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Effect of Guluronic Acid (G2013), As a New Anti-inflammatory Drug on Gene Expression of Pro-inflammatory and Anti-inflammatory Cytokines and Their Transcription Factors in Rheumatoid Arthritis Patients

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ABSTRACT

Rheumatoid arthritis (RA) as a long-term autoimmune disease is characterized by pain, swelling and joints destruction. The therapeutic efficacy of Guluronic acid (G2013) (patented, DEU: 102016113017.6) was reported in phase I/II clinical trial in RA patients. In this study, we aimed to evaluate the effect of G2013 as a novel non-steroidal anti-inflammatory drug (NSAID) with immunosuppressive property on genes expression of anti-inflammatory and pro-inflammatory cytokines and their transcription factors in the blood sample of RA patients.

This study was performed on 12 patients with RA who had an inadequate response to conventional treatments which were disease-modifying anti-rheumatic drugs (DMARDs), NSAID, and biologics. G2013 was administered orally at a dose of 500 mg twice daily for 12 weeks. Before and after the treatment of patients with drug G2013, the peripheral blood mononuclear cells (PBMCs) were isolated for evaluating the gene expression level of interleukin 10 (IL10), interleukin 22 (IL22), interferon γ (IFNγ), and transcription factors specific to the T helper cell lineages, forkhead box P3 (Fox-P3), Aryl hydrocarbon receptor (AHR) and T-box–containing protein expressed in T cells (T-bet) using the real-time PCR method. Since these cytokines have a key role in the progression of RA and disease condition expected induction of IFNγ, AHR, IL22, T-bet, and reduction of IL10, Fox-P3.

Results indicated a significant reduction in the level of IFNγ, AHR and a significant induction in IL10, Fox-P3 gene expression in comparison with the control group.

In conclusion; the results of this investigation showed a part of the immunological mechanism of G2013 as a novel anti-inflammatory that could reduce pro-inflammatory cytokine and their transcription factors. Furthermore, it increased the anti-inflammatory cytokine and its transcription factor (clinical trial identifier: IRCT2016092813739N5).

Keywords: Guluronic acid (G2013); Non-steroidal anti-inflammatory drug (NSAID); Rheumatoid arthritis

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INTRODUCTION

The most common autoimmune inflammatory arthritis among the elderly population is rheumatoid arthritis (RA).[1] This chronic inflammatory disorder is characterized by inflammatory cell infiltration into the synovium, synovial hyperplasia, angiogenesis, and cartilage and bone damage.[2] This defense mechanism of mistaken orchestra leads to deformities and disability which has a significant negative impact on daily activities like work and household tasks. Moreover, it could decrease health-related quality of life and increase mortality.[3] The prevalence of rheumatoid arthritis is about 1% which is also three times higher among women population than men.[4] It should be noted that both genetic and environmental factor has a great role in the pathogenesis of disease and decision for its therapeutic approach.[5,6] Many studies and clinical trials revealed the adverse effect of prolonged usage of conventional therapy in RA patients and also their ineffectiveness.[7] Several studies reported that these anti-inflammatory and disease-modifying anti-rheumatic drugs (DMARDs) have a severe side effect.[8,9] On the other side, biologics treatment strategies like anti-tumor necrosis factor-alpha (anti-TNFα) mostly increases the risk of infection.[10,11] Therefore finding a harmless agent for the treatment of RA could be an important and critical point.

In autoimmune diseases T cells have an important role in disease process and development, so that, T helper 17 and 1 (Th17, Th1) are involved in the pathogenesis of inflammation, whereas the T regulatory cells (Treg) have a protective role against autoimmune diseases .[12,13] Therefore an effective therapeutic agent should be able to balance the level of Treg and Th17 and Th1 in the target organ.

