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Neuroprotection for Retinal Ganglion Cells

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Retinal ganglion cells (RGCs) are important components of the visual pathway and are responsible for conveying retinal information through their axons, passing through the lateral geniculate nucleus and projecting into the visual cortex, thereby cognizing about the visual world. RGCs are energetically expensive dynamic cells that are highly susceptible to the same neuropathological mechanisms shared among other neurodegenerative conditions. These include deficits in mitochondrial subtleties, defects in axonal transport, and redox imbalance, eventually culminating in apoptosis and necrosis and resulting in RGC death. Degeneration of RGCs has significant impact on vision and is the leading cause of irreversible blindness. RGC degeneration is a prominent etiology underlying ophthalmic complications in patients with glaucoma, optic neuropathy and demyelinating disease.

Recently, the tetracycline antibiotic, minocycline has been investigated for potential neuroprotective effect on microglia activation-related degeneration of RGCs in both retinas using the unilateral optic nerve crush mouse model. It was demonstrated that minocycline can protect RGCs from secondary degeneration in the contralateral eye, while not influencing the primary RGC degeneration in the injured eye. Also, minocycline was shown to inhibit microglial activation, but the effect occurred considerably in the outer plexiform layer in both the ipsilateral and contralateral eyes following the crush injury. In the microglia-treated animals, there was decreased microglial cell body area and circumference in the inner and outer plexiform layers in both eyes, while an increased number of nodes as well as length of branches in the contralateral inner plexiform layer was observed. It is noteworthy that all these characteristics were attended by a significant difference in the a- and b-wave amplitudes of electroretinogram after minocycline treatment, greatly improving the function of photoreceptor cells and bipolar cells in the contralateral eye. It was reasonably suggested, therefore, that minocycline partially inhibited the microglia activation and preserved the function of retinas. Minocycline has been designated as a neuroprotective agent, having an inhibitory effect on contralateral eye microglia activation. This has been corroborated by an another study, in which minocycline rescued RGCs in the contralateral retina but produced no effect on the injured retina. Although, in contrast, more authors have acknowledged that minocycline has a neuroprotective proclivity in the injured eye. Nevertheless, minocycline did disclose its potential in protecting the retina against secondary degeneration.

Consequently, minocycline can be a suitable conjunctive therapy choice in unilateral retinal diseases or traumatic brain injury conditions affected with optic nerve injury.

A neuroprotective agent can be beneficiary if it antagonizes the cytotoxic processes triggered by the neurotoxicological insult, along with substantiating the endogenous neuronal protective system. The current neuroprotective strategies are ineffective and produce only partial results, as these are focused on single pathways in neurodegeneration, thus necessitating a revisiting of designing such neuroprotective modalities that possess multitarget impacting propensity in the neuronal cell death cascades. In consequence, a neuroprotective agent may prove to be useful in improving survival and function of the non-regenerative RGCs against intracellular and environmental stresses. In view of this, a clinical neuroprotective strategy was developed in addition to lowering of intraocular pressure in patients with glaucoma. Consequently, a reliable neuroprotective agent for attenuating RGC degeneration is exceptionally timely.

A neuroprotection approach can be applied to restore vision in patients due to RGC damage or as preventive approach in individuals having a high risk of developing RGC degeneration. Preclinical animal models have proved useful in sorting out potential neuroprotective agents versus RGC degeneration-like conditions. These approaches have even been shown to be valuable in detecting potential activity of compounds having neuroprotective affinity for RGCs and their beneficial proclivity has led to the prediction of clinical effectiveness. These include brimonidine, citicoline, triamcinolone acetonide, erythropoietin, prednisolone, methylprednisolone, idebenone, anti-vascular endothelial growth factor antibodies (bevacizumab, ranibizumab), and cyclosporine. An important aspect of RGC neuroprotection is the involvement of neurotrophic family of growth factors, including the neurotrophin family, the glial cell-line derived neurotrophic factor and ciliary neurotrophic factor antibodies (bevacizumab, ranibizumab), and cyclosporine. An important aspect of RGC neuroprotection is the involvement of neurotrophic family of growth factors, including the neurotrophin family, the glial cell-line derived neurotrophic factor and ciliary neurotrophic factor antibodies (bevacizumab, ranibizumab), and cyclosporine. The efficiency of these endogenous neuroprotective systems in preventing RGC degeneration is crucial and these tend to have value as the main mechanistic targets of potential approaches and technologies encompassing RGC neuroprotection.

The important findings underlying the degeneration of RGCs comes from the use of appropriate animal models, including heredity models (C57BL/6J × Krd+/+), transgenic models (Bax−/−, C3H × C57BL/6), and inducible models (N-methyl-D-aspartate [commonly referred to as NMDA], experimental autoimmune encephalomyelitis, optic nerve crush injury, transient ischemia, and light-induced retinal cell degeneration). Although these models have made a major contribution to the field of ophthalmology and vision research, the progress in this area of research has been mired by innate translational problems. These include the rarity of human pathological tissues, accurate modelling of complex multifactorial disease state, variability in disease phenotypic expression,
differences in outcome measures, inclination in the time-course of retinal degenerative changes in animals, apparent differences in ocular anatomy and physiology of tear flow and mixing compared to humans, the greater surface-to-volume ratio, increased basal metabolic rate and polyunsaturated nature of cellular membranes of small animals. Canine studies have shown some promise in improving the predictive power of preclinical ophthalmic research; nonetheless, they are presented with inherent limitations, including mixed genetic background affecting the variability in disease characteristics, in addition to associated economic and ethical challenges. It is worthwhile to bear in mind that these are important matters in extrapolating experimental data from bench to clinic. This may explain why many effective neuroprotective agents in preclinical research fail in clinical trials.

In the clinic, a true parameter of RGC neurodegeneration is currently a challenge, as surrogate measures are utilized to estimate the integrity of RGCs. In this respect, translation of potential RGC neuroprotective strategies into clinical ophthalmic practice remains an issue. Another confounding factor is that in animal studies, neuroprotective agents are administered prior to the RGC damage. This limits their translational relevance when relating any beneficial effect to therapeutic intervention in patients who are already diagnosed with retinal disease. It should also be kept in mind that RGCs exhibit tremendous diversity and RGC types appear to differ profoundly in their ability to survive insults. In the case of minocycline, oral treatment appeared to have potential efficacy in increasing visual acuity and reducing macular edema in patients with diabetes mellitus. Ostensibly, minocycline may well have a neuroprotective value; though in consideration of the foregoing discussion and the failure to achieve any convincing therapeutic benefits with other neuroprotective agents, uncertainty prevails about minocycline’s clinical efficacy in RGC degenerative conditions. Additionally, there are cases of minocycline-induced intracranial hypertension and papilledema, marked confound the prospective efficacy of minocycline in patients with retinal degeneration. This emphasizes the duality of challenges coupled to preclinical investigation of neuroprotective agents and their therapeutic effectiveness; so, clinically, combinatorial approaches should be considered to maximize the neuroprotection of RGCs.

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Conflict of interest

The author has no conflicts of interest to declare.

References

Network Pharmacology Analysis: A Promising Approach for the Research of Traditional Chinese Medicine

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Network pharmacology is a new strategy to explore the molecular mechanisms underlying the action of traditional Chinese medicine (TCM) formula by analyzing the biological network of multi-biological processes and signaling pathways, and identifying the key components of druggable targets.1,2 Network pharmacology approaches have been applied in elucidation of the potential therapeutic effects and action of TCM in several chronic diseases, such as chronic kidney disease, diabetes, rheumatoid arthritis and cancer.1,3 Using network pharmacology analysis, Peng4 predicted that Xia Sang Ju (XSJ) granule, a traditional TCM, might exert its anti-hypertensive effects by targeting 11 genes, among which ESR2 and SLC6A2 were the uppermost hypertension-related targets. The author further revealed that XSJ might affect multi-biological processes and multi-pathways to prompt their anti-hypertensive effects, which coincided with the TCM formula’s therapy concept of multiple compounds, targets, and pathways.5 The findings open new avenues for the application of network pharmacology in the study of TCM formula. With the concept of integrity, comprehensiveness and systematic approach, network pharmacology provides new strategies and approaches for the study of TCM formula, because it may change TCM from experience-based medicine to evidence-based medicine.2 However, we have to understand that current network pharmacology has limitations. First, the results of network pharmacology analysis are affected by the accuracy and integrity of the related databases. In addition, the efficacy of a TCM formula is dependent on the levels of absorbed constituents in vivo.5,6 Thus, it would be more credible and complete if the compounds of XSJ selected in the study were screened from the serum samples for pharmacological experiments. Second, the data from network pharmacology analysis are predictive and static.2 Although the study validated by a virtual strategy, the results still need further experimental validation in vivo or in vitro to exclude potential false positives and observe the dynamic process of XSJ for anti-hypertensive actions. Third, the research on TCM formula employed network pharmacology and mainly provided qualitative analysis results, and there was little reported on the quantity of these constituents.2 In the future, how to achieve a quantitative analysis will be a new challenge for the development of network pharmacology.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Manuscript writing (WRX, XYY), and supports of administration or intellectual content (LHW, XXH).

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Network Pharmacology Analysis Uncovers the Potential Anti-Hypertensive Mechanisms of Xia Sang Ju Granule

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Abstract

Background and objectives: Xia Sang Ju (XSJ) granule, a Chinese drug and herbal tea made up of Prunellae spica (Xia Ku Cao), Mori folium (Sang Ye), and Flos Chrysanthemi Indici (Ye Ju Hua), is commonly used for fever, headache, and sore throat. The underlying pharmacological mechanism of XSJ on hypertension treatment is described here, based on network pharmacology.

Methods: The compounds in XSJ were searched using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (commonly known as TCMSP), and the active components, according to oral bioavailability and drug likeness, were screened. Compounds targets were predicted by the SwissTargetPrediction web server, while hypertension targets were collected from the Online Mendelian Inheritance in Man (commonly known as OMIM) and GeneCards databases. The interaction of targets was analyzed by STRING. The compound-compound target network was constructed by Cytoscape. Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes (commonly known as KEGG) pathways were analyzed by the Database for Annotation, Visualization and Integrated Discovery (commonly known as DAVID).

