Identifying a Small Molecule Blocking Antigen Presentation in Autoimmune Thyroiditis

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We previously showed that an HLA-DR variant containing arginine at position 74 of the DRβ1 chain (DRβ1-Arg74) is the specific HLA class II variant conferring risk for autoimmune thyroid diseases (AITD). We also identified 5 thyroglobulin (Tg) peptides that bound to DRβ1-Arg74. We hypothesized that blocking the binding of these peptides to DRβ1-Arg74 could block the continuous T-cell activation in thyroiditis needed to maintain the autoimmune response to the thyroid. The aim of the current study was to identify small molecules that can block T-cell activation by Tg peptides presented within DRβ1-Arg74 pockets. We screened a large and diverse library of compounds and identified one compound, cepharanthine that was able to block peptide binding to DRβ1-Arg74. We then showed that Tg.2098 is the dominant peptide when inducing experimental autoimmune thyroiditis (EAT) in NOD mice expressing human DRβ1-Arg74. Furthermore, cepharanthine blocked T-cell activation by thyroglobulin peptides, in particular Tg.2098 in mice that were induced with EAT. For the first time we identified a small molecule that can block Tg peptide binding and presentation to T-cells in autoimmune thyroiditis. If confirmed cepharanthine could potentially have a role in treating human AITD.

Autoimmune thyroid diseases (AITD), Graves disease (GD), and Hashimoto thyroiditis (HT), are among the most common autoimmune disorders, afflicting up to 5% of the United States population (1). They are characterized by infiltration of the thyroid by lymphocytes reactive to thyroid antigens and production of thyroid-specific antibodies (2). Complex interaction of genetic susceptibility factors, environmental triggers, and epigenetic alterations leads to the breakdown of tolerance, resulting in AITD (3–5). Currently, there is no satisfactory therapy except for hormone replacement therapy in HT or thyroid suppression or ablation in GD (6–10). To develop new therapies for AITD a better understanding of their etiology is needed. We have been studying the etiology of AITD using a reverse-genetics approach, i.e. dissecting the mechanisms causing disease through unbiased genetic screening studies. These studies led to the identification of a specific HLA-DR pocket sequence that is strongly associated with AITD (11). The presence of arginine at position 74 of the DRβ chain renders the individual highly susceptible to AITD, whereas glutamine at position 74 is protective (12). These data were confirmed by other groups (13). The presence of DRβ1-Arg74 (from here on we refer to the HLA-DR3 containing arginine at position 74 as HLA-DRβ1-Arg74) results in a more positively charged P4 pocket. With this structural change in the pocket, the selectivity and binding of pathogenic peptides is affected, conferring higher risk for disease (12).

Besides the HLA genes, two thyroid-specific genes, the thyroglobulin (Tg) and thyrotropin receptor (TSHR) genes also contribute to the etiology of AITD (2). Thyroglobulin is the most abundant thyroidal protein (14), and it is the precursor to thyroid hormones T3 and T4. All forms of AITD (GD and HT) are characterized by the development of Tg antibodies in the majority of patients, and recent mouse data suggest that Tg is the primary target of the autoimmune response in AITD (15). Furthermore, our group has reported a statistical interaction between HLA-DR3 (DRB1*03) and a Tg variant (W1999R), resulting in a combined odds ratio of 6.1 for GD (16). With DRβ1-Arg74, the primary HLA-DRβ1 sequence variant associated with GD, this gene-gene interaction between Tg and HLA-DR became stronger and resulted in a combined odds ratio of 15.0 for GD (17). We then showed that this statistical interaction reflected a biological interaction, whereby the DRβ1-Arg74 pocket facilitates the presentation of pathogenic Tg peptides to T-cells. Indeed, we identified four peptides (Tg.1951, Tg.2098, Tg.1571, and Tg.726) that showed strong and specific binding to DRβ1-Arg74 but much weaker binding to the protective variant DRβ1-Gln74 (18).

In view of the important interaction between these Tg peptides and the HLA-DRβ1-Arg74 peptide binding pocket, we hypothesized that blocking the presentation of these peptides to autoreactive T-cells that escaped tolerance could be used to treat AITD. Such a targeted therapy could prevent the continuous activation of T-cells against thyroid antigens that is necessary to maintain the autoimmune response in AITD and...
might reverse it. Therefore, the aim of this study was to identify small molecule inhibitors that can block Tg peptide presentation by HLA-DRβ1-Arg74 as a potential new treatment modality forAITD.

