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Targeted Inhibition of Heat Shock Protein 90 Suppresses Tumor Necrosis Factor- α and Ameliorates Murine Intestinal Inflammation

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Abstract

Inflammatory bowel diseases are chronic intestinal inflammatory diseases thought to reflect a dysregulated immune response. Although antibody-based inhibition of tumor necrosis factor- α (TNF- α) has provided relief to many inflammatory bowel diseases patients, these therapies are either ineffective in a patient subset or lose their efficacy over time, leaving an unmet need for alternatives. Given the critical role of the heat shock response in regulating inflammation, this study proposed to define the impact of selective inhibition of heat shock protein 90 (HSP90) on intestinal inflammation. Using multiple preclinical mouse models of inflammatory bowel diseases, we demonstrate a potent anti-inflammatory effect of selective inhibition of the HSP90 C-terminal ATPase using the compound novobiocin. Novobiocin-attenuated dextran sulfate sodium-induced colitis and CD45RB^{high} adoptive-transfer colitis through the suppression of inflammatory cytokine secretion, including TNF- α . In vitro assays demonstrate that CD4⁺ T cells treated with novobiocin produced significantly less TNF- α measured by intracellular cytokine staining and by enzyme-linked immunosorbent assay. This corresponded to significantly decreased nuclear p65 translocation by Western blot and a decrease in nuclear factor- κ B luciferase activity in Jurkat T cells. Finally, to verify the anti-TNF action of novobiocin, 20-week-old TNF^{ARE} mice were treated for 2 weeks with subcutaneous administration of novobiocin. This model has high levels of circulating TNF- α and exhibits spontaneous transmural segmental ileitis. Novobiocin treatment significantly reduced inflammatory cell infiltrate in the ileal lamina propria. HSP90 inhibition with novobiocin offers a novel method of inflammatory cytokine suppression without potential for the development of tolerance that limits current antibody-based methods.

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Keywords

Heat shock protein 90; colitis

Inflammatory bowel diseases (IBD) including Crohn's disease and ulcerative colitis represent a spectrum¹ of intestinal inflammatory diseases with an increasing incidence worldwide. IBD is characterized by chronic, debilitating, uncontrolled intestinal inflammation. Regrettably, current therapies induce sustained remission in less than 50% of IBD patients,² defining an unmet need for newer therapeutics in IBD.

Intestinal inflammation is underpinned by the activation of infiltrating inflammatory cells with a concomitant surge of protein synthesis. These responses are overseen by the ubiquitous heat shock response. Heat shock proteins (HSP) are activated under cellular stress, including chemicals, heat, and exposure to cytokines.³ On expression, these HSP act as chaperones both to facilitate normal protein folding and limit proteotoxicity and to stabilize proteins and prevent degradation. We have previously described the upregulation of one such protein, HSP90, within the intestine in response to intestinal inflammation.⁴ HSP90 inhibitors have demonstrated anti-inflammatory benefits in murine models of arthritis,⁵ multiple sclerosis,⁶ sepsis,⁷ and uveitis,⁸ and so we chose to assess potential protective effects in murine models of IBD.

We have previously described a therapeutic effect of the N-terminal ATPase targeting HSP90 inhibitor 17-AAG in murine IBD models.⁹ Our overarching hypothesis is that different HSP90 inhibitors may attenuate inflammation through disparate pathways. Although 17-AAG functions depended on the transcription of factor heat shock factor 1 (HSF1), our current studies describe an anti-inflammatory mechanism of action for the HSP90 inhibitor novobiocin that is both distinct and novel. In these current studies, novobiocin failed to induce the same HSF1-dependent effects in vitro, consistent with it acting via an alternate, previously undescribed mechanism in murine IBD models. Novobiocin binds to the C-terminal ATPase to either inhibit HSP90 function and/or induce a conformational change, resulting in the loss of HSP90 activity.¹⁰ Definitively, identifying which protein chaperoned by HSP90 is targeted by novobiocin is problematic because there are currently more than 100 known HSP90 client proteins.¹¹ Nevertheless, a number of HSP90-associated anti-inflammatory pathways have been identified. Although HSPs have been shown previously to drive the activation of the anti-inflammatory gene *IL-10*,¹² they are also believed to be involved in the repression of proinflammatory cytokines, such as interleukin (IL)-1b, TNF- α , and interferon- γ (IFN- γ).^{7,13,14} The effects on cytokines involved in inflammation are generally felt to act through HSF1. Data presented in this study would suggest that novobiocin acts via a different mechanism. HSP90 inhibitors are thought to disrupt the complex of HSP90 with a range of other proteins, such as inhibitor of κ B kinase- α (IKK- α) and receptor-interacting protein, leading to degradation of IKK- α and downregulation of nuclear factor- κ B (NF- κ B) signaling.¹⁵ This is consistent with our hypothesis that multiple distinct mechanisms of action may exist for different HSP90 inhibitors.

