Rosuvastatin calcium and pravastatin sodium regulate the antioxidant system in zebrafish liver by inhibiting the PI3K/Nrf2/ARE signaling pathway

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Abstract

Rosuvastatin calcium (ROV) and pravastatin sodium (PRA) are commonly used drugs for the treatment and prevention of cardiovascular diseases, but as a result, they have been reported to be present worldwide in aquatic environments. However, little is known about the effects of ROV or PRA on fish antioxidant systems and the underlying molecular mechanisms. In this study, zebrafish were exposed to ROV or PRA for 48 h, to explore their effects on the antioxidant response system in zebrafish liver and its possible molecular mechanism. The results showed that the contents of reactive oxygen species (ROS)and glutathione (GSH) increased significantly after ROV exposure, while the activities of catalase (CAT)and superoxide dismutase (SOD) were significantly inhibited. Glutathione peroxidase (GPx) and glutathione peroxidase (GST) were activated after exposure to 5 mg/L ROV, and malondialdehyde (MDA) content increased after 0.5 mg/L ROV exposure. Phosphoinositide 3-kinase (*PI3K*) and *CAT* were activated at the mRNA level only after 5 mg/L ROV exposure, while nuclear factor erythroid 2-like 2 (Nrf2) and SOD were inhibited at the protein level. After adding PI3K activator 740Y-P, the protein inhibition of Nrf2 and SOD was reversed. After PRA exposure, the contents of ROS, GSH and MDA were activated, the activities of CAT and SOD were significantly inhibited, and GST was activated only after 10 mg/L PRA exposure. At the mRNA level, 10mg/L PRA activated *PI3K*, *CAT* and *GPx*, while Nrf2 and SOD were inhibited at the protein level. Similarly, after the addition of the PI3K activator 740Y-P, inhibition of both Nrf2 and SOD protein was abolished. Further molecular docking simulations showed that ROV and PRA could spontaneously dock with PI3K and form stable interactions through hydrogen bonds. In summary, both ROV and PRA induce significant oxidative stress in zebrafish liver, and both ROV and PRA act as PI3K inhibitors to block the activation of the nuclear factor erythroid 2-like 2/antioxidant response element (Nrf2/ARE) signaling pathway, thereby reducing the antioxidant capacity in zebrafish liver.

Introduction

Cardiovascular disease is one of the leading causes of death, killing more than 18 million people worldwide each year, and is its incidence expected to continue to increase in the coming decades (Collaborators 2020).In China, the number of people with high blood pressure, dyslipidemia or diabetes has reached hundreds of millions, and the number of people who need lifelong multi-drug treatment or prevention of cardiovascular disease is increasing (China 2022). Rosuvastatin calcium (ROV) and pravastatin sodium (PRA) are commonly used drugs for the treatment and prevention of cardiovascular diseases (Ruscica, Ferri et al. 2021). Studies have found that ROV and PRA entering the body cannot be fully absorbed and metabolized (Wishart, Knox et al. 2006). At the same time, there is a lack of effective elimination methods following ROV and PRA treatment, resulting in both these drugs continuously entering and accumulating in the aquatic environment, resulting in aquatic toxicity (Santos, Ruivo et al. 2016).

It has been reported that different concentrations of ROV and PRA have been detected in aquatic environments around the world, at concentrations in the range of mg/L (Lee, Peart et al. 2009, Tete, Nyoni et al. 2020). However, the concentration levels of statins detected in different cities are

different (Zhang, Zhao et al. 2020). This is mainly related to the size of the city. For example, in the sewage treatment plants in Guangzhou and Foshan, the concentration of PRA is higher than the average level in China (Huang, Wu et al. 2018). The concentration of atorvastatin is also higher than the national mean in the river basin of Ningbo City, Zhejiang Province (Tang, Sun et al. 2021). It has been reported that lower concentrations of statins in the environment can affect some aquatic organisms, including invertebrates and vertebrates (Ribeiro, Torres et al. 2015, Falfushynska, Sokolov et al. 2019). Recent studies have shown that exposure to ROV solution can lead to cardiac malformations and yolk sac edema in zebrafish larvae. Meanwhile exposure to PRA solution can lead to disruption of head development, cardiac malformations, and tail dysplasia in zebrafish larvae (Han, Ma et al. 2022). In addition to fish, there are also reports of toxicological effects of statin exposure in crustaceans. Gilroy et al. (Gilroy, Joel S Klinck et al. 2014) found that ROV exposure led to increased mussel closure time and decreased feeding rate. Statins have high environmental stability and lipophilicity, which promotes the accumulation of statins in organisms and aggravates the harm caused to aquatic organisms by statins and their metabolites (Falfushynska, Sokolov et al. 2019). The effect of statins on the antioxidant system is mainly manifested in the anti-oxidative stress effect of statins on pathological animal models (Ren, Zhou et al. 2021). However, little is known about the molecular mechanism by which environmental ROV and PRA affect the antioxidant system of aquatic organisms such as fish. Liver is an important organ in statin metabolism (Meurer and Cohen 2020), and pollutants in the environment have been shown to cause liver damage in zebrafish (Zhao, Meng et al. 2020). At the same time, the liver is the most important organ that regulates redox metabolism and is the place where key antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), Glutathione peroxidase (GPx) and glutathione peroxidase (GST) are produced to eliminate reactive oxygen species (ROS) (Moreno, Pichardo et al. 2005, Dong, Zhu et al. 2013).Therefore, it is necessary to study the effects of ROV and PRA on the antioxidant system of zebrafish liver.

In recent years, zebrafish has been widely used in toxicological studies as an attractive vertebrate model to evaluate the toxic effects of various pollutants. In this study, 4 month-old AB strain zebrafish were exposed to different concentrations of ROV (0.05, 0.5 and 5 mg/L) and PRA (0.1, 1 and 10 mg/L) for 48 h. The antioxidant response was evaluated by measuring the activities of antioxidant enzymes (CAT, SOD, CuZn-SOD, GPx and GST) and the contents of ROS, glutathione (GSH) and malondialdehyde (MDA). In order to understand the molecular mechanism of the Phosphoinositide 3-kinase/ nuclear factor erythroid 2-like 2/antioxidant response element (PI3K/Nrf2/ARE) signaling pathway regulating the antioxidant system in zebrafish liver, we analyzed the mRNA expression levels of *PI3K, Nrf2, CAT, SOD* and *GPx* and the protein expression levels of Nrf2 and SOD. The protein expression levels of Nrf2 and SOD in ROV and PRA after adding the PI3K activator 740Y-P were also analyzed. Finally, the interaction between ROV and PRA and the important molecule PI3K activated by Nrf2, was studied by homology modeling and molecular docking.

