Enantioselective induction of oxidative stress by acetofenate in rat PC12 cells

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Abstract

In a non-chiral environment, the enantiomers of a racemate possessed the identical physico-chemical properties, but in the biological systems they possessed different activities. Considering that the involvement of oxidative damage has been implicated in the toxicities of various pesticides, this study investigated the possibility of enantioselective oxidative stress and cytotoxicity induction by acetofenate (AF) which contains an asymmetrical center on PC12 cells. The results of the cytotoxicity assay indicated that S-(+)-AF presented more toxic effects than R-(–)-AF and (+)-AF. It also demonstrated that S-(+)-AF possessed the strongest effects in induction of reactive oxygen species (ROS) production, decrease in superoxide dismutase (SOD) and catalase (CAT) activities, and increase in malondialdehyde (MDA) level. These results suggested that AF and its enantiomers could induce enantioselective cytotoxicity in PC12 cells mediated by oxidative stress. Therefore, the assessment in environmental safety and new chiral pesticide development should consider enantioselectivity.

Key words: chiral; organochlorine pesticides; acetofenate; enantioselectivity; cytotoxicity; oxidative stress

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Introduction

Chirality is an important concept in many fields of chemistry. Chiral compounds usually have chiral centers and thus consist of enantiomers. Enantiomers of a chiral compound have identical physical-chemical properties and thus appear as a single compound in standard analysis. However, due to their stereoselective interactions in biological systems, enantiomers often behave drastically differently in their toxicity and environmental fate (Garrison, 2006; Jin et al., 2009; Lewis et al., 1999; Lin et al., 2006; Liu et al., 2005; Wang et al., 2007; Zhao et al., 2009a; Zhou et al., 2007). It was reported that about 25% of agrochemicals are chiral in 1996 (Williams, 1996), and this ratio is yet increasing as compounds with more complex structures are introduced. And most of the chiral pesticides are still applied in their racemic forms nowadays, as equimolar mixtures of enantiomers.

Organochlorine pesticides (OCPs) were used heavily on farmlands in the world several decades before. Many of the OCPs, such as α-hexachlorocyclohexane (α-HCH), cis- and trans-chlordane (TC, CC), heptachlor (HEPT), and dichlorodiphenyltrichloroethane (DDT), have been banned or severely restricted due to their persistence, bioaccumulation, and negative impacts on human, animal and plant lives (Aigner et al., 1998; Wang et al., 2009b). Several OCPs contain chiral centers, such as o,p′-DDT. Recent studies also demonstrated the occurrence of enantioselectivity of chiral OCPs in environmental fate (Kurt-Karakus et al., 2005), carcinogenesis (Ali et al., 2005) and endocrine disrupting activity (Hoekstra et al., 2006; Wang et al., 2009a). Especially for DDT, there were many studies investigating its negative impacts on biological system. For example, Pérez-Maldonado et al. (2004) reported there are close relationship between induction of apoptosis and the levels of DDT and its metabolites in blood of exposed children. They also demonstrated that DDT could induce apoptosis in human peripheral blood mononuclear cells, along with the generation of intracellular reactive oxygen species (ROS) (Pérez-Maldonado et al., 2005). In addition, there are also several investigations on enantiomeric selectivity in estrogenic activity of DDT. McBlain et al. (1976, 1977) found that R-(−)-enantiomer was a more active estrogen comparing with the corresponding S- (+)-enantiomer in rats and Japanese quail. Significant differences in estrogenic potential were found between the two enantiomers of o,p′-DDT in the MCF-7 human breast carcinoma cell proliferation (Wang et al., 2009a). In these regards, it is necessary to investigate negative effects of OCPs to biological system, especially for their enantioselective toxicity.

