Physiological Indicators of Paeoniflorin and Caffeine in Modulating the Motility of *Caenorhabditis elegans* via Adenosine Receptor

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ABSTRACT

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| **Aims:** Paeoniflorin exhibits a broad range of in vitro and in vivo pharmacological effects. However, its potential as a drug targeting adenosine receptors remains largely unexplored.  **Methodology:** In this study, the wild-type N2 strain and the Ador-1 gene knockout EG6890 strain of *Caenorhabditis elegans* were used to compare the effects of different drug concentrations on nematode locomotion and feeding behavior. Four behavioral indicators—swimming, head swing, body bending, and pharyngeal pumping—were quantitatively assessed. 0.03, 0.3, and 3 mM paeoniflorin and 0.05, 0.5, and 5 mM caffeine were involved for physiological evaluation. Additionally, molecular docking analysis was performed to evaluate the interaction between paeoniflorin and the adenosine receptor, Ador-1 protein.  **Results:** The results demonstrated that paeoniflorin (0.03–3 mM) inhibited motility in a dose-dependent tendency (P<0.01). Caffeine significantly enhanced the movement ability of N2 nematodes at low concentrations (0.05–0.5 mM) (P<0.01), whereas higher concentrations (5 mM) exerted an inhibitory effect. Molecular docking analysis revealed that paeoniflorin exhibited a binding energy of -6.9 kcal/mol with the Ador-1 protein, confirming its targeting capability. Specific interaction analysis showed that paeoniflorin formed van der Waals interactions with SER6 of Ador-1, π–π and amide–π stacking interactions with GLY5 and TYR271, C–H bonding with TRP68, and alkyl and π–alkyl interactions with LEU267, VAL8, and LEU272.  **Conclusion:** This study highlights the critical role of nematode adenosine receptors in the drug's mechanism of action and provides an experimental foundation for receptor-based drug screening using the nematode model. |

***Keywords:*** *Caenorhabditis elegans; Ador-1 adenosine receptor; paeoniflorin; caffeine*

1. INTRODUCTION

Adenosine receptors are G protein-coupled receptors with seven transmembrane domains. Based on their affinity for adenosine and the type of G protein they couple with, they are classified into four subtypes: A1, A2a, A2b, and A3. These receptors are primarily expressed on the surface of tissue cells in vertebrates, including humans [1]. Many physiological effects are primarily mediated through binding to adenosine receptors (ARs) [2]. Therefore, numerous adenosine receptor agonists, antagonists, and traditional Chinese medicines have been identified and utilized [3].

Paeoniflorin, a monoterpenoid glycoside, is isolated from the traditional Chinese medicinal plants Paeonia lactiflora (red peony) and Paeonia suffruticosa (white peony). It serves as the primary bioactive component of peony, with the molecular formula C₂₃H₂₈O₁₁ [4]. Studies have demonstrated that paeoniflorin exhibits diverse pharmacological effects both in vitro and in vivo, including antioxidant [5], anticonvulsant [6], neuroprotective [7], antitumor [8], and immunomodulatory properties [9]. However, study on paeoniflorin as a modulator of adenosine receptors remains limited.

Experimental studies in mammals have established that caffeine is a non-selective antagonist of adenosine receptors. Its core chemical structure contains methylxanthine, which confers its ability to act as a non-selective adenosine receptor antagonist [10]. Caffeine has been shown to provide preventive and therapeutic benefits for neurodegenerative diseases such as Parkinson’s and Alzheimer’s, as well as exert protective effects against cerebral ischemia and hypoxia [11]. Additionally, it plays a role in mitigating neuroinflammatory disorders and other related diseases [12].

*Caenorhabditis elegans* was a multicellular organism to have its entire genome sequenced [13]. As a model organism, it is widely utilized for research on antioxidants, aging, age-related diseases, and longevity mechanisms, as well as for identifying drug compounds with potential lifespan-extending effects. Many of its genes and signaling pathways are evolutionarily conserved between nematodes and humans. Moreover, *C. elegans* presents no ethical or regulatory concerns, making it a widely used biological model in biomedical research [14-16].

Several compounds, including adenosine and salidroside, have been identified as agonists of the ador-1 receptor in nematodes [17]. This study aims to establish a drug-culture experimental model in *C. elegans* to investigate the role of nematode adenosine receptors in the pharmacological mechanisms of receptor-targeting drugs. It seeks to verify the antagonist effect of caffeine and explore the potential of the traditional Chinese medicine paeoniflorin in either antagonizing or stimulating adenosine receptors.

