

# Potential Therapeutic Agents Against Retinal Diseases Caused by Aberrant Metabolism of Retinoids

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The retinoid (visual) cycle is a complex enzymatic pathway that operates in the retina for the regeneration of 11-*cis*-retinal (11-*cis*-Ral), the inherent visual chromophore indispensable for vision. Deficiencies in the retinoid metabolism are involved in pathologic mechanisms of several forms of retinal diseases including age-related macular degeneration, Stargardt's disease, and Leber's congenital amaurosis, for which no effective cures presently exist. Nevertheless, the interference of abnormal retinoid metabolism with chemicals has been considered to be a promising strategy aimed at alleviating these retinal dysfunctions. Moreover, since gene therapy is gaining increasing importance in clinical practice, the modulation of key enzymes implicated with the retinoid cycle at a genetic level will hold great promise for the treatment of patients with degenerative diseases of the retina.

Keywords: retinoid visual cycle, retinoids, metabolism, visual chromophores, photoreceptor, gene therapy, retinylamine, RPE65, retinal pigment epithelium

Perception of light is extremely important in visual function or vision. Photoisomerization of opsin-bound visual chromophore 11-*cis*-retinal (11-*cis*-Ral) to all-*trans*-retinal (ATRAL) in vertebrate rod photoreceptors triggers visual phototransduction events. The 11-*cis*-Ral regeneration, a fundamental step in restoring bleached visual pigment rhodopsin (Rh) to its dark-adapted state, is a critical process called the retinoid (visual) cycle leading to vision. The retinoid cycle takes place in the rod outer segments (ROS) and the retinal pigment epithelium (RPE). All-*trans*-retinol (ATROL), also known as vitamin A, serves as a key metabolite of the retinoid metabolism. It forms from the reduction of ATRAL with retinol dehydrogenases (RDHs) in the cytoplasm of the ROS, and is then transferred to the RPE where it is ultimately converted back to 11-*cis*-Ral.<sup>1</sup> Furthermore, food intake is an additional source of vitamin A in the visual cycle.

Disruption of the retinoid metabolism is correlated with the etiology and pathogenesis of several forms of retinal diseases, including age-related macular degeneration (AMD), Stargardt's disease (STGD), Leber's congenital amaurosis (LCA), retinitis pigmentosa (RP), congenital stationary night blindness, fundus albipunctatus, cone-rod dystrophy (CRD), and retinitis punctata albescens.<sup>2-4</sup> The retinoid cycle arrest will lead to an insufficient supply of visual chromophores for phototransduc-

tion, or an excess buildup of toxic retinoid-derived by-products in the RPE.<sup>3,4</sup> Hence, it may be feasible to treat retinal disorders by mediating the visual cycle with the purpose of sustaining the retinoid metabolism balance.

Replacement therapy is one of the strategies for preventing the degeneration of the retina. Indeed, supplementation of vitamin A and its derivatives has proved effective in animal models<sup>5-9</sup> and human patients<sup>10,11</sup> with retinal degenerative diseases. In addition, chemical<sup>5,12-16</sup> and genetic<sup>17-19</sup> methods have been developed to treat retinal degeneration by affecting functional enzymes in the retinoid cycle, some of which have been under the inspection of clinical trials.<sup>20-25</sup> However, these pharmaceutical approaches still need to be assessed in terms of pharmacokinetics and pharmacodynamics before clinical significance is confirmed. Here we provide a review of retinal diseases related to aberrant metabolism of retinoids and summarize recent advances in the development of their therapeutic agents.

## THE METABOLISM OF RETINOIDS IN THE RETINA

The metabolism of retinoids in the retina is essential for vision. The retinoid (visual) cycle is a complex enzymatic pathway for retinoids to metabolize and function continuously within the





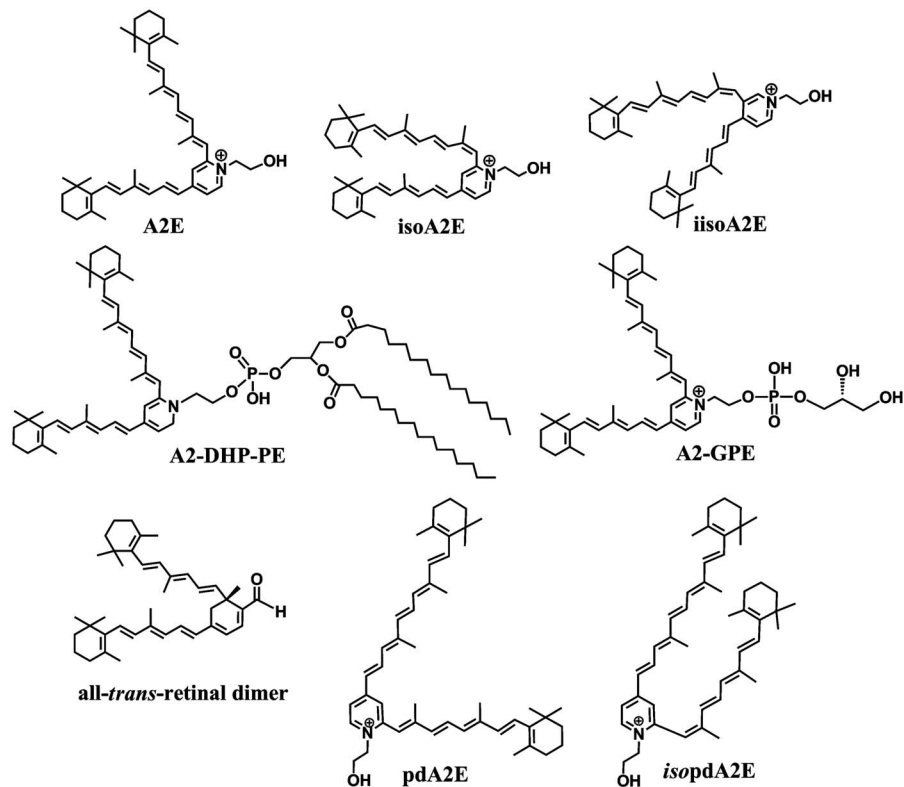


FIGURE 2. Retinal-derived lipofuscin compounds associated with AMD and STGD.

