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Development of a safe antiparasitic against scuticociliates (*Miamiensis avidus*) in olive flounders: new approach to reduce the toxicity of mebendazole by material remediation technology using full-overlapped gravitational field energy

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Abstract

The olive flounder (*Paralychthys olivaceus*) is a representative farmed fish species in South Korea, which is cultured in land-based tanks and accounts for approximately 50% of total fish farming production. However, farmed olive flounder are susceptible to infection with parasitic scuticociliates, which cause scuticociliatosis, a disease resulting in severe economic losses. Thus, there has been a longstanding imperative to develop a highly stable and effective antiparasitic drug that can be rapidly administered, both orally and by bath, upon infection with scuticociliates. Although the efficacy of commercially available mebendazole (MBZ) has previously been established, this compound cannot be used for olive flounder due to hematological, biochemical, and histopathological side effects. Thus, we produced material remediated mebendazole (MR MBZ), in which elements comprising the molecule were remediated by using full-overlapped gravitational field energy, thereby reducing the toxicity of the parent material. The antiparasitic effect of MR MBZ against scuticociliates in olive flounder was either similar to or higher than that of MBZ under the same conditions. Oral (100 and 500 mg/kg B.W.) and bath (100 and 500 mg/L) administrations of MBZ significantly ($p < 0.05$) increased the values of hematological and biochemical parameters, whereas these values showed no increase in the MR MBZ administration group. In addition, there were no histopathological side effects, such as atrophic degeneration or hyaline droplet degeneration, whereas these were observed when MBZ was administered. Thus, we report that the material remediation method using full-overlapped gravitational field energy can be used to reduce drug toxicity.

Keywords Mebendazole · Parasite · Scuticociliate · Olive flounder · Material remediation technology

Jung-Soo Seo and Na-Young Kim contributed equally to this work as first authors. These authors contributed equally to this work.

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Introduction

The olive flounder (*Paralychthys olivaceus*) is a representative farmed fish species in South Korea, which is cultured in land-based tanks and accounts for 50% of total fish farming production (KOSTAT 2017). Scuticociliatosis, a parasitic disease caused by invasive ciliates (class: Scuticociliatida), has the largest detrimental impact on the production of this fish. Since 1986, when it was first detected in farmed olive flounder in Japan, scuticociliatosis has been reported to cause mass mortality mostly in fry and juveniles (Yoshinaga and Nakazoe 1993; Moustafa et al. 2010). In South Korea, scuticociliatosis was first identified from an olive flounder farm in Jeju Island in 1990, and now causes serious economic damage to the olive flounder farms nationwide every year, showing a mortality trend similar to that in Japan (Jin et al. 2003, 2007; Kang et al. 2015). Some 46 to 57% of the recent cumulative damage has been attributed to scuticociliatosis (Kim et al. 2012; Jee et al. 2014). Therefore, scuticociliatosis is a serious infectious parasitic disease that needs to be efficiently controlled in the early stage of olive flounder farming.

Since the first report of scuticociliates as parasites of seahorse (*Hippocampus erectus*), they have been reported to infect species in various taxonomic groups of marine animals, causing serious damage. Representative examples of the species affected by scuticociliates are as follows: *Uronema marinum* in nine species (California sheepshead wrasse *Pimelometopon pulchrum*; cunner *Tautogolabrus adspersus*, Atlantic sea horse *Hippocampus erectus*, Indo-Pacific sea horse *H. kuda*, garibaldi *Hypsypops rubicunda*, tomato drop butterfly *Chaetodon unimaculatus*, diagonal butterfly *Chaetodon auriga*, copper-band butterfly *Chelmon rostratus*, and royal coral man *Heniochus acuminatus*) that are cultured in aquaria (Cheung et al. 1980); *Philasterides dicentrachi* in turbot (*Scophthalmus maximus*) and sea bass (*Dicentrarchus labrax*) (Ramos et al. 2007; Budino et al. 2012); *Anophryoides haemophila* in the American lobster (*Homarus americanus*) (Cawthorn 1997; Athanassopoulou et al. 2004); *Uronema nigricans* in southern bluefin tuna (*Thunnus maccoyii*) (Munday et al. 1997); *Uronema* sp. in silver pomfret (*Pampus argenteus*) (Azad et al. 2007); and *Miamiensis avidus* in starfin flounder (*Verasper moseri*) (Ito and Kasai 2015). In South Korea, *M. avidus* (= synonym of *P. dicentrarchi*) in olive flounders was identified as the dominant species with the strongest pathogenicity (Jee et al. 2001; Kim et al. 2004; Jung et al. 2007; Song et al. 2009). Scuticoiliates invade and infect not only the surface of the body or gills but also internal organs (e.g., brain, kidney, spleen, spinal cord), and therefore it is imperative to prevent the brain or internal organs from becoming infected by these parasites through prompt treatment following early diagnosis (Jin et al. 2009; Harikrishnan et al. 2012).

Drugs/chemicals that are known to date to have antiparasitic activity on scuticociliates include trichlorphon, pyrimethamine + sulphaquinoxaline, amprolium, monesin, doxycycline, oxytetracycline, formalin, copper sulfate, hydrogen peroxide, antiprotozoals, fluoroquinolones, indomethacin, Jenoclean (97% zeolites + 3% citric acid), resveratol, and benzalkonium chloride + bronopol (Novotny et al. 1996; Iglesias et al. 2002; Jee et al. 2002; Quintela et al. 2003; Paramá et al. 2004; Paramá et al. 2007; Harikrishnan et al. 2010; Jin et al. 2010; Budino et al. 2012; Park et al. 2014). However, most studies on these drugs/chemicals have been based on in vitro experiments to test efficacy, whereas there have been few studies that have demonstrated their antiparasitic effects through both in vitro and in vivo experiments. For olive flounder farms in South Korea, the government granted item permissions for the use of formalin (37% formaldehyde) in 2006 and hydrogen peroxide (35% hydrogen peroxide) in 2015, and these agents were proven to be effective by multiple studies (cited above) as aquaculture drugs to treat scuticociliate infection, and were subsequently commercialized (NIFS 2016). Although these antiparasitic bath treatments were effective in controlling external scuticociliate infection to some degree, it was impossible to treat scuticociliate infection of internal organs using these drugs. Thus, there has been a longstanding urgent need to develop a highly safe and effective antiparasitic drug that can be used via both oral and bath administrations in olive flounder farms.

Mebendazole (MBZ, Fig. 1), a synthetic benzimidazole, has been used worldwide as an anthelmintic in both human and veterinary medicine to effectively treat various helminth infections (Choi et al. 2014; Werff et al. 2014). In addition, MBZ has antiparasitic effects on monogeneans that are parasitic on the gills of various freshwater and marine aquatic species, such as the common carp (*Cyprinus carpio*), European eel (*Anguilla anguilla*), goldfish (*Carassuis auratus*), tambaqui (*Colossoma macropomum*), and black rockfish (*Sebastes schlegeli*) (Goven and Amend 1982; Buchmann et al. 1993; Kim et al. 1998; Waller and Buchmann 2001; Chagas et al. 2016). Furthermore, MBZ has also been used to effectively exterminate microsporidia in sticklebacks (*Gasterosteus aculeatus*) (Schmahl and Benini 1998). In contrast, MBZ was shown to be ineffective against eel nematodes (Taraschewski et al. 1988) and monogeneans in red porgy (*Pagrus pagrus*) (Katharios et al. 2006). Similarly, an in vitro study on the antiparasitic effect of MBZ on *P. dicentrachi* isolated from farmed turbot and sea bass revealed no active efficacy (Iglesias et al. 2002). To date, however, there have been no reports on the antiparasitic effect of MBZ on *M. avidus* isolated from olive flounders.

The mechanism underlying the antiparasitic action of MBZ is the inhibition of microtubule formation, which selectively and irreversibly inhibits the glucose uptake of parasites in the intestines (Laclette et al. 1980; Barrowman et al. 1984). To date, information about the activity or toxicity of most

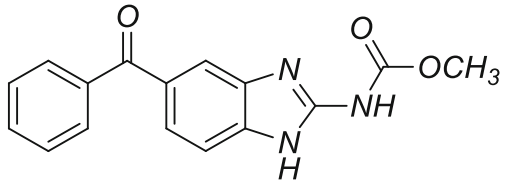

Fig. 1 Structure of mebendazole

molecular drugs, including MBZ, has been obtained through molecule–molecule interactions with other molecules such as drug targets (including enzymes, receptors, and nucleic acids) in the body or pathogens. In contrast to this molecular approach, we, for the first time, approached the problem of MBZ toxicity from the perspective of the individual elements comprising molecules. In other words, the molecular formula of MBZ, an organic compound, is $C_{16}H_{13}N_3O_3$, and the molecule composed of C, H, O, and N was altered by remediation of each element, which had no effect on interactions with known targets. Subsequently, MR MBZ was produced, which retained the efficacy of the parent material, but had lower toxicity, as demonstrated experimentally.

Chemical elements comprise all matter (including living organisms). Newton's law of universal gravitation explains that there are gravitational forces between all objects. These mutual gravitational forces confer energy to the interacting objects. For example, there is gravity between the earth and the moon, and each has an influence on the other through energy exchange. In this case, elements, molecules, and matter that are at a point on the earth where gravity is working could receive energy that is exchanged by gravity. As there are numerous stars and planets in the universe, their gravities can overlap, and full-overlapped gravitational field (FOGF) energy will be present in the FOGF formed in this way. When energy is pulled by the gravity of the earth, matter containing numerous elements on the earth will receive the energy. It is predicted that this occurs mostly through the non-material parts (coexisting with matter of matter that are mostly unexplored, and FOGF energy reception via the non-material part could improve the material part of matter, which would contribute to normalization of the material part. Hence, it was hypothesized that the toxicity of molecules in living organisms after administration of elements that are unable to normally receive FOGF energy via the non-material part would be reduced by making FOGF energy reception normal. Regarding this hypothesis, we were able to develop MR MBZ using FOGF energy, which retained the efficacy of MBZ but had lower toxicity, as confirmed experimentally.

