Identification of Pharmacological Modulators of HMGB1-Induced Inflammatory Response by Cell-Based Screening

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Abstract

High mobility group box 1 protein (HMGB1), a highly conserved, ubiquitous protein, is released into the circulation during sterile inflammation (e.g. arthritis, trauma) and circulatory shock. It participates in the pathogenesis of delayed inflammatory responses and organ dysfunction. While several molecules have been identified that modulate the release of HMGB1, less attention has been paid to identify pharmacological inhibitors of the downstream inflammatory processes elicited by HMGB1 (C23-C45 disulfide C106 thiol form). In the current study, a cell-based medium-throughput screening of a 5000+ compound focused library of clinical drugs and drug-like compounds was performed in murine RAW264.7 macrophages, in order to identify modulators of HMGB1-induced tumor-necrosis factor alpha (TNFα) production. Clinically used drugs that suppressed HMGB1-induced TNFα production included glucocorticoids, beta agonists, and the anti-HIV compound indinavir. A re-screen of the NIH clinical compound library identified beta-agonists and various intracellular cAMP enhancers as compounds that potentiate the inhibitory effect of glucocorticoids on HMGB1-induced TNFα production. The molecular pathways involved in this synergistic anti-inflammatory effect are related, at least in part, to inhibition of TNFα mRNA synthesis via a synergistic suppression of ERK/IKK activation. Inhibition of TNFα production by prednisolone+salbutamol pretreatment was also confirmed in vivo in mice subjected to HMGB1 injection; this effect was more pronounced than the effect of either of the agents administered separately. The current study unveils several drug-like modulators of HMGB1-mediated inflammatory responses and offers pharmacological directions for the therapeutic suppression of inflammatory responses in HMGB1-dependent diseases.

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Introduction

High mobility group box 1 protein (HMGB1) was initially considered a nuclear protein regulating gene transcription. However, data emerging over the last decade identified its separate role as a pro-inflammatory cytokine that is released actively and passively from cells during inflammation and injury [1–3]. According to a current classification, the immune response can be regulated by endogenous danger signals (damage-associated molecular patterns; DAMPs; alarmins) as well as exogenous pathogen-associated molecular patterns (PAMPs). In this context, HMGB1 has been identified as a bona fide DAMP (i.e. a mediator released during sterile inflammatory processes), as well as a mediator released during PAMP-associated inflammatory events (e.g. sepsis and septic shock), which participates in the pathogenesis of the delayed inflammatory response, organ injury and contributes to disease mortality [1–3].

Significant work has focused on the molecular mechanisms of HMGB1 release and on the therapeutic neutralization of HMGB1, either by antibodies, or by inhibiting its binding to its receptors RAGE and TLR4 [1–7]. Several compounds have been identified that attenuate the release of HMGB1, including glucocorticoids, chloroquine, gold salts, nicotinic receptor agonists, ethyl pyruvate and inhibitors of poly(ADP-ribose) polymerase [3,8–11]. However, the inflammatory cellular responses downstream from HMGB1 are less understood, and no systematic survey has been conducted to characterize these pathways or to identify their pharmacological modulators. One determinant of the bioactivity of extracellular HMGB1 is based on the redox status of its three conserved thiol groups. The all thiol confirmation has been shown to facilitate the binding of CXCL12 to CXCR4 and thus exhibit chemokine-like properties [12]. The C23-C45 disulfide C106 thiol confirmation binds to the CD14/MD2/TLR4 receptor complex [13,14] and demonstrates cytokine-like properties. Using a cell-based medium-throughput screening approach, the goal of the current study was to identify drug-like compounds that down-regulate the cytokine-like activity of HMGB1-induced inflammatory processes in murine macrophages in vitro.

Methods

Materials and Reagents

A comprehensive screening set of 5,546 compounds was gathered comprising the NIH Clinical Collection (446 phase I–
III trial compounds) from BioFocus (South San Francisco, CA), the FDA Approved Library (640 FDA approved bioactive compounds) from Enzo Life Sciences (Farmingdale, NY), the Prestwick Chemical Library (1200 marketed drugs in Europe) from Prestwick Chemical (Washington, DC), the US Drug Collection (1040 clinical trial stage USP drugs), the International Drug Collection (240 compounds marketed in Europe or Asia but not in the US) and Killer Plates (160 toxic substances) from MicroSource Discovery Systems (Gaylordsville, CT), the LO-PAC1280 (1280 various biologically active compounds) from Sigma-Aldrich (Saint Louis, MO) and the Natural Products (640 natural compounds and derivatives) from TimTec LLC (Newark, DE). The compounds were dissolved at 10 mM in dimethylsulfoxide (DMSO) and dilutions were made either in DMSO or in phosphate-buffered saline (PBS, pH 7.4) to obtain 0.5% final DMSO concentration. HMGB1 (C23-C45 disulfide C106 thiol form) was prepared as previously described [15] and diluted in OptiMEM I medium (Invitrogen, Carlsbad, CA). Unless specified otherwise, all other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Cell Culture

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, Logan, UT) containing 4.5 g/l glucose supplemented with 10% fetal bovine serum (FBS, PAA Laboratories Inc, Westborough, MA), 100 IU/ml penicillin and 100 μg/ml streptomycin (PBS, pH 7.4) to obtain 0.5% final DMSO concentration. HMGB1 (C23-C45 disulfide C106 thiol form) was prepared as previously described [15] and diluted in OptiMEM I medium (Invitrogen, Carlsbad, CA). Unless specified otherwise, all other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).
tomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂ atmosphere. Prior to HMGB1 stimulation the culture medium was replaced with OptiMEM I reduced serum medium (Invitrogen, Carlsbad, CA).

Screening Assay

RAW 264.7 cells (100 000/well) were plated into 96-well tissue culture plates and cultured overnight. Culture medium was replaced with OptiMEM prior to adding compounds. Test compounds were supplied at 10 mM in dimethyl sulfoxide (DMSO) and were diluted in DMSO and in phosphate buffer saline (PBS) to reach 3 μM final concentration (and 0.5% DMSO) in the culture medium. The Natural Products Library was screened at 1 μg/ml final concentration. Compounds were administered in 1/20 volume 1 hour prior to HMGB1 treatment. In the combined screen the cells received additional dexamethasone (3 μM) treatment. HMGB1 was added at 5 μg/ml final concentration in 1/10 volume and the cells were incubated for 18 hours at 37°C in 5% CO₂ atmosphere. Supernatant was collected to measure TNFα secretion and LDH release.

Viability (MTT Assay) and LDH Release Measurements

The MTT assay and LDH activity measurements were performed as previously described [16]. Briefly, the cells were incubated in medium containing 0.5 mg·mL⁻¹ 1,3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Calbiochem, EMD BioSciences, San Diego, CA) for 1 hour at 37°C in 5% CO₂ atmosphere. The converted formazan dye was dissolved

Figure 2. Concentration- and time-dependence of the HMGB1-induced inflammatory response and reduction in cell viability in RAW 264.7 macrophages. RAW 264.7 cells were treated with the indicated amount of HMGB1 for 24, 48 or 72 hours. A: Cell viability was measured with the MTT assay and B: TNFα secretion was measured in the supernatant. doi:10.1371/journal.pone.0065994.g002