in the RPE can bind 11-*cis*-Rol to speed up the isomerization by regulating the reaction equilibrium.<sup>3,28,43</sup> 11-*cis*-Rol, after binding to CRALBP, is oxidized by retinol dehydrogenase 5 (RDH5) to form 11-*cis*-Ral.<sup>28</sup> The latter can be then transported through IPM with the aid of IRBP, and move back into the ROS disk lumen where a fresh dark-adapted Rh forms, followed by initiation of a new retinoid cycle. In addition to its fate in converting into 11-*cis*-Ral, 11-*cis*-Rol can also be esterified by LRAT to form 11-*cis* retinyl esters, which can be stably stored and are convenient for use when chromophore supplementation is needed.<sup>28,44,45</sup>

Alternatively, a portion of free ATRal that evades reduction will react with phosphatidylethanolamine (PE) in the ROS disk lumen, thus resulting in the formation of all-*trans* *N*-retinylidene-phosphatidylethanolamine (all-*trans* NR-PE).<sup>32,46,47</sup> It should be mentioned here that this is a readily reversible reaction in vivo, producing an equilibrium mixture of all-*trans* NR-PE and free ATRal in the ROS disk lumen.<sup>29</sup> The acidic pH of the ROS disk lumen can trap the protonated form of all-*trans* NR-PE in the lumen leaflet of the disc membranes<sup>48,49</sup> and thus, all-*trans* NR-PE cannot cross the membrane by itself.<sup>36</sup> ABCA4 transporter is important to decrease the amassment of all-*trans* NR-PE in the ROS disk lumen by flipping all-*trans* NR-PE from the disk membrane to the photoreceptor cytoplasmic side.<sup>48,49</sup> With the assistance of this “inward” flippase all-*trans* NR-PE is capable of being dissociated into ATRal and PE in the cytoplasm where ATRal will be further reduced to ATRol by RDH8, and thus the retinoid cycle can continue without excess buildup of all-*trans* NR-PE.<sup>32,49,50</sup>

Loss or decrease in ABCA4 activity results in the formation of a series of retinal-derived lipofuscin adducts (Fig. 2). The latter compounds form in the ROS and accumulate in the RPE with time via phagocytosis, thereby causing damage to both photoreceptors and RPE.<sup>28,36</sup> In all of these pigments, A2E

and its isomers, all-*trans*-retinal dimer, A2-DHP-PE, A2-GPE, and pdA2E and its isomer, have been isolated and identified structurally.<sup>32,46,51–58</sup> Because of their relatively high physiological significance, the in vitro cytotoxicity and phototoxicity of A2E have been extensively investigated.<sup>59–64</sup> It is of note that retinal-derived lipofuscin fluorophores accumulate to a less extent in eyes of wild-type mice than *Abca4* gene knockout mice.<sup>53,59</sup> Although ATRal is thought to play a leading role in the formation of RPE lipofuscin,<sup>34</sup> Boyer et al.<sup>65</sup> demonstrate that, in the absence of light exposure, the primary source of lipofuscin deposits is 11-*cis*-Ral rather than ATRal. 11-*cis*-Ral, like ATRal, is highly toxic owing to its highly reactive aldehyde moiety, and it will positively undergo detoxification by either reduction to retinol or sequestration within retinal-binding proteins in vivo. In addition to all-*trans* NR-PE, ABCA4 is also responsible for the translocation of 11-*cis* NR-PE, the Schiff-base conjugate of 11-*cis*-Ral and PE, from the lumen to the cytoplasmic leaflet of disk membranes. The transport function of ABCA4—together with chemical isomerization of 11-*cis*-Ral to ATRal, which is followed by reduction to ATRol via RDHs—can prevent the buildup of excess 11-*cis*-Ral and 11-*cis* NR-PE as well as the formation of toxic retinal-derived products as found in ABCA4-deficient mice and individuals with STGD.<sup>66</sup>

Previous reports<sup>3,18,29,43,67,68</sup> indicate that the onset and progression of some ocular diseases are definitely related to aberrant metabolism of retinoids in the retina (Table 1). Strategies that can directly fix the dysfunction of retinoid metabolism include retinoid replacement, retinoid cycle regulation, and genetic interference. Yet, it should be borne in mind that heterogeneity is strikingly common in retinal diseases, and the final ocular status is likely attributed to more than one abnormal participant in the retinoid cycle.<sup>18</sup>

TABLE 1. Human Retinal Diseases Related to Aberrant Metabolism of Retinoids

Relative Proteins	Physiological Functions	Associated Eye Diseases
ABCA4	It flips NR-PE out to the cytoplasm, thus promoting ATRal clearance in the outer segment disk lumen	Stargardt's disease Fundus flavimaculatus Age-related macular degeneration Retinitis pigmentosa Cone-rod dystrophy
RDH12	It serves as one of the ATRol dehydrogenases other than RDH8	Leber's congenital amaurosis Retinitis pigmentosa
LRAT	It carries out the esterification of ATRal for storage and further isomerization, and esterifies 11- <i>cis</i> -Ral for storage	Leber's congenital amaurosis Retinitis pigmentosa Congenital retinal blindness Hereditary epithelial aplasia Retinal aplasia
RPE65	It isomerizes all- <i>trans</i> -retinyl esters to 11- <i>cis</i> -Rol	Leber's congenital amaurosis Retinitis pigmentosa
RGR	It is likely to be associated with the phototransduction signaling cascade	Retinitis pigmentosa Choroidal sclerosis
CRALBP	It binds to 11- <i>cis</i> -Rol, and promotes the RPE65-mediated isomerization process	Retinitis pigmentosa Retinitis punctata albescens Bothnia dystrophy Newfoundland rod-cone dystrophy
RDH5	It acts as the major 11- <i>cis</i> -Rol dehydrogenase	Fundus albipunctatus Cone dystrophy
MERTK	It is required for the phagocytosis of photoreceptor outer segments by the RPE	Retinitis pigmentosa Cone-rod dystrophy
Rh	It is crucial for phototransduction	Retinitis pigmentosa Congenital stationary night blindness

RGR, retinal G-protein-coupled receptor.

