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Therapeutic Benefit of a Dissociated Glucocorticoid and the Relevance of In Vitro Separation of Transrepression from Transactivation Activity

Maria G. Belvisi,¹ Sharon L. Wicks, Clifford H. Battram, Stephen E. W. Bottoms, Jane E. Redford, Paul Woodman, Thomas J. Brown, Stephen E. Webber, and Martyn L. Foster

Glucocorticoids (GCs) are the mainstay of asthma therapy; however, major side effects limit their therapeutic use. GCs influence the expression of genes either by transactivation or transrepression. The antiinflammatory effects of steroids are thought to be due to transrepression and the side effects, transactivation. Recently, a compound, RU 24858, has been identified that demonstrated dissociation between transactivation and transrepression in vitro. RU 24858 exerts strong AP-1 inhibition (transrepression), but little or no transactivation. We investigated whether this improved in vitro profile results in the maintenance of antiinflammatory activity (evaluated in the Sephadex model of lung edema) with reduced systemic toxicity (evaluated by loss in body weight, thymus involution, and bone turnover) compared with standard GCs. RU 24858 exhibits comparable antiinflammatory activity to the standard steroid, budesonide. However, the systemic changes observed indicate that transactivation events do occur with this GC with similar potency to the standard steroids. In addition, the GCs profiled showed no differentiation on quantitative osteopenia of the femur. These results suggest that in vitro separation of transrepression from transactivation activity does not translate to an increased therapeutic ratio for GCs in vivo or that adverse effects are a consequence of transrepression. *The Journal of Immunology*, 2001, 166: 1975–1982.

Glucocorticosteroids (GCs)² are the most effective treatment currently available for the treatment of many atopic (e.g., atopic dermatitis, allergic rhinitis) and inflammatory diseases (e.g., asthma, rheumatoid arthritis) (1). However, the severe systemic side effects associated with the long-term use of oral steroids have limited the doses that can be given, and therefore their therapeutic usefulness, and has led in the case of asthma to the development of inhaled steroids. Currently available inhaled GCs, including budesonide, fluticasone propionate, and mometasone furoate, have a reasonable therapeutic ratio possessing significant antiinflammatory activity with minimal side effects due to the removal of the swallowed fraction of the drug by hepatic metabolism. However, these drugs can be directly absorbed from the lung mucosa, and therefore systemic side effects can often be observed at high doses (2). Furthermore, patient compliance issues and poor device training particularly in the old and young often limit the topical steroid dose given. Therefore, currently the search is on for new oral GCs with an improved therapeutic ratio.

The GC receptor (GR) is a member of the nuclear receptor superfamily, which, on binding to their cognate ligands, directly or

indirectly regulates the transcription of target genes (3–6). There are at least three distinct mechanisms by which GCs can regulate gene transcription. First, GCs can bind to a cytosolic GR, which translocates to the nucleus and, in the case of positive regulation, transactivates through *cis*-activating palindromic GC response elements (GREs) located in the promoter region of responsive genes. Second, GCs are able to bind to negative GREs, resulting in the repression of gene transcription. However, GCs decrease the transcription of genes involved in the inflammatory process that have no identifiable GREs in their promoter regions, suggesting that an alternative mechanism must mediate this inhibitory effect. In fact, there is now evidence to suggest that GCs may control inflammation predominantly through the transrepression of transcription factors, such as AP-1, NF- κ B, and NF-AT, that regulate inflammatory gene expression. Furthermore, there is increasing acceptance of the hypothesis that the side effects of steroids are likely to be due to transactivation of genes through binding of the GR dimers to DNA, whereas the antiinflammatory effects result from the binding of a single GR to transcription factors or coactivators resulting in gene repression, i.e., transrepression.

The development of a novel GC, which discriminates between the two main activities of GCs, i.e., transactivation and transrepression, could be predicted to exhibit increased specificity for antiinflammatory activity and consequently an improved therapeutic ratio in vivo. Such a compound, RU 24858, which exhibits this separation of activities in vitro, has recently been described (7). However, the in vivo correlates of this in vitro profile remain unexplored. In this study, we have examined whether the improved in vitro profile results in the maintenance of antiinflammatory activity (evaluated in the rat Sephadex model of lung edema with steroids dosed orally (p.o.) 24 h and 2 h before Sephadex (intratracheally)) with reduced systemic toxicity (evaluated by a loss in body weight, thymus involution, and bone turnover following 7 days p.o. treatment) compared with the standard steroids, prednisolone and

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² Abbreviations used in this paper: GC, glucocorticoid; ACP, acid phosphatase; ALP, alkaline phosphatase; GR, GC receptor; GRE, GC response element; I- κ B, inhibitory protein that dissociates from NF- κ B; p.o., oral administration; TRAP, tartrate-resistant ACP.

budesonide. A preliminary report has been presented in abstract form at the American Thoracic Society meeting (8).

Materials and Methods

Measurement of Sephadex-induced lung edema in the rat

To investigate whether the dissociated GC, RU 24858, could be active as an antiinflammatory drug *in vivo*, we studied its effect in the rat Sephadex model of lung edema (9). The effects observed were compared with the activity of the standard oral GCs, prednisolone and budesonide.

Male Sprague Dawley rats were weighed and randomly allocated into study groups to receive compound or vehicle (distilled water containing 0.5% carboxymethylcellulose containing 0.2% Tween 80). RU 24858 (0.3, 1, 3, 10, 30, 100, 300 mg/kg), budesonide (0.03, 0.1, 0.3, 1, 3, 10 mg/kg), prednisolone (1, 3, 10, 30, 100, 300, 1000 mg/kg), or vehicle was administered orally by gavage in a dose volume of 2 ml/kg 24 and 2 h before Sephadex/vehicle administration. Sephadex (5 mg/kg) or vehicle (saline) was administered intratracheally under halothane anesthesia (4% in oxygen for 3 min). Twenty-four hours later, the animals were killed by overdose with Euthatal (10 ml/kg i.p.). The heart and lungs were removed en bloc, and wet lung weights were determined and corrected for 100 g initial body weight.