Figure 3. Time-dependence of the HMGB1-induced suppression of cellular bioenergetics in RAW 264.7 macrophages. RAW 264.7 cells were exposed to HMGB1 (5 μg/ml) for 24, 48 or 72 hours. Cellular bioenergetic parameters were measured with Seahorse extracellular fluid analysis. A: Time-dependent decrease in basal cellular respiration (Oxygen Consumption Rate, OCR). (**p<0.01 compared to vehicle treated cells) B: Time-dependent decrease in maximal cellular respiration. (†p<0.05 and ‡p<0.01 compared to vehicle treated cells) C: Representative tracing comparing cellular respiration (Oxygen Consumption Rate) in response to sequential administration of pharmacological modulators of cell metabolism in vehicle-treated cells or cells treated with HMGB1 for 72 hours. Basal Respiration, Calculated ATP Turnover, Proton Leak and Maximal Respiration areas are indicated and demonstrate a marked suppression of cellular bioenergetic parameters. doi:10.1371/journal.pone.0065994.g003
in isopropanol and the absorbance was measured at 570 nm. Serial dilution of the cells was used to calculate the count of viable cells. Viability values are shown as percent values relative to vehicle treated controls. LDH release was measured by mixing cell culture supernatant (30 μl) with 100 μl LDH assay reagent containing 110 mM lactic acid, 1350 mM nicotinamide adenine dinucleotide (NAD<sup>+</sup>), 290 mM N-methylphenazonium methyl sulfate (PMS), 685 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) and 200 mM Tris (pH 8.2). The changes in absorbance were read kinetically at 492 nm for 15 min (kinetic LDH assay). LDH activity values are shown as Vmax (mOD/min).

**TNFα ELISA**

Supernatant was diluted 10 times in PBS containing 1% bovine serum albumin (BSA) and the TNFα levels were determined with a commercially available ELISA kit (R&D Systems, Minneapolis, MN) on a robotic system comprising of a plate washer (EL406, Biotek, Winooski, VT), a dispenser (MicroFlo, Biotek, Winooski, VT), a pipetting station (Precision, Biotek, Winooski, VT), an incubator (Cytomat 2G, Thermo Electron Corporation, Asheville, NC) and plate reader (Synergy 2, Biotek, Winooski, VT) connected with a robotic arm (Twister II, Caliper Life Sciences Inc, Hopkinton, MA).

**RNA Isolation, Gene Expression Measurements**

Total RNA was isolated from RAW 264.7 cells exposed to HMGB1 or vehicle for 1.5 or 6 hours using a commercial RNA purification kit (SV total RNA isolation kit, Promega, Madison, WI). 2 μg RNA was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) as previously described [17]. 1 μg RNA was used according to the manufacturer’s protocol for gene expression measurements using the Toll-like receptor signaling pathway real-time PCR array (PAMM-0018ZD, SA Biosciences, Frederick, MD) on CFX96 thermocycler (Biorad, Hercules, CA) and analyzed with the tool provided by SA Biosciences. A full list of the genes investigated is deposited in Table S1. Taqman assay for TNFα was performed using a commercial assay (TNFα assay ID: Mm00443260_g1, Life Technologies, Carlsbad, CA) using GAPDH (VIC/MGB Probe, Applied Biosystems, Foster City, CA) control as normalizer.

**Western Blotting**

Cells were lysed in denaturing loading buffer (20 mM Tris, 2% SDS, 10% glycerol, 6 M urea, 100 μg/ml bromophenol blue, 200 mM β-mercaptoethanol) freshly supplemented with 2 mM sodium vanadate, 100 mM sodium fluoride, 20 mM beta-glycerophosphate and protease inhibitors (Complete Mini EDTA-free, Roche Applied Science, Indianapolis, IN). Lysates were sonicated, boiled and resolved on 4–12% NuPage Bis-Tris acrylamide gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose. Membranes were blocked in 10% non-fat dried milk and probed overnight with phospho-ERK1/2 (Cell Signaling, Boston, MA), phospho-p38 or phospho-IκB antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA). After incubation with peroxidase conjugates the blots were detected on a CCD-camera based detection system (GBox, Syngene USA, Frederick, MD) with enhanced chemiluminescent substrate. To normalize signals, membranes were stripped in 62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol at 60°C for 30 min, blocked and re-probed with antibodies against ERK1/2, p38 and IκB. The signals were
Table 1. List of hit compounds identified in the primary screen.

| Name                    | Library             | Biological activity                      | TNFα production (% of HMGB1 stimulation) | viability (%) |
|-------------------------|---------------------|-----------------------------------------|-----------------------------------------|---------------|
| piperlongumine          | Natural Products    | anti-inflammatory, antioxidant          | 0                                       | 94            |
| indapamide              | ENZO FDA            | sulphonamide diuretic                   | 0                                       | 92            |
| parthenolide            | Prestwick           | MAP kinase inhibitor anti-inflammatory  | 2                                       | 92            |
| stattic                 | LOPAC               | STAT3 activation inhibitor              | 14                                      | 125           |
| parthenolide            | LOPAC               | MAP kinase inhibitor anti-inflammatory  | 20                                      | 104           |
| U0126                   | LOPAC               | inhibitor of MEK1 and MEK2 (MAP kinase) | 20                                      | 92            |
| rubescensin A           | Microsource Killer  | antibacterial, antineoplastic           | 21                                      | 93            |
| 2',4'-dihydroxyflavone  | Natural Products    | flavone                                 | 21                                      | 83            |
| ethylnorepinephrin      | Microsource US Drug | adrenergic agonist, bronchodilator     | 22                                      | 112           |
| parthenolide            | LOPAC               | MAP kinase inhibitor anti-inflammatory  | 23                                      | 102           |
| dexamethasone           | Prestwick           | glucocorticoid steroid                 | 23                                      | 100           |
| betamethasone           | Prestwick           | glucocorticoid steroid                 | 24                                      | 90            |
| metaproterenol          | Prestwick           | beta-adrenergic agonist, bronchodilator | 26                                      | 87            |
| budesonide              | Prestwick           | glucocorticoid steroid                 | 27                                      | 96            |
| dexamethasone           | Microsource US Drug | glucocorticoid steroid                 | 29                                      | 107           |
| hydrocortisone base     | Prestwick           | glucocorticoid steroid                 | 29                                      | 115           |
| tolnaftate              | Prestwick           | antifungal                              | 29                                      | 90            |
| fluocortisone           | Prestwick           | mineralocorticoid, glucocorticoid       | 30                                      | 98            |
| dexamethasone           | Microsource US Drug | glucocorticoid steroid                 | 30                                      | 106           |
| fenoterol hydrobromide  | Prestwick           | beta-adrenergic agonist, bronchodilator | 31                                      | 100           |
| tyloxapol               | Microsource US Drug | polymeric nonionic detergent            | 31                                      | 92            |
| triamcinolone           | Prestwick           | glucocorticoid steroid                 | 31                                      | 92            |
| 6-alpha-methylprednisolone | Prestwick          | glucocorticoid steroid                 | 31                                      | 110           |
| flumethasone            | Microsource US Drug | glucocorticoid steroid                 | 31                                      | 109           |
| ST057244                | Natural Products    |                                          | 32                                      | 85            |
| isoalantolactone        | Natural Products    | sesquiterpene lactone                  | 32                                      | 101           |
| ritodrine               | Prestwick           | beta, adrenergic agonist, tocolytic     | 32                                      | 98            |
| imipenem                | Prestwick           | antibiotic                              | 32                                      | 101           |
| clenbuterol             | Prestwick           | beta-adrenergic agonist, bronchodilator | 32                                      | 98            |
| prenylamine             | Prestwick           | Ca**+ channel blocker, vasodilator     | 33                                      | 87            |
| hydrocortisone          | Natural Products    | glucocorticoid steroid                 | 33                                      | 113           |
| lidocaine               | Prestwick           | Na**+ channel blocker, local anesthetic | 33                                      | 91            |
| prednisolone            | Prestwick           | glucocorticoid steroid                 | 33                                      | 107           |
| flurandrenolide         | Microsource US Drug | glucocorticoid steroid                 | 33                                      | 116           |
| prednisolone            | Microsource US Drug | glucocorticoid steroid                 | 34                                      | 110           |
| bethamethasone          | Microsource US Drug | glucocorticoid steroid                 | 34                                      | 129           |
| fluticasone             | Prestwick           | glucocorticoid steroid                 | 34                                      | 78            |
| clenbuterol             | Microsource Intl Drug | beta2-adrenergic agonist, bronchodilator | 35                                      | 111           |
| ibudilast               | LOPAC               | phosphodiesterase IV inhibitor         | 35                                      | 101           |
| Bay 11-7085             | LOPAC               | IκB phosphorylation inhibitor, inhibitor of NF-κB | 35                                      | 94            |
| fluocinolone            | Microsource US Drug | glucocorticoid steroid                 | 35                                      | 115           |
| tulobuterol             | Microsource Intl Drug | beta-adrenergic agonist, bronchodilator | 35                                      | 111           |
| clobetasol propionate   | Prestwick           | glucocorticoid steroid                 | 36                                      | 146           |
| flurandrenolide         | Prestwick           | glucocorticoid steroid                 | 36                                      | 107           |
| prednisolone            | Natural Products    | glucocorticoid steroid                 | 36                                      | 106           |
| flunisolide             | Microsource US Drug | glucocorticoid steroid                 | 36                                      | 109           |
| dexamethasone           | Natural Products    | glucocorticoid steroid                 | 37                                      | 104           |
| flumethasone            | Prestwick           | glucocorticoid steroid                 | 37                                      | 133           |
### Table 1. Cont.