Experimental Procedures

Virtual Screen on HLA-DRβ1-Arg74 (DR3)—Virtual screening on DR3 was conducted on a structure obtained in MD simulations of the complex with the peptide Tg.1951, as described previously (18). The HLA protein is a representative structure of the complex from which the Tg.1951 peptide has been removed. Three independent screenings have been conducted to probe pockets 1 (P1), 4 (P4), and 9 (P9). Two libraries have been used in the virtual screening: one consisted of a diverse collection of ~150,000 compounds available through the Structure-Based Drug Discovery Core (SBDD) of the Experimental Therapeutics Institute at the Icahn School of Medicine at Mount Sinai; the second library consisted of ~3,000 compounds from the FDA library that includes FDA approved medications, over the counter medications, abandoned drugs, and other compounds previously shown to be safe for use in humans. The virtual screening was conducted with AutoDock4, Autodock-Vina (19), and eHits (20). We used a program DOCKRES (21) (written in the SBDD) to select the top ranked compounds and investigate the distribution of their scores in assessing the point beyond which their scores become indistinguishable. On this basis we have selected ~2500 compounds from the docking results in each pocket and clustered them using a binary Tanimoto coefficient as the definition of the distance that measures their chemical similarity (22). Each cluster contains molecules that are similar to each other, but are maximally different from compounds in other clusters. Representative compounds in the clusters were selected from the complete library to be tested for in vitro binding in biochemical assays.

Production of Recombinant HLA-DR Containing DRβ1-Arg74—HLA-DRβ1-Arg74 protein (containing arginine at position 74 on the β chain) was produced using the baculovirus system. We designed 2 constructs for both the α and β chains of HLA-DR. The β chain construct contained the extracellular portion of the DRB1*0301 β chain fused to the coiled-coil region of the basic leucine zipper domain of JunB, and the α chain construct contained the extracellular portion of the DR α chain fused to the coiled-coil region of the basic leucine zipper domain of Fos. A tobacco etch virus protease cutting site was introduced in each chain to allow removal of the dimerization motif. The JunB and Fos dimerization motifs allowed the protein to dimerize and form the final HLA-DRβ1-Arg74 protein. The α chain has a His6 tag, whereas the β chain has a V5 epitope tag for purification purposes. β, outline of the in vitro binding inhibition assay used to test small molecule inhibition of peptide binding to HLA-DRβ1-Arg74. Recombinant HLA-DRβ1-Arg74 protein was incubated with biotinylated peptide (APO), either with or without small molecules. The ELISA plate was coated with L243 antibody, which captured the HLA-DRβ1-Arg74. If the tested small molecule blocked the HLA-DRβ1-Arg74 pocket, it prevented the binding of the peptide. Europium-streptavidin was added and gave the fluorescence signal. The level of fluorescence signal was reduced when APO binding to HLA-DRβ1-Arg74 was inhibited by the tested small molecules.

In Vitro Screening of Small Molecules—The virtual screen of 150,000 compounds yielded 57 top scoring small molecules, which were then tested in vitro. These 57 compounds were purchased from Chembridge (San Diego, CA) or from Microsource Discovery Systems (Gaylordsville, CT). The compounds were dissolved in 100% DMSO (American Type Culture Col-
antibody. Blocking was done using 2.5% BSA in PBS at room temperature for 1 h. After washing 4 times, 100 μl of the preincubated complex (HLA-DRβ1-Arg74-protein-AP0-small molecules) were added onto the plate and shaken at slow speed for 2 h at room temperature. After washing 4 times, DELFIA Eulabeled streptavidin (PerkinElmer) diluted in DELFIA assay buffer (PerkinElmer) was added for 30 min and shaken at slow speed at room temperature. After washing 6 times, DELFIA Enhancement Solution was added for 1 h or until the optimal signal was reached. Time-resolved fluorescence was measured using the BMG reader (BMG Labtech, Cary, NC). The experiment was performed in triplicates. As negative control we added biotinylated myelin basic protein (MBP)-(87–99) to DR2 expressing cells and assess the inhibitory effect of cepharanthine. Inhibition was analyzed by flow cytometry. As a negative control, cells expressing HLA-DR2 were preincubated with cepharanthine or 40% Captisol® (Ligand Pharmaceuticals, Inc., La Jolla, CA) (the vehicle used to dissolve cepharanthine in this experiment), before 0.5 μg/mL Tg.2098 was added. After 24 h incubation, cells were stained with PE mouse anti-human HLA-DR (BD Biosciences) and APC streptavidin to detect binding of the peptide to VAVY cells and the inhibitory effect of cepharanthine on peptide binding.

