drug after oral administration, in both murine melanoma and an orthotopic human prostate tumor model.[23,24]

More specifically, the goals of the present study were to: (a) synthesize two new aminoCPOs: *N*-[-2-(1-pyrrolidino)ethyl]carbamoylphosphonic acid (PYR-CPO) and *N*-[-2-(1-piperidino)ethyl] carbamoylphosphonic acid (PIP-CPO) (Scheme 1), (b) compare the activity of PYR-CPO and PIP-CPO with that of *cis*-ACCP in reducing TNFα levels by stimulated macrophages and stimulated lymphocyte cells, an abundant cellular sources of

Abbreviations: BUD, budesonide; *cis*-ACCP, *cis*-2-aminocyclohexylcarbamoylphosphonic acid; CPOs, carbamoylphosphonates; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; MMP, metalloproteinases; MTT, 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PIP-CPO, *N*-[-2-(1-piperidino)ethyl] carbamoylphosphonic acid; PYR-CPO, *N*-[-2-(1-pyrrolidino)ethyl] carbamoylphosphonic acid; TACE, TNFα-converting enzyme; TNFα, tumor necrosis factor alpha.

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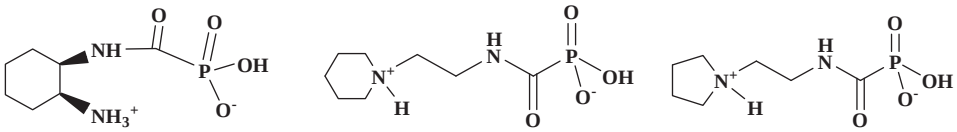

Scheme 1. The aminoCPOs used: *N*-[-2-(1-piperidino)ethyl]carbamoylphosphonic acid (PIP-CPO), *N*-[-2-(1-pyrrolidino)ethyl]carbamoylphosphonic acid (PYR-CPO) and *cis*-2-aminocyclohexylcarbamoylphosphonic acid (*cis*-ACCP).

TNFα,[25,26] (c) assess the cytotoxicity of the three molecules and (d) explore their mode of action.

In this study, we examined the anti-TNFα activity of two novel aminoCPOs, together with the previously studied *cis*-ACCP,[23] The two new CPOs, PIP-CPO and PYR-CPO, were synthesized by phosphonoformylation of 2-(1-piperidino)ethylamine or 2-(1-pyrrolidino)ethylamine, respectively, using triethyl phosphonothiolformate, as previously described.[22,23,27,28] Detailed description of the organic synthetic procedures can be found in the Supporting information section.

The effect of the aminoCPOs on TNFα secretion was assessed in peritoneal macrophages isolated from thioglycolate-stimulated mice. One hundred μM of each of the three compounds was added concomitantly with LPS (to promote TNFα secretion) and their effect was compared to the effect obtained after similar treatment with 10 μM of the steroid drug budesonide (BUD). The anti-inflammatory activity of steroids is associated with reduced lymphocyte proliferation.[29] After incubation with the activated macrophages and centrifugation, supernatants were collected and TNFα levels were measured by ELISA. Figure 1 shows that TNFα levels were reduced by 60–70% compared to the activated cells. To negate impairment on cell viability as a possible cause for lowering the TNFα levels, MTT assays were performed in parallel, and the secreted TNFα levels were normalized to the MTT readout. Figure 1B shows that the cells retained their viability in the presence of the tested aminoCPOs, similarly to the lack of cytotoxicity of BUD.

To verify that the effect of the compounds was not cell type-restricted, their ability to attenuate TNFα secretion was also tested in freshly isolated splenocytes, treated with LPS. A similar reduction in TNFα levels was observed for all three compounds (Fig. 2A). Significant reduction in TNFα secretion was also observed at a lower concentration (10 μM) with a lesser effect of PYR-CPO compared with PIP-CPO. In general, the activated macrophages were less sensitive to the low concentration of the aminoCPOs in terms lowering TNFα secretion (data not shown). It is concluded that the effect of aminoCPOs on TNFα secretion is in general more pronounced in activated lymphocytes.

