# High-Throughput Screening of Chemical Libraries for Modulators of Gene Promoter Activity of *HLA-B2705*: Environmental Pathogenesis and Therapeutics of Ankylosing Spondylitis

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***ABSTRACT.*** ***Objective.*** Ankylosing spondylitis (AS) is a highly heritable disease with *HLA-B27* being the strongest susceptible gene. In order to survey the environmental triggers for arthritis development, we used a high-throughput technique to screen the effects of 12,264 chemicals on the *HLA-B27* gene promoter.

***Methods.*** Promoter reporter transfectants 293T-HLA-B27 and HeLa-HLA-B27 were tested using robotics with 12,264 chemicals. Chemicals that modulated *HLA-B27* promoter activity > 150% or < 40% were selected for further evaluation of IC50/EC50 and cell viability.

***Results.*** The primary screening using the 293T-HLA-B27 promoter reporter cell line yielded 5.1% hits that either suppressed (556 chemicals) or enhanced (68 chemicals) the *HLA-B27* promoter activity. A secondary reconfirmation screening was carried out with these 624 candidates using HeLa-HLA-B27 promoter reporter cells under several different culture conditions. The yield of positive candidates was 130, of which 47 were derived from natural products. Based on the bio-information of those positive natural products, 21 chemicals were selected for analysis by dose-response IC50/EC50 experiments. Eight compounds showed potential pharmacological activities. Two suppressors are both derived from an herbal medicine (*lei gong teng*) that has been used for decades to treat immune diseases. The 6 activators all belonged to a group of chemicals known as flavonoids, widely distributed among dietary fruits and vegetables.

***Conclusion.*** Several common dietary products that contain certain flavonoids might be environmental risk factors for AS; the Chinese traditional herb *lei gong teng* might be a potential drug for patients who are *HLA-B27*-positive. These results provide new research directions for the pathogenesis and therapeutics of AS. (First Release April 1 2011; J Rheumatol 2011;38:1061–5; doi:10.3899/jrheum.101109)

*Key Indexing Terms:*
ANKYLOSING SPONDYLITIS FLAVONOID *LEI GONG TENG*
NATURAL PRODUCTS ENVIRONMENTAL PATHOGENESIS

Ankylosing spondylitis (AS) is a highly heritable disease. Both genetic and environmental influences play a role in the pathogenesis of AS[1]. Among genes, *HLA-B27* demonstrates the strongest association, being observed in over 90% of patients with AS, with an OR of 171, and contributing to 16% of the overall genetic risk[2,3,4]. In contrast, no strong environmental triggers have been identified. To date there has not been any study that addresses the role of natural products in AS. The major obstacle is that there is an astronomical number of naturally occurring chemicals. Without a strong clue, high-throughput screening (HTS) techniques will be necessary. We used HTS to survey natural chemicals using the promoter of the *HLA-B27* gene as a target[5].

For screening reagents, we used > 600 chemicals derived from natural products. As a comparison, we also screened about 4000 drugs/research reagents approved by the US Food and Drug Administration and 8640 chemicals synthesized as potential pharmaceutical reagents. We reason that natural products that can enhance the promoter activity of *HLA-B27* are candidates for further study as environmental factors for modulating the arthritis. Drugs, synthetic chemicals, and chemicals derived from natural products that suppress the promoter activity of *HLA-B27* are candidates for therapeutic agents in AS.

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## MATERIALS AND METHODS

*List of chemical libraries*. A total of 12,264 chemicals derived from chemical libraries were purchased through the University of California at Los Angeles (UCLA) Molecular Screening Shared Resources (UCLA MSSR; www.mssr.ucla.edu/). These chemical libraries were the BioMol Library (www.mssr.ucla.edu/lib.html), the MicroSource SPECTRUM collection (www.msdiscovery.com/spectrum.html), the Prestwick Chemical Library (www.prestwickchemical.fr/index.php?pa=26), and a "Druggable compound set" of the UCLA MSSR laboratory (www.mssr.ucla.edu/lib.html). All the libraries and the individual chemicals are commercially available. Of these 12,264 chemicals, more than 600 are derived from natural products and at least 20 are flavonoids.

