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Antithyroid Drugs Inhibit Thyroid Hormone Receptor-Mediated Transcription

Kenji Moriyama, Tetsuya Tagami, Takeshi Usui, Mitsuhide Naruse, Takuo Nambu, Yuji Hataya, Naotetsu Kanamoto, Yu-shu Li, Akihiro Yasoda, Hiroshi Arai, and Kazuwa Nakao

Department of Medicine and Clinical Science (K.M., T.N., Y.H., N.K., Y.L., A.Y., H.A., K.N.), Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan; and Division of Endocrinology and Metabolism (K.M., T.T., T.U., M.N.), Clinical Research Institute, Kyoto Medical Center, National Hospital Organization, Kyoto 612-8555, Japan

Context: Methimazole (MMI) and propylthiouracil (PTU) are widely used as antithyroid drugs (ATDs) for the treatment of Graves' disease. Both MMI and PTU reduce thyroid hormone levels by several mechanisms, including inhibition of thyroid hormone synthesis and secretion. In addition, PTU decreases 5′-deiodination of T_4 in peripheral tissues. ATDs may also interfere with T_3 binding to nuclear thyroid hormone receptors (TRs). However, the effect of ATDs on the transcriptional activities of T_3 mediated by TRs has not been studied.

Objective: The present study was undertaken to determine whether ATDs have an effect on the gene transcription regulated by T_3 and TRs *in vitro*.

Methods: Transient gene expression experiments and GH secretion assays were performed. To elucidate possible mechanisms of the antagonistic action of ATDs, the interaction between TR and nuclear cofactors was examined.

Results: In the transient gene expression experiments, both MMI and PTU significantly suppressed transcriptional activities mediated by the TR and T_3 in a dose-dependent manner. In mammalian two-hybrid assays, both drugs recruited one of the nuclear corepressors, nuclear receptor corepressor, to the TR in the absence of T_3. In addition, PTU dissociated nuclear coactivators, such as steroid receptor coactivator-1 and glucocorticoid receptor interacting protein-1, from the TR in the presence of T_3. Finally, MMI decreased the GH release that was stimulated by T_3.

Conclusions: ATDs inhibit T_3 action by recruitment of transcriptional corepressors and/or dissociation of coactivators. This is the first report to show that ATDs can modulate T_3 action at the transcriptional level. ***(J Clin Endocrinol Metab* 92: 1066–1072, 2007)**

THYROID HORMONES REGULATE growth, development, and critical metabolic functions. They exert these effects through complex biological pathways, which offer a wealth of opportunity to intervene pharmacologically in thyroid hormone signaling at numerous steps. These include biosynthesis, cell-specific uptake, or export of thyroid hormone as well as nuclear targeting and actions, which are exerted through thyroid hormone receptor (TR) binding and histone acetylation. Such processes represent potentially important pharmacological targets for the drug therapies of thyroid hormone abnormalities, especially hyperthyroidism.

Some compounds having thionamide structure, such as thiourea and thiouracil, inhibit thyroid function. Clinically used antithyroid drugs (ATDs) include methimazole (1-methyl-2-mercaptoimidazole; MMI), and propylthiouracil (6-propyl-2-thiouracil; PTU) to treat Graves' hyperthyroidism (Fig. 1). ATDs have intrathyroidal and extrathyroidal actions. The chief intrathyroidal actions include inhibition of iodine oxidation and organization and iodotyrosine coupling, among others. The main extrathyroidal action is inhibition of conversion of T_4 to T_3 by PTU, but not MMI (1, 2). Thus, the reduction in thyroid hormone production induced by the drugs is central to these actions.

Furthermore, ATDs are known to influence oxygen consumption, or peripheral metabolic suppression, although the mechanisms are not fully understood (3, 4). To date, a number of studies was performed to elucidate how ATDs suppress peripheral metabolism. ATDs can affect gene expression and modulate functions of some cell types (5). Although ATDs were not effective in the binding affinity of T_4 to serum thyroxine binding globulin, they inhibited T_3 binding to the hepatic nuclear extracts (6). However, the effect of ATDs on the transcriptional activities of T_3 mediated by TRs has not been studied in detail. The present study was undertaken to determine whether ATDs have an effect on the gene transcription regulated by T_3 and TRs *in vitro*.

