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Belatacept Normalizes Specific Epigenetic-Cytokine Crosstalk in an Animal Model of Concanavalin A-Induced Acute Hepatic Injury

Doaa Khalid Ibrahim[1,2]*, Yassir Mustafa Kamal Al Mulla Hummadi[2], Huda Jaber Waheed[2]
[1]*Department of Pharmacology and Toxicology, College of Pharmacy, University of Mosul, Mosul, Iraq;* [2]*Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq*
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Abstract
Background: Acute liver injury is still a major therapeutic problem. Studies have focused on the epigenetic environment that controls liver injury. MicroRNAs have become essential post-transcriptional regulators for the production of pro-inflammatory signaling cascades. ***Objective***: This study evaluated the therapeutic potential of belatacept in hepatic injury by measurement of hepatic miRNA-155 and miRNA-223 and assessed its impact on IL-1β expression. ***Methods***: Concanavalin-A was used to induce liver injury in Wistar rats, which were treated with prednisolone, belatacept, or a combination therapy. Hepatic expression of targeted miRNAs was quantified using RT-qPCR. Hepatic IL-1β was measured by the Western blotting technique. ***Results***: The findings of this study identified that belatacept significantly normalized the expression of miRNA-155 and miRNA-223 ($p<0.05$). This epigenetic recovery was correlated with the stabilization of hepatic IL-1β level ($p<0.01$), Furthermore, belatacept demonstrated a therapeutic potency superior to prednisolone in mitigating liver injury, while combination therapy showed no significant synergistic advantage, suggesting a saturation of the common protective pathways. ***Conclusions***: Belatacept ameliorates hepatic injury by targeting the miRNA-155/Akt/IL-1β pathway. Overall, miRNA-155 may offer a successful biomarker for treatment monitoring, and the miR-155/Akt pathway is a promising target for improving management of liver injury.

Keywords: Belatacept; Interleukin-1β; Liver injury; MiRNA-155; MiRNA-223.

بيلاتاسيبت يقوم بتطبيع التداخل الوراثي والسيتوكيتي المحدد في نموذج أصابات الكبد الحادة في الحيوانات المختبرية الناتجة عن كونكافالين-أ

الخلاصة
الخلفية : لا تزال الإصابة الحادة بالكبد تمثل مشكلة علاجية كبيرة. وقد ركزت الدراسات على البيئة فوق الجينية التي تتحكم في إصابة الكبد. أصبحت للحوامض النووية الريبية الدقيقة عناصر أساسية في التنظيم بعد النسخ لإنتاج شبكات الإشارات الالتهابية. **الهدف** :هدفت هذه الدراسة إلى تقييم الإمكانات العلاجية للبيلاتاسيبت في إصابة الكبد من خلال قياس الحوامض النووية الريبية الدقيقة 155 و 223 الكبديين وتقييم تأثيره على تعبير انترليوكين-1 بيتا. **الطرق** :تم استخدام كونكانغالين A لتحريض الاصابة الكبدية في جرذان ويستر، والتي عولجت بالبريدنيزولون، البيلاتاسيبت، أو العلاج المشترك. تم قياس التعبير الكبدي للـ miRNAs المستهدفة باستخدام تقنية RT-qPCR، بينما تم قياس IL-1β الكبدي بواسطة تقنية الواسترن بلوت. **النتائج** :أظهرت نتائج هذه الدراسة أن البيلاتاسيبت يعيد بشكل كبير التعبير miRNA-155. و miRNA-223 ($p < 0.05$) الى مستواه الطبيعي. ثم ربط هذا التعافي الإبيجينيتيكي بتثبيت مستوى IL-1β الكبدي($p < 0.01$) ، علاوة على ذلك، أظهر بيلاتاسبت قدرة علاجية تفوق البريدنيزولون في التخفيف من إصابة الكبد، بينما لم يظهر العلاج المشترك أي ميزة تآزرية كبيرة، مما يشير إلى تشبع المسارات الوقائية المشتركة.**الاستنتاج** :يقوم بيلاتاسبت بتحسين إصابة الكبد من خلال استهداف مسار miRNA-155/Akt/IL-1βبشكل عام، قد يوفر miRNA-155 علامة بيولوجية ناجحة لمراقبة العلاج ومسار miR-155/Akt كهدف واعد لتحسين إدارة إصابة الكبد.