*Deployment of chemicals*. Screening procedures were carried out in the UCLA MSSR laboratory. Chemical libraries were mechanically deployed in multiwell plates as dimethylsulfoxide solutions at an initial concentration of 10 mM and stored at –80°C. Plate-to-plate dilutions were performed vertically by an ORCA robotic arm. Cell transfers to 384-well plates were carried out with 384-pin arrays (Matrix Technologies, Hudson, NH, USA; Genetix, Hampshire, UK).

*Construction of HLA-B27 promoter reporter*. Details of the construction of the *HLA-B27* luciferase reporter have been published[5]. In brief, we used the pGL4.14-luc2 reporter vector (Promega, Madison, WI, USA), and contained a 432-bp fragment from the 5' sequence of the *HLA-B27* genomic DNA (GenBank accession no. M12967) from position –419 base pair through the transcription initiation site. This was stably transfected into the HeLa and the 293-T cell lines. The B27 promoter in both clones responded positively to culture with tumor necrosis factor-α (TNF-α) and interferon-ß (IFN-ß). In secondary screening, TNF-α and IFN-ß were added into the cultures at final concentrations of 20 ng/ml and 1000 units/ml, respectively. Valproic acid was added to reach a final concentration of 1 mM.

*Luciferase and viability assays*. Luciferase and viability assays in 96-well plates followed standard procedures, as described[5]. This was modified for high-throughput chemical library screening. For HTS, 4000 cells in 50 $\mu$l of media were added to each well of 384-well white plates (Matrix Technologies), and incubated for 24 hours at 37°C in 5% $CO_2$. To each well was then robotically added a chemical in a 0.5 $\mu$l volume to reach a final concentration of 10 $\mu$M, and the cultures were carried out for another 24 h at 37°C. Luciferase activities were read by a Wallac 1420 plate reader 5 min after adding Britelite reagents (Perkin Elmer, Waltham, MA, USA).

All chemical screenings were carried out in triplicate. Effects were expressed as percentage inhibition or enhancement in comparison to cells incubated with solvent alone. To minimize false-positives, only those chemicals inducing > 40% inhibition or 150% enhancement were regarded as potential candidates. Cell viability was measured by the ATPlite Assay (Perkin Elmer, Shelton, CT, USA) using the Analyst HT luminometer (Molecular Devices, Sunnyvale, CA, USA).

*Dose response and determination of half-maximal effect concentrations*. Dose response of each chemical was tested in triplicate in the HTS system using 9 serial 2-fold dilutions ranging from 9 nM to 50 $\mu$M. EC50, IC50, Emax, and the 95% CI of these values were calculated by nonlinear regression analysis (GraphPad Prism, La Jolla, CA, USA). The equation for calculation is

$$Y = [(A - D)/(1 + (x/C) \text{ ^ } B)] + D$$

Cell viability for each concentration was also assessed in parallel.

## RESULTS

*Primary screening*. In the first screening, 12,264 chemicals were screened with 293-T cells stably transfected with the *HLA-B27* promoter reporter. A total of 624 chemicals showed positive results (Table 1).

*Secondary screening*. The 624 chemicals identified in the first screening were submitted to a second screening. In this rescreening, instead of using 293-T transfectant cells, we used HeLa transfectant cells. The HeLa transfectant cells were cultured in 3 different conditions. In the first condition, they were cultured with media alone. In the second, they were cultured with TNF-α plus IFN-ß, which would enhance the *HLA-B27* promoter by binding to transcription factors. In the third, they were cultured with valproic acid, which would enhance the promoter activity of the *HLA-B27* by epigenetic effect. From these 3 different culture conditions, we identified 130 chemicals that either enhanced or suppressed the *HLA-B27* promoter activity compared to cultures with solvents alone (Table 2).

