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Potency and specificity of amiloride and its analogues on branchial sodium fluxes in freshwater trout and goldfish

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ABSTRACT

There is a consensus that electroneutral Na^+/H^+ exchangers (NHEs) are important in branchial Na^+ uptake in freshwater fish. There is also widespread belief, based on mammalian data, that EIPA [5-(N-ethyl-N-isopropyl)-amiloride]], and HMA [5-(*N*,*N*-hexamethylene)-amiloride)] are more potent and specific in blocking Na^+ uptake than amiloride. We evaluated this idea by testing the three drugs at 10^{-7} to 10^{-4} M, *i.e.* 0.1 to 100 μM in two model species, rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*), using $^{22}Na^+$ to measure unidirectional Na^+ influx and efflux rates. In both species, the potency order for inhibiting unidirectional Na^+ influx was HMA > amiloride > EIPA (IC_{50} values in the 10–70 μM range), very different from in mammals. At 100 μM, all three drugs inhibited Na^+ influx by >90% in both species, except for amiloride in goldfish (65%). However, at 60–100 μM, all three drugs also stimulated unidirectional Na^+ efflux rates, indicating non-specific effects. In trout, HMA and EIPA caused significant increases (2.1- to 2.3-fold) in efflux rates, whereas in goldfish, significant efflux elevations were greater (3.1- to 7.2-fold) with all three drugs. We conclude that the inhibitory potency profile established in mammals does not apply to the NHEs in fish gills, that non-specific effects on Na^+ efflux rates are a serious concern, and that EIPA and HMA offer no clear benefits in terms of potency or specificity. Considering its much lower cost, we recommend amiloride as the drug of choice for *in vivo* experiments on freshwater fishes.

1. Introduction

Many aspects of Na^+ uptake by the gills of freshwater teleosts remain uncertain, but there is general accord that electroneutral Na^+/H^+ exchangers (NHEs) on the apical surfaces of mitochondrial-rich cells play an important role in most species, at least in circumneutral waters (reviewed by Kovac and Goss, 2024). Early evidence came from the demonstration that amiloride, a "K^+-sparing" diuretic drug developed by Cragoe et al. (1967), which potently inhibits NHEs in mammalian systems (Benos, 1982), also potently inhibited branchial Na^+ uptake in freshwater rainbow trout (Kirschner et al., 1973; Perry and Randall, 1981; Perry et al., 1981; Wright and Wood, 1985). Later, concerns were raised that in mammals, certain isoforms of NHE were amiloride-resistant, and also that amiloride would inhibit Na^+ channels as well as NHEs (reviewed by Masereel et al., 2003). This led to the development of amiloride analogues that in mammals were much more potent in inhibiting NHEs, with much reduced potency in blocking Na^+ channels (Kleyman and Cragoe Jr., 1988). The most successful of these, such as 5-(*N*,*N*-hexamethylene)-amiloride (HMA) and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), bore substituent groups on the 5-amino nitrogen atom. Both of these compounds had >2 orders of magnitude greater potency on NHEs than amiloride, and > 1 order magnitude lower potency than amiloride on Na^+ channels in mammals (Kleyman and Cragoe Jr., 1988).

HMA and EIPA were subsequently tested on intact fish in freshwater and proved to be effective inhibitors of branchial Na^+ uptake in most (*e.g.* Wood et al., 2002; Preest et al., 2005; Esaki et al., 2007), but not all reports (*e.g.* Boisen et al., 2003). Brix et al. (2018) have provided a detailed list of studies that have used EIPA to examine Na^+ uptake in freshwater fish. Unfortunately, we are aware of no *in vivo* studies where the potencies of all three drugs (HMA, EIPA, amiloride) have been compared against one another at the same concentrations, and only two

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(Esaki et al., 2007; Boyle et al., 2016), where amiloride and EIPA were compared at the same concentration. In larval zebrafish, results were equivocal (Esaki et al., 2007), whereas in larval trout, EIPA and amiloride appeared to have similar potencies in blocking Na^+ uptake (Boyle et al., 2016). The findings of Boisen et al. (2003) and Brix and Grosell (2012) who compared unequal concentrations of the two drugs in adult zebrafish and pupfish respectively were also equivocal as to relative potencies. When rainbow trout NHE isoforms were expressed in a cell line and tested *in vitro,* amiloride was more potent than EIPA on a kidney isoform of NHE (NHE3a), whereas results for a gill isoform (NHE3b) were again equivocal (Blair et al., 2021).