## POTENTIAL THERAPEUTIC AGENTS FOR TREATING ABERRANT METABOLISM OF RETINOIDS

### Retinoid Replacement Therapy

11-*cis*-Ral chromophore is an indispensable participant for phototransduction (Fig. 1) and accordingly, a continuously adequate supply of 11-*cis*-Ral is required to produce visual pigments, maintain vision, and preserve photoreceptor function.<sup>69,70</sup> Lack of 11-*cis*-Ral can ultimately lead to complete loss of vision.<sup>11</sup> The abnormality in either biosynthesis or regeneration of the retinoids can cause a deficiency of 11-*cis*-Ral in the retina. Since the function of LRAT and RPE65 in the visual cycle is of utmost concern to replenish 11-*cis*-Ral, it is not surprising that mutations of LRAT and RPE65 are detected among hereditary retinal dystrophies such as LCA and RP.

Although only approximately 10% of LCA patients<sup>10,18,71</sup> and less than 5% of RP patients<sup>18</sup> have mutations in LRAT or RPE65, replacement therapy with the retinoids (Table 2) is still beneficial for these specific patients. Direct supplementation of 11-*cis*-Ral in *Rpe65* gene knockout (*Rpe65*<sup>-/-</sup>) mice has shown improved photoreceptor function.<sup>72</sup> Nevertheless, 11-*cis*-Ral is vulnerable, which has led researchers to discover 9-*cis*-Ral as a more effective candidate.<sup>73,74</sup> Indeed, 9-*cis*-Ral can be readily synthesized and has the ability to bind with opsin to form 9-*cis* Rh, which will trigger the phototransduction signaling cascade as well when activated by light.<sup>73-75</sup> However, the photoreceptor sensitivity of 9-*cis* Rh will be 3-fold lower than that of control Rh containing 11-*cis*-Ral chromophore because of the 3-fold lower quantum efficiency of 9-*cis* Rh than of 11-*cis* Rh<sup>76</sup> and the unavoidable consequence even after full regeneration of the pigment with 9-*cis*-Ral. Despite that, Gearhart and coworkers<sup>77</sup> have demonstrated that 9-*cis*-Ral can improve visual performance in *Rpe65*<sup>-/-</sup> dogs. Moreover, a series of 9-*cis*-retinoids, including

9-*cis*-retinyl acetate, 9-*cis*-retinal succinate, 9-*cis*-retinal palmitate, and 9-*cis*-retinol, are also studied for their therapeutic effects in several animal models characterized by retinoid deficiency. The data indicate that 9-*cis*-retinyl acetate exhibits better stability and lower reactivity, making it suitable for oral administration.<sup>3,5</sup> QLT091001, a synthetic 9-*cis*-retinyl acetate that can be converted into 9-*cis*-retinal in the vertebrate body, is the most anticipated candidate for replacement therapy and is undergoing clinical trials for retinoid deficiency-caused retinal disorders, such as LCA with LRAT or RPE65 mutations.<sup>10</sup> Furthermore, 9-*cis*- $\beta$ -carotene, a precursor of 9-*cis*-retinoids, is an additional promising replacement agent. Oral 9-*cis*- $\beta$ -carotene has been proved quite effective in patients with fundus albipunctatus<sup>78</sup> and RP,<sup>79</sup> and it exhibits no adverse effects in humans owing to its long history as an over-the-counter medication. However, patient heterogeneity may impede uniform efficacy by oral 9-*cis*- $\beta$ -carotene, and the long duration (2–3 months) of drug administration also poses a practical problem for its wider use. Accordingly, future treatments will focus on 9-*cis*- $\beta$ -carotene-based restoration in combination with other approaches that can enhance the survival of impaired photoreceptors. Although the teratogenicity of retinoid replacement therapy has been proposed because of its possible effect on nuclear retinoic acid receptor (RAR)/retinoid x receptor (RXR), no similar adverse effects are present in mouse models<sup>3</sup> or clinical trials.<sup>10</sup> Considering that the subjects of clinical trials are mostly young and early-onset LCA patients, follow-up observation and safety evaluation should be further evaluated.

### Chemically Synthesized Regulators of the Retinoid Metabolism

The foregoing description has indicated that the etiology of some retinal diseases is associated with the retinoid deficiency.



