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Current Therapeutic Research

journal homepage: www.elsevier.com/locate/cuthrePossible Therapeutic Effect of Trilostane in Rodent Models of Inflammation and Nociception[☆]David Tung, PhD^{1,*}, John Ciallella, PhD², Heather Hain, PhD², Peter H. Cheung, PhD¹, Saurabh Saha, MD, PhD¹¹ BioMed Valley Discoveries, Kansas City, Missouri² Melior Discovery, Inc, Exton, Pennsylvania

ARTICLE INFO

Article history:

Accepted 26 September 2013

Key words:

3 β -hydroxysteroid dehydrogenase
inflammation
trilostane

ABSTRACT

Background: Trilostane was identified in an in vivo screen of compounds in a lipopolysaccharide model of inflammation to support a repurposing effort. There is no previous documentation of any anti-inflammatory effects of trilostane.

Objective: The aim of this study was to elucidate the novel pharmacologic activity of trilostane in a series of inflammation and nociception signal-finding models.

Methods: Anti-inflammatory effects of trilostane were evaluated in lipopolysaccharide-induced systemic and lung inflammation models and in a 2,4-dinitrofluorobenzene-induced delayed-type hypersensitivity (DTH) model in the mouse ear. The analgesic activities of trilostane were evaluated in a hot plate nociception model as a function of paw-withdrawal latency and in the formalin-induced nociception model with a behavioral end point. In all studies, trilostane was administered 15 minutes before challenge. In the DTH model, the animals were given a second dose 24 hours after the first dose.

Results: Trilostane inhibited tumor necrosis factor- α and monocyte chemoattractant protein-1 production in the lipopolysaccharide-induced systemic and pulmonary inflammation models. It also significantly reduced ear swelling in the 2,4-dinitrofluorobenzene-induced DTH model. In the hot plate nociception model, trilostane increased the latency of paw-licking behavior. Trilostane also significantly reduced the duration of pain behaviors in the late phase of the formalin-induced inflammatory pain model.

Conclusions: These signal-finding studies suggest that trilostane has novel anti-inflammatory and analgesic properties.

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Introduction

The drug discovery process is a financially intensive and time-consuming process with a high failure rate.¹ Many of the failures are due to safety issues that arise in Phase I human studies. One novel approach to lessen this risk is by “repurposing” existing US Food and Drug Administration (FDA)-approved drugs for alternative indications.^{2,3} These drugs have well-documented safety profiles in humans, which can potentially speed up the drug discovery process and mitigate risks.⁴ It has long been recognized that many existing drugs have off-target effects, which may be beneficial for secondary indications that the drug was not originally intended to target.

However, these additional activities are often not well characterized. These considerations have led to efforts to repurpose existing drugs for novel indications. A number of investigators have screened panels of existing drugs against alternative indications and have identified new indications for these drugs.⁵

Although there are a number of anti-inflammatory drugs available in the United States that target a variety of pathways, there is still an unmet market for new drugs that have fewer adverse effects and are more efficacious.⁶ In an effort to identify new anti-inflammatory agents, we screened a library of FDA-approved compounds in several mouse models of inflammation.

Trilostane, an inhibitor of 3 β -hydroxysteroid dehydrogenase, was found to inhibit tumor necrosis factor- α (TNF- α) production in a systemic lipopolysaccharide (LPS) challenge model during our screening process. We then confirmed this signal in a pulmonary LPS challenge model and a delayed-type hypersensitivity model induced by 2,4-dinitrofluorobenzene (DNFB). Because pain is a frequent component in inflammatory disease, we further evaluated trilostane in a thermal nociceptive pain model and an inflammatory pain model. Our observations are summarized in this brief report.

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Methods

The anti-inflammatory effects of trilostane were studied in 3 animal models of inflammation: systemic and pulmonary LPS-induced inflammation models and a DNFB-induced delayed-type hypersensitivity model. The analgesic properties of trilostane were studied in the hot plate nociception model and the formalin-induced inflammatory pain model. All in vivo protocols were approved by the Institutional Animal Care and Use Committee of Melior Discovery, Inc (Exton, Pennsylvania).

Animals

Male CD-1 (ICR) mice (Ace Animals, Boyertown, Pennsylvania) 8 to 10 weeks of age were used in all models. The animals were housed 6 per cage and kept on a standard 12-hour light cycle. The mice were provided food and water ad libitum. Six animals were used in each group.