Generally, antigenic stimulation and cytokine signaling could activate and differentiate T cells into various T helper cell subsets. Basically, interferon-γ (IFNγ) producing Th1 cells had been considered to play a predominant role in the development of RA.[14] Since this dimer soluble cytokine is a component of a 'pathogenic' Th1 response which it could regulate Th1 development independently.[15] Moreover, recently it has been indicated that human Th17 cells can differentiate towards IFNγ-producing cells in vitro.[16] Here, it should be noted that T-box–containing protein expressed in T cells (T-bet) as a transcription factor is essential for Th1 polarisation.[17] Meanwhile, the differentiation of naive CD4+ T cells to Th1 cells depends on the expression of the transcription factor T-bet and then the T-bet directly activates the production of IFNγ.[18] Therefore, in RA patient the increasing level of IFN-γ and T-bet are observed mostly.[19,20]

The role of interleukin 22 (IL-22) in autoimmunity and inflammation appears to be greatly pro-inflammatory cytokine and augmented osteoclast differentiation by promoting the nuclear factor of activated T cells in RA patients. Furthermore, IL22 plays a pivotal role in bone destruction in patients with RA. Through these matters, Aryl hydrocarbon receptor Ah, a major transcription factor of T helper 22 (Th22) cells, was highly expressed [21,22] in patients. Therefore, the increases of IL22 and Aryl hydrocarbon receptor (AHR) are seen in RA patients [23,24] and a suitable treatment could be able to reduce their level of expression.

In contrast, the level of interleukin 10 (IL10) as an anti-inflammatory cytokine and forkhead box P3 (Fox-P3) as a transcription factor of Treg decrease in RA patients.[25,26] It is reported that Fox-P3 and Treg cells in RA patients lose their suppressive capacity by inhibiting the transcriptional activity of Fox-P3 through dephosphorylation by TNF thus Fox-P3 is needed to maintain the suppressive capacity of Treg cells and its induction is a goal for autoimmune diseases.[27]

Here, the effect of Guluronic acid (G2013) as a novel non-steroidal anti-inflammatory drug (NSAID) on 12 RA patients is evaluated (clinical trial identifier: IRCT2016092813739N5). G2013 is one of the components of Alginic acid which has a low molecular weight with the patent number of DE-102016113017.6. Previous studies on the experimental models of multiple sclerosis and the TLR signaling pathway have been reported the anti-aging feature with anti-inflammatory and immunosuppressive properties of this drug.[28,29,30,31]Moreover, its safety and efficacy have been assessed in the preclinical study on BALB/C mice during the acute and sub-chronic study[30], as well as phase I/II clinical trial of this drug in RA patients which has been showed its high safety when the drug is used orally.[32] In this study, the G2013 as a safe agent without any toxicity on the Gastrointestinal (GI) tract and kidney function were orally administrated in RA patients to evaluate its anti-inflammatory properties based on the assessments of IL-10, IL22, AHR, IFNγ, Fox-P3, and T-bet gene expression.

PATIENTS AND METHODS

Ethics Statement

The study was approved by the Ethics Committee of Tehran University of Medical Sciences (TUMS) with permission reference number (IR.TUMS.VCR.REC.1395.621-2016-09-14) and was conducted under the guidelines established by the American College of Rheumatology (ACR) and Helsinki manifest and its later amendments or comparable ethical standards. The written informed consent was obtained from all patients.

G2013 Preparation

Guluronic acid (G2013) as a small molecule ($C_6H_{10}O_7$) with the molecular weight of 194.139 Da was extracted from sodium alginate (Sigma-Aldrich, St Louis, MO, USA) based on the Fattahi et al (2015) method.[33] The purity of G2013 was approved by characterizing this hydrolytic product using Fourier transform infrared (FTIR) spectroscopy and carbon-13 nuclear magnetic resonance (13C-NMR) spectroscopy.

Clinical Characterization of Patients and Control

In this study, 12 patients (only female) with active RA who had an inadequate response to conventional drugs including DMARDs, NSAIDs, and biologics were selected for 12 weeks clinical trial at the Department of Rheumatology, Loghman Hakim Hospital. In this clinical trial, patients were enrolled by a one-blind randomized selection. The control group is healthy individuals who were matched on sex and age with the patients' group. The age of the selected patients was 18-80 years, and the range of disease duration was at least 5 years. Treatment of these patients with G2013 was based on the American College of Rheumatology (ACR) criteria for RA. At the baseline, although all patients were treated with DMARDs (methotrexate, hydroxychloroquine), steroids (prednisone) and NSAIDs for at least 6-month before the study, however the disease score (disease activity score in 28 joints [DAS28]) was completely high (DAS28≥5). Before starting this clinical trial, the patients were informed of this study and asked to sign an informed consent. Afterward, the patients received the follow-up clinic appointment at baseline, 4 weeks and 12 weeks at the Department of Rheumatology Loghman Hakim Hospital, Tehran, Iran, and the Division of Rheumatology Research, Rheumatism Center. Further follow-up was arranged by telephone for assessing the adverse event of G2013 every week.