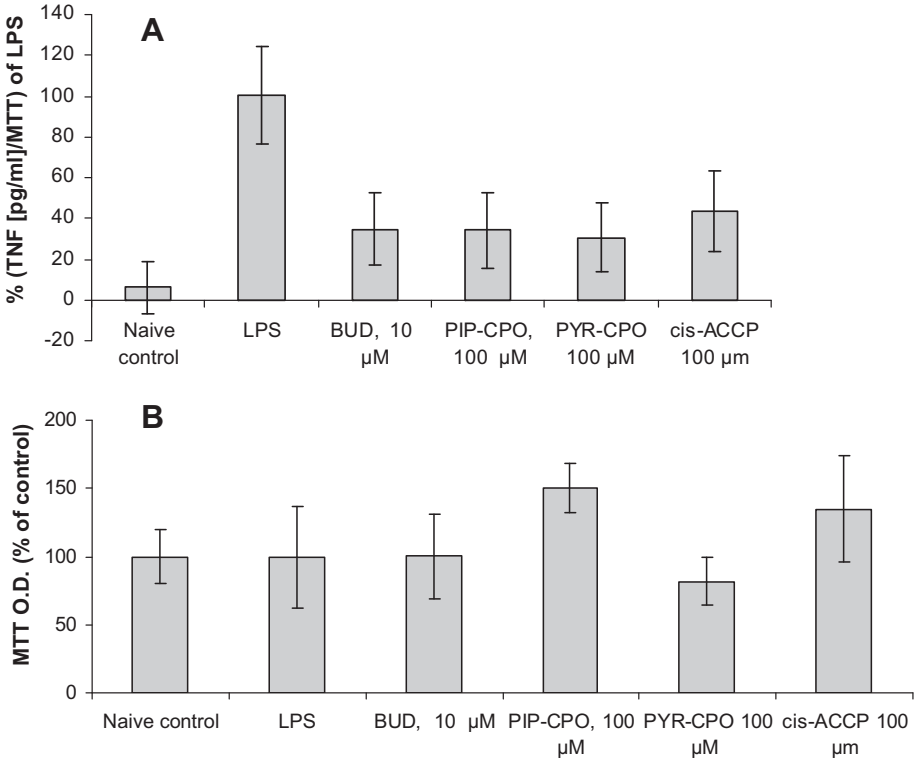

Figure 1. AminoCPOs reduce TNFα secretion in LPS-activated mouse peritoneal macrophages. (A) Supernatant levels of TNFα after incubation of LPS-stimulated macrophages from the mouse peritoneum with 100 μM of PIP-CPO, PYR-CPO and *cis*-ACCP or 10 μM of BUD (positive control). Results are normalized to cell viability, as determined by the MTT assay. (B) Cytotoxicity of 100 μM of PIP-CPO, PYR-CPO and *cis*-ACCP as determined by the MTT assay. Shown are the average results of three different experiments ± S.E.M.

