Supplementary Note 4: Details and follow-up for selective synergy examples

Individual examples of selective synergy

Here we provide examples of selective synergy drawn from our combination screening that represent six different therapeutic areas of research. Among these are cases of both therapeutic selectivity, with potentially useful potency windows between models of medical efficacy and toxicity, and mechanistic selectivity, where differential response profiles across phenotypes can be used to highlight particular mechanisms or prioritize indications for candidate therapies (Fig. 4; Fig. S14; Suppl. Data 8).

A clear example of therapeutic selectivity is the antibacterial synergy between ribavirin, a metabolism inhibitor, and disulfiram, a metabolic drug used for alcohol avoidance therapy. The combination has almost no effect on human cell viability (Fig. 4). Ribavirin and disulfiram are each active on metabolic targets (inosine monophosphate dehydrogenase for ribavirin, and mitochondrial aldehyde dehydrogenase 2 for disulfiram) at high doses in both primary smooth muscle cell viability and *Staphylococcus aureus* proliferation assays, but the strong antibacterial synergy is completely absent in the human cell toxicity model. Similarly, there is a strong antiviral synergy of cepharanthine, an anti-inflammatory drug with antiviral potential[1], and benzamil, a potent inhibitor of ion transport channels[2] (Fig. S14). However, there is no corresponding synergy against host cell viability, even though the single agents are active at high doses in both assays. Finally, the anti-inflammation synergy between prednisolone and nortriptyline against secretion of tumor necrosis alpha (TNF-α) from stimulated peripheral blood mononuclear cells (PBMC) has no increased toxic effect as measured by PBMC metabolic viability (Fig. 5, details to follow).

An example of mechanistic selectivity in the cancer area is the strong synergy between LY 294002 and camptothecin (Fig. 4) that occurs preferentially in lung-derived H460 over colon-derived Colo-205 cells. Camptothecin acts on topoisomerase (TOP) to impede DNA availability for gene expression, while LY 294002 is a specific inhibitor of phosphoinositide 3 kinase (PI3K) signaling. In the screen from which this combination was drawn, we found evidence of synergy in H460 for 21/32 TOP + PI3K combinations tested, 12 of which had similar levels of selectivity over Colo-205 (data not shown), suggesting that the synergy results from coordinated activity on each drug's primary target. This interaction can be used to identify cotherapeutic opportunities for treating cancers that rely especially heavily on these targets. Against anthrax toxicity, potential post-infection treatments for anthrax are suggested by the synergy between manganese sulfate and the hypertension drug methyldopa in a toxin survival assay that shows no antibacterial activity (Fig. S14). Because neither agent has known or observed activity in bacteria (here a close relative of *Bacillus anthracis*), the anti-toxin selectivity can be attributed to host factors. Finally, from our cardiovascular screens, the anticancer microtubule inhibitor paclitaxel and the vasodilator forskolin synergistically inhibit smooth muscle cell proliferation while sparing toxicity on endothelial cells (Fig. S14), pointing to possible uses for reducing the risk of thrombosis with drug-eluting stents[3].

For all of our examples, the single drugs have unrelated indications or modes of action, suggesting that multi-target mechanisms predominate. Because this is a natural outcome of screening combinations of large, diverse sets of chemical agents, screening for synergy is very effective for finding unexpected interactions between functional pathways in cellular systems.

Anti-inflammatory synergy between glucocorticoids and tricyclic antidepressants

It has long been a goal of the pharmaceutical industry to dissociate the powerful anti-inflammatory activity of glucocorticoid receptor (GR) agonists such as prednisolone, budesonide and dexamethasone from their side effects under chronic treatment including hyperglycemia, osteoporosis, adrenal insufficiency, skin thinning, glaucoma and depression[4]. Numerous GR activators have been designed that maintain the transcriptional repressive action that drives

anti-inflammatory activity without inducing DNA-binding dependent gene activation or suppression that contributes to side effects (eg, deflazacort[5], RU40066[6], AL-438[7], and ZK-216348[8]). Unfortunately, these agents still retain unwanted glucocorticoid side effects. For example, ZK-216348 has anti-inflammatory activity similar to prednisolone, and its induction of the gluconeogenesis gene tyrosine aminotransferase (TAT) is threefold lower than prednisone[8], however ZK-216348 retains the suppression of endogenous corticosterone (CORT) that is also associated with side effects[4]. Thus glucocorticoid dissociation may benefit from an approach other than medicinal chemistry.

