

Estimation of the modulatory roles of thieno [2,3-c] pyrazole compounds versus the toxicity of 4-nonylphenol in African catfish (*Clarias gariepinus*)

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Abstract

Abstract

The endocrine disrupting substances represent major challenge to aquaculture and the most widespread one is 4-nonylphenol (4-NP). Pyrazole possess an interesting broad spectrum of pharmacological actions. Therefore, this study was designed to investigate the therapeutic potential of five novel thieno [2,3-c] pyrazole compounds in African catfish (*Clarias gariepinus*) on the hematotoxic and electrolyte disruptor influences of 4-NP. The hemato-electrolyte disturbance induced by 4-NP was well proved by many examined endpoints. On the other hand, thienopyrazole compounds exhibited the ability to modulate the previous toxicological impact by different levels based on the modification in structures and properties. This study provided insight into the endless therapeutic treasures of newly synthesized complexes, and a driving force for its application on the other clinically relevant problems in fish.

Keywords:- 4-nonylphenol; *Clarias gariepinus*; blood; electrolyte; pyrazole

1. Introduction

Pyrazole is considered a privileged structure in drug design which motivating the researchers to put it in the area of attention long ago. Their cores are used as

multitalented scaffolds to develop innovative compounds with a great diversity of biological activities as antipyretic [1], anti-inflammatory, and anti-oxidant [2]. [3] discovered the usefulness of benzimidazole-2-pyrazole carboxylates as orally efficacious stimulators of erythropoietin secretion. An experimentally based rationale for selecting the current compounds is based on the fact that pyrazole possess a potent antioxidant activity giving motivation to hypothesize that these tested derivatives may be able to combat oxidative stress-induced hemato-electrolyte disturbance which is associated with 4-NP which has been proven to be fish toxicant [4].

Endocrine disrupting compounds include a wide variety of chemicals, such as natural estrogens, phytoestrogens, industrial chemicals, and pesticides [5]. Among them, 4-nonylphenol (4-NP) is a widespread xenoestrogen contaminating aquatic environments [6]. It is a product derived from alkylphenol ethoxylates that are used to produce non-ionic surfactants, detergents, emulsifiers for agrochemicals, antioxidants for rubber manufacture and as lubricant oil additives [7]. From a physical–chemical point of view, low solubility and high hydrophobicity allowing its accumulation and persistence in environmental compartments that are characterized by high organic content [8]. It evoked much concern as it represents a major challenge to fish signifying in targeting many systems such as immunity hematology [9], and osmoregulatory [10] systems. Also, its health problems surpass to human by accumulating in the lipid of living organisms through the food chain [11].

Hematological outcome endpoints offer a simple and compelling methodological tool for understanding physiological processes, diagnosis of structural and functional status of intoxicated fish, and screening the water quality [12, 13]. African catfish (*Clarias gariepinus*) is considered as a promising model for toxicological studies [14].

The mission of this study is to focus on the potential modulatory role of five modified thienopyrazole derivatives concerning the hematological indexes and electrolyte equilibrium in 4-NP poisoned African catfish (*Clarias gariepinus*) for the first time in a hope of getting novel effective therapeutical strategies against this highly prevalent aquatic toxicant.

2. Materials and methods:

2.1. 4-Nonylphenol

4-Nonylphenol (4-NP) was obtained from Sigma-Aldrich (Schnelldrof, Germany) with purity 99.3%.

2.2. Novel thieno [2,3-c] pyrazole compounds

New synthesized [2,3-c] pyrazole compounds (4-Amino-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carbonitrile, 4-Amino-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carboxamide, 4-Amino-N-(4-methoxyphenyl)-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carboxamide, 4-Amino-N-(4-chlorophenyl)-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carboxamide and 4-(2-Chloroacetamido)-3-methyl-1-phenyl-1H-thieno[2,3-c]pyrazole-5-carboxamide) were used in this study. Their synthetic procedures, physical properties, and spectral data were previously published [14].