2. materials and methods

**2.1 Nematode Strains**

The N2 wild-type *C. elegans* strain was obtained from the Caenorhabditis Genetics Center (CGC). The Ador-1 knockout strain (EG6890) was provided by Dr. Erik Jorgensen’s laboratory at the University of Utah, USA, and is currently maintained in our laboratory.

**2.2 Drug Preparation**

**Paeoniflorin solution**

A stock solution of paeoniflorin (6 mM) was prepared by dissolving 20 mg of paeoniflorin in 6.94 mL of M9 buffer. Serial dilutions were performed by taking 100 μL and 10 μL of the stock solution and diluting each to 1 mL with M9 buffer, resulting in working solutions of 6 mM, 0.6 mM, and 0.06 mM. The final experimental concentrations were adjusted to 3 mM, 0.3 mM, and 0.03 mM.

**Caffeine solution**

A 10 mM stock solution of caffeine was prepared by dissolving 20 mg of caffeine in 10.3 mL of M9 buffer. Serial dilutions were performed by taking 100 μL and 10 μL of the stock solution and diluting each to 1 mL with M9 buffer, yielding working solutions of 10 mM, 1 mM, and 0.1 mM. The final experimental concentrations were set to 5 mM, 0.5 mM, and 0.05 mM.

All prepared drug solutions were stored at 4°C, protected from light.

**2.3 Nematode Treatment**

Eggs of the wild-type N2 strain and Ador-1 knockout strain (EG6890) were obtained via synchronization using the method described by Xie et al. [18]. The nematodes were cultured at 20°C until they reached the L4 larval stage. The two strains were then treated in parallel with paeoniflorin (Aladdin, HPLC ≥ 98%) and caffeine (Aladdin, HPLC ≥ 98%).

**2.4 Physiological indicators observation**

**Swimming Ability**

Nematodes of similar physiological status were selected and transferred to a 96-well plate containing 200 μL of M9 buffer. After allowing movement in the liquid for 10 seconds, body swings were recorded over 30 seconds. Each group included 15 nematodes, with three experimental replicates.

**Head Swing Frequency**

Nematodes of similar status were placed on nematode growth medium (NGM) without OP50 bacteria and allowed to move for 1 minute. The number of head swings was recorded over a 20-second period. A head swing was defined as a movement from one direction to another and back. Each group included 15 nematodes, with three experimental replicates.

**Body Bending Frequency**

Nematodes of similar status were placed on NGM medium without OP50 and allowed to move freely for 1 minute. The number of body bends was recorded over the same period. A single body bend was defined as one full wavelength of sinusoidal movement. Each group included 15 nematodes, with three experimental replicates.

**Pharyngeal Pumping Frequency**

At room temperature, nematodes of similar physiological status were observed under a stereoscope. The pharyngeal pumping frequency was counted three times over a 20-second period for each nematode. Each group included 15 nematodes, with three experimental replicates.

**2.5 Molecular Docking of Paeoniflorin with Adenosine Receptor**

The three-dimensional (3D) structure of the paeoniflorin molecule, identified by its CAS number, was obtained in SDF format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The structure was then imported into ChemBio3D Ultra 14.0 for energy minimization, with the Minimum RMS Gradient set to 0.001. The optimized small molecule was saved in MOL2 format. Subsequently, the molecule was imported into AutoDockTools 1.5.6 for hydrogenation, charge calculation, charge assignment, and the definition of rotatable bonds, and subsequently saved in PDBQT format.

The 3D structure of the adenosine receptor (Ador-1) protein (PDB ID: 5N2R) was retrieved from the Protein Data Bank (PDB). Using PyMOL 2.3.0, crystal water molecules and original ligands were removed. The processed protein structure was then imported into AutoDockTools 1.5.6 for hydrogenation, charge calculation, charge assignment, and atomic type assignment, before being saved in PDBQT format.

Protein binding sites were predicted using POCASA 1.1, and AutoDock Vina 1.1.2 was used for molecular docking. The docking calculations were performed using AutoDock Vina, with all other parameters set to their default values. Upon completion of the Vina operation, the binding affinity score between the target protein receptor and the small molecule ligand was determined based on the calculated docking binding free energy.