Mice—Mice transgenic for DRB1*0301 were originally generated by G. J. Hämerling and co-workers as previously described (25). Briefly, the DR3 (DRA1*0101/DRB1*0301) transgenics were inserted into (C57BL/6 X DBA/2) F1 embryos and the progeny were back-crossed to B10.Q mice. The DR3 mice were crossed with 1-β2 knockout (C57BL/6 X 129) to obtain mice lacking murine MHC class II and expressing human DR3 (26, 27). The transgenic mouse line was maintained by intercrossing. The background non-MHC genes in the DR3 transgenic line was 50% C57BL/10 genes and 50% contribution from CBA, C57BL/6, and 129 genes. These mice were then back-crossed into the NOD background to produce the NOD-DR3 mice (28). We have confirmed the transgene to be HLA-DRβ1-Arg74 positive (29). Mice were bred in a pathogen-free facility (Icahn School of Medicine at Mount Sinai, New York, NY), and expression of HLA-DR3 was tested by PCR using DR3-specific primers: forward primer, 5′-CCACGACAAATCCACACTGAC-3′ and reverse primer 5′-GACAAAATCCACACTCCAC3′. DR3 transgenic mice on the NOD background known to be susceptible to experimental autoimmune thyroiditis (EAT) (28) were used in this study.

**PC61 Antibody Production**—Hybridoma PC61 (anti-CD25) (ATCC; catalogue number TIB-222) were grown and IgG was purified by QED Bioscience (San Diego, CA).

**Induction of Experimental Autoimmune Thyroiditis**—Female NOD-DR3 mice, 4–6 weeks old, were injected subcutaneously with human thyroglobulin (hTg) (Cell Sciences, Canton, MA) in Complete Freund’s Adjuvant (Sigma) to induce EAT as previously described (30). Mice were immunized with hTg on day 0 and boosted on day 7; mice were sacrificed on day 21. For some mice, PC61 (anti-CD25) antibody (ATCC) at 7.5 mg/ml was injected intraperitoneally on day −4 and day 3 to deplete T regulatory (CD25+) cells and augment the autoimmune response to hTg.

**Lymphocytes Isolation**—Spleen and draining lymph nodes were collected from mice upon sacrifice. The spleens and draining lymph nodes were harvested in complete RPMI (Corning) supplemented with 10% FBS (Sigma) and 1 mM sodium pyruvate.

**TABLE 1**

| Peptide  | Sequence                      |
|---------|-------------------------------|
| Tg.1951 | FRKVKIDDEKVKNF                |
| Tg.2098 | LSSVVVPDSRRHFDV              |
| Tg.1571 | EKVEPKVFDANAPVAVRSKPVDPSEF    |
| Tg.726  | CPTPICQLQAEOAFLRTV            |
| Tg.202  | VNTDMDNFDELHYSNRFPD           |
| ScrAPO  | PKDLKVENNFTKGDLSL             |
| Scr2098 | HDLFSRDSVVVVP                 |
| APO     | IPDNLFLKSDGRIKTYLNK           |

The peptide sequences of mice responding to thyroglobulin and thyroglobulin peptides with stimulation index ≥1.5 are listed in Table 1.
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vate (Sigma). They were cut and pressed in a circular motion using a plunger from a 10-ml syringe. The suspension was filtered through a 100-µm cell strainer twice and centrifuged 200 × g for 10 min. The supernatant was discarded. The pellet was washed with RPMI and centrifuged one more time. 5 ml of ammonium/chloride/potassium lysis buffer was added to remove erythrocytes from the spleen. After a 5-min incubation with ammonium/chloride/potassium lysis buffer at room temperature with occasional shaking, cells were centrifuged at 200 × g for 10 min. The pellet was resuspended in RPMI and the cells were counted and plated.

**T-cell Stimulation and CFSE Analysis**—Cells harvested from the spleen and lymph nodes were resuspended at 2 × 10^6 cells/ml in 0.1% BSA/PBS. 1 × 10^5 cells were labeled with 1.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Life Technologies). After incubating for 10 min at 37 °C, the staining was terminated by the addition of 4 volumes of ice-cold RPMI, 10% FBS. After 5 min of incubation on ice, the cells were washed 3 times with fresh RPMI and resuspended in fresh medium for counting. The CFSE-labeled cells were plated at 2 × 10^5 cells/well in 100 µl of medium (RPMI, 10% FBS). The cells were treated with medium, human thyroglobulin (40 µg/ml), the 5 peptides (Tg.1951, Tg.2098, Tg.1571, Tg.726, and Tg.202) (20 µg/ml), the unrelated negative control peptides (NC) (20 µg/ml), or mouse CD3/CD28 beads (Life Technologies) as positive control. The results from the unrelated negative control peptides were averaged. The cells were collected after 5 days for flow cytometry analysis. All experiments were performed in quadruplicates. The results were analyzed using Flowjo (Tree Star, Ashland, OR). The stimulation index was calculated by using the following formula: stimulation index = [% proliferating lymphocytes (hTg, peptide or mitogen-treated)]/[% proliferating lymphocytes (medium-treated)].