During this clinical trial, patients were allowed to use their routine medications, which included methotrexate (15–20 mg weekly), hydroxychloroquine (400 mg daily) and steroids (5–15 mg daily). However, patients were unallowed to use NSAIDs or other pharmacologic treatment during this 12-week follow-up. Based on the preclinical assessment, a minimum dosage (18 mg/kg/d) of G2013 was provided in a gelatinized capsule (500 mg of G2013) for oral administration. Finally, the G2013 capsule was prescribed twice daily for 12 weeks.

Sample Preparation

Blood samples were obtained from 12 normal donors and 12 patients before and after treatment. Afterward, peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored at −80°C. Total RNA was extracted from 2×10^6–5×10^6 cells; using GeneAll® Hybrid-RTM kits (Dongnamro, Songpa-gu, Seoul South Korea.) based on the manufacturer's instructions and placed into 50 μL of RNase-free water. The concentration of total RNA was measured by NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). It was then concentrated or diluted to the concentration <300 ng/μL for cDNA synthesis.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Reverse-transcribed random hexamer primers (Table 1) were used for conventional PCR based on cDNA Synthesis Kit protocol (Takara Co., Ltd., Dalian, China). Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ II (Takara Co., Ltd.) with a specific primer (Sigma-Aldrich) based on the provided guideline. The analysis of IFNγ, IL10, interleukin 22 (IL22), AHR, Fox-P3, T-box–containing protein expressed in T-bet, and β-actin transcripts were carried out in StepOne™ and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The relative quantities of target genes mRNA compared against one internal control, β-actin mRNA was measured by considering the ΔCT method using an amplification plot (fluorescence signal vs cycle number). The difference (ΔCT) between the

mean values in the replicate samples of target genes and β-actin mRNA was calculated. Subsequently, the difference (ΔΔCT) between changes in the expression of target genes and the normal group was calculated and expressed as $2^{-\Delta\Delta CT}$.

Statistical Analysis

Data were representative of three independent experiments. The data were expressed as mean±standard deviation, and the analysis was performed by SPSS software (19.0; IBM Corporation, Armonk, NY, USA). Moreover, the parametric data were subjected to analysis of variance (ANOVA) and the Newman–Keuls test to determine significant differences in the gene expression level of before and after treatment. The statistical significance was classified as $*p<0.05$, $**p<0.01$ or $***p<0.001$. A *p*-value of <0.05 was considered statistically significant.

RESULT

Patient's Response

After 2 weeks of treatment with Guluronic acid (G2013) and also during the time of trial, an improvement in the clinical determinant of patients was reported by the physician based on the ACR criteria. At the end of the clinical trial, a significant reduction was observed in the level of disease activity (DAS28), morning stiffness, rheumatoid factor (RF) and C-reactive protein (CRP) (Table 2). Moreover, in the other relevant clinical laboratory tests such as anti-cyclic citrullinated peptide (anti-CCP), anti-double-stranded DNA (anti-dsDNA) and erythrocyte sedimentation rate (ESR) a non-significant recovery were seen and their levels were near to the normal range (data are not shown).

Effects of G2013 on AHR and IL22 Gene Expression

The results represented that after 12 weeks of therapy with G2013, the gene expression level of AHR as a transcription factor in Th22 for interleukin 22 (IL22) production[34] significantly decreased following treatment with this new drug and reached to the level of the normal group. However, it should be noted that its cytokine, IL22 which is transcripted by the AHR had any significant changes between the before and after the treatment process which could be due to the different pathways for IL22 production (Figure 1).