* ***Corresponding author***: Doaa K. Ibrahim, Department of Pharmacology and Toxicology, College of Pharmacy, University of Mosul, Mosul, Iraq; Email: doaakhalid@uomustansiriyah.edu.iq
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INTRODUCTION

Acute liver injury, which typically progresses from localized cellular damage to a systemic inflammatory response, is still a major therapeutic problem [1,2]. Although the biochemical characteristics of hepatotoxicity are well known, new studies have focused on the underlying epigenetic environment that controls these mechanisms. MicroRNAs (miRNAs) have become essential post-transcriptional brakes that regulate the production of pro-inflammatory signaling cascades among many regulatory levels [3,4]. In hepatocytes, interleukin-1 beta (IL-1β) is essential for an IL-1-driven auto-amplification of inflammation and cell death at the beginning of acute liver injury [5]. When IL-1β binds to IL-1R1, it causes IL-1R1 dimerization and recruits IL-1 Receptor-Associated Kinase 1 (IRAK1). The Nuclear Factor Kappa B (NF-κB) inhibitor, IκBα, is phosphorylated and degraded when IRAK1 activates the TNF Receptor-Associated Factor 6 (TRAF6)/TGFβ-Activated Kinase (TAK1)/IκB Kinase (IKK; IKKα/IKKβ/NEMO complex) signaling cascade. This allows activation of NF-κB [6,7]. Once active, NF-κB coordinates a transcriptional program that uses a variety of methods to promote inflammation. NF-κB stimulates immune cells to produce chemokines, which attract immune cells and create a chronic inflammatory environment by producing pro-inflammatory cytokines, including TNFα, IL-6, IL-1, and IL-17A. Also, NF-κB upregulates nitric oxide (NO)-mediated IL-1β production, creating a paracrine loop that results in positive feedback and activation of NF-κB [8]. There is growing evidence that some miRNAs function as

direct NF-κB activators, such as miRNA-155 [9], while others act as inhibitors such as miRNA-223 [10], although it is yet unclear how novel pharmacological drugs like belatacept selectively modulate these miRNAs. The aim of the current study is to investigate the underlying molecular processes involved. We concentrate on two specific miRNAs (miRNA-155 and miRNA-223) to ascertain if the restoration of these epigenetic regulators drives therapeutic efficacy of belatacept and to assess its subsequent impact on IL-1β protein expression. We seek to identify the epigenetic-cytokine crosstalk that defines hepatic recovery under Belatacept therapy.

METHODS

Animals

Male Wistar rats weighing approximately 175 ± 25 g were obtained from the Animal House of the College of Pharmacy, Mustansiriyah University. The environment of the animal house was normal, with a temperature of about 22 °C, humidity of about 55%, and a 12 h light/dark cycle, with a standard diet.

Chemicals and reagents

The chemicals used in the study with sources are as follows: Concanavalin A (Con A) [Sigma Aldrich Company, USA], belatacept [Bristol, USA], and prednisolone [European Egyptian Pharmaceutical Industries, Egypt]. Presto™ miRNA Purification Kit from Geneaid (Taiwan), Poly A Kit from OZ Biosciences, France], EasyScript SuperMix kit from TransGen Biotech Co., China], SYBR Green PowerUp Master Mix from Applied Biosystems, USA], SDS-PAGE gel kit from Elabscience (USA), and Western blotting detection kit from Elabscience (USA). Primary and secondary antibodies: Interleukin (IL-1β), β-actin, and rabbit IgG [Elabscience, USA].

Study design

This study used thirty rats that are divided equally into five groups according to the treatment protocol: no treatment group (only saline injection); hepatic injury group (administered a single 15 mg/kg dose of concanavalin-A IV injection); standard group (administered a single 15 mg/kg dose of concanavalin-A IV injection, followed by prednisolone 10 mg/kg/d orally for 2 weeks); the test group (administered a single 15 mg/kg bodyweight dose of concanavalin-A IV injection followed by 60 mg/kg bodyweight belatacept intraperitoneally for 2 weeks); and the combination group (treated with a single 15 mg/kg dose concanavalin-A IV injection followed by prednisolone 10 mg/kg/d orally and 60 mg/kg bodyweight belatacept intraperitoneally for 2 weeks).

Sample collection

Animals in the normal and hepatic injury groups were sacrificed 24 hours after the experiment began. Hepatic tissues were promptly kept at -70°C. After two weeks this process was carried out once more for the other two groups (standard and test groups).