In light of this confusing background, the goal of the present study was to directly compare the inhibitory potency of HMA, EIPA, and amiloride across a wide range of concentrations (10^{-7} to 10^{-4} M; *i.e.* 0.1 to 100 μM) on branchial Na^+ transport in two model species in fresh water. The rainbow trout (*Oncorhynchus mykiss*) was selected because of its rich past history in amiloride studies (see above). The goldfish (*Carassius auratus*) was selected as another widely used model species (Krogh, 1938; Maetz and Garcia Romeu, 1964; Cuthbert and Maetz, 1972; Maetz, 1973; Preest et al., 2005), one which is phylogenetically distant from trout. Furthermore, in contrast to the euryhaline trout, the goldfish is a stenohaline freshwater species and one in which there is disagreement as to the effects of amiloride and its analogues (see Discussion).

Most studies to date have evaluated the effects of these drugs only on unidirectional Na^+ uptake rates. However, here we also assessed potential effects on unidirectional Na^+ efflux rates as they could be equally influential on net Na^+ balance. For example, Wood et al. (2002) reported that while both amiloride (100 μM) and HMA (40 μM) greatly inhibited Na^+ influx rate in a freshwater Amazonian stingray, HMA (but not amiloride) also greatly stimulated Na^+ efflux rate. Our overall objective was to select the most potent and specific inhibitor for Na^+ influx rate for use in future *in vivo* flux studies on freshwater fishes.

2. Methods

2.1. Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*, 1.19 ± 0.05 g, $N = 60$) were obtained from Rainbow Springs Trout Hatchery, Thamesford, Ontario) and small goldfish (1.59 ± 0.17 g, $N = 48$) were purchased from a commercial pet store (PetSmart, Hamilton, Ontario). At McMaster University, they were held at 14 °C for several weeks prior to experimentation in flowing, dechlorinated Hamilton tap water, which is moderately hard water from Lake Ontario. Water chemistry was as follows: $Na^+ = 0.8$ mM; $Cl^- = 0.9$ mM; $Ca^{2+} = 1.0$ mM; $Mg^{2+} = 0.2$ mM; $K^+ = 0.04$ mM; titration alkalinity (to pH 4.0) = 1.9 mM; hardness = 120 mg $CaCO_3$ L^{-1}; dissolved organic carbon (DOC) = 2.9 mg L^{-1}; pH = 8.0) The fish were fed every second day to satiation with commercial trout pellets or goldfish flakes, but fasted for 48 h prior to experiments. Experiments were approved under McMaster Animal Research Ethics AUP 06–01-05 and conformed to the guidelines of the Canadian Council on Animal Care.

2.2. Chemicals and analytical methods

Amiloride hydrochloride, 5-(N,N)-hexamethylene amiloride (HMA), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich, St. Louis, MO, USA. DMSO was used to solubilize all three drugs; final DMSO concentration in all exposures, including drug-free controls, was 0.1%.

Water samples were analyzed for total Na^+ concentrations by flame atomic absorption spectrophotometry (Varian AA-220, Palo Alto, USA) using certified standards (Fisher Scientific, Downsview, ON, Canada) and for $^{22}Na^+$ radioactivity using a NaI crystal gamma counter (MINAXI Auto Gamma 5000, Canberra Packard, Vienna, Austria).