Acetofenate (AF), developed as an analogue of DDT, contains a chiral center and is widely used to control mosquitoes and flies both indoors and outdoors in China and other regions of the southeastern Asia nowadays.
Because of its low toxicity, ease of biodegradation, and high efficacy against pests, it becomes a good substitute of banned OCPs (Ali et al., 2005; Okada, 1996; Tebourbi et al., 1998; Williams, 1996). The production of AF in China averages several hundreds tons each year. However, to the best of our knowledge, the information relative to ecotoxicology of this OCP is limited to several publications. The previous study showed that AF possessed enantioselective developmental toxicity in zebrafish (Danio rerio). The S-(+)-AF was more active than R-(-)-AF in inducing the mRNA levels of estrogen receptor alpha expression, yolk sac edema, and pericardial edema, formation of crooked body, and alteration in heartbeat rates (Xu et al., 2008). Meanwhile, Zhao et al. (2009b) investigated the cytotoxicity and oxidative stress induced by AF and its enantiomers. They found that AF could induce apoptosis, ROS generation and DNA damage and result in the alteration of a series of signaling molecules. Furthermore the enantioselectivity in immunoxicity of AF has also been reported in macrophage (Zhao and Liu, 2009). However, no studies have examined the association between enantioselective cytotoxicity and oxidative stress induced by AF and its enantiomers. In the present study, PC12 cell line was used as an in vitro model to investigate the involvement of enantioselective oxidative stress and cytotoxicity of AF. Several endpoints with respect to oxidative stress were measured to understand the role of oxidative damage in cytotoxicity of AF. The overall aim of this study was to gain insight into the mechanism of chiral OCP AF-induced cytotoxicity with respect to enantioselectivity.

1 Materials and methods

1.1 Chemicals and reagents

Analytical standard of racemic AF (97.3%, 2,2,2-trichloro-1-(3,4-dichlorophenyl)-ethyl acetate) was obtained from Xinhuo Technology Institute (Baoding, China). Thiazolyl Blue (MTT) solution (5 mg/mL, phosphate-buffered saline (PBS)) was obtained from Amresco, USA, and fetal bovine serum (FBS) from Shijiqing Reagent Company, Hangzhou, China. The reagent kits for measuring malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and lactate dehydrogenase (LDH) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other chemicals or solvents used in this study were of cell culture, high-performance liquid chromatography (HPLC), or analytical grade. Test solutions at a range of concentrations were prepared in ethanol, with the final solvent content at 0.1% by volume.

1.2 AF enantiomer separation and quantitative analysis

Chiral HPLC analysis of AF has been described in detail in our previously published work (Xu et al., 2008). Individual pure enantiomers (−)-enantiomer and (+)-enantiomer) of AF (Fig. 1) were separated and prepared from the (±)-AF using modified procedures. Briefly, enantiomeric preparation was carried out on a LC-2000 series HPLC system (Jasco, Japan) equipped with a variable-wavelength CD-2095 circular dichroism (CD) detector. Separation was achieved on a Chiralcel® OD column (Daicel, Japan) (250 mm × 4.6 mm) at room temperature and the flow rate of mobile phase (100% n-hexane) was 1.0 mL/min. The signals of UV and CD detectors were recorded at 230 nm. The rotation sign (“+“ or “−“) was indicated by a positive or negative peak on the chromatogram form CD detector. And the absolute configuration of (+)-AF is S-AF, while the absolute configuration of (−)-AF is R-AF, which was established by octant rule. The resolved enantiomers for bioassays were manually collected into separate glass vials at the HPLC outlet, evaporated to dryness under a nitrogen stream, and redissolved in ethanol.

Quantitative analysis of individual enantiomer of AF was carried out on an Agilent 6890N GC (Agilent, USA) equipped with an electron capture detector (ECD) and a HP-5 capillary column. The flow rate of carrier gas (nitrogen) was 1.0 mL/min. The column temperature was held at 160°C. The temperature in the inlet was 260°C.

1.3 Cell culture and treatments

PC12 cells, obtained from the cell bank at the Chinese Academy of Science (Shanghai, China) with the original source being the American Type Culture Collection (ATCC) (Manassas, USA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, China) supplemented with 10% (V/V) FBS at 37°C in a humidified 5% CO₂ atmosphere. The culture media was refreshed every two days, and cells were subcultured at a ratio of 1:4 when they grown to 75%–85% confluence.