Results: Forty-five active compounds were obtained from 359 ingredients present in the XSJ decoction, corresponding to 237 targets. In addition, 189 genes were found to be related to hypertension, of which 11 overlapped with XSJ targeted by 28 compounds and were thus considered therapeutically-relevant. ESR2 was the most frequent gene targeted by the compounds, while NR3C1 showed the most interaction with other genes. These results revealed that the anti-hypertensive activity of XSJ may directly relate to the regulation of several hypertension-associated biological processes and pathways, such as cellular nitrogen compound biosynthetic process, positive regulation of the nitrogen compound metabolic process, steroid hormone biosynthesis, and aldosterone-regulated sodium reabsorption.

Conclusions: These findings provide a reference for further interpretation of the potential mode of action of XSJ against hypertension and serve as an example for elucidation of the Traditional Chinese Medicine concept of “multiple compounds-multiple targets-multiple effects”.

Keywords: Xia Sang Ju granule; Hypertension; Network pharmacology; Multiple compounds-multiple targets-multiple effects.

Abbreviations: DAVID, Database for Annotation, Visualization and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes; OB, oral bioavailability; DL, drug likeness; OMIM, Online Mendelian Inheritance in Man; SMILES, simplified molecular-input line-entry system; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; TCM, traditional Chinese medicine; XSJ, Xia Sang Ju.

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Introduction

Xia Sang Ju (XSJ) granule is a traditional Chinese drug as well as a kind of Chinese herbal tea which is made up of Prunellae spica (Xia Ku Cao), Mori folium (Sang Ye), and Flos Chrysanthemi Indici (Ye Ju Hua). It originates from the classic prescription called Sang Ju Yin that was recorded in the Treatise on Differentiation and Treatment of Epidemic Febrile Diseases (Wen Bing Tiao Bian) by Wu Jutong in the Qing Dynasty. Though XSJ is well-known for treating fever, headache, and sore throat, hypertension is also one of the main functions of XSJ.¹ However, how XSJ plays a part in anti-hypertensive activity remains
unclear, due to the complexity of Traditional Chinese medicine (TCM).

Hypertension is characterized by elevated blood pressure in arteries, and is the most common of the chronic diseases and one of the most important risk factors for cerebrovascular diseases; causing an estimated 7.5 million deaths, it accounts for 12.8% of the total deaths. So far, commonly-used anti-hypertensive drugs include diuretics, beta-blockers, angiotensin converting enzyme inhibitors, calcium channel blockers, angiotensin receptor blockers, etc. Unfortunately, no specific medicine can yet cure high blood pressure.

TCMs are extensively used in eastern countries, as treatments for such chronic diseases as hypertension, diabetes and stroke, and their advantages have been gradually recognized through the increasing number of people who seek natural herbal remedies in western countries. Most existing research is limited to a certain gene target while interpreting the mechanism of a drug, an approach which may ignore the multi-component, multi-target, multi-pathway characteristics of Chinese herbal formulae. Network pharmacology, based on an integrated multidisciplinary concept, is a powerful tool that analyzes the multi-level network of molecular-target-pathway-disease through the interaction between TCM and disease from a holistic perspective.

In this study, firstly the active compounds of XSJ were screened computationally, according to oral bioavailability (OB) and drug likeness (DL) and then the potential compound targets and hypertension-related targets were predicted. Finally the XSJ-compound-hypertension networks were constructed, so as to deeply understand the potential underlying mechanism of the anti-hypertensive effect of XSJ.

Materials and methods

Compounds of XSJ

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (commonly known as TC MSP; http://www.tcmsp.com/tcmsp.php, version 2.3) was used to collect the compound information of XSJ. A total of 60 compounds in Prunellae spica, 269 compounds in Mori folium, and 30 compounds in Flos Chrysanthemi Indici were found. To select the potential active compounds, OB and DL, the most important criteria for drug screening, was set to be ≥30% and ≥0.18, respectively.

Compound targets

To predict the most relevant targets of compounds, the simplified molecular-input line-entry system (referred to as SMILES) format of each compound was input into the SwissTargetPrediction website (http://www.swisstargetprediction.ch/) with the organism limited to Homo sapiens.

Hypertension targets

Genes associated with hypertension were searched from the Online Mendelian Inheritance in Man (commonly known as OMIM) database (http://www.omim.org/) and GeneCards database (https://www.genecards.org/) using the keywords “hypertension” or “high blood pressure”.

Results

Screen of active compounds

In total, 359 compounds in XSJ were obtained from the TC MSP database. After filtering by OB and drug likeness parameters, 11 compounds from Prunellae spica, 29 compounds from Mori folium, and 12 compounds from Flos Chrysanthemi Indici with favorable pharmacokinetic profiles were included for further investigation (Table 1). Specifically, beta-sitosterol and quercetin were found in all three of the herbs, and kaempferol as well as stigmasterol were originated from both Prunellae spica and Mori folium, while luteolin was found in Prunellae spica and Flos Chrysanthemi Indici.

Hypertension network analysis

In total, 189 genes associated with hypertension were obtained from the OMIM and GeneCards databases after elimination of false positives and repetitive genes (Table S1). The interaction of hypertension target genes was analyzed by GeneMANIA (Fig. 1, Table S2) and a network containing 274 nodes and 10,742 edges was constructed. This result showed that 55.08% of genes were co-expressed, and 20.87% were expressed in the same tissue or their products in the same cellular location. Among the genes, 11.02% were found to be involved in physical interaction, while 4.86% were engaged in predicted functional relationships. Up to 3.61% were identified as possibly participating in the same pathway, 3.01% had shared protein domains, and 1.55% had genetic interactions that were functionally associated.

Protein-protein interaction

The STRING database (https://string-db.org/, version 10.5) was used to analysis the protein-protein interaction. Protein names were input and organism was limited to Homo sapiens. Data of protein-protein interactions were obtained and saved as TSV files.

GeneMANIA analysis

A weighted composite functional interaction network for hypertension-related genes were constructed by GeneMANIA (https://genemania.org/). Genes of interest were input and organism was limited to Homo sapiens.

Network construction

All the networks were constructed by Cytoscape software (https://cytoscape.org/, version 3.6.1).

Gene ontology enrichment analysis

Gene ontology enrichment analysis for biological processes and Kyoto Encyclopedia of Genes and Genomes (commonly known as KEGG) pathways were performed by Database for Annotation, Visualization and Integrated Discovery, commonly known as DA VID, 6.8 server (https://david.ncifcrf.gov/).
Table 1. Active compounds in the herbs and their properties

<table>
<thead>
<tr>
<th>Mol ID</th>
<th>Compound</th>
<th>OB, %</th>
<th>DL</th>
<th>Herbs</th>
</tr>
</thead>
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<tr>
<td>MOL000358</td>
<td>Beta-sitosterol</td>
<td>36.91</td>
<td>0.75</td>
<td>Prunellae spica, Mori folium, Flos Chrysanthemi Indici</td>
</tr>
<tr>
<td>MOL000422</td>
<td>Kaempferol</td>
<td>41.88</td>
<td>0.24</td>
<td>Prunellae spica, Mori folium</td>
</tr>
<tr>
<td>MOL004355</td>
<td>Spinasterol</td>
<td>42.98</td>
<td>0.76</td>
<td>Prunellae spica</td>
</tr>
<tr>
<td>MOL000449</td>
<td>Stigmasterol</td>
<td>43.83</td>
<td>0.76</td>
<td>Prunellae spica, Mori folium</td>
</tr>
<tr>
<td>MOL004798</td>
<td>Delphinidin</td>
<td>40.63</td>
<td>0.28</td>
<td>Prunellae spica</td>
</tr>
<tr>
<td>MOL000006</td>
<td>Luteolin</td>
<td>36.16</td>
<td>0.25</td>
<td>Prunellae spica, Flos Chrysanthemi Indici</td>
</tr>
<tr>
<td>MOL006767</td>
<td>Vulgaxanthin-I</td>
<td>56.14</td>
<td>0.26</td>
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</tr>
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<td>43.83</td>
<td>0.76</td>
<td>Prunellae spica</td>
</tr>
<tr>
<td>MOL006774</td>
<td>Stigmast-7-enol</td>
<td>37.42</td>
<td>0.75</td>
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</tr>
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<td>MOL00098</td>
<td>Quercetin</td>
<td>46.43</td>
<td>0.28</td>
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<td>Poriferast-5-en-3beta-ol</td>
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<td>0.75</td>
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<tr>
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<td>0.58</td>
<td>Mori folium</td>
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<td>38.81</td>
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<td>0.79</td>
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</tr>
<tr>
<td>MOL003851</td>
<td>Isoramanone</td>
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<td>0.51</td>
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<td>55.85</td>
<td>0.23</td>
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<td>Moracin C</td>
<td>82.13</td>
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<td>0.38</td>
<td>Mori folium</td>
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<tr>
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<td>53.81</td>
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<tr>
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<td>75.78</td>
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<td>0.51</td>
<td>Mori folium</td>
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<td>Oxysanguinarine</td>
<td>68.96</td>
<td>0.71</td>
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</tr>
<tr>
<td>MOL000729</td>
<td>Arachidonic acid</td>
<td>46.97</td>
<td>0.87</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL001439</td>
<td>Arctiectorigenin A</td>
<td>45.57</td>
<td>0.2</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL001506</td>
<td>Supraene</td>
<td>33.55</td>
<td>0.42</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL003759</td>
<td>Iristectorigenin A</td>
<td>63.36</td>
<td>0.34</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL003975</td>
<td>Icoda-11,14,17-trienoic acid methyl ester</td>
<td>44.81</td>
<td>0.23</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL006630</td>
<td>Norartocarpetin</td>
<td>54.93</td>
<td>0.24</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL007179</td>
<td>Linolenic acid ethyl ester</td>
<td>46.1</td>
<td>0.2</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL007879</td>
<td>Tetramethoxyluteolin</td>
<td>43.68</td>
<td>0.37</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL013083</td>
<td>Skimmira (8CI)</td>
<td>38.35</td>
<td>0.32</td>
<td>Mori folium</td>
</tr>
<tr>
<td>MOL001689</td>
<td>Acacetin</td>
<td>34.97</td>
<td>0.24</td>
<td>Flos Chrysanthemi Indici</td>
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<tr>
<td>MOL001790</td>
<td>Linarin</td>
<td>39.84</td>
<td>0.71</td>
<td>Flos Chrysanthemi Indici</td>
</tr>
<tr>
<td>MOL000359</td>
<td>Sitosterol</td>
<td>36.91</td>
<td>0.75</td>
<td>Flos Chrysanthemi Indici</td>
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<tr>
<td>MOL008173</td>
<td>Daucosterol_qt</td>
<td>36.91</td>
<td>0.75</td>
<td>Flos Chrysanthemi Indici</td>
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Table 1. Active compounds in the herbs and their properties - (continued)