To develop an antiparasitic drug that can be used for both oral and bath administration against pathogenic scuticociliates isolated from olive flounders, we selected a total of 19 antiparasitic drugs, including MBZ (commercially available mebendazole), identified through a literature review and database searches for animal drugs, and performed in vitro tests, which indicated that MBZ was the most effective. Subsequently, although in vivo experiments on MBZ showed a clear antiparasitic effect against scuticociliates, apparent histopathological degradation was observed in the liver and kidney of olive flounders, and there were significant increases in hematological parameters, including glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, and alkaline phosphatase, which are indicators of hepatotoxicity. To address these side effects, we attempted to remove MBZ toxicity using a completely different new method, which involved in the production of material remediated MBZ (MR MBZ) that retains MBZ efficacy but is less toxic. The effects of this derived product were subsequently compared with those of the commercially available MBZ, and it was consequently found that MR MBZ maintained the efficacy of MBZ but had lower side effects.

Materials and methods

Isolation and subculture of scuticociliates (ciliates)

The ciliates used in this study were obtained from the Pathology Research Division of the National Institute of Fisheries Science (NIFS). The ciliates, which were identified as *M. avidus* using species-specific oligonucleotide primers reported by Seo et al. (2013), were isolated from the ascitic fluids of olive flounders in a local farm that had suffered mass mortality, followed by continuous subculture in the laboratory. For subculture, ciliates were inoculated into a culture of the CHSE-214 (Chinook salmon embryo, ATCC CRL 1691) cell for 3–5 days at 22 °C. The CHSE-214 cell line was maintained in Eagle's minimum essential medium (MEM; Sigma-Aldrich, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) at 20 °C under aseptic culture conditions to grow the ciliates.

Candidate antiparasitics

The 19 candidate antiparasitics included six types of benzimidazoles (albendazole, febantel, fenbendazole, MBZ, oxfendazole, and oxibendazole), three types of avermectin derivatives (abamectin, ivermectin, and selamectin), and levamisole, tetramisole, benzyl benzonate, clorsulon, deltamethrin, imidacloprid, moxidectin, piperazine, pyrantel, and trichlorfon. These were all used as pure reagents from Sigma-Aldrich (USA) at 98%–99% active concentrations. Stock solutions (1000 mg/L) of the antiparasitics were prepared by dissolving in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). The stocks were maintained in a refrigerator at 4 °C prior to use in in vitro efficacy tests.

Table 1 Scoring system for assessing compounds for in vitro efficacy against *Miamiensis avidus* from olive flounder

Score	Interpretation	
	Motility	Morphology
3 (high effect)	No sign of mortality or cilia movement	Extensive lysis with few cells intact
2 (moderate effect)	More than 50% of ciliates stationary, but cilia still beating	Cells stationary and irregular; more than 50% of cells round and lysis evident
1 (low effect)	Slowly motile, showing in more than 50% of ciliates; approximately 50% of ciliates stationary, but cilia still beating	Cells round; less than 50% of cells round or irregularly shaped; approximately 50% of cells round or irregularly shaped
0 (no effect)	Highly motile or normal	No change; cells elliptical

In vitro antiparasitic efficacy

The ciliate-inoculated CHSE-214 cell and sterilized artificial seawater (20‰) was cultured at 22 °C in an incubator. After 3–5 days, the ciliates had proliferated to a sufficient number for in vitro efficacy analysis. Efficacy was assessed after mixing ciliates, which grew with sufficient amounts of cells as prey, with antiparasitics. In detail, 100 μL (1×10^5) ciliates cultured in MEM and seawater for 24 h was aliquoted into each well of 96-well plates, and then 100 μL of different concentrations of 19 antiparasitics were dispensed into each of the wells containing ciliates (200 μL in total), followed by mixing. Subsequently, assay mixtures were observed after 1, 2, 4, and 24 h under a light microscope (× 200 magnification). Efficacy was assessed based on a modification of the methods reported by Jee et al. (2002) and Jin et al. (2010). In detail, the motility and morphology of the ciliates in wells were mainly scored as indicated in Table 1. Each efficacy assay was repeated three times.

Experimental fish and conditions

Juvenile olive flounders were purchased from a commercial farm in Pohang, Korea, which underwent disease tests for various fish pathogens (bacteria, virus, parasites), and also had no history of antibiotic administration. Two experiments were conducted to focus on the relationship between effects of MBZ treatment against ciliates, and toxicity effect of only MBZ by oral and bath administration. A total of 325 fishes were used. Fish were selected randomly and divided two groups (n = [illegible] and 225). They were fully acclimatized to fiberglass reinforced plastic aquarium (capacity, 1 ton) equipped with flow-through filtered seawater and aeration in a professional rearing room in the NIFS. Prior to experimentation, morphologically sound fish with similar body weights and body lengths were selected, and acclimated to the experimental aquaria. All the fish were hand-fed a commercial fish diet (Suhyup Feed, Korea) once a day at a constant rate of 2% of their body weight during the acclimation. Water temperature during the experimental period was stably maintained at approximately 20 °C (± 1.0), the optimal water temperature for olive flounders.

Treatment of fish

Fish (average body weight, 22.2 ± 3.6 g) were administered both oral and bath treatments, in each of which the experimental fish were divided into five aquarium groups, including a control (= placebo). Each group contained 20 olive flounders (200-L aquarium), which were intentionally infected with ciliates (average of 10^5/fish) through intraperitoneal injection. At 1 day, MBZ was administered by oral or bath treatment (in vivo test). Thereafter, mortality was measured daily for a total of 20 days, during which time cumulative mortality rates were monitored.

For oral treatment, pharmaceutical feeds were prepared as follows: Commercial powdered mixed feeds were kneaded with distilled water to produce bite-sized amounts for the olive flounders, to which MBZ was added after weighing to give preparations containing 20, 50, 100, and 200 mg/kg body weight (B.W.) of olive flounder. These were prepared similar to herb-type pills and stored in a freezer (− 80 °C) prior to experimentation. These were taken from the freezer on the day of the experiment and forcefully administered by single dose into the stomach of each unanesthetized olive flounder using forceps. For bath treatment, olive flounders were bathed for 1 h in the aquaria that were prepared by dilution of MBZ to final concentrations of 20, 50, 100, and 200 mg/L. Thereafter, the fish were transferred to aquaria pre-filled with fresh seawater, and cumulative mortality rates were monitored as described for the oral treatment. All dead subjects were dissected to check for the presence of ciliates in the body. All experiments were performed for MR MBZ in the same process as MBZ. Efficacy was finally determined in terms of the relative percentage survival (RPS) using the following equation (Kang et al. 2013):

$$\text{RPS} = [1-(\text{percentage of experimental group}/\text{percentage of control group})] \times 100\%$$

For determination of MBZ toxicity, we used a 100 mg/kg B.W. (mg/L) preparation, which showed efficacy in in vitro and in vivo tests, and a 500 mg/kg B.W. (mg/L) preparation. In this experiment, for both oral and bath administrations, we used six aquarium (250 L) groups, including a control, each of which contained 30 olive flounders (average body weight: 30.5 ± 4.3 g). For oral treatment, pharmaceutical feeds containing MBZ, prepared to provide 100 and 500 mg/kg B.W., were given by single-dose administration as described in in vivo test. For bath treatment, 100 and 500 mg/L MBZ preparations were administered by bathing fish for 1 h. The time point immediately after oral and bath treatments was considered 0 h, and thereafter five olive flounders were caught at 6 h, and 1, 2, 7, and 14 days, for blood collection. Experimental procedures for MR MBZ followed the same process as MBZ. The oral and bath treatments were replicated twice.

Hematological parameters and biochemical analysis

After both oral and bath treatments, fish were used for blood collection. For blood collection, approximately 1 mL of blood was collected from the caudal vessel of the tail of each experimental fish using a disposable syringe (26G × 12.7 mm needle) without anesthesia. Of the whole blood, 20 μL was immediately aliquoted into a microtube (Axygen Co. USA, 1.7 mL), treated with 3 μL heparin solution (5000 IU/mL; JW Pharmaceutical, Korea), and well mixed. The remaining blood was collected in a separate microtube. The microtube containing whole blood was incubated at room temperature for approximately 2 h, and was then placed in a refrigerator (4 °C) for 4 h, followed by centrifugation at 5900 RCF and 4 °C for 10 min using an Eppendorf 5415R centrifuge to separate serum. All of this serum was immediately stored at − 80 °C, and subsequently used for blood biochemical analysis within 3 days. Hematocrit (Ht) was measured using the microhematocrit method, whereas hemoglobin (Hb), glucose (GLU), glutamate oxaloacetate transaminase (GOT)/aspartate aminotransferase (AST), glutamate pyruvate transaminase (GPT)/alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total cholesterol (CHO) were analyzed using a FUJI DRI-CHEM 4500 automatic dry-type chemistry analyzer (FUJI PHOTO FILM Co., Japan). The data obtained from these hematological and biochemical analyses were analyzed for significant differences ($p < 0.05$) between mean values with t test using SigmaPlot 8.0. Tissues, including gill, liver, and kidney, were collected from one dissected fish and were used for histopathological assays. The hematological and biochemical assays were replicated twice.