Drug administration

Trilostane (LKT Laboratories, Inc, St Paul, Minnesota) was administered intraperitoneally at 30 mg/kg in all studies. This dose level was based on the highest reported dose of trilostane in mice that did not have any adverse effect.⁷ The vehicle was 0.4% Tween 80 (ICI Americas Inc, Bridgewater, New Jersey) in saline. The positive controls were dexamethasone 20 mg/kg for all the inflammation models or oxycodone 10 mg/kg for the pain models.

Systemic inflammation

LPS (heat-killed *Escherichia coli* 0127:B5; Sigma-Aldrich Corporation, St Louis, Missouri) was prepared in phosphate-buffered saline (PBS) at a concentration of 0.025 mg/mL and injected at 10 mL/kg intraperitoneally for a final dose of 0.25 mg/kg.^{8–11} Trilostane was administered 15 minutes before the LPS challenge. Dexamethasone was used as a positive control and was administered at the same time as the test article. Ninety minutes after the LPS challenge, blood samples were collected via the retro-orbital route. Serum was collected, and TNF- α levels were measured by ELISA (R&D Systems Inc, Minneapolis, Minnesota).

Pulmonary inflammation

Mice were prophylactically dosed with trilostane, dexamethasone, or vehicle 15 minutes before intranasal challenge with LPS. For intranasal administration of LPS, the mice were anesthetized by using an intraperitoneal injection of ketamine/xylazine (8 mg/1.2 mg in 1 mL of PBS) and suspended vertically while 50 μ g of LPS (in 1 mg/mL) was instilled into the nares in 3 boluses.^{12,13} Mice were then held vertically for 5 minutes to allow full penetration of LPS into the lungs. Four hours after the LPS challenge, the mice were killed by carbon dioxide exposure, and the lungs were collected and homogenized in 1 mL of magnesium and calcium-free PBS containing a protease inhibitor cocktail (Sigma-Aldrich Corporation, #P8340). Homogenates were centrifuged at 3000 rpm for 10 minutes, and the supernatant was analyzed for levels of monocyte chemoattractant protein-1 (MCP-1) by ELISA (Thermo Fisher Scientific Inc, Rockford, Illinois).

Delayed-type hypersensitivity

Mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (8 mg/1.2 mg in 1 mL of PBS). Their backs were shaved and sensitized with 0.5% DNFB (in 4:1 acetone/olive oil) twice over 2 consecutive days.^{14,15} Five days after sensitization,

0.2% DNFB was applied with a brush to the dorsal surface of the right ear, while the left ear was painted with acetone as a control. Ear thickness at 3 separate locations was measured before challenge and then at 24 and 48 hours after challenge. Trilostane was administered 15 minutes before challenge and 24 hours after the first dose.

Hot plate analgesia

Mice were treated with trilostane, oxycodone, or vehicle 15 minutes before exposure to the hot plate. The animals were individually placed on a preheated 52°C hot plate (Columbus Instruments, Columbus, Ohio). An open-ended cylindrical plexiglass tube with a diameter of 30 cm was placed on top of the hot plate to prevent the mice from escaping but leaving their paws exposed to the hot plate.^{16–18} Time from placing the animals on the hot plate to the time of the first paw lick was measured with a stopwatch. To prevent tissue damage, the mice were removed from the hot plate after 1 minute regardless of their response.

Formalin-induced analgesia

Mice were treated with trilostane, oxycodone, or vehicle 15 minutes before formalin injection. A total of 20 μ L of 2% formalin was injected into the left hind paw, nearly parallel to the plantar surface of the mid-foot. The animals were observed, and the amount of time spent exhibiting pain behaviors (licking, biting, shaking, tucking, or favoring the injected paw) was recorded. The cumulative amount of time spent exhibiting pain behaviors within each of the six 5-minute increments was analyzed for a total of 30 minutes per mouse.^{19–22} Data were reported as the cumulated duration of pain-related behaviors within each time interval.

Data analysis and statistics

All data are presented as mean (SEM), and ANOVA was used to analyze statistical significance. Analysis of the data was performed by using MS Excel (Microsoft Inc, San Jose, California).