Femur model of GC-induced osteopenia

To investigate whether the dissociated GC, RU 24858, induced systemic toxicity to the same degree as the standard GCs, prednisolone and budesonide, we studied the effect of these compounds in the rat femur model of steroid-induced osteopenia (10).

Male Sprague Dawley rats (190–220 g; Harlan, U.K.) were allowed free access to food and water. Animals were weighed and randomly allocated into study groups to receive either vehicle (distilled water containing 0.5% carboxymethylcellulose and 0.2% Tween 80), RU 24858 (0.3, 1, 3, 10, 30, or 100 mg/kg/day), budesonide (0.3, 1, 3, 10, or 30 mg/kg/day), or prednisolone (1, 3, 10, 30, or 100 mg/kg/day). Compounds were prepared as a suspension in vehicle and orally administered to rats once daily (2 ml/kg) for 7 days. Body weight was measured daily throughout the study. Twenty-four hours after administration of the last dose of compound, rats were killed by i.p. injection of Euthatal (10 ml/kg). Blood was collected into ice-cold tubes by cardiac puncture, and the serum was obtained after centrifugation ($2500 \times g$ for 20 min at 4°C) stored at -20°C until analyzed. The thymus was dissected free of connective tissue and weighed. The left femur was exposed and removed with head intact in the acetabulum by cutting through the pelvic girdle and through the femur shaft above the knee joint. The tissue was then fixed in 10% neutral buffered Formalin for histological assessment.

Femoral head histology

Preparation of histological specimens. Femurs were fixed in 10% neutral buffered Formalin for 7 days, then decalcified in 35% (v/v) formic acid in sodium citrate solution 13% (w/v) for 10 days (four changes). Tissues were washed in water for 48 h, then processed to paraffin wax overnight using an automated tissue processor (Tissue Tek VIP 3000; Bayer Diagnostics, Berkshire, U.K.). Three-micrometer-thick sections were cut (Shandon AS325 retractor microtome) with the trochanter, and the femoral head was included in the section, to allow standardization of tissue orientation and the level of sectioning. The sections were mounted on poly(L-lysine)-coated slides and dried at 37°C overnight. Sections were stained Alcian blue, as described below, using an automated slide stainer (Sakura DRS 601 Diversified Stainer; Bayer Diagnostics), and coverslipped.

Staining technique. Sections were rehydrated and incubated for 20 min with 1% (w/v) Alcian blue 8GX in 1% (v/v) acetic acid containing 10% (w/v) sodium chloride, then counterstained with hematoxylin and 1% (w/v) acid fuchsin/eosin mixture.

Quantitative histology of the femoral head growth plate

The femoral head growth plate was examined under a light microscope using $\times 100$ magnification. Images of the growth plate were captured onto computer (Apple MacIntosh 700) using Image Grabber software (Neotech) and assessed using Optilab software (Graphtek). One image was captured from each tissue section with five measurements of the growth plate width obtained from each calibrated image. Measurements involved drawing a line perpendicular to the growth plate between the edge of the hypertrophic zone distal to the articular cartilage and the end of the proliferating zone, clearly demarcated by the dark blue staining. Regions excluded from measurement were: the edges of the growth plate and naturally occurring gaps

devoid of proliferating cells (which do not exhibit the distinct dark blue coloration). Sections showing evidence of antemortem trauma were also excluded.

Serum bone markers

Serum samples were thawed for 1 h at room temperature and then centrifuged at $2500 \times g$ for 10 min at 20°C. Osteocalcin, alkaline phosphatase (ALP), acid phosphatase (ACP), and the bone-specific tartrate-resistant ACP (TRAP) were all measured from the same sample. Osteocalcin was measured by RIA (Biogenesis, Bournemouth, U.K.) using a modification of the manufacturer's instructions. Briefly, samples were diluted 1/50 in RIA buffer (0.1225 M sodium chloride, 0.01 M sodium dihydrogen phosphate, 0.025 M EDTA tetrasodium salt, 0.1% (w/v) Tween 20, and 0.1% (w/v) BSA, pH 7.4). Standards and samples were incubated overnight with primary Ab, goat anti-rat osteocalcin at 4°C on an orbital shaker. A total of 0.01 μCi of ^{125}I -labeled rat osteocalcin was then added to each tube vortex, mixed, and again incubated overnight, as previously described. The precipitating Ab, donkey anti-goat IgG, was diluted 1/40 in precipitation buffer (0.1 M sodium phosphate buffer, pH 7.4, 2.5% (w/v) polyethylene glycol, 0.05% (w/v) sodium azide). One milliliter was added to each tube and incubated for 2 h at 4°C on an orbital shaker. Tubes were then centrifuged at $1500 \times g$ for 20 min at 4°C, supernatants were decanted off, and pellets were washed with 500 μl of ice-cold deionized water and respun. Pellets were counted for 1 min on a gamma counter (LKB-Wallac 1275 Mini γ).

Enzymes were measured directly after thawing on the IL-Monarch Chemistry System (Instrumentation Laboratory, Warrington, U.K.); ALP using a para-nitrophenyl phosphate reaction and ACP and TRAP using a α -naphthylphosphate reaction; kit instructions were followed.