| Name                  | Library                  | Biological activity | TNFα production (% of HMGB1 stimulation) | viability (%) |
|-----------------------|--------------------------|---------------------|------------------------------------------|---------------|
| albuterol             | Microsource US Drug      | beta-adrenergic agonist, bronchodilator | 37                                        | 100           |
| hydrocortisone        | Natural Products         | glucocorticoid steroid | 37                                        | 109           |
| flunethasone          | Microsource US Drug      | glucocorticoid steroid | 38                                        | 117           |
| nordihydroguaiaretic acid | Natural Products         | from creosote bush, Larrea divaricata | 38                                        | 92            |
| 2,6-dimethoxyquinone  | Microsource Killer       | antibacterial, mutagen | 38                                        | 82            |
| dichlorisone          | Microsource Int Drug     | glucocorticoid steroid | 38                                        | 107           |
| salbutamol            | Prestwick                | beta-adrenergic agonist, bronchodilator | 38                                        | 102           |
| bethamethasone        | Microsource US Drug      | glucocorticoid steroid | 39                                        | 114           |
| hydrocortisone        | LOPAC                    | glucocorticoid steroid | 39                                        | 110           |
| triamcinolone         | ENZO FDA                 | glucocorticoid steroid | 39                                        | 109           |
| desoxymetasone        | Microsource US Drug      | glucocorticoid steroid | 39                                        | 126           |
| mometasone            | Prestwick                | glucocorticoid steroid | 39                                        | 124           |
| Bay 11-7082           | LOPAC                    | IκB phosphorylation inhibitor, inhibitor of NF-κB | 39 | 95 | |
| prednisolone          | Microsource US Drug      | glucocorticoid steroid | 39                                        | 110           |
| fluorometholone       | Prestwick                | glucocorticoid steroid | 39                                        | 104           |
| 3,7,4'-trihydroxyflavone | Natural Products       | flavone             | 39                                        | 81            |
| budesonide            | Microsource US Drug      | glucocorticoid steroid | 39                                        | 110           |
| halometasone          | NIH Clinical Collection  | glucocorticoid steroid | 39                                        | 106           |
| fluorometholone       | Microsource US Drug      | glucocorticoid steroid | 40                                        | 114           |
| triamcinolone         | Microsource US Drug      | glucocorticoid steroid | 40                                        | 115           |
| ST009819              | Natural Products         | levoglucosenone derivative | 40 | 101 | |
| prednisolone          | ENZO FDA                 | glucocorticoid steroid | 40                                        | 124           |
| hydrocortisone        | Microsource US Drug      | glucocorticoid steroid | 40                                        | 115           |
| fluticasone           | NIH Clinical Collection  | glucocorticoid steroid | 40                                        | 93            |
| rimexolone            | Prestwick                | glucocorticoid steroid | 40                                        | 96            |
| isoproterenol         | Prestwick                | adrenergic agonist, bronchodilator | 40                                         | 109           |
| methylprednisolone    | Microsource US Drug      | glucocorticoid steroid | 41                                        | 115           |
| methylprednisolone    | ENZO FDA                 | glucocorticoid steroid | 41                                        | 95            |
| metamopenronol        | Microsource US Drug      | adrenergic agonist, bronchodilator | 41                                         | 104           |
| karanjin              | Natural Products         | from Pongamia glabra, Leguminosae | 41                                         | 100           |
| salmeterol            | ENZO FDA                 | beta2-adrenergic agonist, bronchodilator | 41                                         | 111           |
| betamethasone         | ENZO FDA                 | glucocorticoid steroid | 41                                        | 101           |
| clobetasol            | Microsource US Drug      | glucocorticoid steroid | 41                                        | 107           |
| budesonide            | LOPAC                    | glucocorticoid steroid | 41                                        | 115           |
| isotretinon           | Microsource US Drug      | antiacne, antineoplastic | 41                                         | 115           |
| benzyl isothiocyanate | Microsource Killer       | antineoplastic, antibacterial, antifungal | 41                                         | 89            |
| quinacrine            | Microsource Killer       | anthelmintic, antimalarial, intercalating agent | 42                                         | 77            |
| flunisolide           | Prestwick                | glucocorticoid steroid | 42                                        | 113           |
| ellipticine           | LOPAC                    | cytochrome P450 (CYP1A1) and DNA topoisomerase II inhibitor | 42 | 95 | |
| terbutaline hemisulfate | Prestwick                | beta2-adrenergic agonist, bronchodilator | 42                                         | 96            |
| aclometazine          | Microsource US Drug      | glucocorticoid steroid | 42                                        | 112           |
| methylprednisolone    | Microsource US Drug      | glucocorticoid steroid | 42                                        | 113           |
| 2',3'-dihydroxyflavone | Natural Products         | flavone             | 42                                        | 88            |
| (+)-dehydroabietylamine | Natural Products         | ingredient of rosin amine, from Rosin Gum | 42                                         | 90            |
| triamcinolone         | Microsource US Drug      | glucocorticoid steroid | 43                                        | 113           |
| betamethasone         | Microsource US Drug      | glucocorticoid steroid | 43                                        | 112           |
| isoflupredone         | Prestwick                | glucocorticoid steroid | 43                                        | 99            |
Pharmacological Modulation of HMGB1-induced TNFα Production in vivo

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Texas Medical Branch, Galveston (Permit Number: 1110054). The procedures were performed humanely with minimal suffering. 6–7 week-old Balb/c male mice (The Jackson Laboratory) were pretreated subcutaneously with 20 mg/kg prednisolone, 10 mg/kg salbutamol, the combination of prednisolone and salbutamol (doses as above), or the glucocorticoid receptor blocker mifepristone (30 mg/kg) or the β-receptor antagonist propranolol (10 mg/kg) or vehicle for 3 hours. Mice were injected intraperitoneally (i.p.) with 0.5 mg/mouse HMGB1 and animals were sacrificed 8 hours later. Serum levels of TNFα were measured by ELISA (as above).