Materials and methods

Zebrafish maintenance. Four-month-old AB strain zebrafish were purchased from Shanghai FishBio Co., Ltd. (Shanghai, China). Feeding was carried out under standard conditions of temperature (28 ± 0.5°C) and pH (7.0 ± 1.0) under 14 hours of light/10 hours of darkness (Sprague, Bayraktaroglu et al. 2008). This study was approved by the Ethics Committee of the Experimental Animal Center of North China University of Science and Technology (Number: LX2021079). Before the start of the exposure experiment, zebrafish purchased from the company will be domesticated in a laboratory environment for 15 days to adapt to the laboratory environment, during which the zebrafish mortality rate is less than 1%.

*Chemical treatments.*ROV capsules and PRA capsules were purchased from Lunanbeite Pharmaceuticals Co., Ltd., (Linyi, China) and Lizhu Group Lizhu Pharmaceutical Factory (Zhuhai, China). Zebrafish (six in each group) were randomly placed in a 3 L fish tank and exposed to ROV solution (0.05, 0.5, 5 mg/L) or PRA solution (0.1, 1, 10 mg/L) for 48 h. The exposure solution was completely replaced every 24 h to reduce the impact of zebrafish excreta on the experiment, while maintaining a constant drug concentration. There was no zebrafish death during the experiment. After 48 h of exposure experiment, the quality and length of zebrafish did not change significantly compared with that before the exposure experiment. During the experiment, the activity and alertness of zebrafish were not significantly reduced compared with the control group. The choice of exposure concentration was based on the following two considerations: the low concentration was based on the concentration of statins detected in surface water, river water and wastewater (Conley, Symes et al. 2008, Lee, Peart et al. 2009, André, Liliana J G Silva et al. 2015). The high concentration was determined according to preliminary experiments, mainly considering whether it was sufficient to cause obvious oxidative damage in the liver of zebrafish, which would be helpful to explore the molecular mechanism of the antioxidant effect caused by ROV and PRA. After 48 h of ROV and PRA exposure, zebrafish exposed to different concentrations of statins for 48 h were fished out and euthanised by placing the zebrafish on ice for 20 min (Wallace, Bright et al. 2018). Death of zebrafish was determined when the gill covers stopped moving and the mouth of the fish stopped moving closed. After that, the zebrafish was quickly placed under the body microscope (Sunny Optical Technology (Group) Co., Ltd., Yuyao, China), the zebrafish was dissected with medical anatomical tools, and the liver of the zebrafish was taken out and placed in a pre-cooled centrifuge tube for use.

ROS determination. The changes of ROS in zebrafish liver were measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The dissected zebrafish liver was placed on a nylon mesh and washed with PBS. The cell suspension was collected and centrifuged at 500 × g for 10 min, then the cells were resuspended with diluted DCFH-DA and incubated at 37℃ for 30 minutes. After incubation, the single cell suspension was collected and centrifuged at 1000 × g for 10 min. The supernatant was removed to collect the cell pellet, which was washed twice with PBS to fully remove DCFH-DA that did not enter the cells. After centrifugation at 1000 × g for 5 min, the cell pellet was collected for fluorescence detection. The collected cell pellet was resuspended in PBS, and the cell suspension was added to a black 96-well plate. The fluorescence intensity was measured using a microplate reader, with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

Antioxidant responses. The weight of zebrafish liver tissue was accurately weighed. According to the ratio of weight (g) : volume (mL) = 1:99, 99 times the volume of PBS was added and diluted to create a 1% tissue homogenate. The homogenate was mechanically homogenized with a hand-held homogenizer in an ice water bath, then the homogenates were centrifuged at 3000g for 10 min at 4℃. The supernatant was transferred to a clean test tube, and the enzyme activity (CAT, SOD, CuZn-SOD, GPx and GST), as well as the GSH and MDA content, were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Gene expression analysis. Total RNA was extracted from zebrafish liver tissue using a tissue RNA rapid extraction kit from Beijing Juhemei Biotechnology Co., Ltd. (Beijing, China). The concentration of total RNA was determined using an ultramicro spectrophotometer (Beijing Kaio Technology Development Co., Ltd., Beijing, China). RNA integrity was determined by electrophoresis analysis of 28S and 18S rRNA subunits. The brightness ratio of ribosomal RNA bands was approximately 2:1. Primers were designed using Primer Premier 5.0 (http://www.premierbiosoft.com) software. All gene primer information is shown in Table 1, with β-actin as the internal reference gene. The primers were synthesized and purified by Beijing Ruiboxingke Biotechnology Co., Ltd. (Beijing, China), and frozen at -80°C, then diluted to the required concentration with RNase-Free water. A 2× M5 HiPer SYBR Premix ExTaq kit from Beijing Juhemei Biotechnology Co., Ltd. was used to configure a 20 μL qRT-PCR reaction system with three replicates in each group. The Applied Biosystems 7500 system (Foster City, CA, USA) was used for qRT-PCR. Finally, the relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1 qRT-PCR primer sequences used in this study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
Cat	AGGGCAACTGGGATCTTACA	TTTATGGGACCAGACCTTGG
Sod	GTCCGCACTTCAACCCTCA	TCCTCATTGCCACCCTTCC
Gpx	AGGCACAACAGTCAGGGATT	CAGGAACGCAAACAGAGGG
Nrf2	TCGGGTTTGTCCCTAGATG	AGGTTTGGAGTGTCCGCTA
Pi3k	AAGTTGTGAGCCCAGTCCA	GTTCATACCGTTGTTAGCG

Western blot analysis. Total protein was extracted from the dissected zebrafish liver using RIPA lysis buffer (Beijing Pulilai Gene Technology Co., Ltd., Beijing, China). Each well was loaded with 50 μg of protein, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting, and then blocked with 5% skimmed milk at room temperature for 2 h. Diluted Nrf2 and SOD (GeneTex, Irvine, CA, USA) primary antibodies were added and incubated overnight at 4℃ on a shaker, and then diluted secondary antibodies were added and incubated at room temperature for 2 h. The labeled protein was detected

using ECL luminescent liquid (Zhao, Wang et al. 2020). Finally, Image J software (NIH, Bethesda, MD, USA) was used to standardize the gray value of the target protein to GAPDH expression.