**TABLE 2.** Replacement Therapy in Humans and Laboratory Animals With Retinal Dystrophies Characterized by the Retinoid Deficiency

Replacement Agents	References	
	Animal Models	Human Trials
9- <i>cis</i> -retinal	Van Hooser et al. 2000. <sup>73</sup> Van Hooser et al. 2002. <sup>74</sup> Gearhart et al. 2010. <sup>77</sup>	-
9- <i>cis</i> -retinyl acetate	Batten et al. 2005. <sup>149</sup> Maeda et al. 2009. <sup>5</sup> Maeda et al. 2013. <sup>181</sup>	Koenekoop et al. 2014. <sup>10</sup> NCT00765427 NCT01999764 For LCA and RP, NCT01014052 For LCA and RP, NCT01521793 For RP, NCT01543906
9- <i>cis</i> -retinyl succinate	Batten et al. 2005. <sup>149</sup>	-
9- <i>cis</i> -retinyl palmitate	Batten et al. 2005. <sup>149</sup>	-
9- <i>cis</i> -retinol	Batten et al. 2005. <sup>149</sup>	-
11- <i>cis</i> -Ral	Ablonczy et al. 2002. <sup>72</sup>	-
ATRol	-	Gamel et al. 1993. <sup>182</sup> For RP, NCT00000114 For RP, NCT00065455 For RP, NCT00000116
9- <i>cis</i> - $\beta$ -carotene	Maeda et al. 2011. <sup>183</sup>	Rotenstreich et al. 2010. <sup>78</sup> Rotenstreich et al. 2013. <sup>79</sup> For RP, NCT01256697 For RP, NCT02018692

Conversely, excess accumulation of retinoids or their metabolites as RPE lipofuscin can also be toxic to normal vision function. Increased levels of deleterious lipofuscin due to the disruption of retinoid clearance are pathologic in retinal degenerative diseases such as STGD, RP, AMD, Best vitelliform macular dystrophy, and a subset of CRDs.<sup>3,46,47</sup> A2E, the most studied retinal-derived component of RPE lipofuscin, has been shown to induce pathologic changes in RPE cells, including oxidative stress,<sup>80</sup> mitochondrial apoptosis,<sup>81,82</sup> membrane damage,<sup>59,61</sup> increased photosensitivity,<sup>63,64,82</sup> reduced mitochondrial membrane potential,<sup>83</sup> diminished function of lysosomal enzymes<sup>60</sup> and phagocytosis,<sup>85</sup> increased level of interleukin-1 $\beta$ <sup>62</sup> and VEGF,<sup>84</sup> accumulated advanced glycation endproducts,<sup>85</sup> and RPE65 isomerase inhibition.<sup>86</sup> It is well-known that damage to RPE results in direct degeneration of photoreceptors.<sup>80</sup> Controversy is mounting as to whether a major factor contributing to the death of RPE cells, particularly in the macula, is A2E accumulation. Ablonczy et al.<sup>87,88</sup> and Grey et al.<sup>89</sup> have shown a lack of correlation between the spatial distribution of A2E and lipofuscin fluorescence in the human RPE. A possible explanation for the study disputing the significance of A2E in humans is that the lipofuscin autofluorescence, which was formerly believed to be associated with retinopathy progression,<sup>90,91</sup> is probably a relevant but not causal indicator.<sup>92-94</sup> By comparison with free ATRal, A2E is less cytotoxic and phototoxic to human RPE cells, suggesting that the production of A2E may decrease ATRal toxicity and serves as a protective mechanism to prevent ATRal-caused retinal damage.<sup>95</sup> While it is clear that there are levels of A2E

that are tolerated by RPE cells, intracellular concentrations can be reached above which A2E is certainly damaging to the cells.

Ten regulating agents with different structures (Fig. 3) and therapeutic effects (Table 3) have been experimentally reported to treat retinal diseases characterized by abnormal retinoid metabolism.<sup>15</sup> Although all of these agents manifest inhibitory effects on lipofuscin buildup, a resultant lack of chromophores that comes with slowing of the retinoid cycle could induce ocular problems including insensitive daylight vision, delayed dark adaptation, and night blindness.<sup>3,13</sup> 13-*cis*-retinoic acid, also known as isotretinoin or acutane, is a common drug used for the treatment of acne.<sup>96</sup> However, some patients under isotretinoin treatment develop impaired nocturnal visual sensitivity and diurnal glare sensitivity,<sup>97,98</sup> suggesting that isotretinoin may affect the retinoid cycle to some extent. Studies on the amelioration of retinal dysfunction in laboratory animals reveal that isotretinoin impairs rod function rather than inducing rod death, and causes a slowdown of chromophore recovery after photobleaching,<sup>7</sup> indicative of the inhibitory effect of isotretinoin on the retinoid cycle. Since STGD is a typical hereditary retinal disease characterized by the inability of retinoids to be cleared from photoreceptors and RPE cells, isotretinoin displays a significant inhibitory effect on lipofuscin accumulation in the STGD mouse model.<sup>6</sup> The mechanism underlying isotretinoin-mediated decrease in the levels of RPE lipofuscin may be its ability to inhibit RDH5.<sup>99,100</sup> Besides, evidence from *in vivo* experiments indicates that isotretinoin likely has a role in inhibiting specific isomerization of all-*trans*-retinyl esters to 11-*cis*-Rol by interfering with RPE65 activity<sup>6,101</sup> or all-*trans*-RDHs such as RDH8.<sup>13</sup> Clinical trials such as NCT01445028 and NCT02149615 have investigated the effect of isotretinoin on nerve fiber layer<sup>102,103</sup> and proliferative vitreoretinopathy (PVR),<sup>104</sup> suggestive of the ability of isotretinoin to compromise retinal nerve fiber layer thickness, thereby potentially improving the rate of retinal reattachment in PVR patients. A clinical study of 11 patients has shown that oral isotretinoin treatment can cause ocular side effects and does not improve vision, although it may slow visual acuity loss in AMD patients, characterized by occult subfoveal choroidal neovascularization.<sup>105</sup>

