



In Vitro and In Vivo Evaluation Techniques for ACE Inhibitory Peptides: Challenges and Innovations in Predicting Hypertensive Effects

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Received: 5 August 2025 / Accepted: 30 November 2025
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Abstract

Background ACE inhibitory peptides derived from food sources are natural alternative to manage hypertension then the synthetic ones due to various side effects. To evaluate their therapeutic potential and biological activities, in vivo and in vitro assays are required.

Objective This paper reviews in vitro and in vivo assessment techniques, highlighting their limitations and advancements.

Methodology This review paper is based on literature survey through databases like ScienceDirect, Scopus, PubMed, Google Scholar from 1986 to 2025. in vitro and in vivo techniques discussed to evaluate ACE inhibitory peptides with older studies based on their significance.

Results In vitro methods, such as ACE inhibition assays and enzyme kinetics, provide insights into bioactivity but lack physiological relevance. In vivo studies, while essential for therapeutic validation, face ethical concerns, high costs, and human variability. Innovations like QSAR models, molecular docking, and organ-on-chip systems enhance predictive accuracy. Biomarkers further improve physiological relevance, bridging preclinical and clinical gaps.

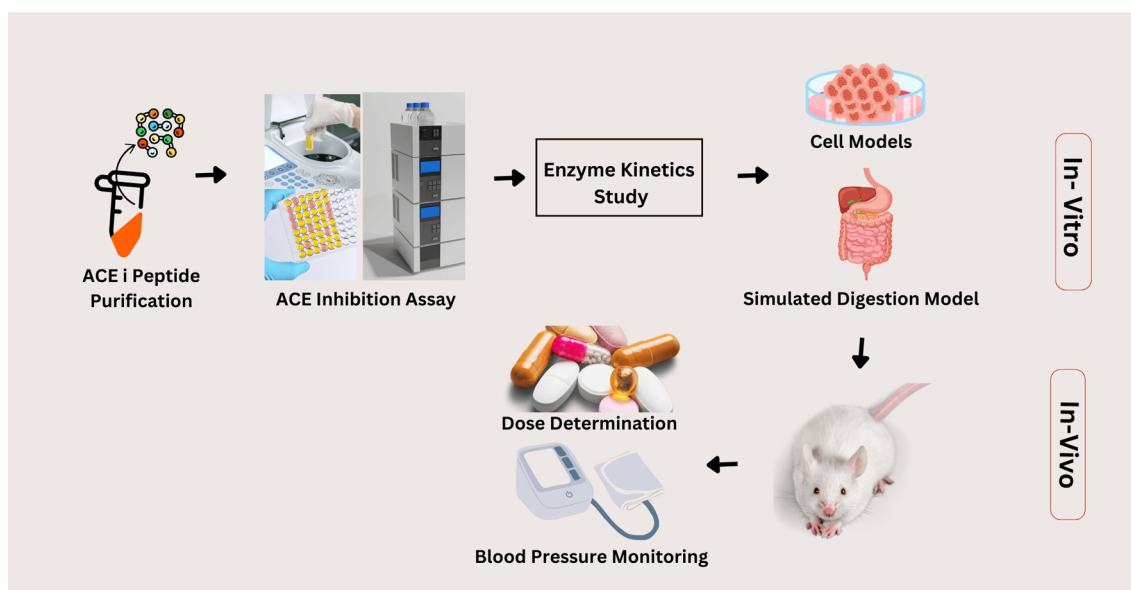
Conclusion The study emphasizes the need for refined methodologies, better bioavailability, and stable formulations. Additionally, regulatory challenges and multi-omics approaches are discussed to enhance peptide evaluation. Overcoming these limitations will facilitate the development of ACE inhibitory peptides as effective antihypertensive agents.

