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Efficient discovery of anti-inflammatory small-molecule combinations using evolutionary computing

Ben G Small[1,2,9], Barry W McColl[3,9], Richard Allmendinger[4], Jürgen Pahle[4], Gloria López-Castejón[5], Nancy J Rothwell[5], Joshua Knowles[4], Pedro Mendes[4,6,7], David Brough[5] & Douglas B Kell[6,8]*

The control of biochemical fluxes is distributed, and to perturb complex intracellular networks effectively it is often necessary to modulate several steps simultaneously. However, the number of possible permutations leads to a combinatorial explosion in the number of experiments that would have to be performed in a complete analysis. We used a multiobjective evolutionary algorithm to optimize reagent combinations from a dynamic chemical library of 33 compounds with established or predicted targets in the regulatory network controlling IL-1β expression. The evolutionary algorithm converged on excellent solutions within 11 generations, during which we studied just 550 combinations out of the potential search space of ~9 billion. The top five reagents with the greatest contribution to combinatorial effects throughout the evolutionary algorithm were then optimized pairwise. A p38 MAPK inhibitor together with either an inhibitor of IκB kinase or a chelator of poorly liganded iron yielded synergistic inhibition of macrophage IL-1β expression. Evolutionary searches provide a powerful and general approach to the discovery of new combinations of pharmacological agents with therapeutic indices potentially greater than those of single drugs.

Acute or chronic (nonresolving) inflammation is a well-established mediator of major diseases including vascular disease (such as atherosclerosis and stroke), chronic obstructive pulmonary disease, cancer, diabetes, obesity, rheumatoid arthritis, psoriasis and inflammatory bowel disease[1,2]. In each of these conditions, the affected individual shows elevated expression of the potent and pleiotropic proinflammatory cytokine interleukin-1β (IL-1β)[2,3]. Pharmacological therapies targeted against IL-1β are largely focused on biologics that are only likely to act extracellularly[4] (for example, anakinra; an IL-1 receptor antagonist), which have shortcomings such as poor central nervous system penetration. Small-molecule modulation of IL-1β may offer benefits in certain conditions, such as in cerebrovascular injury where IL-1β mediates considerable cerebral damage during acute ischemia and excitotoxic insult[5].

The lipopolysaccharide (LPS)-induced Toll-like receptor 4 (TLR4) stimulation of macrophages is a widely used experimental model that mimics key aspects of inflammation including IL-1β expression[6]. Signal transduction induced by TLR4 stimulation proceeds through the activation of a complex array of multiprotein signaling networks (for example, MyD88, TRAF6, p38 MAPK and NF-κB), ultimately resulting in the expression of the *IL1B* gene. Possibly on the basis of the 'one gene, one drug, one disease' paradigm[7], a plethora of reagents has been developed to modulate individual proteins within these signaling networks. However, it is well known that multiple steps must be modulated simultaneously to have meaningful effects on biochemical fluxes[8]. Thus, a single-target approach is unlikely to be optimal in inhibiting the expression of a proinflammatory cytokine such as IL-1β, which has both inherent degeneracy and considerable complexity within the signal transduction network[9]. More generally, there is an increasing recognition of the need to target multiple steps within signaling networks for their effective pharmacological modulation[7].

Combinatorial chemical genetics[10] uses combinations of small molecules that allow dissection of cellular phenomena via their selective modulation of individual biological targets. Despite progress made in high-throughput screening technologies[11], the analysis of even modestly sized chemical libraries is prohibitive because of the combinatorial explosion that occurs in pharmacological space[12] (2^{33} or ~9 billion combinations for all possible combinations of the chemical library explored here). Thus we sought heuristic solutions (that is, reagent combinations) that are good but not provably globally optimal.

The terms 'evolutionary computing' and 'evolutionary algorithms' describe a set of computational approaches based loosely on Darwinian evolution by the natural selection of individuals and populations. In this case the population consists of individuals that each encode a candidate solution to the problem at hand. The 'fitness' of each solution is reflected in the objective function (or functions) designed by the experimenter, but it normally includes the concept that fitter individuals provide more accurate solutions. There may be multiple fitness functions. For instance, a simpler solution may be deemed a fitter solution, and algorithms with multiple objectives (multiple fitnesses), like those in this work, are known as multiobjective evolutionary algorithms. Multiobjective evolutionary algorithms allow for the specification and simultaneous handling of multiple and distinct optimization objectives[13–15]. Based on the fitness (or fitnesses) of each solution, a selection step determines which individuals will be allowed to remain under