**2.6 Statistical Analysis**

GraphPad Prism 8.0.2 was used for statistical analysis and data visualization. Statistical significance was assessed using the t-test, and results were expressed as "mean ± SD." A p-value of <0.05 indicated statistical significance and was marked with "\*", while a p-value of <0.01 indicated highly significant differences and was marked with "\*\*"..

3. results

**3.1 Effects** **and Comparative analysis of Different Concentrations of Paeoniflorin and Caffeine on the Swimming Ability of N2 and EG6890**

The impact of various concentrations of paeoniflorin on the swimming ability of N2 and EG6890 nematodes was assessed (Figure 1A). Results indicate that as the paeoniflorin concentration increased, the swimming frequency of both N2 and EG6890 nematodes significantly decreased (P < 0.01). In the 0.3 mM and 3 mM treatment groups, the reduction in swimming frequency was significantly greater in N2 nematodes than in EG6890 nematodes, suggesting that paeoniflorin may interact with nematode adenosine receptors, thereby reducing motility. The EG6890 strain, which has an ador-1 gene knockout, exhibited significant differences from the N2 strain as paeoniflorin concentration increased (Figure 1a). These findings suggest that the reduction in swimming ability with increasing paeoniflorin concentration is closely associated with adenosine receptors.

The effect of different caffeine concentrations on nematode swimming ability was observed (Figure 1B). Results indicate that under 0–0.5 mM treatment conditions, the swimming frequency of N2 nematodes was significantly higher than that of EG6890 nematodes (P < 0.05). However, at 5 mM caffeine treatment, the swimming ability of N2 nematodes decreased substantially, falling slightly below that of the control group (CK), whereas the EG6890 strain continued to exhibit increased motility. The difference in swimming ability between N2 and EG6890 nematodes increased significantly, suggesting that caffeine likely interacts with adenosine receptors in N2 nematodes. At low concentrations, caffeine appears to enhance nematode activity, whereas at high concentrations, it inhibits swimming frequency (Figure 1b), possibly due to pharmacological side effects leading to reduced motility.



**Fig. 1. Effects and Comparison of different concentrations of paeoniflorin (A, a) and caffeine (B, b) on the swimming ability of N2 and EG6890 nematodes. (\*P < 0.05, \*\*P < 0.01)**

**3.2 Effects and Comparative analysis of Different Concentrations of Paeoniflorin and Caffeine on the Head Swing Frequency of N2 and EG6890**

The effects of different concentrations of paeoniflorin on the head swing frequency of N2 and EG6890 nematodes were evaluated after treatment (Figure 2A). Results demonstrate that as paeoniflorin concentration increased, the head swing frequency of both N2 and EG6890 nematodes significantly decreased (P < 0.01). In the 0.3 mM and 3 mM treatment groups, the reduction in head swing frequency was significantly greater in N2 nematodes than in EG6890 nematodes, suggesting that paeoniflorin may exert a sedative effect on nematodes in both strains. A difference in head swing frequency was observed between the ador-1 gene knockout EG6890 strain and the N2 strain (Figure 2a). However, the difference between paeoniflorin treatment concentrations was not significant (P > 0.05). This indicates that adenosine receptors may not play a critical role in mediating paeoniflorin's effect on head swing frequency.

The impact of caffeine on nematode head swing frequency was assessed after treatment with different concentrations (Figure 2B). Results reveal that as caffeine concentration increased, the head swing frequency of both N2 and EG6890 nematodes initially increased and then decreased. Under 0–0.5 mM treatment conditions, the head swing frequency of N2 nematodes increased more significantly than that of EG6890 nematodes (P < 0.01). However, at 5 mM caffeine treatment, the head swing frequency of N2 nematodes dropped significantly, although it remained higher than that of the control (CK). Combined with the data presented in Figure 2b, the difference in head swing frequency between N2 and EG6890 nematodes increased significantly, suggesting that caffeine likely interacts with adenosine receptors to regulate nematode head swings. In the EG6890 strain, with the exception of the 0.5 mM treatment group, the differences were statistically significant but relatively small. No significant differences were observed between the 0.05 mM and 5 mM treatment groups, suggesting that caffeine has minimal impact on the head swing frequency of EG6890 nematodes. This indirectly supports the hypothesis that caffeine influences nematode head swing frequency through interactions with adenosine receptors.