Materials

Budesonide, prednisolone, Tween 80 (polyoxyethylenesorbitan monooleate), poly(L-lysine) solution, EDTA tetrasodium salt, Tween 20 (polyoxyethylenesorbitan monolaurate), BSA, DMSO, and polyethylene glycol (m.w. 15,000–20,000) were supplied by Sigma (Poole, U.K.). Carboxymethylcellulose (sodium salt), paramat extra paraffin wax, xylene, acid fuchsin, and Alcian blue 8GX were supplied by BDH Merck (Lutterworth, U.K.). Euthatal and Halothane were supplied by Rhone-Merieux (Harlow, U.K.). Neutral buffered Formalin (10%) was supplied by Surgipath Europe (St. Neots, U.K.). Citric acid and formic acid were supplied by Aldrich Chemical (Gillingham, U.K.). Saline (0.9% w/v) was obtained from Fresenius (Basingstoke, U.K.). Hematoxylin and eosin Y were prepared stains from Shandon (Runcorn, U.K.). Osteocalcin assay reagents were supplied by Biogenesis: goat anti-rat osteocalcin, ^{125}I -labeled rat osteocalcin, and donkey anti-goat IgG (5160-4004). Enzyme reagent kits were supplied by Instrumentation Laboratory: ALP, ACP, and TRAP. RU 24858 was synthesized by the Medicinal Chemistry Department at Aventis Pharma (Dagenham, U.K.).

Statistical analysis

All values presented are mean \pm SE of eight determinations. The percentage inhibition of Sephadex-induced lung edema (compared with the Sephadex-administered, vehicle-treated group) was determined for each GC-treated group. The dose-response curve for inhibition of lung edema by GCs was calculated by least squares, nonlinear iterative regression with the 'PRISM' curve-fitting program (Graphpad Instat software program, San Diego, CA). A sigmoidal fit was obtained for the data generated from the Sephadex model. The effective dose causing a 50% reduction of the maximum (ED_{50}) lung edema was calculated.

In the chronic dosing regime, all values presented are mean \pm SE of eight determinations. Dose-dependent decreases in body weight gain, thymus weight, serum osteocalcin, and femur growth plate widths were assessed compared with the vehicle-treated control group. The dose causing a 50% reduction in body weight gain on day 7 (ED_{50}) was calculated. The dose causing a 30% decrease in serum osteocalcin levels and the maximal decrease in femur growth plate width were calculated.

Results

Sephadex-induced lung edema in the rat

Sephadex instillation induced a significant edema response in the lung (between 17% and 36.6% increase in wet weight). This increase in wet lung weight was inhibited in a dose-dependent manner by RU 24858 (0.3–300 mg/kg, p.o.), prednisolone (1–1000 mg/kg, p.o.), and budesonide (0.03–10 mg/kg, p.o.) (Fig. 1). The ED_{50} for inhibition of lung edema with RU 24858, prednisolone,

and budesonide were 0.88, 20.99, and 2.31 mg/kg, respectively. Therefore, the rank order of potency obtained for the antiinflammatory activity of these compounds was RU 24858 > budesonide > prednisolone.

Femur model of GC-induced osteopenia

Dose-dependent decreases were observed in all the main systemic parameters, with the exception of the femur growth plate width, with all three GCs. A significant, dose-related decrease in body weight gain was seen in animals during the 7-day treatment period with RU 24858 (0.3–100 mg/kg, p.o., ED_{50} = 0.6 mg/kg), prednisolone (1–100 mg/kg, p.o., ED_{50} = 1–3 mg/kg), and budesonide (0.3–30 mg/kg, p.o., ED_{50} = 0.55 mg/kg) when compared with vehicle control-treated animals (Figs. 2A, 3A, and 4A; Table I). Thymus involution, observed as a decrease in thymus weight, was produced by 7-day dosing by all three compounds (ED_{50} were obtained for RU 24858, prednisolone, and budesonide of 1, 5, and 0.75 mg/kg, respectively; Figs. 2B, 3B, and 4B; Table I).

Histopathologically, administration of each of the GCs over a 7-day period was associated with hypoplasia and loss of cellularity within the proliferative zone of the femur growth plate (see Fig. 7). In addition, higher doses of steroid elicited apoptosis of cells within the proliferative and hypertrophic zones. These effects were also associated with marginalization of chondrocytes on the proximal aspect of the growth plate. No other pathologies were apparent in any of the sections examined. RU 24858 had a biphasic effect on femur growth width that decreased to a maximal 26% at 3 mg/kg/day (Fig. 6, Table I). prednisolone and budesonide evoked similar decreases in the femur growth plate width (maximal decrease >100 mg/kg/day and maximal decrease at 10 mg/kg/day, respectively; Fig. 6).

Serum biochemistry in the 7-day model

Treatment with all three compounds evoked a dose-related inhibition of serum osteocalcin levels (approximately ED_{30} were obtained for RU 24858, prednisolone, and budesonide of 10, 100, and 12.7 mg/kg, respectively; Fig. 5, Table I). Serum biochemistry demonstrated a dose-dependent decrease in the bone enzyme markers, ALP, ACP, and the bone-specific tartrate-resistant bone isoform (TRAP), with all compounds reflecting a reduction in bone cell turnover (Table II). The systemic effects described above have

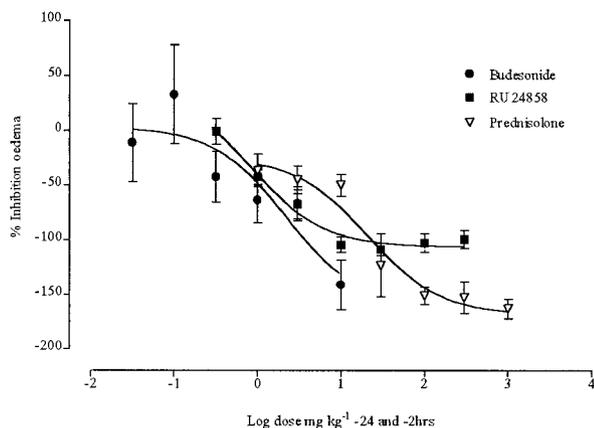


FIGURE 1. Effect of steroids on Sephadex-induced lung edema. Inhibitory effect of RU 24858 (0.3–300 mg/kg, p.o.), prednisolone (1–1000 mg/kg, p.o.), and budesonide (0.003–10 mg/kg, p.o.), administered at 24 and 2 h before Sephadex, on Sephadex-induced changes in wet lung weight in conscious, male Sprague Dawley rats. Data points represent the mean \pm SE of eight rats.

