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A combination of proteasome inhibitors and antibiotics prevents lethality in a septic shock model

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Abstract

Our recent studies with lactacystin, a prototype proteasome inhibitor, have suggested that the proteasome is a key regulator of LPS-induced signaling pathways contributing to the inflammatory process. Moreover, lactacystin protects animals from LPS-induced shock. Therefore, we sought to identify other less toxic compounds that would block the chymotrypsin-like activity of the proteasome or LPS-induced nitric oxide (NO). After screening over 100 natural compounds (based on chemistry and inhibition of LPS-induced biological activities), we now report for the first time that quercetin, like lactacystin (the prototype proteasome inhibitor), and mevinolin are also inhibitors of the chymotrypsin-like activity of the cellular proteasome within living cells. In addition, this study also suggests that mevinolin and quercetin both have relatively potent anti-inflammatory effects on LPS-treated macrophages *in vitro*. Interestingly, both of these compounds behave like lactacystin in that they block LPS-induced NO to a greater extent than TNF- α . The results of our experiments clearly suggest that mevinolin, in combination with the antibiotic imipenem, can provide protection against polymicrobial septic lethality induced by cecal-ligation and puncture in mice. Collectively, these studies strongly support the conclusion that therapeutic targeting of cellular proteasomes, in conjunction with standard antimicrobial therapy, may be of considerable survival benefit in the treatment of septic shock.

Keywords

Inflammation; endotoxic shock; macrophages; statins; Primaxin; cytokines; nitric oxide

INTRODUCTION

An extensive array of lipid A molecules, the biological active component of bacterial lipopolysaccharide (LPS), as well as structurally related analogs, have been isolated and/or synthesized in an effort to identify molecules that would be non-toxic yet retain beneficial biological properties. Monophosphoryl lipid A (MPL) was among the first of several compounds to be discovered that are relatively non-toxic and whose structure is closely related to lipid A. Our laboratory purified and chemically characterized MPL using high-performance

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liquid chromatography and mass spectrometry, and the results from these provided the scientific foundation leading to the initial determination of the complete structure of lipid A.¹⁻⁴ Later, the penta-acyl diphosphoryl lipid A derived from LPS of *Rhodobacter sphaeroides* (RsDPLA) was also purified and characterized in our laboratory. Monophosphoryl lipid A has been shown to function as an effective adjuvant for cancer vaccines and is likely to continue to be used in future vaccine formulations,⁵ while RsDPLA has served as a critical investigational tool, primarily because of its ability to act as an effective non-toxic antagonist of LPS and agonist lipid A moieties in both human and murine cells.⁶⁻¹⁰ A relatively large number of synthetic lipid A analogs based on the structure of RsDPLA were later developed, and some of these are currently being assessed in clinical trials.¹¹⁻¹³ A synthetic analog of RsDPLA is currently in Phase III trials, although preliminary efficacy trials failed to show broad efficacy.

Recently, we have demonstrated that LPS selectively binds to the subunits of the cellular proteasomes. Proteasomes exist as multisubunit complexes within the cell cytoplasm, consisting of a number of distinct, well-characterized, proteins; their primary function within the cell is to degrade, enzymatically, incorrectly folded proteins or those specifically tagged for degradation.¹⁴ Based on those novel findings, we initiated a series of studies, the first of which were *in vitro* models, designed to assess the potential physiological relevance of these LPS:proteasome interactions. To do this, we pretreated the murine-like macrophage cell line, RAW 264.7, with the well-characterized proteasome inhibitor, lactacystin. When these cells were subsequently stimulated with LPS, we observed a dose-dependent inhibition of LPS-induced gene expression and cytokine secretion.^{15,16} The results of these studies strongly suggested an important potential regulatory role for the proteasome in LPS-dependent signaling.^{15,16} In addition to its effect on LPS signaling pathways, lactacystin also blocked the expression of multiple key genes involved in CpG- and peptidoglycan-induced activation pathways.^{17,18} Since all of these agonists function by activating the Toll-like receptors (TLRs),¹⁹ the net effect of proteasome activation, therefore, would appear to be a way of positively regulating TLR-mediated inflammatory responses. By analogy, proteasome inhibition would be predicted to suppress the inflammatory response; consequently, it should be possible to curtail the extent of the inflammatory response leading to Gram-negative septic shock, by inhibiting cellular proteasome activity specifically.

Although lactacystin is a well-known and widely used proteasome inhibitor, that inhibits LPS-dependent inflammatory responses *in vitro*, it is relatively toxic at low concentrations. Consequently, we initiated an investigation for alternative proteasome inhibitors/anti-inflammatory compounds based on their structural relatedness to lactacystin. After screening a variety of different compounds, we have chosen two structurally-related β -lactones, mevinolin (closed ring form, lovastatin) and quercetin, for further investigation. Interestingly, mevinolin, but not quercetin, has been previously shown to be an inhibitor of the cellular proteasomal activity.²⁰ Therefore, we initiated a series of studies to evaluate the anti-inflammatory effects of these compounds *in vitro*. The results of these studies suggest that both mevinolin and quercetin have relatively potent anti-inflammatory effects on LPS-treated macrophages *in vitro*.

To assess the potential clinical significance of these proteasome inhibitors *in vivo*, we also carried out studies in a cecal ligation and puncture (CLP) model of polymicrobial sepsis and septic shock. The results of our experiments clearly suggest that mevinolin, in combination with the antibiotic imipenem, can provide protection against septic shock in a mouse CLP model. Collectively, these studies would strongly support the conclusion that therapeutic targeting of cellular proteasomes, in conjunction with standard antimicrobial therapy, may be of considerable survival benefit in the treatment of life-threatening, bacterial infection. Future

studies, however, will be required before the full consequences of proteasome inhibitors will be completely understood.