Graphical abstract

In vitro and in vivo evaluation techniques for ACE inhibitory peptides

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Keywords ACE inhibitory peptides · In vitro bioactivity assays · In vivo hypertension models · Cellular models · Bioavailability · Predictive modelling · Organ-on-chip

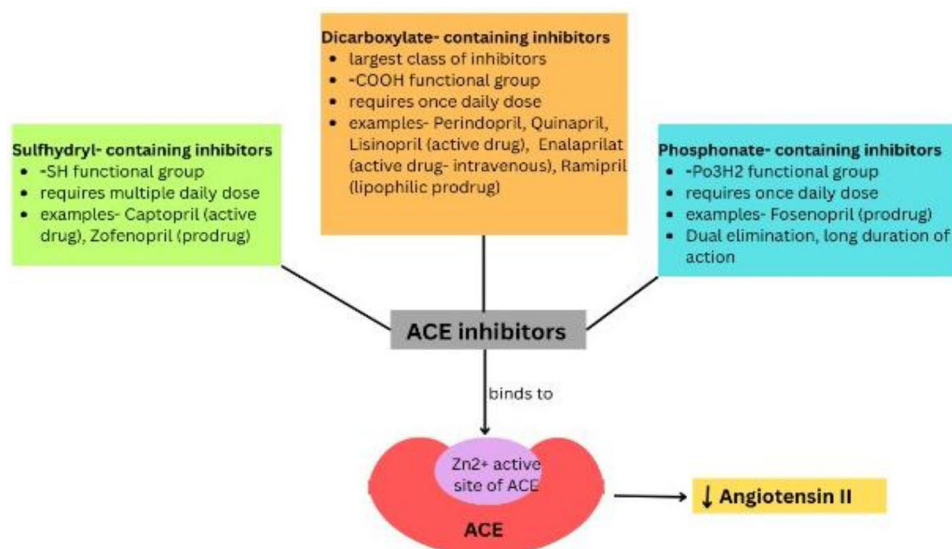
Introduction

Angiotensin-converting enzyme (ACE) plays a crucial role in regulating blood pressure by converting angiotensin I to angiotensin II, a potent vasoconstrictor. Inhibition of ACE reduces angiotensin II levels, promoting vasodilation and lowering blood pressure, making ACE inhibitors essential in the management of hypertension (Ahmad et al. 2023). ACE inhibitory peptides, derived from natural sources such as marine organisms, food proteins, and brewing by-products, offer sustainable alternatives to conventional antihypertensive drugs (Jo et al. 2024; Ribeiro-Oliveira et al. 2022). Marine-derived peptides from fish, molluscs, and algae exhibit diverse structures that enhance ACE inhibition, while peptides from milk (casokinins, lactokinins), soy, and rice also show significant ACE inhibitory effects (Das and Hati 2022; Shukla et al. 2022). Fermented foods enriched with bioactive peptides from lactic acid bacteria improve bioavailability and stability, further enhancing ACE inhibition (Shukla et al. 2022). Brewing by-products, such as spent grains and yeast, offer eco-friendly, cost-effective solutions for hypertension management (Ribeiro-Oliveira et al. 2022). These natural sources of ACE-inhibitory peptides offer promising options for developing functional foods and nutraceuticals to manage hypertension (Ahmad et al. 2023). Whereas synthetic ACE inhibitors are divided into three classes based on the functional group that interacts with the zinc ion in the enzyme's catalytic centre, as shown in Fig. 1. These chemical distinctions affected each class's

pharmacological behaviour, stability, and side-effect profile (Arendse et al. 2022; Zheng et al. 2022). Sulfhydryl-Containing ACE Inhibitors such as captopril and zofenopril contain a thiol (–SH) group that strongly interacts with the zinc ion at the active site, enhancing their inhibitory potential (Özkaya and Yazganoğlu 2014). The presence of the sulfhydryl group in Zofenopril imparts antioxidant and vasoprotective effects. It exhibits high lipophilicity and interacts with the hydrogen sulphide (H₂S) pathway, contributing to vascular relaxation and improved clinical outcomes. However, these same sulfhydryl moieties are responsible for some unwanted effects, including skin rashes, altered taste perception, and other thiol-related side reactions (Borghi et al. 2022; Bucci et al. 2014). The second class of carboxyl-containing ACE inhibitors, such as enalapril, lisinopril, and ramipril, enhances their binding interaction with zinc ion, thereby preventing the conversion of angiotensin I to angiotensin II (Arendse et al. 2022). These drugs are among the most widely prescribed antihypertensive and cardioprotective agents because of their proven efficacy in hypertension management, heart failure, and diabetic nephropathy. Despite their therapeutic benefits, these inhibitors may induce adverse effects like renal dysfunction hyperkalemia, and persistent cough, emphasizing the need for careful dosage regulation and patient monitoring (Regulski et al. 2015; Zheng et al. 2022). Whereas phosphinyl-containing ACE inhibitors, such as fosinopril, have a phosphinic acid group that mimics the natural transition state during peptide bond cleavage, enabling tight and persistent binding with the zinc