2.3. Unidirectional flux measurements

In rainbow trout, each drug was tested at 0, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 6×10^{-5} M, and 10^{-4} M – *i.e.* 0, 0.01, 0.1, 1, 10, 60, and 100 μM - using 2–3 fish at each concentration. The same series was used in goldfish, with omission of 10^{-8} M. Each fish was isolated in an individual, light-shielded container served with aeration, and allowed to settle for 1 h. The volume was 25 mL. $^{22}Na^+$ (0.15 μCi as NaCl, NEN-Dupont, Boston, MA, USA) was then added and allowed to mix for 5 min. Initial and final water samples (1 ml for gamma counting of $^{22}Na^+$, 5 ml for atomic absorption analysis of total Na^+) were taken at the start (0 min) and end (50 min) of the flux period. The fish was then euthanized with an overdose of neutralized MS-222 (Syndel, Vancouver, BC, Canada) and weighed. Therefore, the operative flux volume was 0.019 L, the mean water total Na^+ concentration during the experiments was 800 ± 20 μmol L^{-1}, and the mean specific activity was 8849 ± 30 cpm μmol^{-1}.

Unidirectional and net flux rates of Na^+ were calculated as outlined by Wood (1992). Unidirectional Na^+ influx rate at the gills ($J^{Na}i_n$, in μmol g^{-1} h^{-1}) is positive by convention and was measured by monitoring the disappearance of $^{22}Na^+$ from the water into the fish:

$$J^{Na}{}_{in} = \left([R]_i - [R]_f\right)^{*} V^{*} SA^{-1*} T^{-1*} W \quad (1)$$

where $[R]_i$ is the initial radioactivity in the water (in cpm L^{-1}) at the start of the flux period, $[R]_f$ is the final radioactivity in the water (in cpm L^{-1}) at the end of the flux period, $[Na]_i$ and $[Na]_f$ are respectively the initial and final concentrations of total Na^+ in the water (in μmol L^{-1}), and SA is the mean specific activity (radioactivity per total Na^+) in the water (in cpm μmol^{-1}), calculated from measurements of water radioactivity and total water $[Na^+]$ at the start and end of the flux period.

Net Na^+ flux rate at the gills (J^{Na}_{net}, in μmol g^{-1} h^{-1}) was calculated as:

$$J^{Na}{}_{net} = \left([Na^+]_i - [Na^+]_f\right)^{*} V^{*} T^{-1*} W^{-1} \quad (2)$$

By convention, positive values represent net uptake by the fish, while negative values represent net losses from the fish.

Unidirectional Na^+ efflux rate at the gills (J^{Na}_{out}, in μmol kg^{-1} h^{-1}) is negative by convention and was calculated by difference:

$$J^{Na}{}_{out} = J^{Na}{}_{net} - J^{Na}{}_{in} \quad (3)$$

2.4. Statistical analyses

Data have been expressed as means ± SEM. Within each drug series, data were analyzed by Statistica (version 7.0) with examination for normality by analysis of the distribution of raw residuals, and for homogeneity of variance by the Cochran's C test. All data passed, so means were then compared by One-Way Analysis of Variance (ANOVA) followed by the Fisher's (LSD) test to identify significant differences. The kinetics of inhibition were examined using SigmaPlot for Windows (version 11.0) to perform non-linear regression (logistic three parameter curve-fit) to identify IC_{50} values, representing the concentration of each drug that caused 50% inhibition of unidirectional Na^+ uptake:

$$y = a/(1 + x/b^c))$$

where x is the drug concentration (μM), y is the Na^+ influx rate (J^{Na}_{in}; μmol kg^{-1} h^{-1}), a is the maximum value of Na^+ influx (J^{Na}_{in}; μmol kg^{-1} h^{-1}), b is the IC_{50} value, and c is the Hill coefficient.

The Shapiro-Wilk and constant variance tests were used to verify overall normality and homogeneity of variances. The standard error estimates on IC_{50} values were used to statistically compare them by the Bonferroni test. A significance level of $P < 0.05$ was used throughout. All graphs were drawn in SigmaPlot for Windows (version 11.0).