MATERIALS AND METHODS

Reagents

Highly purified, deep rough chemotype LPS (Re LPS) from *Escherichia coli* D31m4 was prepared as described by Qureshi *et al.*²¹ For tissue culture studies, Dulbecco's Modified Eagle Medium (DMEM), heat-inactivated low-endotoxin fetal bovine serum (FBS), and gentamicin were all purchased from Cambrex (Walkersville, MD, USA). Thioglycollate and mevinolin were purchased from Sigma-Aldrich (St Louis, MO, USA) and RNeasy mini kit from QIAGEN Sciences (Germantown, MD, USA). Lactacystin was purchased from Boston Biochem (Cambridge, MA, USA) and used as described previously.¹⁵ 'Pro-Glo' assays kits for the chymotrypsin-like activity of the proteasome were purchased from Promega (Madison, WI, USA). Quercetin was purchased from Sigma-Aldrich and analyzed by HPLC for purity.

Cell culture and maintenance

The mouse macrophage cell line, RAW 264.7 was maintained in DMEM supplemented with 10% heat inactivated FBS and 10 mg/ml gentamicin at 37°C in a humidified atmosphere with 5% CO₂, as described previously.¹⁵ Cells were cultured in 6-well plates as described in the captions to the figures. Primary, thioglycollate-elicited peritoneal macrophages were prepared from C57BL/6 mice as described previously.¹⁸

Detection of cell viability

Viability of thioglycollate-elicited peritoneal macrophages or RAW264.7 cells treated with LPS and/or mevinolin and lactacystin was determined by a quantitative colorimetric assay with 3-(4,5)-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) as described previously.²²

Measurement of the chymotrypsin-like activity of the proteasome

White plates, 96-well (Fisher, 0877126) and 5×10^3 cells/100 μ l in each well were used. After the addition of quercetin or mevinolin, the cells were incubated in the 37°C incubator with 5% CO₂ for 1 h. After the incubation period, the cells in the 96-well plates were incubated for 20 min at 20–22°C in air prior to the addition of the reagents. The Pro-Glo reagent for the chymotrypsin-like activity was brought to 20–22°C before addition to the wells. Pro-Glo reagent (100 μ l) was added to each well for a total volume of 200 μ l in each well. The plates were covered with a plate sealer and incubated at 20–22°C for 10 min. The relative luminescence units (RLU) of assays were read in the plate luminometer. Chymotrypsin-like activity assays were performed on whole cells, at ambient temperature, by measuring luminescence in a Promega luminometer, according to the manufacturer's directions. The total chymotrypsin-like activities of the cells were measured 1 h after treatment of macrophages with various concentrations of proteasome inhibitors.

Measurement of TNF- α and NO

The levels of TNF- α in cell culture supernatants were determined by Quantikine M ELISA kit (R&D System; Minneapolis, MN, USA) according to manufacturer's instructions. The lower limit of detection for TNF- α in this method is approximately 5.0 pg/ml. Nitric oxide in the supernatants was assayed using the Griess reagent (Sigma) as previously described.

Total cellular RNA isolation and real-time PCR

Real-time PCR was performed with total RNA isolated from the macrophages treated with mevinolin and/or LPS. Total cellular RNA was isolated with RNeasy mini kit according to the

manufacturer's instructions. To quantify cytokine message, reverse transcription and PCR were conducted using a 1-step RT-PCR Kit (Qiagen) according to the manufacturer's instruction. The resulting cDNA was detected and quantified by real-time PCR using SybrGreen dye.

CLP model of septic shock

Female C57BL/6 mice (Charles River, 8-weeks-old, 19 or 20 per group) were anesthetized with ketamine (80 mg/kg) and xylazine (8.5 mg/kg), both injected i.p., and the abdomen opened through a sterile mid-line incision. The cecum was exposed and ligated at the base with care taken to avoid bowel obstruction, followed by a single puncture (all the way through) using a 23-G needle to obtain more quantitative data through better control of percentage mortality and kinetics of death. Cecal contents were gently expressed through the puncture wounds, and the incision closed using 6-0 synthetic sutures. The animals were resuscitated with a subcutaneous injection of saline (1.0 ml) postoperatively. Mice were maintained on a heating blanket during, and for 3 h after, surgery until recovery from the anesthesia. Identical operative and postoperative procedures were followed with the sham-CLP animals with the exception of the actual CLP procedure. Mice were administered inhibitors at 15 h and 1 h prior to CLP surgery i.p., and then administered Primaxin (0.2 mg/20 g mouse; a combination of imipenem, an antibiotic, and ciliastin, which prevents the breakdown of imipenem in the kidney) 8 h after CLP, and every 12 h thereafter, for up to 3 days.

RESULTS

Screening of small molecules for use as potential proteasome inhibitors

Mevinolin (lovastatin; Fig. 1) is most widely known as a cholesterol-lowering compound. The open ring form of mevinolin has been well-established to inhibit competitively β -hydroxy- β -methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol. The closed ring form of mevinolin, however, can function as a proteasome inhibitor.²⁰ Both lactacystin (the prototype proteasome inhibitor) and mevinolin are β -lactones. Therefore, we queried if other naturally occurring compounds with complex lactone structures would also manifest inhibitory activity at the proteasomal level. We screened 100 known drugs or natural compounds that would be predicted (based on their chemistry/structure) to function as inhibitors of proteasomal activity. The effect of these compounds on proteasomes was evaluated by determining their capacity to inhibit both the chymotrypsin-like proteolytic activity of the purified 20S proteasome from rabbit muscle, and LPS-induced NO in RAW 264.7 cells, *in vitro*. Based on the results of these assays, we identified quercetin (a quinolone and a flavonoid) to be a potentially ($K_i < 10 \mu\text{M}$) effective inhibitor.