*Western blot analysis after treatment with 740Y-P.*In order to further verify whether ROV and PRA regulate the antioxidant system in zebrafish liver through the PI3K/Nrf2/ARE signaling pathway, we used 740Y-P, an activator of PI3K (Yimeng Wang, Tianli Tang et al. 2023). The 740Y-P stock solution was prepared with dimethyl sulfoxide (DMSO; Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) and diluted to the final concentration with zebrafish aquaculture water immediately before use. The final concentration of DMSO in the experimental solution was not more than 0.01%. The expression of Nrf2 and SOD in zebrafish liver was analyzed by western blotting following treatment with 200 μg/L of the PI3K activator 740Y-P (MedChemExpress, Monmouth Junction, NJ, USA).

Homology modeling and molecular docking. The zebrafish PI3K (zfPI3K) protein was predicted by Swiss-model (https://swissmodel.expasy.org) (PDB ID:5FI4) (Wu, Huang et al. 2019, Mendonca-Gomes, da Costa Araujo et al. 2021). AutoDock vina1.1.2 (https://autodock.scripps.edu) software was used to simulate the binding of ROV and PRA to zebrafish PI3K protein. The specific docking operation was performed as described in a previous report by Zhao et al. (Zhao, Wang et al. 2020).

Statistical analysis. All data in the present study were reported as the mean ± SEM. Significant differences between the treatment group and the control were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test using GraphPad Prism 8.3.0 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ indicates that any differences were statistically significant ($^{*}p < 0.05$,$^{**}p < 0.01$).

Results

ROS level. Compared with the control group, the levels of ROS in the liver of zebrafish exposed to ROV at 0.5 mg/L ($p < 0.01$) and 5 mg/L ($p < 0.01$) were significantly increased after 48 hours, with levels increased by 178% in the 5 mg/L ROV group. After 48 h of exposure to PRA, the ROS levels in the liver of zebrafish exposed to 0.1 mg/L ($p < 0.05$), 1 mg/L ($p < 0.01$) and 10 mg/L ($p < 0.01$) were all significantly increased (Fig. 1).

Activities of antioxidant enzymes. As shown in Fig. 2A, compared with the control group, exposure to ROV at 0.5 and 5 mg/L had an inhibitory effect on CAT ($p < 0.01$). After exposure to 5 mg/L ROV, the activities of GPx and GST were induced ($p < 0.05$). After treatment with 0.05, 0.5, or 5 mg/L ROV, GSH content was significantly promoted ($p < 0.01$), and 0.5 mg/L ROV also significantly up-regulated MDA activity ($p < 0.01$). However, ROV had no significant effect on SOD or on CuZn-SOD. As shown in Fig. 2B, compared with the control group, after 48 h of PRA exposure, the activities of CAT and SOD were significantly inhibited after 10 mg/L PRA exposure ($p < 0.01$), while the activities of CuZn-SOD and GST were activated ($p < 0.05$), and 1 mg/L PRA increased the content of MDA ($p < 0.01$). At the same time, 10 mg/L ROV increased the content of GSH ($p < 0.05$) and MDA ($p < 0.01$).

Gene expression of antioxidant enzymes. The effects of ROV and PRA exposure on the expression of antioxidant enzyme genes in zebrafish liver are shown in Fig. 3. Compared with the control group, 5 mg/L ROV activated the expression of *Pi3k* ($p < 0.05$) and *Cat* ($p < 0.01$). Lower concentrations of ROV exposure had no significant effect on the mRNA expression levels of *Nrf2*, *Sod* or *Gpx*. A concentration of 1 mg/L PRA activated the expression of *Pi3k* ($p < 0.05$), and 10 mg/L PRA activated the expression of *Pi3k* ($p < 0.01$), *Cat*, and *Gpx* ($p < 0.01$). At the same time, PRA exposure had no significant effect on the mRNA expression levels of *Nrf2* and *Sod*.

Protein expression of antioxidant enzymes. To better understand the effects of ROV and PRA on the antioxidant system in zebrafish liver, the expression levels of Nrf2 and the antioxidant enzyme SOD in the Nrf2/ARE signaling pathway were studied (Fig. 4). Compared with the control group, the levels of Nrf2 protein in the liver of zebrafish exposed to ROV at 0.05, 0.5, or 5 mg/L significantly decreased ($p < 0.01$). At the same time, SOD protein levels were significantly inhibited after exposure to 0.5 or 5 mg/L ROV ($p < 0.01$). After exposure to 1 or 10 mg/L PRA, Nrf2 protein in zebrafish liver was significantly inhibited ($p < 0.01$) in a dose-dependent manner. Meanwhile, the expression of SOD proteins decreased after exposure to 10 mg/L PRA compared to the control ($p < 0.01$).

Protein expression of antioxidant enzymes after 740Y-P treatment. Fish were treated with 5 mg/L ROV along with 200 μg/L 740Y-P. As shown in Fig. 5(A)(C), after adding the PI3K activator 740Y-P, compared with the control group, expression of Nrf2 in the liver of zebrafish was activated ($p < 0.05$), while at the same time, SOD in the liver of zebrafish was also significantly activated ($p < 0.01$). After adding 200 μg/L 740Y-P in the same way to fish treated with 1 or 10 mg/L PRA, Nrf2 was activated in zebrafish liver ($p < 0.01$), while treatment with 0.1, 1, or 10 mg/L PRA along with 200 μg/L740Y-P resulted in a significant increase in the expression level of SOD (p 0.01) (Fig. 5(B)(D)).

