

The cellular entry mechanism and potential target of the novel bioactive peptide AFYRW derived from *Fagopyrum tataricum*

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Research Article

Keywords: peptide AFYRW, bioactive peptides, endocytosis pathway, inhibitor, molecular docking

Posted Date: September 19th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3292024/v1>

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Additional Declarations: No competing interests reported.

Abstract

The novel bioactive peptide AFYRW derived from Tartary buckwheat (*Fagopyrum tataricum Gaertn.*) with strong antioxidant capacity was identified in our previous research, but its cellular entry and targeting mechanism remain unknown. Herein, we employed endocytosis pharmacological inhibition methodologies and determined that AFYRW localized primarily in the nucleus, and NH₄Cl, chloroquine (CQ), and nystatin exposure resulted in a significantly reduced uptake by HepG2 cells. Our results indicate that the cellular uptake of AFYRW depends on caveolae-mediated endocytosis that requires a low pH, but does not involve macropinocytosis or clathrin-mediated endocytosis. In addition, biotin-streptavidin affinity purification was used to screen for interacting proteins, and protein spectrum analysis *in vitro* identified poly (ADP-ribose) polymerase-1 (PARP-1) as a potential target. Subsequent *in silico* molecular docking supports the presence of a peptide–protein interaction between AFYRW and PARP-1. Our findings suggest that the novel bioactive peptide AFYRW could be a potential inhibitor of PARP-1.

1 Introduction

Dietary proteins not only serve as nutrients, but also play important roles in regulating the physiological function of the human body. It has been well established that the physiological effects of dietary proteins are mostly realized by bioactive peptides (BAPs) generally comprised of 2 to 20 amino acid residues, which are produced through protein hydrolysis by endogenous or exogenous proteases and microbial fermentation [1]. BAPs are inactive when present as an integral part of their parent proteins and must be released from their encrypted position by peptide bond hydrolysis to confer health benefits [2]. BAPs have gained increasing attention in the food and pharmaceutical industries because of their inherent functional properties, bioavailability, and direct association with appetite regulation [3–5]. Buckwheat consumption is linked to positive health outcomes in humans. Containing nutrients with diverse biological functions, buckwheat grains have been processed into a variety of products, such as breads, noodles, pasta, beverages, and vinegar, the quality of which may be significantly affected by their protein and peptide properties. In recent decades, a wide range of buckwheat BAP sequences have been identified and characterized as having antihypertensive, antioxidative, antimicrobial, anticancer, antithrombotic, and immunomodulating properties [6, 7]. Buckwheat protein isolates can be processed into BAPs to develop functional ingredients and healthy food products, and great structural diversity of peptides has been found in buckwheat proteins [8]. However, information about the unique properties of buckwheat peptides and their applications is largely scattered, and the mechanisms of BAP actions and remarkable biological activities are not well understood.

Therapeutic peptides must gain access to the cytosol or nucleus in order to elicit any effects [9]. The plasma membrane is a selectively permeable barrier that separates the interior of a cell from its external microenvironment. Endocytosis is a basic cellular process that cells use to take up a variety of molecules. Generally, the electrostatic interactions between the negatively charged cell surface phospholipids and positively charged residues in peptides precede cellular internalization [10]. Because

of their diverse biochemical and therapeutic properties, peptides are a separate class of pharmacological substances, and their conformational flexibility and binding affinity derived from the sequences of amino acids allows them to interact with other proteins to modulate natural processes in the body. Therefore, determining the cellular internalization mechanisms responsible for transporting peptides into the cell can elucidate the subcellular processing of BAPs and their potential downstream effects. Although the structure–function relationships of BAPs are not well established, most share common characteristics, such as richness in hydrophobic amino acids. Thus, computational docking approaches have been utilized to accelerate the process of making accurate predictions of protein structures, surface charges, binding modes, and contact affinities. Using freely accessible bioinformatic binding prediction tools has assisted in the development of novel peptide medicines [11].

In our previous studies, enzymatic hydrolysates of Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) albumin by alkaline protease were isolated and purified, and we obtained three new antioxidant peptides by ultrafiltration and successive chromatography [12]. Among them, the peptide AFYRW, whose sequence is Ala-Phe-Tyr-Arg-Trp with a molecular weight of 741.85 Da, demonstrated the greatest antioxidant capacity, and its IC_{50} values for scavenging hydroxyl radicals ($OH\cdot$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals were 0.65 mM and 0.64 mM, respectively [12]. Additionally, AFYRW exhibited high lipid peroxidation inhibition ability and reducing power. A better understanding of the properties and applications of Tartary buckwheat proteins and BAPs are essential to fully realize the benefits they may have for human health. Moreover, the combination of biochemical and *in silico* experiments is becoming a promising strategy for characterizing and modifying food protein-derived BAPs. Therefore, the present study aims to investigate the cytotoxicity and entry mechanisms of AFYRW using the HepG2 cell line as well as its subcellular targeting. Furthermore, proteins that may interact with AFYRW were screened by biotin–streptavidin affinity purification and mass spectrometry. Lastly, interactions between AFYRW and its potential target protein were predicted with molecular docking. Our study suggests that AFYRW may have broad application prospects for promoting human health, in particular preventing and treating diseases related to oxidative damage.

2 Materials and methods

2.1 Cell culture

The human hepatoma cell line HepG2 was purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). HepG2 cells were cultured in DMEM medium (Thermo Fisher Scientific, US) containing 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Material, Hangzhou, Zhejiang Province, China) and 1% penicillin–streptomycin. The cells were kept at 37°C in humidified incubators with 5% CO_2 .

2.2 Cytotoxicity assay

HepG2 cells were inoculated at a density of 5000 cells per well in 96-well plates and cultured overnight. The cells were treated with different concentrations of AFYRW (TP-CH-3108, Haitaopu Biotechnology, Shanghai, China) for 24, 36, and 48 h. After two washes, 10 μ L of CCK-8 enhanced solution (CCK-8 Counting Kit-8, Dalian Meilun Biotechnology Co., Ltd) was added into the wells. Following CCK-8 incubation for 30 min, the absorbance at 450 nm was recorded by iMark™ Microplate Reader (Bio-Rad Laboratories, US). Experiments were performed at least three times.

2.3 Cellular uptake

HepG2 cells were inoculated at a density of 4×10^4 cells/mL in 24-well plates and cultured overnight. After rinsing with PBS twice, 40 μ g/mL FITC-AFYRW (TP-CH-1847, Haitaopu Biotechnology) was added and incubated for 30 min at 37°C. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA). Subsequently, 4',6-diamidino-2-phenylindole (DAPI) was added to each well and incubated for 5 min, and images were captured by confocal laser microscopy (Olympus Japan)[13]. Experiments were performed at least three times.