2.3. Experimental design

Adult African catfish (*Clarias gariepinus*) were collected at July 2013, and acclimated in aerated recirculating tank containing experimental media water for two months. Prior to the experiments, the fish were examined to be free of external parasites [15]. To test the biological effects of the present chemical compounds, adult fish (six per group) were maintained in 100 L glass aquaria; one group as control, the second exposed to 0.1 mg/l 4-NP, and the other five groups exposed to 0.1 mg/l 4-NP were injected intramuscularly

in the form of emulsion once daily with different tested chemicals compounds as described in our previous publication [14] for two weeks in triplicates per each group. The doses selected for this study were based on the current guidelines and recommendations of bulk chemicals [16]. During the experimental period, fish were fed 5% body weight twice daily with commercial pellets, and the experimental media water was changed every day. Fish measuring the average size about 36.7 ± 1.38 cm in length and the average weight about 424.4 ± 27.34 g.

2.4. Blood analysis

Blood samples (six fish/group) were collected from the caudal vein of fish in heparinized tubes. The red blood cells (RBCs), white blood cells (WBCs) and platelets counts, hematocrit (HCT) value, and Hb content were determined using automated technical analyser (Mindray Bc-2800). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the formulae mentioned by [17]:

Total WBCs were counted using improved Neubaur haemocytometer [18], and differential count (lymphocytes, monocytes, neutrophils, and eosinophils) was displayed by staining blood films with Giemsa stain. The concentrations of HCO_3^- , Na^+ , K^+ , Cl^- , Cu^{+2} , Fe^{+2} and Ca^{+2} were measured in serum using atomic absorption spectrophotometer (GBC Model 300).

2.5. Statistical analysis

The data were expressed as means $\pm$ standard error of the mean. Significant differences between groups were analyzed by one-way ANOVA followed by Duncan post hoc test for multiple comparisons using SPSS software, version 16. Differences were considered statistically significant at $P < 0.05$.

2.6. Ethical statement

All experiments were carried out in accordance with the Egyptian laws and University guidelines for the care of experimental animals. All procedures of the current experiment have been approved by the Committee of Faculty of Science, Assiut University, Egypt.

3. Results

3.1. Macrocytic hyperchromic anemia in African catfish under 4-NP stress, and specificity of thienopyrazole compound 1 in targeting hemoglobin and compound 2 in targeting the rate of erythropoiesis

As shown in table (1), a significant decrease in RBC count and HCT value, and increase in MCV and MCH versus their control levels were observed in African catfish under 4-NP stress. In comparison with 4-NP exposed group, significant decrease in Hb level and MCH were resulted from treatment of 4-NP intoxicated fish with compound 1 in association with 4-NP. On the other hand, compound 2 in combination with 4-NP was the most efficient in correcting RBC count and HCT abnormalities versus 4-NP alone.

3.2. Great disturbances in the cellular fraction of blood by 4-NP, and various recovery patterns exhibited by thienopyrazole derivatives

It was statistically apparent that 4-NP lonely was capable of inducing thrombocytopenia, leucopenia, neutrophilia, eosinophilia, and decreased count of large lymphocytes relative to the control. Compound 2, 3, 4, and 5 were successful in returning platelet count to the control value. Regarding total WBCs count, all thienopyrazole derivatives in conjunction with 4-NP failed to overcome 4-NP-induced leucopenia. Analysis of variance demonstrated that neutrophilia was most significantly prevented by compound 2 and 4, whereas, compounds 3 and 5 reduced such number significantly to a midway between control and 4-NP values. The most significant elevation in large lymphocyte count appeared after treatment of 4-NP poisoned fish with

compound 4, followed in potency by compound 5, 2, and then 3. Small lymphocyte numbers of fish exposed to mixture of 4-NP together with either of compound 2, 4, or 5 were the same as those of either untreated fish or 4-NP intoxicated one while, compound 1 and 3 reduced the numbers to a similar degree as 4-NP alone. In case of monocytes, the treatment itself worsens the condition as indicated by monocytosis in both of 4-NP/compound 1 and 4-NP/compound 2 groups. Conversely, compounds 1, 2, and 3 succeeded in returning eosinophil back to control level.

