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RESEARCH ARTICLE

Neurotoxic responses of rainbow trout (*Oncorhynchus mykiss*) exposed to fipronil: multi-biomarker approach to illuminate the mechanism in brain

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ABSTRACT

Insecticides have potential to non-target organisms, disrupting the healthy functioning of the aquatic environment as they are the ultimate receptor of the aquatic ecosystem. Insecticides, which are widely used in agriculture, have high neurotoxicity on aquatic organisms. In this study, the acute alterations [catalase (CAT), arylesterase (ARE), malondialdehyde (MDA), myeleperoxidase (MPO), paraoxonase (PON), glutathione peroxidase (GPX), superoxide dismutase (SOD), 8-hydroxy-2-deoxyguanosine (8-OHdG) level, caspase-3 activity, and Acetylcholinesterase (AChE) enzyme activity] caused by the different concentrations of Fipronil (FP) insecticide (0.05, 0.1, and 0.2 mg/L) on rainbow trout (*Oncorhynchus mykiss*) brain tissue were investigated. It has been determined that superoxide dismutase –catalase - glutathione peroxidase - paraoxonase and arylesterase enzyme activities were inhibited but MDA and MPO induced depending on the concentration in brain tissue. When compared with the control group, the changes between the pesticide exposed groups were found statistically significant ($p < 0.05$). In brain tissue, while AChE enzyme activity was decreased depending on concentration, caspase-3 activity increased with 8-OHdG level. As a result, it has been determined that FP is a dangerous environmental pollutant for aquatic organisms, even at low concentrations, inducing oxidative stress, damaging the brain tissue of fish and stimulating apoptosis.

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1. Introduction

Fipronil (FP) is a broad spectrum and a systemic insecticide used to control insect pests in urban and agricultural areas. Fipronil, which enters their ecosystem by flowing or seepage from soil into streams, has to moderate persistence in aquatic environments (Beggel *et al.* 2012, Chagnon *et al.* 2015, Menezes *et al.* 2016, Qureshi *et al.* 2016, Gupta and Anadón 2018). This insecticide acts on the central nervous system of insects by selectively binding to gamma-aminobutyric acid (GABA) gated chloride channels and antagonizing the effect of GABA. Gripp *et al.* (2017) reported that fipronil and its metabolites are highly toxic to rainbow trout. In this case, GABA receptor inhibition causes central nervous system toxicity by disrupting the chloride ion control of the neuronal signal (Stehr *et al.* 2006). It has been reported that the application of fipronil in aquatic organisms (in vivo or *in vitro*); not only cause toxicity on DNA, lipids and proteins but also it has neurotoxic, hepatotoxic, and cytotoxic effects (Badgujar *et al.* 2016, Wang *et al.* 2016).

Rainbow trout (*Oncorhynchus mykiss*) is known as a model organism with easy obtaining its tissues and cells. *O. mykiss* is also widely used in carcinogenesis, toxicology, comparative immunology, disease ecology, physiology, and nutrition researches (Thorgaard *et al.* 2002). They are also used as biomarkers of the health indicator of the aquatic environment as they are directly exposed to environmental toxicants. Rainbow trout is one of the most researched fish species cultivated for more than 100 years, given its high economic value. Fish are important biomarkers in aquatic toxicology studies, as they are an important component of the food chain and are easy to obtain. (Wu *et al.* 2014). The most sensitive and complex functioning of energy metabolism in vertebrates is also seen in the brain. The use of the brain in scientific studies contributes to the observation of biochemical exchange processes (Soengas and Aldegunde 2002). Reactive Oxygen Species (ROS) detoxification in the brain is done within certain limits due to the low capacity of producing Glutathione S-Transferase (GSH) in neurons. For this reason, neurons are the cells most affected by ROS increase (Chauhan and Chauhan 2006). In addition to their neurotoxicity, pesticides affect antioxidant defense responses, resulting in cellular damage, increasing oxidative stress. Similarly, detoxification enzyme activities have been reported to provide useful information about the metabolic pathways triggered by the toxic substance (Sandoval-Herrera *et al.* 2019).