*Table 1*. Numbers of positive chemicals during first screening using *HLA-B27* promoter reporter in 293-T cells. Enhancers are chemicals that enhance the luciferase activity to > 150% of baseline; suppressors are those that suppress luciferase activity to < 40% of baseline; positive are either enhancers or suppressors.

| Source of Chemicals | No. Chemicals | No. Enhancers (%) | No. Suppressors (%) | No. Positive (%) |
|---|---|---|---|---|
| BioMol Compound Library | 504 | 1 | 23 | 24 |
| MicroSource SPECTRUM collection | 2000 | 10 | 93 | 103 |
| Prestwick Chemical Library | 1120 | 37 | 122 | 159 |
| UCLA "druggable" compounds | 8640 | 20 | 318 | 338 |
| Totals | 12,264 | 68 (0.6) | 556 (4.5) | 624 (5.1) |

UCLA: University of California at Los Angeles.

Of the 130 chemicals identified to be effective with at least 1 of the culture conditions, 47 were derived from natural products (Table 3).

Finally, we selected 21 chemicals for further testing (Table 4). The selection was based on several factors: lack of toxicity in viability testing, lack of known toxicity to humans or animals, being effective in > 1 culture condition, and being derived from natural products. To this list, we added the following chemicals, which are not in the libraries: epigallocatechin gallate, triptolide, sulforaphene, diallyl disulfide, and resveratrol. These are derived from natural products, and have been reported to carry potential biological effects[6,7,8,9,10].

*Dose response and determination of half-maximal effect concentrations*. The 26 candidate chemicals were tested in the MSSR HTS system for their dose-response relationship using 9 serial 2-fold dilutions ranging from 9 nM to 50 $\mu$M. From these results, we identified 2 chemicals that suppressed the *HLA-B27* promoter activity with IC50 < 10.0 $\mu$M, and 6 chemicals that enhanced the promoter activity with EC50 < 10.0 $\mu$M. None of these chemicals caused > 20% loss of viability even at the highest concentration. The results are shown in Table 5.

*Table 2*. Number of positive chemicals discovered by the second screening using *HLA-B27* promoter reporter in HeLa cells. Enhancers are chemicals that enhance the luciferase activity to > 150% of baseline; suppressors suppress the luciferase activity to < 40% of baseline.

| Source of Chemicals | No. Chemicals | Cell Cultures with | | | | | |
|---|---|---|---|---|---|---|---|
| | | Media Alone | | TNF-α + IFN-ß | | Valproic Acid | |
| | | Enhancers | Suppressors | Enhancers | Suppressors | Enhancers | Suppressors |
| BioMol Compound Library | 24 | 0 | 3 | 1 | 7 | 0 | 7 |
| MicroSource SPECTRUM collection | 103 | 13 | 23 | 7 | 28 | 4 | 32 |
| Prestwick Chemical Library | 159 | 5 | 5 | 3 | 19 | 3 | 17 |
| UCLA "druggable" compounds | 338 | 18 | 3 | 9 | 4 | 17 | 3 |
| Totals | 624 | 36 | 34 | 20 | 58 | 24 | 59 |

TNF: tumor necrosis factor; IFN: interferon; UCLA: University of California at Los Angeles

*Table 3*. Chemicals derived from natural products.

| | |
|---|---|
| 3-hydroxyflavone | Diosmetin |
| 4'-methoxychalcone | Digoxin |
| 5,7-dimethoxyisoflavone | Dihydrocelastrol |
| 6,3'-dimethoxyflavone | Emetine |
| 6,4'-dimethoxyflavone | Harpagoside |
| 7-desacetoxy-6,7-dehydrogedunin | Isoliquiritigenin |
| Apigenin* | Luteolin |
| Apigenin* | Neriifolin |
| Baicalein | Ouabain |
| Biochanin A | Peruvoside |
| Biochanin A diacetate | Piceid |
| Camptothecin | Piperlongumine |
| Cantharidin | Pristimerin |
| Cedrelone | Puromycin hydrochloride |
| Celastrol | Retinol |
| Chrysin | Sanguinarine sulfate |
| Convallatoxin | Staurosporine |
| Cosmosiin | Strophanthidin |
| Cymarin | Thapsigargin |
| Dactinomycin | Tomatine |
| Daunorubicin | Totarol-19-carboxylic acid methyl ester |
| Derrubone | Tyrothricin |
| Derrusnin | |
| Dihydrocelastrol | |

* Apigenin is listed twice because each was derived from a different source.