<table>
<thead>
<tr>
<th>Mol ID</th>
<th>Compound</th>
<th>OB, %</th>
<th>DL</th>
<th>Herbs</th>
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<tbody>
<tr>
<td>MOL008915</td>
<td>Acacetin-7-O-β-D-galactopyranoside</td>
<td>50.19</td>
<td>0.77</td>
<td>Flos Chrysanthemi Indici</td>
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<tr>
<td>MOL008918</td>
<td>Arteglasin A</td>
<td>52.45</td>
<td>0.33</td>
<td>Flos Chrysanthemi Indici</td>
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<tr>
<td>MOL008919</td>
<td>(25,6S,7αR)-2-[(1E,3E,5E,7E,9E,11E,13E,15E)-16-[(4S)-4-hydroxy-2,6,6-trimethyl-1-cyclohexenyl]-1,5,10,14-tetramethylhexadeca-1,3,5,7,9,11,13,15-octaenyl]-(4,4,7α-trimethyl-2,5,6,7-tetrahydrobenzofuran-6-ol</td>
<td>59.52</td>
<td>0.55</td>
<td>Flos Chrysanthemi Indici</td>
</tr>
<tr>
<td>MOL008924</td>
<td>Azuleno(4,5-b)furan-2(3H)-one, 4-(acetylxy)-3α,4,5,6,6a,7,9a,9b-octahydro-6-hydroxy-6,9-dimethyl-3-methylene-, (3αR-(3αalpha,4alpha,6alpha,6aalpha,9aalpha,9bbeta))-</td>
<td>68.44</td>
<td>0.27</td>
<td>Flos Chrysanthemi Indici</td>
</tr>
<tr>
<td>MOL008925</td>
<td>(3αR,4S,6R,6aR,9aR,9bR)-4,6-Dihydroxy-6,9-dimethyl-3-methylene-4,5,6a,7,9a,9b-hexahydro-3αH-azuleno[5,4-d]furan-2-one</td>
<td>40.08</td>
<td>0.19</td>
<td>Flos Chrysanthemi Indici</td>
</tr>
</tbody>
</table>

DL, drug likeness.

Fig. 1. Protein-protein interaction network of hypertension targets.
The SMILES format of each compound was input into SwissTargetPrediction, and predicted compound targets were obtained (Table s3). A compound-compound target network was constructed, consisting of 282 nodes and 703 edges (Fig. 2). These results showed that some target genes may be modulated by many compounds, such as the ESR1, AR, MAPT, CYP19A1, and HMGCR genes. While the AOX1, CTSK, OCD1, SRC, RARA, NOX4, and CDC25B genes are hit by only one compound. Interestingly, both SLC6A4 and P05093 can be regulated by poriferast-5-en-3beta-ol, beta-sitosterol, poriferasterol monoglucoside, stigmast-7-enol, spinasterol, stigmasterol, daucosterol, and sitosterol. This predicted compound-compound target network strengthens the concepts of multi-compound-multi-target of TCM, in which different active components in XSJ may regulate the same targets and one active ingredient may also modulate various targets.

**Hypertension-related compound target network analysis**

Eleven genes with commonalities between hypertension genes and compound targets were found and a hypertension-related compound network was constructed, consisting of 58 nodes and 108 edges (Fig. 3). The schematic representation of the hypertension-related compound target network is shown in Fig. 3. The network shows the complex interactions between 11 hypertension-related genes and 58 compound targets. The network is further visualized using Cytoscape software. The results indicate that the hypertension-related compound target network is a complex and interdependent system, with multiple compounds modulating the same targets. This suggests that the hypertension-related compound target network may play a crucial role in the therapeutic effect of XSJ against hypertension.
target network was constructed (Fig. 3, Table 2), which contained 39 nodes and 39 edges. Among the 28 compounds directly interacting with these genes, 8 of them came from Prunella spica, 18 were from Mori folium, and 5 were from Flos Chrysanthemi Indici. The protein classes for the 11 common genes were obtained from the DisGeNET database. The XSJ and hypertension-related targets’ protein-protein interaction network is shown in Figure 4. ESR2 and SLC6A2, both of which play a role in nucleic acid binding, as receptor and transcription factor, or transporter, were the most frequent genes targeted by the compounds. ESR2 and SLC6A2 are known to be important to cardiovascular physiology and blood pressure regulation.23–27 These results suggested that the anti-hypertension effect of XSJ may be regulated mainly by ESR2 and SLC6A2 (Table 3).

**Biological functional analysis**

Biological functions of the hypertension-related compound targets were annotated to explain the possible mode of action of XSJ in hypertension. Gene ontology enrichment analysis was performed on the 11 targets by DAVID. The top five biological processes were cellular nitrogen compound biosynthetic process, organic cyclic compound biosynthetic process, aromatic compound biosynthetic process, heterocycle biosynthetic process, and nucleobase-containing compound biosynthetic process (Fig. 5a). The significant KEGG pathways included neuroactive ligand-receptor interaction, steroid hormone biosynthesis, aldosterone-regulated sodium reabsorption, PPAR signaling pathway, and thyroid cancer (Fig. 5b). These results elucidated that XSJ may exert anti-hypertension activity through multi-biological processes as well as multi-pathways.

**Discussion**

The escalation of hypertension cases global effects. Coupled with lack of any promising hypotensor, this then requires multiple approaches for treatment, including lifestyle modifications and new drugs. Though XSJ is generally used for treatment of fever, headache, sore throat, and as a beverage for clearing heat, hypertension is also one of the major functions.1 Nevertheless, the mechanism of action for XSJ working on hypertension remains to be fully understood.

During the development of hypertension, endothelin, nitric oxide, and angiotensin II are key factors. Vascular endothelial cells can produce both vasoconstrictor and vasodilator substances for maintaining vasoconstrictor balance and normal tension. Endothelin is the strongest vasoconstrictor and promotes smooth muscle proliferation, while nitric oxide is the main vasodilator substance released by vascular endothelial cells. Endothelin harbors angiotensin-converting enzyme activity that catalyzes the synthesis of angiotensin II; however, angiotensin II can induce expression of the endothelin gene in endothelial cells. Nitric oxide inhibits the production and release of endothelin, and also inhibits the release of renin, which in turn inhibits the production of angiotensin II.28,29

Despite few publications in the publicly available literature describing the anti-hypertension activity of XSJ so far, recent studies have proven that the extracts and some compounds of all three herbs in XSJ have direct or indirect anti-hypertensive effect, consistent with some of the biological processes found in our study. Ethanol extract of Prunella vulgaris L. has been shown to increase the content of nitric oxide, to decrease the content of endothelin and angiotensin II, and finally to reduce blood pressure significantly in a spontaneously hypertensive rat model (e.g., positive regulation of the nitric oxide biosynthetic process, regulation of the systemic arterial blood pressure by endothelin).30 Flavonoid compounds in Mori folium have also been found to expand the coronary vessels, improve myocardial circulation, and reduce blood pressure (e.g., regulation of blood pressure).31 Ethanol extract of Flos Chrysanthemi Indici has shown hypotensive effect in clinical studies.32 Intriguingly, luteolin from Prunella vulgaris L and Flos Chrysanthemi Indici might inhibit vascular smooth muscle cell proliferation and migration, which is pivotal in the development of arterial remodeling during hypertension (e.g., blood vessel remodeling), by suppressing transforming growth factor-β receptor I signaling.33

Luteolin can ameliorate hypertensive vascular remodeling through inhibition of proliferation and migration of angiotensin II-induced vascular smooth muscle cells, a process that is mediated by the regulation of MAPK signaling pathway and the production of reactive oxygen species (e.g., blood vessel remodeling, positive regulation of the reactive oxygen species metabolic process).34 Quercetin from Prunella vulgaris L and Flos Chrysanthemi Indici can attenuate hypertension via reduction in oxidative stress and improving endothelial function, as shown in an acute fluoride-induced hypertension and cardiovascular complications model.35 Furthermore, quercetin was shown to reduce hypertension-induced vascular remodeling, oxidative stress and MMP-2 activity in aorta in the two-kidney one-clip hypertensive Wistar rat model (e.g., blood vessel remodeling).36 Quercetin can also attenuate vascular contraction through the LKB1-AMPK signaling pathway (e.g., regulation of vasoconstriction).37 Delphinidin and quercetin were shown to block the renin-angiotensin system signaling pathway through inhibition of angiotensin-converting enzyme activity and decreasing the production of its mRNA.38 Finally, linarin from Flos Chrysanthemi Indici was shown to directly or indirectly activate macrophages and affect the inhibition of nitric oxide that is responsible for vasoconstriction and hypotension (e.g., vasodilation).39

The current study provided a prediction of the potential mechanism of XSJ as treatment of hypertension, based on a computational approach. There are some limitations of this work. First, the

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Fig. 3. XSJ-hypertension network. Compound targets and hypertension targets are denoted by green hexagons; Prunella spica by blue circles; Mori folium by red circles; Flos Chrysanthemi Indici by yellow circles; Prunella spica and Mori folium by pink circle; Prunella spica and Mori folium and Flos Chrysanthemi Indici by purple circle.
Potential antihypertensive mechanism of XSJ

components in TCM herbs have not yet been completely identified; thus, the databases of compounds are not complete, precluding their ability to represent the integral spectrum of compounds responsible for the anti-hypertension effect. Second, all of the data were based on silico analysis, and as such there may be many false positive and false negative interactions between the found compound-protein and protein-protein interactions. What’s more, the relationship between XSJ and anti-hypertension activity was identified by enrichment analysis. Therefore, the associations presented herein should be further investigated for experimental verification to achieve more accurate and reliable inferences in the future.