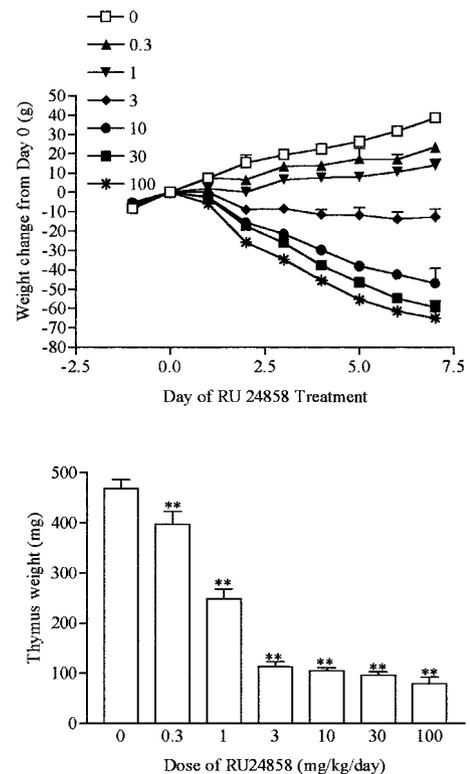


FIGURE 2. Effect of RU 24858 on body weight and thymus weight after 7-day treatment. Change in body weight (A) and thymus weight (B) following once daily, 7-day oral treatment with RU 24858 (0.3, 1, 3, 10, 30, or 100 mg/kg/day). Results are shown as the mean \pm SE of eight rats. Treatment groups were compared with control using ANOVA with a post hoc Dunnett's test (**, $p < 0.01$).

been observed with all three compounds. RU 24858 appears to have a similar potency to budesonide, both of which were more potent than prednisolone (Table II). From the results reported above, the compounds elicit systemic side effects with the following order of potency: budesonide \geq RU 24858 > prednisolone.

In summary, the effect of RU 24858 on osteocalcin levels and on the femur growth plate width was similar to that obtained with budesonide, with RU 24858 being slightly more potent. Prednisolone was significantly less potent at eliciting hormonal side effects and is consistent with its reduced potency, compared with the other steroids, at eliciting antiinflammatory effects.

Discussion

GCs are the mainstay of asthma treatment; however, in many cases, major side effects limit their therapeutic use (1). There is increasing acceptance of the hypothesis that the side effects of steroids are likely to be due to the transactivation of genes through the binding of the GR dimers to DNA, whereas the antiinflammatory effects may be due to the binding of a GR monomer to transcription factors or coactivators, resulting in gene repression. An increased understanding of the molecular mechanisms involved in GC-induced suppression of the inflammatory response has increased the possibility of developing novel, safer GCs.

Many of the proinflammatory genes whose products mediate the inflammatory process in asthma are regulated by the transcription factors AP-1 and NF- κ B. In the early 1990s, a number of groups recognized that the GR can regulate gene transcription by forming protein-protein interactions with these transcription factors without the necessity for DNA binding. AP-1, which is a dimer of *c-jun* and *c-fos*, contributes to the regulation of cytokines and adhesion

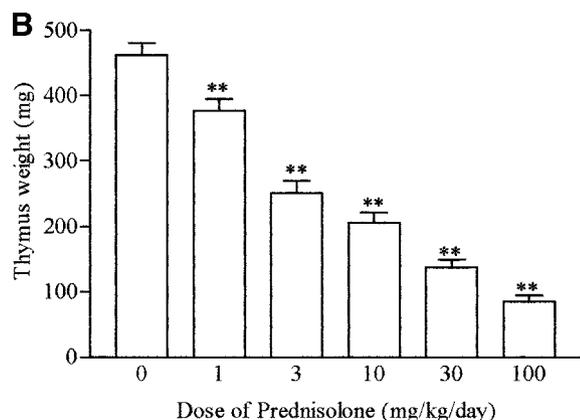
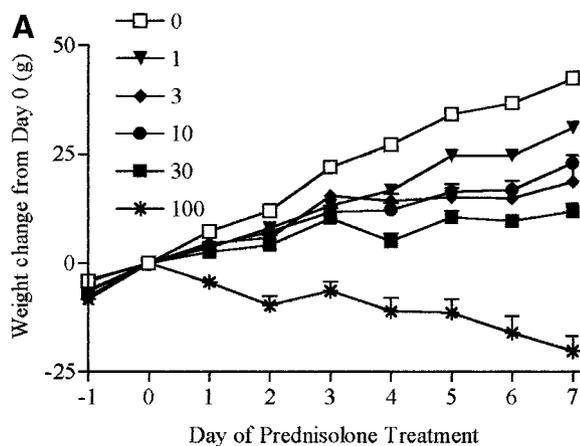


FIGURE 3. Effect of prednisolone on body weight and thymus weight after 7-day treatment. Change in body weight (A) and thymus weight (B) following once daily, 7-day oral treatment with prednisolone (1, 3, 10, 30, or 100 mg/kg/day). Results are shown as the mean \pm SE of eight rats. Treatment groups were compared with control using ANOVA with a post hoc Dunnett's test (**, $p < 0.01$).

molecules. Direct protein-protein interaction between AP-1 and the liganded GR was shown to result in repression of transcriptional activity by blocking the interaction of both transcription factors with their respective response elements (11). Mutation studies of the GR have revealed that this repressive action is most likely mediated by GR monomers rather than dimers. Heck et al. (12) illustrated, through the introduction of mutations in the DNA-binding domain of the GR, that transactivation and transrepression can be dissociated. Mutations resulting in failure of the GR to dimerize and bind DNA were associated with a failure to transactivate GRE-dependent promoters in cell transfection studies. However, repression of the AP-1-dependent promoter by the mutant GR was as effective as the wild-type receptor.