Table 1.Primer sequences for evaluation of gene expression in peripheral blood mononuclear cells of rheumatoid arthritis patients

NO	Gene name	Primer Sequence 5´→3´
1	T-bet	Fwd:5′-TCTCCTCTCCTACCCAACCA-3′
		Rev:5′-CTGACTGCTCGAAACTCAGC-3′
2	INFγ	Fwd:5′-TTTACCGCCTAATGCCCTAA-3′
		Rev:5′-AAATGGCGGATTACGGGATT-3′
3	AHR	Fwd:5′-AGCCACCTTCATCATCCGTCA-3′
		Rev:5′-TGACGTCCAACAGGTGACAGT-3′
4	IL-22	Fwd:5′-CCTGCATTTGACCAGAGCAA-3′
		Rev:5′-TAACGCAGGGGTTCATTTGG-3′
5	Fox-P3	Fwd:5′-GGTTTCCACTGTCTTGCCTG-3′
		Rev:5′-TTGTGAAGGCTCTGTTTGGC-3′
6	IL-10	Fwd:5′-GTTCTTTGGGGAGCCAACAG-3′
		Rev:5′-GCTCCCTGGTTTCTCTTCCT-3′
7	β-actin	Fwd:5′-GCATGGGTCAGAAGGATTC-3′
		Rev:5′-GTCCCAGTTGGTGACGAT-3′

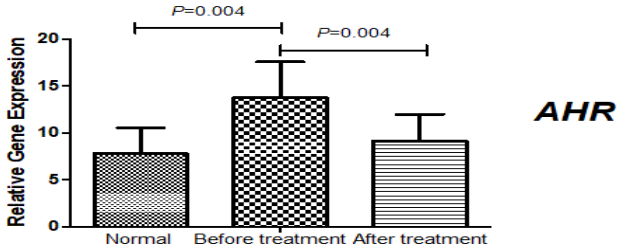
Abbreviations:RA, rheumatoid arthritis; PBMCs, peripheral blood mononuclear cells PCR, polymerase chain reaction; Fwd, forward; Rev, reverse;T-bet, T-box–containing protein expressed in T cells; IFNγ, interferon-γ; AHR, Aryl hydrocarbon receptor; IL22, interleukin 22; Fox-P3, forkhead box P3; IL10, interleukin 10.

Table 2. Changes in primary clinical efficacy end-points in the patients treated with Guluronic acid (G2013) versus those treated with the conventional therapy

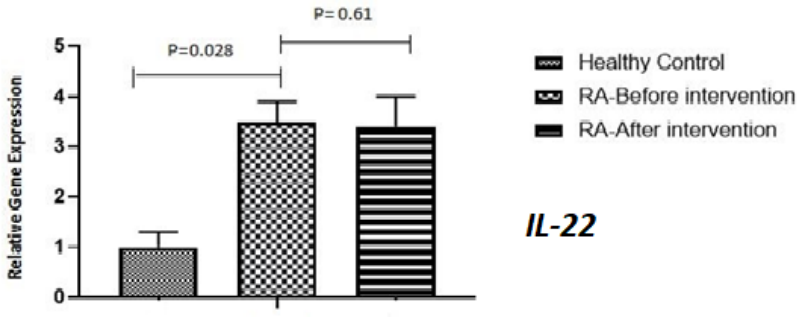
Characteristics	Before (n=12)	After (n=12)	*p*-value
DAS28	5.83±0.98	4.23±0.25	0.003
Morning stiffness (minutes)	50.76±30.45	46.3±27.9	0.063
RF (IU/mL)	68.07±143.73	58.45±124.90	0.39
CRP (mg/L)	16.76±20.82	15±19.20	0.006

The data are presented as means±S.D.

DAS28, disease activity; RF, rheumatoid factor; CRP, C-reactive protein; G2013, Guluronic acid.