Proteasome inhibitors differentially block LPS-induced gene expression of pro-inflammatory genes

The effect of mevinolin and quercetin on LPS-induced cytokine gene expression was assessed *in vitro* by real-time PCR using thioglycollate-induced peritoneal macrophages from C57BL/6 mice. Peritoneal macrophages were treated with medium (NA), LPS (1 ng/ml) alone; vehicle, VH (0.2% DMSO) + LPS, or proteasome inhibitors (either mevinolin or quercetin) + LPS for 4 h. The cells were then harvested and the RNA extracted. The RNA was reverse transcribed and the resulting cDNA was amplified and analyzed by real-time PCR using SybrGreen dye. The panel of genes selected for evaluation in this study included TNF- α , IL-1 β , and iNOS. Lipopolysaccharide-induced activation of the IL-1 β , iNOS and TNF- α gene expression were all inhibited by mevinolin and quercetin, though the TNF- α gene was inhibited to a much lesser extent than IL-1 β and iNOS (Fig. 2). Mevinolin at a 10 μM concentration blocked LPS-induced TNF- α and IL-1 β gene expression by only 20%, whereas, it blocked iNOS by 65%. However, mevinolin (20 μM) blocked TNF- α gene expression by 20% and blocked both IL-1 and iNOS

by 65%. Quercetin (8 μ M), on the other hand, inhibited TNF- α mRNA by 25%, and IL-1 and iNOS mRNA by 80% and 90%, respectively. These results are similar to those achieved previously with lactacystin, which also demonstrates a greater capacity to inhibit iNOS than TNF- α gene expression.¹⁷

Lactacystin, mevinolin and quercetin differentially affect macrophage cell death

Although lactacystin is well-recognized as an effective anti-inflammatory compound, it is relatively cytotoxic to cells because it inhibits proteasomes irreversibly. To assess the differential effects of lactacystin and mevinolin versus quercetin on cellular toxicity, cells of the murine macrophage cell line, RAW 264.7, as well as the peritoneal macrophages of the C57BL/6 mice, were exposed to various concentrations of compounds and cell death monitored at different time points using the MTT assay (Tables 1 and 2). In the RAW264.7 cells, we found that none of these compounds induced significant cell death with up to 4–6 h of exposure. After 24 h, cell death was evident with lactacystin and mevinolin, whereas, with quercetin, all of cells remained viable 24 h after exposure, even up to concentrations of 50 μ M. In the C57BL/6 macrophages, at 8 h of exposure to the compounds, all cells remained viable except for lactacystin at concentrations greater than 5 μ M. After 24 h, cell death is evident with lactacystin but only at 80 μ M with mevinolin.

Proteasome inhibitors preferentially block LPS-induced NO and, to a lesser extent, TNF1- α

We compared the ability of lactacystin, quercetin, and mevinolin to inhibit production of TNF- α and NO in LPS-treated, RAW 264.7 cells (Fig. 3A,B), as well as in peritoneal macrophages from C57BL/6 mice (Fig. 3C,D). In previous experiments with lactacystin, we had documented that it is a potent inhibitor of NO production, and that it also inhibits TNF- α production by LPS-stimulated macrophages, though to a lesser extent.

Importantly, we found that both mevinolin and quercetin were potent inhibitors of NO production by LPS-stimulated murine macrophages, with levels of inhibition similar to that achieved with lactacystin (Fig. 3A). Although the concentrations of these compounds selected for testing do not have appreciable impact on cell viability within the time frame of these experiments (Table 1), it is possible that some of the inhibition observed may be attributable to cell death, especially at higher concentrations of lactacystin and mevinolin (Table 1). It is extremely unlikely that any of the inhibitor effect of quercetin on NO production was attributable to cytotoxicity, as this compound did not cause cell death in RAW 264.7 cells even at the highest concentrations tested. Nitric oxide production in C57BL/6 peritoneal macrophages was tested as well (Fig. 3C). Once again, though it is more likely that some inhibition observed by mevinolin and lactacystin was due to cell death, the concentrations selected (Fig. 3C) did not cause cytotoxicity to the cells (Table 2). Similar to the RAW cells, quercetin did not induce cell death even at the highest concentrations tested.

Quercetin and mevinolin also demonstrated the capacity to inhibit TNF- α production by LPS-stimulated RAW 264.7 cells and C57BL/6 peritoneal macrophages in a dose-dependent manner (Fig. 3B,D). As with lactacystin, however, in inhibition of TNF- α production, the K_i values ranged from 5 μ M, > 40 μ M and > 40 μ M, for lactacystin, quercetin and mevinolin, respectively, whereas those for NO inhibition ranged from < 3.1 μ M, < 8 μ M, and < 3.1 μ M, for lactacystin, quercetin and mevinolin, respectively in the RAW cells. In the C57BL/6 macrophages, K_i values for NO inhibition ranged from < 1.25 μ M, < 40 μ M, and 40 μ M for lactacystin, quercetin and mevinolin, respectively. Inhibition of TNF- α differed slightly in the C57BL/6 cells compared to RAW 264.7 cells, where lactacystin is elevated at very low concentrations, quercetin does not show too much inhibition and the K_i is 20 μ M. These results suggest that all three of these proteasome inhibitors preferentially block LPS-induced NO production, while inhibition of TNF- α production is much less efficient.

