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Modifier Genes as Therapeutics: The Nuclear Hormone Receptor Rev Erb Alpha (Nr1d1) Rescues Nr2e3 Associated Retinal Disease

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Abstract

Nuclear hormone receptors play a major role in many important biological processes. Most nuclear hormone receptors are ubiquitously expressed and regulate processes such as metabolism, circadian function, and development. They function in these processes to maintain homeostasis through modulation of transcriptional gene networks. In this study we evaluate the effectiveness of a nuclear hormone receptor gene to modulate retinal degeneration and restore the integrity of the retina. Currently, there are no effective treatment options for retinal degenerative diseases leading to progressive and irreversible blindness. In this study we demonstrate that the nuclear hormone receptor gene Nr1d1 (Rev-Erbα) rescues Nr2e3-associated retinal degeneration in the rd7 mouse, which lacks a functional Nr2e3 gene. Mutations in human NR2E3 are associated with several retinal degenerations including enhanced S cone syndrome and retinitis pigmentosa. The rd7 mouse, lacking Nr2e3, exhibits an increase in S cones and slow, progressive retinal degeneration. A traditional genetic mapping approach previously identified candidate modifier loci. Here, we demonstrate that in vivo delivery of the candidate modifier gene, Nr1d1 rescues Nr2e3 associated retinal degeneration. We observed clinical, histological, functional, and molecular restoration of the rd7 retina. Furthermore, we demonstrate that the mechanism of rescue at the molecular and functional level is through the re-regulation of key genes within the Nrs2e3-directed transcriptional network. Together, these findings reveal the potency of nuclear receptors as modulators of disease and specifically of NR1D1 as a novel therapeutic for retinal degenerations.

Introduction

Genetic heterogeneity (identical mutations with variable clinical presentation of disease) is a common feature for many Mendelian disorders [1]. While the underlying mechanisms causing such variations are case specific, it is clear that environmental factors, allelic heterogeneity, genetic modifiers, or a combination of these, can have a profound impact on disease expressivity. Genetic modifiers are allelic variants distinct from the disease-causing gene that can alter disease onset, progression or the clinical features for that particular disease [2]. Since genetic modifiers can result in either increasing or reducing disease severity, identification of modifier loci is important for understanding disease pathophysiology, predicting disease progression and developing novel therapeutic strategies. Modifier loci have been mapped for several diseases in both human and mouse, including cystic fibrosis, epilepsy, Huntington’s disease, hearing loss and retinal degeneration [3–14]. The availability of extensive genomic tools and multiple inbred strains of mouse models provide a unique platform to uncover genetic modifiers that strongly influence phenotypic variation in human disease [15]. As such, identification of these modifier genes also provides powerful and novel therapeutics.

While significant disease variability is observed for inherited retinal degenerative diseases, the underlying causes for such variations are largely undiscovered [13,16–19]. Mutations in the nuclear hormone receptor NR2E3, also known as photoreceptor-specific nuclear receptor (PNR), have been associated with several retinal diseases including enhanced S-cone syndrome (ESCS), Goldmann-Favre syndrome and retinitis pigmentosa [20–25]. NR2E3 functions as dual activator and suppressor of gene expression and, together with transcription factors such as NRL, CRX and NR1D1, modulates photoreceptor cell fate and
NR2E3 is also expressed in mature photoreceptors where it regulates expression of genes essential for proper function, such as phototransduction genes [31,32]. The NR2E3 protein contains four evolutionary conserved domains that are shared by the nuclear hormone receptor family; namely the highly variable A/B domain, N terminal DNA binding domain, a flexible hinge region and the ligand-binding and dimerization domain in the C terminus [33,34]. More than 30 disease-causing mutations have been identified in NR2E3, most of which are located within the DNA binding domain and the ligand-binding domain [20–25]. While most NR2E3 mutations have a recessive mode of inheritance, a c.166G>A (p.G56R) mutation in the NR2E3 gene is associated with autosomal dominant retinitis pigmentosa [25]. These data show significant phenotypic variation in patients with NR2E3 associated retinal degeneration. Interestingly, variable clinical presentation is observed even in patients harboring the same mutation and within the same family, suggesting that modifier genes modulate disease outcome in these patients [21,35,36].