*Table 4*. Chemicals selected for third testing.

| Source | Name |
|---|---|
| MicroSource | 3-hydroxyflavone |
| MicroSource | 4'-methoxychalcone |
| MicroSource | 5,7-dimethoxyisoflavone |
| MicroSource | 6,3'-dimethoxyflavone |
| MicroSource | 6,4'-dimethoxyflavone |
| MicroSource | Apigenin* |
| Prestwick | Apigenin* |
| MicroSource | Baicalein |
| MicroSource | Biochanin A |
| MicroSource | Biochanin A diacetate |
| Prestwick | Cantharidin |
| MicroSource | Celastrol |
| MicroSource | Cosmosiin |
| MicroSource | Derrusnin |
| MicroSource | Dihydrocelastrol |
| MicroSource | Diosmetin |
| Prestwick | Harpagoside |
| MicroSource | Isoliquiritigenin |
| MicroSource | Luteolin |
| MicroSource | Piceid |
| MicroSource | Pristimerin |

* Apigenin is listed twice because each was derived from a different source.

## DISCUSSION

Although most patients with spondylitis ask whether diet plays a role in their disease, and many patients use over-the-counter (OTC) natural supplements, no experimentally validated information about diet or the effectiveness of supplements is available[11,12]. The development of AS is unique in requiring the *HLA-B27* gene in the majority of patients. We used an HTS to screen 12,264 chemicals to see whether they modulate the activity of the *HLA-B27* promoter. Our procedure was carried out in the UCLA MSSR laboratory, but such screenings are also commercially available elsewhere. We discovered 2 *HLA-B27* suppressors (celastrol and pristimerin), which are derived from *Tripterygium wildfordii* Hook F (TwHf; *lei gong teng*), also known as the thunder god vine. This is an herbal medicine already used extensively in China for several decades to treat immune and inflammatory diseases. Two placebo-controlled studies in the United States have shown that it has a significant therapeutic effect on rheumatoid arthritis[13,14]. A small open study has also suggested that it might be useful for AS[15]. These phytochemicals are known to have strong antiinflammatory and immunosuppressive effects[7]. Based on such studies and the data from HTS, more extensive clinical trials in AS are warranted.

Interestingly, all 6 activators (apigenin, chrysin, biochanin A, biochanin A diacetate, 6,3'-dimethoxyflavone, 6,4'-dimethoxyflavone) belong to a group of chemicals known as flavonoids. Flavonoids are polyphenolic compounds ubiquitous in foods of plant origin such as fruits, vegetables, tea, cocoa, and wine. Their chemical structure is

*Table 5.* Chemicals with biologically meaningful effective concentrations. All compounds satisfy these criteria for being potentially bioactive and experimentally reproducible: $R^2 > 90\%$, IC50/EC50 < 10 $\mu$M, the upper boundary of 95% CI < 50 $\mu$M, and the dose response curves are sigmoidal; R values are derived from dose-response curves.

| Chemical | IC50 ($\mu$M) | 95% CI | $R^2$ | Maximum Inhibition, % |
|---|---|---|---|---|
| Celastrol | 2.49 | 0.78–4.20 | 0.94 | 94 |
| Pristimerin | 3.25 | 2.96–3.55 | 1.00 | 93 |
| | EC50 ($\mu$M) | 95% CI | $R^2$ | Maximum Activation, % |
| Apigenin | 0.63 | –0.60–1.86 | 0.96 | 193 |
| Biochanin A | 1.33 | 0.81–1.86 | 0.99 | 259 |
| Biochanin A diacetate | 2.31 | 1.50–3.12 | 0.99 | 246 |
| Chrysin | 1.46 | 0.86–2.06 | 0.97 | 244 |
| 6,3'-dimethoxyflavone | 0.09 | 0.01–0.17 | 0.99 | 268 |
| 6,4'-dimethoxyflavone | 0.09 | 0.01–0.17 | 0.99 | 265 |

