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(71) Applicant: KATAIRO GMBH [DE/DE]; Lederstr. 21,
72127 Kusterdingen (DE).

(72) Inventor: SCHRAERMEYER, Ulrich; Burgackerstrasse
1, 72379 Hechingen (DE).

(74) Agent: KÖNIG SZYNKA TILMANN VON
RENESE PATENTANWÄLTE PARTNERSCHAFT
MBB DÜSSELDORF; Mönchenwerther Straße 11, 40545
Düsseldorf (DE).

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(54) Title: REMOFUSCIN (SORAPRAZAN) FOR USE AGAINST LYSOSOMAL DYSFUNCTION AND AGING

(57) Abstract: The invention relates to remofuscin for use against aging, lysosomal dysfunction, for use in stimulating the expression of lysosomal lipase genes and/or lysosomal lipid chaperone genes, and for use in increasing the expression levels of the genes involved in xenobiotic detoxification through nuclear hormone receptors.



"Remofuscin (soraprazan) for use against lysosomal dysfunction and aging"

FIELD OF THE INVENTION

The invention relates to new uses of remofuscin.

BACKGROUND OF THE INVENTION

Remofuscin/soraprazan is a ROS generator, specifically a superoxide generator when illuminated with light as described in EP3855181. ROS can degrade Lipofuscin following intravitreal injection with generators of oxygen radicals such as superoxide ($O_2^{\bullet-}$), peroxy ($OO^{\bullet}$), and hydroperoxy ($HOO^{\bullet}$), in both monkeys and Abca4 - mice.

In the *C. Elegans* model ROS has been shown to induce enhanced autophagy and decreased lifespan in *sin-3* deletion mutants (Meenakshi et al., *Autophagy*. 2018; 14(7): 1239–1255).

Further, in albino mice lipofuscin degradation has been minimal, suggesting that melanin is required for degradation. Melanin is not present in many organs affected by aging.

The object of the invention is to provide new uses of remofuscin, including a medical or non-medical use to extend lifespan or improve lysosomal dysfunction.

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DESCRIPTION OF THE INVENTION

The above-mentioned problem is solved by the invention according to the appended claims. In particular, the problem is solved by using remofuscin for pharmaceutical treatment of aging, or by using remofuscin for non-medical anti aging treatment, cosmetic treatment, or as a lifestyle drug or dietary supplement.

Surprisingly, the inventors have found that the administration of remofuscin can extend lifespan and equally surprisingly reduces ROS.

Specifically, the inventors have found that systemic administration of remofuscin shows a significant anti-aging effect, i.e. remofuscin can be applied systemically with a positive net effect on lifespan. This effect surprisingly appears to require no melanin or light, and is stronger than detrimental effects that are mediated by the remofuscin ROS chemistry on aging.

According to a further aspect of the invention remofuscin can extend lifespan by stimulating the expression of lysosomal lipase genes and/or lysosomal lipid chaperone genes, subsequently increasing the expression levels of the genes involved in xenobiotic detoxification through nuclear hormone receptors and thereby extending lifespan.

According to another aspect of the invention remofuscin can shift energy metabolism from glucose to lipid oxidation, resulting in the upregulation of beta-oxidation genes and reduces the production of ROS, thus contributing to lifespan extension.

In the dietary restriction (DR) state, the lipid catabolism rate increases to supply energy, and lipid catabolism generates lipid metabolites that mediates lipotoxicity.

According to another aspect of the invention remofuscin is used against lysosomal dysfunction.

The disclosure of the invention is not limited to the compound remofuscin but also applies to tetrahydropyridoethers in general.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Effect of remofuscin on the lifespan of *C. elegans* (N2). Worms at the L4 stage (day 1 of the adult stage) fed on the *E. coli* OP50 lawn were treated with various

concentrations of remofuscin. The survival rates were calculated using the Kaplan-Meier method. * $p < 0.05$, *** $p < 0.001$, log-rank test, compared with the NC.

Fig. 2. Effect of remofuscin on aging biomarkers in *C. elegans* (N2). Three-day-old (day 1 of the adult stage) *C. elegans* fed on an *E. coli* OP50 lawn were transferred onto fresh mNGM plates containing *E. coli* OP50 and various concentrations of remofuscin, and the body lengths of 10 worms from each group were measured (A). The pumping rate in the terminal bulb was measured for 1 min every 48 h from day 4 to day 10, and the mean rate of 15 worms from each group was determined (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t-test, compared with the NC.

Fig. 3. Accumulation of lipofuscin in remofuscin-treated *C. elegans* (N2). (A) Images of lipofuscin fluorescence in worms treated with various concentrations of remofuscin on day 14 (scale bar = 100 μm). (B) The lipofuscin fluorescence in the worms was quantified using ImageJ software. Ten worms from each group were used for the measurements. * $p < 0.05$, *** $p < 0.001$, Student's t-test, compared with the NC.

Fig. 4. ROS levels in remofuscin-treated *C. elegans* (N2). The relative ROS levels were measured after 14 days of treatment with remofuscin. The fluorescence signal in each group (more than 80 worms for each measurement) was normalized to the protein concentration in the group. ** $p < 0.01$, Student's t-test, compared with the NC.

Fig. 5. Differential gene expression between the negative control and 200 μM remofuscin-treated groups as determined by RNA microarray analysis. DEG analysis software (ExDEGA v.1.6.8, Ebiogen, KOR) was used to display the gene expression data as a volcano plot (A). The genes in the boxes are related to xenobiotic detoxification shown in Table 2 (fold change ≥ 2.0 and $p < 0.05$). The DAVID functional annotation tool was used to analyze the gene ontology terms (B), and the biological processes associated with the DEGs revealed by microarray analysis are shown as a pie chart (C).

Fig. 6. Expression levels of genes related to xenobiotic metabolism, lysosomes, and NHRs in remofuscin-treated *C. elegans*. Worms were grown on NGM plates containing 0 μM and 200 μM remofuscin for 1 and 5 days, and the gene expression levels were measured by qPCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t-test, compared with the NC (0 μM remofuscin) on each day.

Fig. 7. Expression levels of ech-9 and cyp-35A subfamily members in wild-type (N2) and nhr-234 mutant *C. elegans*. The worms were grown on NGM plates containing 0 μM and 200 μM remofuscin for 5 days, and the gene expression levels were measured by qPCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t-test, compared with the wild-

type (N2) NC and $###p < 0.001$, Student's t-test, compared with the 200 μ M remofuscin-treated wild-type (N2) worms.

Fig. 8. Predicted mechanism by which remofuscin extends the *C. elegans* lifespan. A lysosomal lipase, LIPL-1, activates lipid catabolism, similar to that in a diet restriction (DR)-like state, thereby decreasing the ROS levels and subsequently activating the xenobiotic detoxification process. This sequence ultimately extends the lifespan of remofuscin-treated *C. elegans*. The broken line shows the predicted route based on the previously reported pathway (Chamoli et al., 2014)

EXAMPLES

As shown below remofuscin significantly ($p < 0.05$) extended the lifespan e.g. of *C. elegans* (N2) compared with the negative control. Aging biomarkers were improved in remofuscin-treated worms.

Surprisingly, the expression levels of genes related to lysosomes (lipl-1 and lbp-8), a nuclear hormone receptor (nhr-234), fatty acid beta-oxidation (ech-9), and xenobiotic detoxification (cyp-34A1, cyp-35A1, cyp-35A2, cyp-35A3, cyp-35A4, cyp-35A5, cyp-35C1, gst-28, and gst-5) were increased in remofuscin-treated worms. Moreover, remofuscin failed to extend the lives of *C. elegans* with loss-of-function mutations (lipl-1, lbp-8, nhr-234, nhr-49, nhr-8, cyp-35A1, cyp-35A2, cyp-35A3, cyp-35A5, and gst-5), showing that these genes are associated with lifespan extension in remofuscin-treated *C. elegans*. In conclusion, remofuscin activates the lysosome-to-nucleus pathway in *C. elegans*, thereby increasing the expression levels of xenobiotic detoxification genes resulted in extending their lifespan.

As shown in Fig. 2, remofuscin stimulates the expression of the lysosomal lipase gene lipl-1 and the lysosomal lipid chaperone gene lbp-8, subsequently increasing the expression levels of the genes involved in xenobiotic detoxification through nuclear hormone receptors and thereby extending the *C. elegans* lifespan.

The microarray and qPCR analyses of remofuscin-treated worms in a DR-like state revealed that genes related to lysosomes (lipl-1 and lbp-8), beta-oxidation (echo-9), and xenobiotic detoxification (cyp-34A1, cyp-35A1, cyp-35A2, cyp-35A3, cyp-35A4, cyp-35A5, cyp-35C1, gst-28, and gst-5) are upregulated, which prevented damage caused by lipid metabolites.