Results

LPS-induced systemic inflammation

Systemic LPS challenge was used as a first-tier model to confirm the anti-inflammatory effects of trilostane, which were initially identified in the same model in the screen. The serum TNF- α level serves as a biomarker to gauge the level of anti-inflammatory efficacy. Intraperitoneal challenge with 0.25 mg/kg of LPS resulted in an increase of 3049 pg/mL of TNF- α in the serum by 90 minutes. In the mice that had been pretreated with 30 mg/kg of trilostane before LPS challenge, the TNF- α level was significantly reduced by 64.3% to 1088 pg/mL ($P < 0.05$). The response to trilostane was not as great as that of the positive control (20 mg/kg of dexamethasone), which resulted in background levels of circulating TNF- α (Figure 1A).

LPS-induced pulmonary inflammation

The pulmonary challenge using LPS via intranasal instillation served as a model for local inflammation. MCP-1 levels in lung homogenates served as a biomarker to assess the anti-inflammatory effects of trilostane in this model. In the LPS-challenged animals that were treated with vehicle control, the levels of MCP-1 in the lung increased from undetectable to 12,077 pg/mL at 4 hours' postchallenge. This elevation of MCP-1 was significantly reduced by trilostane pretreatment (by 71% to

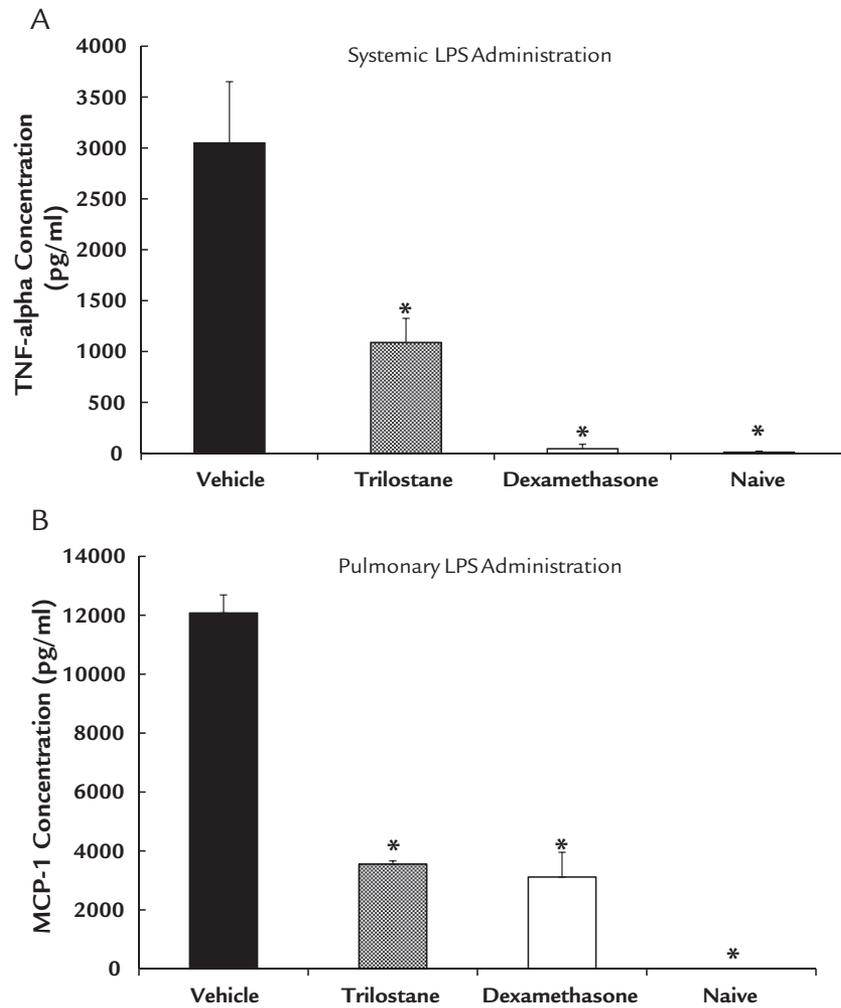


Figure 1. (A) Systemic challenge with LPS led to an increase in serum TNF- α level which was significantly reduced by trilostane treatment. Dexamethasone treatment completely inhibited TNF- α up-regulation (* $P < 0.05$). (B) The animals were given intranasal doses of LPS for pulmonary challenge. At 4 hours post challenge, the lung homogenate MCP-1 level of the vehicle group reached 12077 pg/ml. Trilostane and dexamethasone treatment significantly inhibited this MCP-1 elevation (* $P < 0.0001$).