[1]Doctoral Training Centre, Integrative Systems Biology Molecules to Life, Manchester Centre for Integrative Systems Biology, Manchester Interdisciplinary Biocentre, The University of Manchester, Manchester, UK. [2]School of Chemical Engineering and Analytical Science, The University of Manchester, Manchester, UK. [3]The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, UK. [4]School of Computer Science, The University of Manchester, Manchester, UK. [5]NeuroSystems Section, Faculty of Life Sciences, The University of Manchester, Manchester, UK. [6]Manchester Centre for Integrative Systems Biology, Manchester Interdisciplinary Biocentre, The University of Manchester, Manchester, UK. [7]Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA. [8]School of Chemistry, The University of Manchester, Manchester, UK. [9]These authors contributed equally to this work. *e-mail: dbk@manchester.ac.uk

consideration for the next generation. Some of these individuals are retained because they are simply copied, unchanged, into the subsequent generation(s), but new diversity based on the parents selected is produced by processes analogous to mutation and recombination. The fitnesses of these new individuals are then evaluated as above, and the algorithm continues to cycle through the steps of selection, breeding and fitness evaluation until an acceptable solution is found. Decades of research within the field of evolutionary computing (for example, refs. 16 and 17) have revealed that optimization of multivariate problems can be highly effective using small numbers of experimental tests. Although the present study used an evolutionary algorithm and an adaptive dose-matrix search strategy, we recognize that other kinds of combinatorial optimization approaches might also prove effective.

Using a 'reverse' combination chemical-genetic approach[10], our aim was to optimize combinations of reagents that minimize LPS-induced IL-1β expression in macrophages and simultaneously minimize the number of component reagents in the combination and their propensity to induce macrophage cell death. Subsequently, we sought to optimize reagents to inhibit IL-1β expression at concentrations lower than those of the component reagents used in isolation. This was achieved by application of an evolutionary algorithm–directed, semiautomated robotic assay of IL-1β expression to a dynamic chemical library of a total of 33 reagents (as described in Methods and refs. 18–20). The specific algorithm used here was the indicator-based evolutionary algorithm (IBEA)[21] as in preliminary simulations[22], it proved superior to a variety of other multiobjective optimization algorithms. This was followed by a dose-matrix search of top-ranked reagents resulting from the evolutionary algorithm–directed search. We demonstrate that the evolutionary algorithm converges efficiently on good solutions and that p38 MAPK inhibition along with either IκB kinase inhibition or iron chelation yields synergistic and biologically relevant inhibition of macrophage IL-1β expression.

RESULTS

Rapid convergence of IBEA to near-optimal solutions

Concentration-effect curves for a selection of reagents with known or predicted targets in the IL-1β expression network were determined to identify the most appropriate concentration for use in the evolutionary algorithm (**Supplementary Results**, **Supplementary Fig. 2**). On the basis of these data, we selected a suboptimal dose (3 μM) that would provide scope for observing combinatorial synergy.

The IBEA[21], which directed a semiautomated robotic assay using chemical combinations to inhibit IL-1β expression, was initialized (**Fig. 1**, loop 1; described further in Methods), the IBEA generates subsets of combinations from the library and then assesses the performance of these subsets with respect to inhibition of IL-1β expression, decreases in LDH (a marker for cell death) release and the number of member reagents within the combination. In the present case, we confined the number of experiments in each of the first and subsequent generations to 50, and those for generation 1 were selected randomly by the evolutionary algorithm from the first-generation library of chemicals. Superior combinations were retained and recombined with other library components in successive subsets