Histopathology and microscopy

Autopsy was performed as soon as blood had been collected for blood analysis, in which samples (10 × 5 × 3 mm in size) of tissues such as liver, kidney, and gill were extracted and fixed in a buffered neutral formalin fixative (Sigma) for 24 h. The fixed organ samples were thin-sectioned (approx. 5 × 5 × 1.5 mm) and fixed again in the same fixative (12 h), followed by washing with water, dehydration, clearing, and paraffin infiltration. The organ samples were then embedded in paraffin to prepare paraffin blocks, which were then subjected to thin sectioning at a thickness of 4–5 μm using a microtome (Leica) and subsequently placed on slide glasses and dried. The prepared tissue sections were then stained with Herris's hematoxylin and eosin (H&E) (Kiernan 2008; Llewellyn 2009) to observe morphological changes in the tissues. The tissue sample slides were observed using a light microscope (ZEISS), during which tissue images were captured using an accessory imaging system (ZEISS Software, ZEN).

Production of material remediated MBZ using full-overlapped gravitational field energy

A certain point between the center of the earth and the center of certain outer planet is where the gravities of each interact, where their energies are exchanged, and also where numerous other gravities are working. Therefore, matter composed of elements at this point could receive FOGF energy, named "Dong-ta-ra-con-ching," and in turn more energy will be received by rotation and revolution of the earth. In order to induce this energy into matter, we developed the material remediation installation "Putor" (Fig. 2), which could force synchronization in MBZ, make MBZ normally receive FOGF energy, and reduce toxicity of MBZ. It consisted of the "Eup-cha" and the "Nap-cha" putor program. The Eup-cha putor program induces energy from the center of the earth, whereas the Nap-cha putor program amplifies numerous weak extraterrestrial energies using natural matter, silkworm. To treat MBZ, the Eup-cha putor program was installed under MBZ and the Nap-cha putor program was installed over MBZ.

The principle of this Putor installation is briefly explained. As mentioned in the introduction, elements on the earth receive FOGF energy, and thus living organisms comprised of these elements are also expected to receive FOGF energy. We investigated the capacities of FOGF energy reception in various living organisms, and finally we chose the silkworm because it seemed to receive energy almost all day. We raised them, studied their properties every spring and autumn for 15 years, and could obtain the suitable silkworm, named "Ho-ho-nong", for our purpose. Silkworm excrement was the most appropriate because it was not denatured easily, unlike other parts such as heads, skins, and silk glands that are composed of protein. However, silkworm excrement alone

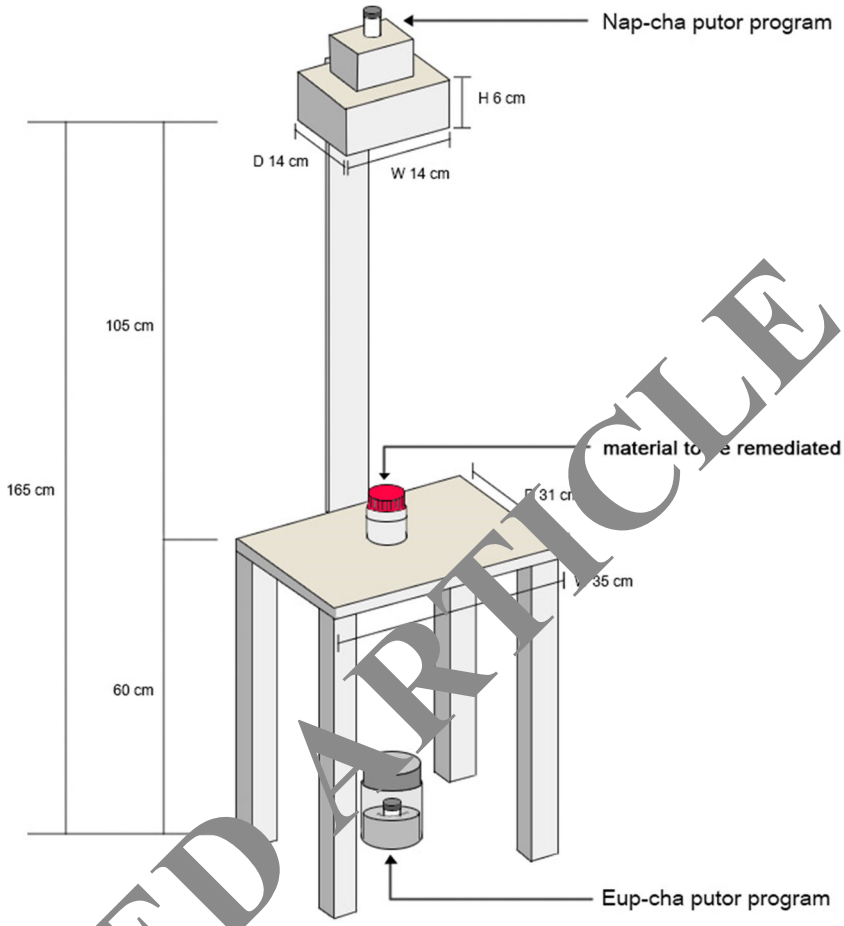

Fig. 2 Dimensions of the material remediation installation "Putor"

was not enough to remediate the destroyed elements, and therefore we placed the excrement at the top and the bottom of several trees and induced amplification, using the energy reception ability of the trees. The Nap-cha and the Eup-cha putor programs of the material remediation installation Putor were produced using this procedure.

Chemistry of MBZ and MR MBZ

^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Unity INOVA 400 MHz in DMSO-d_6 from Sigma-Aldrich (USA). Chemical shifts were expressed in parts per million relative to DMSO-d_6 (^{1}H, 2.5 ppm; ^{13}C, 39.5 ppm). High-resolution mass spectra electrospray ionization (HRMS-ESI) analyses were carried out by using an Agilent technologies 6220 Accurate-Mass TOF LC/MS spectrometer.

Methyl (6-benzoyl-1*H*-benzo[*d*]imidazol-2-yl) carbamate (Mebendazole)

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.09 (brs, 1H), 11.67 (brs, 1H), 7.85 (s, 1H), 7.73–7.70 (m, 2H), 7.65 (m, 1H), 7.58–7.50 (m, 4H), 3.77 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6/TFA): δ 195.0, 152.9, 146.2, 137.5, 133.2, 133.1, 132.7, 129.7, 129.6, 128.7, 126.5, 115.5, 113.4, 53.9.

HRMS (ESI) m/z calculated for $C_{16}H_{14}N_3O_3^+$ $[M + H]^+$ 296.1030, found 296.1032.

Methyl (6-benzoyl-1*H*-benzo[*d*]imidazol-2-yl) carbamate (MR Mebendazole)

^{1}H NMR (400 MHz, DMSO-d_6): δ 11.85 (brs, 2H), 7.85 (s, 1H), 7.72–7.70 (m, 2H), 7.65 (m, 1H), 7.58–7.53 (m, 4H), 3.77 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6/TFA): δ 195.0, 152.9, 146.2, 137.6, 133.4, 133.0, 132.8, 129.8, 129.6, 128.7, 126.6, 115.6, 113, 5, 54.0.

HRMS (ESI) m/z calculated for $C_{16}H_{14}N_3O_3^+$ $[M+H]^+$ 296.1030, found 296.1032.

Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Table 2 In vitro antiparasitic activity of candidate agents against *Miamiensis avidus* under culture conditions in minimum essential medium (MEM)

Compound	Score of motility and morphology (mg/L) at 24 h											
	10	50	100	200	300	400	500	600	700	800	900	1000
Abamectin	0	0	0	0	0	1	3	3	3	3	3	3
Albendazole	0	0	0	0	0	0	0	0	1	1	3	3
Benzyl benzonate	0	0	0	0	0	0	0	0	1	2	2	2
Clorsulon	0	0	0	0	0	0	0	0	0	0	1	1
Deltamethrin	0	0	0	0	0	0	0	0	0	0	0	1
Febantel	0	0	0	0	0	0	0	0	0	0	0	0
Fenbendazole	0	0	0	0	0	0	0	1	1	1	1	3
Imidacloprid	0	0	0	0	0	0	0	0	0	0	0	1
Ivermectin	0	0	0	0	0	0	0	0	0	0	0	0
Levamisole	0	0	0	0	0	0	0	0	0	0	0	0
Mebendazole	0	0	2	3	3	3	3	3	3	3	3	3
Moxidectin	0	0	0	0	0	0	0	0	0	2	3	3
Oxfendazole	0	0	0	0	0	0	0	1	1	1	1	3
Oxibendazole	0	0	0	0	0	0	0	0	0	0	0	0
Piperazine	0	0	0	0	0	0	0	0	0	0	0	0
Pyrantel	0	0	0	0	0	0	0	0	0	0	0	0
Selamectin	0	0	0	0	0	1	2	3	3	3	3	3
Tetramisole	0	0	0	0	0	0	0	0	0	0	0	0
Trichlorfon	0	0	0	0	0	0	0	1	2	3	3	3

Table 3 In vitro antiparasitic activity of candidate agents against *Miamiensis avidus* under culture conditions in seawater