**Future directions**

The associated biological processes and pathways need further investigation for experimental verification.
Table 3. Hypertension-related targets of Xia Sang Ju

<table>
<thead>
<tr>
<th>No.</th>
<th>Target</th>
<th>Uniprot ID</th>
<th>Gene code</th>
<th>Protein class</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Estrogen receptor-beta</td>
<td>Q92731</td>
<td>ESR2</td>
<td>Nucleic acid binding; receptor; transcription factor</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Solute carrier family 6 member 2</td>
<td>P23975</td>
<td>SLC6A2</td>
<td>Transporter</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>Nuclear receptor subfamily 3, group C, member 1</td>
<td>P04150</td>
<td>NR3C1</td>
<td>Nucleic acid binding; receptor; transcription factor</td>
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</tr>
<tr>
<td>4</td>
<td>Nuclear receptor subfamily 3, group C, member 2</td>
<td>P08235</td>
<td>NR3C2</td>
<td>Nucleic acid binding; receptor; transcription factor</td>
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</tr>
<tr>
<td>5</td>
<td>Adenosine receptor A2a</td>
<td>P29274</td>
<td>ADORA2A</td>
<td>Receptor</td>
<td>3</td>
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<tr>
<td>6</td>
<td>Adrenoceptor alpha 2B</td>
<td>P18089</td>
<td>ADRA2B</td>
<td>Receptor</td>
<td>1</td>
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<tr>
<td>7</td>
<td>Maltase-glucoamylase</td>
<td>O43451</td>
<td>MGAM</td>
<td>Hydrolase</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Hypoxia-inducible factor 1, alpha subunit</td>
<td>Q16665</td>
<td>HIF1A</td>
<td>Nucleic acid binding; transcription factor</td>
<td>1</td>
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<tr>
<td>9</td>
<td>11-Beta-hydroxysteroid dehydrogenase, type 1</td>
<td>P28845</td>
<td>HSD11B1</td>
<td>None</td>
<td>1</td>
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<tr>
<td>10</td>
<td>Peroxisome proliferator-activated receptor-gamma</td>
<td>P37231</td>
<td>PPARG</td>
<td>Nucleic acid binding; receptor; transcription factor</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>Corticosteroid 11-beta-dehydrogenase isozyme 2</td>
<td>P80365</td>
<td>HSD11B2</td>
<td>Oxidoreductase</td>
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Fig. 5. Gene ontology functional analysis. (a) Biological processes terms. (b) Significant KEGG pathways.

Conclusions

Investigation for confirmation of the exact mechanism of XSJ in hypertension treatment.
Collectively, the findings presented herein suggest that the compounds in XSJ exert their anti-hypertensive effect via multiple biological processes, such as regulation of blood pressure, blood vessel remodeling, regulation of the nitric oxide biosynthetic process and so on, which is in accord with the TCM therapy concept of “multiple compounds-multiple targets-multiple effects”. Though further experiments are needed to verify this finding, this study revealed the potential anti-hypertensive mechanism of XSJ from holistic and systematic perspectives by using network pharmacology.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/JERP.2020.00008.

Table s1. Hypertension targets.

Table s2. Interaction of genes related to hypertension.

Table s3. Relationships between compounds and targets.

Acknowledgments

The author wishes to thank Zhong Xiaotian and Gao Jiansheng for valuable comments.

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Conflict of interest

None.

References


Introduction

Glaucoma, characterized by the degeneration of retinal ganglion cells (RGCs), is the second leading cause of blindness in the world. Glaucoma can result in both primary and secondary degeneration.

The early death of neurons and glial cells caused by the primary pathological events is termed primary degeneration. In addition, the death of the neuron and glial cells from the following toxic effects of primary degeneration is attributed to secondary degeneration.

Studies have shown that damage to one eye can lead to secondary degeneration in the other eye, for example, unilateral high intraocular pressure in rats induces nerve inflammation, nerve fiber loss and apoptosis of RGCs in the bilateral eyes.

Apart from the RGC degeneration, the degeneration of bipolar and/or photoreceptors was also demonstrated in the intraocular hypertension (IOH) model; specifically, the amplitudes of both a- and b- waves detected by flash electroretinogram (ERG) after injury reduced significantly, while the number of synaptic bands of photoreceptors and the thickness of outer nucleus and plexus layers of the same eye significantly decreased simultaneously.

Hence, both primary and secondary degeneration can happen after damage to an eye, including both the degeneration of RGCs and the impairment of other neurons, such as bipolar cells and/or photoreceptors. Therefore, this study was designed to investigate both the primary and secondary degeneration of RGCs in mice.

Tetracyclines are a broad-spectrum class of antibiotics. Minocycline is a member of the semisynthetic tetracyclines and the most potent agent in the family.

Abstract

Background and objectives: Microglia activation can cause degeneration of retinal ganglion cells (RGCs). This study aimed to investigate the potential therapeutic effect of minocycline on microglia activation-related degeneration of RGCs in both retinas after unilateral optic nerve crush (ONC) in the left eye of male adult C57BL/6 mice.

Methods: First, the primary degeneration of RGCs after unilateral ONC in the left eye and the secondary degeneration of RGCs in the contralateral eye were investigated. Second, microglia activation in both eyes was examined longitudinally at 1, 5 and 14 days post-ONC. Finally, the effects of minocycline treatment on the primary or/and secondary RGC degeneration as well as the function of both retinas (estimated by flash electroretinogram) at 5 days post-ONC were analyzed.

Results: The results indicated that ONC induced the primary RGC degeneration, which was more severe than the secondary RGC degeneration and microglia activation in both eyes. Treatment with minocycline partially inhibited microglia activation, preserved the function of retinas in both eyes, and delayed the secondary degeneration of RGCs in the contralateral retina.

Conclusions: ONC caused RGC degeneration in both eyes of mice. Minocycline treatment delayed the secondary degeneration of RGCs and improved the function of both retinas post-ONC in mice, which were associated with inhibition of microglia activation.

Keywords: Minocycline; Optic nerve crush; Retinal ganglion cells; Microglia; neuroprotection; Electroretinogram.

Abbreviations: Brn3a, brain-specific homeobox/POU domain protein 3A; ERG, electroretinogram; GCL, ganglion cell laye; Iba-1, ionized calcium binding adaptor molecule-1; INL, inner nuclear layer; IOH, intraocular hypertension; IPL, inner plexiform layer; ONC, Optic Nerve Crush; ONL, outer nuclear layer; ONT, optic nerve transection; OPL, outer plexiform layer; PBS, phosphate buffer saline; RGCs, retinal ganglion cells.
activation, cell apoptosis and reactive oxygen species production.\(^9\) Previous studies have shown that minocycline can inhibit the progression of several neurological diseases in rodent models, such as depression, Parkinson’s disease, Alzheimer’s disease, and cerebral ischemia.\(^{10–13}\) Growing data also indicate that minocycline treatment can protect from the injury and death of neuronal cells in some retinal diseases.\(^{14–16}\) In animal studies of glaucoma, such as the optic nerve transection (ONT) model and IOH model, minocycline treatment effectively delayed the death of RGCs in the injured eye,\(^17\) indicating that minocycline inhibited the primary degeneration of RGCs. However, a study of the partial ONT model of rats claimed that minocycline delayed the secondary degeneration of RGCs in the inferior-nasal retinas rather than primary degeneration in the superior-nasal retinas.\(^18\) Thus, the role of minocycline treatment in retinopathy remains inconclusive and needs further investigation.

In many studies related with eye diseases, microglia activation were believed to induce the death of RGCs and a significant negative correlation was found between the number of surviving RGCs and the numbers of activated microglia in the mice administrated using the acute IOH, ONT and optic nerve crush (ONC) model.\(^{19,20}\) The ONC model has been widely used to investigate the pathogenesis of RGC death and to explore new protective treatments for glaucoma.\(^{21,22}\) Although minocycline treatment has shown to partially protect from neuronal death in the crushed eye in ONC mice,\(^23\) it is unclear whether minocycline treatment can attenuate microglia activation in the contralateral eye post-ONC,\(^24\) which can cause the secondary degeneration of RGCs. Thus, this study was designed to explore the degeneration of primary and secondary RGCs and to investigate microglia activation, RGC survival, and retina function (flash ERG examination) in both eyes of mice.

**Materials and methods**

**Animals**

Male C57/BL6 mice (5 weeks-old, 18–22 g) were purchased from Guangdong Province Medical Laboratory Animal Center in Guangzhou, China. All animals were housed in a temperature-controlled room with a 12-hour light/12-hour dark cycle and were supplied with food and water. A total of 32 mice were used in these experiments, with 17 mice used for determination of the surviving RGCs and the numbers of activated microglia in the mice administrated using the acute IOH, ONT and optic nerve crush (ONC) model.\(^{21,22}\) The ONC model has been widely used to investigate the pathogenesis of RGC death and to explore new protective treatments for glaucoma.\(^{21,22}\) Although minocycline treatment has shown to partially protect from neuronal death in the crushed eye in ONC mice,\(^23\) it is unclear whether minocycline treatment can attenuate microglia activation in the contralateral eye post-ONC,\(^24\) which can cause the secondary degeneration of RGCs. Thus, this study was designed to explore the degeneration of primary and secondary RGCs and to investigate microglia activation, RGC survival, and retina function (flash ERG examination) in both eyes of mice.

**Counting of RGCs**

Five photographs (200x200 µm\(^2\)) were captured in each quadrant along the median line, starting from the outer edge of the optic disc to the border of each part and taken at 500 µm intervals at 400x magnification. The numbers of surviving RGCs were counted using ImageJ software (version 1.41; National Institutes of Health, USA). Then, the density values of RGCs (mm\(^{-2}\)) averaged from the total 20 images of each retina were used for comparing the differences among/between various groups.