**Small Molecule Inhibition of T-cell Proliferation**—CFSE-labeled cells were incubated with 1 µM S53 together with human thyroglobulin (40 µg/ml) or Tg.2098 (20 µg/ml) to assess the blockade of antigen presentation in the T-cell stimulation assay by S53. To demonstrate the inhibition is specific, DMSO was used as a control. The cells were processed as described above for flow cytometry analysis.

**Cytokine Assays**—The Milliplex mouse cytokines/chemokine magnetic panel (catalogue number MCYTOMAG-70K, EMD Millipore Corporation, Billerica, MA) was used to assay the cytokines. Splenocytes were plated at 2 × 10^6 cells per well in 500 µl of medium (RPMI, 10% FBS). Supernatants from stimulated lymphocytes were collected 48 h after stimulation with hTg or peptides and stored at −80 °C until the assay was performed. To begin the assay, the 96-well plate supplied in the kit was washed with the wash buffer supplied and the plate was shaken for 10 min at room temperature. Standards and quality controls were added, followed by the samples. The pre-mixed beads (IFN-γ, IL-2, IL-4, and IL-10) were sonicated and vortexed, and then added to the wells. After shaking the plate overnight at 4 °C, the plate was washed twice with wash buffer. Detection antibodies were added for 1 h at room temperature, and streptavidin-phycocerythrin was added for 30 min at room temperature. The plate was washed twice and sheath fluid was added to resuspend the beads for 5 min before reading in Luminex 200 with xPONENT software (Luminex, Austin, Texas).

**Small Molecule Inhibition of Cytokines Production**—Immune- mized mice splenocytes were incubated with 1 µM S53 together with human thyroglobulin (40 µg/ml) or Tg.2098 (20 µg/ml) to assess the inhibition of cytokines production by S53. To demonstrate the inhibition is specific, DMSO was used as a control. Supernatants were collected and the assay was performed as described above.

**Blocking the Induction of EAT by Small Molecule in Vivo**—7 mice were injected with 125 µg of S53 intraperitoneally on days −2 and −1 prior to immunization with hTg (day 0). After days 5 and 6 prior to immunization with hTg on day 7. As a control, 5 mice were injected with the vehicle that was used to dissolve S53 for in vivo studies (ethanol: PEG400 (Fisher Scientific): saline (5:20:75)) on the same days.

**Thyroglobulin and Tg.2098 ELISA**—Sera were collected from mice upon sacrifice and stored in −20 °C until used. Nunc Maxisorp ELISA plate (Thermo Fisher Scientific, Waltham, MA) was coated with 10 µg/ml of hTg (Cell Sciences) or Tg.2098 in bicarbonate buffer (Sigma) at pH 9.6 and incubated overnight. The ELISA plate was washed 4 times with PBS supplemented with 0.05% Tween (Thermo Fisher Scientific) (PBST). After blocking with 2.5% BSA in PBST for 1 h at 37 °C, the plate was washed 6 times with PBST. 100 µl of the diluted sera (1:100 in 0.5% BSA/PBS) were added and incubated for 2 h at room temperature. After washing 6 times with PBST, anti-mouse IgG secondary antibody (Sigma) was added at 1:5000 dilution in 1% BSA/PBST and incubated for 30 min at 37 °C. After washing 4 times with PBST, the freshly prepared para-nitrophenylphosphate substrate (Sigma) was added for 1 h, and the ELISA plate was read at 405 nm using the BMG reader (BMG Labtech).

**Histology**—Thyroids were removed from the mice after sacrifice and put in 10% formalin (Fisher Scientific). After creating paraffin blocks, thyroids were sectioned and stained with hematoxylin-eosin for histological examination (Histology Shared Research Facility at the Icahn School of Medicine at Mount Sinai).

**Statistics**—Prism 5 software was used to perform statistical analysis. Student’s t test (paired t and unpaired t tests, one-tailed, as indicated under “Results”) were used for the comparison of two groups of samples. A p value < 0.05 was considered statistically significant.

**Study Approval**—The project was approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committees (IACUC). All animal studies were carried out according to the guidelines of the IACUC of the Icahn School of Medicine at Mount Sinai.

**Results**

**Virtual Screening of a Library of 150,000 Small Molecules for Blocking HLA-DRβ1-Arg74 Pockets**

We conducted three virtual screenings in the main binding pockets of DRβ1-Arg74 (P1, P4, and P9). We extracted 10,000 top scoring compounds from each of the screens and analyzed the distribution of their scores. We observed that the scores beyond approximately the top 2,500 compounds became very
densely distributed preventing a differential selection of compounds based on their scores. Each set of the 2,500 compounds was clustered based on their chemical similarity into 20 groups (see “Experimental Procedures”) and representative structures were selected from each cluster. These virtual hit compounds were further subjected to in vitro validation.