Compound	Score of motility and morphology (mg/L) at 24 h											
	10	50	100	200	300	400	500	600	700	800	900	1000
Abamectin	0	0	0	0	0	0	0	0	0	2	3	3
Albendazole	0	0	0	0	0	0	0	0	1	1	3	3
Benzyl benzonate	0	0	0	0	0	0	1	1	3	3	3	3
Clorsulon	0	0	0	0	0	0	0	0	0	0	0	0
Deltamethrin	0	0	0	0	0	0	0	0	0	0	0	0
Febantel	0	0	0	0	0	0	0	0	0	0	0	0
Fenbendazole	0	0	0	0	0	0	0	1	1	1	1	3
Imidacloprid	0	0	0	0	0	0	0	0	0	0	0	0
Ivermectin	0	0	0	0	0	0	0	0	0	0	0	0
Levamisole	0	0	0	0	0	0	0	0	0	0	0	0
Mebendazole	0	0	2	3	3	3	3	3	3	3	3	3
Moxidectin	0	0	0	0	0	0	0	0	0	0	0	2
Oxfendazole	0	0	0	0	0	0	0	1	1	1	1	3
Oxibendazole	0	0	0	0	0	0	0	0	0	0	0	0
Piperazine	0	0	0	0	0	0	0	0	0	0	0	0
Pyrantel	0	0	0	0	0	0	0	0	0	0	0	0
Selamectin	0	0	0	0	0	0	1	2	3	3	3	3
Tetramisole	0	0	0	0	0	0	0	0	0	0	0	0
Trichlorfon	0	0	0	0	0	0	0	1	2	3	3	3

Table 4 In vitro antiparasitic activity of material remediated mebendazole (MR MBZ) and mebendazole (MBZ) against *Miamiensis avidus* under culture conditions in minimum essential medium (MEM) and seawater

Compound	Score of motility and morphology (mg/L) at 24 h in MEM											
MR MBZ	10	50	100	200	300	400	500	600	700	800	900	1000
	0	2	3	3	3	3	3	3	3	3	3	3
MBZ	10	50	100	200	300	400	500	600	700	800	900	1000
	0	0	2	3	3	3	3	3	3	3	3	3
Compound	Score of motility and morphology (mg/L) at 24 h in seawater medium											
MR MBZ	10	50	100	200	300	400	500	600	700	800	900	1000
	0	2	3	3	3	3	3	3	3	3	3	3
MBZ	10	50	100	200	300	400	500	600	700	800	900	1000
	0	0	2	3	3	3	3	3	3	3	3	3

Results

Effect of in vitro antiparasitic activity

The antiparasitic activities of the 19 candidate antiparasitic drugs on ciliates in MEM and seawater are presented in Tables 2 and 3, respectively. In terms of efficacy, albendazole, febantel, fenbendazole, MBZ, oxfendazole, oxibendazole, ivermectin, selamectin, levamisole, tetramisole, piperazine, pyrantel, and trichlorfon showed similar activities. In contrast, abamectin, clorsulon, deltamethrin, imidacloprid, and moxidectin showed better activities in MEM than in seawater, whereas benzyl benzonate had a higher efficacy in seawater. In both MEM and seawater, MBZ began to display activity from 100 mg/L (score 2), and had the highest effect at 200 mg/L (score 3). Abamectin showed the second highest effect at 500 mg/L (score 3) in MEM, although its efficacy was low in seawater at 900 mg/L (score 2). Selamectin and trichlorfon were most effective at 700 and 800 mg/L (score 3), respectively, in both MEM and seawater. Moxidectin had highest efficacy at 900 mg/L (score 3) in MEM, whereas it showed low effects in seawater at 1000 mg/L (score 2). The remaining antiparasitic drugs showed low effect (score 1) or no effect (score 0), even at 1000 mg/L. Among 19 candidate antiparasitics, MBZ showed the highest efficacy in an in vitro efficacy test against the pathogenic *M. avidus* isolated from olive flounders.

The in vitro antiparasitic activities of MR MBZ and MBZ are shown in Table 4. In MEM, MR MBZ had a moderate effect (score 2) at 50 mg/L and a high effect (score 3) at 100 mg/L, whereas MBZ showed a moderate effect (score 2) at 100 mg/L and a high effect (score 3) at 200 mg/L. In sterile seawater, MR MBZ and MBZ displayed a high effect (score 3) and a moderate effect (score 2) at 100 mg/L, respectively. Thus, MR MBZ was found to have a better antiparasitic activity in both MEM and seawater.

Treatment effect of MR MBZ and MBZ

For the oral treatment, MR MBZ resulted in 55.0% RPS at 200 mg/kg B.W. and 37.8% RPS at 100 mg/kg B.W. (Fig. 3a), whereas MBZ resulted in 43.5% RPS at 200 mg/kg B.W. and 21.7% RPS at 100 mg/kg B.W. (Fig. 3b). Although both MR MBZ and MBZ showed the highest antiparasitic activity at 200 mg/kg B.W., they began to be effective from 100 mg/kg B.W. For the bath treatment, MR MBZ resulted in 51.1% RPS at 200 mg/L and 42.8% RPS at 100 mg/L (Fig. 4a), whereas MBZ resulted in 44.4% RPS at 200 mg/L and 37.8% RPS at 100 mg/L (Fig. 4b). As in the oral administration, both MR MBZ and MBZ were the most effective at 200 mg/L. However, both resulted in slight increases in RPS at 100 mg/L, which is in contrast to that observed in the oral administration. MR MBZ was, nevertheless, more effective than MBZ in both oral and bath administrations.

Effect of MR MBZ and MBZ on hematological and biochemical parameters

The blood analysis results for MR MBZ and MBZ treatments are presented in Table 5. The MR MBZ group was subjected to single-dose oral treatment at 100 and 500 mg/kg B.W., respectively, and bath treatment at 100 and 500 mg/L for 1 h at each concentration, which were then compared with the respective control groups. For all hematological (Hb and Ht) and biochemical (GOT, GPT, GLU, ALP, and TCHO) parameters analyzed, there were no significant increases ($p < 0.05$). In contrast, in both oral and bath treatments, the MBZ group showed significant increases in hematological and biochemical parameters ($p < 0.05$) in a time series compared with the control group. When orally administered at 100 mg/kg B.W., there were no distinct changes in hematological parameters (Hb and Ht) until the 14th day compared with the control group, whereas there were changes in biochemical parameters. In detail, ALP increased after 6 h, whereas GOT, GPT, GLU, and TCHO showed significant increases a later time

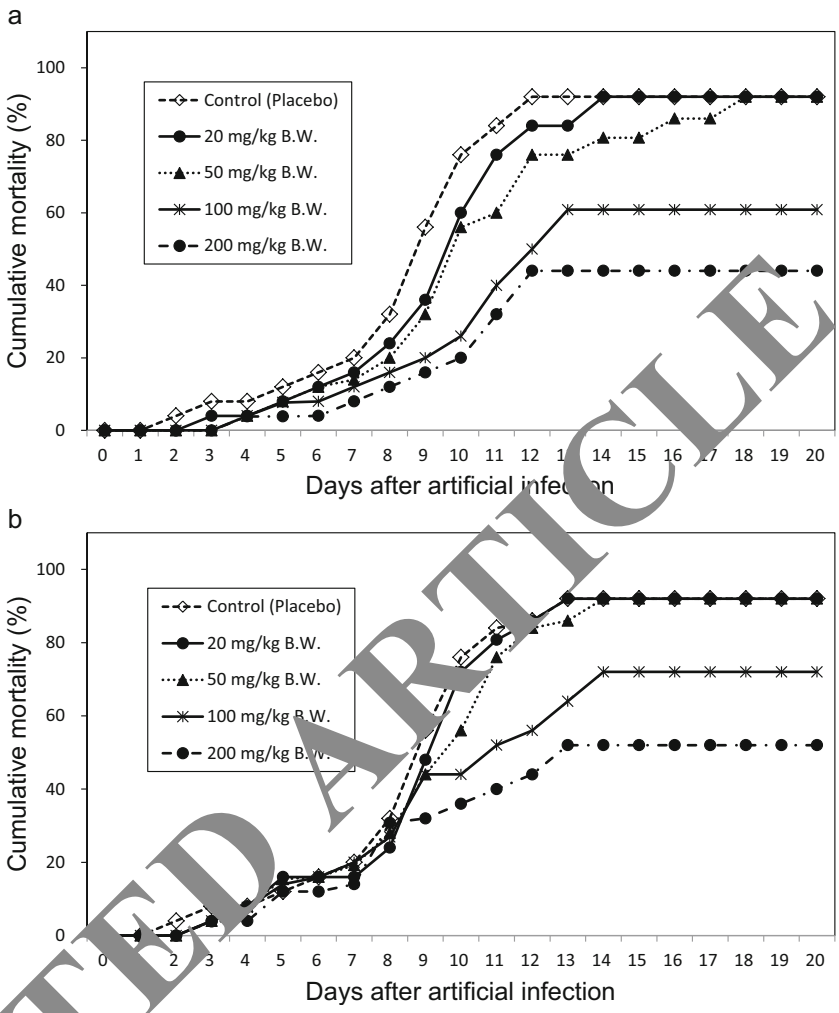

Fig. 3 Oral treatment. Juvenile olive flounders (*Paralychthys olivaceus*) were artificially infected with *Miamiensis avidus* through intraperitoneal injection, and then pharmaceutical feeds containing material-remediated mebendazole (**a**) and mebendazole (**b**), each at concentrations of 20, 50, 100, and 200 mg/kg body weight (B.W.), were given by single oral administration. Thereafter, cumulative mortality rates were measured for 20 days

points. When administered at 500 mg/kg B.W., there was no change in Hb until the 14th day, whereas Ht had increased significantly by the second day. Of the biochemical parameters, GOT, ALP, and TCHO increased significantly from 6 h, and all measured parameters (GOT, GPT, GLU, ALP, and TCHO) had increased significantly by the 14th day. In the 100 mg/L bath treatment, there were no distinct changes in hematological parameters (Hb and Ht) until the 14th day compared with the control group, whereas changes in the patterns of biochemical parameters were similar to those in the group with oral administration at 100 mg/kg B.W. In contrast, in the 500 mg/L bath treatment, Hb, GOT, and ALP increased significantly from 6 h, whereas Hb, Ht, GOT, GPT, ALP, and TCHO showed substantial increases on the second day. Thereafter, the number of parameters showing significant increases gradually declined. Of the hematological parameters, Hb and Ht increased significantly at high treatment concentrations in both the bath and oral administration groups, whereas these trends were not shown in the biochemical parameters. Among all groups, however, there were no mortalities in olive flounders throughout the entire experimental period following both oral and bath treatments.