**Microglia cell analysis**

In the retina, anti-Iba-1-stained microglia were observed by means of a laser confocal microscope and located within the following three layers: ganglion cell layer (GCL); inner plexiform layer (IPL); and outer plexiform layer (OPL) (green color, Fig. 1b). The location of GCL was first determined by the nuclei and cell bodies of RGCs stained by DAPI (blue color) and Brn3a (red color), which appeared arranged in just a single layer on the top (Fig. 1b). The location of the inner nuclear layer (INL) and outer nuclear layer (ONL) was determined by the multilayered nuclei stained by DAPI; the INL was closer to the GCL and the ONL was farther from the GCL, with more nucleus layers than INL as well (Fig. 1b).
Guo R. Minocycline protects retinal neurons

The IPL was between the GCL and the INL, the OPL was between the INL and the ONL, and the microglia (green color) existed in the GCL, IPL and OPL layers (Fig. 1b).

Post-ONC, the changes in morphology of microglia in each layer were observed under a 20× objective and qualitatively analyzed. In addition, the quantitative analysis was conducted by ImageJ software. As it was difficult to count the specific number of microglia in the GCL due to the superposition of the microglial cell bodies and branches, the area of the Iba-1-positive cell bodies in the GCL in each photograph was manually recorded for each animal and the values of all the photographs as the total cell area (Fig. 2a, c and Fig. 3a, c). The numbers of microglia in the IPL and OPL were counted, given that the Iba-1-positive cells were separated by each other and could be discerned clearly in these two layers (Fig. 2a, c and Fig. 3a, c).

Apart from the analysis mentioned above which had been carried out for each animal, additional quantitative analysis was used to further investigate the morphological changes caused by minocycline at a 400× magnification. In the PBS and minocycline groups, the following parameters of microglia cells in the
IPL and OPL were further quantified by Neurolucida software (version 2.7; Micro Brightfield, USA) after the photographs had been taken. The Neurolucida 360 tool was used to reconstruct the morphology of retinal microglia cells (Fig. 4a, c, e, g). Neurolucida Explore was used to analyze the reconstructed cells to obtain relevant information, including cell body circumference, cell body area, node number, total number of branches, total branch length, and average branch length of each cell in each photograph. The average values of each animal were used for comparison.

**ERG**

ERG was performed essentially as described previously. Briefly, the mice were dark adapted overnight; the animals were then anesthesiaized with tribromoethanol (0.2 mL/10 g body weight of 1.25% solution) and placed on a heated platform at 37 °C. Pupils were
dilated with Compound Tropicamide Eye Drops (Santen Pharmaceutical, China). ERGs were recorded with gold-plated wire loop electrodes contacting the corneal surface as the active electrode. Stainless steel needle electrodes were inserted in the skin near the eye and in the tail serving as reference and ground leads, respectively. Following adaptation, animals were stimulated with green flashes of graded intensities of 0.1, 1.0 and 10.0 cd.s/m², followed by light adapted for 5 min under bright green background (20 cd/m²) prior to recording of photopic responses to green flashes of 10.0 cd.s/m².

Statistical analysis

All data are presented as mean ± standard error of the mean. Statistical analysis was performed by GraphPad Prism (version 6.0; GraphPad Software, USA). The data between two groups were compared using unpaired t-test and the data among multiple groups were compared using one-way ANOVA and post hoc Tukey’s multiple comparison test. Statistical significance was defined as p < 0.05.

Results

Survival of RGCs post-ONC in mice

In the normal C57 mice, the mean density of RGCs was 3,993 ± 94 RGCs/mm² in the left eye and 4,100 ± 113 RGCs/mm² in the right eye (Fig. 5a). At day 1, 5 and 14 post-ONC, the corresponding mean densities of surviving RGCs were 3,667 ± 55 RGCs/mm².
Fig. 4. Morphological changes of microglial cells shown by Iba1 staining in the IPL and OPL in both eyes observed under a microscope with a 400× magnification. (a) Neurolucida software was used to reconstruct and compare the morphology of microglia in the IPL of the crushed eyes of mice; the original images are on the left, and the right side shows reconstructions. (b) Microglia cells in the IPL of the crushed eye of the PBS-treated ONC mice had larger cell area than that of the minocycline-treated ONC mice. (c) Neurolucida software was used to reconstruct and compare the morphology of microglia in the OPL of the crushed eye of mice; the original images are on the left, and the right side shows reconstructions. (d) Microglia cells in the IPL of the crushed eye of the PBS-treated ONC mice had larger cell area than those in the minocycline-treated ONC mice. (e) Neurolucida software was used to reconstruct and compare the morphology of microglia in the IPL of the contralateral eye of mice; the original images are on the left, and the right side shows reconstructions. (f) Microglia cells in the IPL of the contralateral eye of the PBS-treated ONC mice had longer cell circumference, less node number and ends' number, and shorter length of dendrite and mean length of dendrite than those in the minocycline-treated ONC mice. (g) Neurolucida software was used to reconstruct and compare the morphology of microglia in the OPL of the contralateral eye of mice; the original images are on the left, and the right side shows reconstructions. (h) Microglia cells in the OPL of the contralateral eye of the PBS-treated ONC mice had longer cell circumference and larger cell area than those in the minocycline-treated ONC mice. The data were tested by unpaired t-test. The data were expressed as mean ± standard error of the mean. NS, no significant difference. *p < 0.05 and **p < 0.01. The number of animals in the normal group, PBS group and minocycline group were 4, 4, 7, respectively.
Fig. 5. Number of surviving RGCs was counted as Brn3a-positive cells in the healthy mice and ONC mice at different time-points post-ONC. (a) The typical immunofluorescent staining images of the RGCs (Brn3a-positive, red color) in the healthy and ONC mice. (b–d) Quantitative analysis of RGCs. (e) The percentages of surviving RGCs in the crushed and contralateral eyes at 5 and 14 days post-ONC. The mean number of RGCs in normal mice were set to 100%. The data in (b) were assessed by unpaired t-test. The data in (c, d) were assessed by one-way ANOVA and post hoc Tukey’s multiple comparison test. The data shown are representative images (magnification × 400) and expressed as mean ± standard error of the mean. NS, no significant difference. **p < 0.01, ***p < 0.001, and ****p < 0.0001. The number of animals in the normal, 1-day ONC, 5-day ONC and 2-week ONC groups were 4, 3, 4 and 6, respectively.
mm², 1,781 ± 124 RGCs/mm² and 375 ± 196 RGCs/mm² in the left crushed eye of mice, while those in the right/contralateral eye were 3,900 ± 190 RGCs/mm², 3,195 ± 123 RGCs/mm² and 3,046 ± 145 RGCs/mm², respectively (Fig. 5a). There was no significant difference in the numbers of RGCs between the left and right eye of normal mice (Fig. 5b). In the crushed eye, the number of RGCs decreased significantly at days 5 and 14 post-ONC but not day 1 (Fig. 5c). The percentage of surviving RGCs was 45 ± 3 % and 9 ± 1% at days 5 and 14 post-ONC, respectively (Fig. 5e). In the contralateral eye, the numbers of RGCs decreased significantly at days 5 and 14 post-ONC but not day 1 post-ONC compared with the normal right eye (Fig. 5d). There was no significant difference in the numbers of surviving RGCs in contralateral eye between days 5 and 14 post-ONC (Fig. 5d), and the percentage of surviving RGCs were 75 ± 3 % and 74 ± 4 % at days 5 and 14 post-ONC, respectively (Fig. 5e).

Qualitative and quantitative analyses of retinal microglia post-ONC

First, the morphological changes of microglial cells post-ONC were observed. During the transition from resting state under normal condition to active state after injury, the microglia were observed to undergo significant morphological changes in the cell body and the process. Microglia cells in the GCL, IPL and OPL of normal mice were characterized by small cell bodies and slender branches, as shown by the white arrows in Figure 2a and c. During the timeline of days 1, 5 and 14 post-ONC, the cell bodies of microglia cells in the GCL, IPL and OPL of the injured eye gradually grew larger and the branches became shorter and thicker over time. In the IPL at day 5 post-ONC and the OPL at day 14 post-ONC, the microglia cells formed a dense mosaic pattern (Fig. 2a). The author found that two different shapes of microglia appeared post-ONC, i.e. the rod-shaped cell body with either branch at ends, as shown by the red arrow in Figure 2a, and the amoeba-like cells without protrusions, as shown by the yellow arrow in Figure 2a. During the timeline of days 1, 5 and 14 post-ONC, the cell bodies of microglia cells in the GCL, IPL and OPL of the contralateral eye also grew larger and the branches also became shorter and thicker over time. The author also observed the amoeba-like microglia cell, as shown by the yellow arrow, and the microglia with rod-shaped cell body, as shown by the red arrow in Figure 2c. In addition, the qualitative analysis showed that the activation patterns of microglia cells in the injured eye and contralateral eye were different. In the GCL, the branches of microglia in the contralateral eye were more clearly visible than those in the injured eye at day 1 post-ONC (Fig. 2a, c). In the IPL, the branches of microglia cells in the contralateral eye were still slender at day 5 post-ONC, while those in the injured eye were shorter and thicker (Fig. 2a, c).

Secondarily, the quantitative analysis post-ONC was done. In the crushed eye, the total area of microglial cell bodies increased significantly at both day 5 and day 14 post-ONC but not at day 1 post-ONC compared with the normal group (Fig. 2b). In the IPL and OPL, the number of microglial cells increased significantly at both day 5 and day 14 post-ONC but not at day 1 post-ONC compared with the normal group (Fig. 2b). In the contralateral eye, the total area of microglial cell bodies increased significantly from day 5 to day 14 post-ONC (Fig. 2d). In the IPL, the numbers of microglial cells had no significant changes during the whole 2-week period post-ONC, while the numbers of microglial cells in the OPL increased significantly at day 5 post-ONC but not at day 1 and day 14 post-ONC (Fig. 2d).