C. elegans has 8 lysosomal lipases, LIPL-1 to LIPL-8, among which LIPL-4 plays a role in the lifespan extension of *C. elegans* (Folick et al., 2015). Overexpression of LIPL-4 induces the expression of the lipid chaperone protein LBP-8, and mitochondrial beta-

oxidation is activated via NHR-49 and NHR-80 to promote lipid catabolism. ROS in mitochondria (mtROS) activate the transcription factor JUN-1, and the LIPL-4 and LBP-8 signaling pathways induce antioxidant targets and oxidative stress tolerance, thereby extending the *C. elegans* lifespan (Ramachandran et al., 2019). However, remofuscin did not increase the expression level of *jun-1* in *C. elegans*. The *C. elegans* genes directly or indirectly involved in lipid catabolism, especially LIPL-1, respond to the DR state and control lipid metabolism (O'Rourke and Ruvkun, 2013).

Below it is shown that the expression levels of genes related to a lysosomal lipase (*lipl-1*), a lipid binding protein (*lbp-8*), and beta-oxidation (*ech-9*) are increased in remofuscin-treated worms. In addition to *jun-1*, the transcription factors *nhr-49* and *nhr-27* play roles in the longevity of *C. elegans* by reducing the mitochondrial electron transport chain (Khan et al., 2013). Unlike mammals, which have only 48 NHR genes, *C. elegans* has 284 NHR genes that play roles in various processes, including lipid and xenobiotic metabolism. In particular, NHR-49, a homolog of mammalian hepatocyte nuclear factor 4 (HNF4) that functions similarly to peroxisome proliferator-activated receptors (PPARs), is known to transcriptionally regulate many genes related to beta-oxidation, including acyl-CoA synthetase, enoyl-CoA hydratase, and carnitine palmitoyl transferase (Pathare et al., 2012; Van Gilst et al., 2005). However, the effect of NHR-49 on *ech-9* (enoyl-CoA hydratase gene) remains debatable (Chamoli et al., 2014; Van Gilst et al., 2005).

Although the expression level of the *nhr-49* gene was not increased, that of *ech-9* was increased in *C. elegans* treated with remofuscin. In addition, the *nhr-49* deletion mutant failed to extend the lifespan of remofuscin-treated worms, indicating the possible role of NHR-49 in the longevity of remofuscin-treated *C. elegans*. The gene expression of nuclear receptor NHR-234, which is known to cooperate with NHR-49, was significantly upregulated in remofuscin-treated *C. elegans* and that the *nhr-234* mutant failed to extend the lifespan of the worms. Unlike the wild-type (N2) worms, those with *nhr-234* deletion mutants (VC1806) did not exhibit increased expression levels of the genes related to lipid metabolism (*ech-9*) and xenobiotic stimulus responses (*cyp-35A2*, *cyp-35A3*, and *cyp-35A4*), which may be regulated by NHR-239. Thus, NHR-234 with or without NHR-49 plays a role in the longevity of remofuscin-treated worms by regulating *ech-9* expression and thereafter altering the expression of genes related to xenobiotic detoxification.

Endogenous lipofuscin, the lipid-containing product resulting from the oxidation of unsaturated fatty acids composed of digested lipid-containing lysosomal residues, accumulates over time and can be a xenobiotic.

According to the invention, remofuscin acts as an exogenous xenobiotic agent. Dependence on fatty acid oxidation for energy sources leads to the formation of lipophilic endotoxins and, in turn, activates xenobiotic detoxification genes (Lindblom and Dodd, 2006; McElwee et al., 2004).

Xenobiotic detoxification occurs in three phases: phase I (cytochrome p450 enzymes (CYPs) chemically modify endotoxins), phase II (UDP-glucuronosyl transferases and glutathione S-transferases (GSTs) make them more soluble), and finally, phase III (modified endotoxins are emitted into the extracellular space by ATP-binding cassette transporters) (Lindblom and Dodd, 2006; Sharom, 2011). The expression levels of *cyp* (cytochrome p450 enzymes) and *gst* (glutathione S-transferases) genes were increased in remofuscin-treated *C. elegans*. GST functions as an antioxidant in detoxification reactions and inhibits ROS generation. In addition, GST stops or slows lipofuscin formation and cleaves the existing lipofuscin (Vekshin and Frolova, 2018). The transcription factors NHR-8, AHR-1, and PHA-4 regulate the expression of genes related to xenobiotic metabolism. NHR-8 is required for xenobiotic resistance and may regulate the expression of cytochrome P450 genes in *C. elegans* (Lindblom et al., 2001), AHR-1, which is related to CYP-35A subfamily members, regulates lipid signaling (Aarnio, 2014), and PHA-4 induces the expression of xenobiotic detoxification genes (Chamoli et al., 2014). *nhr-8* deletion mutants failed to extend the lifespan of remofuscin-treated worms, which means that genes related to xenobiotic detoxification activated by remofuscin conferred *C. elegans* with longevity.

Based on the results a pathway that is associated with lifespan extension in remofuscin-treated *C. elegans* is disclosed in Fig. 8. Remofuscin increases the expression of lysosomal lipase and induces lipid catabolism (beta-oxidation), subsequently activating the xenobiotic detoxification response and extending the *C. elegans* lifespan.

In addition, remofuscin-treated worms enter a DR-like state by decreasing their pharyngeal pumping rate, which is followed by a reduction in ROS levels through fatty acid beta-oxidation, thereby contributing to their lifespan extension. Lysosomal signaling from LIPL-4 to LBP-8 followed by NHR-49 and NHR-80 promotes the longevity of *C. elegans* (Savini et al., 2019); however, the signal herein was observed from LIPL-1 to LBP-8, followed by NHR-234 and/or NHR-49.

Remofuscin is also known as a potent and reversible inhibitor of the H^{+}/K^{+} -ATPase proton pump in cynomolgus monkeys (Julien and Schraermeyer, 2012). Although the proton pump which is inhibited by remofuscin is not present in the RPE in the eyes of human, proton pump inhibitors are known to increase the lysosomal pH, thereby activating transcription factor EB (TFEB), a master transcriptional regulator of

lysosomal biogenesis, and inducing lysosomal exocytosis and autophagy (Nociari et al., 2017). Remofuscin binds to lipofuscin (Julien-Schraemeyer et al., 2020) and is a superoxide generator when illuminated with light (Katairo and Takeda, 2021). Superoxide might help to degrade the polymeric lipofuscin into smaller units which then are transported out of the lysosomes by exocytosis. TFEB is an ortholog of HIH-30 in *C. elegans*, and HIH-30 is known to function as a transcription factor of *lipl-1* in *C. elegans* in a DR-like state (O'Rourke and Ruvkun, 2013).

Additionally, LIPL-1 degrades lipids in the lysosomal lipophagy process.

Lifespan extension derives from many factors, such as diet restriction (DR) and strengthened immunity, in a wide range of taxa ranging from yeast to primates (Hwangbo et al., 2020; Kurz and Tan, 2004). DR reduces the body size of *Caenorhabditis elegans*, which is associated with an extended lifespan (Bishop and Guarente, 2007). Aging induces morphological and metabolic changes, such as body size alteration and lipofuscin accumulation, which thus serve as biomarkers of aging (Pincus and Slack, 2010). Lipid metabolism is also altered over time, and aging and longevity are thus regulated by lipid signaling (Mutlu et al., 2021). Many of the pathways regulating lifespan are linked to lipid metabolism, and lipids act as signaling molecules in longevity signaling pathways.

In *C. elegans*, a reduced pharyngeal pumping rate is associated with a lower food intake, which results in a DR-like state despite an abundant supply of food. DR is one of the most influential environmental interventions that extends the lifespans of a variety of species (Greer et al., 2007). Beta-oxidation genes are upregulated in a DR-like state, consequently reducing the amount of stored fat, which leads to lower reactive oxygen species (ROS) levels. ROS are known as a major cause of aging and oxidative damage (Page et al., 2010).

Increased fatty acid beta-oxidation induces the expression of xenobiotic detoxification genes to clear lipophilic endotoxins produced during lipid catabolism, and the resulting metabolic shift increases the longevity of *C. elegans* (Chamoli et al., 2014). In the DR state, lysosomes play an important role in the early catabolic steps of lipid degradation (Settembre and Ballabio, 2014). *C. elegans* has eight lysosomal lipases, LIPL-1 to LIPL-8 (Seah et al., 2016); LIPL-4 has been extensively studied because it plays important roles in autophagy, fat metabolism, and lysosomal activity, which are linked to longevity in *C. elegans* (Lapierre et al., 2011; Wang et al., 2008; Watts and Ristow, 2017). Among the lysosomal lipase genes, *lipl-1* is the most upregulated in the fasting state, and its sequence is similar to that of human lysosomal acid lipase (BLAST scores 9e-78) (O'Rourke and Ruvkun, 2013).