3. Results

Under control conditions (0.1% DMSO only), rainbow trout maintained Na^+ balance, such that Na^+ influx (J_{in}^{Na}) and Na^+ efflux (J_{out}^{Na}) rates were virtually identical, and Na^+ net flux rate (J_{net}^{Na}) was close to zero. Amiloride had no effects on these parameters at concentrations of 0.01, 0.1, and 1 μM (Fig. 1A). At 10 μM, amiloride caused a significant 35% inhibition of J_{in}^{Na} but had no effect on J_{out}^{Na}, so J_{net}^{Na} became negative, but not significantly different from the control value. At both 60 and 100 μM, amiloride virtually eliminated J_{in}^{Na} (90% inhibition) and did not significantly reduce the J_{out}^{Na}. However, J_{net}^{Na} became significantly negative at both 60 and 100 μM amiloride (Fig. 1A).

In trout, EIPA exhibited a similar pattern to amiloride, but appeared to be slightly less potent in inhibiting J_{in}^{Na}, and slightly more potent in stimulating J_{out}^{Na} (Fig. 1B). J_{in}^{Na} remained unchanged at EIPA concentrations up to and including 10 μM, and then fell significantly by 70% at 60 μM and by 90% at 100 μM. J_{out}^{Na} started to increase and J_{net}^{Na} become negative at 1 μM and 10 μM EIPA, though only the latter was significantly different from the control rate. At both 60 and 100 μM EIPA, significant stimulations of J_{out}^{Na} by 2.1- to 2.3-fold resulted in highly negative values of J_{net}^{Na}.

In trout, HMA exhibited very similar effectiveness to amiloride in blocking J_{in}^{Na}, with the first significant inhibition (40%) at 10 μM, and 90% and 100% reductions at 60 and 100 μM respectively (Fig. 1C). However, HMA appeared to be slightly more potent than amiloride in stimulating J_{out}^{Na} which increased significantly by 2.3-fold at both 60 and 100 μM. J_{net}^{Na} became significantly more negative than the control rate at 10, 60, and 100 μM HMA (Fig. 1C).

Goldfish, similar to trout, maintained Na^+ balance under control conditions (0.1% DMSO only), with approximately equal J_{in}^{Na} and J_{out}^{Na}, such that J_{net}^{Na} was close to zero (Fig. 2). Amiloride had no effect on J_{in}^{Na} at 0.1 and 1 μM, but resulted in significant reductions of 30%, 60%, and 65% at 10, 60, and 100 μM respectively (Fig. 2A). J_{out}^{Na} became highly negative, with a 3.1-fold increase at 60 μM and a 7.2-fold increase at 100 μM, resulting in highly negative J_{net}^{Na} values at both concentrations.

In goldfish, as in trout, EIPA was less potent than amiloride in inhibiting J_{in}^{Na}, which remained unchanged at concentrations up to and including 10 μM (Fig. 2B). At 60 μM and 100 μM, J_{in}^{Na} was reduced by 30% and 95% respectively. J_{out}^{Na} increased by 5.4-fold at 100 μM EIPA, resulting in a highly negative J_{net}^{Na} (Fig. 2B).

In goldfish, HMA had no effect on J_{in}^{Na}, at 0.1 and 1 μM, but caused a significant 45% inhibition at 10 μM (Fig. 2C). At 60 and 100 μM HMA, the reductions in J_{in}^{Na} reached 70% and 90% respectively. At these two concentrations, HMA also caused significant 3.1-fold and 5.7-fold stimulations of J_{out}^{Na}. J_{net}^{Na} first became significantly negative at 10 μM, and strongly negative at 60 and 100 HMA (Fig. 2C).

The logistic three parameter regressions used to generate IC_{50} values for 50% inhibition of J_{in}^{Na} (Table 1) are shown in Supplementary Fig. S1 for trout and Supplementary Fig. S2 for goldfish. In both species, the order of effectiveness was the same, with HMA being the most potent (lowest IC_{50}) and EIPA the least potent (highest IC_{50}). All values were in the range of 10 to 70 μM and differences were significant only for goldfish (Table1).