Minocycline treatment delayed the secondary degeneration of RGCs in the contralateral eye

According to the results of the ONC model, the numbers of RGCs significantly decreased at 5 days post-ONC in both eyes, and the microglial cells were intensively activated at the same time. Therefore, day 5 post-ONC was chosen for the minocycline study in mice. The average densities of RGCs in the left eye of the normal C57 mice, PBS-treated mice (control group) and minocycline-treated mice were 3,994 ± 93 RGCs/mm², 1,681 ± 115 RGCs/mm² and 1,808 ± 72 RGCs/mm², respectively (Fig. 6a). Compared with the normal group, the densities of RGCs in the PBS group and minocycline group decreased significantly but there was no significant difference between the PBS group and minocycline group (Fig. 6b). In the right contralateral eye, the average densities of RGCs in the normal C57 mice, PBS-treated mice (control group) and minocycline-treated mice were 4,119 ± 98 RGCs/mm², 3,075 ± 228 RGCs/mm² and 3,813 ± 75 RGCs/mm², respectively (Fig. 6a). Therefore, the results showed that the densities of RGCs in the minocycline group were significantly higher than those in the PBS group in the contralateral eye (Fig. 6b).

Minocycline treatment reduced the activation of microglia in both eyes

Microglia cells in the GCL, IPL and OPL of normal mice were characterized by small cell bodies and slender branches, as shown by the white arrow in Figure 3a and c. Compared with the normal mice, the microglia cells in both eyes of the PBS-treated ONC mice and minocycline-treated ONC mice had larger cell bodies and shorter branches (Fig. 3a, c). This kind of change was similar to that in the retina layers of the mice without PBS or minocycline treatment for day 5 post-ONC (Fig. 2a, c).

The effects of minocycline on microglia activation for 5 days post-ONC in the GCL, IPL and OPL were estimated according to the procedure described in the methods. In the left (crushed) eyes, minocycline treatment inhibited the activation of microglia in the OPL but not in the GCL either IPL compared with the PBS group (Fig. 3b) and the microglia cell density returned to the normal level in the OPL. In the contralateral eye, minocycline treatment also inhibited the activation of microglia in the OPL compared with the PBS group (Fig. 3d) and maintained the microglial cell number in the OPL at the similar level to that in the normal group (Fig. 3d). In the IPL, there was no significant difference in the microglial cell numbers between either two groups in the contralateral eye (Fig. 3d), and this result was similar to that in the IPL of the mice without PBS or minocycline treatment for the 5 days post-ONC (Fig. 2d). These results showed that minocycline could inhibit microglia activation in the OPL in both retinas post-ONC in mice. Although minocycline could not reduce the microglial cell number in the IPL in both eyes, it seemed that the morphology was different between the PBS and minocycline groups for this layer as well as the OPL. Therefore, in order to confirm this issue, detailed quantitative analysis of microglia cell morphology in the IPL and OPL was conducted with the Neurolucida 360 and Neurolucida Explore tools.

The analysis with Neurolucida showed that minocycline significantly reduced the cell body area in the IPL and reduced the microglial cell circumference in the OPL in the left eye (Fig. 4b, d). In the contralateral eye, minocycline decreased the microglial cell circumference and increased the node number, the total number of branches, and the total and the average length of branches of microglial cells in the IPL (Fig. 4f), and reduced microglial cell...
Effects of minocycline on the ERG of both eyes

To examine retinal function in mice, we performed ERG. Retinal neurons in different group of mice responded to light flashes both under dark adapted (scotopic) (Fig. 7a, f) and under light-adapted (photopic) conditions (Fig. 7d, i). In the crushed eye, compared with the normal mice, both a- and b-wave amplitude of scotopic conditions decreased in the PBS-treated mice at 1.0 and 10.0 cd s/m² stimulation. Compared with the normal mice, the a-wave amplitude of scotopic conditions had been decreased in minocycline-treated mice at 1 cd s/m² stimulation, the b-wave amplitude of scotopic conditions had been decreased in minocycline-treated mice at 0.1 and 10.0 cd s/m² stimulation. There were no significant differences in a-wave and b-wave amplitude between PBS-treated mice and minocycline-treated mice at any stimulation (Fig. 7b, c, e). These results indicated that the minocycline treatment did not improve the function of photoreceptor cell and bipolar cell in the crushed eye of ONC mice.

In the contralateral eye, compared with the normal mice, the a-wave amplitude of scotopic had been decreased in PBS-treated mice at 1.0 cd s/m² stimulation, the b-wave amplitude in contralateral eye of scotopic had been decreased in PBS-treated mice at 0.1, 1.0 and 10.0 cd s/m² stimulation. There were no significant differences in a- and b-wave amplitude between normal mice and minocycline-treated mice at any stimulation. After minocycline treatment, there was a significant difference in a-wave amplitude between minocycline-treated mice and PBS-treated mice at 1.0 cd s/m² stimulation; there was also a significant difference in b-wave amplitude between minocycline-treated mice and PBS-treated mice at 0.1, 1.0 and 10.0 cd s/m² stimulation (Fig. 7g, h, j). These results indicated that the minocycline-treatment had improved the...
Fig. 7. Effects of minocycline on the ERG of both eyes. (a) The typical ERG record curve of crushed eye in the normal mice, PBS-treated mice and minocycline-treated mice under scotopic condition. (b, c) The statistics of a- and b-wave amplitude in the crushed eye of normal mice, PBS-treated mice and minocycline-treated mice under scotopic condition. (d) The typical ERG record curve of crushed eye in the normal mice, PBS-treated mice and minocycline-treated mice under photopic condition. (e) The statistics of b-wave amplitude in the crushed eye of normal mice, PBS-treated mice and minocycline-treated mice under photopic condition. (f) The typical ERG record curve of contralateral eye in the normal mice, PBS-treated mice and minocycline-treated mice under scotopic condition. (g and h) The statistics of a- and b-wave amplitude in the contralateral eye of normal mice, PBS-treated mice and minocycline-treated mice under scotopic condition. (i) The typical ERG record curve of the contralateral eye in the normal mice, PBS-treated mice and minocycline-treated mice under photopic condition. (j) The statistics of b-wave amplitude in the contralateral eye of normal mice, PBS-treated mice and minocycline-treated mice under photopic condition. The data were tested by one-way ANOVA followed by Tukey’s multiple comparison test. The data are expressed as mean ± standard error of the mean. NS, no significant difference. *p < 0.05 and **p < 0.01. The number of animals in the normal group, PBS group and minocycline group were 4, 4 and 6, respectively.
function of photoreceptor cells and bipolar cells in the contralateral eye of ONC mice.

Discussion

This study confirmed secondary degeneration. First, not only did the number of surviving RGCs significantly decrease in the crushed eye post-ONC but also in the intact contralateral eye. Therefore, the results suggest that secondary degeneration leads to RGC death in the contralateral eye, which is consistent with previous reports of loss of RGCs in the contralateral eye of a glaucoma model. Second, in addition to RGC secondary degeneration, ERG results suggest that photoreceptors and/or bipolar cells also have dysfunction in crushed and contralateral eyes. This is another secondary degeneration confirmation at present study. It is tempting to envision a degenerative cascade in glaucoma, in which a substantial reduction in RGCs leads to final trans-synaptic loss of bipolar cells and finally photoreceptors in retrograde progression.

Activation of microglia may cause secondary degeneration. Indeed, several studies have shown that the contralateral eye also responds to injury by increasing glial cells and altering gene expression. These studies are consistent with the results of immunofluorescence staining observation and manual analysis. After optic nerve compression injury, microglial cells were activated in the operative eye and contralateral eye of mice. This is mainly manifested not only by a significant increase in the number but by morphological changes. The cell body of microglia becomes larger and the branches become shorter and thicker. Some studies have shown that glial responses are transmitted to the contralateral retina through optic crossover in the damaged retina. In another way, it is suggested that inflammation in one eye can activate RGC fibers and cause inflammation in both superior colliculus, which, in turn, may activate inflammation in the other eye through retrograde transport of RGC fibers. Due to these controversies, more research and validation are needed to understand how signals from the damaged side are transmitted to the contralateral retina.

As mentioned earlier, minocycline, as a neuroprotective agent, can play a protective role for RGCs in many glaucoma models. In this study, microglial activation of crushed and contralateral eyes was inhibited after minocycline injection, and photoreceptor cells and bipolar cells responded to light strongly. In the contralateral eye, the number of RGCs increased significantly compared with the normal eye, indicating that minocycline protected secondary degeneration of contralateral retinal RGCs after ONC. So, dose this protective effect act directly on the damaged optic nerve by inhibiting microglia or by minocycline? Use of SC560 (a kind of known microglial activation inhibitor) to demonstrate that inhibition of microglial activation protects RGC has suggested that minocycline protects the retina by inhibiting microglial activation and proliferation.

In this study, the author observed the presence of primary and secondary degeneration. Microglia are activated in both the comminuted and contralateral eyes, although the operative eye is more activated than the contralateral one, minocycline has a more significant inhibitory effect on the contralateral eye microglia activation than the surgical eye, the protective effect of secondary degeneration of contralateral eye was more significant. In a rat model with partial transection of the optic nerve, minocycline is ineffective for primary degeneration but effective for secondary degeneration. Does minocycline protect against secondary degeneration rather than act? This may be related to the dosage used as well as (or) the degree and the type of injury. For example, in an ONC mouse model, the role of minocycline in surgical eyes was studied; the results showed that minocycline protected the RGCs of crushed eyes at days 4 and 7 post-ONC, with 45 mg/kg intraperitoneal injection given on the first day and 22.5mg/kg was subsequently given during the following days. While in my study design, this operation was changed to twice daily with 45 mg/kg dosage; low doses of minocycline appeared to provide protection in the model of retinal degeneration ischemia-reperfusion injury, and high doses of minocycline showed an injury effect. In an optic nerve transection model, low and high doses did not play a protective role, which had used minocycline according to the type and extent of injury and selecting the appropriate dose. The issue should be clarified more clearly when the multiple doses were used in future study.

Future directions

More insightful experimentation efforts should be exerted to help further explain the potential mechanisms on primary and secondary degeneration. In addition, more precise appropriate dosage of minocycline in neuroprotection should also be discussed, thereby helping the field to achieve a future treatment for RGCs.

Conclusion

In this study, the author found that minocycline can effectively protect the degeneration of RGCs which died from secondary degeneration in the contralateral eye, although it cannot prevent the primary degeneration of RGCs in the injured eye. This indirectly indicates that the mechanism of secondary degeneration and primary degeneration may be different. It is also possible that the dose used does not prevent more severe primary degeneration.

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Data sharing statement

No additional data are available.

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Conflict of interest

The author declares no conflict of interest.