Lactacystin and quercetin selectively block cellular chymotrypsin-like activity of the proteasome

We studied the effect of lactacystin and quercetin on the chymotrypsin-like activity of the macrophage proteasome *in vitro* using a novel Pro-Glo assay kit. Commercially available kits for assaying the other proteasomal activities, such as trypsin-like and post-glutamase are not available as yet. RAW 264.7 cells were treated without or with the inhibitors for 1 h, and the total chymotrypsin-like activity of the proteasomes measured. Pro-Glo reagent was added to each well. Briefly, LLVY-luciferin-linked peptides are avidly taken up (in specialized cell permeability buffers) and retained by cells; however, when acted upon by chymotrypsin-like activity of the proteasome, aminoluciferin is released from the cells and can be easily quantified using a luciferase-based assay (see Materials and Methods). The relative luminescence units (RLU) of assays were read in the plate luminometer. We found that, like lactacystin (Fig. 4A), quercetin also inhibited chymotrypsin-like activity of the proteasome in this assay (Fig. 4B). It is interesting to note that a major difference between these two compounds is that lactacystin is a much more potent inhibitor of chymotrypsin-like activity (K_i 0.25 μ M), as compared to quercetin (K_i 4.5 μ M). Mevinolin is known to inhibit the activity of the proteasome as well, although the exact mechanism is not understood.²⁰

Proteasome inhibitors and antibiotics block septic shock in a cecal-ligation and puncture (CLP) mouse model

Collectively, the data summarized above provide strong evidence that quercetin and mevinolin have the capacity to inhibit production of pro-inflammatory cytokines by LPS-stimulated macrophages. Therefore, we undertook a series of studies to determine the extent to which these compounds could protect against septic shock following CLP *in vivo*. Briefly, C57BL/6 mice were pretreated twice with mevinolin (4 μ g/mouse, based on a preliminary dose response experiment) at 15 h and 1 h prior to CLP, and then administered imipenem (0.2 mg/mouse) 8 h after CLP, and every 12 h thereafter for up to 3 days. Mortality was monitored over this 3-day time period and it was found that overall mortality was considerably reduced in mevinolin-treated mice relative to untreated mice. Under the conditions of this experiment, CLP resulted in 95% 3-day mortality in untreated mice; this was reduced to 70% with mevinolin alone, 58% with Primaxin alone, and 35% with a combination of mevinolin and Primaxin (Fig. 5).

Similarly, C57BL/6 mice were pretreated twice with quercetin (40 μ g/mouse, based on a preliminary dose response experiment) at 15 h and 1.0 h prior to CLP surgery, and then administered Primaxin (0.2 mg/mouse) 8 h after CLP, and every 12 h thereafter for 3 days. We monitored mortality over this 4.5-day time period and found that overall mortality was also considerably reduced in quercetin-treated mice. The CLP procedure resulted in 83% 4.5-day mortality in untreated mice, and this was reduced to 67% with quercetin alone, 50% with Primaxin alone, and 17% with a combination of quercetin and Primaxin (data not presented). These results show, for the first time, that a combination of mevinolin or quercetin and antimicrobial therapy significantly reduces mortality, compared to antimicrobial therapy alone, in this model of polymicrobial sepsis. These results strongly support the potential for proteasome inhibitors to reduce mortality of septic shock when used in combination with appropriate antimicrobial therapy.

DISCUSSION

We have previously demonstrated that the LPS-induced activation of early genes in murine macrophages can be inhibited by low doses of the proteasome inhibitor, lactacystin. For those experiments, we pretreated, LPS-responsive C3HeB/FeJ primary peritoneal macrophages with lactacystin for 1 h, followed by stimulation with LPS, and analyzed RNA using Affymetrix micro-array analysis and RT-PCR with Southern blot analysis. The results of these micro-array

analyses provided novel information indicating that approximately 90% of LPS-induced early genes in macrophages are lactacystin-sensitive and are, therefore, at least potentially proteasome-dependent.¹⁷

Our published results with lactacystin also demonstrate that this compound is able to prevent LPS-induced shock in galactosamine-sensitized mice.¹⁵ Lactacystin, is known to block the chymotrypsin-like activity of the proteasome irreversibly at low concentrations *in vitro* and, therefore, can be toxic at physiological concentrations. However, lactacystin is a relatively expensive reagent. Therefore, we initiated a search for alternative proteasome inhibitors/anti-inflammatory compounds based on structural relatedness to lactacystin. Initially, we selected two lead compounds, mevinolin and quercetin, both of which are β -lactones. Mevinolin was previously reported to be an inhibitor of proteasomal activity,²⁰ but similar information was not available for quercetin. We determined the *in vitro* effects of mevinolin and quercetin, and have shown that both of these compounds are relatively potent inhibitors of pro-inflammatory responses by LPS-stimulated macrophages *in vitro*.

Results reported here demonstrate that these compounds, especially quercetin, may, in part, be biologically active at the proteasome level, since these compounds also block the chymotrypsin-like activity of the proteasome. Additionally, both quercetin and mevinolin are potent inhibitors of NO and IL-1 β production by LPS-stimulated macrophages, with TNF- α being inhibited to a lesser extent. These results are entirely consistent with our previously published results with lactacystin,¹⁷ suggesting that proteasome inhibitors modulate production of NO more effectively than they inhibit TNF- α production.