In Vitro Testing of Small Molecules Identified by the Virtual Screen

57 small molecules predicted by the virtual screen to block the HLA-DRβ1-Arg74 pocket were tested in vitro using a DELFIA immunoassay developed by our group utilizing the APO peptide, the strongest known peptide binder to HLA-DRβ1-Arg74 (see “Experimental Procedures”). Of the 57 compounds screened, 11 small molecules showed >50% inhibition of APO binding to HLA-DRβ1-Arg74 (Fig. 2a). One of the small molecules, S53, was from the FDA library, and, therefore, we selected it for further analysis (see below). Supplemental Table S1 lists the details and chemical structure of S53 (shown in black in Fig. 2a).

The docked structure of S53 is shown in Fig. 2b. The top-ranked position of S53 originated from screening pocket 1, but because this compound is relatively large we found that it is anchored in more than one pocket spanning pockets P2 and P3 as well. Such an arrangement of the inhibitory compound adds to its ability to inhibit effectively the binding of peptides to HLA-DRβ1-Arg74.

Tg.2098 Is the Major Tg Peptide Triggering Autoimmune Thyroiditis in Humanized NOD-DR3 Mice

EAT was induced in 15 female NOD-DR3 mice by immunizing them with hTg. After development of EAT, splenocytes of immunized mice were stimulated with: 1) hTg; 2) hTg peptides-Tg.1951, Tg.2098, Tg.1571, Tg.726, and Tg.202 that were previously shown to strongly bind to HLA-DRβ1-Arg74 (18) (for Tg.202 data not shown); 3) unrelated peptides (negative con-
trols); or 4) anti-mouse CD3/CD28 beads (positive control). 73.3% (11/15) of the NOD-DR3 mice injected with hTg responded to more than one peptide (stimulation index >1.5) and 26.7% (4/15) of the mice responded to only 1 peptide. 46.6% (7/15) responded to 2 peptides; 6.7% (1/15) responded to 3 peptides; 13.3% (2/15) responded to 4 peptides; and 6.7% (1/15) responded to 5 peptides (Fig. 3). This suggested that multiple epitopes exist. Interestingly, among the 7 mice that responded to 2 peptides, 6/7 responded to Tg.2098 (Fig. 3, inset), suggesting that Tg.2098 is a primary epitope in this mouse model. There could be epitope spreading happening causing responses to other Tg epitopes, although further evidence is needed. The percentage of mice responding to each peptide is shown in Table 2. 86.7% (13/15) of the mice responded to Tg.2098, followed by Tg.726 (46.7%), Tg.202 and Tg.1571 (40%), and Tg.1951 (13.3%). This confirmed our hypothesis that hTg-injected mice could respond to multiple Tg epitopes at the same time. The average stimulation indexes of T-cells from the 15 immunized NOD-DR3 mice in response to different peptides are shown in Fig. 4a. Tg.2098 showed the highest stimulation index, which was similar to that of the hTg native protein (Fig. 4a). Similarly, Tg.2098 induced the strongest cytokine responses in immunized NOD-DR3 mice that developed EAT again supporting Tg.2098 as a major hTg epitope (Fig. 4b). 100% of the immunized mice were positive for hTg antibodies at sacrifice demonstrating that they all developed humoral responses that are the most sensitive biomarker of EAT. Sera also showed significant reactivity against Tg.2098 (p = 0.0064) (Fig. 4c). These data support the notion that Tg.2098 is a primary Tg epitope presented by HLA-DR1-Arg74. 10 of 15 NOD-DR3 mice injected with hTg showed varying degrees of thyroid lymphocytic infiltration. 90% of mice that developed lymphocytic infiltration also had T-cell responses to Tg.2098.

Supplemental Fig. S2 shows histological sections of a normal mouse thyroid tissue (supplemental Fig. S2, A and B) compared with thyroid tissue from a mouse immunized with hTg (supplemental Fig. 2, C and D). There are more round-shaped follicles in the normal mouse thyroid tissue (B) compared with the hTg-injected mouse (D) under the same magnification (100×). The round follicles in B are more tightly packed together, whereas in D, the follicles are smaller and irregular in shape. This is due to thyroid follicles being disrupted by infiltrating lymphocytes as shown in the figure.

Further Analysis of S53 (Cepharanthine)

Of the 11 compounds that showed significant inhibition of peptide binding to HLA-DR1-Arg74 only S53 (cepharanthine, supplemental Table S1) was from the FDA library (i.e. it was previously shown to be safe for use in humans). Moreover, cepharanthine is an over the counter medication that is approved in several countries (albeit not in the United States), and therefore, we proceeded to analyze it further, as its pharmacokinetics, toxicity, and safe doses in humans are known. First, we tested its potency to inhibit peptide binding in our in vitro ELISA. We determined the potency of cepharanthine by testing the inhibition of APO binding to HLA-DR1-Arg74 at decreasing cepharanthine concentrations. These studies were performed at cepharanthine concentrations of 0.0125 to 0.2 mM. Cepharanthine inhibited APO binding to HLA-DR1-
Arg74 in a dose-dependent manner, with approximate IC_{50} of 0.08 mM (Fig. 5a). The compound identity of cepharanthine used in our experiments was confirmed using mass spectrometry (supplemental Fig. S1).