To determine the effect of AFYRW concentration and incubation time on cellular uptake, HepG2 cells were inoculated at a density of 5×10^5 cells/mL in 6-well plates and cultured overnight. 40 μ g/mL FITC-AFYRW was added to each well and incubated for 0, 15, 30, and 60 min; alternatively, FITC-AFYRW of designated concentrations was added to each well and incubated for 15 min. The cells were then washed in ice-cold PBS and resuspended in 1 mL of PBS. Cells were measured using flow cytometry (BD FACSCelesta), and the ratios of positive cells were analyzed by FlowJo version 10. Experiments were performed at least three times.

2.4 Uptake inhibition

To study the effect of various inhibitors on the uptake of AFYRW, HepG2 cells were preincubated with the following inhibitors individually at 37°C, at concentrations that were nontoxic to the cells. Different concentrations of NH_4Cl or chloroquine (CQ) were co-incubated with HepG2 cells for 1 h, and different concentrations of chlorpromazine (CPZ), amiloride (AMR), or nystatin were co-incubated with HepG2 cells for 2 h. Cells were then incubated with 40 μ g/mL FITC-AFYRW at 37°C for 15 min. The fluorescence intensity of each group was measured using flow cytometry.

2.5 Preparation of nuclear proteins and affinity precipitation

Nuclear proteins were extracted from HepG2 cells using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Biotin-AFYRW (TP-CH-2243, Haitaopu Biotechnology) and streptavidin-labeled magnetic beads (Suolaibao Technology, Beijing, China) were then used to collect the target proteins. Specifically, 40 μ g of biotin-AFYRW (1 mg/mL) was incubated with nuclear protein lysates for 1 h at 4°C. Streptavidin-labeled magnetic beads were then added and incubated for 1 h at room temperature. The supernatant was discarded, and the precipitated beads were collected for eluates. The eluted proteins were run on 10% of SDS gel electrophoresis before being subjected to Coomassie brilliant blue staining.

2.6 Protein spectrum analysis

For the in-gel tryptic digestion[14], Gel pieces were destained with 50 mM NH_4HCO_3 in 50% acetonitrile (v/v), followed by washing with 100 μL of 100% acetonitrile for 5 min. Disulfide bonds of proteins were reduced by incubating the gel pieces with 10 mM dithiothreitol and incubated for 1 h at 56°C. Gel pieces were then dehydrated in 100% acetonitrile; liquid was removed, and proteins in the gel pieces were alkylated with 55 mM iodoacetamide for 45 min at room temperature in the dark. Gel pieces were then washed with 50 mM NH_4HCO_3 and dehydrated with 100% acetonitrile. Subsequently, the proteins in the gel pieces were digested with 10 ng/ μL trypsin and resuspended in 50 mM NH_4HCO_3 on ice for 1 h. Excess liquid was removed and gel pieces were digested with trypsin at 37°C overnight. Peptides were extracted with 50% acetonitrile/5% formic acid, followed by 100% acetonitrile. Peptides were dried to completion and resuspended in 2% acetonitrile/0.1% formic acid and dissolved in 0.1% formic acid (solvent A), directly loaded onto a homemade reversed-phase analytical column (15-cm length, 75- μm i.d.) on an EASY-nLC 1000 UPLC system. The peptides were then subjected to an NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo Fisher Scientific) coupled online to the UPLC. The resulting MS/MS data were processed using Proteome Discoverer 1.3.

2.7 Peptide–protein molecular docking

According to the results of mass spectrometry, the protein with the highest score was selected and docked with HPEPDOCK (<http://huanglab.phys.hust.edu.cn/hpepdock/>) according to the method described previously [15]. Briefly, the X-ray crystal structure of the protein was obtained from the Protein Data Bank (PDB ID: 4ZZZ), and the sequence of AFYRW was entered in FASTA format. The model with the best docking ranking was selected for visual analysis using Discovery Studio Visualizer 2019.

2.8 Statistical analysis

All control and experimental values were represented as means $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used in statistical comparisons during multiple samples, and *p*-values smaller than 0.05 were considered significant. All statistical analyses were performed using SPSS version 20.0.0 and GraphPad 6.0.

3 Results

3.1 Cytotoxicity assessment of AFYRW

To assess the cytotoxicity of AFYRW to the cell, we performed CCK-8 assay and hemolysis analysis on HepG2 cells. In the CCK-8 assay, HepG2 cells were treated with AFYRW at concentrations of 25 to 200 $\mu\text{g}/\text{mL}$ for 24, 36, and 48 h, and no significant changes in cell growth were observed (Fig. 1).

3.2 Subcellular localization of AFYRW

To confirm whether AFYRW could be taken up by cells and identify its potential subcellular location, HepG2 cells were incubated with 40 µg/mL FITC-AFYRW for 30 min. Using laser confocal microscopy, fluorescence signals were observed in the cells and mainly located in the nucleus (Fig. 2A), suggesting that AFYRW may play a role in modulating nuclear function and could potentially be used as a nucleus-targeting carrier.

To determine the effect of AFYRW dosage and incubation time on cellular uptake, HepG2 cells were incubated with 40 µg/mL FITC-AFYRW for 0–60 min, or alternatively with FITC-AFYRW at concentrations ranging from 0 to 100 µg/mL for 15 min. The cellular uptake of FITC-AFYRW as measured by flow cytometry displayed a time- and concentration-dependent manner (Fig. 2B and 2C).

3.3 Cellular uptake of AFYRW via endocytosis

To evaluate whether HepG2 cells take up AFYRW through endocytosis or direct membrane fusion, lysosomotropic agents NH₄Cl or CQ were incubated with HepG2 cells for 1 h, and the uptake of FITC-AFYRW by HepG2 cells was subsequently detected by flow cytometry. Cellular endocytosis requires low pH conditions [16]. When treated with CQ (an intracellular acidification inhibitor) or NH₄Cl (a lysosomal affinity weak base), the intracellular pH level is disrupted and endocytosis is blocked. We found that compared with the control group, the uptake of FITC-AFYRW was inhibited by 30% after 100 mmol/L NH₄Cl pretreatment ($p < 0.05$) (Fig. 3A), and inhibited by 15% ($p < 0.05$) and 20% ($p < 0.01$) after 50 µmol/L and 100 µmol/L CQ pretreatment, respectively (Fig. 3B), which were dose dependent. The number of positive cells counted by flow cytometry after treatment with NH₄Cl or CQ is shown in Fig. 3C or 3D. Together, these data suggest that AFYRW gained cellular entry using a pH-dependent and thus most likely an endosomal pathway.