Acetylcholinesterase is a hydrolase enzyme, predominantly found in muscle tissue and the nervous system of organisms. It acts an important role in the neurotransmission and widely

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used enzyme in the determination of the neurotoxic effects of xenobiotics in the aquatic ecosystem (Kim and Lee 2018). The research have shown that Acetylcholinesterase (AChE) activity is blockaged by metals, pesticides, pharmaceuticals and other pollutants in aquatic medium (Rhee *et al.* 2013, Ezeoyili *et al.* 2019), and at pesticides exposed organisms AChE activity acts as a neurotoxicity indicator (Amiard-Triquet 2009). Pesticides have a very specific mode of action that inhibits cholinesterase enzyme (ChE) activity in the nervous system. This makes it important to determine AChE activity in toxicity tests (Sandoval-Herrera *et al.* 2019).

The brain is a crucial organ to study the unfavorable effects of oxidative damage. Oxidative stress in tissues can occur as; (i) enhanced ROS generation, (ii) reduced ROS elimination, and (iii) incompatible combination between generation and elimination (Lushchak 2016). Bioaccumulation of toxic substances and producing free oxygen radical's reactions trigger redox, but also produce other ROS' s. To inhibit the toxic effects of ROS, organisms balance free radicals using both enzymatic and non-enzymatic antioxidants. However, when ROS production exceeds the contents of cellular antioxidants, it reasons oxidative stress and damage. This situation causes biochemical changes in fish tissues (Narra *et al.* 2017).

Oxidative stress-induced by FP can damage macromolecules such as DNA, proteins and lipids. Afterward oxidative stress, cell death, MDA and DNA damage, and protein peroxidation occur by apoptotic or necrotic mechanisms (Wang *et al.* 2016). The most common and susceptible of twenty-three oxidative base spoilage caused by ROS in DNA is 8-Hydroxy-2′-Deoxyguanosine (8-OHdG) (Koç and Akçay 2018). Caspase-3 is an important molecule in the regulation of both mitochondrial and apoptotic pathways. (Gao *et al.* 2013). Apoptosis occurs in two different ways; receptor-mediated apoptotic pathway and mitochondrial-mediated apoptotic pathway; both of them work with caspase-3 as effector caspase (Piner and Üner 2012). Caspase-3 can be activated by ROS construction and therefore can induce cell apoptosis and necrosis (Junn and Mouradian 2001).

Although FP is low environmental toxic, it is an effective phenylpyrazole insecticide with high toxicity for non-target organisms such as fish. To develop new, effective, low-toxic insecticides, a detailed toxic mechanism needs to be determined. For this purpose, biomarkers are often used in ecotoxicology to investigate toxic effects occurring at low concentrations. In this study, a multi-biomarker (AChE activity, oxidative stress, 8-OHdG, and caspase-3) approach was tried to determine the toxicity mechanism created by the FP insecticide in rainbow trout brain tissue.

2. Materials and methods

2.1. Experimental design

160 rainbow trouts (*Oncorhynchus mykiss*) (120 ± 5 g; 20 ± 2 cm) which were produced in Atatürk University Inland Fisheries Application and Research Unit, taken into the trial medium to undergo a 14-day acclimation process in the toxicology center unit. Eight aquariums (40 × 75 × 50 cm/150 l volume) including the control group were used in the study, which was designed as two repetitions of three different doses and two repetitions. The trial chemical (FP) was purchased from Akdeniz Chemistry, (Erzurum).

Table 1. Experimental groups.

Experimental groups	Fipronil concentration
Control	0.00 mg/L
D1	0.05 mg/L
D2	0.1 mg/L
D3	0.2 mg/L

During trial, the physicochemical properties of the water were measured, and determined as suitable for the optimum criteria's of the trout. It was determined as pH: 7.0, dissolved oxygen: 8 − 9°mg/L, temperature: 10.5 ± 0.5 °C and total hardness: 220°mg/L.

The doses were applied according to LC_{50} (0.246°mg/L) value by Chandler *et al.* 2004. Accordingly, the application doses were determined as: D1 (0.05°mg/L) 20% of LC_{50}, D2 (0.1°mg/L) 40% of LC_{50}, and D3 80% (0.2°mg/L) of LC_{50} and experimental design showed in Table 1.