(A)

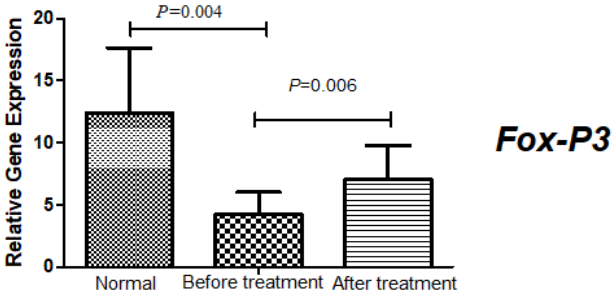


(B)

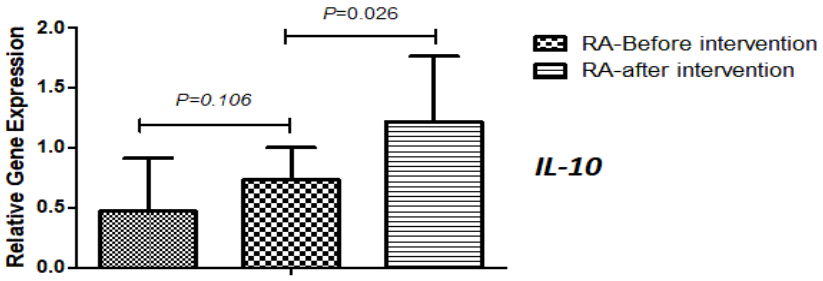
Figure 1. A: AHR gene expression in normal, before and after treatment groups with G2013. B: IL22 gene expression in normal, before and after treatment groups with G2013

Notes: RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of AHR and IL22 genes expression in the above-described conditions. Data are representative of three independent qPCR experiments (level of significance:*p*<0.05).

Abbreviations: PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; AHR, Aryl hydrocarbon receptor; G2013, Guluronic acid; IL22, interleukin 22.



(A)



(**B**)

Figure 2. A: Fox-P3 gene expression in normal, before and after treatment groups with G2013. B: IL10 gene expression in normal, before and after treatment groups with G2013

Notes: RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of Fox-P3 and IL10 gene expression in the above-described conditions. Data are representative of three independent qPCR experiments (level of significance: $p<0.05$).

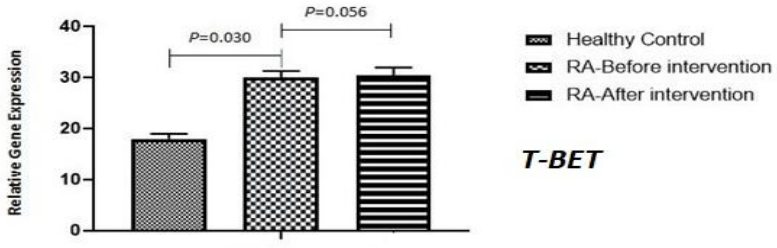
Abbreviations: PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; Fox-P3, Forkhead box P3, G2013, Guluronic acid; IL10, interleukin 10.

Effects of G2013 on Fox-P3 and IL10 Gene Expression

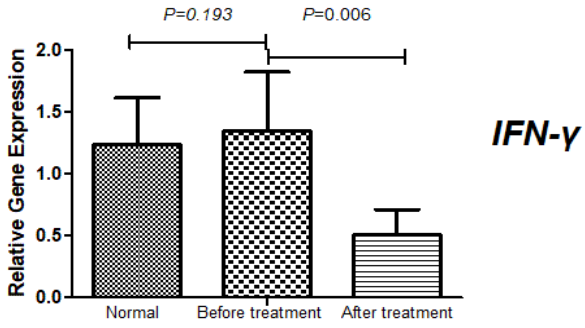
The Fox-P3 as a transcription factor for IL10[35] showed a significant remarked high gene expression level in the normal group compared to the patients' group which has a lower level of expression. Moreover, after treatment of G2013 the level of IL 10 induced significantly compared to the before treatment. Meanwhile, the results indicated that the gene expression of IL10 had a significant increase after G2013 therapy (Figure 2).

Effects of G2013 on T-Bet and IFNγ Gene Expression

As figure 3 illustrates, the IFNγ gene expression level was high in the patient group, whereas after treatment with G2013 it showed a significant reduction. While the level of T-box–containing protein expressed in T-bet had no significant difference between the treated and untreated group, although, the level of T-bet among the normal group was low in comparison with the patients.



(**A**)



(B)

Figure 3. A: T-bet gene expression in normal, before and after treatment groups with G2013. B: IFNγ gene expression in normal, before and after treatment groups with G2013

Notes: RNA extraction from PBMCs and quantitative reverse transcription PCR were performed to evaluate the variation of T-bet and IFNγ genes expression in the above-described conditions. Data are representative of three independent qPCR experiments (level of significance: $p<0.05$).