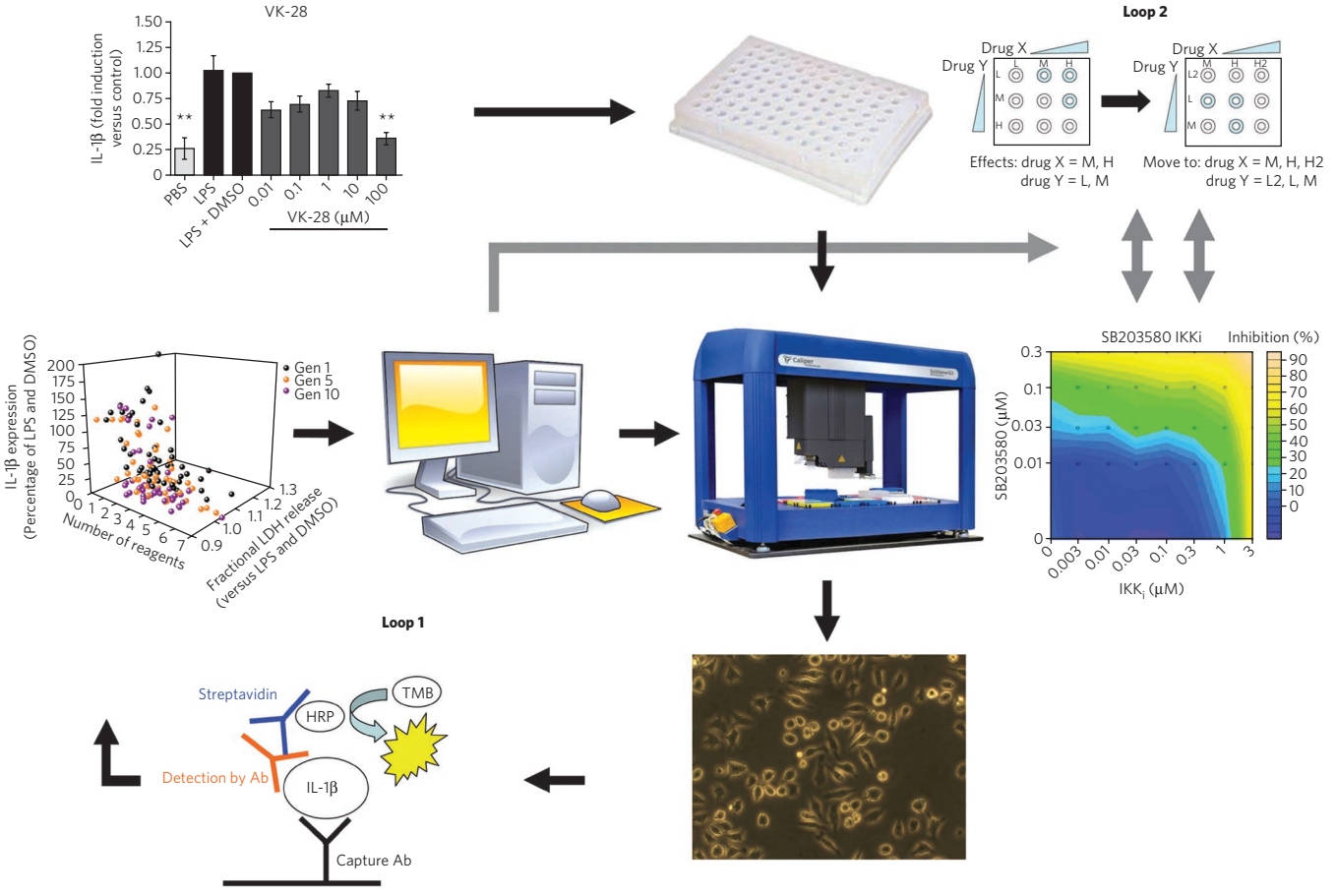

Figure 1 | Combinatorial evolutionary inhibition of IL-1β expression. Known drugs were tested alone before being used at a single concentration (3 μM) in a chemical library (loop 1, clockwise). Initialization of IBEA creates a random selection of combinations that are incubated with stimulated cells before measurement of cell death (LDH release) and IL-1β expression. Evaluation of these data against the number of compounds in the combination (n = 3 for all data) is performed by IBEA before a new generation of combinations is computed and tested. After 11 generations, concentration-dependent optimization (loop 2) of five top-ranked reagents was undertaken. Synergy was detected in new dual combinations. L, low; M, medium; H, high; PBS, phosphate-buffered saline; TMB, 3,3′,5,5′-tetramethyl benzidine; HRP, horseradish peroxidase.

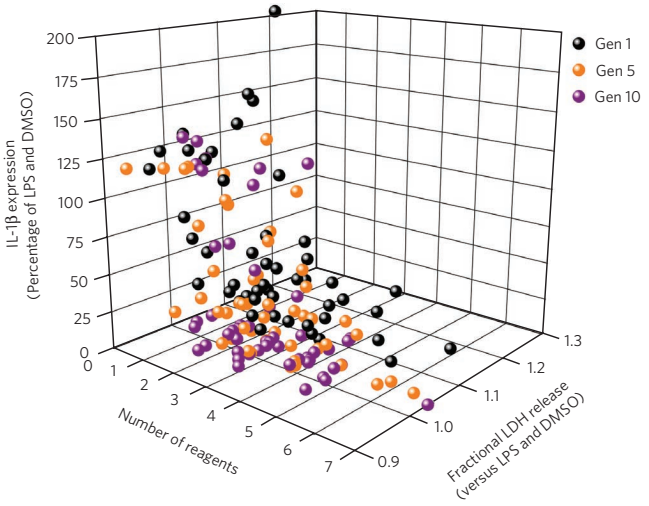

Figure 2 | Combinatorial multiobjective optimization of reagents inhibiting IL-1β production. Analysis of successive generations (generations 1 (initialization), 5 and 10) of reagent combinations reveals their convergence to a subset of highly effective combinations reflecting the inhibition of IL-1β expression with concomitant decreases in LDH release and the number of member reagents. All data presented are the means of three determinations. Data points appearing as zero on the axis labeled "Number of reagents" reflect positive control responses (LPS, 1 μg ml^{-1}; DMSO, 0.5% v/v). n = 3 for all data.

and assessed iteratively until satisfactory combinations were found. Assays of successive generations of chemical combinations from a dynamic chemical library (a total of 33 reagents, each at a concentration of 3 μM; described in **Supplementary Methods**) revealed their convergence toward a set of highly effective cocktails (**Fig. 2**; **Supplementary Fig. 3** and top rows of **Supplementary Tables 3** and **4** describe data on the individual generations). **Supplementary Spreadsheet 1** gives all of the data on both reagent combinations and experimental measurements in tabular form.

Convergence of solutions derived from the IBEA's search of the chemical library was assessed by measuring the population average rank of the IBEA hypervolume (**Supplementary Methods**) and the three objective functions (IL-1β expression, number of component reagents within combinations and LDH release) (**Fig. 3**). Both the observed plateau in IL-1β expression between generations 9 and 10 and very marginal decreases in LDH decrease (**Fig. 3**, top and bottom left, respectively) led us to halt the IBEA-directed search. By this stage, almost all IL-1β expression had been ablated by some combinations with negligible toxicity, although these solutions were not provably globally optimal.