Binding mode of ROV and PRA toward zfPI3K. After performing molecular docking simulation of ROV and zfPI3K protein 100 times, the results showed that ROV entered zfPI3K mainly through hydrogen bonding and hydrophobic interaction. ROV interacted with GLY451, ASN428, TYR392, ALA442, ASN380, THR462 and other residues at the active site of zfPI3K, and formed four hydrogen bonds with GLY451 and ASN345 on zfPI3K. The results showed that the optimal binding site of ROV and zfPI3K was GLY451, and the calculated docking energy was -3.1 kcal/mol (Fig. 6(A)(B)(C)). After repeating molecular docking simulation between PRA and zfPI3K protein 100 times, the results showed that PRA entered zfPI3K mainly by hydrogen bond and hydrophobic force. PRA interacted with amino acids GLU135, SER464, GLY451, GLU103, LYS656, GLN859 and other residues at the active site of zfPI3K, and formed two hydrogen bonds with GLU135 and four hydrogen bonds with TYR432, THR679, SER464 and ASP133. The molecular docking results showed that the optimal binding site of PRA and zfPI3K was GLU135, and the calculated docking energy was -4.49 kcal/mol. (Fig. 6(D)(E)(F)).

Discussion

In recent years, ROV and PRA have been frequently detected in aquatic environments (Ellesat, Tollefsen et al. 2010, Verlicchi, Galletti et al. 2013). In previous studies, ROV and PRA were mainly found to play an anti-oxidative stress role in the treatment of patients (Kattoor, Pothineni et al. 2017). However, the antioxidant reaction system and potential molecular mechanism of ROV and PRA as environmental pollutants affecting aquatic organisms are still unclear. In this study, we demonstrated that ROV and PRA exposure significantly reduced the antioxidant capacity of adult zebrafish. In addition, a possible potential molecular mechanism was proposed.

CAT, SOD and GPx are considered as first line of defense against ROS (Sadasivam, Kim et al. 2022). SOD catalyzes the conversion of $O^{2-}$to H_2O_2, which is then decomposed into H_2O by CAT and GPx. SOD activation is usually accompanied by an increase in CAT and GPx activity to protect cells from oxidative stress (Zheng, Zhu et al. 2016). If the first antioxidant reaction system of CAT or SOD cannot eliminate excess ROS, the risk of oxidative damage increases, thereby reducing enzyme activity and even leading to enzyme degradation (Zheng, Yuan et al. 2016). In this study, ROS levels increased sharply after exposure to either ROV or PRA, indicating that oxidative stress was occurring in the liver of zebrafish. Exposure to 5 mg/L ROV significantly reduced the activity of CAT, while 10 mg/L PRA also significantly reduced the activity of both CAT and SOD, which may be the result of excessive production of ROS leading to the consumption of antioxidant enzymes. In contrast, exposure to 5 mg/L ROV significantly increased the activity of GPx, while exposure to 10 mg/L PRA had no significant inhibitory effect on the activity of GPx. This may be due to the fact that CAT and GPx are effective scavengers of hydrogen peroxide, and the antioxidant effect of GPx is replaced by CAT (Zhang, Li et al. 2016). After ROV and PRA exposure, the changes of GPx mRNA level were basically consistent with those of the protein, indicating a synchronous reaction from molecular level to biochemical level. Usually, the translation reaction should follow the transcription reaction (Nikinmaa and Rytkonen 2011), however, the gene expression levels of CAT and SOD are inconsistent or even opposite to their enzyme activities, which may be related to a negative feedback mechanism (Gaaied, Oliveira et al. 2019). GST is a biotransformation enzyme that catalyzes the binding of GSH to exogenous substrates to reduce oxidative stress. GPx catalyzes H_2O_2 to H_2O, and GSH supplements the reduction of GPx in this process (Sadasivam, Kim et al. 2022). After 48 h exposure to 5 mg/L ROV or 10 mg/L PRA, the activities of GST and GSH increased significantly, indicating that oxidative damage may promote the process of converting H_2O_2 into H_2O. The increase of ROS content leads to the formation of lipid decomposition products, in which the lipid oxidation end product MDA affects the activity of key enzymes in mitochondria, so the level of MDA indirectly reflects the degree of cell damage (Jia, Cen et al. 2019). Usually, the change trend of ROS is consistent with that of MDA content (Dong, Zhu et al. 2013). Our results in this study showed that the content of MDA increased sharply after exposure to 0.5 mg/L ROV or to 1 or 10 mg/L PRA, which also reflected that exposure to ROV or PRA could cause oxidative damage. However, in this study, the increasing trend of MDA content after exposure to 5 mg/L ROV was not obvious compared with the control group. Previous studies have shown that increased GPx activity inhibits or reduces lipid peroxidation (Budni, Zomkowski et al. 2013, Du, Zhu et al. 2014). Therefore, we speculated that the lack of an apparent increase in MDA content after exposure to 5 mg/L ROV may be due to the increase of GPx activity.

