Thiazolidine Diones, Specific Ligands of the Nuclear Receptor
Retinoid Z Receptor/Retinoid Acid Receptor-related Orphan
Receptor α with Potent Antiarthritic Activity*

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Rat adjuvant arthritis is a chronic T cell-dependent autoimmune disease with many similarities to rheumatoid arthritis. We have identified a class of thiazolidine diones (TDs)1 with potent antiarthritic activity at daily oral doses between 0.01 and 1 mg/kg and was shown to specifically inhibit the retinoid Z receptor/retinoid acid receptor-related orphan receptor α (RZR/RORα) in low nanomolar concentrations. This receptor is a novel member of the superfamily of ligand-inducible transcription factors, and we have recently identified the pineal gland hormone melanatonin as a natural ligand. Structure-activity relationship studies with 13 closely related analogues of CGP 52608 revealed a striking correlation between RZR/RORα activation and antiarthritic activity. We therefore suggest that nuclear signaling via RZR/RORα is a key mechanism in mediating the antiarthritic effects of these thiazolidine diones and may open a novel therapeutic approach for the treatment of rheumatoid arthritis and other autoimmune diseases. The existence of a nuclear melatonin receptor may lead to a better understanding of the immunomodulatory actions of melatonin.

During the search for novel immunomodulating compounds, we discovered thiazolidine diones (TDs)1 with potent antiarthritic activity in rat adjuvant arthritis. This chronic T cell-dependent autoimmune disease has many similarities to rheumatoid arthritis such as progressive joint destruction, enhanced T cell responses (Th1), and pronounced cytokine-mediated acute-phase reactions (1–7). It was known that these TDs inhibit the growth of various experimental tumors. Hormone-dependent tumors were especially sensitive, and these TDs reduced serum levels of several pituitary hormones (4–7). Down-regulation of pituitary function was therefore suggested to be the underlying mechanism, although the fact that hormone-independent tumors were also inhibited (8) could not be explained. Subsequently, a subgroup of these TDs, which specifically inhibited epidermal growth factor receptor kinase and c-Src kinase, was identified, and this mechanism was thought to be important for the oncostatic effects (9).

We describe in this report the first in vivo results for structurally related novel TDs. The lead compound CGP 52608 and active analogues suppress chronic inflammation and joint destruction in arthritic rats at daily oral doses between 0.01 and 1 mg/kg. Although these TDs exhibit antiarthritic as well as antitumor and hormone-suppressive properties, they do not inhibit epidermal growth factor receptor kinase and c-Src kinase.2

Searching again for a molecular target, we hypothesized that these TDs may influence transcriptional regulation as is known for ligands of nuclear hormone receptors such as retinoids, vitamin D₃, thyroid hormones, and steroids (10). The superfamily of nuclear hormone receptors contains numerous orphan receptors, for which no ligand has been identified so far (10, 11). Their physiological role is poorly understood, and the discovery of specific ligands is always a key in understanding their regulatory functions. An important example was the identification of 9-cis-retinoic acid as being a ligand of retinoid X receptors (12).

We found recently that our lead compound, CGP 52608, was able to specifically bind to and activate the nuclear receptor RZR/RORα in low nanomolar concentrations (13). The RZR/ROR family is a novel subclass of orphan nuclear receptors (14–17) with three subtypes (α, β, and γ) and four splicing variants of the α-subtype, each with a characteristic tissue distribution (for review, see Ref. 18). The most recently identified thymus orphan receptor (TOR) is closely related to RZR/RORα and was suggested to be involved in the process of thymic maturation, differentiation, and selection of T cells (19).

The characteristic oncostatic, hormone-suppressive, and immunopharmacological properties of the synthetic RZR/RORα ligand CGP 52608 enabled us to identify the pineal hormone melanatonin as being a natural ligand of RZR/ROR receptors (13, 20). The existence of a nuclear signaling pathway for melanatonin was further confirmed by the discovery of the first natural RZR/RORα-melanatonin responding gene, human 5-lipoxygenase (21).

We describe in this report the structural requirements for CGP 52608 analogues to activate RZR/RORα. Thirteen closely related analogues were tested for receptor-mediated gene activation in a potent Drosophila model system and for inhibition of adjuvant arthritis to determine whether we could find any correlation between RZR/RORα activation and inhibition of this experimental autoimmune disease.

