Anesthetic Activity of Acetylated MS-222 in Tilapia (*Oreochromis Niloticus*)

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ABSTRACT

A group of 8 tilapia (*Oreochromis niloticus*) were anesthetized once per week for six consecutive weeks, using tricaine methanesulfonate (MS-222) in the water. Time for the fish to reach anesthesia decreased significantly over the first four weeks, and then plateaued at about 27% below the first anesthetic exposure. These results suggest induction of the liver enzymes that convert MS-222 into metabolites, one or more of which have higher anesthetic activity than the parent compound. Major metabolites of MS-222 have been identified as part of regulatory studies evaluating residue persistence in food fishes. One of these metabolites, N-acetyl-3-aminobenzoic acid ethyl ester, which is acetylated MS-222, was selected for testing of anesthetic activity in tilapia. This report shows results of the testing, and speculates as to the potential utility of the acetylated metabolite of MS-222 as an alternate anesthetic agent in fish.

Key Words: tilapia; fish; anesthesia; MS-222; tricaine

INTRODUCTION

Tricaine methanesulfonate (MS-222; 3-aminobenzoic acid) is a widely used anesthetic/ tranquilizing agent for fish, reptiles, and amphibians, and currently the only anesthetic licensed by the United States Food and Drug Administration (FDA) for use in food fish. It is supplied as a fine, white crystalline powder with a molecular composition of $C_9H_{11}O_2N + CH_3SO_3H$ and molecular weight 261.31. MS-222 possesses moderately high stability (145 ^oC MP) and is clear, colorless, and highly soluble (to 11%) in aqueous solutions [1]. Solutions of pure MS-222 become acidic due to the formation of methane sulfonic acid. Therefore, MS-222 should be buffered to saturation with sodium bicarbonate before use as an injectable, to avoid metabolic imbalances in the anesthetized animals [2, 3].

During procedures in our laboratory that involved weekly bleeding of anesthetized tilapia (*Oreochromis niloticus*), a trend of reduced time to anesthesia in successive weeks was suspected. A naïve group of tilapia was therefore established and anesthetized once per week for six consecutive weeks, which verified that time to anesthesia was significantly reduced upon repeat MS-222 exposures. This result suggested induction of liver enzymes with the repeated exposures, which more rapidly produced an unrecognized active metabolite of MS-222 that had higher anesthetic potency than the parent compound. In the present report, we speculate as to the identity of a putative more active MS-222 metabolite, and show data for testing of its anesthetic potential in tilapia.

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METHODS

Eight tilapia (39 ± 2.7 g) were removed from a holding tank and placed into an 80-L tank with filtered, aerated, and dechlorinated water. Temperature and lighting conditions were 26 ºC and 12/12 hr light/dark cycle. Fish were fed a commercial fish diet of floating pellets (Zeigler Bros., Inc., Gardners, PA) at approximately 2% body weight daily. Ammonia and nitrite levels were monitored weekly, and tanks were cleaned as needed. A two-week acclimation period was allowed before initiating weekly MS-222 anesthesia. These fish had not previously been anesthetized with MS-222.

MS-222 (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving 1.2 g in 6.0 L of dechlorinated water (thus 200 mg/L), in a 40-L bucket. Fish were anesthetized by simultaneous immersion of all eight fish in the MS-222 solution, with time to anesthesia recorded as the time when vertical equilibrium was lost and the fish rotated approximately 90 degrees in the water (Stage III anesthesia). Time to anesthesia in the eight fish was recorded in this manner for six consecutive weeks. Unless otherwise indicated, "anesthesia" in this report refers to Stage III anesthesia with the loss of vertical equilibrium.

Major reported metabolites of MS-222 in fish include 3-aminobenzoic acid, N-acetyl-3-aminobenzoic acid, and N-acetyl-3-aminobenzoic acid ethyl ester [4] (**Figure 1**). The N-acetyl-3-aminobenzoic acid ethyl ester metabolite, or acetylated MS-222 (MS-222A) was selected as the likely more active metabolite of MS-222, and synthesized for testing. Synthesis of MS-222A was as follows. Ethyl 3-aminobenzoate mesylate (MS-222) (5.0 g, 19 mmol) was dissolved in 200 mL water, and 2 mL Ac_2O was added with stirring, followed immediately by 1.65 g NaOAc (19 mmol). The clear solution became milky, and it was left stirring for 30 minutes. The pH was then adjusted to ~2 with a few drops of 12 M HCl, and the solution was put at 4 ºC for the product to complete precipitation. The precipitate was filtered, washed with water, and left to dry, giving 3.66 g (93.7%) of white powder. 1H-NMR showed that this was the expected acetyl product.