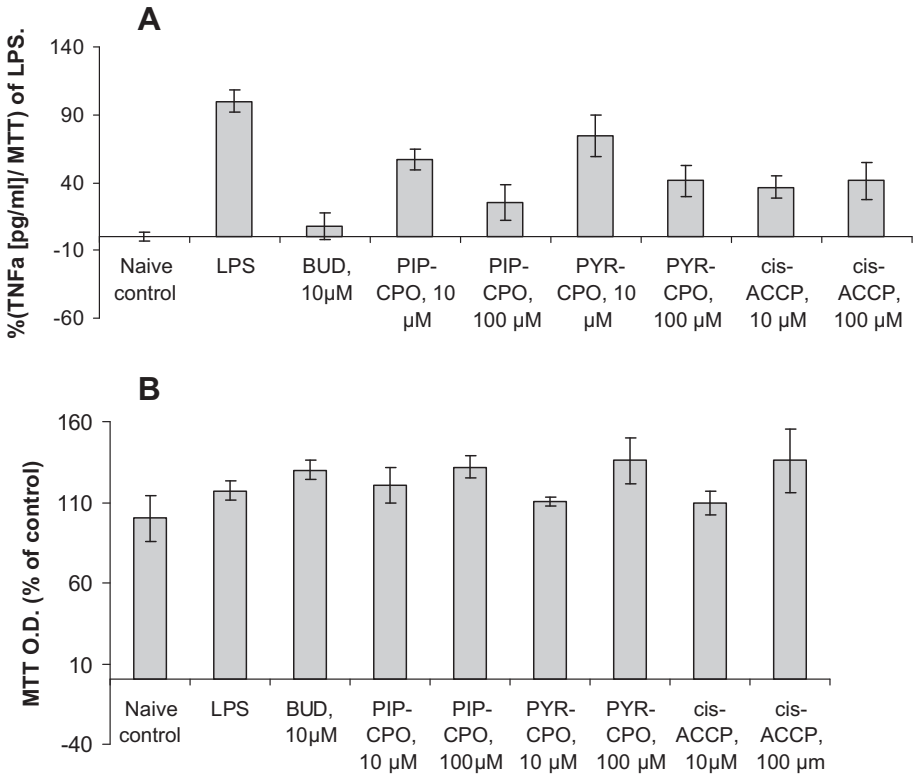

Figure 2. AminoCPOs reduce TNFα secretion in LPS-activated splenocytes. (A) Supernatant levels (normalized to cells viability) of TNFα after incubation of LPS-stimulated mouse splenocytes with 100 or 10 μM of PIP-CPO, PYR-CPO and *cis*-ACCP, or 10 μM of BUD (positive control). (B) Cytotoxicity of LPS-stimulated mouse splenocytes treated with 100 and 10 μM of PIP-CPO, PYR-CPO and *cis*-ACCP, as determined by the MTT assay. Shown are the average results of three different experiments ± S.E.M.

In addition to the MTT test, the fraction of dead cells in the cell cultures used was evaluated by flow cytometry, which was set to measure the distribution of forward and side scattering of the cells, as a reliable means of cell membrane integrity. The activated cells were analyzed after 24 or 48 h of incubation with the three compounds, in a comparative study which also included freshly isolated cells, analyzed prior to the incubation (time = 0). Gated cells viability was ascertained by propidium iodide staining. In these cytotoxicity tests the lipophilic aminophosphonate was employed as a positive control. No significant alterations in the forward and side scattering distribution for the splenocytes treated with the aminoCPOs could be observed. In contrast, BUD was found to exert higher toxicity than the tested molecules (lower number of live cells, Fig. 3A).

Cells proliferation analysis revealed that while BUD decreased the number of live cells, no significant reduction was observed after the incubation (24 h) with the aminoCPOs, compared with activated cells. Longer incubation (48 h) caused a minute reduction, with a better readout for the aminoCPOs compared with BUD, suggesting that the aminoCPOs did not affect proliferation (Fig. 3B). Similar viability measures were obtained when RAW 264.7 cells were exposure to the three compounds (Fig. 3C). Overall, the aminoCPO compounds reduced TNFα secretion in a mechanism that did not compromise cell survival and proliferation.

Were the reduced TNFα levels caused by TACE inhibition of the cytokine transmembrane anchorage of the aminoCPOs? To address this question the enzymatic activity of a purified recombinant TACE was measured in the presence of increasing concentrations of PIP-CPO and PYR-CPO. Figure 4 shows no significant reduction in the catalytic activity of the recombinant TACE. Still, the aminoCPOs could interfere with the LPS-mediated activation process of the cells by affecting TNFα synthesis. To check this possibility total RNA was extracted from the activated cells and converted to cDNA. A comparative RT-PCR analysis for TNFα in the presence and absence of PIP-CPO, was performed, using GAPDH as the house keeping gene. The PCR conditions were carefully calibrated to allow quantification of the PCR product at the logarithmic phase of the PCR profile, where sensitivity was maximal. A consistent reduction (about 30%) in TNFα mRNA levels was observed (Fig. 5), suggesting that the main effect of the aminoCPOs was, indeed, mediated by reducing mRNA levels in the activated cells.