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1 The abbreviations used are: TDs, thiazolidine diones; CAT, chloramphenical acetyltransferase; RZR/ROR, retinoid Z receptor/retinoid acid receptor-related orphan receptor; VDR, vitamin D₃ receptor; TRα, thyroid hormone receptor; RAR, retinoid acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor.
MATERIALS AND METHODS

Compounds—Melatonin (N-acetyl-5-methoxytryptamine) was obtained from Fluka. Thiazolidine diones and S 20098 (Servier; N-acetyl-2-(7-methoxynaphthalin-1-yl)ethylamine) were synthesized by Chemical Research, Ciba-Geigy (Basel, Switzerland).

Synthesis of TDs—A detailed description of the general synthesis of TDs is given in Refs. 22–24. Most of the TDs shown in Figs. 1 and 2 were synthesized as demonstrated in Scheme I via route C for monosubstituted compounds with a hydrogen at N-4 (CGP 52608, CGP 53065, CGP 52528, CGP 53079, CGP 52113, and CGP 52749) or via routes D and E for disubstituted compounds (CGP 55706, CGP 55707, and GP 50468), starting from the corresponding thiosemicarbazone. CGP 55066 was made similarly by using methyl isocyanate in the last step. CGP 53079 was prepared by cyclizing with bromoisobutyric acid instead of bromoacetic acid. Scheme II shows the synthesis of the N-2-substituted derivative CGP 55644 (part 1), CGP 56753 (part 2), and the six-membered ring analogue CGP 58238 (part 3).

DNA Constructs—The pBLCAT2 (25)-derived chloramphenicol acetyltransferase (CAT) reporter constructs, containing, in the XbaI site in front of a thymidine kinase promoter, natural response elements for RZR/ROR (21), the vitamin D3 receptor (VDR) (26), the thyroid hormone receptor (T3R) (27), and the retinoid acid receptor (RAR) (28), have been described recently; their core sequences are given in Fig. 3. The cDNAs of human RZR/RORα, human VDR, chicken T3Rα, human RARα, and human RXRα have been subcloned into the expression vector pSG5 (Stratagene).

Cell Culture, Transfection, and CAT Assays—Drosophila SL-3 cells (2 × 10^6 cells/well in a 6-well plate) were grown overnight in Schneider’s medium (Life Technologies, Inc.) without fetal calf serum. Liposomes were prepared by incubating 2 μg of the reporter plasmid, 1 μg of receptor expression vectors, and 1 μg of the reference plasmid pCH110 (Pharmacia Biotech Inc.) with 11 μg of N-(1,2,3-dioleoyloxy)propyl-N,N,N-trimethylammonium methysulfate (Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 μl. After dilution with 0.9 ml of Schneider’s medium, the liposomes were added to the cells. 4–8 h after transfection, 500 μl of Schneider’s medium supplemented with the indicated ligand was added. After a further 16 h, the cells were harvested, and CAT assays were performed as described (29). CAT activities were normalized to β-galactosidase activity, and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced controls. Each condition was analyzed at least in triplicate, and data are shown as means ± S.D.

Rat Adjuvant Arthritis—Adjuvant arthritis was induced as described previously (30). Briefly, male Lewis rats (LEW/TIF, SPF, 150–180 g of body weight, five animals/group) were immunized by an intraplantar injection of Freund’s complete adjuvant (0.2 mg of heat-killed Mycobacterium butyricum (Difco) in 0.05 ml of paraffin oil (Riedel de Haen)) into the left hind paw (day 0). This procedure induced arthritis in 100% of the animals. Disease progression was followed by plethysmographic edema measurements of the injected hind paw (primary lesion) and the noninjected hind paw (secondary lesion), and joint destruction was assessed by radiography on day 32. Lesions in the tarsometatarsophalangeal and carpometacarpophalangeal regions of hind and fore paws were assessed by scoring as described (30). Test compounds were given...
orally to five animals/group between 8.00 and 10.00 a.m. from days 0 to 30 (or as indicated) in 10 ml/kg of 0.75% methylcellulose. Normal and arthritic control animals (n = 10) received the vehicle. Differences between groups were statistically evaluated by Student’s paired t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001 (asterisks are indicated on Figs. 5–7)).