Effect of MR MBZ and MBZ on histopathological changes

Figure 5 shows the tissues of normal liver (a) and kidney (b) in the control group without administration of mebendazole. Representative histopathological characteristics of groups administered with MR MBZ and MBZ are shown for comparison in Figs. 6 and 7, respectively. For the MR MBZ administration group (Fig. 6), there were no abnormal lesions in liver and kidney tissues during the experimental period from the first day (a, b) to the 14th day (c, d) after oral administration with 100 mg/kg B.W. and from the first day (6E, 6F) to the 14th day (6G, 6H) after bath treatment for 1 h with 500 mg/L of MR MBZ, which was similar to the control group (5A, 5B). For the MBZ administration group (Fig. 7), however, slight atrophy was found in hepatocytes and renal tubule epithelial cells, and eosinophilic hyaline droplet degeneration, which is

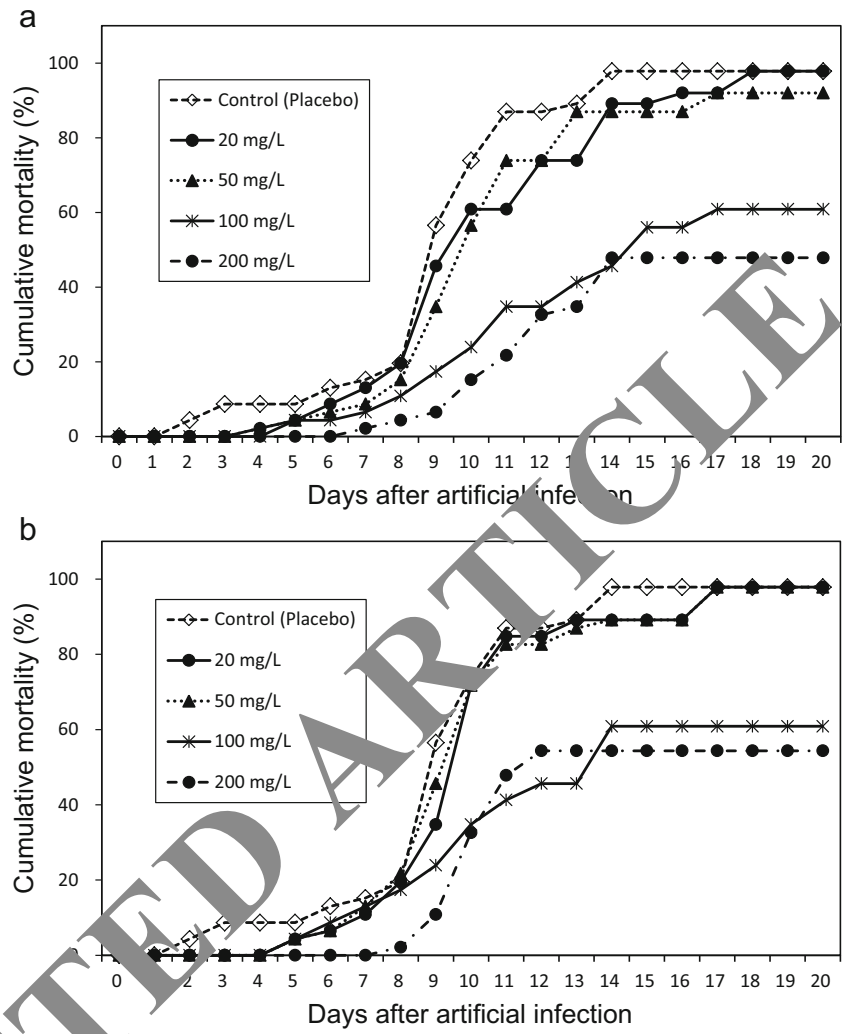

Fig. 4 Bath treatment. Juvenile olive flounders (*Paralychthys olivaceus*) were artificially infected with *Miamiensis avidus* through intraperitoneal injection, and then bath treated in pharmaceutical water tanks containing material-remediated mebendazole (**a**) and mebendazole (**b**), each at concentrations of 20, 50, 100, and 200 mg/L, for 1 h. Thereafter, cumulative mortality rates were measured for 20 days

suspected to indicate protein degradation, was partly observed in the cytoplasm of renal tubule epithelial cells from 1 day after oral administration with 100 mg/kg B.W. of MBZ (a, b), whereas control group tissues (a, b) were found to be normal. At the same concentration, cellular atrophy was clearly observed in hepatocytes and renal tubule epithelial cells until the 14th day, and hyaline droplet degeneration was still clearly visible in the cytoplasm of renal tubule epithelial cells (c, d). After bathing with 500 mg/L of MBZ for 1 h, atrophy was clearly observed in hepatocytes and renal tubule epithelial cells on day 1 (e, f). Hepatocytes, in particular, clearly showed atrophy (e), and hyaline droplet degeneration in renal tubule epithelial cells was also partly observed (f). On day 14 after bathing with 500 mg/L MBZ for 1 h, there was degeneration of hepatocytes and renal tubules similar to that observed at 1 day after bathing in the same concentration (e, f), as well as atrophy of hepatocytes and overall severe degradation in some samples (g). In addition, we observed degeneration of renal tubule epithelial cells, renal tubules, glomeruli, and neighboring interstitial cells comprising hemopoietic tissues (h). In contrast, no abnormal histopathological degradation was observed the gills of fish in any of the experimental groups. Since there were no significant concentration-dependent differences in histopathological characteristics with time after oral and bath administrations of MBZ, we have not shown the pathological tissues for each administration group. Overall, high MBZ concentrations rather than low concentrations and bath treatment rather than oral administration tended to result in relatively clear degradation.

Discussion

Scuticociliates are well-known opportunistic pathogens that show substantially high pathogenicity toward various marine fish. In Korea, approved bath treatments (e.g., formalin) have been used to control scuticociliatosis because commercially antiparasitic drugs are very limited by law. Especially, formalin efficacy has been gradually reduced in olive flounder farms. Iglesias et al. (2002) reported that 52 candidate

Table 5 Changes in hematological and biochemical parameters observed in a time series after single-dose oral treatment (mg/kg body weight (B.W.)) and bath treatment (mg/L) for 1 h with material remediated mebendazole (MR MBZ) and mebendazole (MBZ), given to juvenile olive flounder (*Paralychthys olivaceus*)