2.2. Preparation of the brain tissue

After acute exposure, the brain tissues of the fish in the whole application and control groups were removed. All of the samples taken were homogenized (1% *v/v*) in 0.1 M phosphate buffer (pH 7.4) containing Triton-X 100 using a homogenizer and then centrifuged at each enzyme demand at 4 °C.

2.3. Biochemical biomarkers in the brain

2.3.1. Neurotoxic biomarker: AChE activity

The enzyme acetylcholinesterase catalyzes the breakdown of acetylcholine to thiocoline with acetate. AChE activity was assignment by measuring the changes in the absorbance of 412°nm wavelength of the yellow-colored compound formed by the reaction of thiocoline formed with DTNB by 5-thio-2-nitrobenzoic acid (Ellman *et al.* 1961).

2.3.2. ROS, antioxidant defenses, and oxidative damage

Catalase (CAT) is depend on the enzymatic degradation of H_2O_2 substrate with catalase at 240 nm absorbance reduction (Aebi 1984). Glutathione peroxidase (GPx) was measured by reading the absorbance difference during the oxidation of NADPH to $NADP^+$ at a wavelength of 340°nm (Beutler 1984). Superoxide Dismutase (SOD): depend on measuring the color absorbed at 560°nm with nitro-blue tetrazolium (NBT) of superoxide radicals released by xanthine oxidase in the presence of xanthine. (Sun *et al.* 1988). Paraoxonase is used as a matter for PON activity. The activity is the assurance of the absorbance alter of 4-nitrophenol formed by paraoxon hydrolysis at 37 °C and 412°nm spectrophotometric per unit time (Gülcü and Gürsü 2003). Phenylacetate (Sigma Co, UK) is used as a matter to specify Arylesterase (ARE) activity. It was specified by the changing the absorbance of phenol formed at 270°nm. 17,100 and 1310 M^{-1}/cm molar absorption coefficients were used to calculate PON and ARE activities,

respectively (Gülcü and Gürsü 2003, Alak *et al.* 2019). Malondialdehyte (MDA): The absorbance of the pink-red color formed by the reaction of the most stable of lipid peroxidation products (MDA) with thiobarbutyric acid is based on spectrophotometric evaluation (Luo *et al.* 2006). Myeloperoxidase (MPO); is reside in the kinetic altitude of the absorbance of the yellowish-orange complex originate from the oxidation of o-dianiside with MPO in the existence of hydrogen peroxide at a wavelength of 460 nm (Uçar *et al.* 2020).

2.3.3. DNA and apoptosis biomarkers: 8-OHdG and caspase-3

In supernatants obtained after centrifugation of brain tissue samples, brain 8-OHdG level [Fish (8-OHdG) Catalog No: 201–00-0041/SunRed)] and caspase-3 activation [Fish (CASP3) ELISA Kit (Catalog No: 201–00-0031) (SunRed trade)] were measured (Alak *et al.* 2018).

2.4. Statistical analyses

The outputs of the research were evaluated using SPSS software and the differences between the groups were determined by using Duncan test ($p < 0.05$).

3. Results

The toxic mechanism of FP insecticide, which is acutely administered to rainbow trout in 3 different doses [D1 (0.05 mg/L) 20% of LC_{50}, D2 (0.1 mg/L) 40% of LC_{50}, and D3 80% (0.2 mg/L) of LC_{50}], illuminating with different findings from the level of oxidative stress to apoptosis, as well as neurotoxicity was studied.

3.1. Assessment of AChE activity

It was determined that AChE activity was inhibited in the treatment groups due to the concentration increase in acute application to rainbow trout brain tissue (Figure 1) ($p < 0.05$).

3.2. Assessment of antioxidant activity in brain tissue of O. mykiss

In the obtained results; a decrease was assigned in ARE, PON, CATand GPx activities, depending on the dose compared to the control group, while MDA and MPO values increased. These results were considered statistically significant ($p < 0.05$) (Table 2).

3.3. Assessment of caspase-3 and 8-OHdG in brain tissue of O. mykiss

8-OHdG levels were measured in the brain tissue of rainbow trout. An increase was determined in all groups compared to the control group in acute application due to the increase in concentration. This increase was found significant at $p < 0.05$ level (Table 3). However, the highest increase in the 8-OHdG level and Caspase-3 activity was obtained in D3 (0.2 mg/L) group.