Materials and Methods

Reagents

The chemical structures of PTU, MMI, and T_3 are shown in Fig. 1. ATDs were purchased from Sigma-Aldrich, Corp. (St. Louis, MO). T_3 was purchased from Nakalai Tesque Inc. (Kyoto, Japan).

Plasmid constructions

Expression vectors containing wild-type human TRβ1 (pCMX-hTRβ1) and human TRα1 (pCMX-hTRα1) were provided by K. Ume-

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Abbreviations: AF, Activation function; ATD, antithyroid drug; CoA, coactivator protein; CoR, corepressor protein; DBD, DNA binding domain; FBS, fetal bovine serum; GRIP, glucocorticoid receptor interacting protein; LBD, ligand-binding domain; Luc, luciferase; MMI, methimazole; NCoR, nuclear receptor corepressor; PTU, propylthiouracil; SMRT, silencing mediator of retinoid and thyroid receptor; SRC, steroid receptor coactivator; TK, thymidine kinase; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

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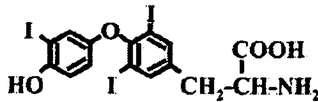
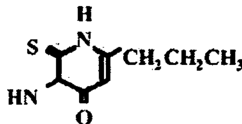
A Triiodothyronine (T3)



B Propylthiouracil (PTU)



C Methimazole (MMI)

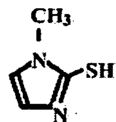


FIG. 1. The structure of T_3 and two antithyroid drugs, propylthiouracil and methimazole.

sono (Salk Institute, San Diego, CA) (7). The ligand-binding domain (LBD) of TRα1 or TRβ1 was fused to the DNA binding domain (DBD) of Gal4 in-frame in pSG424 (8). The Gal4-NCoR (residues 1552–2453), Gal4-SRC1 (residues 213-1061), and Gal4-GRIP1 (residues 480-1462) constructs contain the indicated TR interaction domains of these proteins (9). The VP16 construct for TRβ contains the LBD of the receptor downstream of the VP16 activation domain in-frame in pCMX (9). The reporter plasmids, TRE-TK-Luc (9) and ME-TK-Luc (10), contain two copies of a palindromic thyroid hormone response elements (TRE) and malic enzyme, respectively, upstream of the thymidine kinase (TK) promoter in the pA3-luciferase vector (Luc). TSHα-Luc contains 846 bp of the 5′-flanking sequence and 44 bp of exon I from the human glycoprotein hormone α-subunit gene in pA3-Luc (9). The Gal4 reporter plasmid, UAS-E1BTATA-Luc, contains five copies of the Gal4 recognition sequence (UAS) upstream of E1BTATA in pA3-Luc (11). The pRL-TK vector (Promega Corp., Madison, WI) comprised of the tk promoter and *Renilla* luciferase cDNA was used as an internal control.

Cell culture

TSA201, a clone of human embryonic kidney 293 cells (12), human hepatoblastoma HepG2 cells, and rat pituitary GH3 cells were maintained in DMEM, containing penicillin (100 U/ml) and streptomycin (100 μg/ml) with 10% fetal bovine serum (FBS; Invitrogen, Corp., Carlsbad, CA). For hormone and drug treatment, the medium was changed to phenol red-free DMEM (Nikken Biomedical Laboratory, Kyoto, Japan) containing 10% charcoal-stripped FBS (13).

Transient expression assays

TSA201 and HepG2 cells were grown in phenol red-free DMEM (Nikken Biomedical Laboratory) with 10% charcoal-stripped FBS and were transfected using the calcium precipitation method (14). After exposure to the calcium phosphate-DNA precipitate for 8 h, phenol red-free DMEM with charcoal-stripped FBS was added, in the absence or presence of compounds and/or T_3. Cells were harvested for measurements of luciferase activity, according to the manufacturer's instructions (dual-luciferase reporter assay system; Promega). The transfection efficiencies were corrected with the internal control. Both firefly and *Renilla* luciferase activities were measured to monitor the transfection efficiency and cytotoxicity of the added materials. The firefly activity obtained by the T_3-specific promoter was divided by the *Renilla* activity obtained by the nonspecific promoter in each well. Results are expressed as the mean ± SD from at least three transfections, each performed in triplicate. Data were analyzed by ANOVA with *post hoc* Dunnett's tests to compare with the control.