4. Discussion

The most important conclusion of this study is that the relative potencies of these three agonists on NHEs in the gills of freshwater fish were quite similar (all within only a 7-fold range; Table 1) with HMA > amiloride > EIPA. Boyle et al. (2016) reported that amiloride and EIPA exhibited approximately equal potencies in blocking Na^+ uptake in larval trout, and this also appeared to be the case in some of the trials of Brix et al. (2018) on adult pupfish. All of these results are very different from those established for mammalian NHEs where both HMA and EIPA are >100-fold more potent than amiloride, and the NHE3 isoform is particularly amiloride-resistant (Kleyman and Cragoe Jr., 1988;

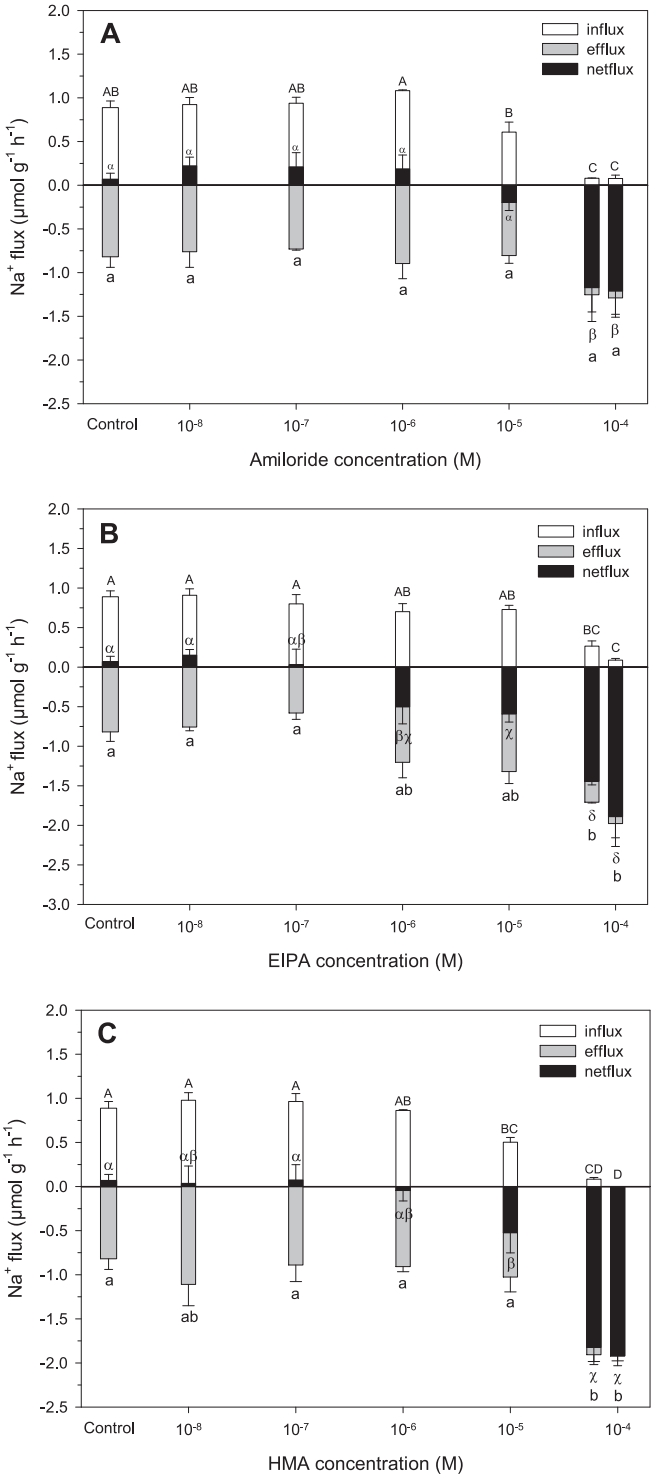

Fig. 1. The effects of various concentrations of (A) amiloride, (B) EIPA, and (C) HMA on unidirectional sodium influx rate (J_{in}^{Na}; upward white bars), unidirectional sodium efflux rate (J_{out}^{Na}; downward grey bars), and sodium net flux rate (J_{net}^{Na}; black bars) in intact rainbow trout *in vivo* in fresh water. Means ±1 SEM. For J_{in}^{Na}, means sharing the same upper-case letter are not significantly different, for J_{out}^{Na}, means sharing the same lower-case letter are not significantly different, and for J_{net}^{Na}, means sharing the same Greek letter are not significantly different, all at $P < 0.05$.