These data are also consistent with more recent results which have also provided evidence that macrophages from mice with targeted mutations in proteasome genes exhibit differential LPS-induced inflammatory responses with respect to TNF- α and NO (Qureshi *et al.*, submitted). In those studies, we evaluated responses of macrophages derived from mice with targeted mutations in genes that encode for several of the proteasome subunits relative to macrophages from wild-type mice. We compared LPS-induced levels of TNF- α and NO in peritoneal macrophages obtained from those mice. We observed significantly reduced LPS-induced secretion of NO, while TNF- α levels in culture supernatants of LPS-stimulated macrophages from the knockouts were essentially unchanged or even increased slightly with LMP7/MECL1 double (proteasome subunits) knockouts as compared to the controls. Collectively, these findings strongly support the concept that proteasomes are critically important for LPS-induced NO production.

Mevinolin is one of several statins, a group of cholesterol-lowering compounds that act by inhibiting HMG-CoA reductase, thus reducing production of mevalonate, a precursor for cholesterol biosynthesis.²³ Statins, however, have also been reported to have other effects, including anti-inflammatory properties,^{24,25} blockage of LPS-induced production of cytokines and NO in different cell types,^{26,27} anti-thrombotic effects,^{28,29} improvement of endothelial and microvascular functions and modulation of eNOS, induction of apoptosis,^{30–33} suppression of lymphocyte functions, and inhibition of proteasome activity.²⁰ Cerivastatin, for example, has been shown to improve survival in LPS-induced shock model,³³ and simvastatin improves survival in the mouse CLP model,³⁴ although most of these mice died eventually. Thus, while inhibition of HMG-CoA reductase activity is clearly an important property of statins, other anti-inflammatory properties of these compounds might be equally important. We are currently trying to study the mechanisms by which statins exert their anti-inflammatory effects.

Quercetin, which is derived from a variety of plants and is sold as an ingestible health supplement, has been widely reported to have multiple beneficial properties on human health,

including reduction in inflammation, allergies, cardiovascular disease, ulcers and gastroenteric disorders, cancer and diabetic complications.³⁵ Despite the many putative beneficial effects of this nutritional supplement, the mechanisms by which quercetin actually reduces inflammation and exerts other protective effects is currently unclear.³⁵ Structurally, quercetin is a flavonoid, consisting of three benzene rings with five hydroxyl groups attached to the two rings (Fig. 1). Several structural analogs of quercetin have been synthesized and tested, but quercetin is the most potent of these compounds.

Biologically, quercetin has been shown to inhibit several enzymes, such as MAPK in human epithelial carcinoma cells,³⁶ the phosphorylating activity of Rous sarcoma virus transforming gene product,³⁷ ATP-utilizing enzymes in which quercetin competes for the ATP-binding site and GTP-dependent phosphorylation reaction, phospholipase A₂,³⁸ platelet and sarcoplasmic-reticulum Ca₂₊-ATPase,³⁹ lipoxygenases and cyclooxygenases in human platelets,⁴⁰ and phospholipase C in rat mast cells.⁴¹ In addition to these effects, quercetin also affects NO synthase activity of the three isoforms of the enzyme^{42,43} and TNF- α .⁴⁴ These biological effects of quercetin have been reviewed recently.³⁵ Quercetin is also a potent inhibitor of oxidative stress, NF- κ B activation, and iNOS over-expression in livers of streptozotocin-induced diabetic rats.⁴⁵ Finally, quercetin inhibits the phorbol-12,13-dibutyrate-induced cell aggregation/adhesion of human mononuclear leukocytes.⁴⁶ These latter effects of quercetin have been attributed to inhibition of cellular ATPases.

We have now assessed the anti-inflammatory properties of mevinolin and quercetin and evaluated the potential clinical significance of these compounds as therapeutic modulators on the pathogenesis of septic shock. Based upon results of *in vivo* studies in a CLP model of polymicrobial sepsis and septic shock, our studies clearly suggest that both mevinolin and quercetin, in combination with the antibiotic imipenem, can prevent septic shock in a mouse CLP model. This is, to our knowledge, the first time that such a combination has been used in a sepsis model. However, since this therapeutic treatment involved pretreatment of mice with these compounds, it is clear that these compounds should also be tested concurrently with antibiotics and after CLP to identify the mechanisms by which these drugs operate *in vivo*; such experiments are currently in progress.

There is currently a relative paucity of non-antibiotic pharmaceutical agents (with the exception of activated protein C, which appears to be effective only in some patients) with demonstrated efficacy for preventing or treating septic shock in humans. It is now clear that LPS triggers a complex pro- and anti-inflammatory cytokine cascade and that a relatively large number of mediators are likely to be involved in the manifestations of septic shock syndrome. Thus, intervention at one point of the cascade is not likely to be therapeutically beneficial. In response to Gram-negative bacterial infections, vertebrate hosts generate an inflammatory response that is designed, at least in part, to eradicate the invading bacteria. However, LPS and other microbial products are frequently sufficiently abundant as to induce systemic inflammation, potentially progressing to septic shock and death. Septic shock and death can occur, therefore, even when effective antimicrobial therapy has been utilized to eliminate or suppress the infection, as LPS, and other microbial constituents released from decomposing bacteria continue to fuel the inflammatory response through their interactions with TLR4, TLR9 or other TLRs, on inflammatory cell types and on epithelial and endothelial cells.