GH secretion and assay

For GH assays, GH3 cells, derived from the rat pituitary tumor cell line, were seeded into 6-well plates at 1.5×10^4 cells/well. T_3 (1 nM) as a physiological concentration and/or 10 μM MMI was added on the day after the medium was replaced. In the case of 10 μM PTU, cell survival was inhibited and the assay was abandoned. Culture media were collected after 2 d of incubation and GH was measured by ELISA (rat GH enzyme-immunoassay system; GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instructions. After harvesting the supernatant, cell numbers were counted to evaluate cell proliferation. Results are expressed as the mean ± SD from at least five experiments, each performed in quadricate. Data were analyzed by ANOVA with *post hoc* Dunnett's tests to compare with the control.

Results

ATDs suppressed transcriptional activities mediated by TRα1 and TRβ1

The chemical structure of T_3, PTU, and MMI are shown in Fig. 1. ATDs are known as thionamides, which contain a sulfhydryl group and a thiourea moiety within a heterocyclic structure. Takagi *et al.* (6) reported that ATDs inhibit specific binding of T_3 to its receptor, perhaps due to an interaction with cysteine residues of the receptor.

We examined whether ATDs antagonize T_3-induced TR activation. Transient expression experiments were performed using TSA201 cells, which are a derivative of human embryonic kidney 293 cells. The LBD of TRα1 or TRβ was fused to the DNA binding domain of the yeast transcription factor, Gal4, and was cotransfected with a Gal4 reporter gene, UAS-E1BTATA-Luc. We determined the effects of ATDs on various physiological concentrations of T_3. In the presence of 10 μM PTU, increasing amounts of T_3 were added to the medium, and transcriptional activity was measured. PTU had no significant effects on TR-mediated transcriptional activity in the absence of T3 (Fig. 2) and in the absence of Gal4-TR (see Fig. 6A). However, PTU suppressed the activity mediated by Gal4-TRα1 up to 36% and Gal4-TRβ up to 39% of the respective control levels in the presence of T_3. The maximum suppression by PTU was obtained at the concentration of 1 nM of T_3. Similar results were obtained using MMI (data not shown).

The inhibitory effects of ATDs were also examined in the context of native receptors. A T_3-responsive reporter gene, TRE-TK-Luc, was cotransfected with full-length TRs (Fig. 3A). ATDs did not affect the activity of TRE-TK-Luc alone without TR. In the presence of 1 nM T_3, dose-dependent inhibition of transcription mediated by TRα1 and TRβ1 was observed with both ATDs. PTU suppressed the TRα1-mediated activity by 45% and TRβ1-mediated activity by 39% and MMI suppressed TRα1-mediated activity by 45% and TRβ1-mediated activity by 53% of the respective T_3 effect.

In a reciprocal manner, another group of negatively regulated genes was stimulated by TRs in the absence of T_3 and was repressed in response to T_3 (14). The effects of ATDs on the TSHα promoter were examined as a model of a negatively regulated gene. As shown in Fig. 3B, although PTU did

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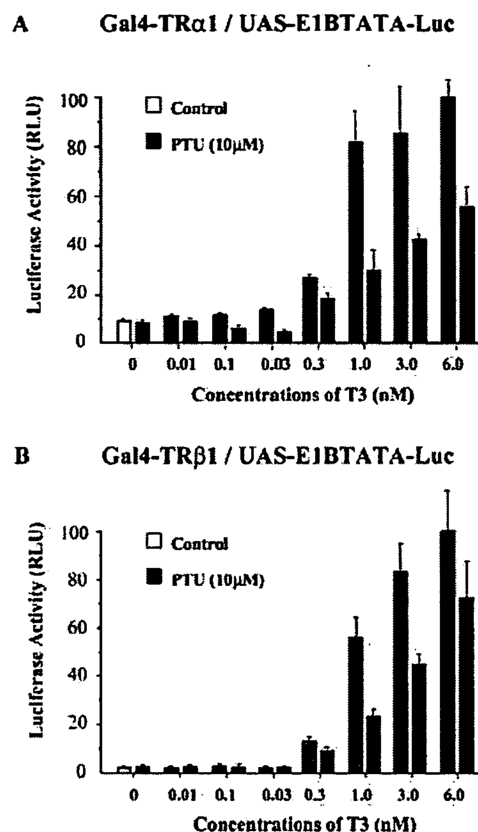

FIG. 2. ATDs suppress TR-mediated transcription in the presence of a physiological range of T_3. Gal4-TRα1 (A) or Gal4-TRβ1 (B; 50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the absence or presence of 10 μM PTU. RLU, Relative light units. The firefly activity obtained by UAS-E1BTATA-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc.

not affect the activity of TSHα-Luc alone without TR, PTU increased the transcriptional activity, which was suppressed by 1 nM T_3 up to the control level. Similar results were obtained using MMI (data not shown).