Abbreviations: PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; T-bet, T-box–containing protein expressed in T cells; G2013, Guluronic acid; IFNγ, interferon- γ.

DISCUSSION

In RA, the disordered immune system attacks the synovium and subsequently it results in inflammation and swelling which is the main problem among patients.[36] Generally, a hyperactive immune system can be seen on joints and also inflammatory reactions spread to the other organ and tissue.[37] To reduce this unbearable inflammation several lines of treatment are used including DMARDs and NSAIDs.[38] It should be noted that when these drugs could not reduce RA inflammation and symptoms.[39,40] The biologic agents such as Etanercept, Rituximab and etc. are prescribed.[41] Treatment with DMARDs has several side effects including nausea, vomiting, diarrhea, liver and blood problem. On the other sides, biologic agents can increase the risk of infection.[42] However, some patients do not respond to these medications. Therefore, Guluronic acid (G2013) as a novel and natural anti-inflammatory drug was used during 3 months clinical trial and showed a suitable response in the proposed gene expression as well as clinical and Para-clinical results.

In general, auto-reactive T cells produce a wide range of chemical agents such as cytokines and other inflammatory mediators. Among these agents, IL10 as a potent anti-inflammatory cytokine can reduce some symptoms in RA patients;[43] therefore, its induction is beneficial for reducing the inflammation. Moreover, previous studies indicated that IL10 has an immune regulatory role in RA and its increase has been observed in the improvement process. In this study, the results indicated that in G2013-treated patients IL10 has a higher level compared to untreated control. It showed that G2013 has an immune regulatory effect. Following the IL10 cytokine, Fox-P3 as a transcription factor in regulatory T cell (T-reg) which has a role in suppression of auto-immune disease should decrease among RA patients and an effective treatment induces its increase.[43] Our data demonstrate G2013 could represent a significant induction in the gene expression level of Fox-P3.

The cytokines network in RA is divided into two groups: the anti-inflammatory and pro-inflammatory cytokines.[44,45] A balance between these two groups could be considered as an important therapeutic goal.[46,47] As mentioned in the above paragraph the anti-inflammatory cytokines have a critical role in the improvement of RA since they can inhibit the synthesis of inflammatory cytokines. Therefore, their induction could be a suitable way for the treatment of RA. Thus, the reduction of inflammatory cytokines is essential to reduce RA symptoms. In this study, the results indicated that IFNγ as an inflammatory cytokine reduced significantly after treatment with G2013 therapy while the level of T-bet as its transcription factor has not changed by G2013 therapy. It means that other upstream factors may control IFNγ production

like the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway.

Besides, newly identified Th22 and its cytokine IL-22 plays a central role in the immunopathogenesis of rheumatic disease.[21] The IL22 inflammatory cytokine[48] was screened in this study but the result showed no effect on its level of production after treatment with G2013, whereas, AHR[49] as its transcription factor reduced significantly. This finding represented that more factors other than AHR are controlling IL22 production like RAR-related orphan receptor gamma (RORC). Since, previous findings reported the down-regulation of either transcription factors, AHR or the RORC could affect IL-22 production.[50] Moreover, several studies have indicated that IL-22 could be co-expressed by Th17.[51, 52] Thus, its activity might influence the activity of Th17 transcription factors. For further conclusion, we need to screen different genes expression through the pathway of IL-22 production.

The immunological assessment in this clinical trial represented the potent anti-inflammatory, immunosuppressive and immune-modulatory properties of G2013 in 12 RA patients. Since the level of pro-inflammatory cytokine and their transcription factors decreased after treatment meanwhile G2013 could potentially induce the level of gene expression of anti-inflammatory cytokine and its transcription factor.

Based on the clinical assessment and gene expression results, it can be concluded that oral administration of G2013 as a novel natural drug with immunosuppressive and immune-modulatory properties could reduce the RA development and symptoms. Moreover, this finding brings a perspective view for further trials with a higher number of patients and also other areas in autoimmune diseases.

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