*N*-(4-hydroxyphenyl)retinamide, also known as fenretinide or 4-HPR, has been used as a chemotherapeutic agent to treat cancer.<sup>3</sup> It has also been discovered that fenretinide can affect the serum vitamin A level.<sup>106,107</sup> Physiologically, vitamin A from the diet will bind to retinoid-binding protein (RBP) in the liver and stay in blood after the complex is bound to transthyretin (TTR), because the latter complex will be large enough to avoid glomerular filtration.<sup>108,109</sup> Treatment with fenretinide in laboratory animals, however, will displace vitamin A from RBP, and the newly formed complex will not bind to TTR, thus reducing the levels of retinoids and RBP in serum by rapid elimination of the RBP-fenretinide complex in urine, and inhibiting the chromophore biosynthesis within the retina.<sup>110</sup> In the STGD animal model, fenretinide effectively decreases lipofuscin accumulation by reducing vitamin A in serum.<sup>110</sup> The actual mechanism of fenretinide-mediated decrease of lipofuscin levels has been questioned. Some researchers point out that the retina, with strong ability to store retinoids, is highly resistant to peripheral vitamin A deprivation,<sup>4</sup> whereas others claim that *RBP*<sup>-/-</sup> mice display a significant ocular phenotype.<sup>111</sup> Since Rh regeneration is still preserved in *IRBP* gene knockout mice,<sup>112,113</sup> RBP is also likely to be present in the IPM area,<sup>3</sup> thereby revealing its function under certain pathologic circumstances. Furthermore, Golczak and coworkers<sup>13</sup> argue against the role of RBP binding in the regulatory effect of fenretinide. On the other hand, the mechanism of action for chemopreventive activity of fenreti-

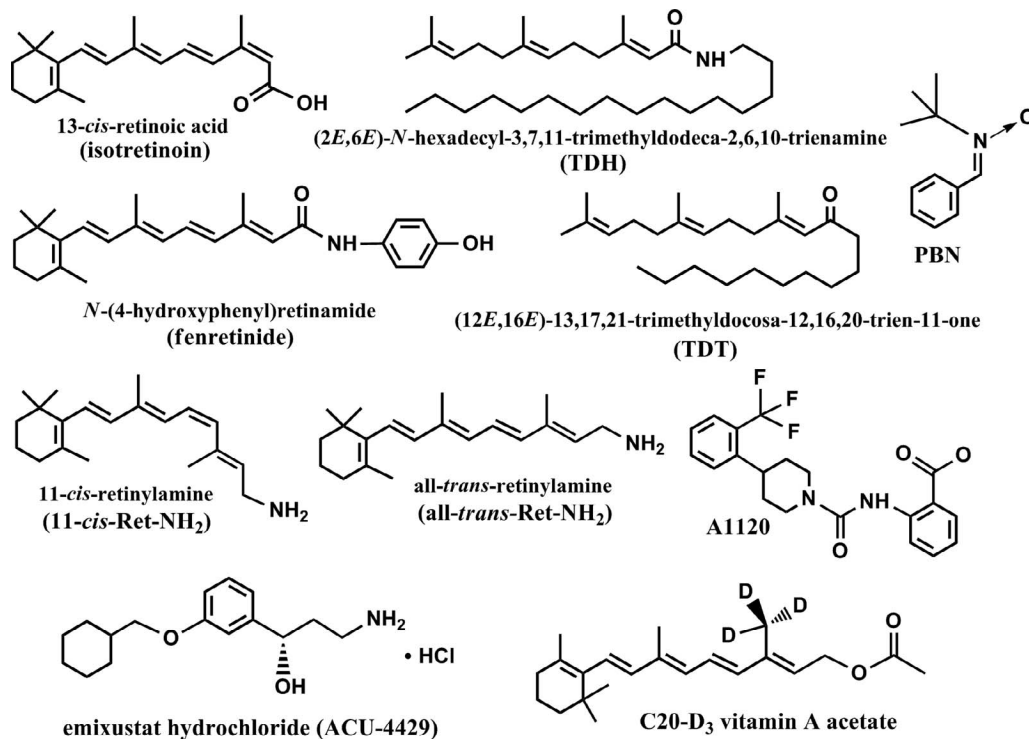


FIGURE 3. Structures of 10 regulators of abnormal retinoid metabolism.

nide is unrelated to its function as a RBP4 ligand but seems to be associated with its ability to generate reactive oxygen species and induce apoptosis in malignant cells.<sup>114</sup> Despite the ambiguous mechanism of fenretinide in the retina, a phase II clinical trial with a 2-year observation shows decelerated lesion growth and later onset of choroidal neovascularization in AMD patients.<sup>115</sup> In addition, as a traditional drug for systemic use, fenretinide is thought to have less teratogenicity than isotretinoin<sup>3,110</sup> and exhibits no clinical signs of systematic vitamin A loss except for a delayed dark adaptability.<sup>116,117</sup>

Retinylamine, also known as Ret-NH<sub>2</sub>, is a positively charged primary amine-containing retinoid. It has the ability to inhibit chromophore regeneration and rod function

recovery,<sup>12</sup> probably by suppression of the RPE65-mediated isomerization process or blockage of retinal-derived by-product accumulation by directly binding excess free ATRal.<sup>13</sup> Retinylamine can acquire exact structures as 11-*cis*-Ret-NH<sub>2</sub>, 9-*cis*-Ret-NH<sub>2</sub>, 13-*cis*-Ret-NH<sub>2</sub> and all-*trans*-Ret-NH<sub>2</sub>, among which 11-*cis*-Ret-NH<sub>2</sub> exerts the strongest inhibition on the RPE65-induced isomerization process.<sup>12</sup> Compared to isotretinoin and fenretinide, Ret-NH<sub>2</sub> displays greater and more prolonged inhibitory effect on chromophore biosynthesis, as well as less influence on RAR/RXR activation<sup>13,118</sup> and cone function.<sup>12</sup> Being a substrate also for LRAT, Ret-NH<sub>2</sub> undergoes *N*-amidation to be stored in the liver or RPE retinosomes for further release,<sup>4,12</sup> but the resulting amidated metabolites exhibit a weaker effect