RESULTS

Specific Activation of RZR/RORα by TDs—To test RZR/RORα-mediated transcriptional activation by TDs, we used transiently transfected Drosophila SL-3 cells as reporter cells. This cell line is devoid of mammalian nuclear receptors and is a well-characterized system for analyzing nuclear signaling processes (31, 32). SL-3 cells are very robust cells that survive more than 2 days in the absence of serum. Since RZR/ROR shows in the presence of serum a rather high constitutive activity (13, 15, 20), it was more effective to analyze drug-induced transcriptional activation under serum-free conditions.

SL-3 cells were transfected with the expression vector for human RZR/RORα and a thymidine kinase-CAT reporter construct containing the RZR/ROR response element of the human 5-lipoxygenase gene (21). It is interesting to note that melatonin causes a transcriptional activation from this heterologous promoter construct in the Drosophila system, whereas in human B lymphocytes, it causes repression (21).

Fig. 3A shows the effect of 13 TDs (Figs. 1 and 2) on RZR/RORα-mediated gene activation in comparison with the effect of the natural RZR/ROR ligand melatonin. All compounds were tested at 100 nM concentrations under serum-free conditions. Five TDs (CGP 52608, CGP 52528, CGP 53065, CGP 53079, and CGP 58238) and melatonin stimulated RZRα-mediated gene activity 5–7-fold, whereas seven TDs (CGP 52113, CGP 52749, CGP 55644, CGP 55706, CGP 55707, CGP 56753, and GP 50468) showed <1.5-fold induction and were therefore considered to be inactive.

The specificity of gene activation by TDs and melatonin was tested on typical vitamin D₃, thyroid hormone, and all-trans-retinoic acid signaling pathways. From DR3-, DR4- and DR5-type response elements, which were cotransfected with RXRα and human VDR, chicken T₃Rα, or human RARα, respectively, we obtained only significant gene activation with the specific ligands 1,25-dihydroxvitamin D₃, thyroid hormone, and all-trans-retinoic acid, but not with 12 of the 13 TDs or melatonin (Fig. 3, B–D). An exception was CGP 55066, which moderately
but nonspecifically activated all four nuclear signaling pathways.

We then performed dose responses with the five specifically RZR/RORα-activating TDs and melatonin for reporter gene activation via the 5-lipoxygenase RZR/ROR response element. The same experimental conditions as described for Fig. 3A were used, and graded concentrations of compounds between $10^{-11}$ and $10^{-6}$ M were used for stimulation (Fig. 4). We obtained typical sigmoidal dose-response curves that provided EC50 values between 0.5 and 6.0 nM for TDs and of 1.0 nM for melatonin.

Antiarthritic Activity of TDs in Rat Adjuvant Arthritis—We first performed detailed investigations with the structural lead compound CGP 52608 in rat adjuvant arthritis and found that this compound was active under prophylactic as well as therapeutic test conditions. Here we show the effects of prophylactic treatment of arthritic rats with low doses of CGP 52608 (Fig. 5). Drug treatment was started at the day of arthritis induction (day 0) and was continued up to day 38. The inflammatory edema in both hind paws was dose-dependently inhibited, and highly significant suppressive effects were measured with oral doses of 0.1 and 1 mg/kg. Destructive processes in the joints of the injected hind paw (primary lesions) and the non-injected paws (secondary lesions) at day 32 were also significantly inhibited.

We then tested all TDs (Figs. 1 and 2) using the same experimental protocol as described for Fig. 5 and sought to determine whether we could find any correlation between antiarthritic activity and RZR/RORα activation. The results are summarized in Table I. All five RZR/RORα-activating TDs significantly suppressed the inflammatory edema and inhibited joint destruction at oral doses of 0.1 and 1 mg/kg (Figs. 5–7). On the other hand, four out of seven receptor-inactive TDs (CGP 52749, CGP 55707, CGP 56753, and GP 50468) and the nonspecific compound CGP 55066 were inactive up to the highest doses tested (5 or 10 mg/kg per os). The remaining three compounds (CGP 55644, CGP 52113, and CGP 55706) were either distinctly less potent or only active during the late phase of arthritis. The N-2 methylated compound CGP 55644 was inactive up to 1 mg/kg, but exhibited weak antiarthritic