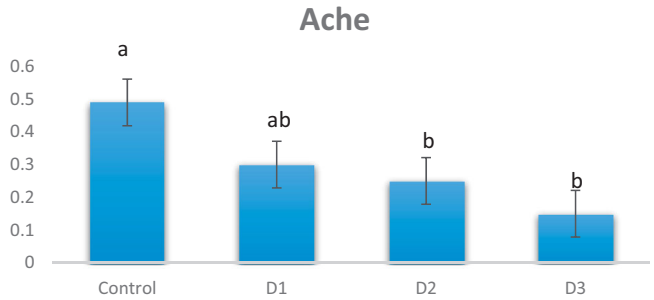

Figure 1. Effect of FP on AChE in rainbow trout's brain (mean ± SE) $n = 20$.

4. Discussion

The brain is an important element of the central nervous system, which is very sensitive to oxidative damage. In the healthful brain where the redox balance is advanced, the resulting ROS is useful and even necessary. However, redox balance disorder causes a condition known as oxidative stress, which is closely related to neurodegeneration (Samet and Wages 2018).

Amino-aminobutyric acid (GABA) receptors are a class of receptors that respond to the neurotransmitter GABA, the inhibitory compound in the central nervous system of vertebrates (Wang *et al.* 2016). FP interferes in the nervous system by selectively binding to gamma-aminobutyric acid (GABA)-gated chloride channels and antagonizing the effect of GABA (Stehr *et al.* 2006, Qu *et al.* 2016). Stimulation of the nervous system results in neuronal hyper-excitation, stroke, and death due to the accumulation of GABA in synaptic connections (Gunasekara *et al.* 2007). GABA-mediated neurotransmission takes part in the modulation of several neural pathways in fish development. It has been reported that the affinity of FP to fish GABA receptors is similar to that found in insects and can be highly toxic to fish (Zhang *et al.* 2018, Dallarés et al. 2020). Acetylcholine is one of the most common neurotransmitters in the body. It is released from various parts of the central nervous system, motor neurons of the somatic nervous system, pre-post ganglionic parasympathetic neurons and preganglionic sympathetic neurons in the autonomic nervous system (Ganong 2003). The biomarker evaluated in our study is Acetylcholinesterase (AChE), a crucial enzyme in the nervous system. AChE ends the nerve impulses by supplying hydrolysis of the neurotransmitter acetylcholine (Lionetto *et al.* 2013). In accordance with research, the effect of insecticides is relavent to reversible or irreversible neutralization of AChE and lead to cholinergic poisoning (Silva *et al.* 2013, Serafini *et al.* 2019), acetylcholine accumulation and hyper-stimulation of receptors (Colovic *et al.* 2013). It can be said that FP administration destroys the brain tissue cholinergic neurons (Tian *et al.* 2018), reduces hydrolysis of ACh, crucial neurotransmitter for synaptic cleft (Baldissera *et al.* 2017), and as a result, inhibits AChE activity. Inhibition of AChE activity may have been caused by the direct effect of FP on

Table 2. Effect of FP on the oxidative stress levels in *O.mykiss*' brain (mean ± SE).

Groups	SOD* (EU/mg protein)	CAT* (EU/mg protein)	GP_X* (EU/mg protein)	MDA* (μmol/l)	PON* (U/ml)	ARE* (U/ml)	MPO* (U/ml)
Control	1.11 ± 0.24^{a}	0.76 ± 0.02^{a}	0.65 ± 0.20^{a}	0.24 ± 0.05^{c}	106.5 ± 18.5^{a}	67.6 ± 2.3^{a}	14.7 ± 3.3^{c}
D1	0.82 ± 0.09^{b}	0.55 ± 0.09^{b}	0.43 ± 0.14^{b}	0.41 ± 0.07^{b}	57.3 ± 11.4^{b}	66.9 ± 2.8^{a}	38.7 ± 8.8^{b}
D2	0.77 ± 0.12^{b}	0.57 ± 0.16^{b}	$0.26 \pm 0.04b^{c}$	0.38 ± 0.06^{b}	39.0 ± 6.5^{b}	55.8 ± 8.2^{b}	34.5 ± 2.2^{b}
D3	0.78 ± 0.10^{b}	0.26 ± 0.14^{c}	0.18 ± 0.05^{c}	0.86 ± 0.13^{a}	16.8 ± 10.0^{c}	51.8 ± 2.4^{b}	73.5 ± 14.5^{a}

Lowercase superscripts (a,b,c) indicate significant differences among the same colon within each experimental treatment group, $p < 0.05$. $n = 20$.*$p < 0.05$.