Remofuscin extends the lifespan of *C. elegans*

Remofuscin significantly ($p < 0.05$) increased the MLS of wild-type *C. elegans* (N2) in a dose-dependent manner compared with that of the NC (0 μM remofuscin) (Table 1). Compared with those of the NCs, the MLSs of *C. elegans* (N2) treated with 50 μM , 100 μM , and 200 μM remofuscin were increased by 9.9%, 14.6%, and 20.4%, respectively. The survival rates of the worms treated with remofuscin were higher than those of the untreated worms after 5 days (Fig. 1).

Table 1

The mean lifespans (MLSs) of *C. elegans* (N2) treated with various concentrations of remofuscin. * $p < 0.05$, *** $p < 0.001$, log-rank test, compared with the NC (0 μM remofuscin).

Remofuscin (μM)	No. of total worms (dead/censored)	MLS $\pm$ SE (days)
0	135 (116/19)	12.11 $\pm$ 0.70
50	135 (112/23)	13.31 $\pm$ 0.72*
100	135 (117/18)	13.88 $\pm$ 0.75***
200	135 (111/24)	14.58 $\pm$ 0.80***

Effects of remofuscin on age-related biomarkers in *C. elegans*

To investigate the effect of remofuscin on lifespan extension in *C. elegans*, age-related biomarkers (body length and pharyngeal pumping rate) were measured. The body lengths of the live worms were measured every 24 h until 6 days of age. While the body length increased with age in all the groups, those of the remofuscin-treated groups were significantly shorter than those of the NC group (Fig. 2A).

A reduced pharyngeal pumping rate indicates a decrease in feeding, which can induce a DR-like state (Onken and Driscoll, 2010). The pharyngeal pumping rates of the remofuscin-treated groups were significantly ($p < 0.05$) decreased compared with that of the NC group (Fig. 2B). Regardless of whether *C. elegans* was treated with or

without remofuscin, the pharyngeal pumping rate decreased until 6 days; however, the pumping rates of worms treated with 100 μ M and 200 μ M remofuscin at 8 and 10 days were nearly similar or even higher than those at 6 days. In contrast, the pharyngeal pumping rate of the NC group continuously decreased over 10 days.

Lipofuscin accumulation is decreased in remofuscin-treated *C. elegans*

Lipofuscin accumulation serves as a biomarker of aging, and remofuscin significantly decreased the lipofuscin levels in *C. elegans* treated with 100 μ M and 200 μ M remofuscin on day 14 (Fig. 3). These results show that remofuscin affects lifespan extension in *C. elegans*.

Effects of remofuscin-induced ROS reductions in *C. elegans*

To investigate the inhibitory effect of remofuscin on ROS generation in *C. elegans*, the total ROS levels in worms treated with remofuscin were measured. The ROS levels in *C. elegans* grown on plates containing 100 μ M and 200 μ M remofuscin for 14 days were significantly ($p < 0.05$) decreased compared with those in the NC group (Fig. 4).

Microarray analysis of remofuscin-treated *C. elegans*

To investigate the mechanism by which remofuscin promotes longevity, microarray analysis of wild-type (N2) worms treated with 0 μ M (NC) and 200 μ M remofuscin for 14 days was performed. A total of 31,383 genes were identified, among which 340 and 103 genes were significantly ($p < 0.05$) upregulated (≥ 2 -fold) and downregulated (≤ 0.5 -fold), respectively, between the NC and 200 μ M remofuscin-treated worms (Fig. 5A). Based on the terms represented in the GO database, the differentially expressed genes (DEGs) were divided into three categories: biological process (BP), cellular component (CC), and molecular function (MF). GO analysis showed 10 major functional categories in the BP group, 8 categories in the CC group, and 7 categories in the MF group based on the criterion for identifying differentially expressed genes by microarray analysis (fold change ≥ 2.0 and $p < 0.05$) (Fig. 5B). Among these genes, those related to response to xenobiotic stimuli were highly expressed in the 200 μ M remofuscin-treated worms compared with the NC worms. Xenobiotic detoxification is known to affect longevity in *C. elegans*, and based on the total significance (Fig. 5C), we focused on the genes related to xenobiotic stimulus responses. The genes related to xenobiotic metabolism (GO: 0006805) (cyp-13A2, cyp-34A1, cyp-35A1, cyp-35A2, cyp-35A3, cyp-35A4, cyp-35A5, cyp-35C1, gst-28, gst-5, ugt-65, pgp-14, and folt-2) were significantly ($p < 0.05$) increased (>2 -fold) compared with those in the NC group (0 μ M remofuscin) as determined by microarray analysis (Table 2).

Table 2

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Microarray analysis revealed that xenobiotic metabolism-related genes were upregulated in *C. elegans* (N2) treated with 200 μ M remofuscin. Fold changes were calculated between the control and remofuscin (200 μ M)-treated *C. elegans*.

Gene name	Wormbase ID	Brief description	Fold change	<i>p</i> -value
<i>cyp-13A2</i>	WBGene00011676	Phase I detoxification, cytochrome P450	2.387	0.018
<i>cyp-34A1</i>	WBGene00011698	Phase I detoxification, cytochrome P450	5.424	0.007
<i>cyp-35A1</i>	WBGene00015399	Phase I detoxification, cytochrome P450	18.131	0.014
<i>cyp-35A2</i>	WBGene00015400	Phase I detoxification, cytochrome P450	8.568	0.007
<i>cyp-35A3</i>	WBGene00019565	Phase I detoxification, cytochrome P450	110.931	0.004
<i>cyp-35A4</i>	WBGene00016786	Phase I detoxification, cytochrome P450	76.458	0.000
<i>cyp-35A5</i>	WBGene00019473	Phase I detoxification, cytochrome P450	30.956	0.009
<i>cyp-35C1</i>	WBGene00007362	Phase I detoxification, cytochrome P450	14.545	0.038
<i>gst-28</i>	WBGene00001776	Phase II detoxification, glutathione S transferase	3.080	0.033
<i>gst-5</i>	WBGene00001753	Phase II detoxification, glutathione S transferase	2.171	0.008
<i>ugt-65</i>	WBGene00008583	Phase II detoxification, UDP-glucuronosyl transferase	2.662	0.026
<i>pgp-14</i>	WBGene00004008	Phase III detoxification, a member of the ABC transporter family	2.299	0.022
<i>folt-2</i>	WBGene00018138	Phase III detoxification, folate transmembrane transporter	2.821	0.022

Effects of remofuscin on the expression levels of genes related to lysosomes and xenobiotic metabolism.

Based on the microarray results, the expression levels of genes related to lysosomal lipases (*lipl-1*), long-chain fatty acid transporters (*lbp-8*), fatty acid beta-oxidation (*echo-9*), and xenobiotic detoxification (*cyp-35A* subfamily, *gst-5*, and *gst-28*) in *C. elegans* (N2) treated with 0 and 200 μ M remofuscin for 1 day and 5 days were measured by qPCR. Table 2 shows that the expression levels of genes related to xenobiotic metabolism, especially *cyp35A* subfamily genes, were significantly increased in remofuscin-treated worms compared with NC worms (Fig. 6). Mostly, the gene expression levels were higher at 5 days than at 1 day. In addition, the expression levels of *lipl-1*, *lbp-8*, and *echo-9* were significantly increased in remofuscin-treated worms. Although the differences were not statistically significant, the levels of several nuclear hormone receptor (NHR) genes, transcription factors of cytochrome p450 family genes, were increased by more than 2-fold as determined by microarray analysis (Table S2). Among them, the levels of only *nhr-210* and *nhr-234* were significantly increased in remofuscin-treated worms compared with the NC worms as determined by qPCR analysis (Fig. 6)

Longevity assay of loss-of-function mutants

To elucidate the mechanism by which remofuscin extends the *C. elegans* lifespan, loss-of function mutants for genes related to xenobiotic detoxification were used for longevity assays. The loss-of function mutants available from the CGC and NBRP were selected based on the qPCR results. In addition, although the expression levels of the transcription factor genes (*nhr-49*, *nhr-8*, *ahr-1*, and *pha-4*) related to lipid metabolism and xenobiotic stimulus responses in *C. elegans* were not upregulated in remofuscin-treated worms compared with the NC (Fig. S1), loss-of function mutants were used to assess lifespan extension based on previous reports that they regulate the expression of genes related to lipid and xenobiotic metabolism (Chamoli et al., 2014; Folick et al., 2015; Lindblom et al., 2001; Ménez et al., 2019; Ramachandran et al., 2019). Remofuscin treatment failed to extend the lifespans of worms exhibiting loss-of-function mutations of genes related to lipid metabolism (*lipl-1* and *lbp-8*) and xenobiotic detoxification (*cyp-35A1*, *cyp-35A2*, *cyp-35A3*, *cyp-35A5*, and *gst-5*) (Table 3, Fig. S2). Interestingly, remofuscin failed to extend the lifespan of *C. elegans* harboring transcription factor (*nhr-49*, *nhr-8*, and *nhr-234*) mutants but not that of worms with the *nhr-210* mutant. Although the *ahr-1* and *pha-4* deletion mutants extended the lifespans of 100 μ M and 200 μ M remofuscin-treated worms, respectively, their overall MLSs were decreased by remofuscin treatment compared with the wild type.