A particular strength of this algorithm is the ability to add and remove reagents to and from the library during the evolution of the combinations[23] (Reagent Removal/Addition and Data Analysis in **Supplementary Methods**), and generations 10 and 11 explored these a little further. We also noted that many of the more successful reagent combinations contained the p38 MAPK inhibitor SB203580.

Post hoc analysis of combinatorial chemical space

The search of combinatorial chemical space yielded 51 and 188 reagent combinations that showed inhibition of IL-1β expression greater than or equal to either 95% or 70% of the control response, respectively (**Supplementary Tables 3** and **4**, top rows) across all generations. We chose to explore the inhibitory activities of other reagents independently of SB203580 because these effects might have been 'masked' in the evolutionary algorithm by the dominance of SB203580 (70%, or 35/50, of sampled combinations at generation 10 contained SB203580). Thus, a '*post hoc*' analysis of all data was conducted to assess the fitness contributions of single reagents (their overall score against our defined objectives) alone and in two- and three- component combinations (in the presence and absence of SB203580) for all reagents (**Fig. 4**, described further in Methods). Top-ranked component reagents included the p38 MAPK inhibitor (SB203580), a sphingosine kinase inhibitor (SKI-II), a statin (simvastatin), an iron chelator (SIH) and an inhibitor (BMS-345541) of the inhibitory κB kinase (IKKi). Inhibitors of p38 MAPK and IKK are well established as inhibitors of IL-1β expression, and (in addition to its effects on HMG-CoA reductase) simvastatin is a known anti-inflammatory[24]. An abundance of evidence also implicates poorly liganded iron in inflammatory processes[24,25]. However, the appearance of SKI-II may have been unexpected, although there is evidence for the involvement of at least one sphingosine kinase in inflammation[26]. Despite the appearance of both wortmannin and the mitochondrial uncouplers in the *post hoc* analysis, these compounds were not pursued further because of their lack of specificity and potential toxicity, respectively. Our observation of substantial inhibition of IL-1β expression with only pairs of inhibitors (**Supplementary Tables 3** and **4**, bottom) led us to study the concentration-dependent, pair-wise optimization of the top-ranked reagents.

Pairwise search reveals combinatorial synergism

The search over defined concentration ranges of all pairs of five top-ranked reagents was assessed using an adaptive dose-matrix search protocol (**Fig. 1**, loop 2; **Supplementary Fig. 4**). Briefly, this protocol adaptively changes the concentrations of chosen reagents, as described in Methods (SB203580 and SIH in **Supplementary Fig. 4** versus the same combination in **Fig. 5**). Reagents were assessed alone and in pairs (**Fig. 5**). Modes of pharmacological effect driven by reagent combinations have their own nomenclature[27–29]. Hence, additivity is the linear superposition of two different reagent effects, and synergy is nonlinear (excess) inhibition from a reagent combination beyond that expected for simple additivity[27]. Evidence of synergy (**Fig. 5c** and **f** show IKKi and SIH, respectively) was determined by subtracting the predicted additive effects (**Fig. 5b** and **e** show IKKi and SIH, respectively) of each combination (based on single-reagent efficacy) from the actual experimental data (**Fig. 5a** and **d** show

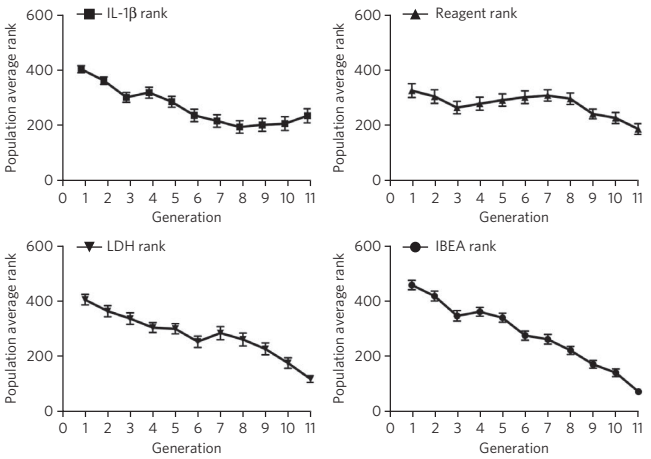

Figure 3 | Analysis of the evolutionary multiobjective optimization of the inhibition of IL-1β production. Population average rank for inhibition of IL-1β expression (top left), number of component reagents in combinations (top right), LDH release (bottom left) and overall IBEA hypervolume (bottom right). The IBEA hypervolume is a composite (described in Methods) of the performance of the different generations with regard to the three objectives, where a smaller number indicates better performance. Error bars, s.e.m.