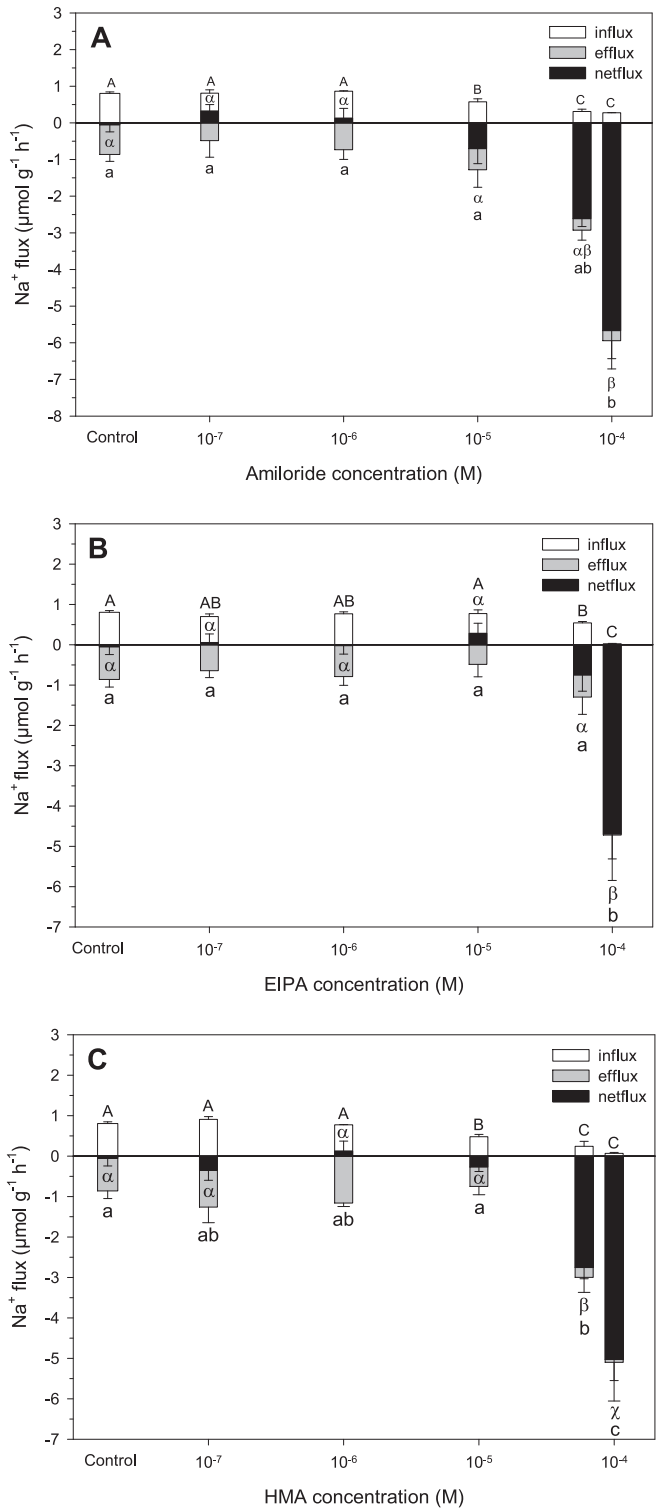

Fig. 2. The effects of various concentrations of (A) amiloride, (B) EIPA, and (C) HMA on unidirectional sodium influx rate (J_{in}^{Na}; upward white bars), unidirectional sodium efflux rate (J_{out}^{Na}; downward grey bars), and sodium net flux rate (J_{net}^{Na}; black bars) in intact goldfish *in vivo* in fresh water. Means ±1 SEM. For J_{in}^{Na}, means sharing the same upper-case letter are not significantly different, for J_{out}^{Na}, means sharing the same lower-case letter are not significantly different, and for J_{net}^{Na}, means sharing the same Greek letter are not significantly different, all at $P < 0.05$.