Table 3

The mean lifespans of *C. elegans* with loss-of-function mutants. n, The number of total worms; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, log-rank test, compared with the NC.

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Nematode type	Remofuscin (μ M)	MLS $\pm$ SE (days) (n)
FX1954 <i>lipl-1</i> (tm1954)	0	10.03 $\pm$ 0.60 (107)
	50	10.35 $\pm$ 0.62 (113)
	100	10.12 $\pm$ 0.67 (101)
	200	10.30 $\pm$ 0.64 (115)
VC4077 <i>lbp-8</i> (gk5151)	0	11.12 $\pm$ 0.56 (125)
	50	11.32 $\pm$ 0.57 (123)
	100	11.13 $\pm$ 0.62 (122)
	200	11.34 $\pm$ 0.64 (114)
FX1290 <i>nhr-210</i> (tm1290)	0	10.43 $\pm$ 0.57 (117)
	50	10.71 $\pm$ 0.64 (113)
	100	11.36 $\pm$ 0.68** (109)
	200	11.78 $\pm$ 0.70*** (112)
VC1806 <i>nhr-234</i> (gk865)	0	10.55 $\pm$ 0.58 (120)
	50	10.54 $\pm$ 0.63 (114)
	100	10.65 $\pm$ 0.61 (119)
	200	10.51 $\pm$ 0.62 (116)
FX30306 <i>nhr-49</i> (tm7967)	0	5.83 $\pm$ 0.33 (109)
	50	5.71 $\pm$ 0.32 (109)
	100	5.80 $\pm$ 0.31 (111)
	200	6.03 $\pm$ 0.34 (115)
FX19275 <i>nhr-8</i> (tm1800)	0	10.09 $\pm$ 0.53 (117)
	50	10.34 $\pm$ 0.58 (104)
	100	10.25 $\pm$ 0.57 (107)
	200	10.02 $\pm$ 0.56 (110)
FX01722 <i>ahr-1</i> (tm1722)	0	11.00 $\pm$ 0.66 (109)
	50	11.08 $\pm$ 0.66 (107)
	100	11.61 $\pm$ 0.69* (105)
	200	11.37 $\pm$ 0.67 (113)
FX4598 <i>pha-4</i> (tm4598)	0	9.29 $\pm$ 0.62 (109)
	50	10.04 $\pm$ 0.61 (114)
	100	10.46 $\pm$ 0.56 (120)
	200	10.53 $\pm$ 0.59* (118)
VC875 <i>cyp-35A1</i> (ok1414)	0	7.58 $\pm$ 0.44 (101)
	50	8.09 $\pm$ 0.46 (100)
	100	7.51 $\pm$ 0.47 (100)
	200	7.29 $\pm$ 0.40 (106)
FX21842 <i>cyp-35A2</i> (tm11844)	0	9.68 $\pm$ 0.58 (107)
	50	10.11 $\pm$ 0.59 (113)
	100	10.20 $\pm$ 0.62 (111)
	200	10.34 $\pm$ 0.61 (113)
RB2046 <i>cyp-35A3</i> (ok2709)	0	9.41 $\pm$ 0.64 (117)

Role of NHR-234 in the longevity mechanism of remofuscin

NHR-234 cooperates with NHR-49 to induce the transcription of target genes related to lipid metabolism. Remofuscin-treated nematodes upregulated *nhr-234* expression compared with that in the control group, and the *nhr-234* mutant failed to extend the lifespan of the worms. To investigate the effect of NHR-234 on the expression of genes related to lipid metabolism and xenobiotic detoxification proposed to be downstream of NHR-234 in *C. elegans* treated with remofuscin, the expression levels of *ech-9*, *cyp-35A1*, *cyp-35A2*, *cyp-35A3*, *cyp-35A4*, and *cyp-35A5* in the *nhr-234* deletion mutants were analyzed by qPCR. The expression levels of *ech-9*, *cyp-35A2*, *cyp-35A3*, and *cyp-35A4*, but not *cyp-35A1* and *cyp-35A5*, were not increased in the *nhr-234* deletion mutants treated with remofuscin (200 μ M) compared with the wild-type (N2) worms, indicating that NHR-234 might regulate their expression (Fig. 7).

Materials and methods

Bacterial strains and culture conditions

Escherichia coli OP50 was obtained from the Caenorhabditis Genetics Center (CGC, USA) of the University of Minnesota and used as a food for *C. elegans*. *E. coli* OP50 was grown in Luria-Bertani (LB) broth (Ambrothia, Daejeon, Korea) at 37 °C overnight with shaking, collected by centrifugation at 3,000× g for 10 min, washed in sterile M9 buffer, and diluted to a final concentration of 0.1 mg (wet weight) per microliter in M9 buffer (Zhao et al., 2013).

Nematode strains and growth conditions

C. elegans Bristol strain N2, provided by the CGC, was used as the wild-type strain. The mutant strains VC1806 *nhr-234* (gk865), VC4077 *lbp-8* (gk5151[*loxP* + *myo-2p::GFP::unc-54* 3' UTR + *rps-27p::neoR::unc-54* 3' UTR + *loxP*]), VC875 *cyp-35A1* (ok1414), RB2046 *cyp-35A3* (ok2709), and RB2063 *gst-5* (ok2726) were provided by the CGC, and FX1954 *lip1-1* (tm1954), FX1290 *nhr-210* (tm1290), FX30306 *nhr-49* (tm7967), FX19275 *nhr-8* (tm1800), FX01722 *ahr-1* (tm1722), FX4598 *pha-4* (tm4598), FX21842 *cyp-35A2* (tm11844), and FX22344 *cyp-35A5* (tm12345) were provided by the National Bioresource Project (NBRP) for nematodes at Tokyo Women's Medical University (Tokyo, Japan). Worms were maintained and propagated in peptone-free modified nematode growth medium (mNGM) at 25 °C according to standard techniques (Stiernagle, 2006). *E. coli* OP50 was spread on mNGM in 90-mm-diameter Petri dishes as food for the worms. A sodium hypochlorite-sodium hydroxide solution (Sigma Aldrich, St. Louis, MO, USA) was used to obtain viable eggs as previously described (Sulston and Hodgkin, 1988). The eggs were transferred onto fresh mNGM plates seeded with *E. coli* OP50 and incubated at 25 °C until the L4 stage (3-day-old worms), and all experiments used the L4 stage (3-day-old worms) as day 1 of the adult stage to control the reproductive system in *C. elegans* (Komura et al., 2013).

Assay of the *C. elegans* mean lifespan

Remofuscin (kindly provided by Professor Ulrich Schraermeyer, Universität Tübingen, Tübingen, Germany) was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) and administered final concentrations of 0 μ M (control), 50 μ M, 100 μ M, and 200 μ M. An equal amount of DMSO (final concentration, 0.2%) was added as the control. 5-Fluoro-2'-deoxyuridine (FUdR, Sigma Aldrich) (50 μ M) was added to the plates (Gruber et al., 2009), which were then seeded with *E. coli* OP50. The *C. elegans* mean lifespan (MLS) assay was conducted by transferring 15 young adult (L4 stage) worms onto mNGM/FUdR plates containing *E. coli* OP50 and treated with remofuscin at the indicated concentrations. The plates were incubated at 25 °C, and the live and dead worms were counted every 24 h. Worms were considered "dead" when they did not respond to a gentle touch with a worm picker. Nematodes that crawled off the plates and died in a non-natural manner, such as by bagging or adhering to the plate wall, were not included in the analysis (censored) (Schmeisser et al., 2013). The worms were transferred every two days to maintain a sufficient food source. All experiments were conducted three times independently at least in triplicate, and more than 100 worms were scored.