3.4 Cellular uptake of AFYRW does not involve macropinocytosis or clathrin-mediated endocytosis pathway

To further characterize the endocytic pathways of AFYRW, HepG2 cells were pretreated with different concentrations of the macropinocytosis inhibitor AMR or the clathrin-dependent endocytosis inhibitor CPZ for 2 h, and the uptake of FITC-AFYRW by HepG2 cells was quantified. We found that compared with the control group, there was no significant difference in fluorescence intensity after pretreatment with AMR at a concentration up to 200 µmol/L (Fig. 4A), which indicates that cellular entry of AFYRW did not require macropinocytosis. However, the uptake of AFYRW increased slightly after pretreatment with 30 and 50 µmol/L of CPZ (Fig. 4B), suggesting that clathrin may not be an essential pathway for AFYRW translocation across the cell membrane. When CPZ suppressed the clathrin-mediated pathway, other endocytic pathways might be upregulated to promote the entry of AFYRW into cells, a phenomenon consistent with descriptions by Natarajan *et al.* [17, 18].

3.5 AFYRW enters HepG2 cells through the caveolin-mediated endocytosis

To determine the role of caveolae in AFYRW entry, we used the cholesterol sequestrator nystatin to block endocytosis that is mediated by caveolin. We pretreated HepG2 cells with increasing concentrations of nystatin for 2 h, and the uptake of FITC-AFYRW by HepG2 cells was examined. As shown in Fig. 5, pretreatment with 1.25–50 µg/mL nystatin significantly reduced the uptake of peptide AFYRW dose-dependently as compared to the control group. The fluorescence intensity declined by 20% after pretreatment with 12.5 µg/mL nystatin and declined by 25% with 50 µg/mL. Collectively, these results suggest that caveolae-mediated endocytosis is the pathway via which AFYRW enters the cells.

3.6 Screening of nucleoproteins by biotin-streptavidin interaction

The nucleoproteins incubated with biotin-AFYRW were magnetically sorted by biotin-streptavidin interaction, separated by SDS-PAGE, and stained by Coomassie brilliant blue R-250, and the strips with proteins interacting with AFYRW were obtained (Fig. 6A). The protein most reliably interacting with AFYRW was identified as poly (ADP-ribose) polymerase-1 (PARP-1), and the results of mass spectrometry (MS) analysis were shown in Fig. 6B–D. PARP-1 plays critical roles in many biological processes and is a target for anticancer therapies. PARP-1 uses nicotinamide adenine dinucleotide (NAD⁺) as a substrate to catalyze the transfer of ADP-ribose units to nuclear target proteins during oxidative stress and DNA damage [19]. We next used molecular docking to quickly assess whether PARP-1 was a potential target for AFYRW.

3.7 Peptide–protein molecular docking

Molecular docking is a popular and powerful computation tool that has been used for protein–protein docking, small molecular–protein docking, and peptide–protein docking [20]. Diverse docking schemes are utilized for different situations [21], and HPEPDOCK, a peptide–protein docking server based on a hierarchical algorithm [22], was employed in this study. The X-ray crystal structure of PARP-1 was derived from the protein database (PDB ID 4ZZZ) [23], and peptide AFYRW was inputted into the docking program in FASTA format (Fig. 7A). PARP-1 has three structural regions: an N-terminal DNA-binding domain, a central self-modifying domain, and a C-terminal catalytic domain. The active site of PARP-1 can be generally divided into two sites (the acceptor site and the donor site), of which the nicotinamide-ribose binding site (NI site) is considered to be the binding site of inhibitors [24]. The docking results showed that AFYRW interacted with eight amino acid residues of PARP-1 (Fig. 7B), mainly through hydrogen bonds (Arg878, Tyr907, Met890, Gln763), pi–pi accumulation (His862), electrostatic attraction (Asp766), and pi–alkyl interactions (Ala898, Lys903). These included several key amino acids (Ala898, Tyr907, Lys903, and Asp766) that were reported to inhibit the activity of PARP-1 [25], indicating that AFYRW may play a role as an inhibitor of PARP-1.

4 Discussion

In this study, we employed the endocytosis pharmacological inhibition strategies and treated HepG2 cells with a variety of inhibitors to determine the specific mechanism by which *Fagopyrum tataricum*-

derived peptide AFYRW enters cells. Endocytosis is an energy-dependent process in which extracellular substances (such as proteins, peptides, microorganisms, and nanoscale systems) are engulfed into the cell through deformation and movement of plasma membrane. Generally, endocytic pathways can be classified into two types: phagocytosis (for large nanoparticles) and pinocytosis (for fluids and solutes). Pinocytosis is usually divided into clathrin-dependent endocytosis (CDE) and clathrin-independent endocytosis (CIE). CIE involves three distinct mechanisms: the caveolae-mediated endocytic pathway, the clathrin/caveolae-independent endocytic pathway, and the macropinocytosis pathway. Clathrin/caveolae-independent endocytosis can be further divided into Cdc42, RhoA, flotillin, and Arf6 pathways [26]. Macropinocytosis, as a unique pathway of endocytosis, usually mediates the entry of macromolecules or viruses into cells. To prevent macropinocytosis, we exposed HepG2 cells to AMR, which suppresses macropinocytosis by reducing Na^+/H^+ exchange at the cell surface and blocking Rac1 and Cdc 42 signal transduction [27]. As expected, our results demonstrated that although cellular uptake of AFYRW required active endosomal acidification, AMR did not significantly alter FITC-AFYRW fluorescence intensity, indicating that macropinocytosis was not responsible for its cellular entry. The clathrin-dependent endocytosis pathway, which exists in all mammalian cells, is the primary way cells obtain nutrients. CPZ is a cationic amphipathic and the most important drug that inhibits the clathrin-dependent endocytosis pathway. CPZ relocates clathrin and adaptor protein complex-2 (AP2) from the cell surface to the intracellular endosomal membrane, causing the plasma membrane to collapse and form vesicles, thereby blocking CDE [28, 29]. When HepG2 cells were pretreated with CPZ, the uptake of AFYRW did not decrease, but instead increased, which may result from a compensatory phenomenon based on CDE changes [30]. It has been similarly reported in the literature that some individual treatments with endocytic inhibitors revealed higher cellular uptake compared to the untreated group [18]. It is possible that AFYRW enters cells via multiple pathways simultaneously, and disrupting one endocytic pathway might cause the upregulation of other active pathways. The effect of incubation time with endocytosis inhibitors may be cell type-dependent, and a careful optimization would thus be required for each *in vitro* system. Caveolin is rich in cholesterol and sphingolipid, so the endocytosis pathway mediated by caveolae can be studied by cholesterol-sensitive reagents such as methyl- β -cyclodextrin (m- β -CD) or nystatin. M- β -CD was commonly used as a caveolae-mediated endocytosis inhibitor by sequestering cholesterol, which in turn perturbed plasma membrane fluidity in lipid rafts. However, it has been reported with growing evidence that m- β -CD is not a specific inhibitor, as it also can also inhibit CDE and macropinocytosis [31]. We thus chose nystatin, which is more specific towards caveolae-mediated endocytosis. A significant and concentration-dependent reduction in the signal intensity of fluorescently positive cells was observed upon 12.5 or 50 $\mu\text{g}/\text{mL}$ nystatin pretreatment, indicating a block in AFYRW uptake. Taken together, our data suggest that AFYRW enters HepG2 cells by relying on caveolae-mediated endocytosis. It would be interesting to further explore whether cells are also using an energy-dependent pathway that is unaffected by the chemical inhibitors tested in this study over the incubation period.