Masereel et al., 2003). In freshwater fish, current evidence points to the isoforms NHE2, NHE3a, and NHE3b as potential contributors to the apical step of Na^+ uptake, with the most focus on NHE3b (reviewed by Kumai and Perry, 2012, and Kovac and Goss, 2024). A potentially confounding factor is that mammalian NHEs usually function at the high Na^+ concentrations typical of body fluids (50–200 mM), whereas fish gill NHEs normally function at very low Na^+ levels typical of fresh water (0.8 mM in the present study). This is well known to affect IC_{50} values (Masereel et al., 2003). However, Blair et al. (2021) expressed rainbow trout NHE3a in AP-1 cells bathed with 135 mM Na^+ and reported that the IC_{50} for amiloride was 9 μM while that for EIPA was 44 μM, very similar to the present values determined *in vivo* in fresh water (Table 1). Unfortunately, these workers could not determine IC_{50} values for NHEb expressed in the same AP-1 system but noted that amiloride seemed to be more effective than EIPA. Overall, our data strongly support the conclusions of Blair et al. (2021) and Kovac and Goss (2024) that the well-established pharmacological inhibitory profiles of amiloride analogues in mammals cannot be applied to the NHEs in fish gills. Indeed, this may explain why phenamil, another amiloride analogue that is highly selective for the epithelial Na^+ channel (ENaC) in mammals (Kleyman and Cragoe Jr., 1988) effectively blocks Na^+ uptake in many freshwater fish, despite the fact that ENaC is not present in teleost genomes (Kumai and Perry, 2012; Kovac and Goss, 2024).

A second conclusion is that the effects of HMA, EIPA, and amiloride on Na^+ transport in goldfish were generally similar to those in rainbow trout (Table 1), with the same potency order (HMA > amiloride > EIPA) despite their differences in phylogeny and euryhalinity. Cuthbert and Maetz (1972) originally reported that branchial Na^+ transport in goldfish was insensitive to amiloride (up to 750 μM), and Sandbichler and Pelster (2004) reported that intracellular pH regulation in goldfish gill cells cultured *in vitro* was less sensitive to amiloride than in a previous report on trout gill cells in culture (Pärt and Wood, 1996). However, Preest et al. (2005) tested four amiloride analogues (including EIPA and HMA, though at unequal concentrations) and found all to be effective in blocking Na^+ uptake in goldfish *in vivo*. The reasons for these discrepancies are unclear, but the current results agree with those of Preest et al. (2005). One interesting feature of the goldfish response was that 100 μM amiloride caused only a 65% inhibition of J_{in}^{Na} (Fig. 2A) in contrast to the ≥90% inhibitions seen at the same concentrations of EIPA and HMA in this species ((Fig. 2B,C), and with all three drugs in rainbow trout (Fig. 1A,B,C). As pointed out by Kumai and Perry (2012), a possible explanation is that part of J_{in}^{Na} may occur *via* a sodium-chloride co-transporter (NCC) in goldfish as in another cyprinid, the zebrafish. Preest et al. (2005) reported that both 100 μM furosemide and Cl^--free media reduced J_{in}^{Na} in the goldfish, whereas there is no evidence for the participation of NCC in the rainbow trout (Kovac and Goss, 2024).

A third, very important conclusion is that at concentrations which effectively inhibit Na^+ influx (J_{in}^{Na}), all three drugs may greatly stimulate Na^+ efflux rates (J_{out}^{Na}). In rainbow trout, this effect was modest at 60 and 100 μM amiloride, where only non-significant 1.5-fold increases in J_{out}^{Na} occurred (Fig. 1A), but at 60 and 100 μM EIPA (Fig. 1B) and 60 and 100 μM HMA (Fig. 1C), the effects were much larger (2.1- to 2.3-fold increases.). Very few previous studies have measured the effects of these drugs on J_{out}^{Na}, but our results agree with those of Wood et al. (2002) on freshwater Amazon rays where 40 μM HMA stimulated Na^+ efflux rate by about 7-fold, whereas 100 μM amiloride caused only a non-significant 1.5-fold increase. Kirschner et al. (1973) and Wright and Wood (1985) also found no significant effect of 100 μM amiloride on J_{out}^{Na}, in adult rainbow trout. Actions on Na^+ efflux rates in goldfish were more serious, with significant 3.1- to 7.2-fold increases in J_{out}^{Na} caused by all three drugs at 60 and 100 μM (Fig. 2A,B,C). These effects on J_{out}^{Na} are perhaps not surprising, given the wide range of non-specific, non-target effects of amiloride-type drugs that have been reported (Kleyman and Cragoe Jr., 1988; Masereel et al., 2003). The mechanisms behind these actions in fish gills remain unknown; we speculate that these compounds my affect Na^+-coupled volume regulatory processes in gill epithelial