MS 222 or MS-222A, 500 or 1000 mg each (experiments 1 and 2, respectively), were dissolved in 2.5 ml dimethyl sulfoxide (DMSO) and added separately to buckets containing 5 L filtered, aerated, and dechlorinated water. The concentration of MS-222 or MS-222A in respective buckets was therefore 100 mg/L (experiment 1) or 200 mg/L (experiment 2). An additional bucket was prepared that contained fresh water plus 2.5 ml DMSO (vehicle control). Tilapia that had not been previously anesthetized were sequentially (individually) placed into the test buckets, N=5, and the time to anesthesia was recorded.

Intracoelomic injection of buffered MS-222 is used for anesthesia or euthanasia in reptiles and amphibians, in both research and clinical settings [5]. As the 2nd comparison of anesthetic potential, tilapia (N=4) were anesthetized by intracoelomic injection, 600 mg/kg, with either buffered MS-222 or MS-222A. MS-222A lacks the sulfonate group that leads to sulfonic acid production in an aqueous environment, has a pH of 7.5 of when dissolved in water, and therefore was not buffered before injection.

Immediately following intracoelomic anesthetic injections, tilapia were placed into five gal buckets containing about two gal fresh water. The time required for these fish to enter Stage II, plane 2 anesthesia, characterized by narcosis involving muscle relaxation and slowed opercular and mouth movement [2], was recorded. Not all fish progressed into Stage III anesthesia, however, time was also recorded for the fish that reached this level. Stage II, plane 1, light narcosis was observed in a few of the fish, characterized by a very short excitement phase involving rapid swimming and increased opercular and mouth movement, and not recorded as a timed observation.

Upon reaching Stage III anesthesia, all fish in this report were immediately returned to fresh water tanks containing no anesthetic, for recovery. Fish that did not progress beyond Stage II anesthesia were in a similar manner returned to fresh water tanks containing no anesthetic, for recovery. All procedures in this report were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (IACUC), before initiation of the studies.

RESULTS

Time to anesthesia decreased significantly with repeated exposures of tilapia to 200 mg/L MS-222 in the water (**Figure 2**). Mean time to anesthesia was 160 seconds on the first exposure of tilapia to MS-222, and decreased by 27% to 117 seconds at week 4. Mean time to anesthesia at weeks 4, 5 and 6 plateaued at 117, 114 and 118 seconds, respectively, suggesting

maximal metabolic enzyme induction had been reached.

Fish individually (sequentially) exposed to 100 mg/L MS-222 in the water tended to require more time to reach anesthesia with each subsequent fish (**Table 1**), suggesting partial depletion of MS-222 by earlier fish. Dissolving MS-222 in DMSO before addition to water, did not affect time to anesthesia. Fish in fresh water containing the DMSO vehicle only, showed no signs of anesthesia. None of the fish exposed to 100 mg/L MS-222A in the water reached Stage III anesthesia.

Fish individually exposed to 200 mg/L MS-222 in the water also tended to require more time to reach anesthesia with each subsequent fish tested (**Table 2**). None of the fish exposed to 200 mg/L MS-222A reached Stage III anesthesia. Both the 100 and 200 mg/L MS-222A fish reached Stage I anesthesia rapidly, including melanomacrophages induction not seen in MS-222 fish, however, this endpoint, with the lack of further anesthesia progression, was not anticipated before the exposure and therefore not timed.

Fish injected with 600 mg/kg MS-222, N=4, reached Stage II, plane 2 anesthesia with mean time of 1 min 1 sec (**Table 3**). Three of these 4 fish did not progress to Stage 3 anesthesia. Fish injected with 600 mg/kg MS-222A, N=4, reached Stage II, plane 2 anesthesia, with a mean time 0 min 46 sec. Time to Stage II anesthesia was therefore numerically but not significantly reduced with MS-222A injection. All four MS-222A fish progressed to Stage III anesthesia, and two of the four continued into deepening anesthesia, and were classified as Stage IV (anesthetic overdose and death). One of the two fish classified as Stage IV, however, recovered after about 20 minutes, and therefore was reclassified to very deep Stage III.