Cepharanthine Blocks Tg.2098 Binding to VAVY Cells Expressing HLA-DR3

To confirm the ELISA results we performed binding inhibition studies using the VAVY B-cell line that is homozygous for DR3 (confirmed to contain DR3/H9252 Arg74). Biotinylated Tg.2098 was incubated with VAVY cells in the presence of cepharanthine (dissolved in 40% Captisol®/H23041 (Ligand Pharmaceuticals, Inc.), which is not toxic to cells) or in the presence of 40% Captisol® alone (negative control). The binding of Tg.2098 to HLA-DR3-positive VAVY cells was 42.7% when incubated without cepharanthine, whereas the negative control peptide (a scrambled Tg.2098 peptide) showed 2.1% of binding to HLA-DR3-positive VAVY cells. In the presence of 0.025 mM cepharanthine, the binding of Tg.2098 to VAVY cells was reduced to 16.6% (Fig. 5b, panels A and B), confirming our ELISA results and suggesting that cepharanthine can block the presentation of Tg.2098 within HLA-DR3 to T-cells. We confirmed that S53 blocked specifically the binding of peptides to DR3 and did not block other DR pockets by showing that S53 did not block the binding of MBP-(87–99) (known specific binder to DR2) from binding to DR2 (Fig. 5b, panels C and D).

Cepharanthine Blocks T-cell Activation by Tg and Tg.2098

T-cell Proliferative Responses—To test if cepharanthine can block T-cell activation by Tg.2098 presented within HLA-DR3 we induced EAT in NOD-DR3 mice by immunizing them with Tg as previously described (30). Immunized mice splenocytes were incubated with hTg with or without cepharanthine; as negative control we incubated the splenocytes with vehicle (DMSO) alone. Cepharanthine (final concentration 0.001 mM) significantly blocked the activation of T-cells of hTg-immunized mice by both hTg (p = 0.0005, one-tailed t test, paired) and Tg.2098 (p = 0.0079, one-tailed t test, paired), but DMSO has no effect (Fig. 5c). Supplemental Fig. S3 shows the representative flow cytometry results from one of the hTg-injected mice, showing the proliferation under different conditions. To rule out the possibility that cepharanthine could block the expression of surface markers, we performed an experiment incubating cepharanthine with splenocytes and checked for CD4/CD25 expression. Our results suggested that cepharanthine did not block CD4/CD25 expression at both resting and stimulating conditions (supplemental Fig. S4).
FIGURE 5. a, dose-response curve of S53 inhibition of APO binding to HLA-DRβ1-Arg74. S53 inhibited binding in a dose-dependent manner, at concentrations ranging from 0.2 to 0.0125 mM. The IC₅₀ is ~0.08 mM. b, Tg.2098 was incubated with VAVY cells expressing HLA-DR3 (positive for DRβ1-Arg74) and with either (A) 40% Captisol® (vehicle in which cepharanthine was dissolved) or (B) S53 (cepharanthine) dissolved in 40% Captisol®. Inhibition of binding was analyzed by flow cytometry. 42.7% binding to HLA-DR3-positive VAVY cells was observed when Tg.2098 was incubated with 40% Captisol® (panel A, upper left-hand quadrant). With cepharanthine, the binding was decreased to 16.6% (panel B, upper right-hand quadrant). MBP-(87–99) was incubated with cells expressing HLA-DR2 either with Captisol (C) or S53 (D) dissolved in 40% Captisol. 28.8% binding to HLA-DR2-positive cells was observed when MBP-(87–99) was incubated with 40% Captisol (panel C, lower left-hand quadrant). With cepharanthine, the binding was slightly increased to 33.5% (panel D, lower right-hand quadrant). c, inhibition of T-cell proliferation by S53 (cepharanthine). T-cell proliferation was analyzed by the CFSE assay after stimulation with hTg and Tg.2098 with or without addition of S53. S53 significantly decreased proliferation induced by hTg (p = 0.0005, paired t test, one-tailed) and Tg.2098 (p = 0.0079, paired t test, one-tailed), whereas DMSO alone had no effect. Stimulation with CD3/CD28 beads was used as positive control, and stimulation with unrelated peptides was used as negative control (NC). (*, p < 0.05; **, p < 0.01; ***, p < 0.001). d, inhibition of IFN-γ production by S53 (cepharanthine) in splenocytes obtained from mice induced with EAT. Shown is the percentage of inhibition, after stimulation with hTg (A) and Tg.2098 (B) with addition of S53. S53 caused significant inhibition of IFN-γ production in splenocytes from mice induced with EAT and stimulated with Tg (A) or Tg.2098 (B) when compared with DMSO alone (p = 0.002 for hTg and p < 0.001 for Tg.2098, paired t test, one-tailed). (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
Cytokine Production—Interferon γ production was similarly inhibited by 38.7% when hTg was incubated with splenocytes from immunized mice together with cepharanthine (p = 0.002, paired t test, one-tailed) (Fig. 5d, panel A), and by 48.8% when Tg.2098 was incubated with splenocytes together with cepharanthine (p < 0.0001, paired t test, one-tailed) (Fig. 5d, panel B). To rule out a nonspecific suppression of cytokines by cepharanthine we also tested IL-2, IL-4, and IL-10 levels. The production of none of these cytokines was significantly altered by cepharanthine. In fact, IL-10 levels were nonsignificantly decreased, a change that would be expected to augment T-cell responses, not inhibit them.