3554 pg/mL; $P < 0.0001$). This level of inhibition is statistically identical to that provided by the positive control of dexamethasone 20 mg/kg (Figure 1B).

DNFB-induced delayed-type hypersensitivity

The immunomodulatory effects of trilostane were examined in a contact hypersensitivity model induced by DNFB. The animals were sensitized to DNFB and then challenged on the right ear 5 days later. Ear thickness increase was used as an end point. After the initial sensitization, there was no increase in the ear thickness. On challenge 5 days later, the DNFB-challenged animals that were treated with vehicle control experienced an increase in ear thickness of 76% to 0.34 mm. The trilostane-treated animals exhibited less swelling, with a maximum thickness of 0.27 mm at 48 hours. This finding constitutes a statistically significant 19% reduction ($P < 0.05$) compared with the vehicle-treated group. The animals that were treated with the positive control (dexamethasone) exhibited baseline levels of swelling (Figure 2). As a measure of the animals' health, their weight was measured before and after the dosing period. There was no decrease in weight, and the weight of the treatment group was not significantly different from the vehicle-treated animals.

Hot plate nociception

The hot plate nociception model was used as a first-line signal-finding model for evaluating the analgesic potential of test

compounds. In this model, vehicle-treated mice began exhibiting paw-licking behaviors at 10.8 seconds after exposure to the hot plate. Trilostane significantly delayed the onset of this behavior to 16.5 seconds ($P < 0.05$). This finding represents a 34.2% increase in

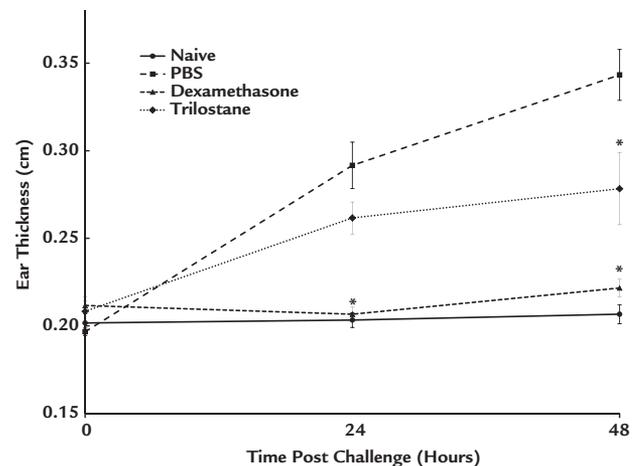


Figure 2. At 5 days post DNFB sensitization, the animals were challenged topically with DNFB on their right ears. Trilostane significantly reduced the resulting ear swelling at 48 hours post treatment, but the effect was not significant at 24 hours. Dexamethasone treatment completely inhibited the swelling response at all time points (* $P < 0.05$).