The inhibitory effects of PTU were also examined at high concentrations of T_3. In the presence of 100 nM of T_3, which is almost 20 times greater than concentrations found in blood samples of patients with severe hyperthyroidism, the effects of PTU were eliminated (Fig. 4). Similar results were obtained using MMI (data not shown).

ATDs suppressed transcriptional activities mediated by endogenous TRs

We next studied the effects of ATDs using a cell line that contains physiological amounts of endogenous TRs. The reporter gene regulated by the ME-TRE, ME-TK-Luc, was transfected into human hepatoblastoma, HepG2. No significant effects were observed by 48 h incubation with 10 μM PTU or MMI on the ME-TK-Luc activity in the absence of T_3. One nanomole T_3 stimulated the activity by 1.8-fold, compared with that without T_3, and ATDs inhibited its increase by 70% (Fig. 5).

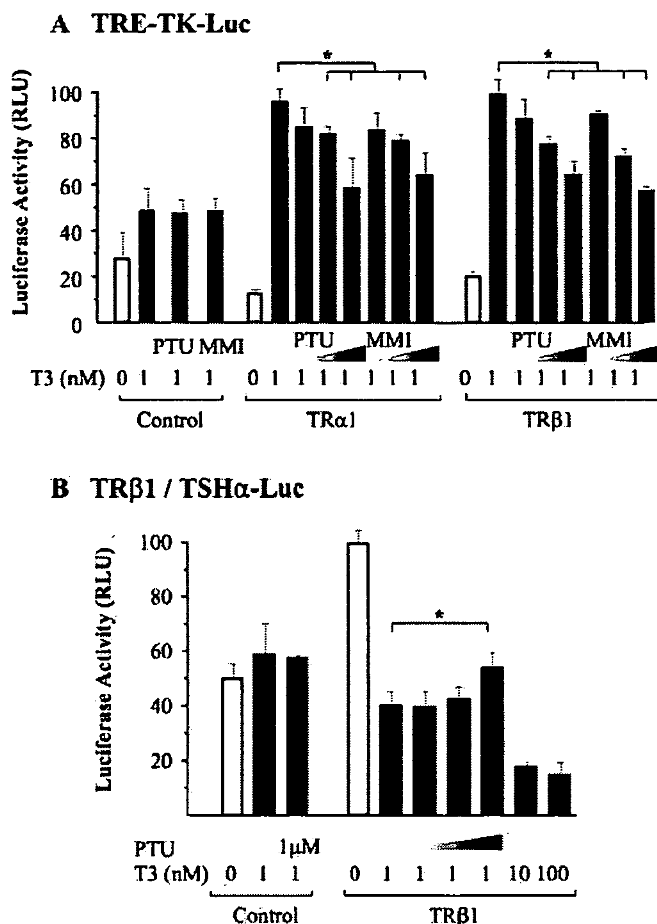

FIG. 3. The inhibitory effects of ATDs on the gene transcription mediated by native TR. A, The CMX, CMX-TRα1, or CMX-TRβ1 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TRE-TK-Luc, in the absence or presence of 1 nM T_3 and increasing amount of ATDs. B, The CMX or CMX-TRβ1 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TSHα-Luc, in the absence or the presence of 1 nM T_3 and increasing amount of PTU (1 nM, 100 nM, and 10 μM). RLU, Relative light units. The firefly activity obtained by TRE-TK-Luc or TSHα-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. *, $P < 0.05$.

ATDs recruit corepressor and dissociate coactivator

Transcriptional repression of the positively regulated genes by unliganded TR is mediated by interacting with corepressor proteins (CoRs) such as nuclear receptor corepressor (NCoR) (15) and silencing mediator of retinoid and thyroid receptors (SMRT) (16). CoRs might also be involved in the basal activation of negatively regulated genes (14). In the other hand, in the presence of T_3, liganded TR activates transcription of the positively regulated genes and inhibits that of the negatively regulated genes by interacting with coactivator proteins (CoAs) (17) such as steroid receptor coactivator (SRC)-1 (18) and glucocorticoid-interacting protein (GRIP)-1 (19). Using a mammalian two-hybrid assay, the effect of ATDs on the TR-CoR or TR-CoA interaction was examined. The TR interaction domain of CoRs or CoAs was fused to the Gal4-DBD. The LBD of TRβ was fused to the transcriptional activation domain of VP16 to allow detection of the interaction between the Gal4-CoR/CoA and VP16-TR.