In these studies, we examined the therapeutic benefit of the C-terminal targeted inhibition of HSP90, using novobiocin in multiple murine models of IBD. First, we investigated whether inhibition of HSP90-attenuated chemically induced dextran sodium sulfate (DSS) colitis. Using a combination of enzyme-linked immunosorbent assay (ELISA), flow cytometry, and histologic scoring, we assessed intestinal inflammation after novobiocin treatment. Due to the chronic relapsing nature of IBD, it is widely recognized that CD4⁺ T cells represent an attractive target for IBD therapeutics. As such, we chose to further evaluate the anti-inflammatory potential in T-cell-dependent model. Therefore, we assessed if HSP90 inhibition could reverse established adoptive-transfer colitis using a similar approach as with DSS colitis. We then investigated the capacity of novobiocin to suppress proinflammatory signaling pathways in T cells in vitro by assessing production and secretion of TNF- α by ELISA and flow cytometry, respectively. We reinforced these findings by assessing nuclear localization of NF- κ B subunit p65 by Western blot in concert with NF- κ B luciferase assays performed in human Jurkat T cells. Finally, we assessed the anti-inflammatory properties of novobiocin in a chronic ileitis model driven by the over-expression of the canonical IBD-associated cytokine TNF- α .

MATERIALS AND METHODS

Mice

A colony of C57BL/6J mice were maintained in house kept under specific pathogen-free conditions. RAG1^{-/-} (2216) and HSF1^{-/-} mice (129/SvEvTac; 10543) were obtained from Jackson laboratories (Bar Harbor, ME); HSF1^{+/+} littermates were used as controls for HSF1^{-/-} mice. TNF^{ARE16} mice were generously provided by Dr Jesús Rivera-Nieves, University of California, San Diego. A colony of TNF^{ARE} mice were then bred in house with age-matched and sex-matched wild-type (WT) littermates used as controls. Fecal samples from these mice were consistently negative for *Helicobacter*, protozoa, and helminths. All animals were handled according to procedures approved by the University of Colorado Institutional Animal Care and Use Committee.

Chemically Induced Acute Murine Colitis Model

Mice were treated with DSS ad libitum (36–50 kDa, 3% wt/ vol; MP Biomedicals, Santa Ana, CA) in drinking water for 7 days. Water-alone groups were included as a control. DSS groups received daily intraperitoneal injections of novobiocin (40 mg/kg) or vehicle dimethyl sulfoxide. At the time of killing, spleen, mesenteric lymph nodes, and segments of colonic tissue were excised for flow cytometric analysis of leukocyte subsets, colon lengths were assessed, and colon sections were processed for histologic examination, RNA extraction, and cytokine analyses to assess disease activity. Histologic examination was evaluated and scored by a trained pathologist blinded to the conditions of the experiments (P.J.) according to a previously described scoring system.¹⁷

Adoptive-transfer Murine Colitis Model

Naive T cells from Foxp3-GFP donor mice were isolated by magnetic enrichment of CD4⁺ cells from splenocytes (CD4 T-cell isolation kit; Miltenyi Biotec, Auburn, CA) followed by fluorescence-activated sorting of CD4⁺CD45RB^{High}Foxp3^{Neg} naive T cells. RAG1^{-/-} mice

received 5×10^5 naive T cells by intraperitoneal injection. Establishment of colitis was determined by weekly weight monitoring. On the establishment of disease, mice were treated with novobiocin ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) or vehicle (dimethyl sulf-oxide), receiving daily injections for 10 to 12 days. In accordance with Institutional Animal Care and Use Committee policy, experiments were terminated early if mice experienced 15% weight loss.