Various mechanisms have been invoked to explain the transrepressing actions of steroids. Although evidence exists for the direct protein interaction through the leucine zipper (B-zip) region of AP-1 and the DNA-binding domain of the GR resulting in failure of DNA binding, this is unlikely to be the sole mechanism (13). Furthermore, it is now known that GCs can change chromatin structure and that this property may be essential for their gene-regulatory activity. DNA is wound around histone proteins to form nucleosomes and the chromatin fiber in chromosomes. Increased transcription is associated with uncoiling of DNA wound around histone. This is secondary to the acetylation of the histone residues by the enzymatic action of coactivator molecules such as the

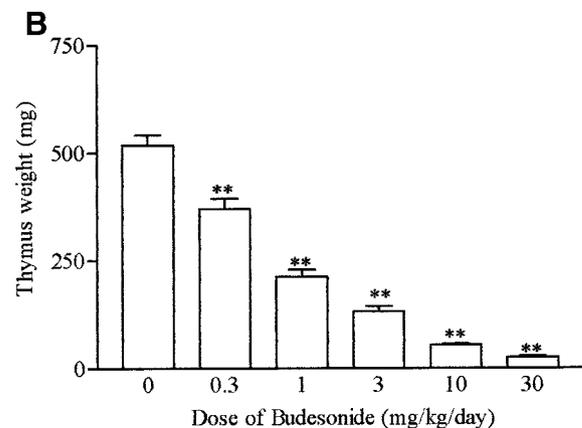
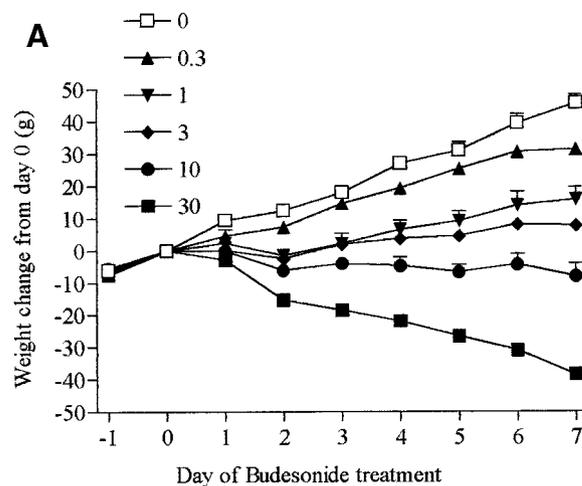


FIGURE 4. Effect of budesonide on body weight and thymus weight after 7-day treatment. Change in body weight (A) and thymus weight (B) following once daily, 7-day oral treatment with budesonide (0.3, 1, 3, 10, or 30 mg/kg/day). Results are shown as the mean \pm SE of eight rats. Treatment groups were compared with control using ANOVA with a post hoc Dunnett's test (**, $p < 0.01$).

CREB-binding protein, which is activated by the binding of transcription factors such as AP-1 and NF- κ B (14). Because binding sites on these coactivator molecules may be limited, this may result in competition between transcription factors and the activated GR for the limited binding sites available, resulting in an inhibition of inflammatory gene transcription. GC-mediated repression of proinflammatory gene transcription may also occur by deacetylation of histone, resulting in tighter coiling of DNA and reduced access of transcription factors to their binding sites.

The physiological significance of the DNA-binding and dimerization-independent transrepressive actions of the GR is revealed in studies in homozygous mice carrying a dimerization- and DNA-binding defective mutant of GR (GR^{dimm}). DNA binding and transcriptional regulation of genes containing GREs and nGREs were confirmed to be unresponsive to GCs, whereas repression of AP-1-mediated gene expression was shown to be intact. The GR^{dimm} homozygotes appeared normal and survived to adulthood in sharp contrast to mice, which were deficient in their GR. This suggests that GRE-mediated gene regulation is not essential for survival or development, whereas the ability of GR to transrepress transcription factors is essential (15–17). However, these studies do not address whether in these mutants GCs are capable of regulating the activity of other transcription factors such as NF- κ B, and thereby repress the expression of proinflammatory genes, e.g., cytokines.

Table I. ED_{50}/ED_{30} values for systemic effects of 7-day oral steroid treatment

Study	ED_{50} (mg/kg/day)		Approximate ED_{30} (mg/kg/day)	Dose that Achieved a Maximum Response (mg/kg/day)
	Loss of body weight gain	Thymus involution	Serum osteocalcin	Femur growth plate width
RU24858	0.6	1.0	10	3
budesonide	0.55	0.75	12.7	10
prednisolone	1–3	5	100	>100

Furthermore, although GR^{dim/dim} mice are resistant to GC-induced thymus involution, it is not clear whether these mice would demonstrate an antiinflammatory effect in the lung following GC treatment. These studies suggest that different domains of the GR are responsible for different actions, but can this be exploited pharmacologically, leading to agents with more selective greater antiinflammatory activity compared with current drugs? The evidence is that different steroids can preferentially elicit different responses from the GR. Of considerable importance to the development of more selective steroids is the consideration as to whether different mechanisms can be invoked by different ligands following activation of the GR (18).