Time (days)	Experimental groups		Hb (g/dL)	Ht (%)	GOT (U/L)	GPT (U/L)	GLU (mg/dL)	ALP (U/L)	TCHO (mg/dL)
6 h	Control		7.5 ± 1.3	25.2 ± 4.6	25.4 ± 2.1	2.6 ± 0.5	13.8 ± 3.0	161.0 ± 30.6	149.6 ± 28.5
	MR MBZ	100 oral	6.8 ± 1.0	25.5 ± 1.0	25.3 ± 2.5	2.2 ± 1.6	12.3 ± 3.3	152.6 ± 55.8	136.2 ± 21.1
		500 oral	6.2 ± 0.5	27.2 ± 5.1	21.5 ± 5.3	2.6 ± 2.1	18.3 ± 5.7	161.0 ± 77.9	157.5 ± 16.8
		100 bath	7.4 ± 2.5	23.4 ± 4.6	23.0 ± 8.7	2.2 ± 0.4	12.5 ± 2.6	152.2 ± 42.4	133.6 ± 28.9
		500 bath	6.2 ± 1.3	22.8 ± 2.3	24.0 ± 7.0	2.0 ± 0.8	15.5 ± 2.2	147.0 ± 23.4	13[illegible] ± 21.7
	MBZ	100 oral	8.2 ± 0.3	26.0 ± 1.8	29.3 ± 5.3	3.8 ± 1.0	16.8 ± 4.6	224.5 ± 21.7*	148.8 ± 23.2
		500 oral	9.0 ± 0.8	30.4 ± 1.5*	41.9 ± 5.7*	4.0 ± 1.4	17.5 ± 4.4	313.0 ± 2[illegible]*	240.3 ± 10.2*
		100 bath	8.6 ± 1.4	26.0 ± 2.2	29.8 ± 3.6	3.0 ± 1.4	13.0 ± 2.6	209.5 ± [illegible]6.2	203.3 ± 35.6*
		500 bath	9.0 ± 0.6*	29.3 ± 2.9	30.5 ± 2.6*	3.5 ± 0.6	17.3 ± 3.6	23[illegible]5 ± 49.4*	263.3 ± 32.7
1 day	Control		7.6 ± 1.8	27.2 ± 4.9	23.1 ± 5.7	2.6 ± 0.9	14.6 ± 4.3	16[illegible] ± 28.8	154.4 ± 26.3
	MR MBZ	100 oral	7.1 ± 0.5	25.0 ± 3.7	25.3 ± 5.5	2.2 ± 1.1	15.6 ± 3.2	156.0 [illegible].9	144.4 ± 36.3
		500 oral	8.3 ± 3.3	27.0 ± 1.8	23.3 ± 1.9	2.0 ± 1.2	19.5 ± [illegible]3	[illegible]2 ± 23.0	144.8 ± 31.7
		100 bath	7.5 ± 0.5	24.7 ± 1.5	22.5 ± 4.2	2.4 ± 1.1	12.4 ± [illegible]	159.4 ± 29.9	125.2 ± 26.3
		500 bath	8.0 ± 1.5	24.8 ± 3.7	24.0 ± 2.6	2.2 ± 0.8	[illegible]2 ± 3.1	149.6 ± 27.2	111.2 ± 7.9
	MBZ	100 oral	8.3 ± 1.1	29.2 ± 3.3	30.6 ± 3.5*	3.6 ± 1.3	22[illegible] ± 3.4*	200.0 ± 43.9	156.2 ± 49.4
		500 oral	9.5 ± 1.1	33.2 ± 2.4*	39.3 ± 4.3*	5.8 ± 1.5*	[illegible].0 ± [illegible].0*	216.7 ± 41.8	223.4 ± 50.6*
		100 bath	8.3 ± 0.6	30.4 ± 4.7	28.8 ± 3.3	3.6 ± 0.9	1[illegible] ± 3.3	236.7 ± 32.3*	246.3 ± 40.4*
		500 bath	9.2 ± 1.5	33.8 ± 4.8	39.5 ± 4.0*	5.2 ± [illegible]	17.0 ± 3.7	239.7 ± 63.2	185.0 ± 24.9
2 days	Control		7.9 ± 1.5	27.0 ± 2.6	22.1 ± 4.8	2.7 ± [illegible].1	13.0 ± 4.4	154.8 ± 19.0	132.2 ± 28.9
	MR MBZ	100 oral	7.7 ± 0.4	26.2 ± 5.0	25.6 ± [illegible]	3.7 ± [illegible].2	10.0 ± 2.8	154.0 ± 44.2	116.6 ± 23.7
		500 oral	8.1 ± 1.2	23.7 ± 0.6	23[illegible]5.1	[illegible]3.3 ± 1.3	11.8 ± 3.0	157.0 ± 54.7	140.6 ± 26.4
		100 bath	7.2 ± 1.5	24.7 ± 2.3	24.3 ± [illegible]	2.3 ± 1.3	11.5 ± 2.6	134.2 ± 52.8	142.2 ± 35.7
		500 bath	8.1 ± 1.1	25.7 ± 2.[illegible]	26.0 ± 3.[illegible]	2.7 ± 0.6	17.8 ± 6.5	148.5 ± 66.6	133.2 ± 55.4
	MBZ	100 oral	8.4 ± 1.3	30.3 ± [illegible].6	[illegible]5.5 ± [illegible].1*	11.3 ± 4.0*	20.2 ± 3.7*	198.3 ± 28.7*	223.3 ± 50.1*
		500 oral	9.5 ± 1.1	31[illegible] ± 3.1*	4[illegible] ± 3.0*	28.8 ± 4.7*	28.8 ± 2.6*	218.0 ± 40.8*	193.3 ± 28.5*
		100 bath	8.7 ± 0.5	27.8 ± [illegible].7	30.5 ± 4.2*	4.6 ± 1.5	17.6 ± 3.6	176.0 ± 39.0	207.3 ± 77.0
		500 bath	10.9 ± 0.[illegible]*	31.8 ± 2.9	36.3 ± 4.7*	7.5 ± 1.9*	18.0 ± 4.7	199.0 ± 32.8*	212.7 ± 11.6*
7 days	Control		7.4 ± [illegible].2	25.0 ± 3.5	24.0 ± 1.8	2.5 ± 0.4	14.2 ± 5.5	154.6 ± 38.2	139.0 ± 36.9
	MR MBZ	100 oral	7.2 ± 1.[illegible]	27.0 ± 3.2	24.5 ± 2.6	2.5 ± 1.9	19.0 ± 5.2	142.4 ± 41.8	149.3 ± 25.7
		500 oral	[illegible].1 ± 2.6	27.2 ± 2.2	25.8 ± 3.6	2.3 ± 1.0	18.0 ± 5.5	141.4 ± 32.4	157.4 ± 32.1
		100 bath	8.[illegible] ± 0.[illegible]	27.4 ± 2.4	24.0 ± 2.6	2.4 ± 0.5	15.6 ± 4.8	153.2 ± 52.2	156.4 ± 41.1
		500 ba[illegible]	8.[illegible] ± 1.9	26.8 ± 3.3	23.3 ± 2.5	2.0 ± 1.2	15.8 ± 3.9	130.6 ± 39.5	153.0 ± 34.0
	MBZ	1[illegible] or[illegible]	[illegible].9 ± 1.3	29.4 ± 4.0	44.3 ± 4.6*	19.7 ± 3.2*	24.4 ± 3.6*	178.3 ± 46.6	267.3 ± 57.6*
		500 [illegible]	8.8 ± 1.1	29.4 ± 4.9	56.8 ± 3.3*	22.8 ± 4.1*	26.4 ± 4.2*	206.4 ± 39.2	281.7 ± 19.9*
		100 bath	8.5 ± 0.7	27.0 ± 5.3	38.0 ± 5.6*	10.3 ± 2.5*	19.2 ± 4.1	209.3 ± 27.2	233.7 ± 61.7*
		[illegible]0 bath	9.3 ± 0.5*	29.0 ± 3.2	37.0 ± 4.4*	17.5 ± 2.4*	21.0 ± 4.2	218.5 ± 27.1*	223.7 ± 72.1
14 days	Control		7.5 ± 1.5	25.2 ± 2.6	23.0 ± 3.9	2.8 ± 0.8	12.8 ± 2.3	157.6 ± 39.5	136.8 ± 17.5
	[illegible]R MBZ	100 oral	7.9 ± 1.5	26.3 ± 4.9	23.3 ± 3.5	3.0 ± 0.8	11.5 ± 3.7	167.8 ± 20.7	155.6 ± 31.6
		500 oral	7.3 ± 0.8	26.4 ± 3.3	24.3 ± 4.3	2.8 ± 0.5	11.3 ± 1.7	158.8 ± 68.4	145.8 ± 26.2
		100 bath	7.4 ± 0.6	27.8 ± 2.5	25.0 ± 3.0	2.8 ± 1.0	14.8 ± 4.4	157.2 ± 30.4	134.4 ± 57.5
		500 bath	7.8 ± 1.4	28.3 ± 2.9	25.2 ± 3.6	3.2 ± 0.8	11.5 ± 4.5	140.2 ± 39.3	118.4 ± 32.6
	MBZ	100 oral	9.2 ± 1.5	29.2 ± 3.4	30.5 ± 2.6*	11.5 ± 1.3*	21.8 ± 5.6*	175.6 ± 28.6	216.7 ± 45.3*
		500 oral	9.1 ± 1.3	29.0 ± 3.4	34.7 ± 4.9*	15.7 ± 1.5*	32.3 ± 4.2*	222.3 ± 29.2*	265.3 ± 12.7*
		100 bath	8.9 ± 1.3	29.7 ± 0.6	30.0 ± 2.0*	7.8 ± 1.7*	16.5 ± 3.9	187.4 ± 28.0	296.7 ± 21.2*
		500 bath	9.1 ± 1.3	29.2 ± 2.8	34.0 ± 4.6	12.2 ± 3.6*	17.2 ± 4.0	196.2 ± 48.2	271.0 ± 12.8*

*$p < 0.05$ (t test)

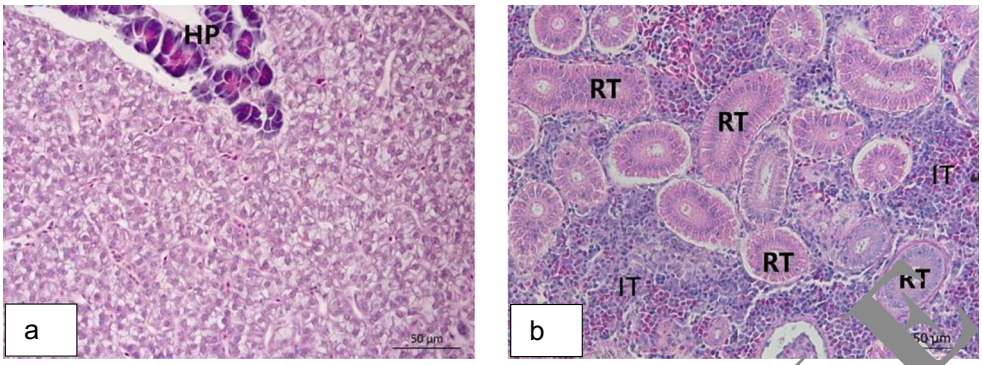

Fig. 5 Histopathological photographs of normal liver (**a**) and normal kidney (**b**) of olive flounder (*Paralychthys olivaceus*) in the control group without administration of mebendazole (H&E stain, scale bar = 50 μm). Hepatopancreatic tissue (HP), interstitial tissue (IT), renal tubule (RT)