Lymphocyte Isolation

Single-cell suspensions were obtained by gently pressing the mesenteric lymph nodes (MLN) or spleen against a $70\text{-}\mu\text{m}$ cell strainer. Splenic red blood cells were lysed by 3-minute incubation in ammonium chloride lysing reagent (ACK Lysis Buffer; Invitrogen, Carlsbad, CA). Intestinal segments were opened along the mesentery and rinsed off the luminal contents with phosphate-buffered solution before cutting into 1-cm sections in phosphate-buffered solution containing 15 mM HEPES and 1 mM EDTA with vigorous agitation on a vortex mixer. The tissue was then passed through a $70\text{-}\mu\text{m}$ tissue strainer, and the process was repeated until the wash remained clear. The remaining lamina propria was digested in 1 mg/mL collagenase type VIII (C9722; Sigma Aldrich, St Louis, MO) for 10 minutes in an orbital shaker at 270 rpm and 37°C . Tissues were vortexed briefly and filtered to remove any remaining undigested material, and cells were counted before flow cytometric evaluation.

Cytokine Production Assays

Tissue explants (0.5 cm^2) were cultured for 24 hours in Dulbecco's Modified Eagle Medium (without sodium pyruvate; Cellgro Manassas, VA; supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 IU penicillin, and $100 \mu\text{g/mL}$ streptomycin; Invitrogen) culture; supernatants were then analyzed for the presence of cytokines using the Milliplex Cytokine Multiplex Assay system (Logan, UT).

Flow Cytometry

Cells from indicated compartments were incubated with fluorescent rat antimouse antibodies, including against mouse CD4 (RM4-5), CD25 (PC61.5), CD44 (IM7), CD45 (30-F11), CD62L (MEL-14), TNF- α (MP6-XT22), and Foxp3 (FJK16S) or their respective isotype controls. Live cells were identified using Live/Dead fixable Aqua dye from Invitrogen. Foxp3 staining was performed according to manufacturer's instructions (eBiosciences, San Diego, CA). Cells were stained, washed, and fixed with 2% paraformaldehyde and analyzed using the FACS Canto system (Becton–Dickinson Immunocytometry Systems, San José, CA). Postanalyses were performed using FlowJo software (Tree Star, Inc, Ashland, OR). Intracellular cytokine staining for TNF- α was performed, as previously described.¹⁸

In Vitro Stimulation Assays

Freshly isolated CD4^+ from WT mice were stimulated with plate-bound anti-CD3 antibody ($1 \mu\text{g/mL}$; eBioscience) and soluble anti-CD28 ($1 \mu\text{g/mL}$; eBioscience) with (out) novobiocin ($100 \mu\text{M}$) for 24 hours. Nuclear and cytoplasmic protein extracts were then isolated using the NE-PER extraction kit (Thermo Scientific, Waltham, MA) according to

manufacturer's instructions. Western blot of p65, AKT, and GAPDH (Cell Signaling, Danvers, MA) using denaturing conditions were performed on 4% to 15% gradient gels (Bio-Rad; Hercules, CA).

T-Cell Proliferation Assays

CD4⁺CD25⁺ regulatory T cells (Tregs) were isolated by negative selection of CD4⁺ T cells, followed by positive selection of CD25⁺ cells using the MACS Treg isolation kit (Miltenyi Biotec). Fifty thousand CellTrace Violet-labeled (Invitrogen) CD4⁺CD25^{Neg} effector T cells/well were stimulated with anti-CD3 monoclonal antibody in the presence of irradiated syngeneic antigen-presenting cells from WT mice; percentage proliferation was determined by the CellTrace profile of live effector cells at 72 hours.

Luciferase Assays

Early passage Jurkat cells were plated at 0.5×10^6 cells per well in a 6-well format and were transfected using Lipofectamine LTX with 2.5 μ g of DNA per well of NF- κ B luciferase/reporter construct. After 24 hours of transfection, cells were replated to 2.5×10^5 cells/well in a 96-well plate and treated with increasing concentrations of novobiocin or antihuman CD3/CD28 Dynabeads (8 μ L/well; Invitrogen) or both for 24 hours. Cells were then centrifuged, supernatants removed, and cells lysed in 25 μ L of passive lysis buffer (Promega, Madison, WI) before reading using the dual luciferase reporter assay system, and luciferase activity was measured on a GloMax-Multi Microplate Multimode Reader (Promega).

Statistics

Statistical analyses were performed using Student's *t* test or repeated measures analysis of variance with Graphpad Prism Data Analysis software (GraphPad Software, La Jolla, CA). Data were expressed as mean $\pm$ standard error of the mean. Statistical significance was set at $P < 0.05$.