PI3K is composed of lipids and Ser/Thr kinases. When an exogenous substance activates PI3K, it promotes the dissociation of Nrf2 in the cytoplasm (Deshmukh, Unni et al. 2017). Nrf2 is a protein composed of 589 amino acids and is a redox-sensitive transcription factor expressed in many tissues such as spleen, heart, kidney and liver (Jaramillo and Zhang 2013). Dissociated Nrf2 accumulates and stabilizes in the cytoplasm and is transferred to the nucleus where it binds to ARE (Sivandzade, Prasad et al. 2019), ARE is a cis-regulatory module located in the promoter region of several antioxidant enzymes and cytoprotective proteins such as CAT, SOD, and GST (Loboda, Damulewicz et al. 2016). When Nrf2 binds to ARE, it activates the expression of downstream antioxidant enzymes to maintain the balance of the antioxidant system. Our data showed that there was no significant difference in the expression level of Nrf2 mRNA in the liver of zebrafish after ROV or PRA treatment compared with the control group. However, in terms of protein, Nrf2 protein levels were significantly reduced, and SOD protein expression was also significantly reduced. The reason why the mRNA and protein expression levels of Nrf2 are not very consistent may be that the decrease of Nrf2 protein leads to an increase of mRNA translation to supplement this consumption (Wu, Huang et al. 2019). Previous studies have shown that PI3K regulates the Nrf2/ARE signaling pathway, and PI3K inhibitors prevent Nrf2 activation (Deshmukh, Unni et al. 2017). In addition, the PI3K/AKT pathway has been shown to be an upstream activator of Nrf2 signaling (Li, Dong et al. 2014). Western blot results showed that both ROV and PRA significantly down-regulated the expression of Nrf2 protein, and this inhibitory state was abolished after addition of the PI3K activator 740Y-P, therefore, we speculated that ROV and PRA can be used as PI3K inhibitors to regulate PI3K/Nrf2/ARE signaling pathway in zebrafish liver. In recent years, computer methods have been applied to explore the binding form of small drug molecules to receptor protein macromolecules, and simulate the interaction between ligands and receptors through molecular docking. Visualization of this interaction has been successfully applied to elucidate the molecular mechanism of toxic effects (Zhang, Chen et al. 2022). Our molecular docking results showed that both ROV and PRA were well docked with zfPI3K, in which ROV formed four hydrogen bonds with GLY451 and ASN345, while PRA formed two hydrogen bonds with GLU135 and four hydrogen bonds with TYR432, THR679, SER464 and ASP133. Further docking energy analysis showed that the optimal binding site of ROV to zfPI3K was GLY451, and the calculated docking energy was -3.1 kcal/mol, while the optimal binding site of PRA to zfPI3K was GLU135, and the calculated docking energy was -4.49 kcal/mol. In general, negative docking energy indicates that the ligand and the receptor can spontaneously bind (Morris, Huey et al. 2009, Trott and Olson 2010). Therefore, we speculate that inhibition of the Nrf2/ARE pathway by ROV and PRA may be attributed to their direct interaction with zfPI3K as inhibitors.

In summary, our results clearly show that exposure to ROV and PRA causes oxidative stress in the liver of zebrafish, which can be proved by changes in ROS, antioxidant enzyme activity, mRNA and protein levels. Both ROV and PRA inhibit the Nrf2/ARE pathway as PI3K inhibitors. Our results will help improve the understanding of the mechanism of ROV and PRA in causing oxidative stress at the molecular level, and provide new insights into the toxicity of ROV and PRA in fish.

Declarations

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Authors' contributions

XW: Design of the study, performing the experiments, data analysis, writing the first draft. QM: Performing the experiments, data analysis. XL: Performing the experiments, data analysis. JS: Performing the experiments. RL: Performing the experiments. YZ: Data analysis, manuscript correction. CD: Funding acquisition, project management, resources, experimental design, data analysis, manuscript revision.

Competing interests

The authors declare that they have no competing interests.

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Figures

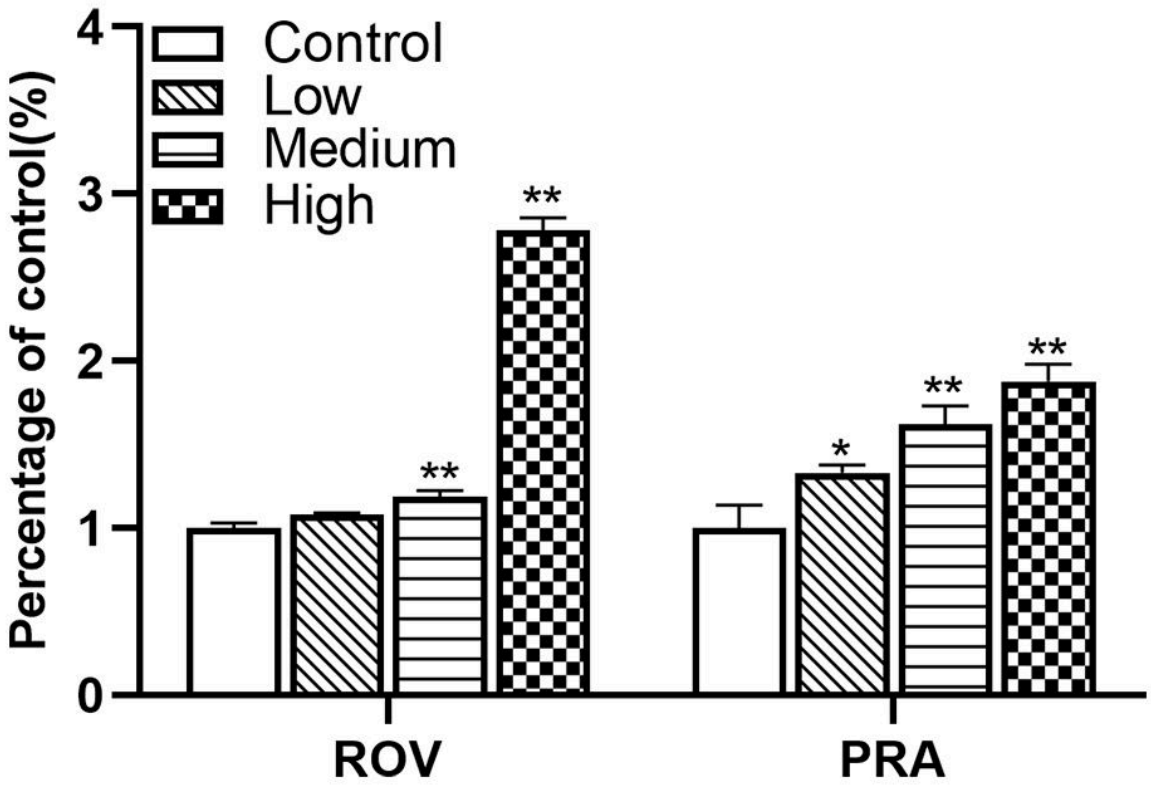

Figure 1

Effect of rosuvastatin calcium on ROS content in zebrafish liver, mean ± standard error (n = 3 replicates), *represents $p < 0.05$ compared with the control group, **represents $p < 0.01$ compared with the control group.