TABLE 3. Mechanism and Clinical Trials of Synthesized Regulators Against Aberrant Retinoid Metabolism

Regulating Agents	Potential Targets	Influence on the Levels of Retinoids in the Retina					Human Trials on Retinal Diseases
		11- <i>cis</i> -Ral	11- <i>cis</i> -Rol	11- <i>cis</i> -Retinyl Ester	All- <i>trans</i> -Retinyl Ester	Bisretinoids	
Isotretinoin	RDH5, RPE65, RDH8	↓↓	↑	↑↑	↑↑	↓↓	For PVR, NCT01445028
Fenretinide	RBP	↓↓	↓	UK	UK	↓↓	For AMD, NCT00429936
Ret-NH <sub>2</sub>	RPE65, free ATRal	↓↓	↓↓	↔	↑↑	↓↓	-
TDT	RPE65	↓↓	↓	↔	↑↑	↓↓	-
TDH	RPE65	↓↓	↓	↔	↑↑	↓↓	-
A1120	RBP	↓↓	↓	↓	↓	↓↓	-
PBN	RPE65	↓↓	↓↓	↑	↑	↓↓	-
ACU-4429	RPE65	↓↓	↓↓	↑	↑	↓↓	For AMD, NCT01002950 For AMD, NCT01802866 For AMD, NCT02130531
C20-D <sub>3</sub> -vitamin A acetate	NR-PE					↓↓	NCT02402660 NCT02230228

UK, unknown; ↑, increased; ↑↑, significantly increased; ↓, decreased; ↓↓, significantly decreased; ↔, unchanged.

on the retinoid cycle.<sup>12</sup> Interestingly, Ret-NH<sub>2</sub> can be oxidized into retinol and later be esterified into retinyl esters by LRAT, and then stored in retinosomes in vivo.<sup>119</sup> As for the free ATRal-binding mechanism, a therapeutic study using more than 20 Food and Drug Administration-approved drugs with primary amine groups indicates that primary amines could protect against retinal degeneration without any evidence of inhibition of the retinoid cycle enzymes.<sup>120</sup> Rather, the formation of a reversible Schiff base from ATRal and primary amines decelerates the accumulation of retinal-derived lipofuscin while slowly releasing ATRal into the retinoid cycle to alleviate adverse effects such as delayed dark adaptation.<sup>13,120</sup> Although no clinical trials with Ret-NH<sub>2</sub> have been performed, advances have been made to synthesize and evaluate Ret-NH<sub>2</sub> analogues with a strong RPE65 inhibition activity, a high ATRal-binding ability, and a superior LRAT affinity.<sup>14</sup> Chemical modifications of Ret-NH<sub>2</sub> have been carried out for future drug development. Zhang et al.<sup>14</sup> demonstrate that the conformation of the  $\beta$ -ionone ring is a critical structural feature for LRAT substrate recognition. Indeed, replacements within the  $\beta$ -ionone ring, together with elongation of the double-bond conjugations and a variety of substitutions of the C9 methyl, do not abolish the LRAT-mediated acylation, thereby revealing broad substrate specificity for LRAT. As for the inhibition of RPE65 enzymatic activity, an altered  $\beta$ -ionone ring structure, characterized by the absence of methyl groups and the presence of one bulky group at the C9 position, will weaken its inhibition effect on RPE65. Polyethylene glycol<sup>121</sup> and polylactic acid nanoparticle technologies<sup>122</sup> are also being explored to enhance the pharmacokinetic properties of Ret-NH<sub>2</sub>. Then, Ret-NH<sub>2</sub> could be a more promising candidate for clinical use in retinal degenerative diseases, with higher solubility in water, lower level of accumulative toxicity in liver, prolonged duration of retina protection, and a convenient way of drug administration, without strong RAR/RXR activation or severe ocular adverse effects. In addition to STGD and AMD, the application of Ret-NH<sub>2</sub> has also been expanded to treat early diabetic retinopathy (DR) in mice because photoreceptors have been identified as major contributors to the vascular damage in DR.<sup>123</sup>

Two farnesyl-containing isoprenoids, TDT and TDH, exert inhibitory effects on the biosynthesis of visual chromophores to approximately the same extent as isotretinoin, but these two chemicals exhibit more persistent inhibitory effects on the chromophore biosynthesis and a remarkable specificity to block RPE65 activity.<sup>124</sup> Evidence has indicated the role of the farnesyl moiety as a posttranslational modification device in the phototransduction cascade.<sup>125-127</sup> A1120 has previously been designed to treat diabetes mellitus. As a nonretinoid RBP antagonist, A1120 shares similar effect and mechanism with fenretinide, but it demonstrates more potent activity and less RAR/RXR activation.<sup>128,129</sup> Although A1120 effectively diminishes lipofuscin buildup, this adduct, unlike other retinoid metabolism regulators, does not lead to a significant delay of rod function after photobleaching.<sup>128</sup> Such a phenomenon indicates that A1120 may possess the ability to ameliorate ocular adverse effects such as night blindness and delayed dark adaptation. To enhance the RBP4-binding affinity and metabolic stability, chemical modifications of A1120 have drawn much attention.<sup>130-132</sup> Using A1120 as a template, bicyclic-octahydrocyclopenta[c]-pyrrolo analogues have been synthesized that display more favorable RBP-binding potency and inhibition effects on lipofuscin formation.<sup>131,132</sup>  $\alpha$ -Phenyl-*N*-tert-butyl nitron (PBN), a free radical spin trap, may have the capacity to inhibit RPE65 activity, and to protect photoreceptor cells by means of free radical scavenging and *c-fos* suppression.<sup>15</sup> Although a delayed recovery of rod response function is observed in laboratory animals treated with PBN, the cone

visual cycle is not significantly affected, such that the extent of night blindness after PBN treatment is supposed to be slight.<sup>15</sup>