The synergy between nortriptyline, a tricyclic antidepressant (TCA), and the glucocorticoid prednisolone (Fig. 5) offers the hope that dissociation might be achieved using a multi-target approach. The anti-inflammatory synergy is likely to act through each drug's primary target because it persists when either agent is replaced by a related drug (Fig. S15; Suppl. Data 8). The same synergistic effect ($\boldsymbol{S} > 5$ standard errors) was observed in 59 of 63 GCR-SLC6A targeting combinations we have tested in the TNFα assay at sufficient doses (data not shown). To validate our mechanistic hypothesis, we investigated the effects of perturbing the main components of the autocrine pathway in combination with GC. First, we directly increased extracellular norepinephrine (NE), modeling the effect of TCA treatment, and recovered the observed synergy (Fig. S15; Suppl. Data 8). Next, we applied an ADRB agonist, which should also strengthen the autocrine pathway, and found that this also increased the synergistic anti-inflammatory response at high concentrations. Finally, we used an ADRB inhibitor to weaken the NE-mediated pathway, and as expected, there was no synergy with GC.

Due to differential expression of GCR, SLC6A2, and ADRB2, we expect the synergy with tricyclic antidepressants to increase the therapeutic window over glucocorticoid-associated toxicities. While all three genes are have comparable levels in lymphoid cells, ADRB2 is expressed 3-10 fold lower[9] in tissues, such as liver and the pituitary gland, that mediate major GC-associated adverse effects[4]. This differential expression weakens the NE-mediated pathway, and should attenuate the GC-TCA synergy in toxicity-related tissues. Indeed, the amplification of anti-inflammatory effect seen in rodents with this combination does not show a corresponding rise in glucocorticoid-associated toxicity at similar doses (Fig. 5). The generality of this synergy (Fig. S15), and the low prednisolone dose (~100 nM) required in combination (~0.3 mg/kg in rats, below levels that induce side effects[8]), suggest that the TCA-corticosteroid combination could show improved dissociation in therapeutic contexts.

The potential therapeutic selectivity of the combination was further investigated using *in vivo* models of glucocorticoid-induced toxicity in rats (Suppl. Data 9). In an intra-nasal ovalbumin challenge model for asthma, both nortriptyline and budesonide, a synthetic glucocorticoid used in asthma, had minimal effects as single agents compared to vehicle-treated controls. The combination, however, suppressed infiltration of pro-inflammatory eosinophils to the level seen with unchallenged control animals (Fig. 5). The anti-inflammatory activity of the combination was significantly different from the components alone ($\boldsymbol{p} < 0.05$, ANOVA) consistent with the synergistic interaction observed between budesonide and nortriptyline *in vitro* (Fig. S15). The synergy between nortriptyline and prednisolone was further confirmed in a rat model for carrageenan-induced inflammatory pain in an experiment designed to detect synergy over the drug-with-itself standard (Fig. S16). This effect was durable across multiple time points and was also observed in a similar formalin-induced pain model (data not shown). By contrast, in a liver toxicity model monitoring TAT mRNA levels, high dose prednisolone (5 mg/kg) significantly increased expression ($\boldsymbol{p} < 0.05$, ANOVA), while the combination was not distinguishable from vehicle (Fig. 5). This lack of toxicity prevailed under chronic dosing at levels comparable to or exceeding those that synergized in the anti-inflammatory *in vivo* studies.

These early studies demonstrate the promise of multi-target approaches towards dissociating the anti-inflammatory effects in steroids from their associated side effects, by taking advantage of biological complexity.