RESULTS

HSP90 Inhibition Attenuates Chemically Induced Acute Murine Colitis

To evaluate the role of the C-terminal HSP90 inhibitor, novobiocin, on acute chemically induced murine colitis, 10-week-old WT mice were treated with novobiocin or vehicle for 7 days while receiving DSS ad libitum in drinking water. Treatment with novobiocin significantly attenuated weight loss in the DSS colitis model, as measured by repeated measures analysis of variance (Fig. 1A). This effect coincided with a decreased colon shortening, a surrogate marker of colitis, in novobiocin-treated mice compared with vehicle (Fig. 1B).

A pathologist, blinded to the experimental conditions, performed histologic assessment of colitis, which identified a significant decrease in tissue injury and inflammatory indices in novobiocin-treated colons relative to vehicle controls (Fig. 1C). This reduction in tissue injury, improved maintenance of crypt architecture, and protection of epithelial integrity along with the reduction in inflammatory infiltrate are depicted in the representative

hematoxylin and eosin micrographs (Fig. 1D). Taken together, these results support the hypothesis that inhibition of the C-terminal ATPase on HSP90 could exert anti-inflammatory benefits in murine models of IBD.

HSP90 Inhibition Alters Cytokine Secretion Profile from Colonic Explants

Based on our preliminary findings, we chose to assess the effect of novobiocin on a range of inflammatory cytokines released during colitis. Cytokine expression analysis from colonic explants from DSS colitic mice treated with novobiocin demonstrated a significant reduction in proinflammatory cytokine production following HSP90 inhibition. Release of IL-1 α was significantly attenuated after novobiocin treatment (Fig. 2). Similarly, levels of IL-2, IL-4, IL-17, and IFN- γ secretion were higher in vehicle-treated mice relative to novobiocin-treated colons. Furthermore, the expression of TNF- α , known to be critical in IBD, was significantly lower in explant cultures from novobiocin-treated animals relative to vehicle-treated mice (Fig. 2). Therefore, novobiocin seems to mediate an anti-inflammatory effect partly via decreased proinflammatory cytokine production.

HSP90 Inhibition Alleviates Established Colitis in CD45RB^{High} Adoptive-transfer Model

Given the chronic nature of IBD, we chose next to assess novobiocin function in treating established colitis with a significantly longer disease time course. On disease development, defined by significant weight loss, colitic mice were treated with intraperitoneal novobiocin for 2 weeks. Colitis improved (using weight loss as an indicator) in animals treated with novobiocin (Fig. 3A) compared with vehicle alone. Postmortem analysis revealed that novobiocin-treated mice displayed significantly less colonic shortening compared with the vehicle control mice (Fig. 3B). The protective effect of HSP90 inhibition also resulted in a visible decrease in inflammation compared with vehicle controls. Both active and chronic inflammatory indices decreased after novobiocin treatment (Fig. 3C). Furthermore, total inflammatory indices significantly decreased with novobiocin administration. Tissue from HSP90-treated mice also displayed significantly less disruption of crypt architecture, as shown by representative hematoxylin and eosin micrographs (Fig. 3D). Overall, the administration of novobiocin reduced both macroscopic and microscopic evidence of inflammation in a chronic T-cell-mediated model of established colitis.

Decreased Inflammatory Cell Infiltrate and Cytokine Production After Novobiocin Treatment

Based on the reduction in inflammatory cell infiltrate seen by histologic examination, cell populations were evaluated from the above adoptive-transfer colitis experiments, and treated groups were assessed for the presence of CD4⁺Foxp3⁺ cells. Lymphocytes were isolated from the spleen, MLN, and lamina propria and evaluated by flow cytometry. We noted that there was a relative increase in the percentage of CD4⁺Foxp3⁺ cells in the lamina propria of mice treated with HSP90 inhibitor novobiocin compared with vehicle-treated mice (Fig. 4A). Furthermore, cytokine secretion analysis of explant cultures from CD45RB^{High}-recipient mice demonstrated a similar pattern to those of DSS colitic mice, with a significant decrease in IFN- γ from vehicle-treated controls to novobiocin-treated mice (Fig. 4B). Novobiocin-treated mice also displayed significantly decreased TNF- α secretion relative to vehicle controls. In contrast, vehicle-treated and novobiocin-treated mice produced

comparable levels of IL-10. Consistent with our findings in DSS colitis, novobiocin-treated mice secreted significantly less TNF- α in chronic CD45RB^{High} colitis compared with vehicle controls.