Consequently, a novel treatment strategy that combines antimicrobials (to decrease bacterial multiplication) and proteasome inhibitors (to generally suppress inflammation) should, therefore, function to decrease the incidence of shock associated with both Gram-negative and Gram-positive bacterial infections. Based on our present data, low-dose proteasome inhibitors, in combination with antibiotics, probably lead to effective killing of bacteria, and also cause reductions in inflammatory mediators by blocking proteasomal activity, rather than

antagonizing one cytokine at a time, as accomplished with other failed anti-cytokine approaches. High-dose corticosteroids have not been shown to be particularly effective, in all likelihood because they block almost all inflammatory responses of the host; in contrast, proteasome inhibitors appear to be far more selective. Additional studies will however, be required before the full potential therapeutic benefit of proteasome inhibitors can be completely understood and evaluated.

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References

1. Qureshi N, Takayama K, Heller D, et al. Position of ester groups in the lipid A backbone of lipopolysaccharides obtained from *Salmonella typhimurium*. *J Biol Chem* 1983;258:12947–12951. [PubMed: 6355099]
2. Qureshi N, Takayama K, Ribi E. Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*. *J Biol Chem* 1982;257:11808–11815. [PubMed: 6749846]
3. Qureshi, N.; Takayama, K. Structure and function of lipid A. In: Iglewski, BH.; Clark, VL., editors. *The Bacteria*. New York: Academic Press; 1990. p. 319-338.
4. Zähringer, U.; Lindner, B.; Rietschel, ET. Chemical structure of lipid A: recent advances in structural analysis of biologically active molecules. In: Brade, H.; Opal, SM.; Vogel, SN.; Morrison, DC., editors. *Endotoxin in Health and Disease*. New York: Marcel Dekker; 1999. p. 93-114.
5. Rockwell, CE.; Morrison, DC.; Qureshi, N. Lipid A-mediated tolerance and cancer therapy. In: Jeanin, J-F., editor. *Tolerance in cancer therapy*. Bioscience Landes; 2008. In press
6. Takayama K, Qureshi N, Beutler B, et al. Diphosphoryl lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023 blocks induction of cachectin in macrophages by lipopolysaccharide. *Infect Immun* 1989;57:1336–1338. [PubMed: 2784418]
7. Kirkland TN, Qureshi N, Takayama K. Diphosphoryl lipid A derived from lipopolysaccharide (LPS) of *Rhodopseudomonas sphaeroides* inhibits activation of 70Z/3 cells by LPS. *Infect Immun* 1991;59:131–136. [PubMed: 1898897]
8. Qureshi N, Takayama K, Kurtz R. Diphosphoryl lipid A obtained from the nontoxic lipopolysaccharide of *Rhodopseudomonas sphaeroides* is an endotoxin antagonist in mice. *Infect Immun* 1991;59:441–444. [PubMed: 1987057]
9. Henricson BE, Perera PY, Qureshi N, et al. *Rhodopseudomonas sphaeroides* lipid A derivatives block *in vitro* induction of tumor necrosis factor and endotoxin tolerance by smooth lipopolysaccharide and monophosphoryl lipid A. *Infect Immun* 1992;60:4285–4290. [PubMed: 1398939]
10. Qureshi, N.; Jarvis, BW.; Takayama, K. Nontoxic RsDPLA as a potent antagonist of toxic lipopolysaccharide. In: Brade, H.; Opal, SM.; Vogel, SN.; Morrison, DC., editors. *Endotoxin in Health and Disease*. New York: Marcel Dekker; 1999. p. 687-698.
11. Stutz P, Liehl E. Lipid A analogs aimed at preventing the detrimental effects of endotoxin. *Infect Dis Clin North Am* 1991;5:847–873. [PubMed: 1783772]
12. Rossignol, DP.; Hawkins, LD.; Christ, WJ., et al. Synthetic endotoxin antagonists. In: Brade, H.; Opal, SM.; Vogel, SN.; Morrison, DC., editors. *Endotoxin in Health and Disease*. New York: Marcel Dekker; 1999. p. 699-717.
13. Persing DH, Coler RN, Lacy MJ, et al. Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol* 2002;10:S32–S37. [PubMed: 12377566]
14. Hirsch C, Ploegh HL. Intracellular targeting of the proteasome. *Trends Cell Biol* 2000;10:268–272. [PubMed: 10856929]
15. Qureshi N, Perera P-Y, Splitter G, Morrison DC, Vogel SN. The proteasome as a LPS-binding protein in macrophages. Toxic lipopolysaccharide activates the proteasome complex. *J Immunol* 2003;171:1515–1525. [PubMed: 12874245]