Table 3. Effect of FP on the DNA damage (8-OHdG) and apoptosis (Caspase-3) in *O.mykiss*' brain.

Groups	8-OHdG (ng/ml)*	Caspase-3 (ng/ml)*
Control	0.25 ± 0.02^{b}	1.45 ± 0.08^{a}
D1	0.28 ± 0.02^{ab}	1.55 ± 0.16^{a}
D2	0.29 ± 0.02^{ab}	1.53 ± 0.08^{a}
D3	0.31 ± 0.03^{a}	1.64 ± 0.10^{a}

Lowercase superscripts (a,b) indicate significant differences among.*$p < 0.05$.

the active sites of the AChE enzyme and destroyed its connecting to acetylcholine.

CAT, GPx and SOD, the most important antioxidant enzymes of the defense system, can effectually send away the generated free radicals (Bhattacharjee and Sil 2006) and this enzyme protect the cells against the oxidative damages. At the cellular level, antioxidants are responsible for protecting cells from damage by removing free radicals (El-Murr *et al.* 2015). ROS formation is induced in living things with the toxic substance exposure and also occurs as a result of detoxifying mechanisms initiated by the organism. Also, ROS causes cellular damage by irreversible oxidation of biomolecules. This indicates the oxidative stress occurring in the tissue (Ghazanfar *et al.* 2018). The physiological function of SOD is to preserve cells that metabolize oxygen against the harmful effects of superoxide free radical, such as lipid peroxidation (Lushchak and Bagnyukova 2006). In this study, there was a significant decrease in SOD enzyme in the brain tissue with FP exposure. This reduction can be caused by the release of free radicals inside the cells. The decrease in SOD activity can also be caused by reactive oxygen species inactivating the chemical structure of SOD or by the increase in the number of hydroxyl radicals (Ezeoyili *et al.* 2019). CAT (derived from mitochondria and peroxisomes) catalyzes the decomposition of H_2O_2 into the abundantly produced H_2O and O_2 by phagocytes and maintains the balance between ROS formation and the immune system (Biller and Takahashi 2018). The decrease in CAT activity is thought to be due to superoxide radicals and an increase in cellular hydrogen peroxide. The substrate of the GPx enzyme is Glutathione (GSH), this enzyme catalyzes the reduction of H_2O_2 and lipid peroxides in the cell. Reduced antioxidant defense system mediated by glutathione causes oxidative stress and increased cytotoxicity. It has also been reported that decreases in GSH levels lead to increased free radical production and cellular degradation (Alak *et al.* 2019). Considering the increasing level of MDA, it can be said that lipid peroxidation cannot be prevented because the FP insecticide inhibits the GPx enzyme and therefore cannot remove H_2O_2. Free radicals react easily with unsaturated bonds of cholesterol and fatty acids in the cell membrane, creating lipid peroxidation (Aslankoç *et al.* 2019). In our study, it was assigned that the level of MDA examined as a marker of lipid peroxidation increased compared to the control group. It can be said that this situation increases the production of free radicals and reduces antioxidant activity, as a result of which the balance between antioxidants and free radicals induces oxidative stress. Clasen *et al.* (2012) reported that lipid peroxidation increased in the carp brain after fipronil application. It explains that oxidative stress stimulated by FP can cause lipid peroxidation of cells throughout the entire developmental period, and differentiating cells are much more susceptible to oxidative stress (Lassiter *et al.* 2009). Therefore, it is recommended that LPO is a prevalent phenomenon in the *in vivo* and *in vitro* oxidative stress-stimulate toxicity of FP. ROS increase in the brain suggests that FP may be a threat to homeostasis. Because the overproduction of ROS can interact with many cellular components, including carbohydrates/lipids, enzymes, proteins, and nucleic acids (Serafini *et al.* 2019). Another powerful source of oxidants in the living system is the "Neutrophilic myeloperoxidase" enzyme, which catalyzes hypochloric acid production by oxidation of chloride ions by hydrogen peroxide (Lavelli *et al.* 2000). MPO in the tissue is an oxidative enzyme and is considered as an indicator for neutrophil activity, producing hypochloric acid, which damages nearby tissues (Taoka *et al.* 1997). PON and ARE are enzymes in the esterase group that are encoded by the same gene and whose active centers are similar (Yonar and Harlı oğlu 2019). As a result of the study, a decreased obtained in PON and ARE values depending on the dose. PON has a crucial role in the elimination of potential oxidants due to its ability to hydrolyze lipid peroxidation. This enzyme, which is also a powerful antioxidant, has been reported to decrease in infection (Marsillach *et al.* 2011). ARE results are consistent with the results of paraoxonase activity and may have occurred through similar mechanisms (Alak *et al.* 2019).