Statistical analysis. Data are shown as means ± SEM. One-way ANOVA was applied for statistical analysis and for the determination of significance, the Tukey’s post-hoc test was used. A p value of <0.05 was considered statistically significant. All statistical calculations were performed using Graphpad Prism 4 analysis software. Experiments were performed at least 3 times on different days.

Results

HMGB1 Induces Inflammatory Mediator Production and Cytotoxicity in RAW 264.7 Macrophages

HMGB1 (1–10 μg/ml) induced concentration-dependent tumor necrosis factor α (TNFα) secretion by RAW 264.7 cells, an effect, which was potentiated by INF-γ (Fig. 1A). HMGB1 also reduced cell viability (Fig. 1B); this cytotoxic response became

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**Table 1. Cont.**

| Name               | Library          | Biological activity                         | TNFα production (% of HMGB1 stimulation) | viability (%) |
|--------------------|------------------|---------------------------------------------|------------------------------------------|---------------|
| alclometasone      | Prestwick        | glucocorticoid steroid                      | 43                                       | 147           |
| p-aminobenzoate    | Microsource US Drug | vitamin B₆                                   | 43                                       | 118           |
| dexamethasone      | ENZO FDA         | glucocorticoid steroid                      | 43                                       | 113           |
| indinavir          | NIH Clinical Collection | HIV protease inhibitor                        | 43                                       | 96            |
| etacrynic acid     | Microsource US Drug | diuretic                                   | 43                                       | 96            |
| beclomethasone     | Microsource US Drug | glucocorticoid steroid                      | 43                                       | 112           |
| acinomide          | Microsource US Drug | glucocorticoid steroid                      | 43                                       | 109           |
| MNS                | LOPAC            | Src and 5y kinase inhibitor                 | 43                                       | 93            |
| sulfasalazine      | Prestwick        | prostaglandin 15-hydroxydehydrogenase inhibitor | 44                                       | 97            |
| betamethasone      | Microsource US Drug | glucocorticoid steroid                      | 44                                       | 119           |
| fludrocortisone    | Microsource US Drug | mineralocorticoid, glucocorticoid          | 44                                       | 115           |
| 5,3'-dihydroxyflavone | Natural Products | flavone derivative                        | 44                                       | 99            |
| methyl cholate      | Natural Products | glucocorticoid                             | 44                                       | 97            |
| desonide           | Microsource US Drug | glucocorticoid steroid                      | 44                                       | 115           |
| tosalonel           | Microsource US Drug | alpha adrenergic antagonist, vasodilator | 44                                       | 87            |
| vincristine        | ENZO FDA         | antineoplastic, microtubular polymerization inhibitor | 44                                       | 94            |
| etacrynic acid     | Prestwick        | diuretic                                   | 45                                       | 98            |
| levonordrifen       | Prestwick        | adrenergic agonist, vasoconstrictor         | 45                                       | 116           |
| isoxupredrone       | Microsource US Drug | glucocorticoid steroid                      | 45                                       | 123           |
| 4'-hydroxy-6-methoxyflavone | Natural Products | flavone                                     | 45                                       | 80            |
| fluconolone        | ENZO FDA         | glucocorticoid steroid                      | 45                                       | 106           |
| alprostadil         | Prestwick        | vasoconstrictor, prostaglandin receptor agonist | 45                                       | 82            |
| maprotiline         | Prestwick        | antidepressant, noradrenaline uptake inhibitor | 45                                       | 99            |
| dobutamine          | Prestwick        | beta-adrenergic agonist, bronchodilator     | 45                                       | 92            |
| betamethasone       | Microsource US Drug | glucocorticoid steroid                      | 45                                       | 122           |
| bropemidol         | Prestwick        | antipsychotic, dopamine antagonist          | 45                                       | 85            |

Non-toxic compounds that reduced the HMGB1-induced TNFα production by 2 standard deviation values are listed in order of potency, according to their inhibitory potency for TNFα secretion. The source library of the compounds, their known biological activity and the respective viability values are shown. Viability was measured by the MTT assay. (Abbreviations: MAP kinase: Mitogen-activated protein kinase, U0126:1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene, MEK: mitogen-activated protein kinase kinase, STAT3: Signal transducer and activator of transcription 3, ST057244:1-(2E)-3-(3,4,5-trimethoxyphenyl)prop-2-enoxy)piperidin-2-one, Bay 11-7085: (2E)-3-[(4-(1,1-dimethyl-phenyl)sulfonyl)-2-propenenitrile, Bay 11-7082:3-[(4- methylphenyl)sulfonyl]- (2E)- propenenitrile, ST009819: (2R,3R,13R,14R)-3-[4-(1,1-dimethylethyl)phenyl]sulfonyl]-17,19-dioxa-4-azapentacyclo[14.2.1.0²,²,14.0⁷,⁷,0¹²,¹².0⁷,⁷,0¹²,¹²]nonadeca-5,7(12),8,10-tetraen-15-one, MNS: 3,4-methylenedioxy-b-nitrostyrene, IkB: inhibitor of nuclear factor κB, Src: sarcoma tyrosine kinase, Syk: Spleen tyrosine kinase). doi:10.1371/journal.pone.0065994.t001
Table 2. Compounds that enhance the HMGB1-induced TNFα production of RAW264.7 cells.