3.3. Electrolyte balance disruption in 4-NP poisoned African catfish and continuation of thienopyrazole compound 1 in exhibiting side effects

As shown in table (2), 4-NP toxicity in African catfish was responsible for hyponatremia, hyperkalemia, hyperferremia, and metabolic acidosis as evidenced by decreased serum HCO_3^- level versus normal untreated fish. Levels of serum Na^+ became halfway between those of control and 4-NP poisoned groups following supplementation with compounds 1, 2 or 3 combined with 4-NP. The adverse influences of compound 1 when supplemented with 4-NP were repeated again in the form of exaggerated hypocholesterolemia, hypoferremia, and hypocalcaemia. In contrast, absence of significant differences in serum Cl^- , Fe^{+2} , and Ca^{+2} levels were clear between 4-NP toxicated group and other 4-NP/thienopyrazole combination groups. All thienopyrazole compounds failed to cause any significant changes in serum K^+ and Cu^{+2} levels with respect to 4-NP exposed group. Estimated from serum Na^+ , K^+ , Cl^- , and HCO_3^- levels, anion gap failed to elicit any significant change in 4-NP intoxicated fish versus normal untreated ones. As compared with both of control and 4-NP treated groups, other compounds had no significant impacts on anion gap except compound 1 which incriminated in causing severe metabolic acidosis as manifested by raising this diagnostic endpoint.

4. Discussion:

The present investigation paves the road to get novel and efficient protective modalities against unresolved toxicological conditions in aquatic organisms, and is the first to give spotlight on the ability of the synthetic pyrazole compounds in alleviating toxicity in fish. It is worthy to take into consideration that the differences/similarities in structure/properties of the present compounds were already investigated [14]. 15 days-exposure to 4-NP in this study exhausted haematopoietic potential, as revealed by reduced RBC count, matching with the findings of previous studies [9, 19-21]. The decline in RBCs number may be attributed to inhibition of erythropoiesis, exhaustion of hemopoietic activity of kidney, elimination of RBCs from circulation, and haemolysis [19, 21, 22]. The non-significant change in Hb content may be due to the dose is a critical variable in understanding the changes in Hb content following 4-NP exposure [9]. The marked increase in MCV along with MCH goes hand to hand with the hematological outcomes in African catfish and juvenile common carp [9, 20, 21] suggesting macrocytic hyperchromic anemia. MCH increased due to marked enlargement in RBCs sizes (MCV), however the noticeable increment in MCH failed to compensate reduced total RBCs count resulting in unchanged MCHC which keeps pace with the data obtained from [21]. Thienopyrazole compound 1 or 2 was effective in correcting one aspect of anemic disorders. Compound 2 elevated RBCs number, with subsequent rising in PCV to the same level as control, while compound 1 was superior in reducing MCH towards the control value. Inhibition of hypoxia inducible factor prolyl 4-hydroxylase, and stimulation of erythropoietin secretion may justify the new pyrazoles modulatory impacts as exemplified by other pyrazole derivatives, namely benzimidazole-2-pyrazole carboxylates [3].

Versus normal untreated fish, there was a significant decrement in blood leucocyte count of 4-NP treated ones in parallel with the ability of 4-NP and 17 β -estradiol (E2) in

suppressing lipopolysaccharide-induced leukocyte proliferation [23]. High remarkable drop in the large lymphocyte count of African catfish poisoned with 4-NP is corresponding to the same outcome pattern in fish and mice [20, 24]. Contradictory, remarkable decrements in large and small lymphocyte numbers were obvious following dosing with 4-NP/compound 1, and small lymphocyte numbers in case of 4-NP/compound 3 to similar levels which caused by 4-NP only. Similarly, pyrazole complexes decreased lymphocyte populations in *Leishmania amazonensis*-infected mice and mouse graft-versus-host disease model via suppression of lymphocyte proliferation and Ca^{2+} signals in CD4^{+} and Jurkat T-cells [25-27].

In the present work, 4-NP-induced neutrophilia is matched with previous work demonstrating increment in the granulocyte percentage in female rats exposed maternally to 4-tert-octyphenol [28]. The ability of all thienopyrazole compounds with exception of first one to relieve neutrophilia is compatible with decreased total neutrophil count in horse [29].