Novobiocin-mediated Suppression of TNF- α Production In Vitro

Guided by the consistency of attenuated TNF- α production from intestinal tissues of both colitis models, we chose to focus on the production of TNF- α by CD4⁺ T cells in vitro. We observed that freshly isolated CD4⁺ T cells from WT mice produced significantly less TNF- α measured by ELISA on activation with anti-CD3/CD28 antibodies and treated with novobiocin (10 μ M) compared with those stimulated with CD3/CD28 alone (Fig. 5A). Moreover, similar findings were obtained when isolated CD4⁺ T cells were stimulated for 6 hours with phorbol12-myristate 13-acetate/ionomycin/monensin for 6 hours before TNF- α expression assessment by intracellular cytokine staining (Fig. 5B, C). Given that we had previously demonstrated an anti-inflammatory effect of the HSP90 inhibitor, 17-AAG mediated by the release of the transcription factor HSF1, we wished to assess if the reduced TNF- α production by novobiocin administration was also HSF1 dependent. Interestingly, in vitro suppression of TNF- α production by novobiocin measured by intracellular cytokine staining was unaffected by HSF1 deficiency as in HSF1^{-/-} mice, CD4 T-cell expression of TNF- α was similarly suppressed by novobiocin (Fig., Supplemental Digital Content 1, <http://links.lww.com/IBD/A411>). This is consistent with our overarching hypothesis that distinct HSP90 inhibitors attenuate inflammation via disparate pathways. In addition, in any study where a compound is determined to be anti-inflammatory, one must consider that the compound could be affecting the proliferation of CD4⁺ T cells directly. To address this question, we evaluated proliferation of CD4⁺ T cells in vitro in the presence and absence of novobiocin and determined that there is no effect on proliferation of these cells (Fig., Supplemental Digital Content 2, <http://links.lww.com/IBD/A412>).

Novobiocin Acts Through Disruption of HSP90 Chaperone Function

Previous studies have demonstrated that HSP90 can act to stabilize a number of key proteins associated with inflammatory cytokine production. We began by assessing the expression of a known HSP90 chaperoned protein AKT in response to novobiocin treatment in isolated CD4⁺ T cells. Over 24 hours, novobiocin significantly decreased the quantity of both AKT and IKK- α in the cytoplasm of CD4⁺ T cells by Western blot (Fig. 5D, E). Based on the reduction in IKK- α expression, we proceeded to assess nuclear localization of the NF- κ B p65 subunit under these conditions (Fig. 5D, E). Although novobiocin had little impact on p65 localization under basal conditions, on engagement of the T-cell receptor, the translocation of p65 to the nucleus was greatly diminished by novobiocin treatment. Based on these findings, we sought to assess functional changes in NF- κ B signaling after novobiocin administration (Fig. 5F). Using a readily transfectable human Jurkat T-cell line, we demonstrated that T-cell receptor stimulation induced NF- κ B luciferase that was significantly attenuated by novobiocin in a concentration-dependent manner. Similar findings of concentration-dependent suppression of NF κ B signaling were recapitulated in Caco-2 intestinal epithelial cells (not shown). These data suggest that novobiocin may mediate its anti-inflammatory function partly by the suppression of proinflammatory cytokine signaling and in particular downregulation of TNF- α .

Novobiocin Attenuates Inflammation in a TNF- α – dependent Chronic Ileitis Model

As an extension of our in vitro studies, we chose to assess the capacity of novobiocin to successfully attenuate inflammation in a T-cell associated TNF- α –dependent murine model of IBD. The TNF^{ARE} mice lack a critical 69–base pair AU-rich element (ARE) responsible for messenger RNA stability, leading to the overproduction of TNF- α . These mice recapitulate many features of Crohn's disease including spontaneous discontinuous transmural ileitis along with common IBD-associated extraintestinal manifestations, such as bone catabolism and debilitating arthropathy. TNF^{ARE} mice aged 20 weeks were treated for 2 weeks with novobiocin (40 mg·kg⁻¹·d⁻¹) before the assessment of intestinal inflammation. Ileal sections were scored by a trained pathologist in a blinded fashion (P.J.) with quantification of ileitis subdivided into acute inflammatory indices (an index of granulocytic infiltration), chronic (lymphocytic infiltrate), and a villus distortion index (a measure of the loss of villus architecture associated with ileitis; Fig. 6A). Novobiocin significantly decreased indices of inflammation, greatly reducing inflammatory cell infiltrate, and improved overall tissue architecture (Fig. 6B).