The retinal degeneration 7 (rd7) mouse is a model for Nr2e3 associated retinal disease [37,38]. We utilize the Nr2e3rd7/rd7 mouse model to study the genetic heterogeneity observed in Nr2e3 associated retinal degeneration and to identify genetic modifiers that contribute to such variation. Mice homozygous for the rd7 mutation develop retinal dysplasia, with whorls and rosettes apparent at postnatal day 10 (P10) and retinal spots detectable by fundus examination at eye opening (P14) [37–39]. Similar to patients with Nr2e3 mutations, rd7 mice exhibit significant increase of S-cones and progressive degeneration of rod and cone photoreceptor cells [38]. Our previous studies demonstrated that the rd7 phenotype is highly variable depending on genetic background [40]. We observe complete penetrance in the B6.Cg-Nr2e3rd7/rd7 strain, while suppression occurs in crosses with the genetically divergent and inbred strains AKR/J, CAST/EiJ and NOD,NOG-H2d,1, revealing that modifier alleles are conferring resistance or susceptibility to the Nr2e3rd7 phenotypes [40]. In this study, we identified the nuclear hormone receptor Rev-erb alpha, hereafter referred to as Nr1d1, as a genetic modifier of Nr2e3rd7/rd7. We genetically fine mapped a locus on chromosome 11 linked to Nr2e3rd7/rd7 suppression in the AKR/J background and through sequence analysis, identified two strain-specific variants in the Nr1d1 gene within this locus. Delivery of the Nr1d1 gene to the retinas of B6.Cg-Nr2e3rd7/rd7 neonates rescues retinal spotting and retinal dysplasia associated with Nr2e3 loss, confirming that increased Nr1d1 expression is sufficient for suppressing rd7. Importantly, we show that Nr1d1 delivery results in re-regulation of key genes within the Nr2e3-directed network that are essential for proper photoreceptor function. Our findings uncover NR1D1 as a potential therapeutic target for Nr2e3 associated retinal degeneration that can compensate for Nr2e3 loss by regulating key molecular pathways associated with disease.

Results

Genetic Fine Mapping of rd7 Modifier Locus on AKR/J Chromosome 11

Our previous studies revealed that genetic background strongly influences penetrance of Nr2e3rd7/rd7 phenotypes [37,40]. Specifically, complete suppression of rd7 retinal degeneration was observed in outcrosses of B6.Cg-Nr2e3rd7/rd7 mice to AKR/J, CAST/EiJ or NOD,NOG-H2d,1 mice and several modifier loci that were unique for each strain were identified [40]. Two suggestive quantitative trait loci (QTL) located on chromosomes 7 and 11 were found to be associated with suppression in the AKR/J genetic background [40]. To determine if a single modifier gene is able to ameliorate rd7 associated retinal degeneration, we generated an incipient congenic strain that harbors the AKR/J modifier locus on chromosome 11, named Mor7 for modifier of rd7 by backcrossing F2 progeny from our B6.Cg-Nr2e3rd7/rd7 x AKR/J cross to the C57BL/6J inbred strain for six consecutive generations. Congenic animals carry the modifier loci from AKR/J on a C57BL/6J genetic background. Approximately 65% of the B6.Cg-Mor7/Nr2e3rd7/rd7 mice exhibit significant retinal spotting normally observed in B6.Cg-Nr2e3rd7/rd7 animals, compared to 49% of F2 animals from the initial intercross of B6.Cg-Nr2e3rd7/rd7 x AKR/J, suggesting a single modifier gene is sufficient for rd7 suppression. A genome wide analysis of the congenic F2 animals confirmed that approximately 95% of the B6.Cg-Mor7/Nr2e3rd7/rd7 genome harbor C57BL/6J alleles in the N6 generation. Two-thirds of the B6.Cg-Mor7/Nr2e3rd7/rd7 animals were heterozygotes across the Mor7 locus, indicating that the AKR/J Mor7 allele acts as a dominant protective allele. Consistent with our previous results, the suppressed B6.Cg-Mor7/Nr2e3rd7/rd7 mice harboring the modifier allele showed restored retinal morphology (Figures 1A and B) and normal expression of S-cone opsin (Omp15), compared to affected littermates harboring the susceptible allele (Figures 1C and D). Through our fine mapping analysis, we refined the Mor7 suppressor locus to a 3.3 cM region in chromosome 11. This region is flanked by markers D11Mit145 and D11Mit360 and contains approximately 200 genes.