Before treatment, the cells were seeded in 24-well or 96-well plates (Costar, USA) at a density of 1 × 10⁵ cells/mL and allowed to adhere for 24 hr prior to assaying. FBS was reduced to 1% in the experimental medium to reduce the effect of serum. On the basis of the results of pretests, the cells were treated with dosing medium (the experimental medium along with a range of concentrations of the test compound) at concentrations of 10⁻⁸ to 5 × 10⁻⁵ mol/L for 24 hr for the cell viability assay, 10⁻⁹ or 10⁻⁵ mol/L for 5 hr for generation of the ROS assay, and 24 hr for MDA, SOD and CAT assays. Ethanol (0.1%, V/V) was used as the negative control.

1.4 Assessment of cell viability and LDH release

Cell viability was measured by quantitative colorimetric assay with MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) followed previous
1.6 Assessment of MDA, SOD and CAT

The intracellular generation of ROS was measured using a fluorescence probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma, USA), which is oxidized to the fluorescent form 2',7'-dichlorofluorescin by H$_2$O$_2$ and other ROS. DCFH-DA which is known to cross the cell membranes, is enzymatically hydrolyzed to a nonfluorescent analog, 2',7'-dichlorofluorescein diacetate (DCF-DA), and is trapped in the cell. In the presence of certain ROS intermediates, such as H$_2$O$_2$ or low-molecular weight peroxyl radicals, DCF-H is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) (Lebel et al., 1992). Briefly, after treatment with AF or vehicle for 5 hr, the treated cells were washed three times with ice-cold PBS and then incubated with 10 µmol/L 2',7'-dichlorofluorescein diacetate (DCFH-DA; 100 mmol/L in dimethyl sulfoxide) for 30 min at 37°C. The cellular free radical content was assayed by DCF fluorescence using a fluorescent spectrophotometer (excitation at 485 nm/emission at 535 nm, Tecx Infinite M200, Switzerland).

1.5 Assessment of the cellular ROS content

The intracellular generation of ROS was measured using a fluorescence probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma, USA), which is oxidized to the fluorescent form 2',7'-dichlorofluorescein by H$_2$O$_2$ and other ROS. DCFH-DA which is known to cross the cell membranes, is enzymatically hydrolyzed to a nonfluorescent analog, 2',7'-dichlorofluorescein diacetate (DCF-DA), and is trapped in the cell. In the presence of certain ROS intermediates, such as H$_2$O$_2$ or low-molecular weight peroxyl radicals, DCF-H is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) (Lebel et al., 1992). Briefly, after treatment with AF or vehicle for 5 hr, the treated cells were washed three times with ice-cold PBS and then incubated with 10 µmol/L 2',7'-dichlorofluorescein diacetate (DCFH-DA; 100 mmol/L in dimethyl sulfoxide) for 30 min at 37°C. The cellular free radical content was assayed by DCF fluorescence using a fluorescent spectrophotometer (excitation at 485 nm/emission at 535 nm, Tecx Infinite M200, Switzerland).

1.6 Assessment of MDA, SOD and CAT

We detected MDA, SOD and CAT indexes to investigate the oxidative stress effect caused by AF. These indexes were detected with an assay kit according to the manufacturer’s protocol (Nanjing Jiancheng Co., China). Briefly, the PC12 cells were seeded into 24-well plates and were allowed to attach for 24 hr. The medium was replaced with the experimental medium along with each concentration of the tested AF or vehicle. After 24 hr, the medium was removed and the cells were pooled in a 1.5 mL tube. These cells were centrifuged at 3000 r/min at 4°C for 10 min, and the pellet was resuspended with 1000 µL PBS, freeze-thawed twice at −20°C and centrifuged at 10,000 r/min at 4°C for 15 min. The supernatant was collected for MDA, SOD and CAT assays.

1.7 Statistical analysis

All experiments were repeated three times. Unless otherwise stated, all data were expressed as mean ± SD. Comparison of the values between groups was performed by analysis of variance, and $p < 0.05$ was considered statistically significant.

2 Results

2.1 Enantioomer separation and isolation

Baseline separation of AF was obtained under the chromatographic condition used in this study, and each enantiomer was manually collected. The purity was calculated to be more than 99% for individual enantiomers, as analysed by GC-ECD.