The decrease in inflammatory scores corresponded to a reduction in inflammatory cell infiltrate, observed in digested ileal lamina propria of novobiocin-treated mice relative to vehicle controls (Fig. 7A) along with a decrease in the critical CD4⁺CD44^{High}CD62L^{Low} effector T cells (Fig. 7B, C), which can alone confer disease to T-cell–deficient mice.¹⁹ This effect was largely restricted to the ileal lamina propria as similar differences failed to reach statistical significance in the spleen or MLN. Thus, results from the TNF^{ARE} model recapitulate previous findings that novobiocin can successfully attenuate inflammation in murine models of IBD and extend these findings to include both small and large intestinal IBD models.

DISCUSSION

This study was designed to assess the therapeutic potential of C-terminal ATPase HSP90 inhibition using novobiocin for the treatment of IBD. We had previously demonstrated that a specific N-terminal HSP90 inhibitor provided protection in murine colitis⁴ and were keen to ascertain if targeting specific termini on HSP90 would produce a differential outcome. The results presented herein demonstrate that indeed novobiocin does offer protection in preclinical models of IBD; however, novobiocin offers a distinct mechanism from 17-AAG to mediate this anti-inflammatory outcome. Rather than driving proregulatory pathways as with 17-AAG, novobiocin suppressed inflammation by downregulating proinflammatory cytokine signaling.

Because of its rapid onset and proven use as a relevant model for the translation of mice data to human disease,²⁰ we chose to begin by assessing the therapeutic potential in DSS colitis. Novobiocin attenuated murine colitis and protected against severe weight loss, reminiscent of our previous studies with 17-AAG. However, unlike 17-AAG, novobiocin suppressed proinflammatory cytokine secretion rather than enhancing anti-inflammatory IL-10 production. A marked increased secretion of proinflammatory cytokines from DSS colitis tissue explants was attenuated in novobiocin-treated animals.

Although the DSS data offers considerable insight into the potential benefit of novobiocin administration, this model is not without limitations, such as its phenotype nature and innate immune cell predominance, which fails to completely reflect the nature of human IBD. Moreover, although DSS induces colitis, albeit attenuated, in the absence of T cells,²¹ the importance of CD4⁺ T cells in IBD is well recognized. This prompted us to extend our investigations to a second model of murine colitis for the validation of our previous results. Using the CD45RB^{High} model of adoptive-transfer colitis, we were able not only to target a T-cell-dependent IBD model with a more chronic inflammation profile but also to one in which we could treat established disease and thereby more faithfully reflect the clinical context. Once again, novobiocin greatly attenuated proinflammatory cytokine secretion, consistent with data from the DSS colitis experiments and suggesting a common mechanism of action in both models mediated partly via the reproducible suppression of TNF- α secretion.

Guided by our initial in vivo observations, we chose to assay TNF- α production in isolated CD4 T cells in vitro. Using multiple experimental approaches, we observed that novobiocin attenuated TNF- α production by CD4⁺ T cells through destabilization of IKK- α and increased degradation of the well-established HSP90 chaperone target AKT, leading to reduced nuclear trans-location of the NF- κ B p65 subunit. This is particularly important given the well-established role of NF- κ B translocation in T-cell activation and proliferation.²² Although this novobiocin-mediated effect has not been described in T cells previously, similar undertakings in RAW macrophages showing that a distinct HSP90 inhibitor gambogic acid suppresses TNF- α production over 6 hours along with blocking translocation of RelA/p65 to the nucleus.²³ Interestingly, while inhibition of NF- κ B signaling occurs with the inhibition of HSP90, antagonism of this signaling pathway has also been demonstrated to occur via induction of hsp72.²⁴ The rapid upregulation of hsp90²⁵ in contrast with the delayed sustained upregulation of hsp72²⁶ suggest that the heat shock response may offer both positive and negative mechanisms for tight regulation of the kinetics of this critical pathway to limit persistent inflammation.