Recently, a novel class of synthetic GCs has recently been identified that exert strong AP-1 inhibition, whereas they only weakly activate the GRE-based reporter genes. In addition, these dissociated GCs were shown to inhibit IL-1 β secretion and to display antiinflammatory activity in vivo (7). A subsequent study has demonstrated that these compounds are able to inhibit TNF- α -induced IL-6 secretion in murine fibroblasts and HeLa cells. In addition, these compounds are able to directly interfere with NF- κ B-dependent gene activation without changing the expression level of inhibitor κ B (18). In parallel to NF- κ B-dependent transrepression, transactivation experiments were performed in the same study with GRE-dependent promoter reporter gene variants in murine fibroblasts. In agreement with Vayssière et al. (7), in human HeLa cells and a rat hepatoma cell line, the dissociated steroids RU 24858 and RU 40066 did not substantially stimulate GRE-dependent transactivation in murine fibroblast cells. Surprisingly, however, and in

contrast to previous observations reported by this group in human and rat cells, RU 24782 displayed similar transactivating ability as dexamethasone on the GRE-dependent reporter gene variants in mouse fibroblast cells. These data illustrate the divergent potencies of dissociated steroids in GRE-dependent reporter gene activation in human and murine cells.

In this study, we investigated the activity of one of these dissociated compounds, RU 24858, which claimed to differentiate between the two main actions of GCs (i.e., transactivation and transrepression), while possessing potent in vivo antiinflammatory activity (7). In vitro studies have shown that that this compound exhibits significant AP-1 transrepression while only weakly activating the GRE-based reporter genes. Furthermore, the in vitro antiinflammatory activity of RU 24858 was confirmed by inhibition of IL-1 β secretion from activated monocytes, while the compound was unable to induce tyrosine amino transferase activity, confirming the lack of transactivating activity. However, this study did not address whether RU 24858 could exhibit the same dissociation by demonstrating antiinflammatory properties without steroid adverse side effects in vivo. We have demonstrated that RU 24858 exhibits comparable antiinflammatory activity to the standard steroid, budesonide, in the Sephadex model of lung edema in the rat. We have chosen to study the consequences of GC excess on decrease in body weight, thymus involution, and quantitative osteopenia of the femur growth plate, as these effects can be easily modeled and reproducible measurements obtained in animal models of this kind. Thymus involution is an appropriate parameter to

Table II. Serum biochemistry markers summarizing significant changes to bone enzyme markers^a

	ALP (IU/l)	ACP (IU/l)	TRAP (IU/l)
RU24858 (mg/kg/day)			
0	424 \pm 7	27.7 \pm 1.43	10.6 \pm 0.95
0.3	386 \pm 22 (–9%)	27.3 \pm 1.57 (–2%)	10.3 \pm 0.87 (–3%)
1.0	420 \pm 14 (–1%)	23.7 \pm 2.07 (–14%)	8.7 \pm 1.13 (–18%)
3.0	341 \pm 10 (–19%)	19.9 \pm 1.41** (–28%)	6.9 \pm 0.91* (–35%)
10	308 \pm 22** (–27%)	9 \pm 1.01** (–67%)	1.9 \pm 0.55** (–82%)
30	345 \pm 41 (–19%)	12.8 \pm 1.00** (–54%)	4.0 \pm 0.71** (–62%)
100	209 \pm 15** (–51%)	10.1 \pm 0.77** (–63%)	3.6 \pm 0.46** (–66%)
prednisolone (mg/kg/day)			
0	494 \pm 19	22.1 \pm 0.8	9.4 \pm 0.48
1.0	469 \pm 14 (–5%)	19 \pm 1.5 (–14%)	7.6 \pm 0.72 (–20%)
3.0	479 \pm 44 (–3%)	18.7 \pm 1.2 (–15%)	7.9 \pm 0.59 (–17%)
10	493 \pm 22 (0%)	18.8 \pm 1.0 (–15%)	8.2 \pm 0.60 (–13%)
30	448 \pm 17 (–10%)	16 \pm 0.7** (–28%)	6.9 \pm 0.40** (–27%)
100	262 \pm 19** (–47%)	12 \pm 1.3** (–46%)	4.7 \pm 0.76** (–51%)
budesonide (mg/kg/day)			
0	557 \pm 19	22.7 \pm 1.3	9.6 \pm 0.7
0.3	534 \pm 23 (–4%)	25.6 \pm 2.0 (13%)	11.3 \pm 0.9 (18%)
1.0	514 \pm 34 (–8%)	21.0 \pm 1.1 (–8%)	8.0 \pm 0.5 (–16%)
3.0	414 \pm 6.2** (–26%)	19.9 \pm 1.7 (–13%)	7.4 \pm 0.9 (–22%)
10	384 \pm 35** (–31%)	14.3 \pm 1.5** (–37%)	6.0 \pm 0.9** (–37%)
30	278 \pm 25** (–50%)	12.4 \pm 0.8** (–45%)	4.7 \pm 0.9** (–51%)

^a, Values are shown \pm SEM. *, $p < 0.05$, **, $p < 0.01$ relative to control (ANOVA, Dunnett's test).