Bioactive macromolecular peptides entering the cell via endocytosis need to escape endosomes before they can be delivered to cytoplasm or organelles, and effective delivery of drugs at specific sites is a

prerequisite for pharmacological action and therapeutic effect. Endocytosis is composed of multiple steps: an engulfed membrane embolus is first pinched off, and the endosomes are then delivered to specific vesicles that sort the contents for different destinations, which include diverse organelles, the extracellular environment, and other cells. Consistent with our observation of the subcellular localization of AFYRW, studies have shown that hydrophobic amino acids, such as Phe (F) or Trp (W) hydrophobic aromatic rings, are beneficial for endosome escape [32]. AFYRW contains the hydrophobic amino acids Ala (A), Phe, and Tyr, as well as aromatic amino acids Tyr (Y) and Trp, which might contribute to its endosome escape and nuclear translocation, in addition to its antioxidant properties. Furthermore, basic amino acids are particularly prominent among the nuclear targeting sequences. Nuclear localization signals (NLS), for instance, are a kind of short peptide rich in basic amino acids, whose net positive charge can interact with negatively charged DNA and promote the nuclear targeting of drugs [33]. Arg (R) in AFYRW with its positively charged guanidine may specifically form hydrogen bonds with the phosphate skeleton of DNA/RNA [34]. It would be interesting to explore the therapeutic potential of AFYRW to enhance nuclear delivery in the absence of cytotoxicity.

The peptide AFYRW may play a role in modulating nuclear function, and we utilized biotin-AFYRW-streptavidin affinity purification method to screen for nuclear proteins that interact with AFYRW. After protein spectrum analysis, PARP-1 was identified as the potential target of AFYRW. PARP-1 is a key ribozyme commonly expressed in eukaryotic cells and plays a vital role in DNA damage and repair. When the DNA damage is mild, PARP-1 participates in the process of DNA repair. In the context of high expression of PARP-1 in tumor cells, inhibition of PARP-1 blocks DNA repair, promotes tumor cell apoptosis, and exerts anti-tumor activity [35]. When DNA damage is severe, PARP-1 is over-activated, which leads to the consumption of NAD^+ and ATP; energy deficiency will then slow down glycolysis and cause mitochondrial dysfunction and cell death. Oxidative stress is the main reason for the induction of apoptosis, tissue damage, and pathological changes in the body. Therefore, inhibiting the activity of PARP-1 has become promising for the treatment of neurodegenerative [36], cardiovascular [37], and metabolic diseases [38]. However, currently marketed PARP-1 inhibitors have adverse side effects, and highly potent and selective drugs remain to be discovered. AFYRW has a demonstrated capacity for eliminating radicals [12]; its effectiveness as food-derived peptidic antioxidant prepared from enzymatic hydrolysis of edible protein warrants further investigation given its safety characteristics as a phytocompound.

Peptide drugs can mimic protein-binding domains and competitively inhibit protein-protein interactions with advantages over traditional small molecular inhibitors. The characterization of peptide-protein interactions is necessary for the purposes of clarifying important biological processes and designing peptides or peptidomimetic drugs. Molecular docking is a powerful computing tool that is widely used to predict the binding structure of protein-peptide complexes and can provide leads for targeted inhibitor design. The current peptide-protein docking algorithms can be divided into local and global peptide docking [20, 39]. HPEPDOCK developed a hierarchical flexible global peptide docking method through rapid peptide conformation generation and global docking, which performs well in many protein-peptide complex docking schemes. Therefore, we used HPEPDOCK to determine whether PARP-1 interacts with

AFYRW. Consistent with the biochemical results, the docking analyses revealed that AFYRW interacted with eight amino acid residues of PARP-1 mainly through hydrogen bonds and pi–pi accumulation. So far, most of the PARP-1 inhibitors in clinical and preclinical trials are structural analogues of NAD⁺, which are designed to compete with NAD⁺ at the enzyme active site and bind to the catalytic domain of the enzyme to inhibit self-modification [23]. Our data showed that AFYRW interacts with several key amino acids (Ala898, Tyr907, Lys903, and Asp766) that were reported to inhibit the activity of PARP-1 [25], confirming that AFYRW may be a potential inhibitor of PARP-1. The antioxidant mechanisms of food-derived peptides can be classified into three types: regulation of reactive oxygen species production, inhibition of enzymatic activity, or modification of the antioxidant pathway. Our study suggests the molecular regulation of PARP-1 enzyme activity; however, AFYRW may act on multiple pathways to exert its antioxidant activities.

There are several limitations to this study. First, we only used HepG2 cells as the target and alternative cell lines were beyond the scope of the current study. Second, although our data imply that AFYRW could be a potential PARP-1 inhibitor, the precise mechanisms of AFYRW function remains to be elucidated. Another challenge was that the bioactivity of AFYRW was tested via *in vitro* biochemical assays and cell culture, which should be verified by *in vivo* studies through animal models and human clinical trials. Hence, further work is needed to address these limitations.

5 Conclusion

In the present study, we examined the cell entry mechanism and potential targets of the peptide AFYRW, which is derived from enzymatic hydrolysate fractions of *Fagopyrum tataricum*. Our results show that the uptake of AFYRW into HepG2 cells depends on caveolae-mediated endocytosis that require a low pH, but does not involve macropinocytosis or clathrin-mediated endocytosis. In addition, AFYRW primarily localizes in the nucleus and may act as a potential inhibitor of the nuclear protein PARP-1. Findings of this study can provide new insights for the development and utilization of the novel bioactive peptide AFYRW as nuclear targeting therapeutic drug.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study docking are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there have no competing interests, financial or otherwise.

Funding

This study was supported by Guizhou Provincial Basic Research Program(Natural Science) Grant No.19NSP038; Grant No. ZK[2021]104.

Author contributions

The author's contributions were as follows: Jie Zuo: performed the experiments and drafted the manuscript; Yuheng Jiang, Chengjin Wang, Yi Xiao: performed the experiments; Jiajun Yang, Yan Deng, Lilin Zhang, Qingzhong Xu: performed data analysis and provided guidance for modifying the manuscript; Hongmei Li: conceived the study, designed the experiments and modified the manuscript.