Identification of Nr1d1 as a Genetic Modifier of rd7

We utilized a candidate approach to identify the Mor7 gene responsible for conferring rd7 suppression. Through rigorous in silico analysis using several publicly available resources [http://blast.ncbi.nlm.nih.gov], [http://www.ensembl.org/index.html], [http://pipeline.lbl.gov/cgi-bin/gateway2], [http://www.ncbi.nlm.nih.gov/geo/], we determined that 95 of the approximately 200 genes that lie within the Mor7 locus are expressed in the retina, 10 of which are transcription factors (Figure 1E). We hypothesized that the Mor7 modifier gene functions in the same or parallel pathway as Nr2e3. Three of the identified genes, thyroid hormone receptor alpha (Thralpha), retinoid acid receptor alpha (Rara) and reverb alpha (Nrl1d) are, like Nr2e3, members of the nuclear hormone receptor family. Given that several members of this family have been described as key regulators of retinal development and function, Thralpha, Rara and Nr1d1 were considered strong candidates for Mor7 and their coding as well as upstream regions were sequenced to identify allelic variants between C57BL/6J and AKR/J. While allelic variants were not found in either Thralpha or Rara, two single nucleotide polymorphisms (SNPs) were identified in Nr1d1 at homozygous state (Figure 2).

The SNPs identified in Nr1d1 are located in both the translated and un-translated regions of the gene. A non-synonymous SNP at position 1222hp was identified in Nr1d1, resulting in replacement of the consensus Arginine at position 408 by Glutamine in the AKR/J Nr1d1 protein (Figures 2A and B). This SNP is located within the highly conserved ligand-binding domain (LBD) of the NR1D1 nuclear hormone receptor. Specifically, the SNP lies within the binding domain for the NR1D1 co-repressor N-CoR, also known as X domain [41]. A second SNP was identified at position +105 (Figures 2C and D), within the Nr1d1 promoter region [42,43]. Specifically, the AKR/J genome harbors a thymidine at this position whereas a cytosine residue is found in C57BL/6J. Sequence alignment of the Nr1d1 gene across species revealed that T is the evolutionary conserved allele at this position (Figure 2B). As this SNP resides within the putative promoter
region of Nr1d1, we examined whether Nr1d1 mRNA expression varies in C57BL/6J versus AKR/J retinas. Quantitative real time PCR confirmed that Nr1d1 mRNA expression is upregulated by 3 fold in the AKR/J retina, compared to C57BL/6J (P = 0.0024, Figure 2E). This difference in expression may account for the differences observed in AKR/J vs B6. Thus, the increased levels of Nr1d1 in CAST/Eij and NOD-NOH-2<sup>rb1</sup> may contribute to rd7 suppression; however, the overall modification of the rd7 phenotype in those strains is likely due to the presence of other modifier genes.

**Nr1d1 Delivery Restores Retinal Integrity in rd7**

NR1D1 regulates many processes such as differentiation, metabolism, and the circadian rhythm [44]. Recently, our studies and those of others have demonstrated a role for NR1D1 in the retina. NR1D1 forms a complex with NR2E3, CRX and NRL, key transcriptional regulators of retinal development and function [26]. Importantly, NR1D1 binds the NR2E3 protein directly and acts synergistically to regulate transcription of photoreceptor-specific genes [26]. Further, our work identified a number of genes co-regulated by NR2E3 and NR1D1 in the developing and adult retina [43]. Thus, Nr1d1 is a strong candidate to modify the effects of N<sup>2</sup>e3-associated retinal degeneration.