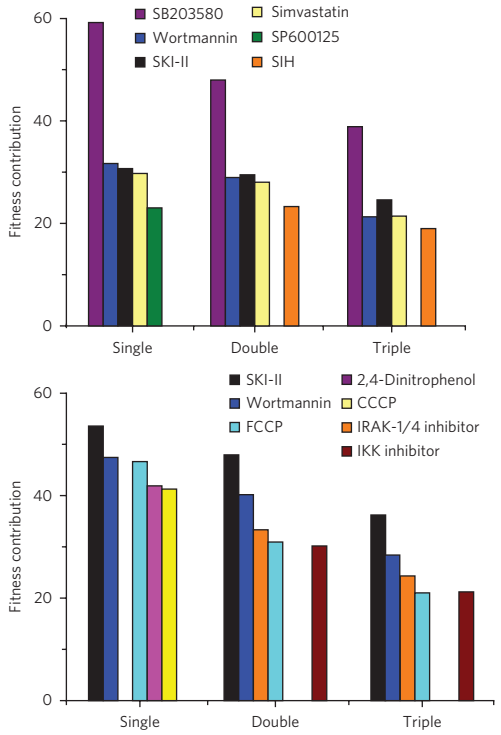

Figure 4 | Rank ordering of component reagents. Analysis of all evolutionary algorithm generations (1–11) in the presence (top) and absence (bottom) of SB203580 yielded a rank order for the fitness contribution (described in Methods) of each reagent within the library. Only five top-ranked reagents are displayed here either alone (single) or in double or triple combinations in the presence and absence of SB203580.

IKKi and SIH, respectively). Synergy was observed for most combinations; however, we noted in some instances that this could in fact be attributed to the loss of a potentiating effect on IL-1β expression that occurred when either of the reagents were used alone. In these cases, the net (that is, resulting) magnitude of IL-1β inhibition was minimal, and therefore we focus here on examples of combinatorial synergy generating biologically relevant levels of IL-1β inhibition. SB203580 (0.1 μM) and IKKi (1 μM) individually inhibited IL-1β expression by 28 ± 7% and 12 ± 9%, respectively (mean ± s.e.m., $n = 7$), and in combination they achieved significantly greater inhibition than either of the individual drugs (59 ± 5%, $n = 7$; $P < 0.02$ and $P < 0.0005$, one-way ANOVA with Tukey's multiple test correction versus SB203580 or IKKi alone, respectively). The inhibitory effect of this combination was 19% greater than that predicted for a purely additive effect, thus demonstrating a marked synergistic interaction at these concentrations (**Fig. 5c**). Similarly, SB203580 (0.1 μM) and SIH (3 μM) individually inhibited IL-1β expression by 31 ± 10% and 19 ± 8%, respectively, ($n = 7$ plates) and combinatorially inhibited IL-1β expression by a significantly greater magnitude (59 ± 4%, $n = 7$ plates, $P < 0.04$ and $P < 0.004$, one-way ANOVA with Tukey's multiple test correction compared to SB203580 and SIH alone, respectively). This combinatorial inhibitory effect was 9% greater than that predicted for pure additivity, therefore indicating a synergistic interaction (**Fig. 5f**). We also observed marked synergistic effects for combinations of IKKi and SKI-II, IKKi and SIH, and SB203580 and SIH (**Supplementary Fig. 4**). To assess whether a triple combination of SB203580, IKKi and SIH could inhibit IL-1β expression beyond the synergy already observed for both paired combinations (that is, SB203580 with either IKKi or SIH), we superimposed increasing concentrations of SIH (0 μM, 0.1 μM, 0.3 μM, 1 μM and 3 μM) onto SB203580 and IKKi dose matrices (**Supplementary Fig. 5**). We did not observe any further synergy with the triple combination.

DISCUSSION

There is a growing recognition that for drugs to be effective, whether singly or in combination, multiple steps must be affected simultaneously[7,10,30,31]. This, however, immediately leads to a combinatorial explosion of experimental possibilities that limits the number of drugs that can reasonably be tested exhaustively. We have applied a multiobjective evolutionary algorithm to the optimization of reagent combinations using a panel of candidate reagents, selected from our own studies and the literature[32], that target the pro-inflammatory IL-1β expression network. The objective assessment of reagent combinations arising from the IBEA-directed search using *post hoc* analysis of reagent fitness contributions is useful as it removes a layer of decision making[20], reducing bias and potentially enhancing 'hidden' phenomena (for example, off-target effects of reagents) that may have beneficial effects on the system output (that is, IL-1β expression). In this regard, it is worth mentioning the increasingly effective use of adaptive dosing regimes in clinical trials of pharmaceutical drugs, both singly and in combination with each other[33].

Combination therapy is now returning to the fore with a greater understanding of pharmacological mechanism being uncovered by advances in parallel measurements of biological endpoints[11,34,35] The Gur-game stochastic search algorithm has been reported to be useful[36,37] in elucidating the antiviral and NF-κB activating efficacy of drug and cytokine combinations, respectively. Briefly, this algorithm functions by generating a random number (for example, one representing a specified antiviral activity) and switching the concentration of component drugs if their efficacy is below this value. In contrast, stochastic and deterministic elements of our search were based on experimental data output and recombination of reagents in new cocktails that had not yet been evaluated. The multiobjective nature of the evolutionary algorithm–driven search presented here allows assessment of a number of biological endpoints. Following this approach with an adaptive dose-matrix–driven search enabled us to search pharmacological space using only the top-ranked 'hits'. Applications of search algorithms and the use of machine learning in the optimization of combinatorial therapies have recently been reviewed[38], and within the categorization used by the authors, our method falls under 'E – Model-free Biological Search'.