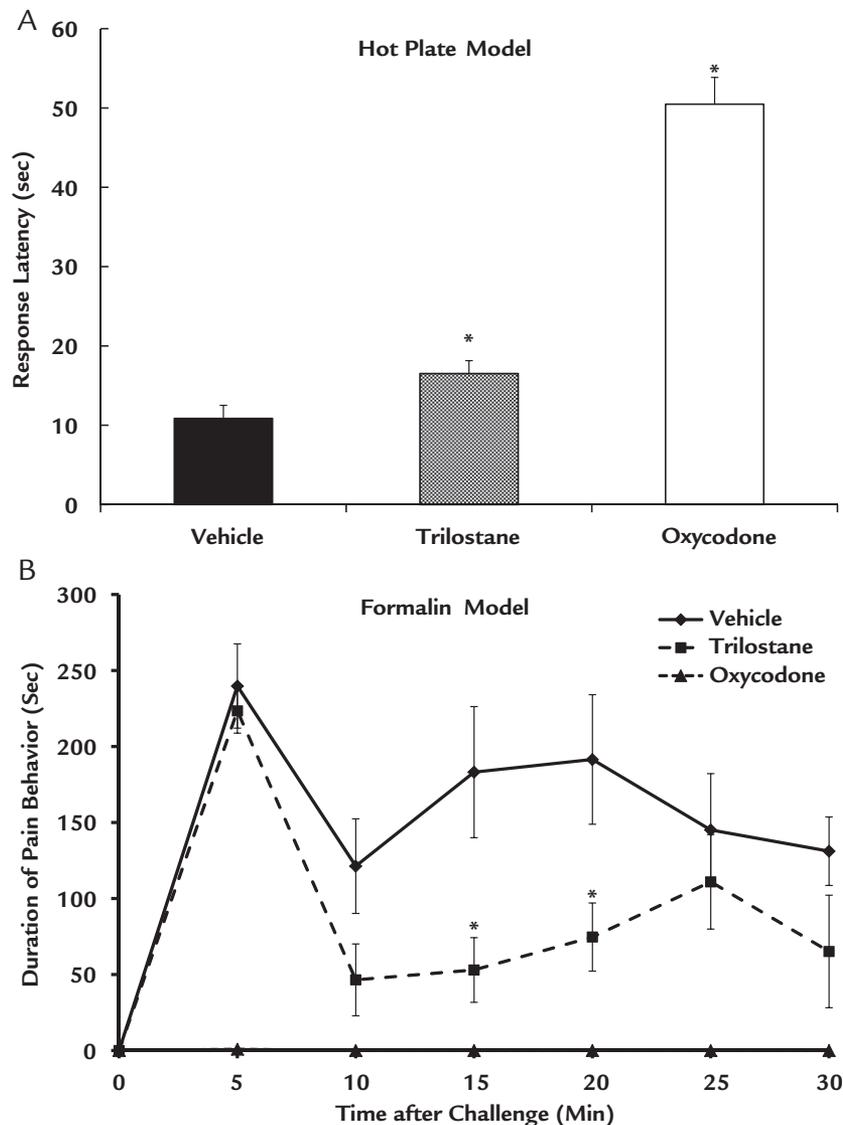


Figure 3. (A) Trilostane or oxycodone treatment was administered 15 minutes prior to the hot plate challenge. The vehicle group took an average of 10.5 seconds to react to the thermal stimulus. Trilostane and oxycodone treatment significantly extended the response time to 16.5 seconds and 50.5 seconds, respectively ($*P < 0.05$). (B) Formalin injection into the footpad resulted in a pain related licking response which diminished and entered into a secondary phase at 10 minutes post challenge. Trilostane pretreatment had no effect on the peak acute phase response, though it did modulate the response slightly towards the end of the acute phase. However, the animals demonstrated significantly reduced response at the 15 and 20 minutes segments during the secondary phase. Oxycodone treatment completely inhibited the acute and secondary phase response ($*P < 0.05$).

latency. The animals treated with the positive control (oxycodone) exhibited an increased latency of 500% to 50.5 seconds (Figure 3A).

Formalin-induced nociception

Formalin injection into the mouse paw produces a pain-related irritation that elicits a licking response. The frequency of this response can be used as a measure of a test compound's analgesic property. The response can be divided into an acute phase (phase I) that lasts for 10 minutes, followed by a late phase (phase II) that lasts for ~30 minutes after the injection. The vehicle-treated animals exhibited a cumulative licking response of 239.8 seconds within the first 5 minutes. Trilostane pretreatment had no effect on the peak of the acute phase response; however, between the 5- and 10-minute segment of the acute phase, the cumulative duration of response was reduced to 121.3 seconds. Although trilostane decreased this response duration to 46.5 seconds, this effect was not statistically significant. However, in the late phase,

trilostane significantly decreased the cumulative response ($P < 0.05$) in the 10- to 15-minute and the 15- to 20-minute time segments to 53.0 seconds and 74.7 seconds, respectively, compared with 121.3 seconds and 183.2 seconds for the vehicle-treated group. This outcome constitutes a 62% and a 71% reduction, respectively. The positive control, oxycodone, completely abolished the licking behavior in phases I and II (Figure 3B).