In vivo electroporation was performed to deliver Nr1d1 alleles from either C57BL/6J or AKR/J into the retina of neonatal rd7 mice and determine whether NR1D1 can modulate rd7 associated retinal degeneration. The vector used has GFP to detect expression at the site of delivery. One month after delivery, GFP expression was present in both the outer nuclear layer, composed of the cell bodies of rod and cone photoreceptors, and in the inner nuclear layer of the retina (Figure 3). One month after injection, animals were examined clinically by indirect ophthalmoscopy for detection of the characteristic rd7 pan-retinal spotting. While spotting of the fundus was clearly observable in the eyes electroporated with the control GFP vector at P30.5, electroporation of GFP.Nr2e3<sup>rb6</sup> resulted in suppression of the phenotype (Figures 4C and D). Delivery of either GFP.Nr1d1<sup>rb6</sup> (B6 allele, without the LBD SNP) or GFP.Nr1d1<sup>AKR/3</sup> (AKR/J allele, with the LBD SNP) also resulted in rescue of the pan-retinal spotting phenotype (Figures 4E and F). Further, the absence of retinal spotting correlated with absence of retinal dysplasia in histological sections (Figures 4G-J). A subset of the electroporated animals were aged to 4 months and electroretinograms (ERGs) were performed to examine visual function. Significant improvements were observed in both scotopic (dark-adapted) and photopic (light-adapted) ERG response were observed in B6.Cg-Nr1d1<sup>rb7/rd7</sup> eyes injected with GFP.Nr1d1<sup>AKR/3</sup>, compared to GFP injected eyes (Figures 4K and L). These studies demonstrate that the dosage of Nr1d1 is sufficient for rescue of N<sup>2</sup>e3-associated retinal disease irrespective of the allelic variant in the ligand-binding domain; thus the promoter SNP is likely the protective allele mediating rescue of disease.

**Nr1d1 delivery results in the molecular rescue of rd7 misregulated genes**

NR1D1, a regulator of circadian clock metabolism, also functions as a cofactor of NR2E3 and regulates key transcriptional regulators of retinal development and function [32]. Thus, Nr1d1 is sufficient for rescue of N<sup>2</sup>e3-associated retinal disease irrespective of the allelic variant in the ligand-binding domain; thus the promoter SNP is likely the protective allele mediating rescue of disease.
retina of untreated rd7 animals (left eye, n = 3) compared to C57BL/6J, consistent with previous reports (p = 0.004, Figure 5). GFP.Nr1d1AKR/J delivery to the right eye of the same rd7 animals resulted in a significant reduction in Opn1sw expression (p = 0.035, Figure 5). Furthermore, Opn1sw levels in GFP.Nr1d1AKR/J injected eyes were not significantly different from those present in wild-type C57BL/6J retinas (p = 0.86), indicating that Nr1d1 delivery rescues Opn1sw expression to near normal levels.

Figures 2A-F show strain specific alleles and differential expression of Nr1d1. (A) C57BL/6J and AKR/J chromatograms of polymorphisms identified in the ligand-binding domain of Nr1d1. (B) ClustalW2 sequence alignment of amino acid sequences from C57BL/6J, AKR/J, rat, chimpanzee and human. Stars indicate identity in all sequences, while dots indicate conserved amino acids. (C) C57BL/6J and AKR/J chromatograms of polymorphisms identified in the Nr1d1 5' UTR region. (D) ClustalW2 sequence alignment across species reveals the consensus is in accordance with AKR/J sequence. Stars indicate nucleotide conservation in all species. (E) Nr1d1 relative expression in P30.5 AKR/J and C57BL/6J retinas (mean ± SD of mean, n = 3, p = 0.0024). (F) Nr1d1 relative expression in P30.5 C57BL/6J, CAST/EiJ and NOD.NOH-H2nb1 retinas (p<0.05).

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**Discussion**

Photoreceptor biogenesis and homeostasis are directed by key transcription factors that modulate expression of gene networks in an ordered fashion both during development and in the mature retina. Moreover, specific combinations of those transcription factors to regulatory regions are an important mechanism of suppressing Nr2e3 associated retinal degeneration by redirecting the biological networks that modulate photoreceptor development and function.
tightly regulated and are a major determinant of circadian gene expression program [47,48]. In the retina, both \(N2e3\) and \(Nr1d1\) mRNA levels oscillate over a 24-hour period [43]. The retina contains an intrinsic circadian clock that regulates many aspects of retinal physiology [49]. More than 2,500 retinal genes have been identified to have circadian expression, including key photoreceptor genes regulated by \(N2e3\) and \(Nr1d1\) [50]. Therefore, it is likely for changes in \(Nr1d1\) concentration to result in altered photoreceptor gene expression. We show that delivery of \(Nr1d1\) to the \(rd7\) retina re-regulates two key genes required for proper retinal function: \(Opn1sw\) and \(Gnat2\). Both \(Opn1sw\) and \(Gnat2\) function in the phototransduction process whereby the retina converts light to an electrical stimulus and as such are essential to vision. We chose to examine these two genes in rescued eyes because they are significantly misregulated in \(rd7\) animals and are known direct targets of \(NR2E3\). Our data strongly suggest that \(NR1D1\)-mediated regulation of key gene networks disrupted by \(NR2E3\) loss contributes to rescue of retinal integrity and function in \(rd7\) animals.