The beneficial implications of novobiocin in both murine models of colitis is somewhat complicated by the dependence of both models on bacterial flora. Novobiocin also possesses antibiotic activity as a DNA gyrase inhibitor that binds to the ATPase active site located on the GyrB subunit and inhibits transduction of DNA gyrase in prokaryotes.²⁷ In either DSS colitis or adoptive-transfer colitis, reduced bacterial loads suppressed the induction of colitis, although not previously established colitis.^{28,29} Nevertheless, studies using concomitant administration of broad-spectrum antibiotics and DSS observed either an increase in proinflammatory cytokine production relative to controls or no difference in disease severity depending on the source of C57BL/6 mice used,³⁰ consistent with our hypothesis that the beneficial effect of novobiocin is not due to its antibiotic function alone. In addition, the fact that we see alterations in TNF- α secretion in our in vitro studies where bacteria do not play a role would suggest that the anti-TNF- α effect seen with novobiocin is not a result of the antibacterial effect of novobiocin.

Having demonstrated the benefit of novobiocin in murine colitis models, we chose to further validate these findings in a truly chronic T-cell-dependent model that offers 2 additional

benefits: first, that it is a TNF- α -dependent model and therefore ideal for testing a compound that we hypothesize exerts an anti-inflammatory effect mediated partly by TNF- α suppression, and second, previous studies in TNF^{ARE} mice have demonstrated that quercetin, an alternative DNA gyrase inhibitor, fails to attenuate inflammation in mice.³¹ Once again, novobiocin attenuated intestinal inflammation in this model, unlike quercetin, consistent with its beneficial effect not being dependent on its role as a DNA gyrase inhibitor. Given the evidence, it is enticing to speculate that this ameliorating effect was largely due to its ability to suppress NF- κ B signaling instead. Although HSP90 has been well documented to inhibit NF- κ B signaling by the destabilization of the IKK complex, preventing release of p65, our studies describe an additional mechanism whereby blockade of AKT stabilization allows degradation of this kinase and provides the additive inhibitory function to account for the significant decrease in both lucif-erase activity and TNF- α production seen in T cells in vitro. Broad multicenter trials on antibiotic use in pediatric IBD³² along with retrospective studies have not only highlighted the therapeutic benefit of antibiotics for IBD treatment but also indicated that they are currently a mainstay therapy.^{33,34} Whereas clinical applications for HSP90 inhibitors are generally somewhat limited because of their narrow therapeutic range, novobiocin has been proven safe in a number of clinical phase 1 trials.^{35,36} Given the favorable track record of novobiocin as an antibiotic for more than 50 years,³⁷ these newly described novel anti-inflammatory mechanisms mediated by novobiocin support its application to the treatment of IBD. In conclusion, although antibiotics are commonly used as a primary treatment of IBD, with biologics such as anti-TNF- α antibodies reserved for the treatment of severe disease, novobiocin may offer an alternative approach that may combine the therapeutic properties of both of these common strategies without the potential for the development of a loss of response that often occurs with anti-TNF- α antibodies. Moving forward, the findings presented herein provide tractable evidence to reexamine novobiocin administration, with its pleiotropic effects as a potential therapy for the treatment of mucosal diseases, such as IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

HSP90 inhibition attenuated chemically induced murine colitis. A, Weight loss of mice treated with vehicle or novobiocin during DSS colitis. Mean percent weight loss $\pm$ standard error of the mean from $n = 8$ mice. $***P < 0.001$ based on t tests of individual time points. Results representative of 3 independent experiments. B, Colon length of mice after DSS colitis, measured postmortem. C, Histologic evaluation of indices of inflammation (injury and inflammation) by a trained pathologist (P.J.) in a blinded fashion. $**P < 0.01$. D, Representative micro-graphs of colonic hematoxylin and eosin sections from mice in each treatment group. Black scale bar indicates 100 μm .

FIGURE 2.

Suppressed proinflammatory cytokine profile from the inflamed colon after HSP90 inhibition. Multiplex analysis of IL-1α, IL-2, IL-4, IL-17, IFN-γ, and TNF-α expression from 24-hour culture of colonic explants from DSS colitic mice treated with novobiocin. Mean ± standard error of the mean for n = 4 mice. ** $P < 0.01$; *** $P < 0.001$. Results representative of 3 independent experiments.

FIGURE 3.

HSP90 inhibition attenuates established adoptive-transfer colitis. A, Weight-loss curve of RAG1^{-/-} mice adoptively transferred CD45RB^{High} cells treated with vehicle or novobiocin on establishment of disease. * $P < 0.05$; *** $P < 0.001$. B, Postmortem measurement of colon length of CD45RB^{High} cell recipient with(out) treatment with novobiocin (40 mg⁻¹·kg·d⁻¹). Mean $\pm$ standard error of the mean. ** $P < 0.01$. C, Histologic evaluation of indices of active and chronic inflammation in CD45RB^{High} model of colitis by a trained pathologist in a blinded fashion. ** $P < 0.01$. D, Representative micrographs of colonic hematoxylin and eosin sections from mice in each treatment group. Black scale bar indicates 100 μ m. Results representative of 3 independent experiments with $n = 8$ mice per treatment group.