The MLS was estimated using the following equation (Wu et al., 2006):

$$\text{MLS} = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} d_j$$

In the equation, j is the age (day), d_j is the number of worms that died during the day interval (x_j, x_{j+1}), and N is the total number of worms. The standard error (SE) of the estimated MLS was calculated using the following formula.

$$\text{SE} = \sqrt{\frac{1}{N(N-1)} \left(\sum_j \frac{x_j + x_{j+1}}{2} - \text{MLS} \right)^2 d_j}$$

Measurement of body length

Worms at the L4 stage (day 1 of the adult stage) were transferred onto mNGM plates (60 mm Petri dish) containing various concentrations of remofuscin and seeded with 5 mg (wet weight) of *E. coli* OP50 in M9 buffer. The plates were incubated at 25 °C, and the body lengths of live worms were measured every 24 h until 6 days of age. In total, 10 worms per group were measured. *C. elegans* were imaged with a stereomicroscope (Olympus SZ61, Tokyo, Japan) and a ToupCam (UCMOS05100KPA, ToupTek, Hangzhou, China), and the images were analyzed by using ToupCam software. The

area of the worm's projection was estimated automatically and used as an index of body length. Three independent experiments were conducted for each group.

Measurement of the pharyngeal pumping rate

A pharyngeal pumping rate assay was performed on mNGM plates seeded with *E. coli* OP50 and treated with various concentrations of remofuscin. Three-day-old worms (L4 stage) were transferred onto mNGM plates containing various concentrations of remofuscin and incubated at 25 °C, and the number of contractions in the terminal bulb of the pharynx was counted every 48 h for 1 min using an Olympus CKX41 inverted microscope (400×). Three independent experiments were conducted, and 15 worms were included in each group for each measurement.

Measurement of lipofuscin accumulation

The autofluorescence of lipofuscin in 14-day-old adult *C. elegans* was measured as an aging index. Randomly selected worms from each group were placed onto 5% agar pads coated with 10 mM sodium azide (Junsei Chemical, Tokyo, Japan) in M9 buffer for anesthetization. Images of lipofuscin autofluorescence at a blue excitation wavelength (405–488 nm), which captures 4',6-diamidino-2-phenylindole (DAPI), were acquired with a laser confocal scanning microscope (Olympus Ix81-FV1000) (Zhao et al., 2013). Fluorescence was quantified using FV10-ASW1.1 software (Olympus) to measure lipofuscin accumulation. Three independent experiments were conducted, and 10 worms were included in each group for each measurement.

Measurement of ROS

The ROS levels in *C. elegans* treated with 0 μ M, 50 μ M, 100 μ M, and 200 μ M remofuscin were measured for 14 days. Randomly selected worms from each group were washed twice with M9 buffer, after which the supernatant was removed, and the remaining worm pellet was suspended in 100 μ L of M9 buffer. The worm pellet (100 μ L) and 100 μ L of 50 mM 2',7'-dichlorofluorescein diacetate (H2-DCF-DA, Sigma Aldrich) were added to the wells of a black 96-well plate. The ROS levels were measured with a fluorescence microplate reader (SpectraMAX GEMINI EM, Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 485 nm and 520 nm, respectively, at 90 min after activation. The fluorescence signal in each group, which included more than 80 worms for each measurement, was normalized to the protein concentration in each group. Three independent experiments were conducted.

Microarray analysis

Worms fed *E. coli* OP50 for 14 days on NGM plates containing 0 μ M and 200 μ M remofuscin were collected and washed twice with M9 buffer, after which total RNA was isolated from whole worms using TRIzol (Invitrogen, Carlsbad, CA, USA) according to a previously described method (Greer et al., 2007). For each RNA, the synthesis of

target cRNA probes and hybridization were performed using an Aligent LowInput QuickAmp labeling kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Amplified and labeled cRNA was purified on a cRNA Cleanup Module (Agilent Technologies), and labeled cRNA targets were quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). After checking the labeling efficiency, the cRNA was fragmented by adding 10X blocking agent and 25X fragmentation buffer and incubating at 60 °C for 30 min. The fragmented cRNA was resuspended in 2X hybridization buffer and directly pipetted onto assembled *C. elegans* oligo microarrays (Agilent, 44K). The arrays were hybridized at 65 °C for 17 h using a hybridization oven (Agilent Technologies). The hybridized microarrays were washed according to the manufacturer's protocol (Agilent Technologies). The hybridized images were scanned using an Agilent DNA microarray scanner and quantified with Feature Extraction 10.7 software (Agilent Technologies). Raw intensity data were globally normalized (Cheadle et al., 2003). All data normalization and selection of differentially expressed genes (fold change) were performed using GeneSpring GX 7.3.1 (Agilent Technologies). The criterion for the identification of genes with significantly altered expression was a p value < 0.05 compared with the negative control. The RNA sequencing data were deposited in the NCBI Gene Expression Omnibus (GEO) database (accession code: GSE144059). For the pie chart and volcano plot, Excel-based Differentially Expressed Gene Analysis (ExDEGA, eBiogen, Seoul, Korea) software was used to analyze the microarray data according to classified Gene Ontology (GO) terms. Genes with a p-value < 0.05 and a fold change of 2.0 compared with the negative control were defined as significantly changed genes.

Quantitative real-time polymerase chain reaction (qPCR)

C. elegans fed *E. coli* OP50 for 1 and 5 days on NGM plates containing 0 μ M and 200 μ M remofuscin were collected and washed twice with M9 buffer. Total mRNA was isolated from whole worms using TRIzol (Invitrogen) as previously described (Greer et al., 2007). The RNA was converted into cDNA using a RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific, Wilmington, DE, USA) and then amplified by qPCR using SYBR Green (KAPA Biosystems, Wilmington, MA, USA) and a QuantStudio 6 Flex Real Time PCR machine (Applied Biosystems, Foster City, CA, USA). For qPCR, an initial step at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s were performed, and melting curve analysis was performed. The experiments were independently conducted at least three times, and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The internal control gene *act-1* was used to normalize the gene expression data. The sequences of the primers used are listed in Table S1.

Statistical analysis

In the lifespan assay, the Kaplan-Meier method and the log-rank test were used to calculate the MLS and p-values, respectively (Zhao et al., 2013). In the other experiments, the significance of comparisons between the negative control (NC) and remofuscin-treated groups was calculated by using Student's t-test. Significance was defined as a p-value less than 0.05 in all experiments. If the data were not normally distributed, the Mann-Whitney U test was used (Komura et al., 2013).

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Claims:

1. Remofuscin for use against aging.
2. Remofuscin for use against lysosomal dysfunction.
3. Remofuscin for use disease with insufficient expression of lysosomal lipase genes and/or lysosomal lipid chaperone genes.
4. Remofuscin for use against xenobiotic toxic effects by increasing the expression levels of the genes involved in xenobiotic detoxification through nuclear hormone receptors.
5. Remofuscin for regular, preferably weekly or daily use according to one of claims 1-4.
6. A tetrahydropiridoether for use according to one of claims 1-5.
7. Use of Remofuscin for life extension or to increase xenobiotic detoxification.