Our study illustrates that modifier genes capable of modulating a disease state provide viable therapeutic options with broad applicability. We provide evidence that rescue of disease can be achieved by delivering a modifier gene rather than replacing the disease-causing gene. Gene therapy clinical trials have resulted in tremendous success for treating patients with Leber’s congenital amaurosis (LCA), an inherited retinal disease [51–55]. These studies demonstrated the safety and efficacy of gene transfer through adeno-associated viral (AAV) delivery and have led to great advancements towards the use of gene therapy in the clinic. We predict that exploiting modifier genes as candidates for gene therapy may significantly broaden the therapeutic potential of AAV to treat retinal diseases and other diseases altered by genetic modifiers.

In summary, this study demonstrates that modifier genes play an integral role in disease presentation and as such can be used as powerful tools for gene therapy that can alter both disease progression and outcome. Future studies will focus on exploring the applicability of using \(Nr1d1\) gene delivery for treating \(N2e3\)-associated retinal degeneration at advanced stages of disease, as well as retinal disease with other genetic causes. Specifically, this therapeutic approach has a powerful mechanism to treat diseases caused by mutations in different genes that converge on specific nodes or pathways within a common signaling network and as such has a much broader impact than single gene replacement therapy. Further, novel drugs and therapeutics distinct from gene therapy can be developed to exploit the use of genetic modifiers in the clinic.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use and procedures were approved by the University of Nebraska Medical Center Animal Care and Use Committee and the Schepens Eye Research Institute Animal Care and Use Committee (Permit Number: S302-0614) in compliance with the Animal Welfare Act Regulations. All efforts were made to minimize animal suffering.

Animal Maintenance

Animals were housed in vivariums at the Schepens Eye Research Institute and the Nebraska Medical Center. C57BL/6J and AKR/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine.
Nr1d1 Prevents Nr2e3 Associated Retinal Disease
Figure 4. Gene delivery of Nr1d1 suppresses pan-retinal spotting, retinal dysplasia and function in Nr2e3<sup>rd7/</sup>rd7 mice. (A–F) Fundus photographs of control and rd7 injected retinas: (A) B6 (uninjected), (B) rd7 (uninjected), (C) GFP injected, (D) GFP.Nr1d1<sup>B6</sup> injected, (E) GFP.Nr1d1<sup>AKR/J</sup> injected, (F) GFP.Nr1d1<sup>B6</sup> injected. (G–J) DAPI staining (blue) shows rescue of defects in retinal morphology 30 days after electroporation into rd7 neonatal retinas. (G) GFP control, (H) Nr2e3<sup>rd7/</sup>rd7 injected, (I) Nr1d1<sup>AKR/J</sup> injected, (J) No injection to the left eye served as a negative control. 1 m. (K, L) Representative scotopic (K) and photopic (L) electroretinograms from animals 4 month after injection with GFP (blue) or GFP.Nr1d1<sup>AKR/J</sup> (red).

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Harbor, ME. B6.Cg-Nr2e3<sup>rd7/</sup>rd7 has been previously described [40]. B6.Cg-Mor</sub>Nr2e3<sup>rd7/</sup>rd7 mice were generated by out-crossing B6.Cg-Nr2e3<sup>rd7/</sup>rd7 × AKR/J F<sub>2</sub> mice to C57BL/6J, followed by backcrossing of the F<sub>2</sub> progeny to C57BL/6J for six consecutive generations. Genotyping for the Nr2e3<sup>rd7/</sup>rd7 mutation was performed as previously described [38].