16. Qureshi N, Vogel SN, Van Way C III, Papasian CJ, Qureshi AA, Morrison DC. The proteasome. A central regulator of inflammation and macrophage function. *Immunol Res* 2005;31:243–260. [PubMed: 15888915]
17. Shen J, Reis J, Morrison DC, et al. Key inflammatory signaling pathways are regulated by the proteasome. *Shock* 2006;25:472–484. [PubMed: 16680012]
18. Shen J, Gao JJ, Zhang G, et al. Proteasome inhibitor, lactacystin blocks CpG DNA- and peptidoglycan-induced inflammatory genes, cytokines and mitogen-activated protein kinases in macrophages. *Shock* 2006;25:594–599. [PubMed: 16721267]
19. Sabroe I, Read RC, Whyte MKB, Dockrell DH, Vogel SN, Dower SK. Toll-like receptors in health and disease: complex questions remain. *J Immunol* 2003;171:1630–1635. [PubMed: 12902458]
20. Rao S, Porter DC, Chien X, Herliczek T, Lowe M, Keyomarsi K. Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. *Proc Natl Acad Sci USA* 1999;96:7797–7802. [PubMed: 10393901]
21. Qureshi N, Takayama K, Mascagni P, Honovich J, Wong R, Cotter RJ. Complete structural determination of lipopolysaccharides obtained from deep rough mutant of *Escherichia coli*: purification by high performance liquid chromatography and direct analysis by plasma desorption mass spectrometry. *J Biol Chem* 1988;263:11971–11976. [PubMed: 3136169]
22. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986;89:271–277. [PubMed: 3486233]
23. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986;232:34–47. [PubMed: 3513311]
24. Kwak B, Mulhaupt F, Myit S, et al. Statins as a newly recognized type of immunomodulator. *Nat Med* 2000;6:1399–1402. [PubMed: 11100127]
25. Bustos C, Hernandez-Presa MA, Ortego M, et al. HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in rabbit model of atherosclerosis. *J Am Coll Cardiol* 1998;32:2057–2064. [PubMed: 9857893]
26. Guijarro C, Kim Y, Schooner CM, et al. Lovastatin inhibits lipopolysaccharide-induced NF- κ B activation in human mesangial cells. *Nephrol Dial Transplant* 1996;11:990–996. [PubMed: 8671958]
27. Pahan K, Sheik FG, Namboodiri AMS, Singh I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia and macrophages. *J Clin Invest* 1997;100:2671–2679. [PubMed: 9389730]
28. Prueffer D, Scalia R, Lefer AM. Simvastatin inhibits leukocyte–endothelial cell interactions and protects against inflammatory processes in normocholesterolemic rats. *Arterioscler Thromb Vasc Biol* 1999;19:2894–2900. [PubMed: 10591666]
29. Mayer J, Eller T, Brauer P, et al. Effects of long term treatment with lovastatin on the clotting system and blood platelets. *Ann Hematol* 1992;64:196–201. [PubMed: 1581407]
30. Wada H, Mori Y, Kaneko T, Wakita Y, Nakase T, Hosomi N. Elevated plasma levels of vascular endothelial cell markers in patients with hypercholesterolemia. *Am J Hematol* 1993;44:112–116. [PubMed: 8266915]
31. Jones KD, Couldwell WT, Hinton DR, et al. Lovastatin induces growth inhibition and apoptosis in human malignant glioma cells. *Biochem Biophys Res Commun* 1994;205:1681–1687. [PubMed: 7811252]
32. Tan A, Lvrey H, Dahm C, Polunovsky VA, Rubins J, Bitterman PB. Lovastatin induces fibroblast apoptosis *in vitro* and *in vivo*. A possible therapy for fibroproliferative disorders. *Am J Respir Crit Care Med* 1999;159:220–227. [PubMed: 9872842]
33. Ando H, Takamura T, Ota T, Nagai Y, Kobayashi K-I. Cerivastatin improves survival of mice with lipopolysaccharide-induced sepsis. *J Pharmacol Exp Ther* 2000;294:1043–1046. [PubMed: 10945857]
34. Merx MW, Liehn EA, Janssens U, et al. HMG-CoA reductase inhibitor simvastatin profoundly improves survival in a murine model of sepsis. *Circulation* 2004;109:2560–2565. [PubMed: 15123521]

35. Middleton E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharm Rev* 2000;52:673–751. [PubMed: 11121513]
36. Bird TA, Schule HD, Delaney PB, Sims JE, Thoma B, Dower SK. Evidence that MAP (mitogen-activated protein) kinase activation may be a necessary but not sufficient signal for a restricted subset of responses in IL-1 treated epidermoid cells. *Cytokine* 1992;4:429–440. [PubMed: 1337984]
37. Graziani Y, Erickson E, Erikson RL. The effect of quercetin on the phosphorylating activity of the Rous sarcoma virus transforming gene product *in vitro* and *in vivo*. *Eur J Biochem* 1983;135:583–589. [PubMed: 6311542]
38. Lee T-P, Matteliano ML, Middleton E. Effect of quercetin on human polymorphonuclear leukocyte lysosomal enzyme release and phospholipids metabolism. *Life Sci* 1982;31:2765–2774. [PubMed: 6818414]
39. Fischer TH, Campbell KP, White GC. An investigation of functional similarities between the sarcoplasmic reticulum and platelet calcium-dependent adenosine triphosphatase with the inhibitors quercetin and calmidazolium. *Biochemistry* 1987;26:8024–8030. [PubMed: 2962642]
40. Rao GH, Radha E, White JG. Irreversible platelet aggregation does not depend on lipoxygenase metabolites. *Biochem Biophys Res Commun* 1985;131:50–57. [PubMed: 3929780]
41. Cockcroft S. Phosphatidylinositol metabolism in mast cells and neutrophils. *Cell Calcium* 1982;3:337–349. [PubMed: 6186378]
42. Chiesi M, Schwaller R. Inhibition of constitutive endothelial NO-synthase activity by tannin and quercetin. *Biochem Pharmacol* 1995;14:103–108.
43. Mu MM, Chakravorty D, Sugiyama TJ, et al. The inhibitory action of quercetin on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophage cells. *Endotoxin Res* 2001;7:431–438.
44. Nair MP, Mahajan S, Reynolds JL, et al. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF- κ B system. *Clin Vac Immun* 2006;13:319–328.
45. Dias AS, Porawski M, Alonso M, Marroni N, Collado PS, Gonzalez-Gallego J. Quercetin decreases oxidative stress, NF- κ B activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. *J Nutr* 2005;135:2299–2304. [PubMed: 16177186]
46. Patarroyo M, Jondal M. Phorbol ester-induced adhesion (binding) among mononuclear leukocytes requires extracellular Mg^{++} and is sensitive to protein kinase C, lipoxygenase and ATPase inhibitors. *Immunobiology* 1985;170:305–319. [PubMed: 2935488]

Fig. 1. Structures of lactacystin, *clasto*-lactacystin β -lactone, mevinolin open ring form, mevinolin closed ring form, and quercetin.