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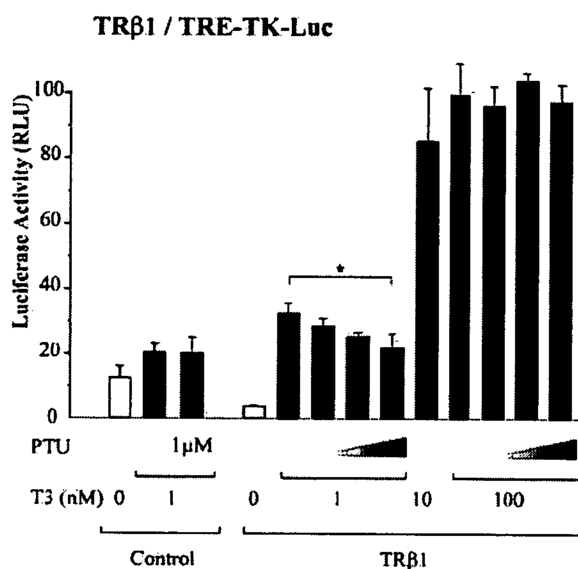

FIG. 4. High amount of T_3 blocks the inhibitory effects of ATDs on the gene transcription mediated by native TR. The CMX or CMX-TRβ1 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TRE-TK-Luc, in the absence or the presence of 1 or 100 nM T_3 and an increasing amount of PTU (1 nM, 100 nM, and 10 μM). RLU, Relative light units. The firefly activity obtained by TRE-TK-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. *, $P < 0.05$.

As shown in Fig. 6A, both PTU and MMI had no significant effects on the activity mediated by Gal4-DBD and VP16-TR. PTU enhanced TR-NCoR interaction but not TR-SMRT interaction in a dose-dependent manner (Fig. 6B). In contrast, PTU inhibited TR-SRC1 interaction and, to a lesser degree, TR-GRIP1 interaction in a dose-dependent manner (Fig. 6D). Similarly, MMI enhanced TR-NCoR interaction but not TR-SMRT interaction and inhibited TR-GRIP1 interaction but not TR-SRC1 interaction (Fig. 6, C and E).

ATDs inhibited endogenous hormone secretion induced by T_3

T_3 stimulates endogenous GH gene transcription mediated by endogenous TRs in rat pituitary tumor cells (20–22).

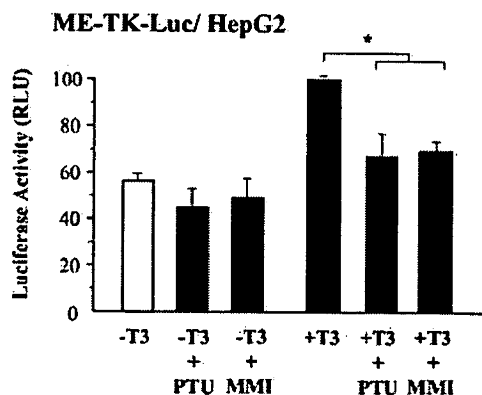

FIG. 5. ATDs suppress transcriptional activities mediated by endogenous TRs. The ME-TK-Luc (100 ng) was transfected into HepG2 cells and incubated with or without 1 nM T_3 and/or 10 μM PTU or MMI for 48 h. RLU, Relative light units. The firefly activity obtained by ME-TK-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. *, $P < 0.05$.

The production of GH was measured by ELISA in the culture media of GH3 cells. Forty-eight-hour incubation with 1 nM T_3 stimulated the GH production by 2.7-fold (Fig. 7). Addition of 10 μM MMI significantly decreased the GH release to 82% of that with 1 nM T_3 alone. In the case of 10 μM PTU, cell death was induced (data not shown).

Discussion

The ATDs have been a mainstay of treatment of patients with Graves' hyperthyroidism (23). The ATDs are heterocyclic compounds known as thionamides that contain a thioureylene group (Fig. 1). Two kinds of drugs of this type are available at present: one is MMI and carbimazole (1-methyl-2-thio-3-carbethxy-imidazole), which is rapidly metabolized to MMI, and the other is PTU. These drugs cause goiter in animals, which is due to the stimulation of the thyroid by the pituitary, consequent to pharmacological inhibition of thyroid hormone production (24, 25). Thus, derivatives of thiourea and thiouracil have been used as antithyroid drugs for more than 60 yr.