Discussion

Despite intense efforts in the pharmaceutical industry to discover new treatments for inflammatory diseases and pain, there is still a major unmet need for patients who are refractory to current therapeutic options or cannot tolerate the substantial adverse effects.⁶ One potential source of new therapeutic agents is the existing pharmacopeia. Most existing drugs have off-target activities or the intended target is present in additional organs,

both of which can be beneficial in additional indications. There are several examples of such repurposing successes. For example, sildenafil was identified as a treatment for erectile dysfunction despite its lack of efficacy for angina.²³ In recent years, several groups have sought to identify and characterize the off-target activities of existing drugs by screening libraries of these compounds in their models of interest. These programs have resulted in the approval of miltefosine for the treatment of visceral leishmaniasis,²⁴ ceftriaxone as a potential treatment for amyotrophic lateral sclerosis,²⁵ and astemizole as an antimalarial agent.²⁶

We sought to identify potential new treatments for inflammatory diseases and their associated pain by screening our library of FDA-approved drugs by using several animal models of inflammation. The anti-inflammatory activity of trilostane was first identified in an LPS-induced systemic inflammation model, in which trilostane significantly inhibited serum TNF- α levels post-LPS challenge. TNF- α is 1 of the key mediators involved in inflammatory diseases. This homotrimeric cytokine is first synthesized as a membrane-bound protein, then cleaved by proteases to release the soluble cytokine. It is produced by all inflammatory cells and by many noninflammatory cells.²⁷ Two key membrane-bound TNF receptors can be cleaved into soluble forms: TNFR1 and TNFR2. TNFR1, expressed on almost all nucleated cells, preferentially binds soluble TNF- α , even though it is activated by both the soluble and membrane-bound TNF- α .²⁸ The expression of TNFR2 is mostly restricted to endothelial cells and some immune cells, such as T cells, B cells, monocytes, and natural killer cells.^{27,29} TNFR2 can only be fully activated by membrane TNF- α and not by soluble TNF- α , even though it can bind both forms.^{30,31} The concentration of soluble receptors can mediate TNF-dependent biologic effects by scavenging soluble TNF- α . Due to the high binding affinity of the soluble form of TNFR2 to TNF- α , it can decrease TNF-mediated activities by scavenging the cytokine that can potentially interact with TNFR1.³² Therefore, a decrease in serum TNF- α levels as observed in the present study is potentially due to inhibition of TNF- α release or the scavenger effect of the soluble receptors.

This observation in the systemic LPS challenge study led to a series of subsequent signal-finding studies to elucidate its full anti-inflammatory potential. The anti-inflammatory effects of trilostane were further confirmed in a tissue-specific LPS-induced pulmonary inflammation model using MCP-1 as a biomarker. The MCP-1 level in lung tissue was significantly attenuated by trilostane treatment. To further expand the understanding of trilostane's anti-inflammatory activity, we also tested the agent in a delayed-type hypersensitivity model, in which we found that DNFB-induced ear swelling was significantly reduced by trilostane.

Because pain is often a co-factor in inflammatory diseases, and there is a large unmet medical need for novel pain medications, our goal was to determine if trilostane has any analgesic effects. Unfortunately, the inflammation models used in this set of studies do not have a strong pain component; therefore, we explored the analgesic effects of trilostane in several nociception models. We found that trilostane treatment produced a significant increase in latency of the response to thermal stimulus from a hot plate. However, the response was relatively small compared with that generated by oxycodone. Similarly, in the formalin-induced nociception model, trilostane treatment resulted in a significant reduction in pain response behaviors; oxycodone completely attenuated any pain-related behavioral response in the animals, however.

Although trilostane treatment did have a significant effect on this pain response during certain time segments in the late phase of the model, the magnitude of the response was not as potent as that of oxycodone, and it failed to affect the magnitude of the

acute phase response. The dose level for trilostane was selected based on the highest dose published for mice in the literature that did not have adverse effects.⁷ Because oxycodone is the standard of care for many pain indications, the comparatively mild effects of trilostane suggest that it would not be competitive as an analgesic agent.