Recently, two papers have cited the pairing of reagent combinations as either indicative or predictive of higher-order effects[39,40]. The latter paper[40] used a series of chemotherapeutic reagents to monitor for additivity, antagonism or synergy of combinations on yellow fluorescent protein–tagged protein dynamics in the H1299 cell line. The authors propose that a linear superposition of weighted sums from the effect single drugs have on protein dynamics can predict higher-order (that is, combinatorial) effects for these reagents, although they were unable to demonstrate this for wortmannin (an inhibitor of phosphoinositide 3-kinase (PI3K)).

The former paper[39] looked for an enhancement in the intracellular concentration of Ca^{2+} in platelets using a pairwise agonist scanning approach of six reagents at three different concentrations. Using these data, the authors trained a neural network model to predict higher-order effects and were successful in doing so. However, the rapid and nontranscriptional signal transduction required for Ca^{2+} mobilization may not entirely reflect multiprotein signaling networks, which involve extensive cross-talk and feedback loops that modulate responses on time scales ranging from minutes to hours and beyond.

In the present case, we observed substantial inhibition of IL-1β production by the p38 MAPK inhibitor SB203580 that was enhanced synergistically by either the IKK inhibitor BMS 345541 or the iron chelator SIH[41]. Perhaps surprisingly, the triple combination of SB203580, IKKi and SIH did not reveal additional effects beyond those observed for pairwise combinations of SB203580 and either of the other two reagents. The effects of iron chelation are of special