The main effect of ATDs is to inhibit the synthesis of T_4 and T_3 as an intrathyroidal action, including inhibition of iodine oxidation and organization, inhibition of iodotyrosine coupling, possible alteration of thyroglobulin structure, and inhibition of thyroglobulin biosynthesis. As an extrathyroidal action, PTU but not MMI inhibits conversion of T_4 to T_3. During the last 3 decades, reports have been accumulating on extrathyroidal actions of the ATDs, especially of the thionamide group, causing several undesirable side effects. Bandyopadhyay *et al.* (5) reviewed extrathyroidal actions of antithyroid thionamides in animals and humans. Thionamides inhibit lactoperoxidase, which contributes to the antibacterial activities of a number of mammalian exocrine gland secretions that protect a variety of mucosal surfaces. These drugs stimulate both gastric acid and pepsinogen secretions, thereby augmenting the severity of gastric ulcers and preventing wound healing. Severe abnormalities may develop in blood cells and the immune system after thionamide therapy. They may cause agranulocytosis, aplastic anemia, and purpura along with immune suppression. Olfactory and auditory systems are also affected by these drugs. Thionamide affects the sense of smell and taste and may also cause loss of hearing. Thionamide also affects gene expression and modulate the functions of some cell types.

After administration of ATDs to patients with Graves' disease, it usually takes 2 or 3 d to start decreasing serum level of T_4 and T_3. However, O_2 consumption or other peripheral metabolic indexes indicated that ATDs exerted an immediate effect *in vivo* (4, 5). Besides a specific effect of PTU as a 5'-deiodinase inhibitor effectively preventing T_3 generation, those actions were reported to persist in the presence of both of ATDs. Because thyroid hormones directly activate the expression of the human and mouse uncoupling protein-3 genes through a thyroid response element in the proximal promoter region, peripheral metabolism was controlled most partly by thyroid hormone level (26). An immediate effect of both of ATDs might be involved in the inhibition of transcriptional activities by thyroid hormone.

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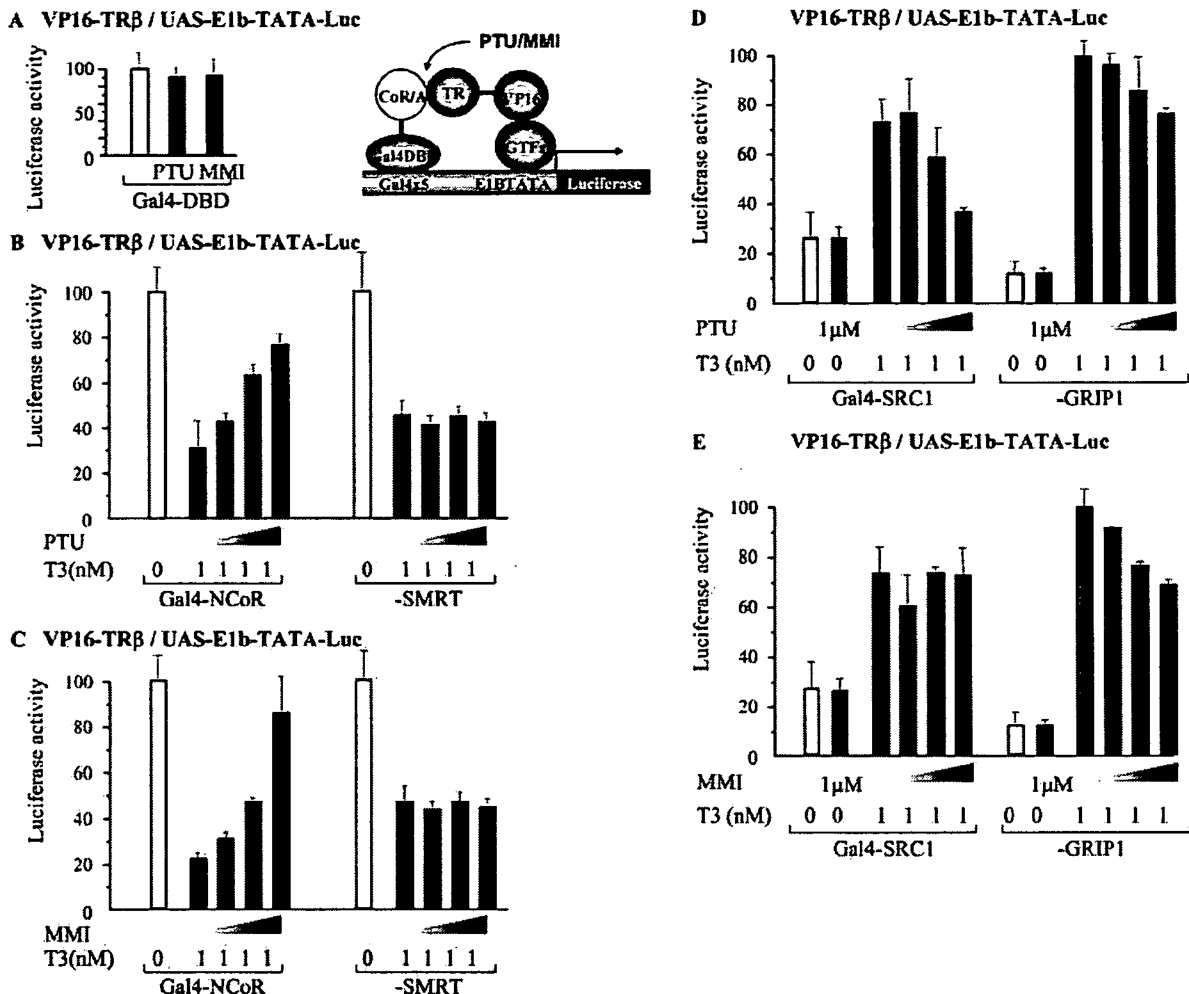