| Name                  | Library         | Biological activity                                         | TNFα production (% of HMGB1 stimulation) | viability (%) |
|-----------------------|-----------------|-------------------------------------------------------------|------------------------------------------|---------------|
| beta-escin            | Prestwick       | an increaser of calcium permeability, venous insufficiency drug | 351                                      | 101           |
| thapsigargin          | LOPAC           | sarco-endoplasmic reticulum Ca2+-ATPase inhibitor           | 336                                      | 13            |
| niclosamide           | Microsource Killer | ananthelmintic, uncouples oxidative phosphorylation       | 335                                      | 67            |
| wortmannin            | LOPAC           | phosphatidylinositol 3-kinase inhibitor                     | 330                                      | 93            |
| calcimycin            | LOPAC           | Ca2+ ionophore                                              | 327                                      | 1             |
| gossypol              | LOPAC           | proapoptotic, binds calmodulin, PKC inhibitor, anti-HIV     | 321                                      | 88            |
| niclosamide           | LOPAC           | ananthelmintic, uncouples oxidative phosphorylation         | 302                                      | 39            |
| tyrphostin A9         | LOPAC           | PDGF tyrosine kinase receptor inhibitor                     | 289                                      | 51            |
| rottlerin             | LOPAC           | mitochondrial uncoupler                                     | 261                                      | 99            |
| S-azacytidine         | Prestwick       | antineoplastic, pyrimidine antimitabolite                   | 206                                      | 86            |
| vinblatrine           | Microsource US Drug | antineoplastic, spindle poison           | 204                                      | 112           |
| imiquimod             | Prestwick       | immunomodulator, activator of Toll-Like Receptor 7         | 243                                      | 84            |
| S-azacytidine         | Microsource Killer | antineoplastic, pyrimidine antimitabolite                   | 241                                      | 59            |
| niclosamide           | Prestwick       | ananthelmintic, uncouples oxidative phosphorylation         | 239                                      | 58            |
| vincristine           | Microsource US Drug | antineoplastic, microtubular agent                  | 211                                      | 87            |
| ivermectin            | ENZO FDA        | antiparasitic                                               | 206                                      | 86            |
| gossypol              | Microsource Killer | proapoptotic, binds calmodulin, PKC inhibitor, anti-HIV     | 204                                      | 112           |
| rottlerin             | Natural Products | mitochondrial uncoupler                                     | 196                                      | 77            |
| pararosaniline        | Microsource US Drug | ananthelmintic, antischistosomal                           | 195                                      | 89            |
| podophyllotoxin       | Natural Products | antineoplastic, microtubular agent                          | 188                                      | 78            |
| colchicine            | Microsource US Drug | antineoplastic, microtubular agent                  | 187                                      | 79            |
| cantharidic acid      | LOPAC           | protein phosphatase 1 and 2A inhibitor                     | 177                                      | 37            |
| podophyllotoxin       | Microsource US Drug | antineoplastic, microtubular agent                          | 171                                      | 84            |
| fluvasatin            | ENZO FDA        | statin, HMG-CoA reductase inhibitor                         | 168                                      | 127           |
| tyrphostin AG 879     | LOPAC           | tyrosine kinase inhibitor with potent effects on TrkA       | 165                                      | 65            |
| itavastatin           | NIH Clin. Collection | statin, HMG-CoA reductase inhibitor                   | 163                                      | 105           |
| simvastatin           | Prestwick       | statin, HMG-CoA reductase inhibitor                         | 162                                      | 106           |
| fluvasatin            | Prestwick       | statin, HMG-CoA reductase inhibitor                         | 162                                      | 111           |
| 8-azaguaine           | Prestwick       | antineoplastic, purine antimitabolite                      | 159                                      | 74            |
| methiazole            | Prestwick       | antiparasitic                                               | 159                                      | 77            |
| N-oleylidopamine      | LOPAC           | endogenous vanilloid, weak cannabinoid receptor ligand     | 158                                      | 24            |
| oxaliplatin           | ENZO FDA        | antineoplastic DNA crosslinker                              | 157                                      | 94            |
| hexachlorophene       | Microsource Killer | disinfectant, topical anti-infective, anti-bacterial agent | 156                                      | 99            |
| cerivastatin          | NIH Clin. Collection | statin, HMG-CoA reductase inhibitor                   | 156                                      | 100           |
| colchicine            | Microsource Killer | antineoplastic, microtubular agent                          | 155                                      | 77            |
| parbendazole          | Prestwick       | ananthelmintic, microtubular agent                          | 155                                      | 80            |
| S-azacytidine         | LOPAC           | antineoplastic, pyrimidine antimitabolite                   | 155                                      | 61            |
| mevastatin            | LOPAC           | statin, HMG-CoA reductase inhibitor                         | 153                                      | 110           |
| tridihexethyl         | Prestwick       | anticholinergic antispasmodic                               | 153                                      | 96            |
| cerivastatin          | ENZO FDA        | statin, HMG-CoA reductase inhibitor                         | 153                                      | 121           |
| nordihdroguairetic acid | LOPAC           | antioxidant from Larrea divaricata                         | 153                                      | 113           |
| norcantharidin        | LOPAC           | inhibitor of the serine/threonine protein phosphatase 2A   | 151                                      | 108           |
| tannic acid           | Microsource Killer | nonspecific enzyme/receptor blocker                      | 150                                      | 99            |

Compounds augmenting the HMGB1-induced TNFα production by 2 standard deviation values are listed in order of potency, according to their enhancing effect on TNFα secretion. The source library of the compounds, their known biological activity and the respective viability values are shown. Viability was measured by the MTT assay. (Abbreviations: PKC: protein kinase C, HIV: human immunodeficiency virus, PDGF: platelet-derived growth factor, HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A, TrkA: TRK1-transforming tyrosine kinase protein).

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more pronounced at later time points (48 h, 72 h) (Fig. 2) and was associated with a suppression of mitochondrial function (Fig. 3) and caspase activation (Fig. 4). In addition to TNFα, HMGB1 also upregulated multiple pro-inflammatory cytokine (IL1α, IL1β, IL6, TNFβ) and chemokine (CCL2, MCP-1, CXC10) genes, as well as the anti-inflammatory cytokine IL10 (Fig. 1C). The HMGB1-mediated responses were also associated with an upregulation of nuclear factor κB (NF-κB) (Fig. 1C). Moreover, HMGB1 induced a down-regulation of TLR4 and MD2 and upregulation of TLR2, TLR9 and TLR adaptor molecule 1 (TICAM1) (Fig. 1C). Thus, the form of HMGB1 used for the screen exhibited the expected cytokine like properties of C23-C45 disulfide C106 thiol HMGB1.

Identification of Inhibitors of HMGB1-induced TNFα Production by Cell-based Screening

Cell-based screening of a focused library of over 5,000 clinical drugs, natural products and pharmacologically active compounds identified ~2% of the compounds, which suppressed TNFα production, without adversely affecting cell viability (Table 1; Fig. 5). Conversely, a limited number of compounds induced a significant enhancement of HMGB1-mediated TNFα response (Table 2). A full list of the primary screen data is deposited in Table S2.

More than 50% of the hit compounds that inhibited TNFα production were glucocorticoids (Fig. 5; Table 1). Beta-adrenergic agonists represented the second-most common class. The activity of the hit compounds was next confirmed at 3 and 10 μM. Since glucocorticoids and beta agonists showed a clear class action, only a subset of these compounds was retested. Apart from glucocorticoids and beta agonists, the highest inhibitory activity was detected for the NF-κB inhibitors Bay 11-7085 and parthenolide, and the antioxidant piperlongumine. Increasing the concentration of the compounds to 10 μM did not produce more pronounced inhibitory responses, but approximately 15% of the hit compounds became slightly cytotoxic at this concentration (Table 3).

Identification of Pharmacological Potentiators of Glucocorticoids by Cell-based Screening

We hypothesized that synergistic drug combinations may be more effective than single agents in controlling HMGB1-induced inflammatory responses. To identify compounds that potentiate the effect of glucocorticoids, a follow-up screen of the NIH Clinical Collection compound library was conducted in the presence of...
related to the fact that, according to the results of the primary
vention in the presence dexamethasone (Table 4); this effect was
receptor antagonist SCH 23390 (a
glucocorticoid’s effect (Fig. 6, Table 4). In addition, the dopamine
(metabolites were designed to further characterize the pharmacological
properties and underlying mechanisms of the glucocorticoid/beta-
adrenergic synergy. Both prednisolone and salbutamol, on their
own, decreased the HMGB1-induced TNFα secretion (Table 1).