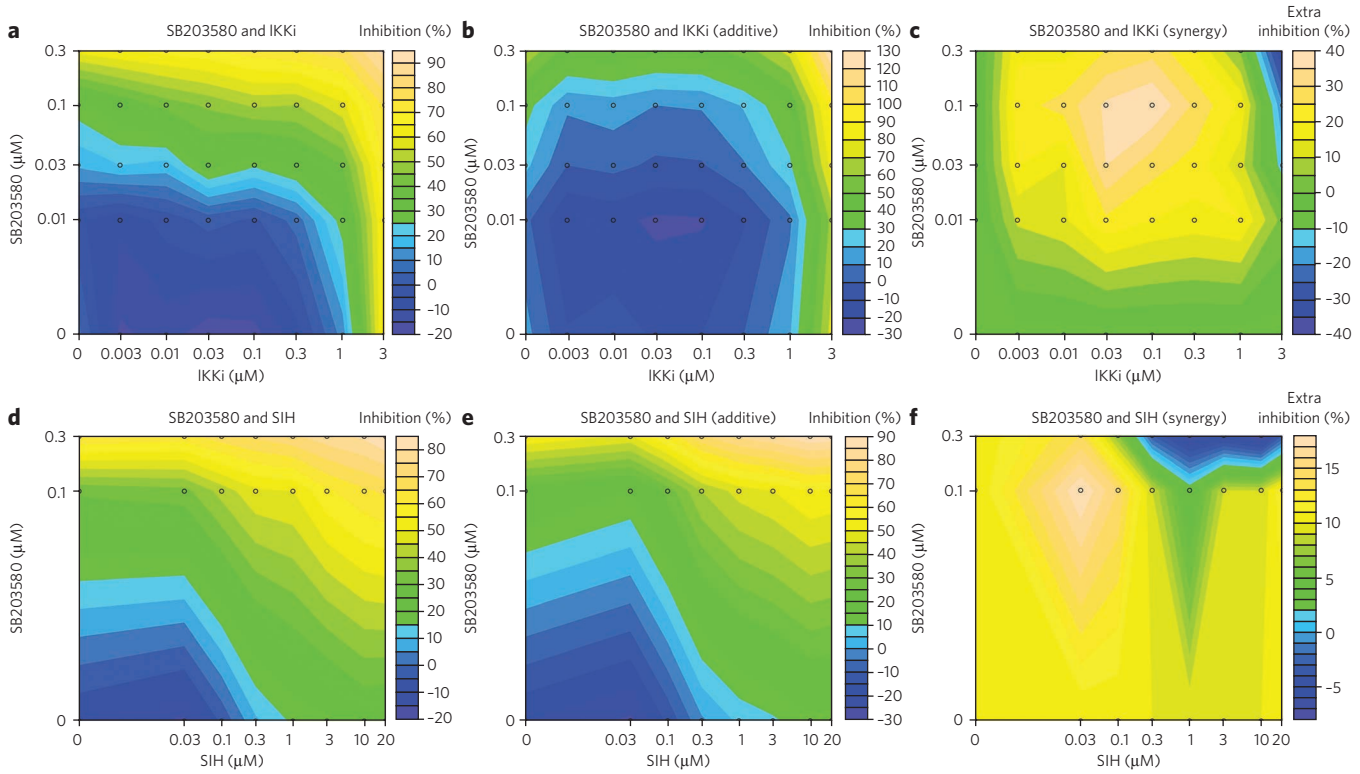

Figure 5 | Concentration-dependent adaptive dose-matrix optimization of paired reagent combinations. Concentration-dependent adaptive dose matrix optimization of paired reagent combinations was achieved by adaptively changing the concentrations of reagents after assessing the inhibition of IL-1β expression. (**a**) A p38 MAPK inhibitor (SB203580) and an Iκ kinase inhibitor (IKKi) were assessed alone and as a paired combination. (**b**) Simple additive effects of the SB203580-IKKi combination. (**c**) Potential synergy of the SB203580-IKKi combination (as in **a**) revealed by subtraction of simple additive effects of the experimental data in **b**, calculated from single-reagent data in the absence of the other reagent. (**d**) A p38 MAPK inhibitor (SB203580) and an iron chelator (SIH) were assessed alone and as a paired combination. (**e**) Simple additive effects of the SB203580-SIH combination. (**f**) Potential synergy of the SB203580-SIH combination (as in **d**) revealed by subtraction of simple additive effects of the experimental data in **e**, calculated from single-reagent data in the absence of the other reagent. Synergistic inhibition of IL-1β expression was revealed with the combinations of SB203580 with IKKi in **c** or with SIH in **f**; synergistic inhibition is considered as 'extra' inhibition relative to the additive inhibitions measured for the individual reagents.

interest here as there is abundant but widespread evidence for the role of unliganded iron in a variety of inflammatory disorders[24,25,42].

p38 inhibitors have been disappointing in clinical trials, not so much because they are not active *in vivo* but because (at the doses used) they lose specificity for the α isoform of p38 and are toxic[43]. A particular benefit of the synergy achieved when a p38 inhibitor such as SB203580 is combined with BMS345541 or SIH, as observed here, is that lower concentrations of inhibitor can be used than when they are used individually (**Fig. 5**). We also note that such inhibitors seem to have not been designed to exploit the specificity of pharmaceutical drug transporters[44]. The efficacy of combinations that did not involve the p38 inhibitor was also noted.

In conclusion, the application of an evolutionary algorithm in conjunction with a semiautomated assay of a dynamic chemical library enables rapid scanning of reagent combinations without the need for initial hypotheses[45] about likely higher-order effects. Our results show that synergistic combinations can be revealed quickly and can survive further experimental scrutiny, leading to pairwise combinations that seem promising to use in practice. The synergism shown by the combination of SB203580 and either IKKi or SIH presented here, in contrast to the comparatively marginal effect of the individual reagents at the same concentrations, shows that pharmacological modification of biological targets and processes may be effected at concentrations that are less likely to be toxic. This has particular relevance to the treatment of chronic inflammatory conditions such as irritable bowel syndrome[46] and chronic obstructive pulmonary disease[47], which involve treatment that is maintained for extensive periods. Our approach is essentially generic, and the time required per generation is determined by the time needed for setting up and running the assays (typically 3 d, one each for cell preparation, combination preparation and ELISA analyses), as the time needed for the algorithm to analyze the results and then choose the cocktails for the next generation was negligible in comparison. Overall, our new method substantially decreases the time taken to triage pharmacologically useful chemical diversity within chemical libraries[48]. Additionally, we demonstrate how combinations of known drugs or reagents could allow them to be repurposed[49] and could provide an elegant adjunct to existing therapeutic strategies in chronic inflammatory conditions.

METHODS

All procedures, protocols and methods were carried out under aseptic conditions where deemed necessary.

Construction and composition of the chemical library. The choice of reagents with which to populate the chemical library searched here was guided in part by Oda and Kitano's TLR signaling network[32] and via identification of suitable ligands from single-reagent studies in peritoneal macrophages. The following pharmacological classes of reagents were used: iron chelators, the zinc chelator TPEN, anti- and pro-oxidants, NADPH oxidase inhibitors, PI3K inhibitors, MAPK pathway inhibitors, NF-κB pathway inhibitors, the tyrosine kinase inhibitor genistein, mitochondrial uncouplers (removed after generation 3), statins and small-GTPase inhibitors. In the evolutionary optimization process, each of these reagents corresponds to a single binary variable indicating whether or not the reagent is included in a combination; the combination itself represents a candidate solution to the problem. Detailed information regarding the construction, storage,

maintenance, and removal and replacement of reagents within the chemical library can be found in **Supplementary Methods**.

Implementation of the IBEA. In order to select a suitable multiobjective evolutionary algorithm for addressing the search of the fitness landscape of the chemical library, a comparison of four evolutionary algorithms—IBEA, SPEA2, NSGA2 with binary tournament and NSGA2 with probabilistic selection[50]—was undertaken[23]. All evolutionary algorithms were assessed on a family of test problems used to simulate the reagent-combination problem. The test problems model a scenario where pharmacological interactions among reagents can be described by single, binary and ternary effects only. A reagent combination can effect minimal IL-1β expression by killing cells; cell death is measured as a large release of LDH. To improve the detection of lethal versus effective or benign combinations, the possibilities of positive, negative and no correlation were considered for these two objectives. The different levels of correlation were realized by assigning certain probabilities to effect values; a probability was first assigned for IL-1β expression, and, dependent on this value, a probability determining an effect value for the LDH release was assigned. Depending on the correlation level, the effect values were drawn uniformly from the interval [−1,0) and/or (0,1].