Acknowledgments

We express our sincere thanks to Prof. Daquan Sun for providing HepG2 cells. We also thank the Research Center for Basic Sciences of Medicine of Guizhou Medical University for providing large-scale instruments and equipments.

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Figures

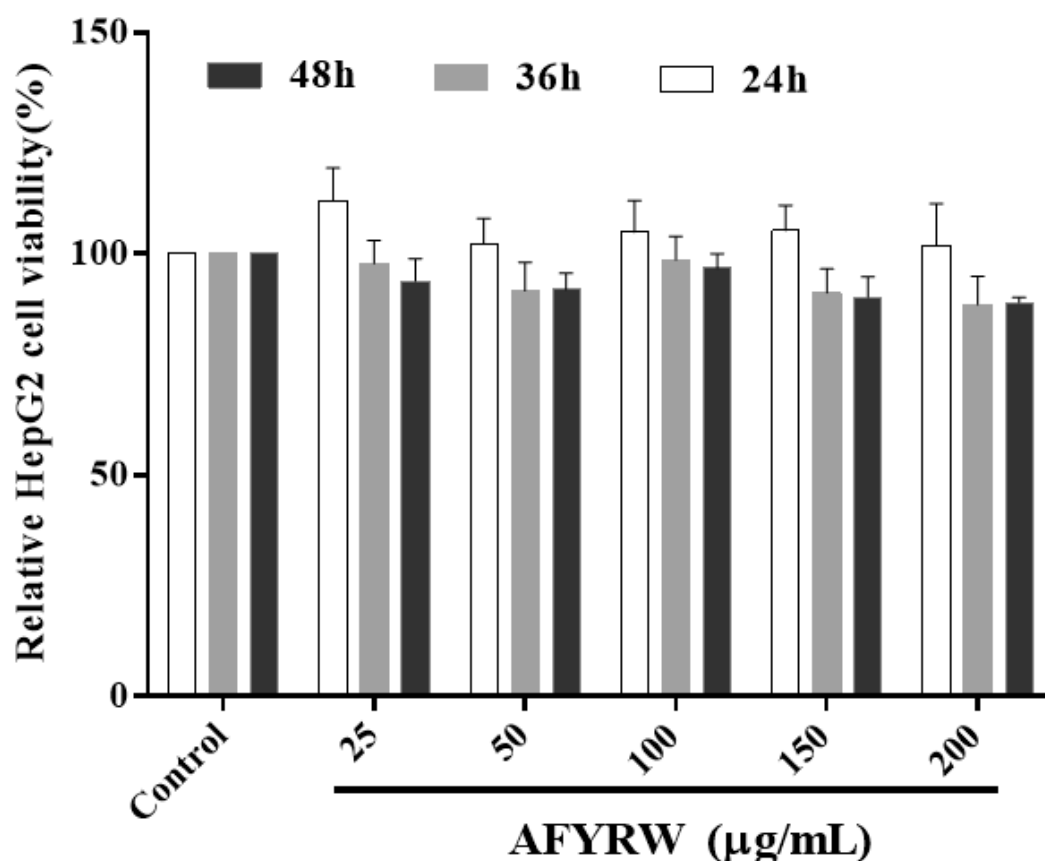


Figure 1

Cytotoxicity evaluation of AFYRW. CCK-8 assay for HepG2 cells treated with AFYRW at the indicated concentrations for 24, 36, and 48 h. The cell viability was quantified and normalized by the signal of the

control group.