DISCUSSION

The observation of reduced time to anesthesia in fish, upon repeated exposures to MS-222, indicated the formation of an unrecognized metabolite of MS-222 that had greater anesthetic activity than the parent compound. The three major metabolites of MS-222 are 3-aminobenzoic acid, N-acetyl-3-aminobenzoic acid, and N-acetyl-3-aminobenzoic acid ethyl ester [4, 6]. 3-aminobenzoic acid is a molecule used for the synthesis of azo dyes, is found in yeasts, and is essential for the growth of many microorganisms [7, 8]. Anesthetic activity of 3-aminobenzoic acid is not suggested by its presence in yeast and use in cell culture media, is not indicated on Material Safety Data Sheets (MSDS) for this chemical (http://www.sciencelab.com/msds.php?msdsId=9922875), and has not otherwise been reported in the literature. N-acetyl-3-aminobenzoic acid is an active ingredient in sun-blocking creams, has no reported numbing effects on skin or other anesthetic activity [9], and anesthetic activity is again not indicated on the MSDS for this chemical (https://www.spectrumchemical.com/MSDS/A0472.pdf).

MS-222 is a sulfonated analog of benzocaine, and was discovered accidentally during attempts to derive a synthetic substitute for cocaine [10, 11]. Benzocaine is structurally very similar to MS-222, having an NH_2 (amino) group in the *para* position as compared to an NH_3^+ group in the *meta* position of water-dissolved MS-222. The acetylated or N-acetylbenzocaine metabolite of benzocaine is rapidly formed *in vivo* by acetyltransferase enzyme activity, has greater anesthetic activity than benzocaine, and is commercially available as a faster-acting drug [12]. N-acetyl-3-aminobenzoic acid ethyl ester (MS-222A) differs from N-acetylbenzocaine by the presence of the N-acetyl group at the *meta* rather than the *para* position. Given the reported enhancement of anesthetic activity of benzocaine by acetylation, and given lack of reported or suspected anesthetic activity of the other major MS-222 metabolites, MS-222A was selected as the likely major metabolite to evaluate for anesthetic activity in fish.

Fish placed into water containing 100 mg/L MS-222A proceeded rapidly to Stage I anesthesia, as characterized by reduced motion and melanomacrophage induction. These fish, however, did not progress beyond Stage I. The concentration of MS-222A was therefore increased to 200 mg/L, with the same result of fish rapidly reaching Stage I anesthesia but progressing no farther. A DMSO vehicle effect limiting the availability of MS-222A was not viewed as likely, however, a second vehicle, 200 □l ethanol, was tested in N=3 fish to answer this concern. The mean time to Stage III anesthesia for 100 mg/L MS-222 was 6 min 15 sec, and for 100 mg/L MS-222A was 10 min 13 sec for two fish that achieved Stage III, while one fish did not progress past Stage I (full data not shown).

The MS-222A fish again rapidly reached Stage I anesthesia using the ethanol vehicle.

The rapid progression of MS-222A fish to Stage I anesthesia, followed by no or lengthier progression to deeper anesthesia, suggested possible lower availability of the more lipophilic, acetylated metabolite in water. Fish were therefore injected with MS-222 or MS-222A, following published guidelines for intracoelomic anesthesia or euthanasia of poikilothermic species [5, 13]. The mean time to Stage II, plane 2 anesthesia was numerically but not statistically more rapid in fish injected with MS-222A. One of four fish injected with MS-222 reached Stage III anesthesia following injection, while all four fish injected with MS-222A reached Stage III. Two of the MS-222A fish were then rated as continuing to Stage IV (overdose death), however, one of these two fish recovered after 20 minutes. When injected into a group of test fish, MS-222A therefore exhibited stronger anesthetic activity than MS-222.

Summary and Conclusions: When injected, MS-222A caused deeper anesthesia than did MS-222. When dissolved in water, MS-222A caused fish to rapidly reach Stage I anesthesia, but these fish did not progress to deeper anesthesia. Halting of anesthetic progression at Stage I may suggest very rapid further metabolism of MS-222A, by the same enzymes that metabolize parent MS-222, but without competition from additional MS-222 in the MS-222A treated fish. Experiments in which fish are pre-treated with mixed-function oxidase (MFO) inhibitors and then exposed to either MS-222 or MS-222A in the water are a logical next step. If MS-222 is metabolized to a more active acetylated form, MFO inhibition should lengthen the time to anesthesia. Further, if MS-222A is a more active anesthetic than MS-222, but is metabolized too rapidly for fish to progress beyond Stage I anesthesia, MFO inhibition should cause rapid progression to deeper stages of anesthesia.