Cepharanthine blocks activation of T-cells to Tg in Vivo

To test the in vivo effects of cepharanthine in the EAT model, we injected mice with S53 (or vehicle as control) intraperitoneally prior to immunizing them with hTg. After sacrificing the mice, splenocytes were isolated and tested for their responses to Tg. Cepharanthine intraperitoneal significantly blocked the hTg-induced activation of T-cells from hTg-immunized mice. The stimulation index was decreased by 41% from 2.27 (vehicle) to 1.34 (S53) (p = 0.0432). Responses to Tg.2098 were not significantly reduced although at baseline responses were not very high (supplemental Fig. S5).

Discussion

AITD are the comonest autoimmune diseases in the United States (1, 31), but their treatment is still largely based on symptom relief and not on reversing the autoimmune response to the thyroid. In particular treatment of GD is not satisfactory, anti-thyroid medications such as Methimazole are associated with serious side effects including liver dysfunction and agranulocytosis (32, 33); thyroidectomy is associated with potential surgical risks; and radioactive iodine was shown to worsen or trigger de novo Graves ophthalmopathy (34). Treatment of HT is relatively straightforward, but up to 15% of HT patients remain symptomatic even after thyroid functions are normalized (35–37). Therefore, better treatment modalities to reverse the autoimmune response targeting the thyroid are needed. In this study we screened a large library of compounds and identified, for the first time, a small molecule, cepharanthine, that can block thyroglobulin peptide presentation by HLA-DRβ1-Arg74 and T-cell activation in a “humanized” (DR3) mouse model of autoimmune thyroiditis. Moreover, cepharanthine showed activity when given to NOD-DR3 mice in vivo. If our results can be extended to humans, cepharanthine or a derivative of it could potentially be used in the future for the treatment of AITD in individuals that are positive for DRβ1-Arg74.

Why did we focus on thyroglobulin peptide presentation? Several lines of evidence support Tg as the key antigen in AITD. First, Tg was found to be strongly linked and associated with AITD (38), and amino acid variants in Tg confer a significant risk for developing both GD and HT (16). Moreover, Chen et al. (15) showed that the development of Tg antibodies preceded the appearance of TPO antibodies in NOD.H-2b4 mice, suggesting that Tg initiates the autoimmune response to the thyroid in NOD.H-2b4 mice and possibly also in human AITD. A potential explanation is that the MHC class II binding sites are dominated by Tg peptides that are more abundant in the thyroid. Indeed, in a study of patients with GD whose thyroids were removed, only Tg peptides were eluted from MHC II proteins purified from their thyroids (39), and no other thyroid antigen-derived peptides were identified. In view of these data McLachlan and Rapoport (40) concluded that immunogenicity of thyroid antigens is more important than breakdown of central tolerance to the development of thyroid autoimmunity. If that is the case then strategies to block Tg peptide presentation by blocking the HLA-DR peptide binding pocket using compounds such as cepharanthine may be a rational approach to treatment and prevention of AITD.

Of several Tg peptides that were identified as strong binders to DRβ1-Arg74 we confirmed Tg.2098 as the major thyroglobulin peptide that triggers AITD. Several previous studies by us and others (29, 39, 41) identified Tg.2098 as the major peptide triggering thyroid autoimmunity. In the present study we confirmed and extended these findings (29). We showed that 87% of NOD-DR3 mice that developed EAT following immunization with whole hTg protein had significant T-cell proliferative and cytokine responses to Tg.2098, whereas responses to other hTg peptides that bound HLA-DRβ1-Arg74 were much weaker. Because mice were immunized with the entire hTg protein this suggested that Tg.2098 was generated from hTg by antigen-presenting cells in the mice and presented by HLA-DR3 (containing HLA-DRβ1-Arg74) to T-cells, generating a memory response to Tg.2098 (39, 41).