**Table 3.** List of confirmed hit compounds.

| Compound         | Biological activity     | Source Library | Primary screen | Hit confirmation |
|------------------|-------------------------|----------------|----------------|-----------------|
|                  |                         |                | TNFα response (%) of HMGB1 stimulation | viability (%) |
| Parthenolide     | NF-κB inhibitor         | Prestwick      | 2              | 92              | 21 ± 2          | 0 ± 0*        |
| Bay 11-7085      | NF-κB inhibitor         | LOPAC          | 35             | 94              | 25 ± 1          | 2 ± 1*        |
| Ethynorepinephrine | beta adrenergic agonist | US Drug        | 22             | 112             | 31 ± 0          | 26 ± 1        |
| Halometasone     | glucocorticoid          | NIH Clinical   | 39             | 106             | 36 ± 2          | 34 ± 1        |
| Salbutamol       | beta adrenergic agonist | Prestwick      | 38             | 102             | 37 ± 1          | 37 ± 6        |
| Dexamethasone    | glucocorticoid          | Prestwick      | 23             | 100             | 39 ± 3          | 30 ± 2        |
| Budesonide       | glucocorticoid          | LOPAC          | 41             | 115             | 41 ± 3          | 38 ± 1        |
| Indinavir        | HIV protease inhibitor  | NIH Clinical   | 43             | 96              | 42 ± 3          | 42 ± 3        |
| Ethacrynic acid  | diuretic                | US Drug        | 43             | 96              | 44 ± 0          | 3 ± 1*        |
| Fluticasone      | glucocorticoid          | NIH Clinical   | 40             | 93              | 44 ± 4          | 43 ± 1*       |
| Hydrocortisone   | glucocorticoid          | LOPAC          | 39             | 110             | 45 ± 1          | 45 ± 3        |
| Metaproterenol   | beta adrenergic agonist | Prestwick      | 26             | 87              | 48 ± 3          | 50 ± 4        |
| Fenoterol        | beta adrenergic agonist | Prestwick      | 31             | 100             | 51 ± 1          | 56 ± 1        |
| Ritodrine        | beta2 adrenergic agonist| Prestwick      | 32             | 98              | 51 ± 4          | 48 ± 6        |
| Terbutaline      | beta2 adrenergic agonist| Prestwick      | 42             | 96              | 51 ± 2          | 50 ± 3        |
| Isoflupredone    | glucocorticoid          | Prestwick      | 43             | 99              | 52 ± 5          | 51 ± 2        |
| Clembuterol      | beta adrenergic agonist | Prestwick      | 32             | 98              | 52 ± 4          | 44 ± 5        |
| MNS              | Src/Syk kinase inhibitor| LOPAC          | 43             | 93              | 54 ± 2          | 9 ± 1*        |
| Ethacrynic acid  | diuretic                | Prestwick      | 45             | 98              | 59 ± 5          | 39 ± 2*       |
| Levonordefrin    | adrenergic agonist      | Prestwick      | 45             | 116             | 64 ± 1          | 56 ± 8        |
| PABA potassium salt | vitamin Bx            | US Drug        | 43             | 118             | 65 ± 1          | 59 ± 1        |
| Isoprotroenol    | adrenergic agonist      | Prestwick      | 40             | 109             | 67 ± 2          | 58 ± 5        |
| Prenylamine      | calcium channel blocker | Prestwick      | 33             | 87              | 69 ± 4          | 59 ± 4        |
| Tylokapal        | surfactant              | US Drug        | 31             | 92              | 78 ± 0          | 57 ± 2        |
| Isotretinoin     | retinoid                | Prestwick      | 41             | 115             | 79 ± 2          | 60 ± 5        |
| Piperlongumine   | antioxidant in peppers  | Nat. Prod.     | 0              | 94              | 80 ± 1          | 39 ± 3*       |
| Lidocaine        | local anesthetic        | Prestwick      | 33             | 91              | not tested      |              |

Hit compounds of the primary screen were retested in replicates at 3 and 10 μM against HMGB1 and LPS and compounds are shown that decreased the HMGB1-induced TNFα production by at least 40% in the hit confirmation experiments. (The majority of the glucocorticoids were not retested during the hit confirmation studies, since all glucocorticoids showed similar activity, confirming their class action.) TNFα production and viability values are shown for the primary screen and the TNFα production is shown for the hit confirmation experiments (Mean ± SD). Compounds that reduced cell viability by at least 25% are labeled with an asterisk. (Abbreviations: Bay 11-7085: (2R,3S)-3-(4-(1,1-dimethylethyl)phenyl)sulfonyl]-2-propenenitrile, MNS: 3,4-methylenedioxy-1H-benzazepine HCl), the antioxidant ebsele and the 61 opioid receptor agonist SB 205607 decreased TNFα secretion in the low nanomolar concentration range: they reached their maximum response at around 100–300 nM, exhibiting a 50% inhibition of TNFα production (Fig. 7). Salbutamol (1 μM), in combination
dexamethasone (3 μM). The screen identified beta2 agonists (salbutamol, salmeterol), the phosphodiesterase (PDE) inhibitor rolipram and as prostaglandin E1 as synergistic enhancers of the glucocorticoid’s effect (Fig. 6, Table 4). In addition, the dopamine receptor antagonist SCH 23390 ([R(+)]-1-(2,3-dihydroxyphenylazo)-2-propyl-1 benzazepine HCl), the structurally related benzodiazepine lorazepam, the antioxidant ebsele and the 61 opioid receptor agonist SB 205607 decreased TNFα production in the presence of the glucocorticoid. As expected, the glucocorticoid receptor antagonist milepristone attenuated the effect of dexamethasone (Table 4). A few drugs (e.g. cerivastatin, vindesine, vinorelbine) increased TNFα production in the presence dexamethasone (Table 4); this effect was related to the fact that, according to the results of the primary screen, these compounds, on their own, increase HMGB1-induced TNFα secretion (Table 1).

**Glucocorticoid/beta-adrenergic Agonist Synergy: Mechanism of Action**

Using prednisolone (a prototypical glucocorticoid) and salbutamol (a prototypical beta 2 adrenergic agonist), follow-up experiments were designed to further characterize the pharmacological properties and underlying mechanisms of the glucocorticoid/beta-adrenergic synergy. Both prednisolone and salbutamol, on their own, decreased the HMGB1-induced TNFα production in the low nanomolar concentration range: they reached their maximum effect at around 100–300 nM, exhibiting a 50% inhibition of TNFα production (Fig. 7). Salbutamol (1 μM), in combination...
Table 4. Compounds of interest identified by screening of the NIH Library in the presence of 3 μM dexamethasone on HMGB1-induced TNFα production.

| Compound                        | Biological activity             | Single compound screen | Steroid combination screen |
|---------------------------------|---------------------------------|------------------------|---------------------------|
|                                 |                                 | TNFα response (% of HMGB1 stimulation) | Viability (%) | TNFα response (% of HMGB1 stimulation) | Viability (%) |
| **Steroid action enhancers (potentiators)** |                                 |                        |                           |                           |
| salmeterol β2 adrenergic agonist | 50                              | 102                    | 21                        | 111                       |
| rolipram phosphodiesterase inhibitor | 60                              | 86                    | 23                        | 109                       |
| prostaglandin E1 prostaglandin | 55                              | 97                    | 23                        | 109                       |
| salbutamol sulfate β2 adrenergic agonist | 51                              | 91                    | 25                        | 110                       |
| lorazepam benzodiazepine        | 50                              | 95                    | 27                        | 108                       |
| SB 205607 δ1 opioid receptor agonist | 53                              | 102                   | 29                        | 110                       |
| R(+)-SCH-23390 HCl D1 dopamine receptor antagonist | 73                              | 96                    | 30                        | 110                       |
| ebselen anti-inflammatory antioxidant | 85                              | 103                   | 34                        | 117                       |
| **Hit compounds in single-compound screen that do not enhance steroid action (non-potentiators)** |                                 |                        |                           |                           |
| fluticasone propionate glucocorticoid steroid | 40                              | 93                    | 43                        | 108                       |
| halometasone monohydrate glucocorticoid steroid | 39                              | 106                   | 44                        | 107                       |
| tadalfil PDE5 inhibitor          | 49                              | 107                   | 44                        | 108                       |
| tropisetron HCl serotonin 5-HT3 antagonist | 46                              | 99                    | 44                        | 110                       |
| indinavir sulfate HIV protease inhibitor | 43                              | 96                    | 48                        | 105                       |
| beclomethasone glucocorticoid steroid | 54                              | 108                   | 49                        | 106                       |
| zaleplon GABA Aγ agonist hypnotic | 52                              | 101                   | 50                        | 109                       |
| desoximetasone glucocorticoid steroid | 52                              | 108                   | 50                        | 111                       |
| pergolide mesylate salt dopamine receptor agonist | 60                              | 99                    | 54                        | 110                       |
| loteprednol etabonate glucocorticoid steroid | 59                              | 104                   | 62                        | 108                       |
| **Compounds that counteract dexamethasone (steroid inhibitors)** |                                 |                        |                           |                           |
| cerivastatin Na statin          | >150                            | 100                   | 83                        | 126                       |
| rutin platelet aggregation inhibitor | 102                             | 97                    | 69                        | 112                       |
| ritonavir HIV protease inhibitor | 112                             | 98                    | 73                        | 113                       |
| vinorelbine bitartrate antineoplastic | 112                             | 78                    | 78                        | 106                       |
| vindesine sulfate mitotic inhibitor | 120                             | 69                    | 96                        | 84                        |
| mifepristone glucocorticoid receptor antagonist | 101                             | 101                   | 92                        | 115                       |
| **Toxic compounds**             |                                 |                        |                           |                           |
| dactinomycin antibiotics        | 1                               | 1                     | 0                         | 2                         |
| triptolide NF-kB inhibitor       | 0                               | 2                     | 0                         | 1                         |
| homoharringtonine 60-S ribosome inhibitor | 0                               | 1                     | 1                         | 1                         |
| idarubicin HCl anti-leukemic drug | 6                               | 2                     | 9                         | 2                         |
| epirubicin HCl anthracycline drug | 12                              | 2                     | 18                        | 15                        |
| doxorubicin HCl anthracycline drug | 45                              | 4                     | 31                        | 32                        |
| topotecan HCl topoisomerase inhibitor | 94                              | 49                    | 45                        | 94                        |
| indarubicin antineoplastic       | 100                             | 72                    | 54                        | 94                        |
| diphenylcyclopropenone local immune response inducer | 114                             | 71                    | 56                        | 100                       |
| artesunate anti-malaria compound | 121                             | 74                    | 75                        | 78                        |
| vincristine sulfate mitotic inhibitor | 130                             | 72                    | 90                        | 80                        |