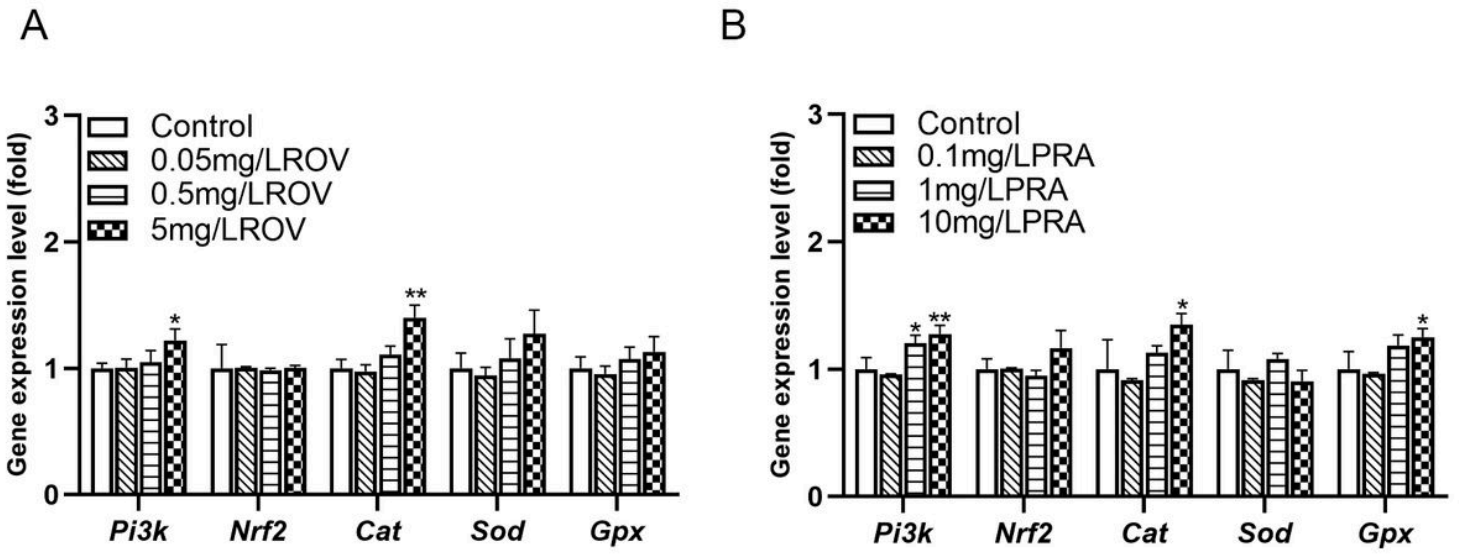

Figure 2

Effects of ROV (A) and PRA (B) on antioxidant enzyme activity as well as GSH and MDA content in zebrafish liver, mean ± standard error (n = 3 replicates), *represents $p < 0.05$ compared with the control group, **represents $p < 0.01$ compared with the control group.

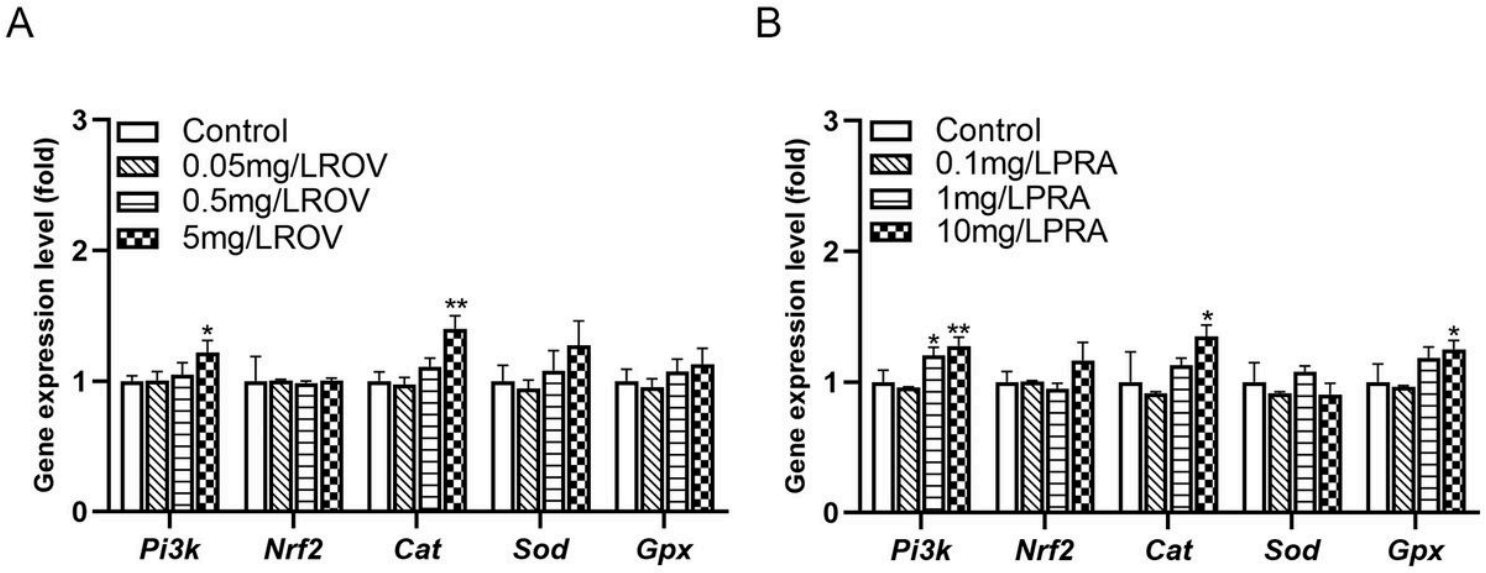

Figure 3

Effects of ROV (A) and PRA (B) on *Pi3k, Nrf2, Cat, Sod* and *Gpx* gene expression in zebrafish liver, mean ± standard error (n = 3 replicates), *represents $p < 0.05$ compared with the control group, **represents $p <$

0.01 compared with the control group.