References

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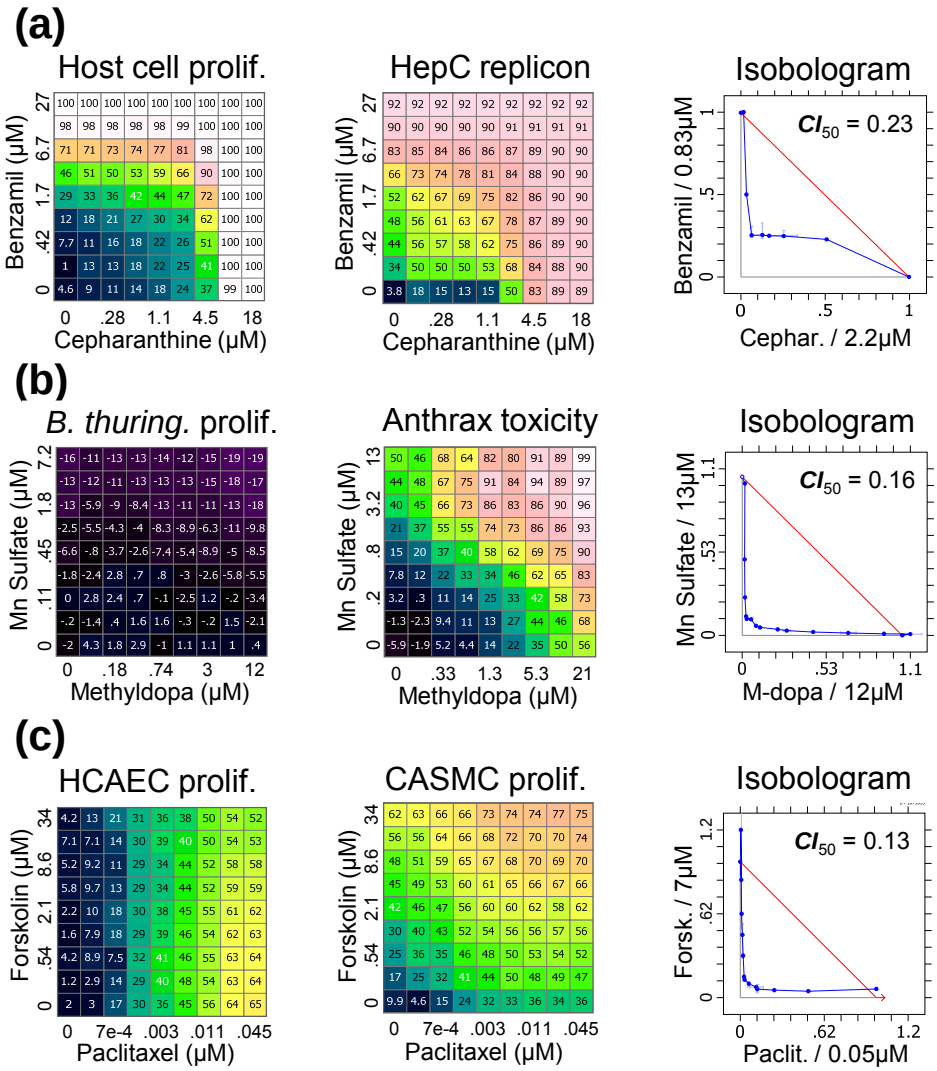

Figure S14. Dose matrices for selective synergy examples. For each combination, the responses for the control (left) and test (center) assays are displayed, with the test isobologram at 50% effect (right). (a) The antiviral synergy of cepharanthine and benzamil shows no detectable synergy against host cell viability, even though the single agents are active at high doses in both assays. (b) Manganese sulfate and the hypertension drug methyldopa produced a strong pro-survival synergy in an anthrax toxin assay. Because neither agent has known or observed activity in bacteria (here a close relative of *Bacillus anthracis*), the anti-toxin selectivity can be attributed to host factors. Finally, in our cardiovascular assays (c), the vasodilator forskolin selectively inhibits smooth muscle proliferation over endothelial cells, and the combination with the microtubule inhibitor paclitaxel is still more selective while reducing paclitaxel's toxicity to endothelial cells at high doses of forskolin.