Compounds of interest are shown with their respective TNFα response and viability values attained in the single compound and combined screens. Drugs that reduced the TNFα response compared to the action of dexamethasone are classified as potentiators. Drugs that decreased the TNFα response by themselves, but showed negligible increase in their activity in combination with dexamethasone are listed as non-potentiators. Compounds that resulted in higher TNFα secretion (>MEAN±2SD) are listed as steroid inhibitors. Compounds that reduced the viability by more than 2 SD (<75% viability) are listed as toxic compounds. doi:10.1371/journal.pone.0065994.t004
with prednisolone, significantly reduced TNFα production already at 10 nM (compared to salbutamol alone); at 100 nM prednisolone the combination reached its full potential (approximately 70% inhibition). Likewise, the combination of prednisolone (1 μM) with 30 nM salbutamol significantly reduced the HMGB1-induced TNFα response (compared to prednisolone alone) and with 300 nM salbutamol the combination reached its full potential (approximately 70% inhibition) (Fig. 7).

HMGB1-induced TNFα secretion was associated with a rapid-onset and marked increase in TNFα mRNA (Fig. 8). Prednisolone and salbutamol each decreased the TNFα mRNA level by 50%; combination of the two compounds synergistically inhibited the transcription of TNFα mRNA (Fig. 8). We next tested whether the early inhibition of TNFα production involves upstream signaling events such as mitogen-activated protein kinase (MAPK) activation and IkB phosphorylation. HMGB1 induced an early and sustained activation of the extracellular signal-regulated kinases 1/2 (ERK1/2, p44 and p42) and p38 and of IkB phosphorylation (Fig. 9). The combination of salbutamol and prednisolone resulted in a partial, but statistically significant inhibition of ERK1 phosphorylation and IkB phosphorylation (Fig. 9). These data indicate the regulation of HMGB1-mediated cellular signaling by the glucocorticoid/beta-agonist combination has an upstream regulatory component.

To further characterize the effect of the glucocorticoid/beta-agonist combination on HMGB1-induced gene transcription, a TLR signaling pathway array was next employed. The responses could be characterized by four distinct expression patterns: a) prednisolone, but not salbutamol inhibiting gene expression, b) salbutamol, but not prednisolone inhibiting gene expression, c) the two compounds synergistically blocking gene expression and d) the two compounds synergistically enhancing gene expression (Fig. 10). The genes which were mostly inhibited by steroids included the interleukins (IL1a, IL1b, IL6, IL10) and PtgS2 (COX-2); the inhibition exerted by the beta-2 agonist was dominant in case of the chemokines Ccl2 and Cxcl10 and TLR2 and TLR9; synergistic inhibition by the glucocorticoid and the beta-agonist was confirmed for TNFα, as well as demonstrated for lymphotixin (Lta) and the TLR adaptor Ticam1 (Fig. 10). Unexpectedly, in a few instances, the steroid and the beta2 agonist led to a synergistic enhancement, as seen with Csf3 (GCSF), CD14, CCAAT/enhancer-binding protein beta (Cebpb), interleukin 1 receptor alpha (Il1R1) and TLR8 (Fig. 10).

Given the fact that both glucocorticoids and beta-receptor agonists represent endogenous hormones of the sympathetic-adrenal-medullary axis, we have next evaluated whether cortisol and/or adrenaline/noradrenaline, at concentrations that are comparable to their endogenous plasma levels, affect HMGB1-induced TNFα production. Cortisol, and, more markedly, the combination of adrenaline and noradrenaline, suppressed the HMGB1-induced TNFα response (Fig. 11).

Glucocorticoid/beta-adrenergic Agonist Synergy in vivo

The combination of prednisolone and salbutamol effectively suppressed HMGB1-induced TNFα production in Balb/c male mice in vivo, and this effect was more pronounced than the effect of either agent alone (Fig. 12). In contrast, the glucocorticoid receptor blocker mifepristone or the β-receptor antagonist propranolol did not enhance HMGB1-induced TNFα production (Fig. 12), suggesting that the response is not under significant control by endogenous glucocorticoids acting on the mifepristone-
sensitive glucocorticoid receptor or by endogenous catecholamines acting on the β-receptor.

Discussion

It is well established that HMGB1 plays a central role in sterile inflammation [1–3]. This screen was undertaken to identify inhibitors of HMGB1-induced, TLR4 dependent TNFα production. The hit compounds emerging from the primary screen included several signal transduction pathway modifiers, such as the IκB phosphorylation inhibitor Bay 11-7085 and the Src/Syk kinase inhibitor MNS. These findings are consistent with a role of NF-κB and kinase activation in HMGB1-mediated cellular signaling. Glucocorticoids and beta-receptor agonist activators of intracellular cAMP (such as such as salbutamol, clenbuterol, metaproterenol, ethylnorepinephrine and ritodrine) were two most prominent drug classes emerging from the screen. Because of their therapeutic potential, and because of the endogenous physiological regulatory implications, these two classes of compounds were subject of follow-up studies (see below). Additional classes of hit compounds included the natural compounds, piperlongumine and parthenolide (the latter compound is known pharmacological actions as a NF-κB and MAP kinase inhibitor). The mechanism of action and potential anti-inflammatory utility of miscellaneous additional compounds that showed inhibitory effects - such as the HIV protease inhibitor indinavir, the local anesthetic lidocaine, the surfactant tyloxapol, the calcium antagonist prenylamine and the diuretic ethacrynic acid - requires further characterization. It is interesting to note that indinavir [18], tyloxapol [19] and lidocaine [20] have previously been demonstrated to suppress TNFα production in various experimental models in vitro, although the underlying molecular pathways have not been fully characterized. It is intriguing to speculate that these compounds may have additional, hitherto unrecognized, secondary modes of pharmacological action (as well as potential therapeutic utility) due to inhibition of HMGB1-mediated inflammatory responses.