2.2 Enantioselective cytotoxicity and LDH release in PC12 cells

Cytotoxicity can be described as the ability of a chemical compound to induce cell death. In this research, the enantioselective cytotoxicity of AF in PC12 cells was determined by type of MTT assays. As shown in Fig. 2, dose-dependent growth inhibition induced by AF was observed in PC12 within the range of 10$^{-8}$ to 5 × 10$^{-5}$ mol/L, and distinct enantioselectivity in cytotoxicity were observed between the two enantiomers (S-(+)-AF, R-(−)-AF). Exposure to 10$^{-8}$ mol/L AF for 24 hr, there is almost no effect on cell viability. However, the concentration of 5 × 10$^{-5}$ mol/L of S-(+)-AF, R-(−)-AF and (±)-AF led to the decrease in cell viability by 50.7%, 27.5% and 38.2%, respectively.

To further investigate the enantioselective cytotoxicity of AF, the release of LDH which is often used as a marker of cell membrane damage was measured. LDH is a tetrameric enzyme that catalyses the interconversion of...
pyruvate and lactate with concomitant interconversion of NADH and NAD⁺ (Yang and Sun, 1998). The results in Fig. 3 showed that the extracellular release of LDH was not obvious at 10⁻⁸ and 10⁻⁷ mol/L of AF, while great elevation in LDH release by AF and its enantiomers was observed at 10⁻⁵ mol/L. The values manifested that S-(-)-AF exhibited more toxic effect to PC12 cells than R-(-)-AF and racemate.

2.3 Enantioselectivity of oxidative stress effect in PC12 cells

Oxidative stress is commonly used to evaluate the imbalance between the concentrations of ROS and the antioxidant defense mechanisms of the body. ROS, which are generated by some chemicals, play an important role in cytotoxicity. ROS levels in PC12 cells were expressed as relative fluorescence intensities. As shown in Fig. 4, PC12 cells incubated with individual enantiomers and racemate of AF exhibited an increase in intracellular ROS levels when compared to the control. In addition, enantioselectivity in production of ROS of AF was observed and the fluorescence intensities of PC12 cells followed the order: S-(-)-AF > R-(-)-AF. And the fluorescence intensities of PC12 cells incubated with S-(-)-AF was 1.17 times higher than R-(-)-AF. No significant difference was observed in inducing ROS production between R-(-)-AF and rac-AF.

SOD and CAT serve as key antioxidant roles in protecting cell from oxidative damage. The specific activity of SOD in PC12 cells was inhibited by racemate AF in a dose-dependent manner, while obvious inhibition by individual enantiomers only happened at the highest concentration. As shown in Fig. 5, at the concentration of 10⁻⁸ and 10⁻⁷ mol/L, the reduction in the SOD activity of PC12 cells was no more than 10%. However, the SOD activities of PC12 cells decreased by 32.9%, 16.8% and 30.2% when exposed to 10⁻⁶ mol/L of S-(-)-AF, R-(-)-AF and rac-AF. Obviously, significant differences were observed in altering SOD activities between the enantiomers of AF at higher concentration. Figure 6 shows that the CAT activity increases slightly at AF concentration of 10⁻⁸ mol/L, whereas at 10⁻⁷ to 10⁻⁵ mol/L CAT activity also decreases in a dose-dependent manner. And the activity of CAT in cells treated with S-(-)-AF is significantly lower than in cells with R-(-)-AF and rac-AF at its highest experimental concentration.

MDA is one of the end products of lipid peroxidation. The lipid peroxidation assay showed a slightly increase in MDA levels in PC12 cells when exposed to 10⁻⁸, 10⁻⁷ and 10⁻⁶ mol/L AF for 24 hr (Fig. 7). At the concentration of 10⁻⁵ mol/L of S-(-)-AF, R-(-)-AF and rac-AF, the MDA levels in cells increased by 1.46-, 1.17- and 1.23-fold, respectively, compared to the control group. Significant difference was observed in altering MDA level between the enantiomers of AF at highest experimental concentration.