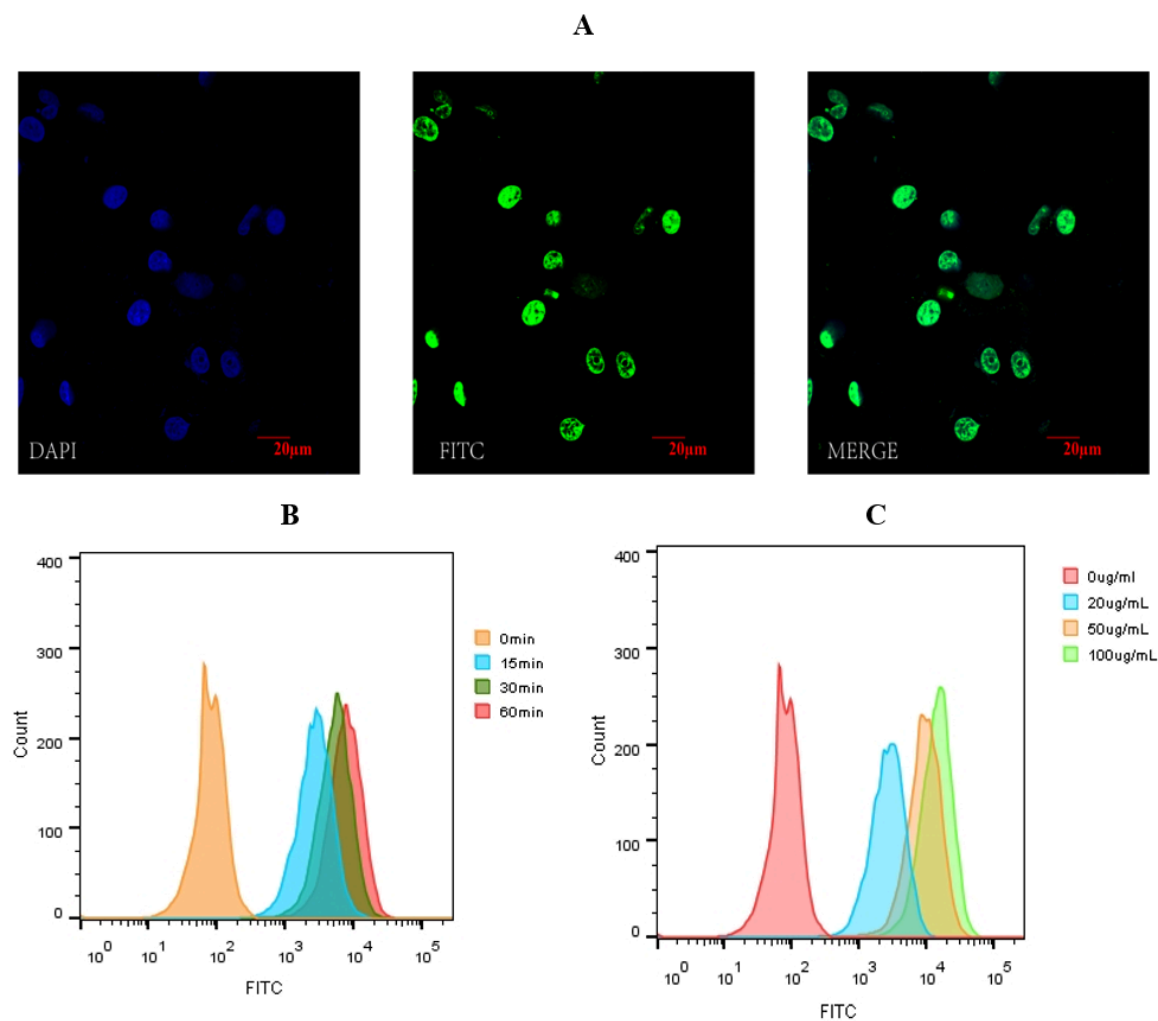


Figure 2

Subcellular localization and cellular uptake of FITC-AFYRW.A. Representative fluorescent images of FITC-AFYRW subcellular localization in HepG2 cells. B. Effect of incubation time on the cellular uptake of FITC-AFYRW measured by flow cytometry. C. Effect of FITC-AFYRW concentration on its cellular uptake measured by flow cytometry.

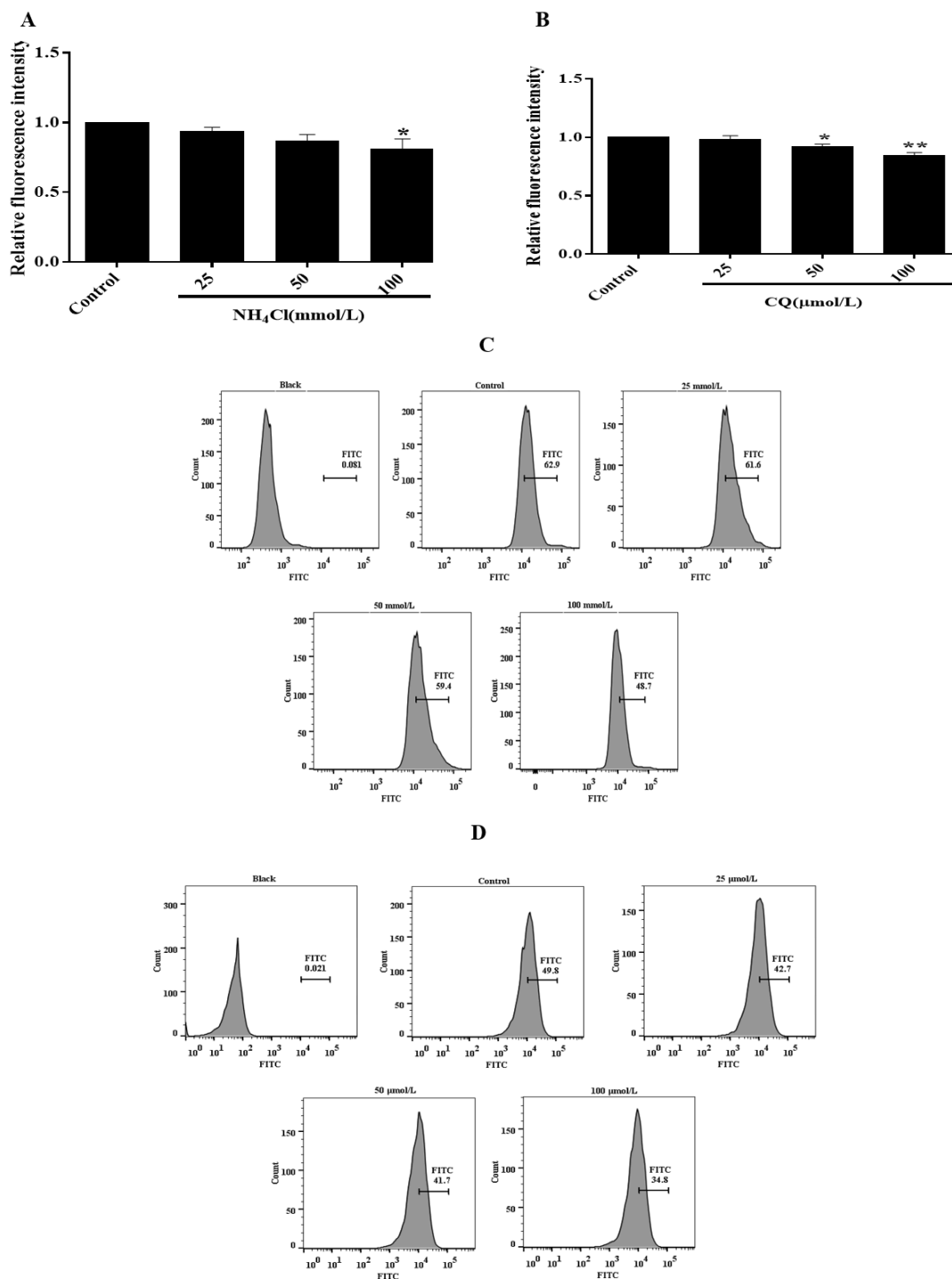


Figure 3

Uptake of AFYRW by cells was inhibited by lysosomotropic agents. A. Fluorescence intensity quantification of FITC-AFYRW after exposing HepG2 cells to NH₄Cl for 1 h. B. Fluorescence intensity quantification of FITC-AFYRW after exposing HepG2 cells to chloroquine (CQ) for 1 h. C. The number of positive cells counted by flow cytometry after treatment with the indicated concentrations of NH₄Cl. D. The number of positive cells counted by flow cytometry after treatment with the indicated

concentrations of CQ. The fluorescence intensity was quantified and normalized by signal of the control group. * $p<0.05$, ** $p<0.01$ versus control group.