Construction of Expression Vectors
cDNA from C57BL/6J or AKR/J mice was used to amplify the Nr1d1<sup>B6</sup>, Nr1d1<sup>AKR/J</sup> and Nr2e3 alleles with the following primers: Nr1d1<sup>F</sup>: TTTTTAAGCTTCATCACACTCCAGGATGTTGT GTC, R: TTTTTAAGCTTCAGCCCGGAAGGACAGCA<sub>and</sub> Nr2e3<sup>F</sup>: TTTTTAAGCTTCAGCCGGAAGGAGCACCGACC<sub>and</sub> R: TTTTTAAGCTTCACATCCATTAGACC. Amplified sequences spanning the whole coding sequence were cloned into the pAcGFP1-N1 plasmid from Clontech. All plasmids were confirmed by direct sequencing using vector and gene-specific primers.

In vivo Electroporation
Nr1d1 allele specific constructs (designated as GFP.Nr1d1<sup>B6</sup> and GFP.Nr1d1<sup>AKR/J</sup>) were delivered by subretinal injection into the right eye of P<sub>0.5</sub>d animals using the electroporation method developed by Matsuda et al. [56]. The Nr2e3 allele from C57B6L/J (GFP.Nr2e3<sup>B6</sup>) was electroporated into rd7 animals as a positive control, while empty GFP expression vector or no injection to the left eye served as a negative control. 1 μg of naked DNA was injected subretinally, followed by electroporation immediately with tweezer electrodes at five 80 V pulses of 50 μs duration, with 950 ms intervals, using a square wave electroporator.

Clinical Examination
Animals were examined by indirect ophthalmology at P30 as previously described [40]. Briefly, pupils of animals were dilated with 1% Atropine and a Keeler Vantage indirect ophthalmoscope with a 60-diopter lens was used for fundus examinations.

Quantitative Real Time PCR
Gene expression analysis was performed using quantitative RT-PCR as previously described [32]. In brief, retinas were dissected rapidly after eye enucleation and placed in Trizol (Life Technologies, Carlsbad, CA) for RNA extraction. Eyes were consistently collected in the early afternoon for each animal in order to eliminate variability due to circadian expression. Two micrograms of total RNA was reverse transcribed using Retroscript (Ambion, Austin, TX). Real-time PCR was performed in technical triplicates with a minimum of three biological replicates using SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). The following primer were used: Nr1d1<sup>F</sup>: CCGGCTCAGCGGTCA- TAATGGA, R: GTTGGCTTGGC GTAGACTGTTT; Opn1<sub>sw</sub><sup>F</sup>: ACCCTCTAACAATGGGTGTTGA, R: GCTGCCGAAGG- GTTTACAGA; Gnat2<sup>F</sup>: CAGGCTGAGCCGAGTACAG, R: CAGGCTGACTTCTCAGCGAGCA; Nr1d1<sup>B6</sup><sup>F</sup>: CCAGCTGGACCGGATTACAG, R: ATGCC- TCCCCTACCAATCTTC, R: CAGGCTGACTTCTCAGCGAGCA. Reactions were quantified using a Roche 480 LightCycler real time PCR instrument. Relative expression levels were normalized to the amount of β-Actin expressed and fold change relative to wild-type C57BL/6J control was calculated using the delta Ct method. Standard error was calculated to determine statistical significance.

Statistical Analysis
Statistical analysis for Figures 2 and 4 was performed using the two-tailed Student's t test, with significance defined as P<0.05. At minimum 3 biological replicates were included in the each experiment.

Electroretinography
Electroretinogram analysis was performed on 7 mice of each strain (4 month-old), as described previously [40]. Mice were anesthetized with an intraperitoneal injection of a saline carrier (10 mg/g body weight) containing ketamine (1 mg/mL) and xylazine (0.4 mg/mL). Mice were dark adapted for at least six hours and then anesthetized prior to recording. Dark-adapted
The data shown were acquired for each stimulus condition with up to 50 records for the strongest signals.

References


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Author Contributions

Conceived and designed the experiments: NBH. Performed the experiments: NBH. Analyzed the data: NBH. Contributed reagents/materials/analysis tools: RB UK MMD PE. Wrote the paper: NBH.