ACU-4429, often in the form of emixustat hydrochloride tablet, is also a small-molecule nonretinoid modulator of the visual cycle that was initially found to serve as an inhibitor of RPE65.<sup>16</sup> (R)-isomer of ACU-4429 shows higher affinity toward RPE65 than its (S)-isomer.<sup>133</sup> Compared to Ret-NH<sub>2</sub>, ACU-4429 possesses stronger inhibitory activity of 11-*cis*-Rol production and could lead to prolonged blockage of the visual pigment regeneration in vivo. Similar to Ret-NH<sub>2</sub>, ACU-4429 is able to sequester ATRal, and the tendency of ACU-4429 to form a Schiff base with ATRal seems to be stronger than that of Ret-NH<sub>2</sub>. Efficient amidation upon incubation of ACU-4429 with bovine RPE microsomes indicates that tissue uptake of the drug can be facilitated by LRAT enzymatic activity, whereas component analysis of an incubation mixture of eye extracts from mice in the presence of ACU-4429 gives rise to a predominantly free form of ACU-4429. Moreover, antiangiogenic properties of ACU-4429 have also been observed in animal models of retinal neovascularization.<sup>134</sup> In clinical trials, safety, tolerability, and effect of oral ACU-4429 on healthy subjects (phase I) have been followed up since 2008. Single-dose (2-75 mg) or multidose (5-40 mg, 14 days) applications manifest a dose-dependent effect of ACU-4429 in rod function inhibition.<sup>16</sup> Ocular side effects such as dyschromatopsia or nyctalopia occur in more than 50% of healthy subjects but are mostly resolved after 2 weeks. And yet, a long-time observation should not be underestimated.<sup>135</sup> Phase II/III clinical trials of ACU-4429 are still under way. In this regard, the latest report shows that oral administration of emixustat hydrochloride tablet (2-10 mg) for 90 days may benefit AMD patients with geographic atrophy lesions.<sup>136</sup> C20-D<sub>3</sub>-vitamin A, a form of deuterated vitamin A, can retard the retinoid cycle without impairing rod function and dark adaptation. Since the rate-determining step in vitamin A dimerization is the cleavage of a C20 carbon-hydrogen bond of the NR-PE Schiff base,<sup>137</sup> replacement of three C20-H bonds with C20-D bonds will result in a primary kinetic isotope effect slowing the formation of A2E and all-*trans*-retinal dimer.<sup>137</sup> Studies carried out in wild-type mice<sup>137</sup> and STGD1 mice<sup>138,139</sup> demonstrate that, when C20-D<sub>3</sub>-vitamin A acts as the sole source of vitamin A intake, there is a greater than 50% decrease in the formation of cytotoxic bisretinoids and a significant reduction of fundus autofluorescence.

## Gene Therapy

Table 1 gives reason to consider gene therapy as a direct modification method for the expression of key enzymes and other functional proteins in the retinoid cycle. Since several retinal diseases, such as hereditary macular degeneration or AMD, are associated with or caused by certain genetic mutations, the repair of responsible genes is a promising approach to strike a normal balance back in retinal environments. Replacement of defective genes and suppression of abnormal mutants are possible ways to cure retinal diseases genetically. It is noted that 95% of STGD, 30% of CRD, and 8% of autosomal recessive RP patients have *ABCA4* mutations,<sup>140</sup> and 6% of LCA patients have *RPE65* mutation.<sup>141</sup> The genetic heterogeneity in the whole population makes gene therapy costly for clinical trials and use. Nevertheless, the same genetic mutations will not all develop into clinical syndromes, which makes it challenging for the application of gene therapy as a preventive measure in progressive eye diseases. Despite the complicated pathologic situation, studies of gene therapy in retinal diseases have been advancing owing to preliminary success of gene augmentation therapy in LCA caused by the *RPE65* mutation.



TABLE 4. Gene Therapies That Repair Aberrant Metabolism of Retinoids for Retinal Dysfunctions

Targeted Gene	Retinal Disease	References		Information on Clinical Trials		
		Animal Model	Human Trial	Trial Number	Phase	Intervention
<i>Rpe65</i>	LCA	147-159	160-162, 164-173	NCT00821340	I	rAAV2-hRPE65
				NCT01496040	I/II	rAAV2/4.hRPE65
				NCT00481546	I	rAAV2-CBSB-hRPE65
				NCT00749957	I/II	rAAV2-CB-hRPE65
				NCT00999609	III	AAV2-hRPE65v2
				NCT00643747	I/II	tgAAG76 (rAAV2/2.hRPE65p.hRPE65)
				NCT00516477	I	AAV2-hRPE65v2
				NCT01208389	I/II	AAV2-hRPE65v2
<i>Abca4</i>	STGD	176,177	-	NCT01367444	I/II	EIAV-derived lentiviral-ABCA4
				NCT01736592	I/II	
<i>MERTK</i>	RP	178-181	-	NCT01482195	I	rAAV2-VMD2-hMERTK

rAAV, recombinant adeno-associated virus; EIAV, equine infectious anemia virus.