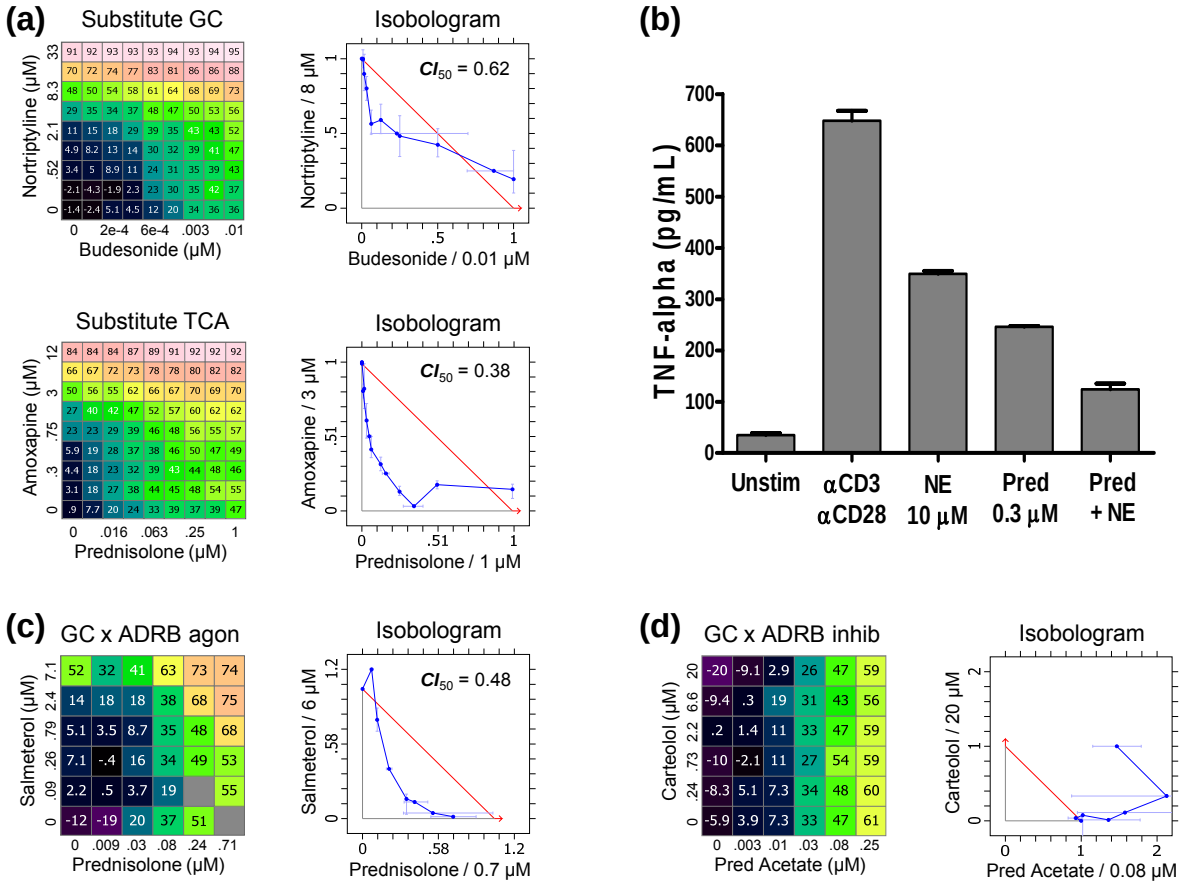

Figure S15. Supporting *in vitro* data for the GC-TCA synergy's selective mechanism. (a) The anti-inflammatory synergy is likely to act through each drug's primary target because it persists when either agent is replaced by a related drug. Also, a synergistic effect (***S*** > 5 standard errors) was observed in 59 of 63 GCR-SLC6A targeting combinations we have tested in the TNFα assay at sufficient doses (data not shown). To validate our mechanistic hypothesis (Fig. 5), we investigated the effects of perturbing the main components of the autocrine pathway in combination with GC. First (b), we directly increased extracellular norepinephrine (NE), modeling the effect of TCA treatment, and recovered the observed synergy. The combination of prednisolone (Pred) and norepinephrine (NE) was statistically different than all other groups (ANOVA $p < 0.001$, SD error bars, Tukey's). Next (c), we applied an ADRB agonist, which should also strengthen the autocrine pathway, and found that this increased the synergistic anti-inflammatory response at high concentrations. Finally (d), we used an ADRB inhibitor to weaken the NE-mediated pathway, modeling the reduced ADRB2 levels seen in tissues related to GC toxicity[9], and as expected, there was no synergy with GC.