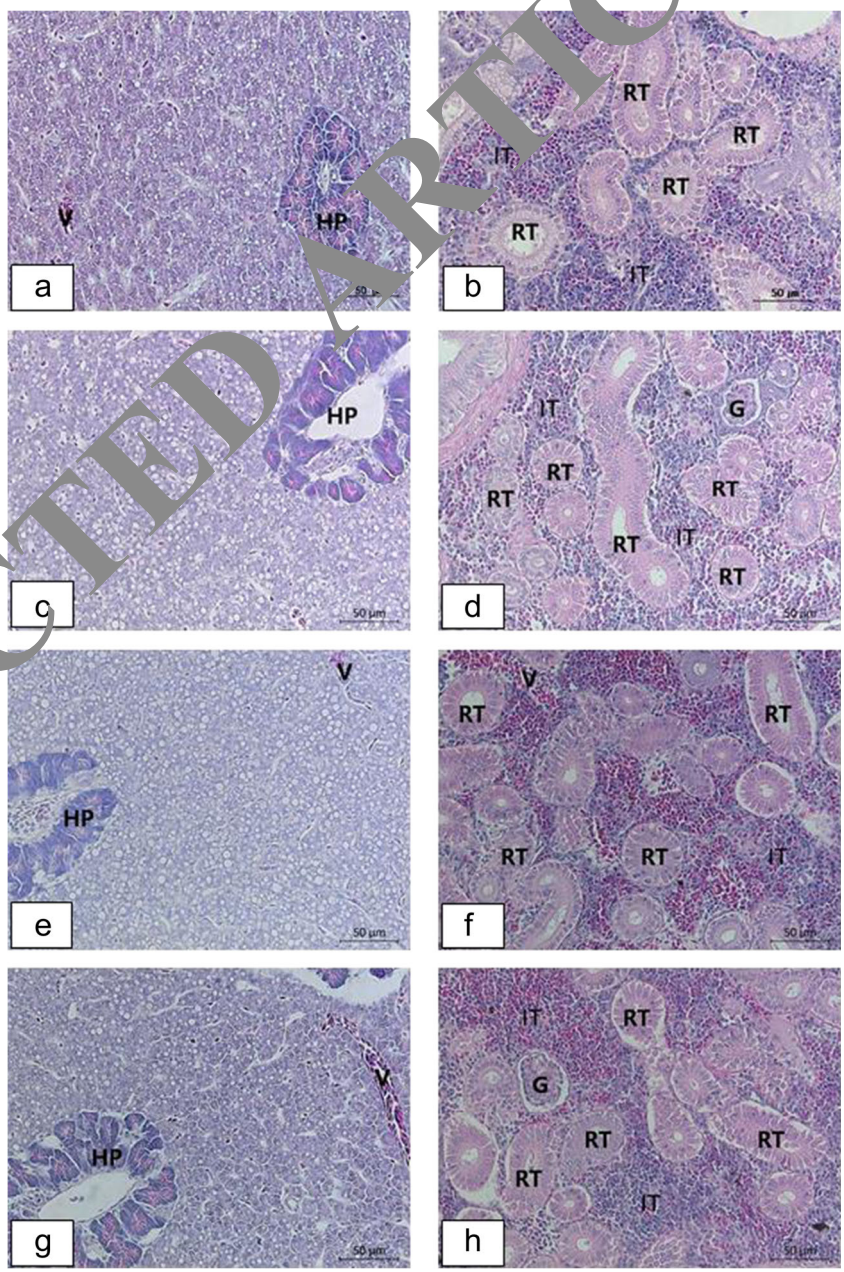

Fig. 6 Representative histopathological photographs of liver and kidney tissues collected from olive flounder (*Paralychthys olivaceus*) administered material remediated mebendazole (MR MBZ) (H&E stain, scale bar = 50 μm). Liver (**a**) and kidney (**b**) 1 day after oral administration at 100 mg/kg body weight (B.W.) Liver (**c**) and kidney (**d**) 14 days after oral administration at 100 mg/kg B.W. Liver (**e**) and kidney (**f**) 1 day after bath administration at 500 mg/L for 1 h. Liver (**g**) and kidney (**h**) 14 days after bath administration at 500 mg/L for 1 h. None of the groups (**a**–**h**) showed abnormal degradation in comparison with the liver (**a**) and kidney (**b**) of the control group. Hepatopancreatic tissue (HP), interstitial tissue (IT), renal tubule (RT), Glomerulus (G), vein (V)

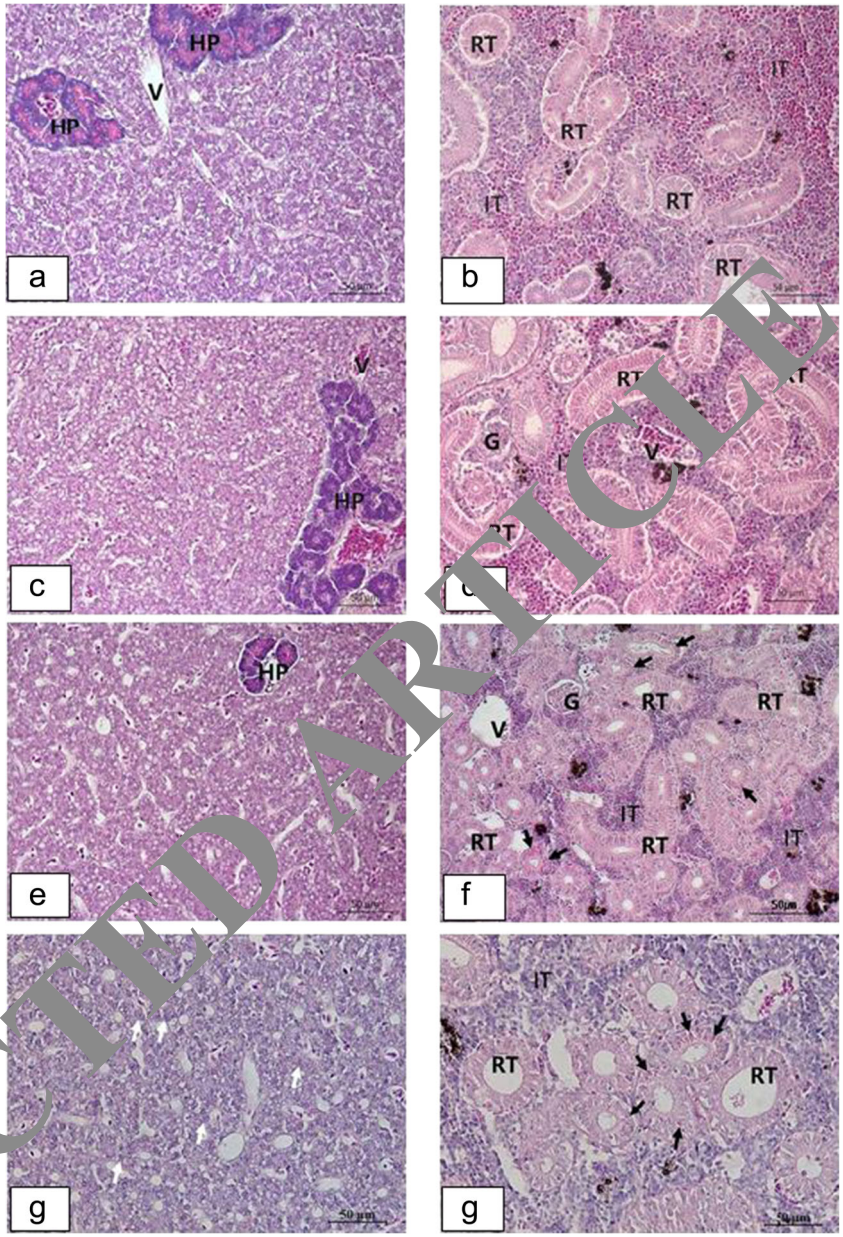

Fig. 7 Representative histopathological photographs of alterations in liver and kidney tissues of olive flounder (*Paralychthys olivaceus*) administered mebendazole (H&E stain, scale bar = 50 μm). Liver (**a**) and kidney (**b**) 1 day after oral administration at 100 mg/kg body weight (B.W.): light atrophy was found in hepatocytes and renal tubule epithelial cells, and eosinophilic hyaline droplet degeneration was partly observed in the cytoplasm of renal tubule epithelial cells. Liver (**c**) and kidney (**d**) 14 days after oral administration at 100 mg/kg B.W.: characteristics shown in (**a**) and (**b**) are still clearly observed. Liver (**e**) and kidney (**f**) 1 day after bath administration at 500 mg/L for 1 h: characteristics similar to those in oral administrations (**c**, **d**) are observed. Liver (**g**) and kidney (**h**) 14 days after bath administration at 500 mg/L for 1 h: there was atrophy of hepatocytes and overall severe degradation in some samples. There was severe degeneration of renal tubule epithelial cells, and degeneration of renal tubules, glomerulus, and neighboring interstitial cells. Hepatopancreatic tissue (HP), interstitial tissue (IT), renal tubule (RT), Glomerulus (G), vein (V), piknosis of hepatocyte (black arrows), eosinopilic droplet (white arrows)

antiprotozoals [illegible] in vitro activity against ciliate *P. dicentrarchi* were isolated from farmed turbot. They estimated susceptibility against ciliates of these antiprotozoals using filtered seawater (salinity 28‰); and albendazole, febantel, ivermectin, MBZ, and trichlorfon did not show antiparasitic activity. MBZ showed good antiparasitic activity in vitro when used in filtered seawater (salinity 20‰) against ciliates, although the others (albendazole, febantel, ivermectin, trichlorfon) were null or very low. Four antiprotozoals showed similar susceptibility effects, but the sensitivity of MBZ did not concur with our results. However, previous researchers did not investigate treatment effect of selected antiprotozoals as an antiparasitic drug in fish infected with ciliates. Therefore, the candidate 19 antiparasitic drugs have been chosen for scuticociliatosis treatment in this study. One of them, MBZ, demonstrated the most effective antiparasitic activity in vitro (Table 2).