Fig. 1

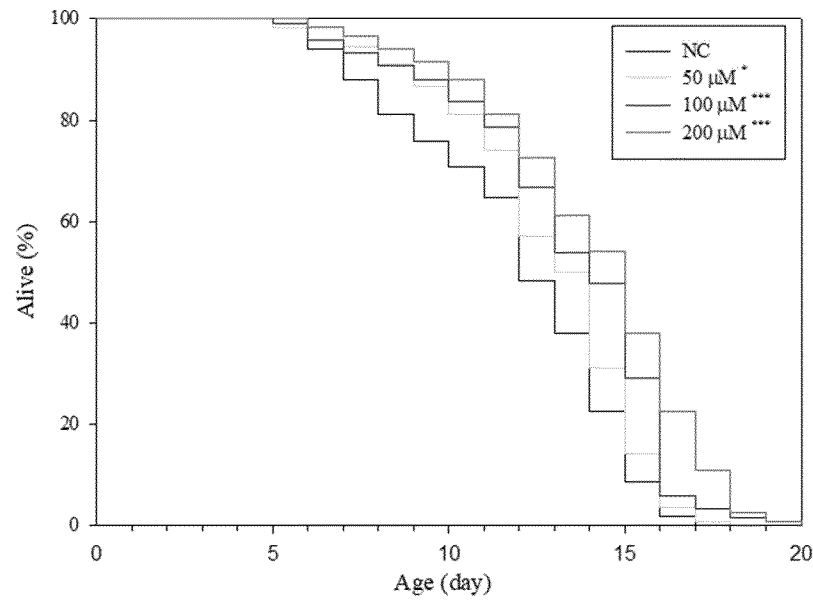


Fig. 2

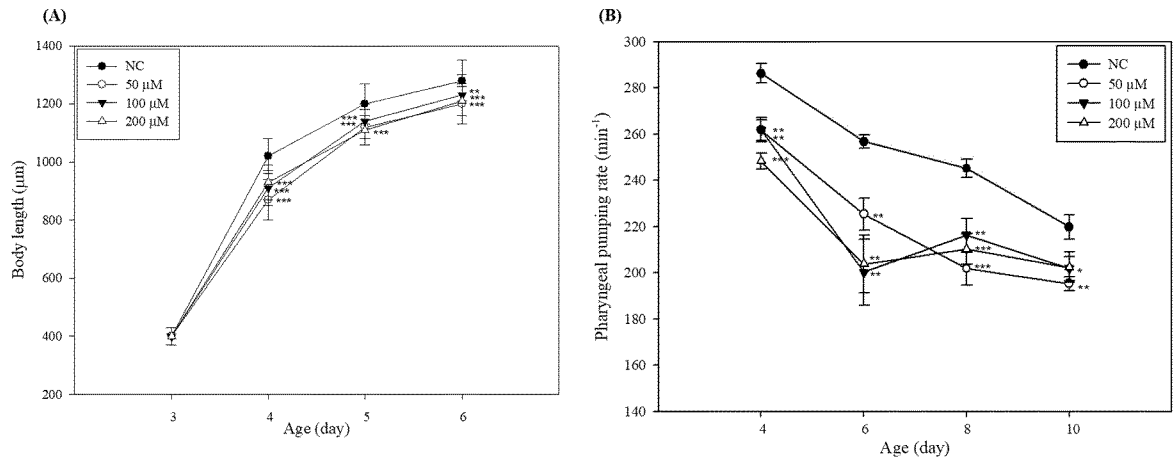


Fig. 3

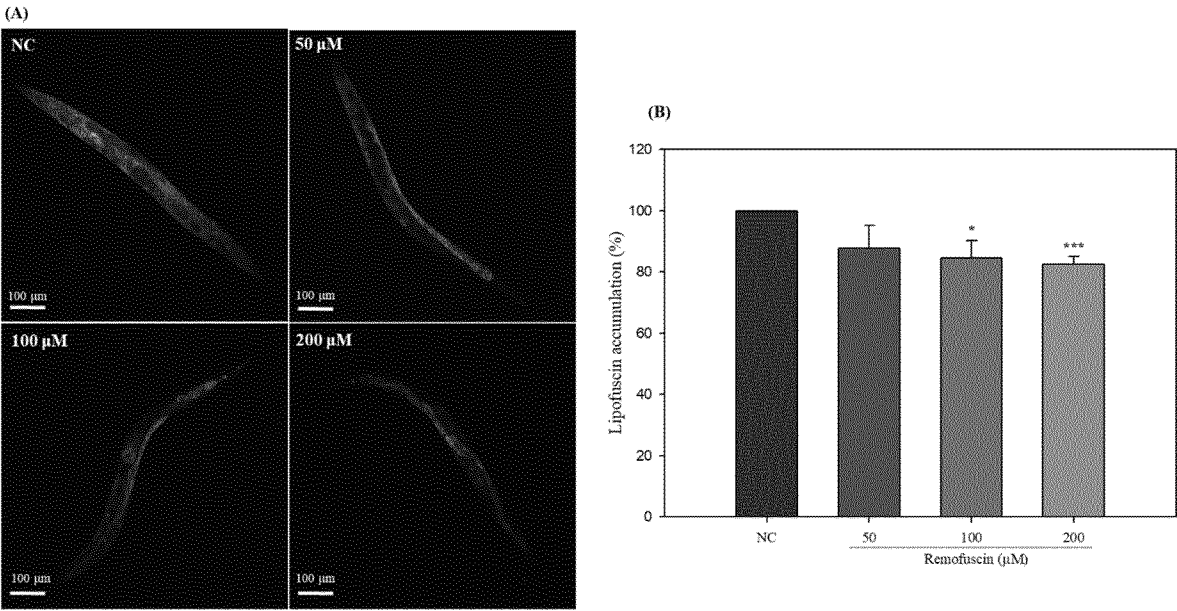
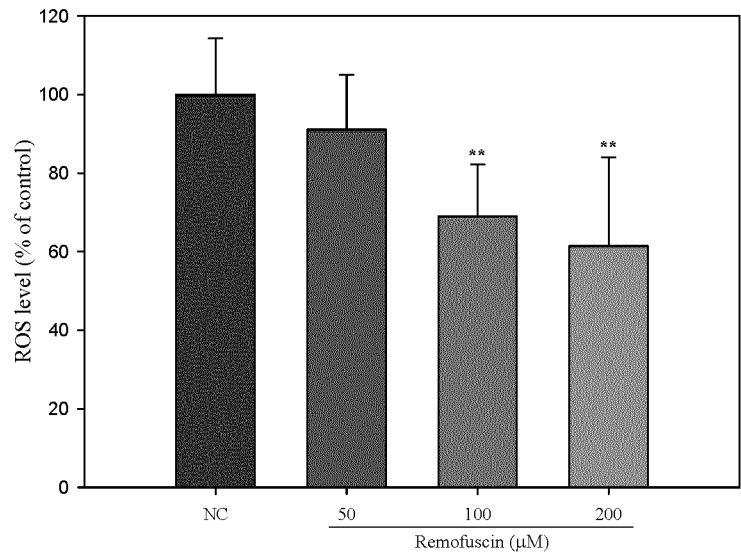
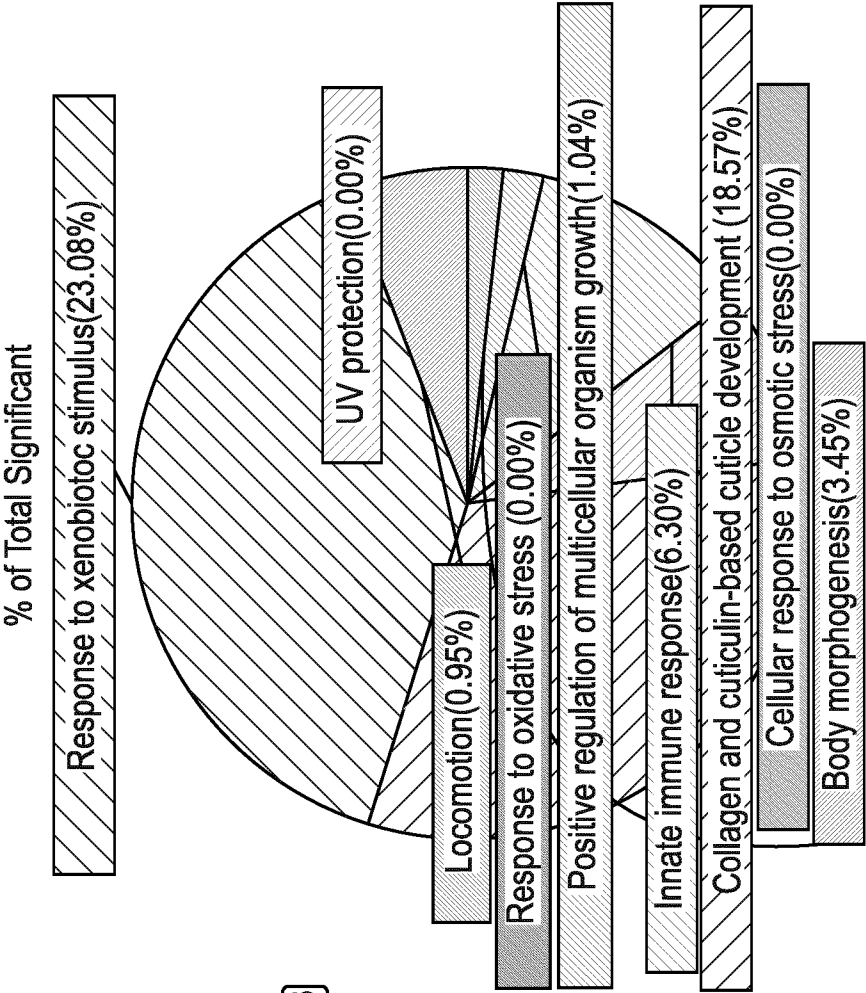
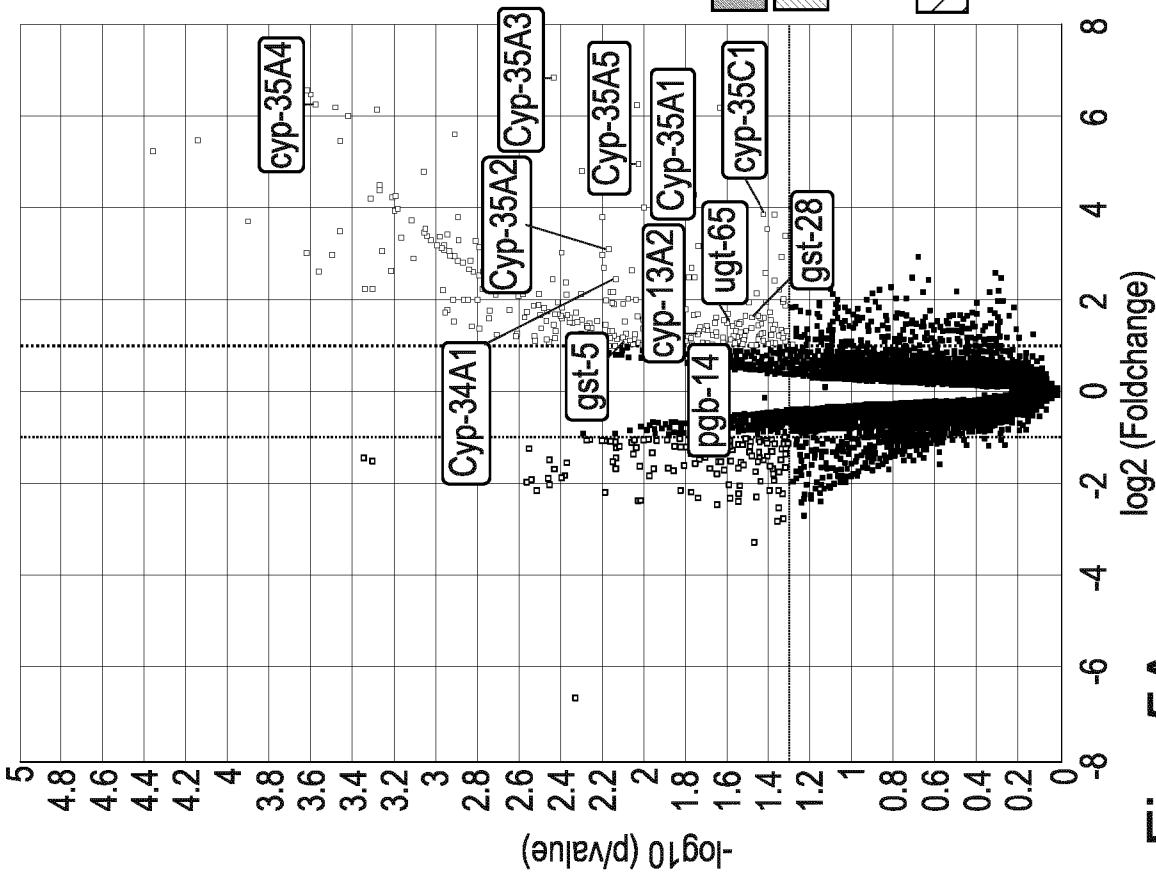