FIG. 6. The effects of ATDs on the interaction between TR and cofactors. The format of the mammalian two-hybrid experiment is shown *between panels A and D*. Gal4 fusion plasmids of indicated cofactors (50 ng) were cotransfected into TSA-201 cells with 100 ng of VP16-TRβ together with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the absence or presence of 1 nM T3. Increasing amounts (1 nM, 100 nM, and 10 μM) of PTU or MMI were added. A, Gal4-DBD. B and C, Gal4-NCoR and Gal4-SMRT. D and E, Gal4-SRC1 and Gal4-GRIP1. RLU, Relative light units. The firefly activity obtained by UAS-E1BTATA-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. GTF, General transcription factor.

Induction of cell proliferation by mitogen or growth factor stimulation leads to the specific and sequential expression of a large number of genes. To date several reports showed that ATDs change mRNA expression level of certain genes. MMI and PTU increase thyroglobulin gene expression and increase thyroid-specific mRNA concentration in human thyroid FRTL-5 cells (27–29). The stimulatory effects of MMI and PTU can be suppressed by iodide and do not occur when protein synthesis is inhibited by cycloheximide. MMI and PTU increased thyroid peroxidase mRNA in cultured porcine thyroid follicles (30). MMI can suppress the interferon-γ-induced increase in HLA-DRα gene expression (31). Fas ligand expression is induced by MMI in follicular cells of thyroid glands obtained from Graves' patients and cultured thyrocytes, resulting in Fas ligand-dependent apoptosis of thyrocytes (32). In the thyroid, the TR expression has been reported (33–35).

ATDs are mainly concentrated in the thyroid gland but more than 70% of ATDs are unevenly distributed in the whole body (36). Therefore, the transcriptional inhibition by ATDs in the periphery may be dependent on the concentration of ATDs in each tissue. The peak serum concentrations of MMI are in the range of 300 ng/ml (2.6 mM) after a 15-mg oral dose (37), and those of PTU are about 3 mg/ml (18 mM) after a 150-mg oral dose (38). As we have shown here, inhibition of T_3 action was observed for GH expression (20–22) in rat pituitary GH3 cells and for malic enzyme expression (10) in human hepatic HepG2 cells. However, the inhibitory effects on cell proliferation of these cells were also seen under the therapeutic concentrations of ATDs.