MiRNA extraction and poly-A tailing for miRNA samples

Using the Presto™ miRNA Purification Kit, miRNA was extracted from frozen hepatic tissue samples (around 20 mg) in accordance with the manufacturer's instructions. Following extraction, the poly(A) tail was attached to the miRNA using a kit from OZ Biosciences in France. The EasyScript One Step gDNA removal and cDNA synthesis SuperMix kit was used to carry out reverse transcription. To guarantee specificity for miRNA analysis, particular stem-loop primers for miRNA-155, miRNA-223, and the internal control (U6 small nuclear RNA) were used. In summary, miRNAs were reverse transcribed in a final reaction volume of 20 µl while the enzyme was inactivated by heat cycling at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes.

Quantitative analysis using RT-qPCR

Using SYBR Green PowerUp Master Mix on a StepOnePlus Real-Time PCR System, the expression levels of miRNA-155 and miRNA-223 were measured. The cDNA template, forward and reverse primers, master mix, and nuclease-free water were all included in the reaction mixture (20 µl). After starting the thermal cycling conditions at 95°C for 10 minutes, there were 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. The comparative Ct method ($2^{-\Delta\Delta CT}$) was used to calculate relative expression levels. The results were presented as a fold change in comparison to the normal group after the data were standardized to the expression of the endogenous reference gene U6 snRNA. Primer oligonucleotides used for miRNAs are shown in Table 1.

Estimation of IL-1β by the Western blotting technique

Total protein was extracted from liver tissues using RIPA lysis buffer (using 0.5 ml of buffer for each 0.1 g of tissue). Lysis of cells was performed on ice for half an hour with centrifugation at 12,000 × g for 15 minutes at 4°C. The supernatant was transferred to a clean tube, and protein concentration was determined using the Bradford Protein Assay Kit according to the manufacturer's instructions.

Table 1: Primer oligonucleotides used for quantitative PCR (qPCR)

Target Gene	NCBI Accession #	Forward (5'→3')	Reverse (5'3')
miRNA-155	MIMAT0000646	AGCCAGCGTTAATGCTAATTGTGAT	CAGTGCAGGGTCCGAGGT
MiRNA-223	MIMAT00004570	AACAAGCGTGTATTTGACAAGCTGA	CAGTGCAGGGTCCGAGGT
U6	NR_004394	CTCGCTTCGGCAGCACATATACT	ACGCTTCACAATTTGCGTGTC

Protein samples were diluted with phosphate-buffered saline to get equal concentrations of proteins in all samples, followed by diluting with SDS loading buffer at a ratio of 4:1 and denaturing at 95°C for 10 minutes, then centrifuging for 2 min at 12,000 rpm and 4°C in a microcentrifuge. Proteins were separated by size using 12% SDS-PAGE gels in an electrophoresis running buffer at 120V. Following electrophoresis, proteins were transferred onto a PVDF membrane using a wet transfer system at 300 mA for 30 minutes. After the transfer was completed, blocking of membranes was done using 5% skim milk in TBS-T for 90 minutes at room temperature. Followed by membranes' incubation with the primary antibodies which are IL-1β antibody (dilution 1:1000) and anti-β-actin antibody (dilution 1:1000), for overnight with shaking at 4°C. After that, membranes were washed three times with TBS-T each for 15 minutes, then were incubated with secondary antibody HRP-conjugated (dilution 1:5000) for 60 minutes at room temperature.

Signal detection and analysis

Protein bands were visualized using enhanced chemiluminescence detection solution and captured using a ChemDoc XRS plus imaging system. Intensities of protein bands were quantified via densitometry analysis using ImageJ software. IL-1β levels were normalized to the intensity of the β-actin to account for variations in protein loading.

Ethical considerations

The handling and work with animals followed the guideline for using laboratory animals that was approved by the Animal Ethics Committee of the College of Pharmacy/Mustansiriyah University (certificate ID 62).

Statistical analysis

The data are expressed as mean ± standard deviation (SD). SPSS 25.0 was used for statistical analysis. GraphPad Prism 10 was used to create graphical representations and data visualization. One-way analysis of variance (ANOVA) was used to examine group differences, and Duncan's multiple range test was used for post hoc comparisons. Hepatic miRNA expression levels and interleukin-1 beta were compared using a Pearson's correlation coefficient (r) analysis to determine the correlation of miRNAs with inflammatory biomarker. Statistical significance was defined as a *p*-value of less than 0.05.