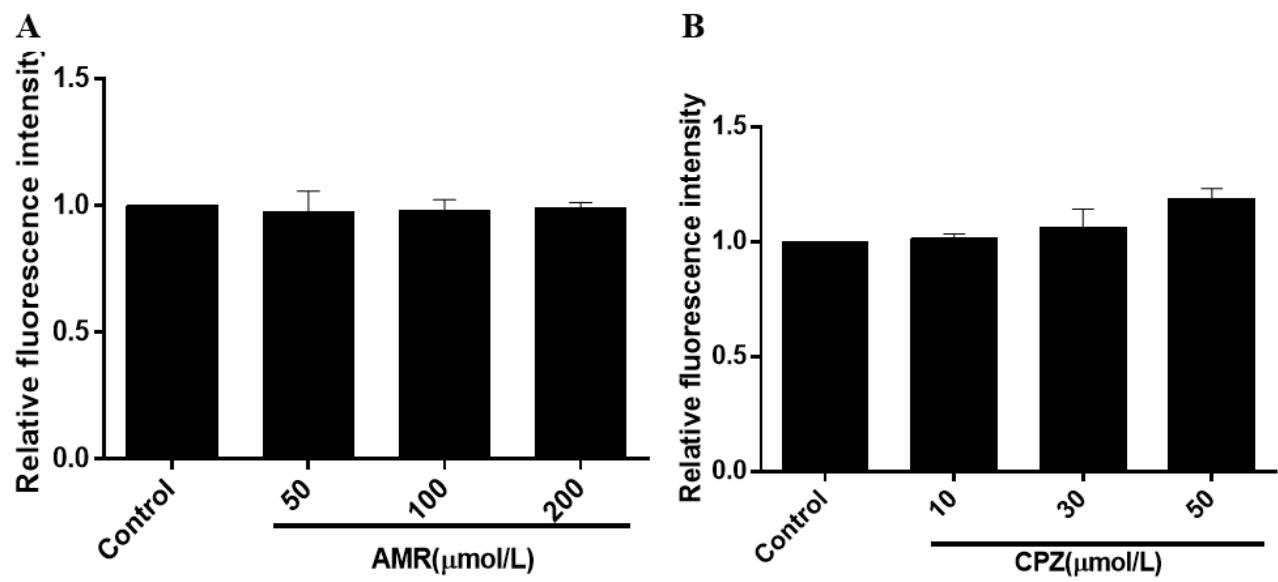


Figure 4

Macropinocytosis and clathrin-dependent endocytosis are not the pathways by which AFYRW peptide enters HepG2 cells. Fluorescence intensity quantification of FITC-AFYRW after exposing HepG2 cells to amiloride (AMR) (A) or chlorpromazine (CPZ) (B) for 2 h. The fluorescence intensity was quantified and normalized by signal of the control group.

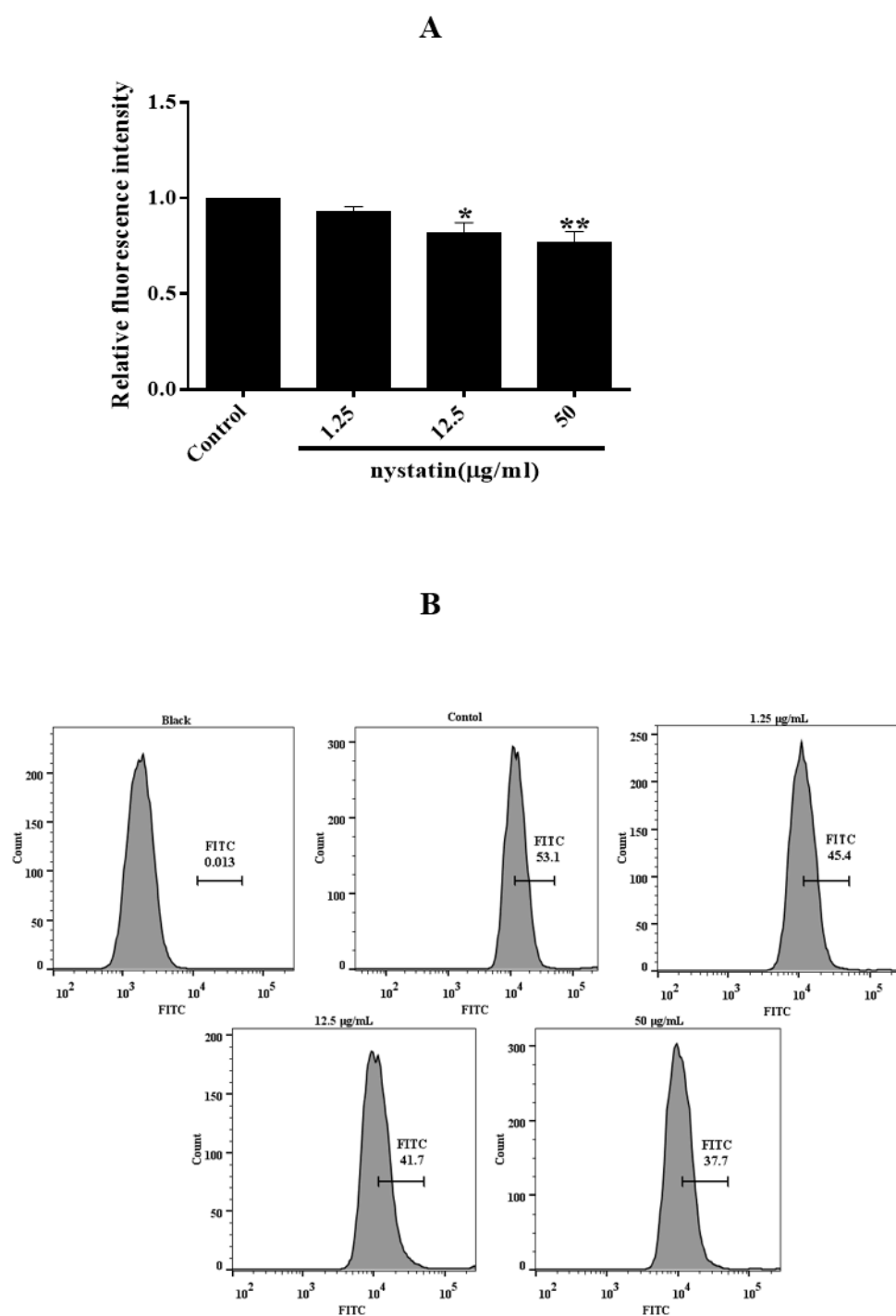


Figure 5

AFYRW peptide enters HepG2 cells through clathrin-independent endocytosis. A. Fluorescence intensity quantification of FITC-AFYRW after exposing HepG2 cells to nystatin for 2 h. B. The number of positive cells counted by flow cytometry after treatment with the indicated concentrations of nystatin. The fluorescence intensity was quantified and normalized by signal of the control group. * $p < 0.05$, ** $p < 0.01$ versus control group.