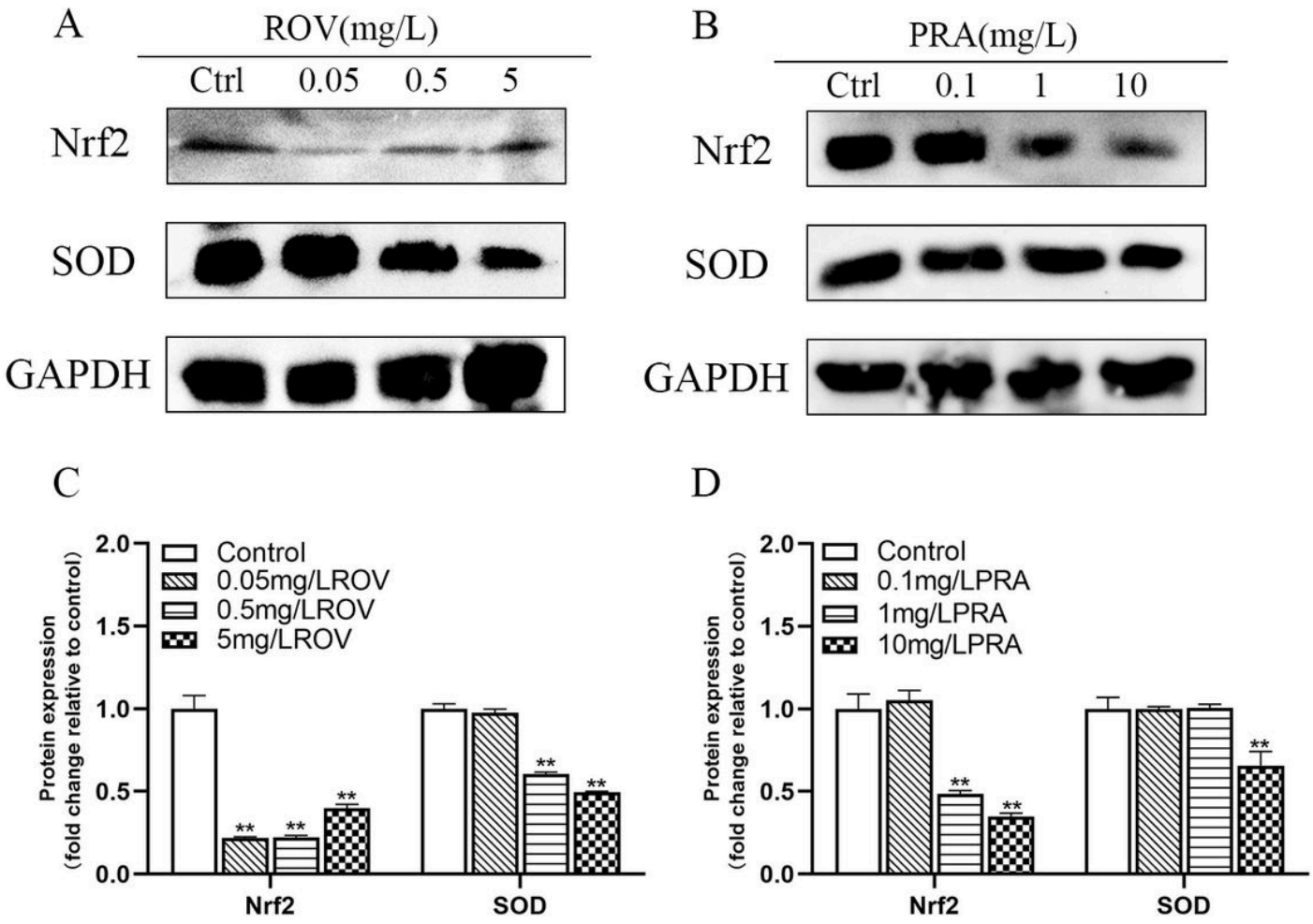

Figure 4

Effects of ROV and PRA on the expression of Nrf2 and SOD in zebrafish liver. Western blot results of Nrf2 (A) and SOD (B) protein expression are shown, and the relative quantitative results of Nrf2 and SOD protein expression are shown as (C) and (D). Mean ± standard error (n = 3 replicates), *means compared with the control group $p < 0.05$, **means compared with the control group $p < 0.01$.