Our data showed that cepharanthine can block thyroglobulin peptide presentation by HLA-DRβ1-Arg74 pockets and suppress T-cell activation by these Tg peptides. Cepharanthine is a plant alkaloid extracted from Stephania cepharantha Hayata. It has been widely used in Japan for more than 40 years for treatment of a diverse group of acute and chronic diseases (42). Cepharanthine is available for both oral and parenteral administration (42). Cepharanthine therapy has been used in various clinical conditions including radiation-induced leukopenia (43–46), thrombocytopenic purpura associated with multiple myeloma (47), alopecia areata (48, 49), and venomous snake-bites (50). So far no major side effects have been reported with cepharanthine therapy, but studies are limited and it cannot be ruled out that with larger studies more side effects will be identified. The mechanisms of action of cepharanthine were not fully understood prior to our study because very few mechanistic studies were previously performed.

Cepharanthine was reported to have antitumor activity, which could be due to inhibition of Ca2+-phospholipid-dependent protein kinase (PKC)-mediated phosphorylation of cytoplasmic proteins causing a reduction in the interaction of these proteins with the plasma membrane (51–53); reversal of multidrug resistance through changes in plasma membrane function (54, 55), and anti-allergic actions, which may be mediated by membrane stabilizing action and stimulation of pituitary-adrenotropic function (56, 57). Its inhibitory effect on dendritic cell activation and function (58) supports our finding that cepharanthine can block the HLA-DRβ1-Arg74 pocket, even though this specific effect has not been tested prior to our study. Because autoimmune responses are initiated when T-cells recognize peptide antigens that are bound to MHC class II an
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attractive approach to treating and/or preventing autoimmunity is by blocking antigen presentation. This approach was first tested with peptides that target the relevant MHC II but do not activate T-cells (59). However, despite early successes in mouse models of autoimmunity (60, 61) a human study in multiple sclerosis showed worsening of disease as the peptide blocker activated pathogenic T-cells (62). Therefore, there is an increasing search for non-peptidic small organic molecules to block T-cell responses because such compounds are much less likely to elicit T-cell activation and worsen disease. Moreover, unlike peptides, small compounds can be used in oral formulations, are more stable than peptides (that undergo proteolytic cleavage), and can penetrate tissues more easily. One of the first studies that tried to identify small molecules for the treatment of autoimmune screened for compounds that could block MBP-(152–165) peptide presentation by HLA-DR1301 as a potential treatment for multiple sclerosis. Using computational structure-based screening, two lead compounds and their analogs were identified (63). Another more recent study identified a compound (PV-267) with high in vitro binding affinity for HLA-DR2, an HLA allele that is strongly associated with multiple sclerosis; PV-267 was able to inhibit human HLA-DR2-restricted T-cell proliferation and cytokines production induced by MBP-(87–99). Moreover, PV-267 was shown to both prevent experimental autoimmune encephalomyelitis and ameliorate disease after its onset (64). Similarly, attempts have been made to identify compounds that could block the type II collagen CII-(263–272) peptide, which binds specifically to HLA-DR4, as a novel therapy for rheumatoid arthritis. Two compounds were identified that blocked T-cell proliferation induced by CII-(263–272) (65). In another study an in silico molecular docking algorithm was used to identify small molecules that could inhibit specific T-cell signaling in the presence of insulin B9–23 peptide; these small molecules could be used to develop novel therapeutic strategies for type 1 diabetes (66). Our identification of cepharanthine as a compound that blocks Tg peptide presentation by HLA-DRB1-Arg74, both ex vivo and in vivo, could also have important clinical implications for the treatment of autoimmune thyroiditis. We have shown that cepharanthine blocked activation of T-cells by thyroglobulin using a humanized mouse model ofAITD. Examination of the binding of cepharanthine to the DRB1-Arg74 pocket revealed that cepharanthine is largely anchored by hydrophobic residues and most importantly it interacts directly with DRB1-Arg74 in the pocket providing a direct explanation for its effectiveness as an inhibitor of peptide binding and presentation (Fig. 6).

In summary, our study confirmed that Tg.2098 is a major thyroglobulin epitope that can bind specifically to HLA-DRB1-Arg74 and induce autoimmune thyroiditis. Moreover, through a combination of virtual and in vitro screening we identified a new compound, cepharanthine, that can suppress T-cell activation in autoimmune thyroiditis, in vivo by blocking thyroglobulin peptide binding to the DRB1-Arg74 pocket and its presentation to T-cells. These data set the stage for the development of peptide blocking compounds as a novel therapeutic approach for treating human AITD. The advantage of this approach is that it is both personalized, in that only individuals that carry

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