**Fig. 2. Effects and Comparison of different concentrations of paeoniflorin (A, a) and caffeine (B, b) on head swing in N2 and EG6890 nematodes. (\*P < 0.05, \*\*P < 0.01).**

**3.3 Effects and Comparative analysis of Different Concentrations of Paeoniflorin and Caffeine on Body Bending in N2 and EG6890**

The effects of various concentrations of paeoniflorin on the body bending of N2 and EG6890 nematodes were observed (Figure 3A). Results indicate that as the paeoniflorin concentration increased, the bending frequency of both N2 and EG6890 nematodes decreased significantly (P < 0.01). In the 0.03–3 mM treatment group, the reduction in bending frequency was significantly greater in N2 nematodes than in the EG6890 group, suggesting that paeoniflorin may act on nematode adenosine receptors to reduce motility. The EG6890 strain, which carries an Ador-1 gene knockout, exhibited significant differences compared to the N2 strain as the paeoniflorin concentration increased (Figure 3a). These findings suggest that the reduction in bending frequency due to paeoniflorin treatment is closely related to adenosine receptor activity.

Following treatment with different concentrations of caffeine, the effects on nematode bending frequency were assessed (Figure 3B). Results indicate that under 0–0.5 mM caffeine treatment, the bending frequency of N2 nematodes was significantly higher than that of EG6890 nematodes (P < 0.01). However, at 5 mM, the bending frequency of N2 nematodes decreased significantly, falling slightly below that of the control group, whereas the bending frequency of EG6890 nematodes continued to increase. However, the difference between the EG6890 and control (CK) groups was not statistically significant (P > 0.05). The significant difference between N2 and EG6890 nematodes suggests that caffeine likely interacts with adenosine receptors in N2 nematodes. While low caffeine concentrations enhance nematode activity, higher concentrations suppress bending frequency (Figure 3b), potentially due to pharmacological side effects that reduce motility.



**Fig. 3. Effects and Comparison of different concentrations of paeoniflorin (A, a) and caffeine (B, b) on body bending in N2 and EG6890 nematodes. (\*P < 0.05, \*\*P < 0.01).**

**3.4 Effects and Comparative analysis of Different Concentrations of Paeoniflorin and Caffeine on Pharyngeal Pumping in N2 and EG6890**

The effects of different concentrations of paeoniflorin on pharyngeal pumping in N2 and EG6890 nematodes were observed (Figure 4A). Results indicate that increasing paeoniflorin concentrations led to a decline in pharyngeal pumping frequency in N2 nematodes, although this decrease was modest. No significant differences were observed between the 0–0.3 mM treatment groups and the control group (P > 0.05). However, the 3 mM treatment group exhibited a significant reduction in pharyngeal pumping frequency compared to the control (P < 0.01). Conversely, the pharyngeal pumping frequency of EG6890 nematodes fluctuated significantly (P < 0.01). The ador-1 gene knockout strain (EG6890) exhibited significant differences from the N2 strain as paeoniflorin concentration increased (Figure 4a). These findings suggest that, in addition to acting through adenosine receptors, paeoniflorin may influence pharyngeal pumping frequency via other pathways at higher concentrations.

After caffeine treatment, the pharyngeal pumping activity of N2 and EG6890 nematodes was observed (Figure 4B). Results show that pharyngeal pumping frequency in N2 nematodes significantly decreased at 0.05 mM but increased significantly at 0.5 mM. However, at 5 mM, pharyngeal pumping frequency declined again (P < 0.01). In EG6890 nematodes, pharyngeal pumping initially increased and then decreased (P < 0.01). Figure 4b illustrates that caffeine’s effect on pharyngeal pumping frequency does not follow a linear trend.

**Fig. 4. Effects and Comparison of different concentrations of paeoniflorin (A, a) and caffeine (B, b) on pharyngeal pumping in N2 and EG6890 nematodes. (\*P < 0.05, \*\*P < 0.01)**

**3.5 Paeoniflorin’s Molecular Docking**

To further investigate the potential interaction between paeoniflorin and the nematode adenosine receptor (Ador-1), molecular docking simulations were performed. A molecular binding model was constructed, interaction modes were analyzed, and binding affinity was calculated. The analysis (Figure 5) revealed that the binding energy between paeoniflorin and Ador-1 was -6.9 kcal/mol, indicating a strong binding interaction. Specific interaction analysis showed that paeoniflorin formed van der Waals interactions with SER6 of Ador-1, π–π and amide–π stacking interactions with GLY5 and TYR271, C–H bonding with TRP68, and alkyl and π–alkyl interactions with LEU267, VAL8, and LEU272.