A significant decrement was noticed in the blood platelet count together with eosinophilia in 4-NP intoxicated African catfish in a harmony with the findings detected by [20]. All thienopyrazole compounds except compound 1 antagonized the thrombocytopenia induced by 4-NP perhaps by prolongation of survival, reduction of turnover, and inhibition of phagocytosis [30, 31]. With exclusion of thienopyrazole compounds 1, 2, and 3, the remaining ones succeeded to overcome the 4-NP-mediated eosinophilia as matched with prevention of eosinophil infiltration in actively sensitized guinea pigs and Brown Norway rats by YM-58483, another pyrazole derivative [32].

In the current experiment, 4-NP caused a significant decrease in serum Na^{+} level in parallel with a significant increase in serum K^{+} level most probably as consequent to inhibition of kidney $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$ activity [10]. Serum Na^{+} levels were increased

following administration of 4-NP-poisoned African catfish with thienopyrazole compound 2 and 3, without significant changes in serum K^+ levels in any compound treated groups. Some derivatives of imidazole and pyrrol, a structurally relevant to pyrazole, represented novel classes of gastric H^+/K^+ ATPase inhibitors [33, 34] indicating consequent loss of K^+ from the digestive tract.

Insignificant alteration in serum Cl^- level of 4-NP-loaded fish is conflicting with obvious increase of chloride cell size and number in rainbow trout [35]. However, this disagreement may be due to variations in the experimental dose-duration setup. In its continuing way to show its dark sides, thienopyrazole compound 1 induced hypochloremia which may attributed to affinity of similar structures as pyrrole to bind with chloride anion [36].

4-NP in this work was responsible for metabolic acidosis which is consistent with previous findings [37] and was characterized by a massive fall in serum HCO_3^- level as a result of endogenous acids accumulation leading to shift in the acid-base balance towards the acidic side [38]. Surprisingly, the remarkable drop in serum HCO_3^- level did not accompany with any significant changes in the anion gap contradicting [37]. This condition referred to non-anion gap acidosis which observed in disorders due to loss of HCO_3^- , such as renal tubular acidosis [39]. Thienopyrazole compound 1 was the superior in increment of anion gap because it caused the highest decrease in serum HCO_3^- and Cl^- levels together with the highest increase in serum Na^+ and K^+ levels.

4-NP-induced metabolic acidosis may be lie behind a marked increment in serum Fe^{2+} level, because pH-dependent affinity of transferrin for iron decreases under acidic conditions [40]. Lowering serum Fe^{2+} level below the control by thienopyrazole compound 1 provoked undesirable effect because iron in combination with some proteins exerts a variety of vital functions [41].

Absence of significant changes in serum Ca^{+2} level following 4-NP stresses is conflicting with other finding [42]. Another adverse impact of thienopyrazole compound 1 was marked hypocalcaemia which could result in increased neuromuscular excitability leading to tetany [43], and compromised bone integrity due to the reduction in available stores of Ca^{+2} [44].

6. Conclusions

The results of this study indicated that, most of the new synthesized thienopyrazole compounds used in this study as compound 2>3>4>5 were effective in relieving the toxicological features associated with 4-nonylphenol exposure regarding electro-hematological markers of the African catfish (*Clarias gariepinus*). More investigation was required for compound 1.

7. References

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Table 1: Impacts of 4-nonylphenol alone and its combination with thienopyrazole compounds on the hematological parameters of African catfish (*Clarias gariepinus*).