Early onset of visual impairment occurs in LCA patients. Gene-based ocular therapy has found application in the treatment of RPE65-related LCA, commonly categorized as LCA2. The genetic augmentation therapy has been tested for its safety and effect in dogs,<sup>142-148</sup> mice,<sup>73,148-152</sup> and primates<sup>153</sup> before starting clinical trials in humans. The protective effect of *Rpe65* gene augmentation has even lasted for 3 years in a canine model.<sup>144</sup> Clinical trials on LCA patients with *RPE65* mutation have been advancing to phase III (Table 4). Importantly, the short-time (less than 12 months) observation shows no local or systemic adverse effects after subretinal injection,<sup>20</sup> and visual function appears to be improved<sup>21, 22</sup> by *Rpe65* gene augmentation. It is also suggested that earlier use of gene therapy results in better visual improvement,<sup>23</sup> probably because a significant decrease of plasticity in the connection between retina and central nervous system is detected in children older than 3 years.<sup>18</sup> Although visual function can be ameliorated, retinal degeneration cannot be retarded by *Rpe65* gene therapy.<sup>154</sup> In addition, prolonged observations of up to 3 years still achieve visual improvement after single-dose treatment with *Rpe65* gene augmentation,<sup>24,25,155-157</sup> but recent studies that observe treated patients for up to 6 years show that improvements in retinal sensitivity are only evident for the first 3 years, peaking at 6 to 12 months after treatment, and then decline.<sup>158,159</sup> Therefore, re-administration of *Rpe65* gene should be considered for persistent therapeutic effects on LCA patients.<sup>160</sup>

Stargardt's disease is another retinal disease with no presently effective treatment. Most of the STGD cases are characterized by juvenile onset and autosomal recessive heredity.<sup>18,161</sup> The autosomal recessive STGD, namely STGD1, is thought to be the most common hereditary macular dystrophy.<sup>162</sup> It was identified by Allikmets et al.<sup>163</sup> who claim that STGD1 is caused by *ABCA4* mutation and is considered to be a monogenic retinal disease. The retinoid metabolism indicates that *ABCA4* is responsible for clearing NR-PE in photoreceptor outer segments, thus blocking lipofuscin formation in the RPE and photoreceptor. Since *ABCA4* is present in rod and cone photoreceptors,<sup>162</sup> different phenotypes of STGD1 can be detected in clinic and in animal models.<sup>140</sup> *Abca4* gene knockout (*Abca4*<sup>-/-</sup>) mice, an animal model of STGD1, have shown phenotype corrections after *Abca4* gene augmentation by lentivirus<sup>164</sup> or nanoparticle<sup>165</sup> vectors. Phase I/II clinical trials for gene-based treatment of STGD1 have initiated<sup>140</sup> but reports are not available yet. Retinitis pigmentosa is a retinal disease that predominantly influences rod function<sup>18</sup> and is associated with more than 60 gene mutations, such as *MERTK* gene mutation. *MERTK* (c-mer protooncogene receptor tyrosine kinase) is required for the

phagocytosis of photoreceptor outer segments by the RPE, and gene transfer of *MERTK* by a virus vector is effective in Royal College of Surgeons (RCS) rats with *MERTK* deficiency by repairing retinal functions and structures.<sup>166-169</sup> Some promising gene therapies that repair aberrant retinoid metabolism in retinal diseases are shown in Table 4, but many preclinical studies must still be performed to discover more potential gene targets. *Rb* gene-related RP has long been studied in the preclinical phase for the feasibility of gene therapy. Despite this common method to replace deficient genes by augmentation,<sup>170</sup> several lines of investigations in Rh-related RP have opted to suppress mutant genes by gene-silencing technology.<sup>171-180</sup>

## CONCLUSIONS

In this review, we have mainly focused on aberrant metabolism of retinoids within the retina and potential agents used for its interference. Today, replacement therapy with vitamin A derivatives is still a developing field with ongoing clinical trials. 9-*cis*-retinyl acetate, also known by its drug name QLT091001, is likely to be a future candidate for LCA treatment. Replacement therapy is merely recommended to be used for retinoid deficiency diseases, and conversely, for retinal diseases caused by abnormal retinoid accumulation, inhibitors of retinoid biosynthesis should be considered. Regulating agents of aberrant retinoid metabolism, which were not discovered and well studied until 2000, are classified into retinoid and nonretinoid compounds. Retinoid compounds include isotretinoin, fenretinide, retinylamine, and C20-D<sub>3</sub>-vitamin A; the retinoid structure allows them to mimic physiological retinoids in the visual cycle without triggering real functions, thus inhibiting the normal retinoid metabolism. But similar to vitamin A derivatives used for replacement therapy, these retinoid-resembling drugs have the potential ability to activate nuclear receptor RAR/RXR, which will affect cell proliferation and cellular functions. Among all these retinoid metabolism modulators, retinylamine and C20-D<sub>3</sub>-vitamin A may be thought of as the most promising agents owing to more effective inhibition activity and less adverse effects. Development of deuterated vitamin A likely solves a long-term problem regarding how to balance the contradiction between inhibiting formation of bisretinoids and retarding the function of photoreceptors. On the other hand, it is proposed that nonretinoid regulating agents would prevent the occurrence of systemic adverse effects by RAR/RXR activation. Although the efficiency of nonretinoid regulating agents has



yet to be confirmed, modifications in chemical structure may broaden their biological significance.

Yet, both retinoid or nonretinoid regulating agents will cause relative deficiency of chromophores in the visual cycle except perhaps for deuterated vitamin A, because their targets are not specific to certain pathologic mechanisms. To tackle the phenomenon, gene therapy is envisioned and is considered to be a promising strategy to alleviate nonspecific and systemic adverse effects induced by the foregoing two categories of therapeutic agents. The challenge with using gene therapy agents remains the presence of genetic and phenotype heterogeneity in retinal diseases, which will hinder the universal use of gene therapy for one type of ocular disease. Moreover, gene repair requires direct contact with targeted structures, and therefore intraocular methods should be applied to treat retinal diseases genetically; it is quite clear that this is far more complicated and risky than taking drugs orally. However, with the advancement in both gene screening technology and exploration of more suitable strategies, we believe that existing obstacles will be removed, and gene/drug therapies will gain greater practical significance.

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