References


A Novel Vitamin D Receptor Agonist, VS-105, Improves Bone Mineral Density without Affecting Serum Calcium in a Postmenopausal Osteoporosis Rat Model

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Abstract

Background and objectives: VS-105, a novel vitamin D receptor agonist with significantly less hypercalcemic side effects than calcitriol, is a useful tool to investigate whether or not a vitamin D receptor agonist at non-hypercalcemic doses could improve bone mineral density (BMD).

Methods: VS-105 and calcitriol were evaluated in an ovariectomized (OVX) osteoporosis rat model and in calvariae bone organ culture.

Results: Treatment of OVX rats by VS-105 (0.1, 0.2 or 0.5 µg/kg, intraperitoneal, 3x/week, for 90 days) significantly improved BMD in the L3 lumbar vertebra in a dose-dependent manner (sham vs. OVX/vehicle: 324 ± 14 vs. 279 ± 10 mg/cm²; VS-105 at 0.1, 0.2 and 0.5 µg/kg: 306 ± 9, 329 ± 12, and 327 ± 10 mg/cm², respectively) without affecting serum calcium (Ca). Calcitriol at 0.1 µg/kg significantly increased BMD but it also increased serum Ca. VS-105 and calcitriol at the test doses significantly suppressed serum parathyroid hormone and promoted tibia bone growth. With respect to biomarkers of bone remodeling, calcitriol and VS-105 both significantly elevated serum osteocalcin. In the calvariae bone organ culture, net Ca release was significantly less in VS-105-treated groups (vs. calcitriol).

Conclusions: VS-105 is efficacious in improving BMD in a dose range that does not affect serum Ca in OVX rats; the improvement in BMD by VS-105 is attributable to increased osteoblastic activity and reduced osteoclastic bone resorption.

Introduction

It is well documented that vitamin D is essential for bone health.1 Vitamin D₃, synthesized in the skin, is not active and needs to be converted to 25-hydroxyvitamin D₃ (25(OH)D₃), and then further hydroxylated by 1-alpha-hydroxylase CYP27B1 to form the active hormone, calcitriol (1,25(OH)₂D₃). Calcitriol, the active metabolite of vitamin D₃, is a secosteroid hormone that, by activating the vitamin D receptor (VDR), regulates a variety of physiological processes and functions in various cells and tissues,2,3 including bone formation, intestinal calcium (Ca) transport, and synthesis of parathyroid hormone (PTH).4–9

Nutritional vitamin D (the inactive precursor of calcitriol) supplementation for the prevention and/or treatment of osteoporosis is advocated by some proponents but the subject remains an area of considerable controversy. However, vitamin D receptor agonists (VDRAs), such as calcitriol, 1α-hydroxyvitamin D₃ (alfacalcidol), and eldecalcitol (ED-71, Edirol®), have been used as therapeutic agents for osteoporosis for many years in a number of countries, albeit not in the USA.10–12 These drugs increase bone mineral density (BMD) and reduce the incidence of bone fracture in patients with osteoporosis.10,11

Despite encouraging clinical experience with VDRAs’ benefits
for the bone, in the USA, they are mainly indicated for managing secondary hyperparathyroidism in chronic kidney disease. The fact that VDRAs are not more widely used for treating osteoporosis is in part due to the hypercalcemic side effects of the current VDRAs; calcitriol, alfacalcidol and also eldecalcitol are known to induce hypercalcemia at therapeutic doses. Data exist to support that calcitriol, alfacalcidol and eldecalcitol exert direct effects on the bone. At the same time, these compounds, given at therapeutic doses, raise serum Ca, which plays an important role in the bone remodeling process. Thus, it is important to investigate whether or not VDRAs could exert beneficial effects on BMD, independent of a change in serum Ca.

We have previously reported that VS-105, a novel VDRA, exhibits a significantly wider therapeutic index than calcitriol, alfacalcidol and paricalcitol, when comparing their efficacies on reducing serum PTH vs. hypercalcemic side effects in the 5/6 nephrectomized uremic rats. The mechanism(s) of action for the less hypercalcemic side effect of VS-105 is attributable to its reduced effect on stimulating intestinal Ca absorption and on releasing Ca from the bone. VS-105 has completed a Phase 1 clinical study involving healthy subjects (Clinicaltrials.gov #NCT03043482); the data show that VS-105 is well tolerated with no drug-related adverse events or other issues. In this report, to investigate whether or not a VDRA can improve BMD independently of raising serum Ca, we compared calcitriol and VS-105 in an OVX rat model of osteoporosis and also in the calvariae bone organ culture. The results suggest that VS-105, in a dose range that does not induce hypercalcemia, is efficacious in stimulating bone formation with reduced bone resorption, leading to increased BMD.

Materials and methods

Materials

VS-105 ((1R,3R)-5-((E)-2-((3αS,7αS)-1-((R)-1-((S)-3-hydroxy-2,3-dimethylbutoxy)ethyl)-7α-methylidencyclohexane-1,3-diol)) and calcitriol (1,25-dihydroxyvitamin D₃) were synthesized by Vidasym (Chicago, IL, USA). The synthesis scheme of VS-105 was published previously. All other reagents used were of analytical grade.

OVX rats

Female Sprague-Dawley rats at 8 months of age underwent a bilateral ovariectomy. Two weeks after the surgery, animals were administered vehicle (5% ethanol + 95% propylene glycol, 0.4 ml/kg), calcitriol, or VS-105 (doses as indicated), 3×/week, intraperitoneally, for 90 days (n = 8–12 per group). The dose range for VS-105 was chosen based on previous studies comparing its efficacy on suppressing serum PTH and its effect on affecting serum Ca. Untreated, age-matched sham rats served as controls. Blood samples were collected on day 0 (24 h before the first dose) and also on day 91 (24 h after the last dosing), and assayed for serum PTH, phosphate (P) and total Ca. At the end of the study, L3 lumbar vertebra and tibia bone samples were collected for further analyses (an approach similar to that used by Fu et al., Uchiyama et al. and Wang et al. for testing the effects of an agent on BMD). All animal studies were conducted under the auspices of the Office of Animal Care and Institutional Biosafety, University of Illinois at Chicago, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Per the NIH guidelines, the number of animals used in this study was according to the approved protocol to achieve a statistically significant group difference. The standard photoperiod for rodent rooms is 14 h of light and 10 h of darkness.

Details of animal grouping

Due to the difficulty in processing the bone samples from these rats, the study was staged in batches such that each batch had <30 rats and each batch always included sham and OVX-vehicle (as control) animals. All drug treatment groups were also represented in each batch. At the end of the studies, the data were compiled. The treatment groups and number of rats per group at the end of the study (a total of 69 rats) were: sham, n = 11; OVX-vehicle, n = 12; VS-105 at 0.1 µg/kg, n = 9; VS-105 at 0.2 µg/kg, n = 9; VS-105 at 0.5 µg/kg, n = 10; calcitriol at 0.02 µg/kg, n = 9; and calcitriol at 0.1 µg/kg, n = 9.

Measurements of physiological parameters

Serum PTH was measured using a rat intact PTH ELISA kit, obtained from Immutopics (San Clemente, CA, USA). Serum Ca was measured using the Stanbio LiquiColor Ca Assay Kit (Boerne, TX, USA). The serum Pi was determined using a Pi colorimetric assay (Catalog #K410-500; BioVision, Milpitas, CA, USA).

Three-dimensional computed microtomography analyses

Lumbar vertebra (L3) samples were fixed in 10% formalin for 3 days and then transferred to 70% alcohol. Three-dimensional computed microtomographic analyses of the L3 samples were performed with a 40 micro-CT (SCANCO Medical AG, Bassersdorf, Switzerland). The x-ray source voltage was 55 kVp, the source current was 145 µA, and the integration time was 300 ms. The scanning resolution was set at a 10-micron voxel size. The Scanco 40 micro-CT was calibrated using a method reported by Mashahtulla et al. A reconstruction of the bitmap dataset was used to build the 3-dimensional images. BMD from micro-CT was mean density of all voxels within the volume of interest. The analysis was conducted in a blinded manner, independently by a micro-CT technician who was not involved in the animal studies.

Bone growth assessment

Bone was fixed in 10% formalin for 3 days and then transferred to 70% alcohol. Three-dimensional computed microtomographic analyses of the L3 samples were performed with a 40 micro-CT (SCANCO Medical AG, Bassersdorf, Switzerland). The x-ray source voltage was 55 kVp, the source current was 145 µA, and the integration time was 300 ms. The scanning resolution was set at a 10-micron voxel size. The Scanco 40 micro-CT was calibrated using a method reported by Mashahtulla et al. A reconstruction of the bitmap dataset was used to build the 3-dimensional images. BMD from micro-CT was mean density of all voxels within the volume of interest. The analysis was conducted in a blinded manner, independently by a micro-CT technician who was not involved in the animal studies.
osteoblastic activity) was measured using an ELISA kit obtained from Immutopics.

**Bone resorption assessment by ex vivo calvariae culture**

The approach was as described in our previous publication. Briefly, 1 week-old mice (CD-1/ICR mice) were sacrificed and the semi-calvariae were removed and prepared for organ culture. Calvariae were incubated in DMEM containing 1 μM indomethacin, 15% heat-inactivated horse serum and 10 μg/mL heparin (resorption medium) overnight, and then transferred to fresh resorption medium without indomethacin and incubated with test agents (at $10^{-10}$–$10^{-7}$ M) for 4 days. The amount of Ca released into the medium was determined. Due to the difficulty in handling these samples, the ex vivo bone culture study was staged such that each batch had <20 samples and each batch always included the negative control (C, no addition of drug) and the positive control (calcitriol at $10^{-8}$ M). All drug treatment groups were also represented in each batch. At the end of these experiments, the data were compiled.

The treatment groups and the number of samples per group at the end of the study were: control (C, no addition of drug), $n = 20$; VS-105 at $10^{-10}$ M, $n = 4$; VS-105 at $10^{-9}$ M, $n = 4$; VS-105 at $10^{-8}$ M, $n = 8$; VS-105 at $10^{-7}$ M, $n = 4$; calcitriol at $10^{-10}$ M, $n = 4$; calcitriol at $10^{-9}$ M, $n = 10$; calcitriol at $10^{-8}$ M, $n = 4$.