Table 1
IC_{50} values [50% inhibition concentrations against sodium influx rate (J_{in}^{Na})], and the logistic three parameter regression equations used to derive these values, for HMA, amiloride, and EIPA in intact rainbow trout and goldfish *in vivo* in fresh water. For IC_{50} values (means ± SEM), means sharing the same letters within a species are not significantly different from one another at $P < 0.05$.

Rainbow Trout		
HMA IC_{50}		10.4 ± 1.5 μM ^{A}
Amiloride IC_{50}	13.8 ± 3.1 μM ^{A}	
EIPA IC_{50}		38.4 ± 12.7 μM ^{A}
HMA		$y = 0.952/(1 + (x/10.362^{1.338}))$; $R^2 = 0.994$; $P = 0.0004$
Amiloride	$y = 0.984/(1 + (x/13.828^{1.646}))$; $R^2 = 0.980$; $P = 0.0029$	
EIPA		$y = 0.801/(1 + (x/38.374^{1.846}))$; $R^2 = 0.957$; $P = 0.0088$
Goldfish		
HMA IC_{50}		10.6 ± 4.6 μM ^{X}
Amiloride IC_{50}	30.7 ± 11.1 μM ^{XY}	
EIPA IC_{50}		67.3 ± 3.6 μM ^{Y}
HMA		$y = 0.917/(1 + (x/10.630^{0.783}))$; $R^2 = 0.984$; $P = 0.0165$
Amiloride	$y = 0.863/(1 + (x/30.688^{0.756}))$; $R^2 = 0.976$; $P = 0.0238$	
EIPA		$y = 0.746/(1 + (x/67.300^{8.515}))$; $R^2 = 0.991$; $P = 0.0087$

cells, resulting in swelling or shrinking, thereby opening up paracellular pathways for Na^+ leakage. Regardless, in future studies, these potential stimulatory effects on unidirectional Na^+ efflux rate should be assessed in any studies using these drugs *in vivo* in fish.

In this context, it is important to be aware that adding drugs to the external environment of a highly complex organism *in vivo* can cause several unknown effects that may also affect Na^+ fluxes. Depending on the degree of protonation of the compounds, which will be a function of the water pH, drugs tested can be lipophilic and thus traverse the gill epithelium, potentially influencing intracellular pathways as well. Furthermore, the degree of protonation may also affect the drugs potencies, as does the external Na^+ concentration. Finally, another aspect to be considered is that multiple pathways are likely available for Na^+ uptake when drugs are tested *in vivo*. Therefore, the possibility that other Na^+ uptake proteins, in addition to NHE, are being affected cannot be ruled out. Our original objective was to select the most potent and specific inhibitor of Na^+ influx rate for use in future *in vivo* flux studies on freshwater fishes. The present results show that EIPA, which has been favored by many previous workers because of its assumed greater potency and specificity, is actually slightly less potent than amiloride. At least in trout, amiloride also causes less disturbance of Na^+ efflux rate. HMA is only very marginally more potent than amiloride, and it too greatly disturbs Na^+ efflux rate. Given that large amounts of these drugs are required to be dissolved in the external water for *in vivo* experiments on whole fish, and that amiloride is generally less than one tenth the price of EIPA and HMA, we recommend amiloride as the drug of choice.

CRediT authorship contribution statement

Adalto Bianchini: Writing – review & editing, Visualization, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chris M. Wood:** Writing – original draft, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2024.111715.

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