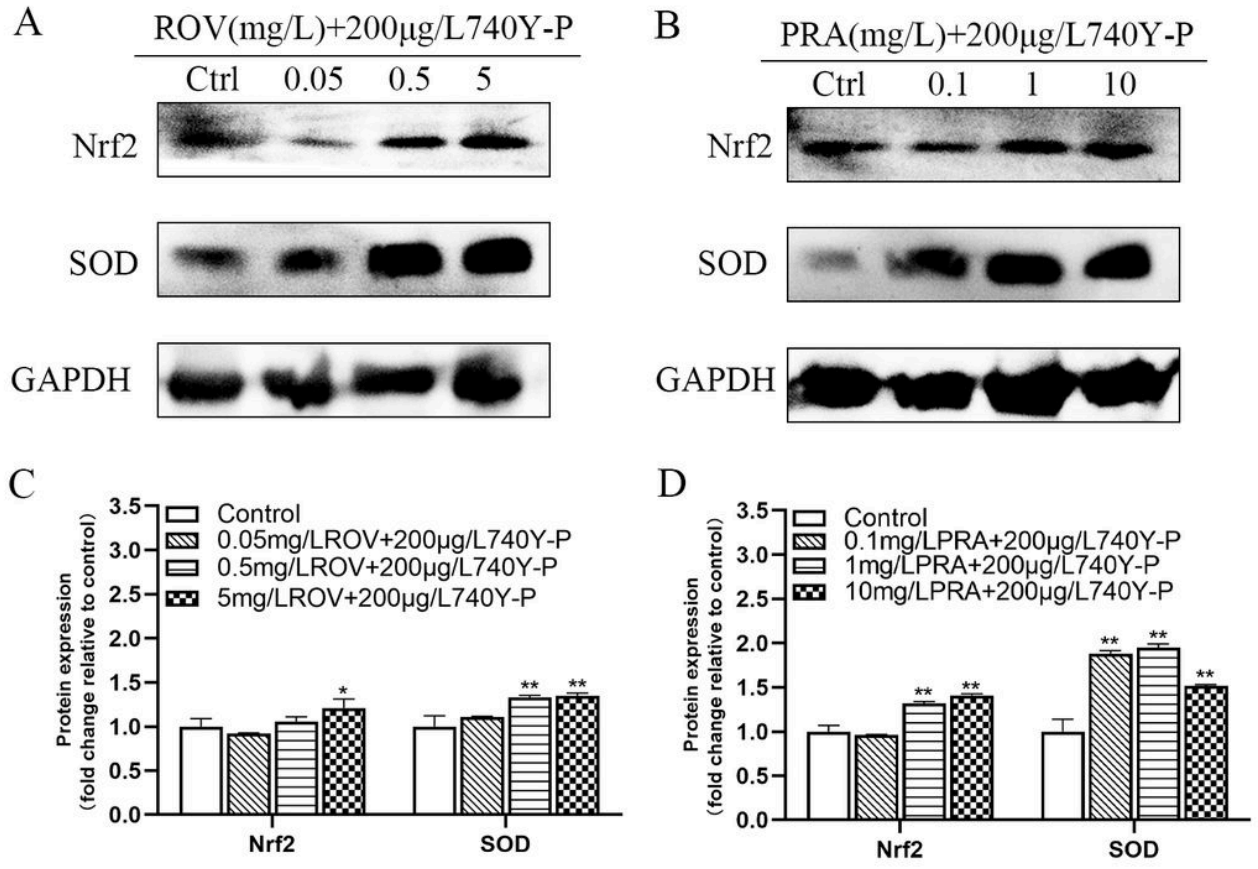

Figure 5

Effects of 740Y-P on Nrf2 and SOD protein expression in zebrafish liver after treatment with ROV or PRA. Western blot results of Nrf2 (A) and SOD (B) protein expression are shown, and the relative quantitative results of Nrf2 and SOD protein expression are shown as (C) and (D). Mean ± standard error (n = 3 replicates), *means compared with the control group $p < 0.05$, **means compared with the control group $p < 0.01$.

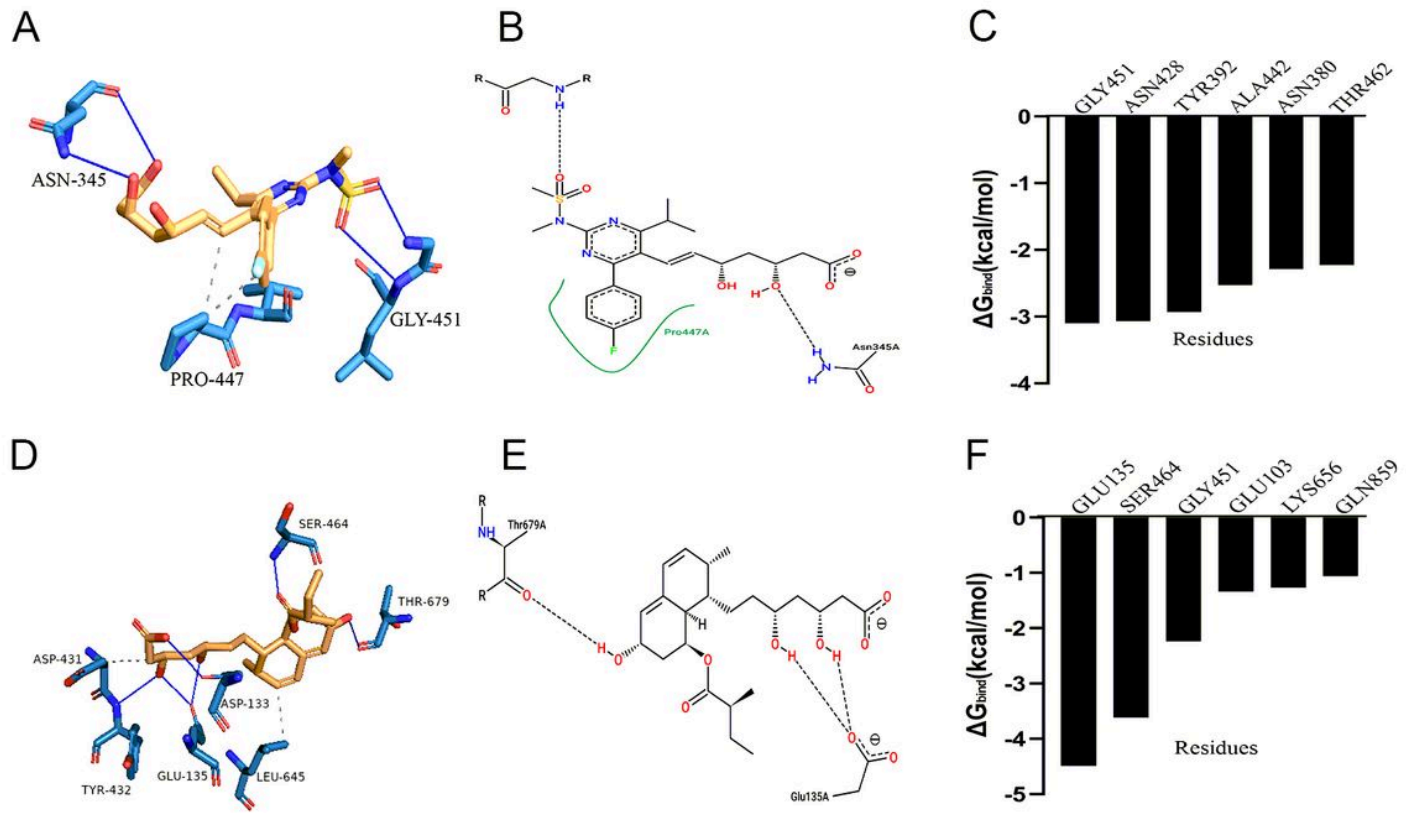

Figure 6

The 3D (A) and 2D (B) structures of ROV binding to zfPI3K, and the 3D (D) and 2D (E) structures of PRA binding to zfPI3 K protein. Blue solid lines in the image represent hydrogen bonds, and gray dotted lines indicate hydrophobic interactions. The main amino acid sites of ROV (C) and PRA (F) binding to zfPI3K are the main amino acid sites.