The oral method, in which an antiparasitic is administered with feed to cultured fish, does not cause various stresses to mass-cultured fish, and is accordingly the preferred treatment for farmed fish (Kang et al. 2013; Chagas et al. 2016). With respect to oral administration, in farmed fish that are infected by parasites, the degree of infection severity varies, such that some individuals are inferior to other fish in terms of prey competition, which makes it difficult for them to take medicated feeds. Since anorexia occurs at the aggravative stage of scuticociliatosis (Iglesias et al. 2002; Kang et al. 2013), it is not possible to apply the same force-feeding method used for

livestock, which makes it difficult to administer effective antiparasitic treatment. Thus, we performed experiments taking into consideration the fact that a combination of both oral and bath methods would be the most effective antiparasitic method. Accordingly, although oral administration is the best method for treating scuticociliate infection, the likelihood of systemic infection caused by a failure to perform treatment at the optimal time should not be overlooked.

In both human and veterinary medicines, it has been reported that short-term therapy and high-dose administration with MBZ can be accompanied by various side effects (Rosenthal 2009). ALT/GPT or ALP values by MBZ treatment, which are indicators of liver injury, have been found to increase (Seitz et al. 1983; Junge and Mohr 1983; Bekhti et al. 1986; Davis et al. 1986; Bekhti and Pirotte 1987). In addition, it has been shown to result in higher levels of GOT, GPT, ALP, indicating abnormal liver function (Shikiya et al. 1990, 1991a, 1991, 1991c, 1992).

Oral administration of MBZ at a single dosage of 50–200 mg/kg B.W. significantly reduced the infestation level of microcotylid monogeneans (*Microcotyle sebastes*) in black rockfish (Kim and Choi 1998; Kim et al. 1998). We attempted to demonstrate that MBZ would be effective for the treatment of *M. avidus* by applying it orally in olive flounder. MBZ-therapeutic diets administered orally at a single dose of 100–200 mg/kg B.W. improved survival rates effectively. Moreover, bath treatment of MBZ at a dose of 100–200 mg/L h.$^{-1}$ was similar that of oral efficacy. These results might constitute useful data to determine the concentration of MBZ treatment in olive flounder as a control for ciliate.

Few hematological and biochemical studies have been observed in fish after MBZ administration. Treatment of MBZ against natural infections with the monogenean helminth (*Anacanthorus penilabiatus*) have been investigated on cultured pacu (*Piaractus mesopotamicus*), and long-term baths over 24 h at 1, 10, and 100 mg/L resulted in a significant increase in Hb and Ht (Marcos et al. 2001). However, oral administration of a feed supplemented with 0.5–2.0 g MBZ/kg dry ration for treatment of tambaqui (*C. macropomum*) naturally infected with monogeneans did not affect Hb and GLU (Chagas et al. 2016). We dealt with toxicity effects of only MBZ administration on olive flounder through blood analysis. None of the studies cited above is directly comparable to the present study in terms of parasites or fish species. Presently, there are no available data in the open literature regarding toxicity effects of MBZ on fish through blood analysis. Fish blood is a pathophysiological indicator of whole body function; and therefore, blood parameters are important in diagnosing the functional status of fish exposed to toxicants (Zutshi et al. 2010). Also, biochemical changes, resulting in altered physiology, are known to more quickly respond to toxicants than any apparent morphological changes. When tissue damage occurs, the intracellular enzymes are leaked into the blood, depending upon the extent of tissue damage (Javed and Usmani 2017). Hence, although MBZ is not a toxic chemical, the blood analysis of toxic chemicals may be used to estimate the side effects of MBZ.

In fishes, changes in the activities of GOT/AST, GPT/ALT, and ALP enzymes, as well as changes in cholesterol lipid composition, are used as important indicators to assess liver damage caused by toxic chemicals (Jyothi and Narayan 1997; Sharma 1999; Jung et al. 2003; Fırat et al. 2011; Fırat and Alıcı 2012; Hoseini et al. 2016). Plasma GOT and GPT activities have frequently been used to detect early signs of damage to the hepatocytes (Min and Kang 2008). In the present study, important indicators for diagnosis of liver damage, including GOT, GPT, ALP, and TCHO, increased significantly ($p < 0.05$) in the MBZ administration group, which appears to support the fact that MBZ is toxic to the liver of olive flounder. In addition, the value of GLU, a well-known stress indicator in fishes, also increased significantly concomitant with stressful conditions (Kim et al. 2000; Cnaani et al. 2004; Biawas et al. 2010; Choi et al. 2015). In the present study, the same rearing environment was used for all experimental groups including the control groups, and in all groups, the fish were not fed for 14 days after MBZ administration. During the experimental period, there were no significant changes in the GLU levels of the control and MR MBZ administration groups, whereas GLU levels increased significantly in the MBZ administration group. Unfortunately, we were unable to determine the root cause of this increase in the present study. However, Kim et al. (2014) reported that olive flounders that were starved for 42 days and those that were supplied with feed showed no significant differences in hematological and biochemical parameters, including Hb, Ht, GOT, GPT, GLU, and TCHO. In addition, sea bass that were starved for over a month showed no difference in plasma GLU levels compared with a fed control group (Chatzifotis et al. 2011). Hence, the significant increase in GLU levels observed in the present study was probably not attributable to the lack of food intake for 14 days after treatment. Therefore, we speculate that the administered MBZ causes stress in olive flounders through metabolic processes such as absorption and distribution. Moreover, hematological parameters of Hb and Ht have been applied in many studies as useful indicators to assess physiological stresses caused by toxic chemicals in fishes (Jung et al. 2003; Carvalho and Fernandes 2006; Sepici-Dinçel et al. 2009). In the present study, the Hb and Ht values for olive flounders that received oral and bath treatments with MBZ increased significantly ($p < 0.05$) as time passed, and we accordingly postulate that MBZ causes physiological stress to olive flounders, leading to the inhibition of oxygen transport in the blood.

Mullet (*Mugil liza*) juveniles (0.15 g ± 0.07 g, salinity 4‰) were bath treated with MBZ using 100 to 5 g/L for 24 h (Führ et al. 2012). No mortality was observed at any concentration, and this concurs with our findings. In their study, serious damage of gill tissue was observed in the fishes exposed to amounts equal to or greater than MBZ 10 mg/L. However, the histopathological evaluation showed no effects on the liver and kidney. They concluded that the therapeutic bath concentration of MBZ may cause gill damage at concentrations of 10 mg/L and greater. In the present study, no specific histopathological degeneration was observed in gill tissues, while atrophy of the hepatocyte and renal tubular epithelium, and eosinophilic hyaline droplet degeneration were found. Interestingly, histopathological characteristics showed remarkable differences. Overall, there are limited studies concerning the effect of MBZ administration on tissue histopathology of fish. Nevertheless, we would predict that species of fish, aquatic parameters of the various habitat conditions, like temperature, pH, conductivity, alkalinity, and salinity, may have affected the histological results (Martins et al. 2001). Both the liver and kidney would be sensitive to damage by MBZ administration, which is partially demonstrated by the histopathological changes in the present study.

As mentioned previously, hematological and biochemical parameters increased significantly in the MBZ administration group, indicating that MBZ causes physiological stresses and hepatotoxicity in olive flounders, whereas no comparable changes were observed in the MR MBZ administration group, and there was no significant increase ($p > 0.05$) when compared with the control group. Consistently, in histological analyses of liver, kidney, and gill tissues of the MBZ administration group, although we observed no abnormal histopathological degradation in gill tissues, we could detect atrophy in hepatocytes and renal tubule epithelial cells and lesions in the cytoplasm of renal tubule epithelial cells such as hyaline droplet degeneration. In contrast, the MR MBZ administration group showed no abnormal lesions in liver and kidney tissues during the experimental period.

Herein, we have introduced a new approach to reduce drug toxicity that differs markedly from the existing approaches. This method enhances materials using FOGF energy, which modifies molecules through remediation of each element within particular molecule. In this way, the toxicity in matter could be reduced. It is expected that this method can address many issues relating to drug toxicity that currently prohibit the development of molecular drugs.

In this study, we produced MR MBZ from pure MBZ (Sigma-Aldrich, M2523-25G; > 98%) using FOGF energy technology. The derived MR MBZ was used to treat olive flounders infected with scuticociliates, either by single oral dose or 1-h bath administration, followed by monitoring of the subsequent efficacy and toxicity. However, under actual farm conditions, it is necessary to repeat oral administrations of MBZ for at least several days in order to prevent reinfection after treatment for scuticociliatosis. Thus, this technology has been applied to commercially available nematocide products for pigs and chickens in South Korea in order to improve the material. Similarly, in the present study, the remediated material was orally administered to olive flounders for five consecutive days. Currently, additional studies are being conducted on hematological, biochemical, and histopathological safety during repeated administrations.

In conclusion, we demonstrated that MR MBZ, produced using FOGF energy, retained the same antiparasitic effects as the parent MBZ against scuticociliates in olive flounders. Furthermore, it had no harmful effects on the hematological and physiological characteristics of olive flounders, and caused no abnormal toxic lesions in the liver and kidney histopathologically. The present study is the first study to demonstrate that the toxicity of MBZ could be reduced by remediation of component elements using the FOGF energy that is present in nature. Regardless of the fact that this new approach was initially examined in the marine environment, it has considerable potential for future application to reduce side effects that can occur in medical products applied in both veterinary and human medicines, and also the side effects that can occur during development of numerous new drugs, consequently resulting in the suspension of development.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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