Fig.4





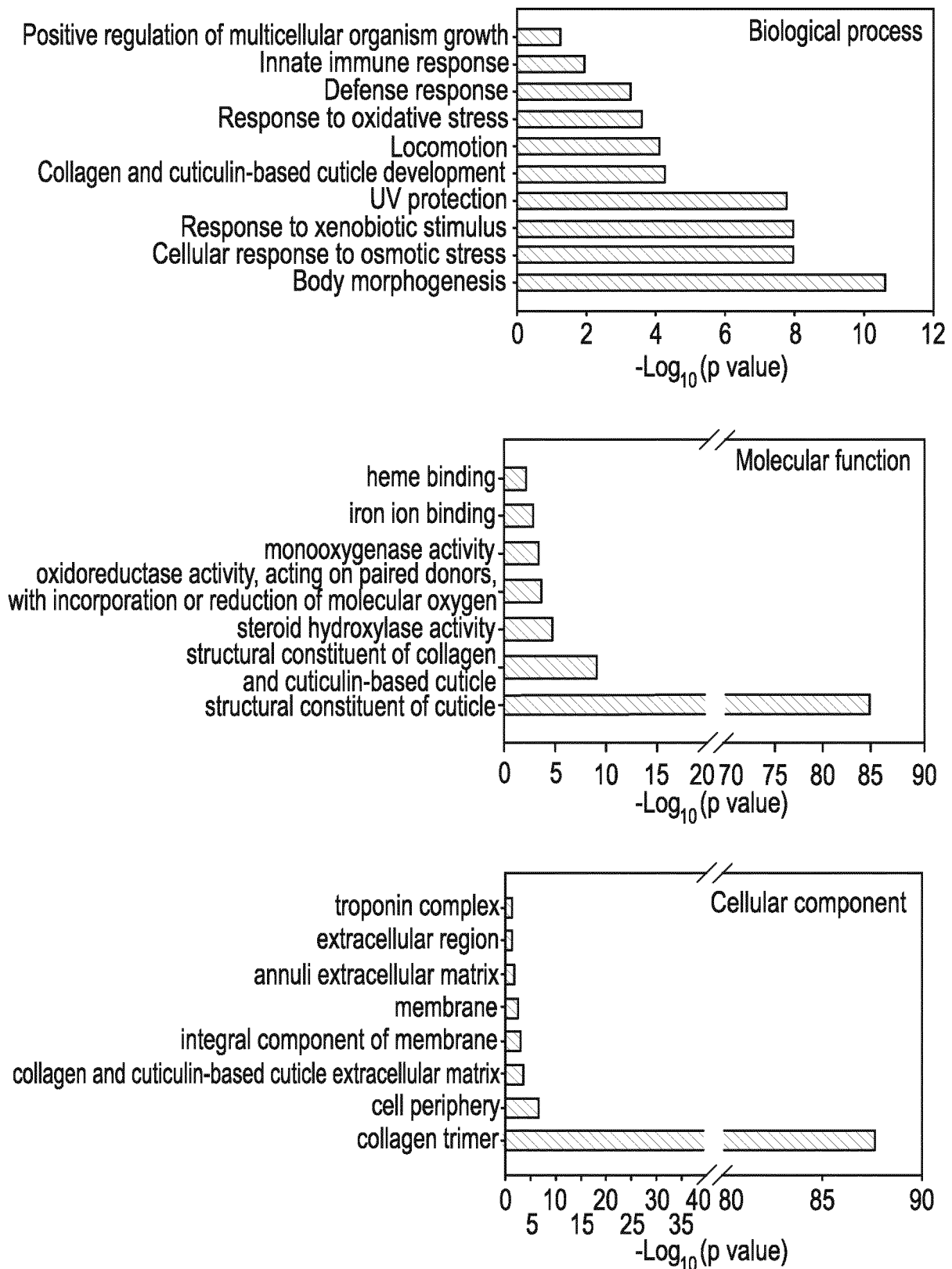
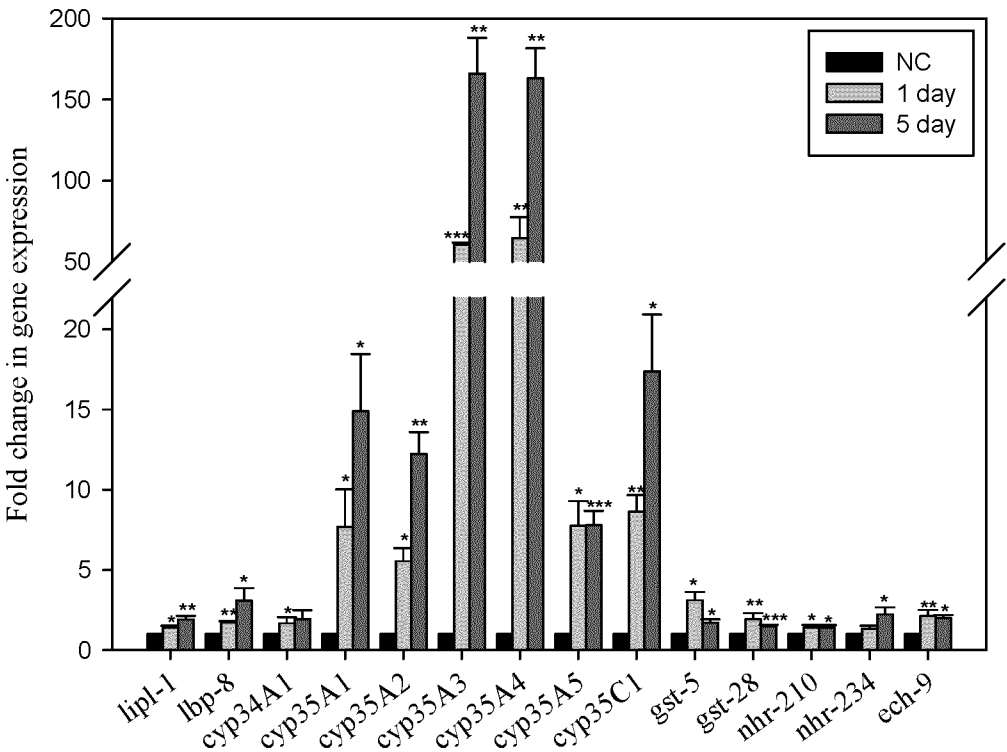