**Fig. 4.** Dose-response curves for thiazolidine diones and melatonin. Drosophila SL-3 cells were transfected with the expression vector for human RZR/RORα and the CAT reporter construct containing the RZR/ROR response element of the human 5-lipoxygenase promoter (21). The cells were treated with increasing concentrations of melatonin or thiazolidine diones (no addition of fetal calf serum) as indicated. CAT activities were determined 16 h later, and stimulation was calculated in comparison with solvent controls. Each point represents the mean of triplicates; the standard deviation was always <10%.
effects at 10 mg/kg, starting around day 20 after arthritis induction. The N-propyl derivative CGP 52113 was active at 1 mg/kg per os from day 12 onward, and CGP 55706 with two methyl groups at N-4 showed significant but delayed inhibition at 0.1 and 1 mg/kg, starting around day 25. The antiarthritic effects of these three receptor-inactive TDs might be explained by prodrug cleavage. Taken together, our results show a good correlation between activation of human RZR/RORα and antiarthritic activity in rat adjuvant arthritis for 13 TDs with closely related structures.

Melatonin and S 20098 in Rat Adjuvant Arthritis—We repeatedly tested the natural RZR/ROR ligand melatonin in doses between 0.001 and 10 mg/kg per os in rat adjuvant arthritis. Although this model always gave reproducible results with TDs as well as with the reference compounds prednisolone and cyclosporin A, we obtained inconsistent results with melatonin. In some of the experiments, melatonin caused significant but not dose-dependent inhibitory effects, whereas in other experiments, the hormone was completely inactive (Table I). We assume that environmental (maybe seasonal) influences and the very short plasma half-life of orally administered melatonin (33) could explain our variable results. It is clear, however, that all five RZR/RORα-activating TDs were much more potent in inhibiting adjuvant arthritis than melatonin, although their EC50 values for RZR/RORα activation in the Drosophila system were comparable (Fig. 3A).

Finally, we investigated a melatonin agonist with specificity for the membrane receptor. We found that the Servier compound S 20098, which is a highly potent melatonin agonist at the membrane receptor (34), was not able to induce transcriptional activation via RZR/RORα under conditions described for Fig. 3 (data not shown) and was inactive in adjuvant arthritis in doses between 0.01 and 10 mg/kg per os (Table I).

**DISCUSSION**

We have discovered a structurally novel class of thiazolidine diones with high potency in inhibiting inflammation and joint...
FIG. 6. Inhibition of primary edema in arthritic rats by RZR/RORα-activating thiazolidine diones. Adjuvant arthritis was induced in Lewis rats, and disease progression was followed by edema measurements of the injected hind paw as described for Fig. 5. Compounds were administered orally from days 0 to 30. ■, adjuvant arthritis control; *, 0.1 per os; □, 1 per os.

FIG. 7. Inhibition of joint destruction in arthritic rats by RZR/RORα-activating thiazolidine diones. Experimental details are as described for Fig. 6. Radiography was performed on day 32. Joint lesions were scored in the injected hind paw (primary lesions (PL)) and in the noninjected hind paw and fore paws (secondary lesions (SL)). n.s., not significant. ■, adjuvant arthritis control; □, 0.1 per os; □, 1 per os.
destruction in rat adjuvant arthritis, which is a chronic T cell-dependent autoimmune disease. Compared with reference compounds with known antiarthritic activity in this model such as corticosteroids, cyclosporin A, cytostatic compounds, and cyclooxygenase inhibitors, these TDs have a distinctly different pharmacological profile. Besides their activity in adjuvant arthritis, they suppress other experimental autoimmune diseases and exhibit oncostatic and hormone-suppressive properties, but they do not cause a systemic immuno- or myelosuppression. They also do not inhibit the activity of the enzymes cytochrome P450, 5-lipoxygenase, and phospholipases A and C. We therefore suggested a novel mechanism of action and have found that the lead compound CGP 52608 specifically activates the orphan nuclear receptor RZR/RORα in low nanomolar concentrations (13).