In pesticide treated groups inhibition was determined in antioxidant enzyme levels connected with concentrations' increase. The enzymes can use safely supporting with MPO and MDA levels for determining the damage. The responses for low and high doses were found more effective for these parameters (MDA, MPO) and shown in Table 2. There was statistical similarity with two parameters in D1 and D2 doses. This situation can be explained by the adaptive response process via hormesis. DNA damage (8-OHdG) and apoptosis (caspase 3) which observed with the result of Table 2 is compatible with Table 3's data.

Hydroxyl radicals synthesized near DNA attack the purine and pyridine bases, causing mutations. Bases most affected by oxidative stress are guanine and cytosine in DNA (David et al. 2004). 8 OHdG concentration, which is the main product of DNA oxidation, is one of the biomarkers used for

oxidative DNA damage (Tian *et al.* 2018). Oxidation of DNA is another effect of the existence of ROS in the cell. This type of harm is critical to cell functions, as it causes mutations. 8-oxoguanin, which is caused by DNA attack by ROS, is the most often assessment marker of DNA damage (Lushchak 2016). The formation of free radicals causes genotoxicity, especially changes in DNA bases. (Stoliar and Lushchak 2012). In terms of DNA damage, it was determined that low dose 8-OHdG increased compared to the control group level. It can be said that this situation leads to DNA damage due to free radical formation and the production of 8-OHdG is induced. Pesticides activate a prevalent range of signaling pathways, promoting apoptosis by activation/modulation of death receptors and DNA damage (internal paths) (Raszewski *et al.* 2015; Alak *et al.* 2019). Caspase is an important effector of apoptosis, playing an crucial role in variety physiological processes (from the sustainability of homoeostasis to the health of tissues and organs) (Qu *et al.* 2018). Apoptosis has a crucial effect on the development and homeostasis of organisms. In the study, caspase 3 activity, an important biomarker of apoptosis, increased compared to the control group. These findings showed that FP triggers intrinsic apoptosis by mitochondrial signal induced by ROS production. Therefore, the toxic effect of FP causes protein inactivation, DNA damage and lipid peroxidation causing oxidative damage to the cell's structural and functional activities. As a result, it can be said that DNA damage associated with oxidative stress triggers apoptosis in fish.

5. Conclusion

In this study, the acute toxicity mechanism formed by the FP insecticide in rainbow trout brain tissue was tried to be illuminated with approaching multi-biomarker (AChE activity, oxidative stress, 8-OHdG and caspase-3) parameters. When the data were interpreted, it was concluded that oxidative stress stimulated by FP in the brain tissue as a result of acute administration affects the antioxidant defense system, inhibiting enzyme activities, causing DNA damage, and lipid peroxidation, causing the cell's structural and functional activities to oxidative damage.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All conditions (stocking density, water parameters etc.) were designed according to the welfare-related assessments, suitable for *O. mykiss*. The trials with the rainbow trout were approved by Ataturk University Local Ethical Committee for Animal Studies (approved in 27/02/2020, Acceptance No:13).

Disclosure statement

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. No potential conflict of interest was reported by the author(s).

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