Fig. 5B

Fig. 6



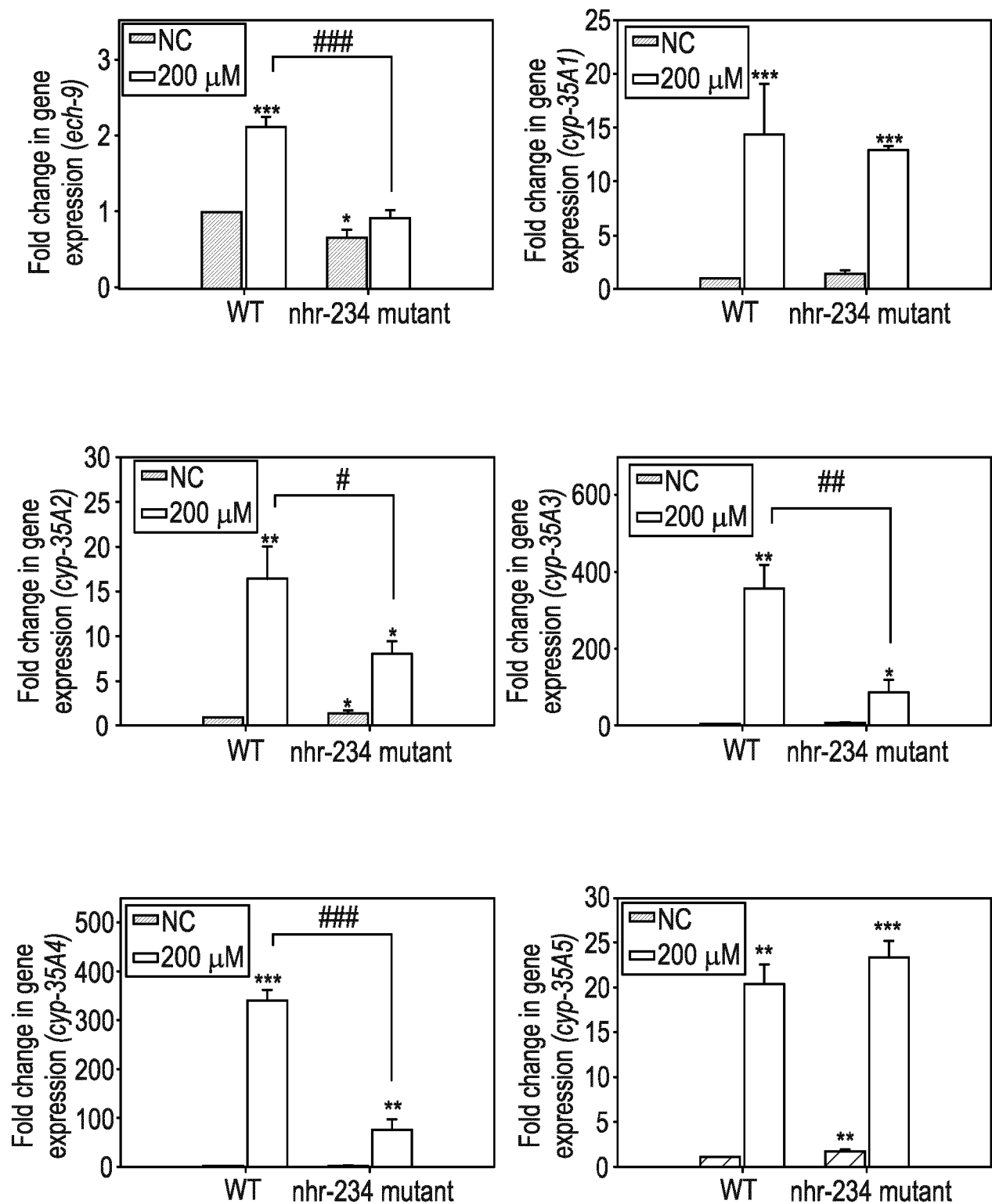
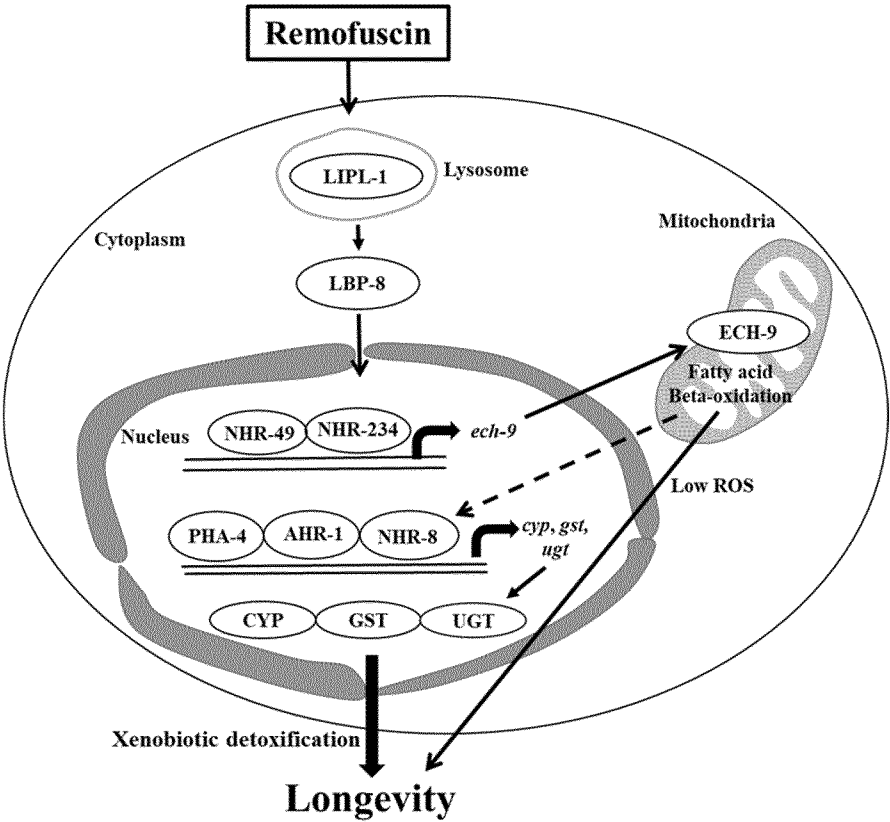


Fig. 7

Fig. 8



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/061710

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/4375 A61P25/28 A61P39/00 A61Q19/08 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A61K A61Q A61P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, SCISEARCH, CHEM ABS Data, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FANG YUAN ET AL: "Removal of RPE lipofuscin results in rescue from retinal degeneration in a mouse model of advanced Stargardt disease: Role of reactive oxygen species", FREE RADICAL BIOLOGY & MEDICINE, ELSEVIER INC, US, vol. 182, 25 February 2022 (2022-02-25), pages 132-149, XP087000600, ISSN: 0891-5849, DOI: 10.1016/J.FREERADBIOMED.2022.02.025 [retrieved on 2022-02-25]	1-4, 6, 7
Y	abstract page 133, column 1, paragraph 3 - column 2, paragraph 5 page 144, column 2, paragraph 2 ----- -/--	1-7
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
12 July 2023		21/07/2023
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Cielen, Elsie

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INTERNATIONAL SEARCH REPORT

International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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X,P	WO 2023/034553 A1 (JAPAN CHEM RES [JP]; ARMAGEN INC [US]) 9 March 2023 (2023-03-09) page 1, paragraph [0001]-[0002] page 8, paragraph [0029]-[0031] page 19, paragraph [0069]-[0070] -----	2-7

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