FIGURE 4.

HSP90 inhibition suppresses inflammatory cytokine production, increasing relative Foxp3 expression in the inflamed colon. **A**, Representative zebra plot of CD4⁺ T cells from the lamina propria of CD45RB^{High} colitic mice treated with vehicle or novobiocin, showing expression of Foxp3 gated on live CD45⁺ cells. **B**, Percentage of CD4⁺ T cells that are Foxp3⁺ from indicated organs of colitic mice. * $P < 0.05$. **C**, Multiplex analysis of IFN- γ , TNF- α , and IL-10 expression from 24-hour culture of colonic explants from CD45RB^{High} adoptive-transfer colitic mice treated with vehicle alone or combined with novobiocin. Mean $\pm$ standard error of the mean for $n = 4$ individual mice. Results representative of 3 independent experiments. * $P < 0.05$.

FIGURE 5.

Novobiocin suppresses TNF- α production via decreased NF- κ B activity. Secretion of TNF- α from isolated CD4⁺ T cells (A) cultured for 24 hours in the presence of vehicle or novobiocin (100 μ M) measured by ELISA. B, Quantification of intracellular expression of TNF- α from CD4 T cells stimulated with phorbol12-myristate 13-acetate/ionomycin/monensin for 6 hours in the presence or absence of novobiocin (100 μ M). C, Representative dot plots of live CD4⁺ T-cell intracellular expression of TNF- α . D, Western blot analysis of nuclear and cytoplasmic expression of AKT, IKK- α , and nuclear p65 expression from isolated murine CD4⁺ T cells stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies for 1 hour in the presence or absence of novobiocin (100 μ M). E, Densitometric evaluation of expression of AKT, IKK- α , and p65 results represent mean $\pm$ standard error of the mean for 3 individual experiments. * P < 0.05; ** P < 0.001; *** P < 0.0001. F, Relative NF- κ B luciferase activity of Jurkat cells stimulated with CD3/28 Dynabeads along with increasing concentrations of novobiocin for 24 hours. Mean $\pm$ standard error of the mean (n 4). * P < 0.05; ** P < 0.01; *** P < 0.001. Results representative of 3 independent experiments.

FIGURE 6.

Novobiocin attenuated chronic murine ileitis in 20-week-old TNF^{ARE} mice. Two-week treatment with novobiocin (40 mg·kg⁻¹·d⁻¹) significantly attenuated histologic indices of inflammation. A, Histologic evaluation of indices of active and chronic inflammation and degree of villus distortion in 20-week-old TNF^{ARE} model of ileitis by a trained pathologist in a blinded fashion. **P* < 0.05. B, Representative micrographs of colonic hematoxylin and eosin sections from mice in each treatment group. Black scale bar indicates 100 μm. Results representative of 3 independent experiments.

FIGURE 7.

Decreased inflammatory cell infiltration in the ileum lamina propria after novobiocin treatment. A, Total cellularity of indicated organs from 20-week-old TNF^{ARE} mice treated with vehicle or novobiocin. Results represent mean ± standard error of the mean for n = 4 individual mice/group and representative of 2 comparable studies. **P* < 0.05. B, Representative zebra plots of the expression of CD44 and CD62L on live CD4⁺ T cells from the ileal lamina propria of TNF^{ARE} mice treated with either vehicle or novobiocin for 2 weeks gated on live CD45⁺ CD4⁺ cells. C, Quantification of relative frequency of effector (CD44^{High}CD62L^{Low}), central memory (CD44^{High}CD62L^{High}), naive (CD44^{Low}CD62L^{High}), and total CD4 T cells by flow cytometry. D, Quantification of effector T-cell expression measured by flow cytometry from indicated organs. E, ELISA analysis of TNF expression from 24-hour culture of ileal explants from 20 week old TNFDARE mice treated with Vehicle or Novobiocin for 2 weeks. **P* < 0.05; ****P* < 0.001. Results represent mean ± standard error of the mean for n = 4 separate animals and reflect similar patterns from 2 independent studies. Results are representative of 3 independent experiments with similar findings.