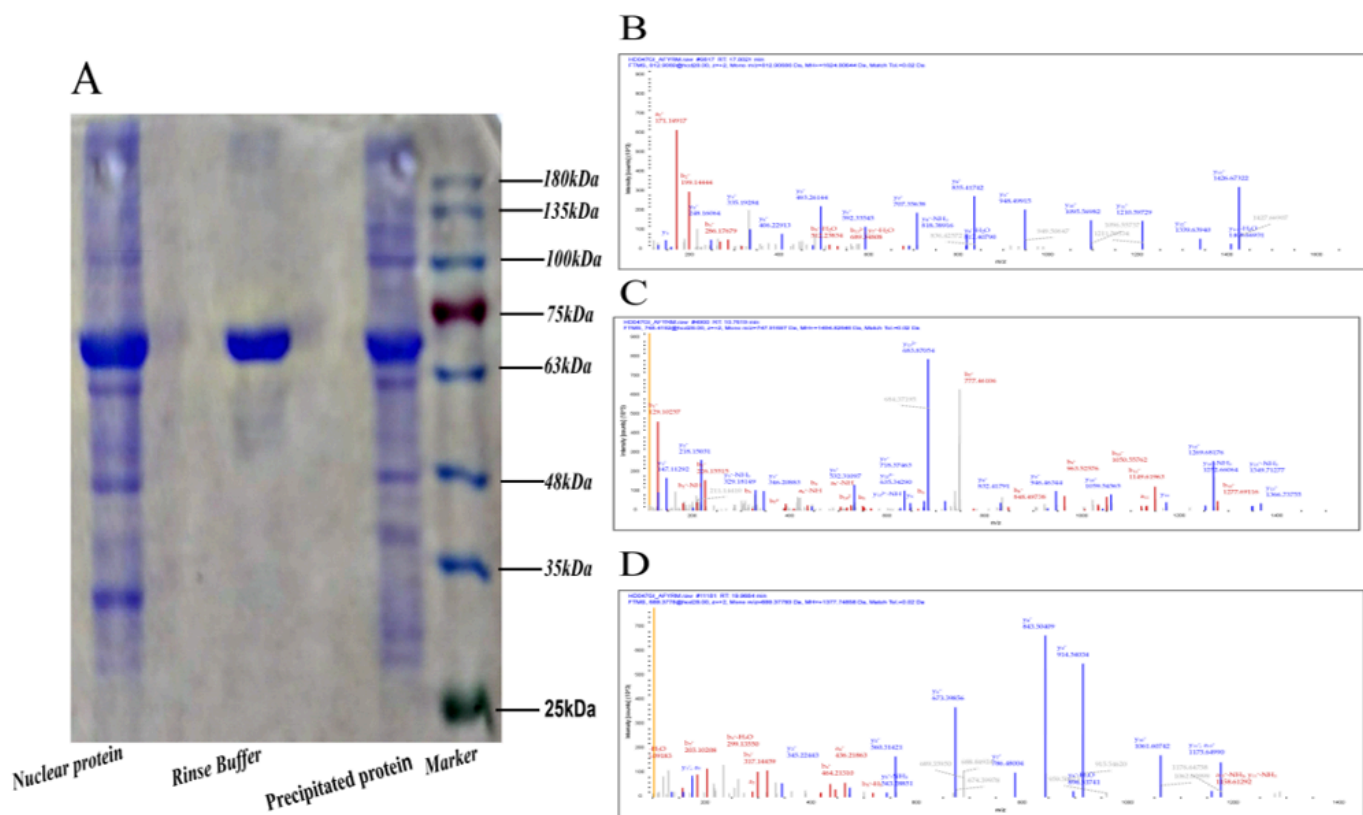


Figure 6

Affinity precipitated nuclear proteins and spectrum analysis. A. Coomassie brilliant blue R-250 staining of nuclear proteins, rinse buffer, and affinity precipitated nuclear proteins. B–D. Mass spectrogram of the potential target protein, Poly (ADP-ribose) polymerase-1 (PARP-1).

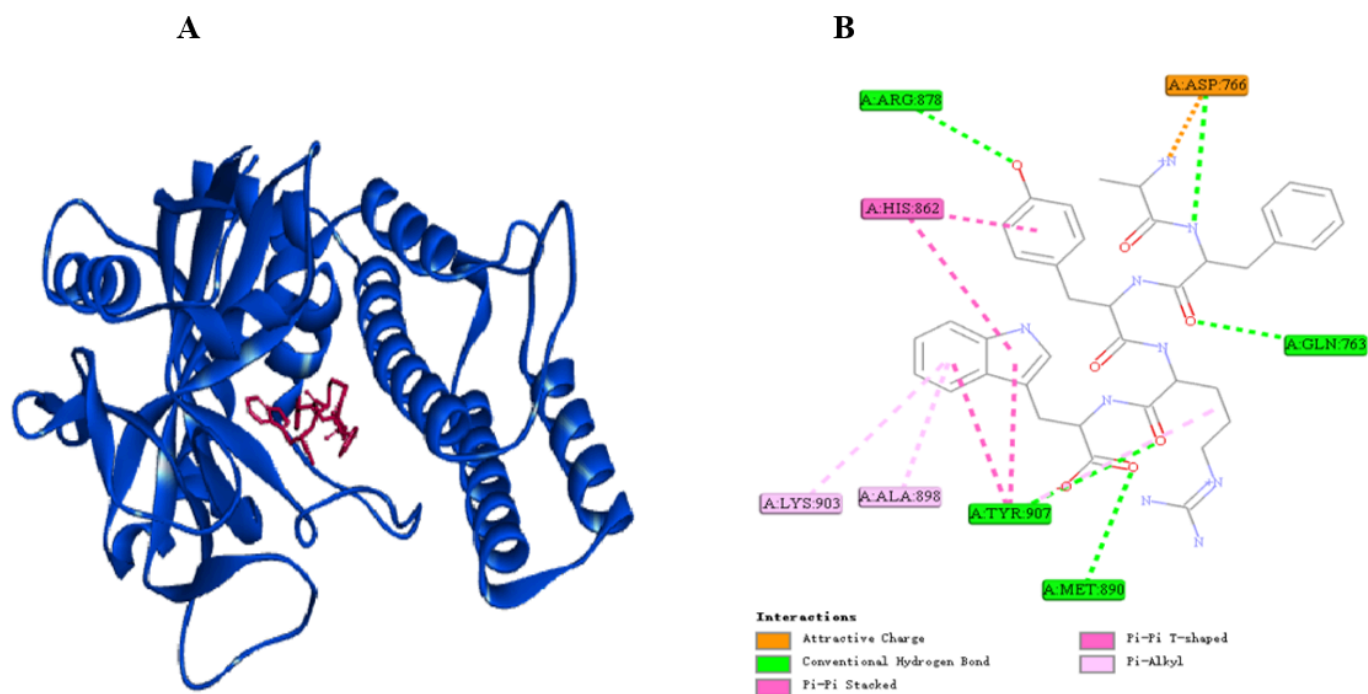


Figure 7

Molecular docking of AFYRW with PARP-1. A. 3D cartoon representation of PARP-1 (blue) in complex with AFYRW (red) in stick model. B. Interactions established after docking AFYRW against PARP-1 showing the specific amino acids involved. Electrostatic attraction, conventional hydrogen bond, pi–pi interaction, and pi–alkyl interaction are represented by orange, green, purple, and pink dashed lines, respectively.

Supplementary Files

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