We identified several different activators of intracellular cAMP signaling as part of the screen for enhancers of the inhibitory effect of glucocorticoids. The enhancers exerted their effects their effects either through beta-adrenergic receptor agonism (such as salbu-
tamol and salmeterol), through prolongation of the intracellular half-life of cAMP (such as the phosphodiesterase inhibitor rolipram) or by activating the cAMP-dependent protein kinase (PKA) (such as prostaglandin E1). While neither the glucocorticoids nor the cAMP-stimulating agents, on their own, produced a complete inhibition of HMGB1-mediated TNFα response, the combination of these two agents yielded a robust inhibition, and did so at low micromolar/nanomolar concentrations. Previous studies have demonstrated synergistic interactions between steroids and beta-agonists in various experimental systems in vitro and suggested that cAMP and glucocorticoids act via distinct upstream pathways, which activate transcription though separate hormone response elements, the glucocorticoid receptor (GR) element (GRE) and the cAMP-response element (CRE), respectively. The site of the synergistic convergence was identified at the level of inhibition of the promoter activation of various pro-inflammatory genes [21–23]. Based on our findings, at least some of the synergistic inhibition of HMGB1-induced signaling by the glucocorticoid/beta-agonist combination occurs upstream from NF-κB activation, upstream from GRE and CRE and upstream from the promoter region of the inflammatory genes studied.

Our analysis of the gene expression profiles using a TLR signaling pathway array demonstrated that the synergistic inhibition of HMGB1-induced TNFα production by the glucocorticoid and the beta agonist does not represent a generalized phenomenon. In the case of several mediators, neither the glucocorticoid tested (e.g. Ccl2, Tlr2, Tlr9, Cd14, Cebpb, Csf3, Tlr8), nor the beta agonist tested (e.g. Il-1a, IL1b, IL6, Csf3, IL1r1) showed any inhibition. In some cases an enhancement was seen (IL1ra, Ptgs2, IL-10). These findings clearly demonstrate that HMGB1-mediated pro-inflammatory mediator production is regulated by glucocorticoids and by cAMP in a fashion that is specific to each gene product, and may be, at least in part, related to individual differences in the steroid and cAMP-responsive elements in individual promoters. Nevertheless, the combination of the beta agonist and the glucocorticoid resulted in a partial suppression for the majority of the genes studied, yielding a shift towards an overall anti-inflammatory phenotype (without suppressing the expression of the anti-inflammatory cytokine IL-10).

HMGB1 signals through numerous receptors, depending upon the molecular conformation of the three cysteines [2,12–14,24–26]. For example, extracellular HMGB1 is post-translationally regulated via redox mechanisms, and the C23-C45 disulfide C106

Figure 8. Prednisolone and salbutamol inhibit the HMGB-induced TNFα production. RAW 264.7 cells were pretreated with prednisolone (1 μM) and salbutamol (1 μM) and then exposed to HMGB1 (5 μg/ml) for various time up to 18 hours. A: TNFα secretion measured in the supernatant is plotted versus exposure length. (MEAN±SD values are shown) B: TNFα mRNA expression, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is shown as fold expression values of vehicle treated cells. (CTL: vehicle treated control, HMGB: cells exposed to HMGB1, Pred: cells pretreated with prednisolone and exposed to HMGB1, Salb: cells pretreated with salbutamol and exposed to HMGB1, Pred+Salb: cells pretreated with both prednisolone and salbutamol and exposed to HMGB1. *p<0.05 HMGB1-treated group compared to vehicle treated control, #p<0.05 compared to single compound treatment).

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thiol conformation binds to and activates the TLR4/MD2 receptor complex in the absence of LPS [12–14]. Here we utilized this recombinant conformation of HMGB1 (purified and characterized as previously described), which primarily signals through TLR4 to induce TNFα. Because RAGE and TLR2 are dispensable for this effect, our studies would not be expected to address signaling mediated through these receptors.

Hormones of the hypothalamic-pituitary-adrenal axis, the sympathetic-adrenal-medullary axis, and the sympathetic and parasympathetic arms of the autonomic nervous system have powerful roles in the control of inflammation [27–31]. Adrenalectomy or pharmacological blockade of endogenous glucocorticoid receptors exacerbates [30], while beta-receptor activation suppresses systemic inflammatory responses [31]. Considering the fact that the biologically active concentrations of glucocorticoids and catecholamines in the current study are in the physiological range, we have also explored whether the HMGB1-mediated inflammatory responses are under the tonic control of these hormones. While the combination of exogenous glucocorticoid and beta agonist inhibited HMGB1-induced TNFα production (thereby...
Figure 10. The interaction of prednisolone and salbutamol in the inhibition of HMGB-induced gene expression. RAW 264.7 cells pretreated with prednisolone (1 μM) and salbutamol (1 μM) were exposed to HMGB1 (5 μg/ml) for 1.5 hours and the expression of TLR-associated genes was analyzed with TLR signaling pathways array. Gene expression normalized to control genes (GAPDH, actin, B2m, Gusb, Hsp90ab1) is shown as fold expression values of vehicle treated cells. (CTL: vehicle treated control, HMGB: cells exposed to HMGB1, Pred: cells pretreated with prednisolone and exposed to HMGB1, Salb: cells pretreated with salbutamol and exposed to HMGB1, Pred+Salb: cells pretreated with both prednisolone and salbutamol and exposed to HMGB1. \( ^\# p < 0.05 \) HMGB1-treated group compared to vehicle treated control, \( ^* p < 0.05 \) compared to HMGB1 group, \( ^{\#} p < 0.05 \) compared to single compound treatment).

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Figure 11. Inhibition of the HMGB-induced inflammatory response by endogenous catecholamines and glucocorticoids at physiological concentrations. RAW 264.7 cells were pretreated with cortisol (0.7 μM), noradrenaline (0.5 ng/ml), adrenaline (0.5 ng/ml), dexamethasone (1 μM) and salbutamol (1 μM) and exposed to HMGB1 (5 μg/ml) for 18 hours. TNFα secretion was measured in the supernatant. (\( ^{1}\ p < 0.05 \) HMGB1-treated group compared to vehicle treated control, \( ^* p < 0.05 \) compared to HMGB1 group, \( ^{\#} p < 0.05 \) cells treated with all compounds in combination versus treated with a combination of two).

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extending the in vitro findings to an in vivo system, blockade of the endogenous glucocorticoid receptors with mifepristone or inhibition of the beta receptors with propranolol failed to potentiate the HMGB1-induced TNFα responses in vivo. Thus, circulating HMGB1 does not result in a more severe inflammatory response in subjects with impairment of their endogenous sympathetic-adrenal-medullary homeostasis, at least in the current in vivo experimental system.

In summary, the current study unveils several drug-like modulators of HMGB1-mediated inflammatory responses and offers pharmacological directions for the therapeutic suppression of inflammatory responses in diseases driven by the HMGB1-TLR4 axis. Glucocorticoids remain a mainstay of therapy for rheumatoid arthritis, as well as many other inflammatory diseases. In rheumatoid arthritis HMGB1 has been shown to play a significant pathogenetic role [2,3]. We hypothesize that the mode of the therapeutic action of glucocorticoids, in addition to inhibiting HMGB1 release [11], also involves an inhibition of HMGB1’s downstream signaling action. Furthermore, we conclude that the synergistic administration of a glucocorticoid and a beta-receptor agonist or (another cAMP-elevating agent) is an effective approach to suppress HMGB1-mediated inflammatory responses in vitro and in vivo.

**Supporting Information**

**Table S1** A full list of the genes investigated in the real-time PCR array experiments. (XLS)

**Table S2** A full list of the primary data produced by the primary cell-based screens. (XLS)

**Author Contributions**

Conceived and designed the experiments: DG PS KM JP YA HY SC TB KT CS. Performed the experiments: DG PS KM JP YA HY SC. Analyzed the data: DG PS KM JP YA HY SC TB KT CS. Contributed reagents/materials/analysis tools: SC KT. Wrote the paper: TB KT CS.

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