**Statistical analysis**

Differences between sham and OVX rats with different treatments were assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test. A t-test with 95% confidence intervals of difference was used to assess differences between two groups. Statistical significance was defined as $p < 0.05$, with $p < 0.001$ indicating highly statistically significant.

**Results**

**Serum PTH, Pi and Ca in OVX rats**

The chemical structures of calcitriol and VS-105 are shown in Figure 1. Figure 2a shows that calcitriol at 0.02 μg/kg had no effect on serum Ca, but calcitriol at 0.1 μg/kg significantly raised the serum Ca level. As a comparison, VS-105 at the test doses did not have significant effects on serum Ca. Serum Ca trended slightly higher on day 91 for the VS-105 0.5 μg/kg group, but the difference (vs. pre-dosing) did not reach statistical significance. VS-105 and calcitriol produced significant suppression of serum PTH at all test doses (Fig. 2b). Both compounds exhibited no significant effect on serum Pi at all test doses (Fig. 2c).

**Micro-CT scanning of L3 vertebrae from OVX rats**

Figure 3a shows representative 3-D micro-CT scans; the quantitative results are summarized in Figure 3b–d. Compared to sham rats, BMD, bone volume/tissue volume and trabecular thickness were significantly reduced in the OVX rats treated with vehicle. Calcitriol at 0.02 μg/kg exhibited a modest effect but the hypercalcemic dose at 0.1 μg/kg produced a significant elevation in the three parameters above the sham level. In comparison, VS-105 improved the three parameters in a dose-dependent manner. When compared with the vehicle group, statistically significant improvement was observed for the VS-105 groups at doses of 0.2 and 0.5 μg/kg. When compared with the sham group, there were no significant differences observed between sham and VS-105 at all three doses.

**Tibia growth plate in OVX rats**

Figure 4a shows representative hematoxylin-eosin stained tibia. The quantitative results are summarized in Figure 4b. Compared to sham rats, the growth plate was significantly smaller in the OVX rats. Treatment with either VS-105 or calcitriol alone at all test doses resulted in significant restoration of the growth plate to the sham level. Tibia samples from the 0.2 μg/kg VS-105 group were collected but not processed since the results are unequivocal that VS-105 at 0.1 μg/kg, similar to the VS-105 0.5 μg/kg dose, already restored the growth plate to the sham level.

**Serum osteocalcin in OVX rats**

As shown in Figure 5, calcitriol at 0.1 μg/kg significantly increased the serum osteocalcin level but the 0.02 μg/kg dose of calcitriol produced no effect. Serum osteocalcin was significantly elevated by VS-105 in a dose-dependent manner.

**Bone resorption in ex vivo calvariae culture**

In the ex vivo calvariae culture (Fig. 6), the effect of calcitriol on Ca release reached a plateau at 1 nM, while VS-105 stimulated Ca release from the bone in a dose-dependent manner (vs. control – no drug). When comparing VS-105 and calcitriol at the same dose
such as 0.1 and 1 nM, it is evident that calcitriol induced significantly more Ca release. These data suggest that there was less bone resorption in the VS-105-treated samples.

Discussion

Calcitriol, alfalcaldiol and eldecalcitrol have been used as therapeutic agents for osteoporosis in several countries (albeit not in the USA) for many years. These drugs exert direct effects on the bone.\(^\text{18,19}\) For example, eldecalcitrol has been shown to possess a strong inhibitory effect on bone resorption.\(^\text{18,19}\) At the same time, these drugs also raise serum Ca.\(^\text{24,29,30}\) Ca is known to impact various factors (e.g., PTH and Pi) involved in the bone remodeling process via a complex yet tightly regulated system.\(^\text{31–33}\) Thus, to delineate the direct vs. indirect (via raising Ca) effect of a VDRA on the bone, VS-105, with its significantly wider therapeutic window than calcitriol, was chosen as a tool to investigate whether or not a VDRA can exert beneficial effects on BMD without raising serum Ca.

In the OVX rat model, our data show that serum Pi was not significantly altered either in vehicle- or drug-treated groups. Interestingly, calcitriol at the two test doses suppressed PTH to a similar level, yet the low calcitriol dose of 0.02 μg/kg exhibited no significant effect on BMD. In comparison, the three test doses of VS-105 suppressed PTH to a similar level, and VS-105 improved bone parameters in a dose-dependent manner. These results suggest that the efficacy of VDRAs on the bone is likely independent of their effects on serum phosphate and/or PTH in this OVX rat model.

Regarding BMD and serum Ca, calcitriol induced hypercalcaemia at 0.1 μg/kg but not at 0.02 μg/kg. Meanwhile, calcitriol showed no significant effect on BMD at 0.02 μg/kg, yet it increased BMD at 0.1 μg/kg to a level significantly higher than that observed in the sham rats. In comparison, VS-105 increased BMD in a dose-dependent manner to a level similar to that in the sham rats without affecting serum Ca. These data suggest that different VDRAs may exhibit differential effects on BMD and serum Ca. Seemingly, there is a correlation between serum Ca and BMD for calcitriol, but such a correlation is not observed for VS-105.

A word of caution should be added based on previous experiences with VDRAs. While VDRAs currently in clinical use for treating osteoporosis demonstrate consistent data when comparing animal studies and human trials, a lack of correlation between preclinical and clinical studies exists at least for one VDRA: 2MD.
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This compound restores various bone parameters, including BMD, to the sham level at non-hypercalcemic doses, and significantly raises BMD above the sham level at the hypercalcemic doses of 2.5–10 ng/kg/day in the OVX rat model. However, in the clinical studies, the drug failed to increase BMD in postmenopausal women with osteopenia. It is suggested that the lack of effect on

Fig. 3. Effects of VS-105 and calcitriol on L3 lumbar vertebra parameters in OVX rats. Rats were treated as described in the "Materials and Methods" (VS-105 at 0.1, 0.2, 0.5 μg/kg; calcitriol at 0.02, 0.1 μg/kg). Lumbar vertebra (L3) samples were fixed and three-dimensional computed microtomography analysis was conducted in a blinded manner. (a) Representative 3-D micro-CT scans of whole vertebra (L3) from each group in similar orientation. (b) BMD. (c) BV/TV. (d) Trabecular thickness. Differences among different treatments were assessed using a one-way ANOVA followed by a Dunnett's post hoc test. *p < 0.01, **p < 0.001 vs. vehicle (OVX-Veh). *p < 0.05, **p < 0.01, ***p < 0.001 vs. sham. BMD, bone mineral density; BV/TV, bone volume/tissue volume; OVX, ovariectomized; Veh, vehicle.

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BMD in the clinical studies is attributable to the dual activity of 2MD on stimulating bone formation and bone resorption. In this study, attempts were made to investigate how VS-105 affects bone resorption vs. bone formation. Osteocalcin (or bone gamma-carboxyglutamic acid-containing protein) is secreted by osteoblasts during the bone formation phase of the remodeling

Fig. 4. Effects of VS-105 and calcitriol on tibia growth plate thickness in OVX rats. Rats were treated and tibia sections were stained as described in the “Materials and Methods”. (a) Representative (hematoxylin-eosin-stained) tibia with the growth plate region (black arrow). (b) Analyzed data for tibia growth plate thickness (expressed as % of sham). Differences among different treatments were assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test. ###p < 0.001 vs. vehicle (OVX-Veh). ***p < 0.001 vs. sham. OVX, ovariectomized.

Fig. 5. Effects of VS-105 and calcitriol on serum osteocalcin in OVX rats. Rats were treated as described in the “Materials and Methods”. Blood samples were collected for the measurement of serum osteocalcin. Unpaired t-test with 95% confidence intervals of difference was performed to assess differences between baseline day 0 (before treatment: white bar) and day 91 (after treatment: black bar). *p < 0.05, **p < 0.01 vs. pre-dosing, same group. OVX, ovariectomized; Veh, vehicle.
It is worth noting that, for serum osteocalcin in the OVX rat model, no significant difference was observed between sham and the OVX rat treated with vehicle; although, serum osteocalcin trended higher in the OVX rats, which is consistent with previous reports such as that by Ma et al. However, Uchiyama et al. reported that serum osteocalcin was significantly increased in the OVX rat, and further increased by eldecalcitol (ED-71) at a hypercalcemic dose (0.2 μg/kg) but not at a non-hypercalcemic dose. Our data show that in the OVX rat, VS-105 increased the serum osteocalcin level in a dose-dependent manner without affecting serum Ca. In comparison, calcitriol significantly increased the serum osteocalcin level only at 0.1 μg/kg but had no effect at 0.02 μg/kg. In the ex vivo calvariae culture used to investigate bone resorption, significantly less Ca release was observed in the VS-105-treated groups.

Previously, we reported that calcitriol induces more Ca release from the bone than paricalcitol in the ex vivo calvariae culture. The data from the current study suggest that there is less Ca release and thus less bone resorption in the VS-105-treated samples.

Conclusions

In summary, in this report, we demonstrate that VDR activation by VS-105 improves bone parameters, including BMD, without causing hypercalcemia in the OVX rat model of osteoporosis. The improvement of BMD by VS-105 is attributable to increased osteoblastic activity and reduced osteoclastic bone resorption.

Future directions

Various vitamin D analogs with similar structural and biological characteristics have been shown to increase BMD and to improve bone strength in the OVX rat model of osteoporosis by their direct effects on osteoblasts and osteoclasts. However, in the clinical setting, different vitamin D analogs seem to perform differently, with at least one vitamin D analog not exhibiting efficacy in improving BMD in postmenopausal women with osteopenia after 1 year of treatment, albeit this compound was efficacious in the OVX rats. Thus, whether VS-105 is useful for the treatment of osteoporosis awaits the results from future clinical trials evaluating VS-105 in postmenopausal women with osteoporosis.

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Data sharing statement

All data used to support the findings of this study are included within the article.

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Conflict of interest

Chen YW, Chen T, Wessale JL and Wu-Wong JR work for Vidasym.

Author contributions

The present study was completed by the collaborative efforts of all authors. The manuscript was written by Wu-Wong and reviewed and approved by other authors.

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