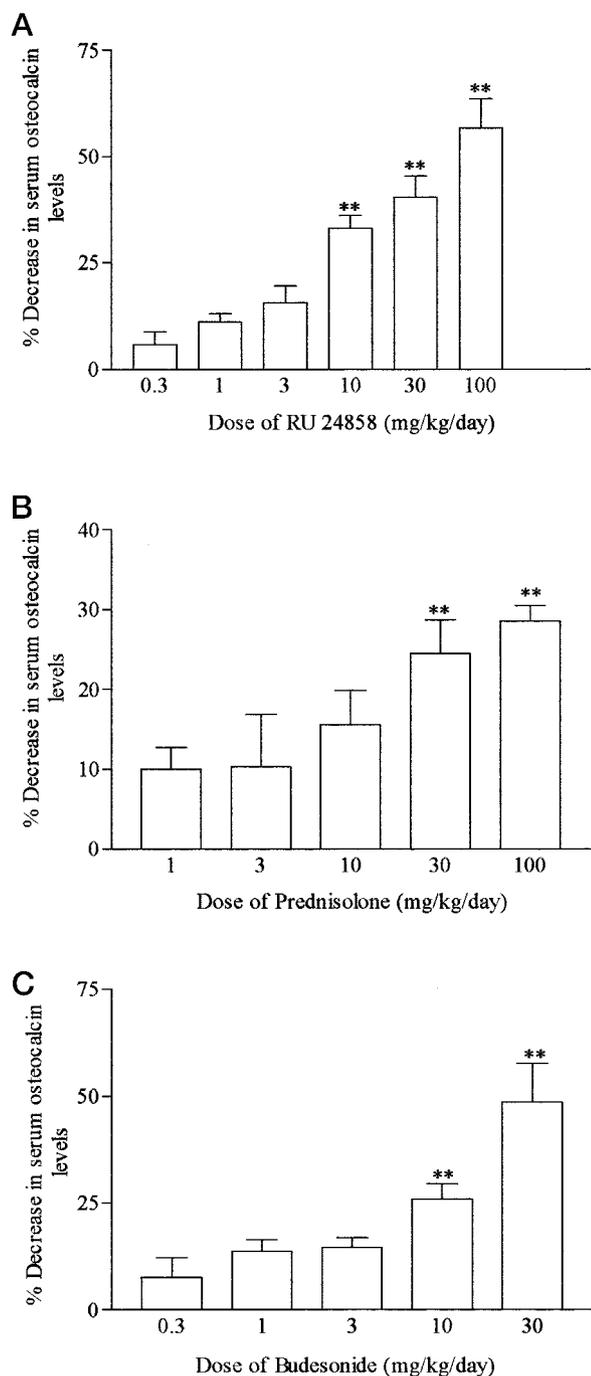


FIGURE 5. Effect of steroids on serum osteocalcin levels following 7-day treatment. Percent decrease in serum osteocalcin levels measured following 7-day oral treatment with RU 24858 (0.3–100 mg/kg/day; A), prednisolone (1–100 mg/kg/day; B), and budesonide (0.3–30 mg/kg/day; C) compared with vehicle control. Results are shown as the mean \pm SE of eight rats. Treatment groups were compared with control using ANOVA with a post hoc Dunnett's test (**, $p < 0.01$).

investigate the ability of GCs to transactivate given that GC-induced thymocyte apoptosis requires GR-mediated gene activation (19).

Interestingly, RU 24858 showed no differentiation, compared with standard steroids, in the ability to induce systemic changes (e.g., loss in body weight, thymus involution) and in the quantitative osteopenia of the femur observed after 7 days treatment. For all steroids tested, depression in systemic osteocalcin levels indicates inhibition of bone turnover via the osteoblast axis. In addition,

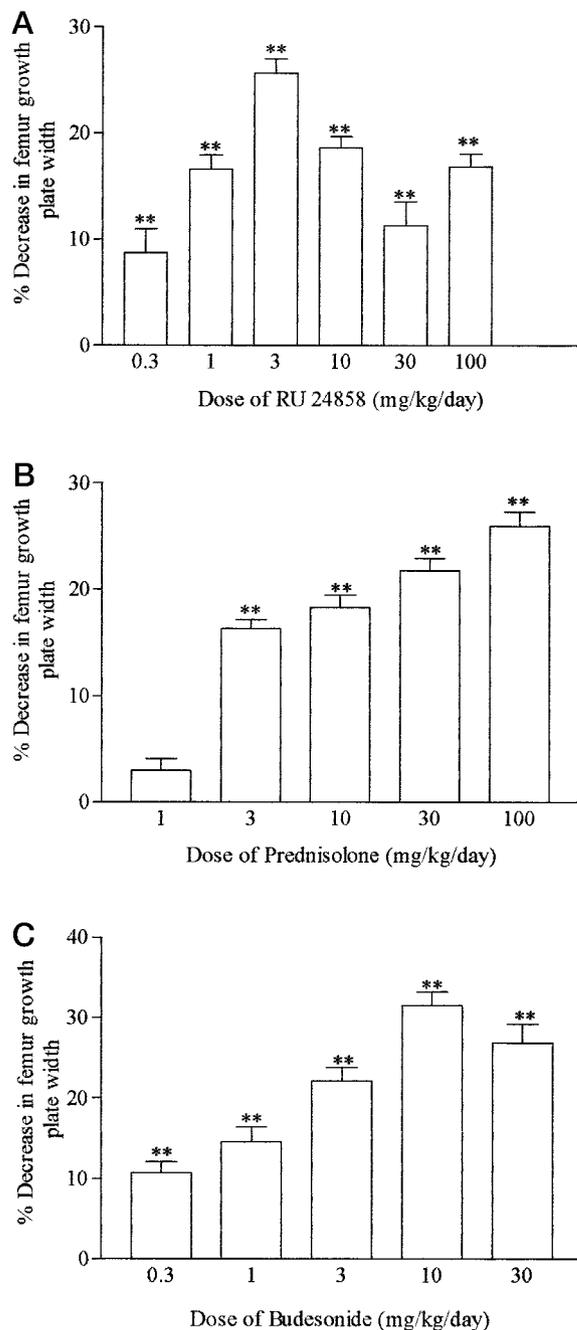


FIGURE 6. Histograms illustrating the effect of steroids on femoral growth plate width after 7-day treatment. Histograms depicting image analysis measurements of femoral head growth plate width from histological sections. Data are expressed as the percent decrease in femur growth plate width following 7-day oral treatment with RU 24858 (0.3–100 mg/kg/day; A), prednisolone (1–100 mg/kg/day; B), and budesonide (0.3–30 mg/kg/day; C) compared with vehicle control. Results are shown as the mean \pm SE of eight rats. Treatment groups were compared with control using ANOVA with a post hoc Dunnett's test (**, $p < 0.01$).