diphenylpropanes (C6-C3-C6), which consist of 2 aromatic rings (A, C) linked through 3 carbons (ring B)[16]. They are subdivided into 6 major subclasses, based on variations in the heterocyclic C-ring. These subtypes include flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. Over 5000 flavonoids have been identified[17]. The estimated daily intake of flavonoids ranges from 100 to 1000 mg/day[18,19]. Interestingly, their absorption requires conversion to aglycones by the colonic microflora[18]. Thus, their bioavailability partly depends on the endogenous bowel flora and varies from 0.2%–20%[20]. After absorption, the flavonoids are conjugated in the liver or metabolized to smaller phenolic compounds. They are excreted either unchanged in feces or as flavonoid metabolites in urine[21].

Flavonoids have been reported to have antioxidant, antiallergic, antiviral, antiinflammatory, and anticarcinogenic activities in *in vitro* studies[22]. Epidemiological data also show an inverse correlation between dietary flavonoid intake and mortality from cardiovascular diseases[23,24].

We discovered that 6 flavonoids could potentially promote *HLA-B27* expression. These 6 are widely distributed in diet, and would be potential environmental factors in the pathogenesis of AS. This would be especially so if their inherent biological effects are also proinflammatory. We describe here some of the known biological effects of 3 popular flavonoids.

Apigenin (4',5,7-trihydroxyflavone) is commonly found in vegetables (e.g., parsley, artichoke, basil, celery), and is the main component of German chamomile, a folk remedy for treating muscle spasms, dermatitis, and upper respiratory infections[25]. It has also been claimed to be a chemopreventive reagent and an inhibitor of cell proliferation[26] and protooncogene expression[27,28]. It is now sold OTC as a sleep aid. Importantly, its antiinflammatory effects are mediated through inhibition of adhesion molecule expression[29], nuclear factor-κB (NF-κB) activity[30], prostaglandin $E_2$, cyclooxygenase (COX)-2 production[31], and the proinflammatory cytokine interleukin 6 (IL-6)[29].

Chrysin (5,7-dihydroxyflavone) is a natural flavone derived from the blue passionflower (*Passiflora caerulea*). It is available as an OTC herbal supplement as an anxiolytic agent, although no controlled data in humans are available. In rat *in vivo* studies[32,33], chrysin induced significant anxiolytic behavior in rats and acted as a ligand of benzodiazepine receptors. Antioxidant[34] and anticancer activities[35] have also been reported. Importantly, chrysin has antiinflammatory effects by inhibiting NF-κB transcriptional activity[36], COX-2 expression, and IL-6 signaling[37].

Biochanin A is a naturally occurring isoflavone, most commonly found in legumes and red clover. The spectrum of its biological activities is wide and includes antioxidative, antiinflammatory, anticancer, and enzyme inhibitory properties[38]. In a randomized, double-blind, placebo-controlled trial[39], 177 women aged 49–60 years were treated with daily 25 mg biochanin A for 1 year. Significant reductions in the loss of lumbar spine bone mineral content and bone mineral density were found compared to the placebo ($p = 0.04$, $p = 0.03$, respectively). Another randomized, double-blind, placebo-controlled study also showed that use of biochanin A (40 mg/day) for 6 weeks could lower the low-density lipoprotein level in middle-aged men ($n = 46$; $p < 0.05$)[40]. It is available as an OTC supplement to relieve vasomotor symptoms in menopause[41], to prevent bone loss, and for cardiovascular protection[42].

There are considerable limits to our study. The results are derived from promoter reporter activity alone. Gene expression depends on many factors, and the factor reported here might be rather minor. The flavonoids and TwHf have antiinflammatory and immunosuppressive effects. Their effect on *HLA-B27*, if any, might not be of importance to the hosts. Finally, our study does not address those patients who do not express *HLA-B27*.

Nevertheless, what should be emphasized is that apigenin, chrysin, and biochanin A are all being purchased in massive quantities as OTC dietary supplements in the United States, and as TwHf in China. Compared to prescription medications, they are subjected to less stringent safety regulation, evidence-based dose recommendation, and quality control. Our study is an indication that they have potential biological effect on *HLA-B27*.

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