Takagi *et al.* (6) demonstrated that thionamides inhibit specific T_3 binding to the hepatic nuclear receptor. MMI and PTU at a pharmacological dose (2 mM) reduced specific T_3 binding to chromatographed nuclear receptors by 84 and 85%, respectively. Scatchard analyses indicated that neither MMI nor PTU significantly altered the affinity constant, whereas they both

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GH3 cell

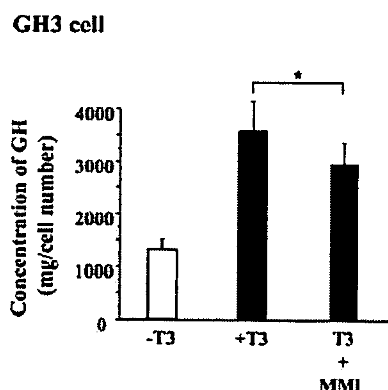

FIG. 7. The inhibitory effects of ATDs on the GH secretion induced by T_3. GH3 cells were incubated with or without 1 nM T_3 and/or 10 μM MMI for 48 h. Culture media were collected and GH was measured by ELISA. *, $P < 0.05$.

decreased maximal binding capacity. We obtained similar results using rat liver nuclear extract (data not shown) (13). Thereafter, there is no study of the actions of ATDs on the transcriptional regulation mediated by T_3 and TR.

In this study we demonstrated that ATDs could impair thyroid hormone action by suppressing its transcriptional activity. Gene suppression is attributed partly to the recruitment of NCoR and dissociation of GRIP1 or SRC1. In the case of positively regulated promoter, TRE-TK, the effect of T_3 was supposed to be inhibited by recruiting NCoR and dissociating GRIP1 or SRC1 by ATDs. In contrast, in the negatively regulated promoter, TSHα, the opposite effect was observed because corepressors may be involved in basal stimulation in the absence of T_3 (14) and coactivators may be involved in T_3-dependent inhibition in the presence of T_3 (17). A number of nuclear cofactors have been cloned, but most of their specific functions are unclear (39). These cofactors were initially studied in the context of the TR and other nuclear hormone receptors. However, every cofactor has multiple interaction domains comprised of subtly distinct LXXLL motif and its combination and seems to interact with multiple receptors in a different way. Each specific ligand also contributes conformational change of the receptor and modulates the cofactor binding to the receptor as an agonist or an antagonist. Indeed, PTU and MMI enhanced recruitment of NCoR but not SMRT to the TR and both drugs dissociated GRIP1, but dissociation of SRC1 occurred solely by PTU. NCoR preferentially bind TR homodimer over TR-RXR heterodimer (40) and TR prefers to recruit NCoR, and retinoid acid receptor prefers to recruit SMRT (41). These preferences are likely due to sequence differences in interacting domains of corepressors (42).

Conformational change of TR induced by ATDs may be subtly different from that induced by T_3, and it may enhance the interaction with specific domain of NCoR. The functional specificity is also reported among SRC family members. For example, progesterone receptor interacts preferentially with SRC1, which recruits cAMP response element-binding protein (CREB) binding protein (CBP) and enhances acetylation of histone H4, whereas glucocorticoid receptor interacts preferentially with SRC2 (GRIP1), which recruits p300/CBP-associated factor and results in histone H3 acetylation (43). PTU and MMI may have some different effect on the interaction between TR and SRC family members via their subtly distinct LXXLL motifs. Every receptor also has more than one activation domain [activation function (AF)-1 and AF-2]. Coactivators may preferentially use specific activation domains, depending on the receptor or activation function (AF-1 *vs.* AF-2) that is mediating the response to hormone (44). AF2 is conserved among nuclear hormone receptor superfamily as ligand-inducible transcription factors (45). We also tested the effects of ATDs on transcription mediated by other nuclear hormone receptors such as estrogen receptor-α and -β. ATDs had no effects on estrogen action (data not shown).

In summary, our findings demonstrate that ATDs, which are the most prevalent drugs to treat Graves' diseases, suppress transcriptional activity by modulating the cofactor recruitment to the TR. Although the clinical and therapeutic significance of our findings remains to be established, our data provide a mechanistic basis for one of the extrathyroidal actions of ATDs. It might be helpful in designing new therapeutic compounds with modifications of existing ATDs to enhance transcriptional inhibition against T_3 action.

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Address all correspondence and requests for reprints to: Tetsuya Tagami, M.D., Ph.D., Division of Endocrinology and Metabolism, Clinical Research Institute, Kyoto Medical Center, National Hospital Organization, Kyoto 612-8555, Japan. E-mail: ttagami@kyotolan.hosp.go.jp.

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