![Fig. 3](image1.png)  
**Fig. 3** Effect of individual stereoisomers and the racemate of AF on extracellular LDH release. PC12 cells were exposed to different concentrations of individual stereoisomers and the racemate of AF for 24 hr, followed by LDH determination. Different letters above adjacent bars indicate a significant difference (p < 0.05, n = 3) between individual enantiomer and racemate. * Indicates a significant difference (p < 0.05, n = 3) between cells treated with AF and negative control.

![Fig. 4](image2.png)  
**Fig. 4** Effects of individual stereoisomers and the racemate of AF on intracellular ROS production. PC12 cells were exposed to different concentrations of individual stereoisomers and the racemate of AF for 5 hr, followed by ROS determination. Different letters above adjacent bars indicate a significant difference (p < 0.05, n = 5) between individual enantiomer and racemate. * Indicates a significant difference (p < 0.05, n = 5) between cells treated with AF and negative control.

![Fig. 5](image3.png)  
**Fig. 5** Effects of individual stereoisomers and the racemate of AF on intracellular SOD production. PC12 cells were exposed to different concentrations of individual stereoisomers and the racemate of AF for 24 hr, followed by determination of SOD activity. Different letters above adjacent bars indicate a significant difference (p < 0.05, n = 3) between individual enantiomer and racemate. * Indicates a significant difference (p < 0.05, n = 3) between cells treated with AF and negative control.
human systems (Pérez-Maldonado et al., 2004, 2005). In containing oxidative damage, in both in vitro and in vivo conditions such as exposure to environmental pollutants. Furthermore, significant differences were observed in ROS production between the two enantiomers of AF. It revealed that S-(+)-AF most likely induced the generation of ROS which probably resulted in oxidative stress to PC12 cells and leading to the cells viability decreasing. This result is consistent with the previous research which suggested that difference in enantioselective intracellular ROS generation may be due to the difference of the rate of biotransformation of enantiomers, particularly as some enzymes.

3 Discussion

Oxidative stress is a common mechanism caused by an imbalance between the production of reactive oxygen and the ability of biological system to readily detoxify the reactive intermediates or easily repair the resulting damage. The study of oxidative stress as a possible mechanism of toxicity for pesticides has become a focus of toxicological research for many investigators, since this biological phenomenon has been involved in the etiology of many human diseases such as neurodegenerative diseases, cancer, and immunosuppression (Coyle and Puttfarcken, 1993; Fahn and Cohen, 1992; Okada, 1996). Moreover, many OCPs have been found possessing negative effects, containing oxidative damage, in both in vitro and in vivo human systems (Pérez-Maldonado et al., 2004, 2005). In this regard, we examined the stereospecific cytotoxicity of AF in PC12 cells, and analyzed participation of enantioselective oxidative stress process in it.

In the present report, the cytotoxic effects of AF and its enantiomers were determined by MTT assay and LDH release assay. The MTT assay is an indicator of the cell’s energy metabolism and mitochondrial activity, while the LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage, which was also used as an index of cytotoxicity. Our data indicated a clear enantioselectivity of AF in reducing cell viability on PC12 cells at higher concentration. Moreover, in a certain concentration range, the toxicity of AF on PC12 cells was observed in a dose-dependent manner. These results are in agreement with previous studies. Zhao and Liu (2009) indicated that S-(+)-AF was more cytotoxic than R-(−)-AF and rac-AF. In addition, cytotoxicity studies with several other OCPs also have been reported. For example, after 6 hr of incubation with 2 × 10⁻³ and 2 × 10⁻⁴ mol/L DDT rat thymocytes viability decreased to 70% and 57% respectively in comparison to unexposed control cells (Tebourbi et al., 1998), whereas in vitro exposure of murine embryos to 0.1 mg/mL o,p′-DDT increased percent cell death by 96 hr of culture (Greenlee et al., 1999). However, the molecular mechanisms of cytotoxicity of OCPs as well as other pesticides have not been elucidated and require further studies. Nonspecific cellular oxidative damage is often observed during toxicity (Okada, 1996). Many studies support that pesticides can induce oxidative damage, as a mechanism of toxic action (Barros et al., 1994; Hassoun et al., 1993). In this study, our results also showed that AF could induced oxidative stress enantioselectively in PC12 cells, including the generation of ROS, and decrease in SOD and CAT activities, and increase in MDA level. Importantly, it is interesting to find the relationship that the enantioselectivity in oxidative damage is consistent with the enantioselectivity cytotoxicity.