**Fig. 5. Binding interactions between paeoniflorin and the Ador-1 adenosine receptor of *C. elegans* (A: structure of the adenosine receptor protein; B: hydrogen bond interactions of paeoniflorin).**

4. discussion

Some studies suggest that paeoniflorin may function as an adenosine receptor agonist, exerting sedative effects in the nervous system through regulation of the adenosine A1 receptor signaling pathway [19]. Conversely, other studies indicate that paeoniflorin can mimic the action of the adenosine receptor antagonist DPCPX [20]. Further studies suggest that paeoniflorin may play a role in combating Alzheimer’s disease by activating A1 receptors while inhibiting A2a receptors [21].

This study utilized *C. elegans* as an effective model organism for drug testing and primarily investigated the effects of two adenosine receptor-targeting compounds, paeoniflorin and caffeine, on the nematode adenosine receptor (Ador-1). The results demonstrated that under standard culture conditions at 20°C, treatment of N2 and EG6890 nematodes with 0.03 mM, 0.3 mM, and 3 mM paeoniflorin led to a concentration-dependent inhibition of motilities, including swimming, body bending, head swing, and pharyngeal pumping (Figure 1A, a; Figure 2A, a; Figure 3A, a; Figure 4A, a). Given that EG6890 is an Ador-1 gene knockout strain, these findings suggest that paeoniflorin acts on the nematode adenosine receptor and likely functions as a receptor agonist.

To further confirm whether paeoniflorin interacts with nematode adenosine receptors, molecular docking simulations were conducted between paeoniflorin and the Ador-1 protein. Interaction analysis revealed a binding energy of -6.9 kcal/mol, indicating a strong binding affinity and supporting the hypothesis that paeoniflorin can target nematode adenosine receptors. In comparison, the binding energy of caffeine with Ador-1 was -6.7 kcal/mol (unpublished data), suggesting a similar but slightly weaker interaction.

Ador-1 is a nematode homolog of human adenosine receptors, including the A1, A2a, and A2b subtypes [22]. Previous studies suggest that paeoniflorin exerts sedative effects in the nervous system by modulating the adenosine A1 receptor signaling pathway [23]. Additionally, paeoniflorin appears to have anti-Alzheimer's properties, potentially through activation of A1 receptors and inhibition of A2a receptors [24].

Caffeine is widely recognized as a non-selective adenosine receptor antagonist with strong affinity for A1 and A2a receptors [25]. Under standard culture conditions at 20°C, caffeine treatments at 0.05 mM, 0.5 mM, and 5 mM exhibited a biphasic effect on locomotion and feeding behavior in N2 and EG6890 nematodes, with low concentrations enhancing activity and high concentrations inhibiting it. These results indicate that caffeine exhibits characteristics of an adenosine receptor antagonist in nematodes. The addition of appropriate caffeine concentrations may enhance nematode motility and feeding behavior, possibly through excitatory effects on the nematode nervous system. This aligns with existing research indicating that caffeine acts as a non-selective antagonist of adenosine receptors A1R and A2AR [26].

5. Conclusion

Our study demonstrates that both paeoniflorin and caffeine significantly influence the locomotion of *C. elegans* in this study. Paeoniflorin exhibited a dose-dependent inhibitory effect on motilities, including swimming, head swing, body bending, and pharyngeal pumping, indicating a negative correlation between concentration and movement. In contrast, caffeine displayed a biphasic effect, wherein low concentrations (0.05–0.5 mM) significantly enhanced locomotion in N2-type nematodes, while high concentrations (5 mM) exerted an inhibitory effect. Molecular docking experiments revealed that paeoniflorin exhibits strong binding affinity for the nematode adenosine receptor (Ador-1), suggesting that its effects on locomotion are mediated through the adenosine receptor pathway. Given that the *C. elegans* adenosine receptor shares homology with human adenosine receptor subtypes, these results highlight the experimental significance of the Ador-1 gene knockout model in the screening and evaluation of potential human adenosine receptor-targeting drugs.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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