Here we show for 13 CGP 52608 analogues a striking correlation between their antiarthritic activity and their ability to activate RZR/RORα (Table 1). Five TDs (Fig. 1) activate RZR/RORα, whereas eight closely related analogues (Fig. 2) are inactive, with the exception of CGP 55066, which has moderate nonselective activity. All compounds shown in Fig. 1 exhibit potent antiarthritic activity, whereas the compounds shown in Fig. 2 are either inactive or distinctly less potent. The resulting structure-activity relationship for receptor activation by this series of CGP 52608 analogues is summarized in Fig. B. Neither propyl (CGP 52113) nor benzyl (CGP 52749) is tolerated as a substituent at the ring amide, whereas allyl (CGP 52608), methallyl (CGP 52528), and propynyl (CGP 53065) activate the receptor. Disubstitution (compared with CGP 52608) at N-4 (CGP 55706 and CGP 55707), as well as an unsubstituted N-4 (GP 50468), results in loss of activity. Both replacement of N-2 by a carbon atom (CGP 56753) or its methylation (CGP 56164) inactivate as well. Some modifications at the ring system (methylation, CGP 53079; homologation to a six-membered ring, CGP 58238) lead to compounds that retain full activity. The sulfur in the thiosemicarbazone part seems to be crucial for activity (CGP 52608 versus CGP 55066). It is important to note that competition experiments with [125I]iodomelatonin and [3H]CGP 52608 (13) indicate that TDs do not undergo a covalent interaction with RZR/RORα and therefore do not form disulfide bridges with cysteine side chains. The finding that compounds with an identical thiosemicarbazone part but different R1 substituents (e.g. CGP 52608 versus CGP 52113 and CGP 52749) are either active or inactive shows that activation of RZR/RORα cannot be simply rationalized by the known ability of thiosemicarbazones to complex different heavy metals. Based on these results, we suggest that these TDs exert their antiarthritic activity and potentially other pharmacological effects at least in part via RZR/RORα-mediated nuclear signaling processes.

It is very interesting that structurally and pharmacologically different thiazolidine diones have been recently identified as ligands of the orphan nuclear receptor PPARγ (35, 36). Since these compounds have antidiabetic and adipocyte differentiation-inducing properties, a pivotal role for PPARγ and its endogenous ligand(s) in adipocyte development and glucose homeostasis is suggested.

Several lines of evidence have shown that the pineal gland hormone melatonin is a natural ligand of RZR/RORα (Refs. 13 and 20 and this paper). Although we were unable to demonstrate consistent therapeutic effects with melatonin in rat adjuvant arthritis, CGP 52608 analogues and melatonin share several characteristic pharmacological features, which point to a common mechanism of action. The best known physiological role of melatonin is its function as a transmitter of photoperiodic information and regulator of seasonal reproduction (37). A functionally active high affinity G protein-coupled melatonin membrane receptor has recently been cloned (38, 39) and is thought to mediate the circadian effects of the hormone (40). However, melatonin additionally exhibits oncostatic effects and influences various immunological and endocrinological functions (41). Similar properties are also known for CGP 52608 analogues. It has been suggested that melatonin is involved in basic mechanisms controlling cell growth and differentiation (42, 43), and nuclear signaling was in fact proposed before the discovery of RZR/RORα (44, 45). It is currently not clear whether nuclear and membrane signaling are functionally linked or whether both pathways mediate different effector functions of the hormone (18, 46). Since CGP 52608 and its analogues do not bind to the melatonin membrane receptor (13), and S 20098, a potent melatonin agonist at the membrane receptor (34), does not activate RZR/RORα (this paper), these compounds may lead to a better understanding of the molecular mechanisms responsible for the pleiotropic functions of the neurohormone. We have shown here that S 20098, which influences circadian rhythms in rats in a manner similar to melatonin (47), has no activity in rat adjuvant arthritis.

The intracellular functional changes following RZR/RORα activation and finally causing the described antiarthritic effects of CGP 52608 analogues are not known. It is interesting that the first identified melatonin/RZR/RORα-responder gene, 5-lipoxygenase, a key enzyme in allergic and inflammatory reactions, is down-regulated by melatonin in human B cells (21). However, we suggest that other target genes central to the immunoinflammatory response are also controlled via RZR/RORα-mediated nuclear signaling. The regulation of many genes is typical for members of the nuclear receptor superfamily and their ligands and is well known for glucocorticosteroids, retinoids, thyroid hormone, and vitamin D, which all have important modulatory effects on the immune system (48, 49). The discovery of RZR/RORα receptors may open new perspectives, and the identification of the first synthetic ligands with interesting pharmacological properties may help to elucidate important physiological functions of these nuclear receptors.

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