RESULTS

The expression of IL-1β was measured in hepatic tissue to evaluate the impact of belatacept on the inflammatory cytokine. Upon comparison between the hepatic injury group and the normal group, we found a significant elevation in the IL-1β level ($p< 0.05$), indicating induced damage and the resulting inflammatory response. While treatment with belatacept resulted in significant reduction in IL-1β level ($p< 0.0001$). Notably, the inhibitory effect of belatacept was stronger than that of prednisolone ($p< 0.001$) as shown in Figure 1, suggesting that belatacept possesses potent anti-inflammatory properties compared to standard clinical treatment.

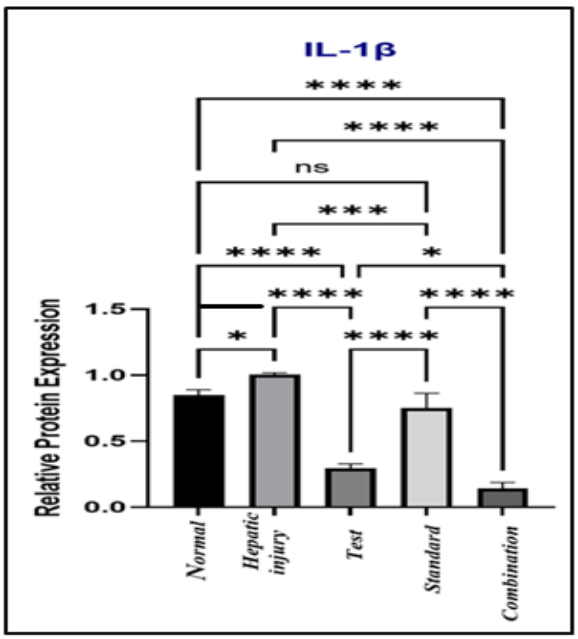

Figure 1: Bar charts showing IL-1β levels across the studied groups.

MiRNAs are vital regulators of the inflammatory response. Specific miRNAs were investigated to evaluate whether Belatacept affects inflammatory response through modulating this epigenetic pathway. Quantitative real-time PCR showed that belatacept significantly restores the expression of selected miRNAs (miRNA-155 and miRNA-223); miRNA-155 was reduced by about 13-fold compared to the untreated group ($p< 0.0001$) as shown in Figure 2A, while miRNA-223 expression was restored by belatacept with a 5-fold increase in expression level in comparison with the injured group ($p< 0.001$) (Figure 2B).

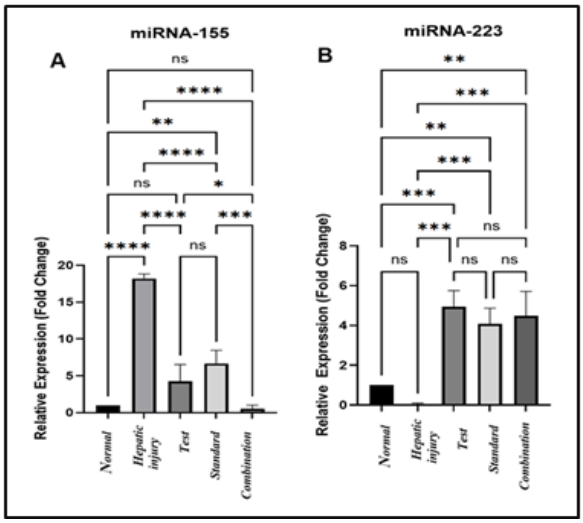

Figure 2: Relative expression (fold change): (A) miRNA-155, and (B) miRNA-223.

This shift in selected miRNAs expression proposes that belatacept may have indirect effects on inflammatory signaling pathways through the post-transcriptional regulation of these pathways. Table 2 shows the correlation between belatacept-induced changes in selected miRNAs and IL-1β levels to evaluate if belatacept affects inflammatory pathways through epigenetic modulation.

Table 2: Pearson correlation coefficients between pro-inflammatory cytokine and hepatic miRNAs

Cytokines/MiRNA	IL-1β (r)	Significance
MiRNA-155	0.978	Significant
MiRNA-223	-0.272	Non-Significant

Note: r represents Pearson's correlation coefficient

The association between the selected miRNAs and pro-inflammatory cytokines was clearly varied, according to a Pearson correlation analysis. IL-1β cytokine showed strong, statistically significant positive relationships with miRNA-155 of the measured hepatic miRNAs (Figure 3). However, there was no significant correlation found between the expression levels of miRNA-223 and the cytokine.