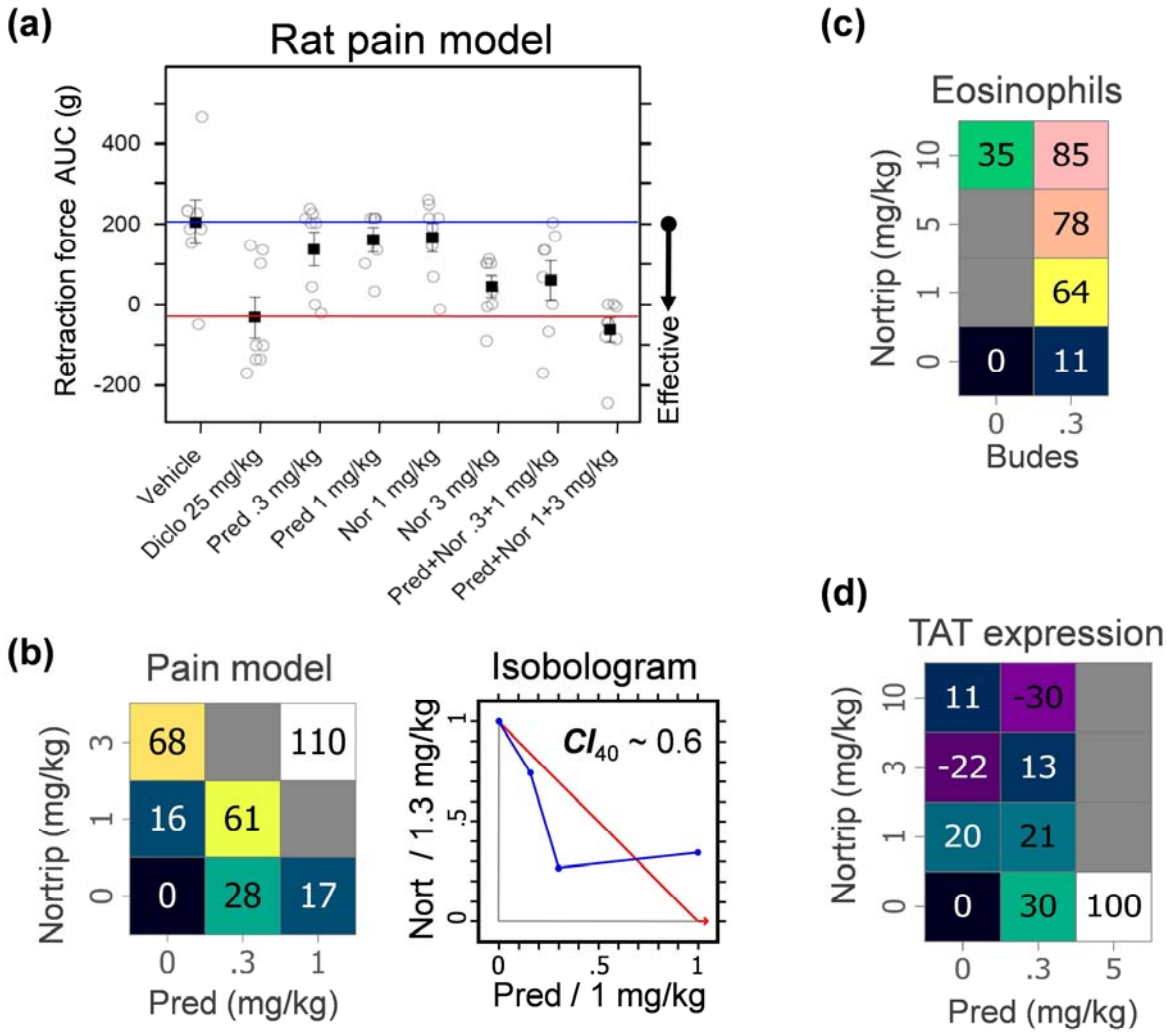

Figure S16. More *in vivo* data supporting GC-TCA synergy in inflammation models. (a) In a carrageenan-induced pain model with rats, prednisolone and nortriptyline produced a synergistic effect (ANOVA $\boldsymbol{p} < 0.05$ over agents) equivalent to a high-dose analgesic. (b) The analgesic synergy is evident in an isobologram after organizing these data into a dose matrix and normalizing to the control ranges (percent change between the vehicle to positive control). (c,d) The data from the *in vivo* models for asthma and toxicity (Fig. 5), similarly organized into dose matrices of effect normalized to control ranges.