Parameters/Groups	Control	4-NP	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
RBC count (million/ μ l)	3.80 $\pm$ 0.06 ^a	2.93 $\pm$ 0.088 ^{cd}	2.98 $\pm$ 0.72 ^{cd}	3.20 $\pm$ 0.058 ^b	3.13 $\pm$ 0.33 ^{bc}	3.07 $\pm$ 0.09 ^{bcd}	2.87 $\pm$ 0.33 ^d
Hb content (mg/dl)	9.43 $\pm$ 0.68 ^a	8.61 $\pm$ 0.23 ^{ab}	7.51 $\pm$ 0.35 ^c	9.01 $\pm$ 0.10 ^{ab}	8.78 $\pm$ 0.20 ^{ab}	8.77 $\pm$ 0.16 ^{ab}	8.42 $\pm$ 0.20 ^b
HCT (%)	38.22 $\pm$ 0.59 ^a	35.15 $\pm$ 0.57 ^{bc}	33.55 $\pm$ 0.87 ^c	37.52 $\pm$ 0.39 ^a	36.79 $\pm$ 0.66 ^{ab}	34.86 $\pm$ 0.92 ^{bc}	34.89 $\pm$ 0.86 ^{bc}
MCV (μ m ³)	98.22 $\pm$ 4.37 ^a	119.97 $\pm$ 3.29 ^a	112.86 $\pm$ 4.25 ^a	117.33 $\pm$ 2.43 ^a	117.46 $\pm$ 2.94 ^a	113.96 $\pm$ 5.66 ^a	121.79 $\pm$ 4.14 ^a
MCH (pg)	24.16 $\pm$ 0.39 ^a	29.37 $\pm$ 0.72 ^a	25.19 $\pm$ 0.55 ^a	28.18 $\pm$ 0.60 ^a	27.98 $\pm$ 0.46 ^a	28.69 $\pm$ 1.37 ^a	29.40 $\pm$ 1.04 ^a
MCHC (g/dl)	24.72 $\pm$ 1.41 ^a	24.52 $\pm$ 0.90 ^a	22.41 $\pm$ 1.22 ^a	24.02 $\pm$ 0.52 ^a	23.89 $\pm$ 0.94 ^a	25.18 $\pm$ 0.42 ^a	24.14 $\pm$ 0.26 ^a
Platelet count (thousands/ μ l)	216.67 $\pm$ 2.67 ^a	204.33 $\pm$ 2.03 ^a	203.67 $\pm$ 4.18 ^c	211.33 $\pm$ 6.06 ^{ab}	206.67 $\pm$ 3.33 ^{ab}	211.33 $\pm$ 0.88 ^{ab}	213.67 $\pm$ 1.20 ^{ab}
WBC count (thousands/ μ l)	12.24 $\pm$ 0.24 ^a	10.50 $\pm$ 0.21 ^b	10.64 $\pm$ 0.76 ^b	11.43 $\pm$ 0.38 ^{ab}	10.87 $\pm$ 0.29 ^b	11.17 $\pm$ 0.03 ^{ab}	11.53 $\pm$ 0.13 ^{ab}
Neutrophil count (%)	11.67 $\pm$ 0.33 ^a	15.67 $\pm$ 0.33 ^a	16.33 $\pm$ 0.33 ^a	13.00 $\pm$ 0.58 ^{bc}	13.67 $\pm$ 0.67 ^b	13.00 $\pm$ 0.58 ^{bc}	13.67 $\pm$ 0.33 ^b
Large lymphocyte count (%)	58.67 $\pm$ 0.33 ^a	53.00 $\pm$ 0.58 ^a	53.67 $\pm$ 0.88 ^{ab}	56.33 $\pm$ 0.88 ^{bc}	55.33 $\pm$ 0.88 ^{cd}	57.67 $\pm$ 0.33 ^{ab}	57.33 $\pm$ 0.33 ^{abde}
Small lymphocyte count (%)	25.00 $\pm$ 0.58 ^a	22.67 $\pm$ 1.67 ^{abc}	21.33 $\pm$ 0.88 ^c	23.00 $\pm$ 0.58 ^{abc}	22.00 $\pm$ 0.00 ^{bc}	24.33 $\pm$ 0.33 ^{bc}	24.00 $\pm$ 0.58 ^{abc}
Monocyte count (%)	2.67 $\pm$ 0.58 ^{bc}	2.00 $\pm$ 0.58 ^a	4.00 $\pm$ 0.00 ^a	3.67 $\pm$ 0.33 ^{ab}	3.00 $\pm$ 0.58 ^{abc}	2.67 $\pm$ 0.33 ^{bc}	2.33 $\pm$ 0.33 ^a
Eosinophil count (%)	2.00 $\pm$ 0.00 ^d	6.67 $\pm$ 0.88 ^a	4.67 $\pm$ 0.33 ^{abc}	4.00 $\pm$ 1.16 ^{bcd}	6.00 $\pm$ 0.58 ^{ab}	2.33 $\pm$ 0.33 ^d	2.67 $\pm$ 0.33 ^{cd}

Results are expressed as means $\pm$ SEM of 6 fish per group.