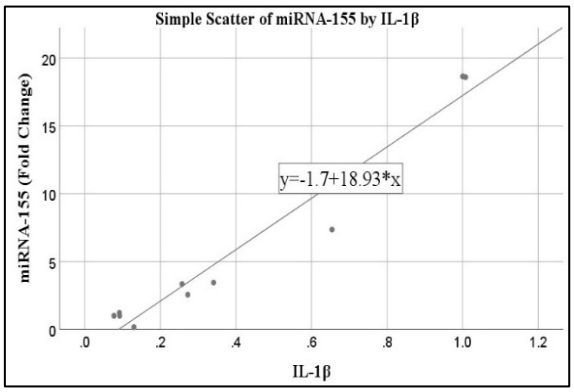

Figure 3: Scatter plot of miRNAs expression and inflammatory cytokine levels with a regression line. Dots represent an individual animal.

DISCUSSION

The present study observed that the therapeutic efficacy of belatacept relies on its ability to restore the miRNA-155/miRNA-223 axis and suppress IL-1β overproduction. CD28 is a potent upstream activator of the phosphoinositide 3-kinases (PI3Ks), and its activation promotes cytokine transcription [11]. Belatacept, a selective costimulation blocker by binding to CD80/CD86 on antigen-presenting cells, blocks this signal and leads to a significant reduction in Akt phosphorylation and activation within the hepatic inflammatory environment. Collectively, belatacept inhibits IL-1β with other cytokines. While its relation to miRNA-155 is emphasized, T-cell activation and hepatic stress are highly inducing miRNA-155 expression. By blocking costimulation, Belatacept suppresses the transcriptional drivers of miRNA-155 [12]. T cell activation has opposite effects on miRNA-223 expression that result in downregulation of miRNA-223 [13]. The restoration of these miRNAs by belatacept therapy indicates that this drug resets genetic programming, not just providing temporary immunosuppression like traditional immunosuppressants such as calcineurin inhibitors. Through this two-miRNA axis, Belatacept encourages a heavy reduction of both the translational activation and post-translational prevention of IL-1β, effectively breaking chronic inflammation's cycle. Our results are strong positive relationship of hepatic miRNA-155 with hepatic IL-1β level. These results demonstrated the biological link between miRNA-155 and IL-1β that occurs through multiple pathways. Firstly, miRNA-155 targets the PTEN-PI3K/AKT pathway by inhibiting PTEN and SHIP1, which are the natural inhibitors of PI3K/AKT phosphorylation that finally results in activation of IL-1β inflammatory effects, so miRNA-155 is considered an indirect activator of IL-1β [14,15]. Secondly, the role of miRNA-155 on the production of IL-1β is through the inhibition of SOCS1 (Suppression Of Cytokine Signaling), a gene that encodes protein inhibitor of inflammatory signaling NF-κB pathway, which finally results in massive increase in IL-1β production [16]. Also, miRNA-155 activates the NLRP3 inflammasome that is necessary for the release of active IL-1β [17]. On the other hand, miRNA-223 showed non-significant correlation with IL-1β, and this observation suggests that miRNA-223 functions through independent regulatory mechanisms that are less sensitive to the specific T-cell costimulation blockade provided by Belatacept.

Study Limitations

This study focused on a selective panel of microRNAs (miRNA-223 and miRNA-155). Although these are important indicators of hepatic damage, there is a wide range of miRNAs. Additional Belatacept epigenetic targets may be found using a more comprehensive, high-throughput method like RNA sequencing. Wistar rats were used in the study as an animal model of acute liver injury. To validate the potential of belatacept as a hepatoprotective agent, more research into human hepatocyte cultures or clinical trials is required.

Conclusion

Therapeutic efficacy of belatacept in mitigating hepatic inflammation and its association with modulation of the MiRNA-155/Akt/IL-1β axis. These observations highlight miRNA-155 as a potential biomarker for monitoring the anti-inflammatory response to treatment of hepatic injury and suggest the miRNA-155/Akt pathway as a target for improving the treatment of liver injury.

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Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

The data that supports the findings of this study are available from the corresponding author upon a reasonable request.

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