All evolutionary algorithms tested were capable of locating combinations of compounds of similar quality in the presence of 80% and 10% variability in IL-1β expression and LDH release measurements, respectively. However, IBEA was best at finding effective compound combinations that contained only a few compounds (although its search was unrestricted and could have used any number of compounds). IBEA was the only evolutionary algorithm tested that was not based on Pareto ranking; rather, it searches for those solutions that maximize their hypervolume within objective space. Initialization of the first generation of reagent compounds in IBEA was conducted by fixing the probability of compound selection to 3/33 to ensure a random selection of compounds from across the library, with, on average, three compounds in a cocktail. **Supplementary Methods** and **Supplementary Table 2** describe other details of the algorithm and its parameters.

Production and assay of reagent combinations. A Sciclone ALH3000 laboratory robot (Caliper Life Sciences) under the indirect control of an IBEA enabled the semiautomated assay of chemical combinations (**Supplementary Methods**) in LPS-stimulated J774.A1 macrophages. The iterative searching and analysis of incremented generations of combinations was conducted via measurements of an IL-1β expression ELISA (R&D Systems; DY401) and LDH release (Promega) (**Supplementary Methods**).

Treatment of peritoneal and J774.A1 macrophages with single reagents and combinations. Peritoneal and J774.A1 macrophages were prepared and cultured (**Supplementary Methods**) for either single-reagent or combinatorial and dose-matrix studies, respectively. Peritoneal macrophages were exposed to either single reagents (0.01 μM–100 μM) or DMSO (0.5% v/v) for 0.5 h before stimulation with LPS (1 μg ml^{-1}). Similarly, J774.A1 macrophages were treated with chemical combinations (3 μM or varying concentrations) or DMSO (0.5% v/v or 0.1% v/v) during the evolutionary algorithm–directed and adaptive dose-matrix search, respectively, for 10 min before stimulation with LPS (1 μg ml^{-1}). After 4 h (peritoneal) or 2 h (J774.A1), aliquots of well supernatants were taken for the measurement of LDH during single-reagent and evolutionary algorithm–driven combinatorial assessment, respectively, (**Supplementary Methods**) before disposal of remaining supernatant, lysis of cells and freezing before measurement of IL-1β expression (**Supplementary Methods**).

***Post hoc* analysis of IBEA search and calculation of reagent fitness.** Calculation of the fitness contribution of a single reagent within a combination was assigned as follows (1):

$$F_i = \bar{X}_1 - \bar{X}_n \quad (1)$$

where F_i is the fitness contribution of any given single reagent (i), and $\bar{X}_1$ and $\bar{X}_n$ are the mean IL-1β expression values of all combinations where the single reagent (i) was present or absent, respectively. Thus, a larger fitness contribution F_i indicates that a reagent is more efficient in decreasing IL-1β expression.

Concentration-dependent optimization of paired reagent combinations using an adaptive dose matrix search protocol. Upon completion and *post hoc* prioritization of reagent combinations from the IBEA search, a concentration-dependent optimization step was implemented. Briefly, to assess the potentially synergistic effects of paired combinations on IL-1β expression, we serially and logarithmically decreased the test concentrations of reagents from those used during the evolutionary algorithm–directed search. Similarly, after this initial optimization step, we extended the scanned concentration ranges of promising combinations by adding test concentrations of reagents at approximate 0.5 $\log_{10}$ spacings within the dose matrix. This allowed effect (that is, IL-1β expression) comparisons at multiple doses of paired reagents. Pseudocolor mappings were performed by linear interpolation between samples; mappings that move away from the blue end of the spectrum within combination response-shape plots indicate synergistic inhibition of IL-1β expression between two reagents (**Fig. 5**, **Supplementary Fig. 4**).

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Author contributions

B.G.S. designed, performed and analyzed the data from the single-reagent, combinatorial, evolution-directed and adaptive dose-matrix studies and wrote the first draft of the manuscript. B.W.M. designed, performed and analyzed the data from single-reagent and evolution-directed combinatorial studies. J.P. suggested and was involved in the design of the concentration-dependent adaptive dose-matrix search protocol of paired combinations and wrote the code in R to produce the combination response-shape plots. G.L.-C. conducted adaptive dose-matrix studies. D.B.K., D.B. and N.J.R. conceived the idea of applying an evolutionary algorithm to select combinations of reagents for inhibition of IL-1β expression; wrote the grant application; investigated and supervised, together with B.G.S.; and advised on experimental design. J.K. supervised R.A., generally advised on evolutionary algorithms and multiobjective optimization and, with R.A., evaluated and selected the evolutionary algorithm (IBEA) applied here. R.A. wrote the IBEA code in Java, helped implement its use and generated *post hoc* reagent-fitness measurements. P.M. supervised B.G.S. and advised on experimental design and data analysis. All authors contributed to the writing of the manuscript and approved its final form.

Competing financial interests

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturechemicalbiology/.

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