tion, bone remodeling via the osteoclast was also attenuated, as evidenced by the depression in ACP. Taken together, these results are consistent with the known effects of GCs on the bone-remodeling axis, i.e., depression of both the key cell types involved (20). In addition, the osteocalcin data would support depression of calcium uptake into the skeleton, which would further enhance these inhibitory effects. Inhibition of bone growth is one of the major limitations of steroid usage. Any steroid, which has an improved

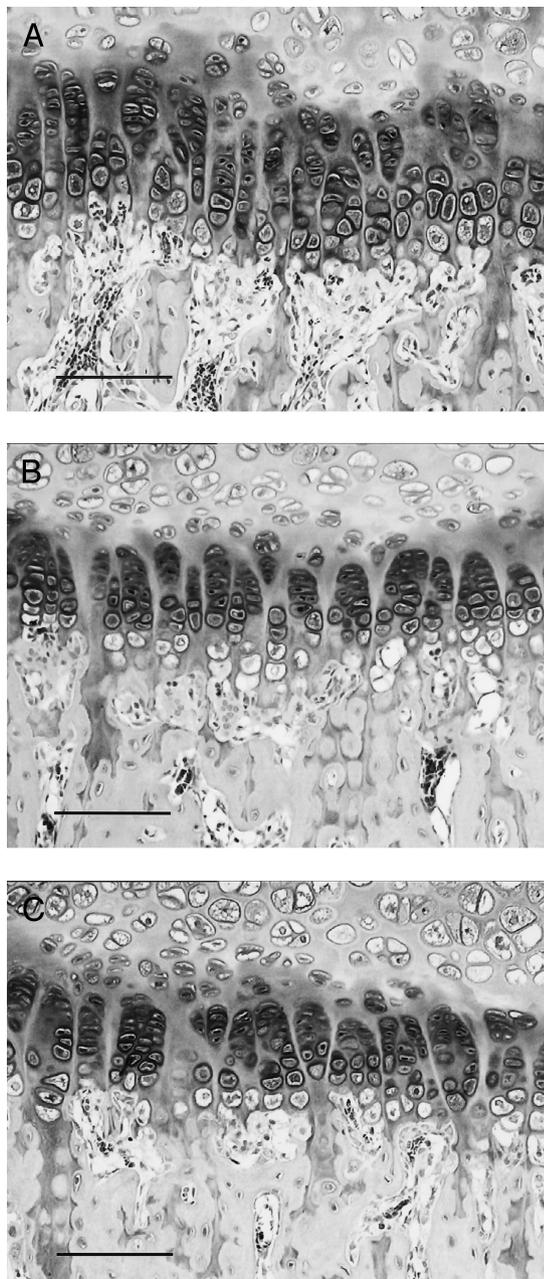


FIGURE 7. Photomicrographs showing the proliferating zone of the femoral head growth plate and the effect on growth plate width following 7-day steroid treatment. Photomicrographs showing the proliferating zone of the femoral head growth plate and a typical steroid osteopenic reaction after vehicle (A), RU 24858 3 mg/kg/day (B), and budesonide 10 mg/kg/day (C) treatment for 7 days. Maturing cartilage can be seen above the growth plate, and cancellous bone with marrow cavities below. Tissue sections were stained with Alcian blue 8GX and counterstained with hematoxylin and acid fuchsin/eosin mixture. Alcian blue-stained sections of decalcified femoral heads demonstrating the femoral head growth plate width. The dark proteoglycan-stained region indicates the proliferative zone, which is the region, measured to assess changes in femur growth plate width. Note the reduction in width of the proteoglycan-stained region with oral steroid treatment. Bars indicate 100 μm .

therapeutic ratio in terms of this parameter, will exhibit an advantage in the market place. Bone changes in this study have the following rank order of potency: RU 24858 \geq budesonide > prednisolone. In addition, these compounds show the same rank order of potency on decrease in body weight and thymus involution.

Down-regulation of NF- κ B DNA binding, accompanied by increased expression of I- κ B α (inhibitory protein that dissociates from NF- κ B) and I- κ B β , precedes thymocyte cell death, suggesting that NF- κ B may be important for the survival of immature thymocytes (21). Dissociated steroids, such as RU 24858, have been shown to directly interfere with NF- κ B-dependent gene activation without changing the expression level of I- κ B (18). These data suggest that the ability of RU 24858 to evoke thymus involution is due to the transrepression of NF- κ B or the ability to induce other genes that mediate cell death. Preliminary evidence describing the GC-induced expression of known and novel genes (e.g., a cDNA clone, encoding a human β -galactoside-binding protein) has been reported that has been shown to be inhibitory to cell growth (19, 22).

In summary, RU 24858 was equipotent compared with budesonide in eliciting side effects, suggesting that *in vitro* separation of transrepression from transactivation activity does not translate to an increased therapeutic ratio for GCs *in vivo*. However, although the parameters we have chosen in this study (e.g., weight loss, thymus involution, osteopenia of the femur growth plate) to represent the undesirable side effects of steroids are appropriate, we have not studied whether RU 24858 exhibits an improved safety profile on other known side effects of GCs. In fact, diminution of any of the other side effects of GCs (e.g., diabetes mellitus, glaucoma, opportunistic infection, behavioral changes) with a GC that retained significant antiinflammatory activity would be an important development.

In conclusion, although the development of selective steroids, which differentiate between transrepression and transactivation mechanisms, remains an interesting approach, whole animal physiological studies have failed to confirm the predicted dissociation between antiinflammatory activity and adverse effects. Whether this indicates that homeostatic mechanisms present in the animal models override selectivity, which argues for biochemical redundancy in the mechanism, or merely reflect limitations of the prototypical tool, RU 24858, is unknown. Alternatively, these data suggest that some of the classical hormonal actions of GCs are a consequence of transrepression rather than transactivation. It is clear that research in this area should give us fundamental insights into the molecular physiology of steroid action. Given the possible therapeutic rewards of a dissociated steroid, the contradictory data generated to date should only serve as a spur to future research in this fascinating area.

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