ROS, such as superoxide anions (O₂⁻), hydroxyl radicals (·OH) and H₂O₂, are formed continuously in cells as a consequence of oxidative biochemical reactions or external factors. It has been implicated as a second messenger in multiple signaling pathways to induce apoptosis in various cell types (Antherieu et al., 2007; Liu et al., 2008, 2009). As a consequence, ROS is also an important biomarker for assessing cytotoxicity (Zhang et al., 2010). We noted that when the exposed concentration of AF increased, the formation of ROS in PC12 also increased, and the percentage of viable cells decreased. Obviously, it is harmful when ROS are produced in excess in certain abnormal conditions such as exposure to environmental pollutants.

Fig. 6 Effects of individual stereoisomers and the racemate of AF on intracellular CAT production. PC12 cells were exposed to different concentrations of individual stereoisomers and the racemate of AF for 24 hr, followed by determination of CAT activity. Different letters above adjacent bars indicate a significant difference (p < 0.05, n = 3) between individual enantiomer and racemate. * Indicates a significant difference (p < 0.05, n = 3) between cells treated with AF and negative control.

Fig. 7 Effects of individual stereoisomers and the racemate of AF on intracellular MDA production. PC12 cells were exposed to different concentrations of individual stereoisomers and the racemate of AF for 24 hr, followed by MDA determination. Different letters above adjacent bars indicate a significant difference (p < 0.05, n = 3) between individual enantiomer and racemate. * Indicates a significant difference (p < 0.05, n = 3) between cells treated with AF and negative control.
show stereoselectivity in the hydrolysis of AF enantiomers (Zhao and Liu, 2009). Since the chiral molecules, such as protein and nucleic acids, are existed all over the life world, stereoselective interactions are common. In addition, there is evidence in literature that overproduction of ROS resulted in oxidative damage of lipids, proteins and DNA (Perandones et al., 1993; Zhang et al., 2008). From our present study, the enantioselectivity in ROS generation maybe contribute to the enantioselectivity of the cytotoxicity in PC12 cells.

SOD and CAT enzymes, as two important antioxidants, process with the aim of eliminating excess oxidant. They provide a repair mechanism that could prevent cellular damage caused by ROS. The result obtained in this study showed that the activity of SOD decreased along with the formation of ROS when the exposure concentration increased. In addition, we noted a biphasic response in the change of CAT activity, with an increase at the concentration of $10^{-8}$ mol/L, followed by a decrease in CAT at the concentration of $10^{-7}$ to $10^{-5}$ mol/L. This phenomenon suggested that the tolerance capacity of the cells to AF depended on the balance of the antioxidant enzymes reducing oxidative stress and the lipid peroxidation favoring oxidative stress. In the study of Li et al. (2006), balance between pro-/anti-oxidant and pro-oxidant state also has been shown. As one byproduct of lipid peroxidation, MDA is also a biomarker of cellular damage following exposure to contaminants. In present study, the level of MDA increased along with the formation of ROS when the exposure concentration increased. It was indicated that the lipid peroxidation of polyunsaturated fatty acids could result in oxidative stress. The mechanisms by which chemicals induce inhibition of some cancer cells growth remain unclear, but it may be due to the lipid peroxidation of polyunsaturated fatty acids, resulting in the production of toxic radicals, and may induce cell death. As a result of a linkage, enantioselective generation of ROS, alteration of SOD and CAT activities, and elevation of MDA level conferred enantioselective cytotoxicity on AF.

4 Conclusions

In conclusion, the present study indicates that AF and its enantiomers are able to induce enantioselective cytotoxicity of PC12 cells in vitro by the generation of oxidative stress, especially at higher concentration. And S-(+)-AF plays an important role in oxidative stress damage, revealing that the enantiomers of chiral pesticides may differ in their biological activity. Therefore, it is really important that the assessment of environmental risk of chiral insecticides should take stereospecificity into consideration.

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