One question that arises from these observations is whether the analgesic effect of trilostane on the formalin-induced pain behavior was due to its anti-inflammatory properties or whether it was a purely analgesic effect. The best evidence for anti-inflammatory-independent analgesic effects of trilostane can be seen in the hot plate-induced nociception model. The onset of the paw-withdrawal response was rapid, and there is no inflammatory component in this short-acting model; however, trilostane was able to affect paw-withdrawal latency. Formalin is a strong irritant and is capable of inducing a potent pain response that results from a combination of nociceptive and subsequent inflammatory effects. Although dissecting out which portion of this response was inhibited by trilostane would be challenging, understanding the mechanism in play at each stage of the model might shed some light on trilostane's mechanism of action. Phase 1 of the model involves the direct activation of nociceptors.³³ The second phase is sensitive to NSAIDs, mild analgesics, prostaglandin synthesis inhibitors,³⁴ and anti-inflammatory steroids (hydrocortisone, dexamethasone), as well as some centrally active drugs such as gabapentin.²⁰ A number of targets have been implicated in late-phase formalin inflammatory pain models. These include casein kinase 2, nitric oxide, nitric oxide synthase, arachidonic acid, cytokines, TNF- α , activation of p38 MAPK, and MAP kinase kinase 3.^{35,36} It is difficult to tell which of these targets, or which combination of them, plays a part in the analgesic effect of trilostane without detailed *in vitro* analyses, which is beyond the scope of this brief report. The attenuation of the pain behaviors observed in the late stage of this model was probably due to a combination of the anti-inflammatory effect and the analgesic effects of trilostane. One good alternative approach would be to demonstrate the analgesic effects of trilostane in a purely nociceptive model without an inflammatory component (ie, the hot plate model) as described in this article. We also attempted to apply a quantitative visual scoring system to the hind paws of the formalin-injected mice in an effort to determine if trilostane demonstrates any anti-inflammatory properties in the formalin-induced hyperalgesia model. Unfortunately, this effort was unsuccessful because the inflammation was not intense enough to generate adequate swelling to make such a scoring system feasible. Due to the small magnitude of swelling, the pharmacologic window was therefore not large enough to observe a trilostane-mediated anti-inflammatory effect.

Trilostane is an inhibitor of 3 β -hydroxysteroid dehydrogenase, which catalyzes the conversion of pregnenolone to progesterone and is critically involved in the production of corticosterone and aldosterone production in the adrenal glands.³⁷ This mechanism of action is not thought to play a role in inflammation or analgesia, suggesting that trilostane may affect other mechanisms involved in these indications. Elucidating the exact mechanism of action of trilostane in inflammatory and nociception would be difficult and is beyond the scope of this brief report.

Glucocorticoid steroids such as dexamethasone are used as a first-line treatment for inflammatory diseases. It is important to determine whether the anti-inflammatory and analgesic activities of trilostane are mediated via glucocorticoid receptors or whether these results indicate that a novel mechanism is involved. In sheep, it is known that trilostane increases the activity of 11 β -hydroxysteroid dehydrogenase,³⁸ and it is not clear whether this effect or other off-target activities in mice are responsible for our findings.

Conclusions

The results of these studies suggest that trilostane has novel anti-inflammatory and analgesic activities in mouse models. To our knowledge, this effect had not been reported previously. It is beyond the scope of this short communication to address the mechanism behind these effects. In April 1994, trilostane was withdrawn by the FDA from human use in the US market. However, it is still available for human use in the United Kingdom for the treatment of breast cancer and Cushing's disease.^{39,40} Patients with postmenopausal breast cancer are usually treated with antiestrogens and aromatase inhibitors. Unfortunately, resistance to these treatments often occurs over time. Trilostane, as an allosteric modulator of the estrogen receptor that targets both the estrogen and growth factor-dependent pathways, offers an alternative approach.³⁹ For the treatment of life-threatening diseases such as cancer, the use of trilostane can be justified. The application of trilostane in pain and inflammatory disease remains to be seen; however, the safety profile of trilostane is likely to prohibit its long-term use in patients with these conditions. Moreover, the effects of the current standard of care in pain and inflammatory disease models are superior to trilostane, making trilostane's long-term use in these disease areas less desirable.

Acknowledgments

This set of studies was sponsored by Biomed Valley Discoveries and performed at Melior Discovery, Inc. Drs. Tung, Cheung, and Saha are employees of Biomed Valley, and Drs. Ciallella and Hain are employees of Melior. All authors participated in the design of the studies and data interpretation. Drs. Ciallella and Hain were responsible for the execution of the studies.

The authors thank Dr. Catherine Magill for her assistance in the manuscript preparation.

Conflicts of Interest

The authors have no conflict of interest regarding the content of this article.

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