Although not determined, it is reasonable to expect that the pK_a, and water solubility of PYR-CPO and PIP-CPO resemble those of *cis*-ACCP, with similar pharmacokinetic and bioavailability outcome. After oral administration, approximately 1% of *cis*-ACCP is absorbed by the paracellular route.[23] As the second pK_a of the aminoCPOs, reported in this study is approximately 5, they possess a single net negative charge at physiological pH.[20] This is probably why *cis*-ACCP will not penetrate into the cells and concentrates in the extracellular fluid where MMPs, its target, is located, providing a rational explanation to the lack of toxicity of the CPOs.[23] Similar trait is reminiscent of the behavior of the anti-osteoporosis drugs bisphosphonates. They are poorly absorbed, but selectively self-target to the bone, where they exert their biological activity.[30] It is reasonable to assume that under local inflammatory conditions, typical to colitis or Crohn's disease, the restricted permeability properties of the aminoCPOs would exert local activity. The high water solubility which obstructs passage through biological membranes may be advantageous in our

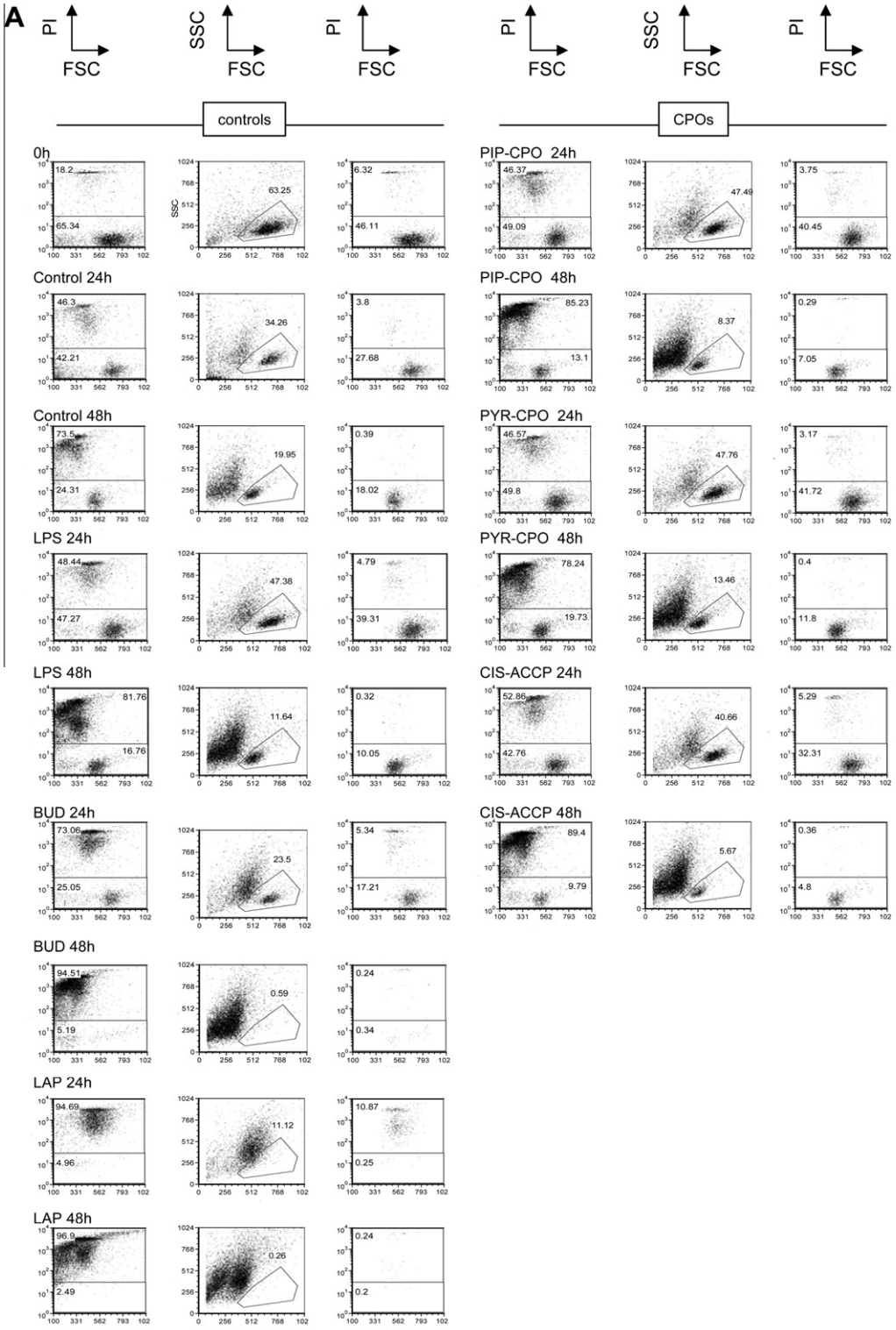

Figure 3. AminoCPOs are neither cytotoxic nor do they impair cell proliferation. Viability of stimulated mouse splenocytes or macrophages after incubation with PIP-CPO, PYR-CPO or *cis*-ACCP, was measured by flow cytometry. Lipophilic aminophosphonates (LAP) served as a positive control for cytotoxicity. (A) Dot plots showing the forward scatter and propidium iodine stain of the entire cell population (left column), forward and side scatter distribution of splenocytes (center column) and propidium iodine staining of the gated splenocytes population (right column) after 0, 24 and 48 h of incubation with the three aminoCPOs. (B) Quantification of the viable splenocytes population. (C) Quantification of the viable RAW 264.7 after 24 h incubation with the three aminoCPOs.

case as demonstrated by the low cytotoxicity observed in our in vitro studies (Figs. 1b and 2b). In conclusion, our data indicate PIP-CPO as a non-toxic lead compound for reduction of TNFα secretion from macrophages and lymphocytes.

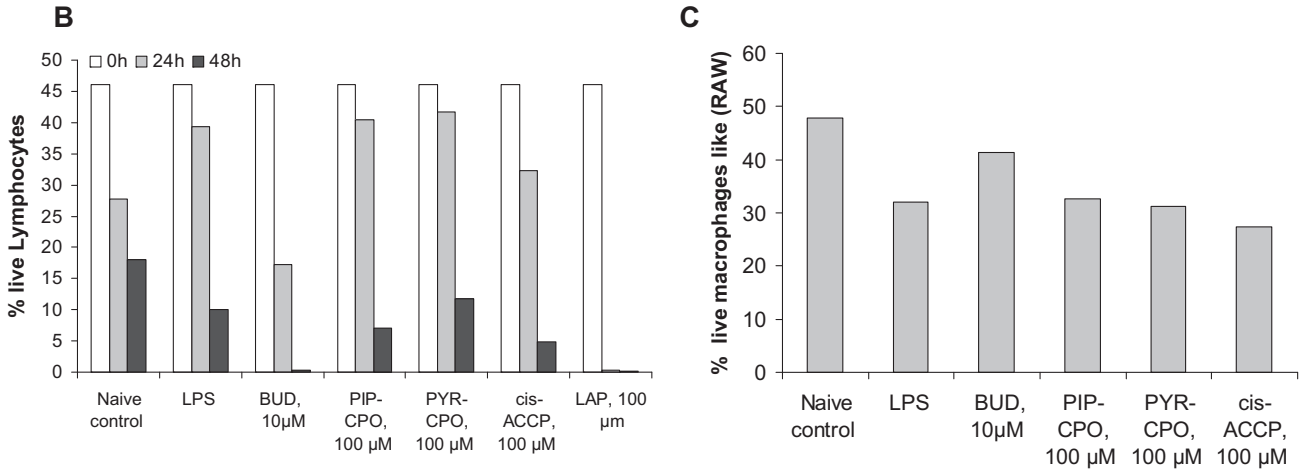

Fig. 3 (*continued*)