Fig. 2.

Mevinolin and quercetin affect LPS-induced cytokine gene expression measured by real-time PCR, in C57BL/6 peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were treated with medium, LPS (1 ng/ml) alone, vehicle (0.2% DMSO) + LPS, or proteasome inhibitors + LPS for 4 h. The cells were then harvested and the RNA extracted. The RNA was reverse transcribed and the resulting cDNA was amplified and analyzed by real-time PCR using SybrGreen dye.

Fig. 3.

Lactacystin, quercetin and mevinolin differentially block LPS-induced TNF- α and NO in RAW264.7 cells as well as in C57BL/6 peritoneal macrophages. (A) RAW264.7 cells were treated with medium containing DMSO (0.2%), LPS (1 ng), and inhibitors (1 h prior to LPS) plus LPS for 4 h. Supernatants were removed and assayed for TNF- α activity using an ELISA assay, as described in Materials and Methods. Standard error bars were so low that they did not show up on the figure. (B) RAW264.7 cells were treated with medium containing DMSO, LPS (10 ng), and inhibitors plus LPS concurrently; in the presence of IFN- γ (IFN- γ , 50 U/ml) for 24 h. Nitric oxide in supernatants was analyzed using the Griess reagent system, as described in Materials and Methods. (C) C57BL/6 peritoneal macrophages were treated with medium containing DMSO (0.2%), LPS (10 ng), and inhibitors (1 h prior to LPS) plus LPS for 8 h. Supernatants were removed and assayed for TNF- α activity using an ELISA assay, as described in Materials and Methods. (D) C57BL/6 peritoneal macrophages were treated with medium containing DMSO, LPS (50 ng), and inhibitors plus LPS concurrently in the presence of IFN- γ (IFN- γ , 50 U/ml) for 24 h. Nitric oxide in supernatants was analyzed using the Griess reagent system, as described in Materials and Methods. All experiments were performed three times.

Fig. 4.

Lactacystin and quercetin block the chymotrypsin-like activity of the proteasome. RAW264.7 macrophages were treated with (A) lactacystin (L) at 0 (DMSO C), 0.25 (L0.25), 0.5 (L0.5) and 1 μM (L1) concentrations, and (B) quercetin (Q) at 0 (DMSO C), 2 (Q2), 4 (Q4), and 8 μM (Q8) concentrations, and then these macrophages were assayed for the chymotrypsin-like proteasomal activity after 1 h. RLU, relative luminescence units.

Fig. 5.

A combination of mevinolin and Primaxin reduces mortality in mice with polymicrobial sepsis. A combination of mevinolin and Primaxin was used for treatment and 19 or 20 mice were used for each group. Eight-week old, female C56BL/6N mice were used for this experiment. Mevinolin (4 μ g, 0.2 mg/g body weight, in 0.4 ml saline containing 0.1% ethanol) was injected into mice intraperitoneally, 15 h and 1 h before surgery; and then administered Primaxin (0.2 mg/mouse) 8 h after CLP, and every 12 h thereafter, for up to 3 days. This combination worked well in CLP mice. A *t*-test was done with a confidence interval of 95% and the results were statistically significant with *P*-values < 0.05. Other conditions are described in the text.

Table 1
Quercetin is considerably less toxic than lactacystin or mevinolin in RAW264.7 cells

	Lactacystin 6	Lactacystin 24	Quercetin 6	Quercetin 24	Mevinolin 4	Mevinolin 24
DMSO	100	100	100	100	100	100
1.56 μ M	100	88			79	78
3.13 μ M	100	69			93	75
6.25 μ M	123	61	112	107	106	50
12.5 μ M	119	35	111	104	91	29
25 μ M	109	3	110	105	100	17
50 μ M	94	0	90	107	123	21

RAW264.7 cells were treated with the indicated concentration of inhibitor for 4, 6, or 24 h, and then viability of cultures were assessed by MTT assay as described in Materials and Methods. The numbers represent percentage viability compared to DMSO controls.

Quercetin and mevinolin are both less toxic compared to lactacystin in thioglycollate-elicited C57BL/6 peritoneal macrophages

Table 2

	Lactacystin 8	Lactacystin 24	Quercetin 8	Quercetin 24	Mevinolin 8	Mevinolin 24
DMSO	100	100	100	100	100	100
0.625 μ M	102	112				
1.25 μ M	96	89				
2.5 μ M	92	51				
5 μ M	70	25	89	100	89	83
10 μ M	63	17	83	108	88	87
20 μ M			92	104	85	88
40 μ M			90	92	87	80
80 μ M			87	90	85	73

Thioglycollate-elicited C57BL/6 peritoneal macrophages were treated with the indicated concentration of inhibitor for 8 h or 24 h, and then viability of cultures were assessed by MTT assay as described in Materials and Methods. The numbers represent percentage viability compared to DMSO controls.