Different letters indicate significance at $p < 0.05$ (one-way ANOVA followed by Duncan post hoc test).

Table 2: Impacts of 4-nonylphenol alone and its combination with thienopyrazole compounds on serum electrolyte levels and anion gap of African catfish (*Clarias gariepinus*).

Parameters/Groups	Control	4-nonylphenol	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
Serum Na ⁺ level (μ g/ml)	125.67 $\pm$ 2.03 ^a	116.33 $\pm$ 2.19 ^a	118.33 $\pm$ 0.33 ^{bc}	124.00 $\pm$ 1.53 ^{ab}	120.00 $\pm$ 0.58 ^{abc}	115.67 $\pm$ 3.71 ^a	116.00 $\pm$ 2.65 ^a
Serum K ⁺ level (μ g/ml)	4.04 $\pm$ 0.09 ^c	4.74 $\pm$ 0.28 ^{ab}	5.17 $\pm$ 0.19 ^a	4.39 $\pm$ 0.26 ^{bc}	4.50 $\pm$ 0.22 ^{bc}	4.33 $\pm$ 0.11 ^{bc}	4.34 $\pm$ 0.15 ^{bc}
Serum Cl ⁻ level (μ g/ml)	94.67 $\pm$ 1.76 ^a	89.67 $\pm$ 0.33 ^{ab}	81.67 $\pm$ 3.18 ^c	91.67 $\pm$ 1.20 ^{ab}	91.00 $\pm$ 0.58 ^{ab}	85.67 $\pm$ 3.90 ^{bc}	89.00 $\pm$ 0.58 ^{ab}
Serum HCO ₃ ⁻ level (μ g/ml)	20.00 $\pm$ 1.00 ^a	13.00 $\pm$ 0.58 ^a	12.33 $\pm$ 0.33 ^c	19.00 $\pm$ 0.58 ^a	16.00 $\pm$ 1.16 ^b	13.67 $\pm$ 0.33 ^c	13.67 $\pm$ 0.33 ^c
Anion gap (mEq l ⁻¹)	15.04 $\pm$ 1.91 ^b	18.40 $\pm$ 1.84 ^{ab}	29.50 $\pm$ 3.05 ^a	17.72 $\pm$ 1.38 ^b	17.50 $\pm$ 0.48 ^b	20.67 $\pm$ 7.41 ^{ab}	17.67 $\pm$ 3.46 ^b
Serum Cu ²⁺ level (μ g/ml)	110.15 $\pm$ 6.01 ^a	98.77 $\pm$ 0.62 ^{abc}	91.63 $\pm$ 2.26 ^c	107.11 $\pm$ 6.50 ^{ab}	106.59 $\pm$ 6.21 ^{ab}	94.80 $\pm$ 0.32 ^{bc}	96.77 $\pm$ 1.15 ^{abc}
Serum Fe ²⁺ level (μ g/ml)	15.60 $\pm$ 0.42 ^a	17.50 $\pm$ 0.30 ^a	12.93 $\pm$ 0.13 ^c	16.43 $\pm$ 0.20 ^{ab}	17.07 $\pm$ 0.37 ^a	16.43 $\pm$ 0.55 ^{ab}	16.83 $\pm$ 0.58 ^{ab}
Serum Ca ²⁺ level (μ g/ml)	52.13 $\pm$ 1.17 ^a	48.42 $\pm$ 0.84 ^a	42.13 $\pm$ 1.16 ^b	52.45 $\pm$ 1.86 ^a	49.81 $\pm$ 0.30 ^a	49.16 $\pm$ 1.51 ^a	48.76 $\pm$ 1.17 ^a

Results are expressed as means $\pm$ SEM of 6 fish per group.

Different letters indicate significance at $p < 0.05$ (one-way ANOVA followed by Duncan post hoc test).

* Anion gap = Na⁺ + K⁺ - Cl⁻ - HCO₃⁻.