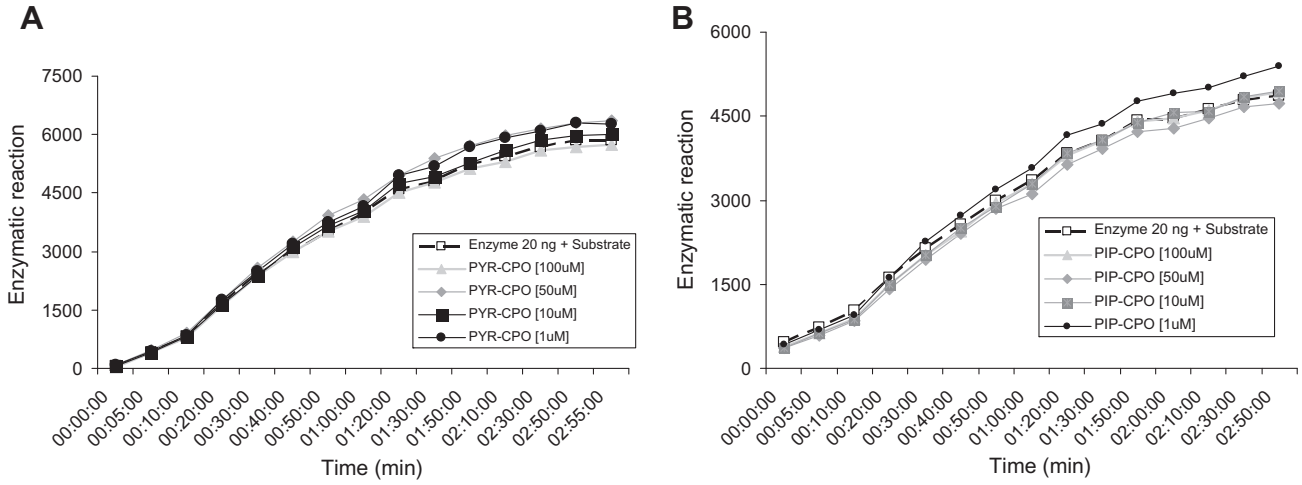

Figure 4. PYR-CPO and PIP-CPO do not affect TACE activity. TACE inhibitory effect of increasing concentrations (1, 10, 50 and 100 μM) of PYR-CPO (A) and PIP-CPO (B), as measured on recombinant TACE (20 ng), using the fluorescent peptide substrate, Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2. Neither PYR-CPO nor PIP-CPO exerted TACE inhibition properties after 3 h incubation with the recombinant TACE.

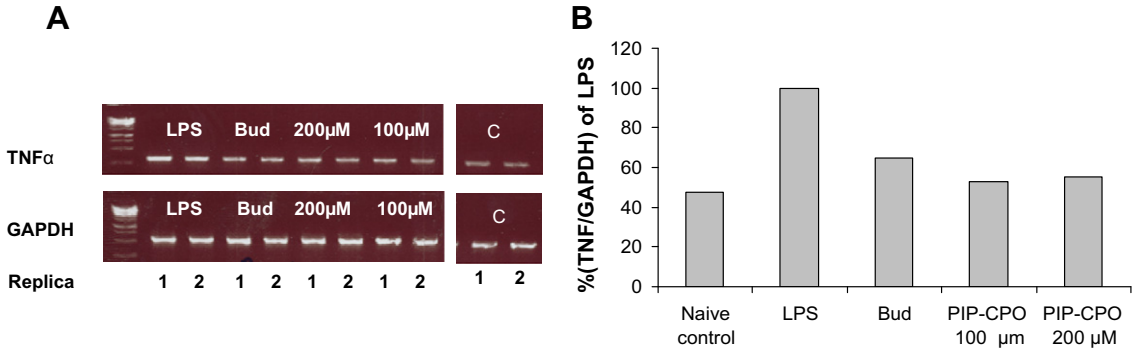

Figure 5. PIP-CPO treatment reduces TNFα mRNA levels. (A) RT-PCR analysis for TNFα mRNA. Shown are mRNA levels in mouse splenocytes after stimulation with LPS in the presence of 100 or 200 μM of PIP-CPO and 10 μM of BUD (positive control). Shown are TNFα and the house keeping gene GAPDH (served as a normalizer). (B) Densitometry quantification of the PCR products normalized to GAPDH.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.048.

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