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Figure Legends

Figure 1. Major metabolites of tricaine methanesulfonate (MS-222)

Figure 2. Time to anesthesia on repeat exposure of tilapia to MS-222

Author Profile

Dr. Holladay obtained his Ph.D. in toxicology from North Carolina State University in 1989. He then spent two years in a postdoctoral appointment at the National Institute of Environmental Health Sciences (NIEHS), studying developmental immunotoxicity of endocrine disrupting environmental chemicals. He has served nationally and internationally as an invited speaker. Dr. Holladay has published over 150 research manuscripts over the past 30 years, and served as Editor of the textbook, Developmental Immunotoxicology, published by CRC Press. He has been a member of numerous research journal editorial boards, was Editor of the journal Advances in Pharmacological Sciences from 2006-2009, and served as Associate Editor for BMC Pharmacology and Toxicology from 2008-2015. He has also served as an expert invited reviewer over 30 times for the U.S. National Institutes of Health (NIH) and the U.S. Environmental Protection Agency (EPA). Awards he has received have included the North Carolina State University Employee of the Year, the North Carolina Governor's Award of Excellence, presented by Governor James Martin, two Merck Foundation Awards for Creativity in Teaching, the Virginia Tech Academy Award for Teaching Excellence, the Norden Distinguished Teaching Award, considered the highest teaching award in veterinary medical education, and the Pfizer Animal Health Award for Research Excellence. Dr. Holladay is presently Department Head, Biosciences and Diagnostic Imaging, at the College of Veterinary Medicine, University of Georgia.

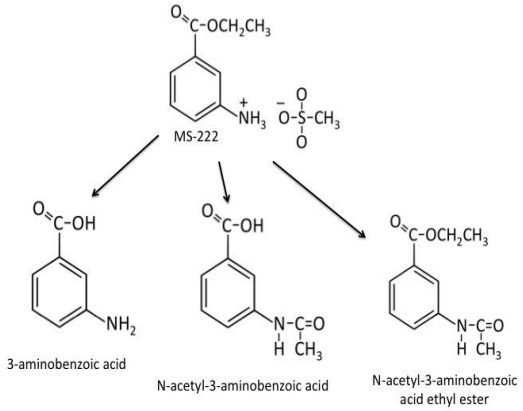

Figure 1. Major metabolites of tricaine methanesulfonate (MS-222).

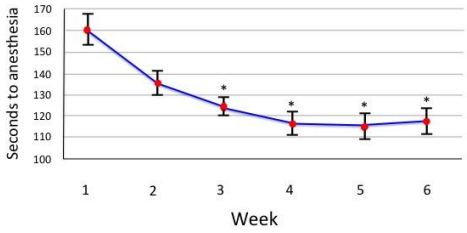

Figure 2. Time to anesthesia on repeat exposure of tilapia to MS-222.

Table 1. Time to anesthesia in tilapia exposed to 100 mg/L MS-222 or MS-222A, using a DMSO vehicle.

MS-222	MS-222 + DMSO	MS-222A + DMSO	DMSO
5:30	6:50	NA	NA
7:09	6:05	NA	NA
5:58	8:40	NA	NA
10:05	8:57	NA	NA
12:13	11:26	NA	NA
Mean 8:11	Mean 8:32		

NA: Stage III anesthesia not attained

Table 2. Time to anesthesia in tilapia exposed to 200 mg/L MS-222 or MA-222A, using a DMSO vehicle.

MS-222	MS-222A
1:40	NA
1:59	NA
2:48	NA
2:57	NA
5:01	NA
Mean 1:43	

NA: Stage III anesthesia not attained

Table 3. Time to Stage II or Stage III anesthesia in tilapia injected with 600 mg/kg MS-222 or MS-222A, using a DMSO vehicle.

MS-222 Stage II	MS-222 Stage III	MS-222A Stage II	MS-222A Stage III
0:48	NA	1:05	2:09
1:14	NA	1:11	1:30
0:20	1:40	0:30	3:46
1:43	NA	0:19	0:39
Mean 1:01	-	Mean 0:46	Mean 2:01

NA: Stage III anesthesia not attained