Fig. 1 Classification of ACE inhibitors based on their functional group interacts with the Zn ion, active site of ACE, leading to inhibition of angiotensin II formation.

Source: Singh et al. 2025; Bawa et al. 2019



ion (Arendse et al. 2022). This interaction leads to strong and long-lasting ACE inhibition. Fosinopril differs from other ACE inhibitors because it is eliminated through both renal and hepatic pathways, making it safer for patients with kidney impairment and does not contain sulfhydryl group inhibitors, side effects like rashes and taste disturbance (Ebzeeva et al. 2021; Özkaya and Yazganoğlu 2014). Due to its positive safety profile and ability to improve hemodynamic parameters and patient well-being, it is recommended as a first-line treatment for multimorbid patients (Fomin 2022). To overcome these limitations, naturally derived ACE inhibitory peptides are necessary. Peptides derived from natural sources, particularly from snake venom, have played an essential role in the discovery and design of pharmaceutical ACE inhibitors. The prominent example is teprotide, which acted as a structural model for the synthesis of captopril, the first clinically approved ACE inhibitor. This example highlights the translational value of natural peptides in guiding the design of synthetic inhibitors for blood pressure control (Kim et al. 2025; Freuville et al. 2024).

A number of major hypertension management guidelines—such as those developed by the European Society of Cardiology (ESC), the Eighth Joint National Committee (JNC 8), and the American College of Cardiology/American Heart Association (ACC/AHA)—emphasize the therapeutic importance of agents that target the Renin–Angiotensin–Aldosterone System (RAAS), including Angiotensin-Converting Enzyme (ACE) inhibitors and Angiotensin II Receptor Blockers (ARBs), along with diuretics, calcium channel blockers (CCBs), and beta-adrenergic blockers. These guidelines recommend treatment that varies according to patient age, ethnicity, and coexisting medical conditions. For instance, race-specific recommendations often suggest calcium channel blockers and

diuretics as first-line treatments for Black populations, as a result of differences in drug responsiveness and cardiovascular risk profiles. However, compliance with these clinical guidelines differs across various geographical regions and population groups, reflecting inconsistency in healthcare practices, physician preferences, and treatment accessibility (McCarthy et al. 2024; Alcocer et al. 2023; Nguyen et al. 2024; Colvin et al. 2020; Antza et al. 2018; Chobanian et al. 2003).

ACE inhibitory peptides primarily inhibit ACE by binding to its active site, preventing the conversion of angiotensin I to angiotensin II, thereby reducing vasoconstriction and lowering blood pressure (Kumar et al. 2024; Xue et al. 2021). These peptides also promote vasodilation by enhancing nitric oxide production within vascular walls, contributing to blood pressure regulation (Shukla et al. 2022). The bioavailability and stability of these peptides are critical to their therapeutic potential, as absorption and retention in the body significantly influence their effectiveness. To improve bioavailability and ensure stability during digestion, techniques like nano- and liposome encapsulation are being explored, which protect peptides from degradation and enhance their delivery and bioactivity (Amorim et al. 2023; Jogi et al. 2021). Furthermore, advancements in peptide modification, such as sustained-release formulations, help prolong the antihypertensive effects, offering more consistent therapeutic outcomes (Cai et al. 2023). Incorporating ACE inhibitory peptides into functional foods aligns with growing consumer demand for natural health products, providing an accessible and non-invasive approach to managing hypertension (Xue et al. 2021; Bhorla and Dhewa 2025).

Bioactive peptides (BPs), short chains of amino acids, have various biological effects, including antioxidant, antimicrobial, antihypertensive, and antidiabetic properties.

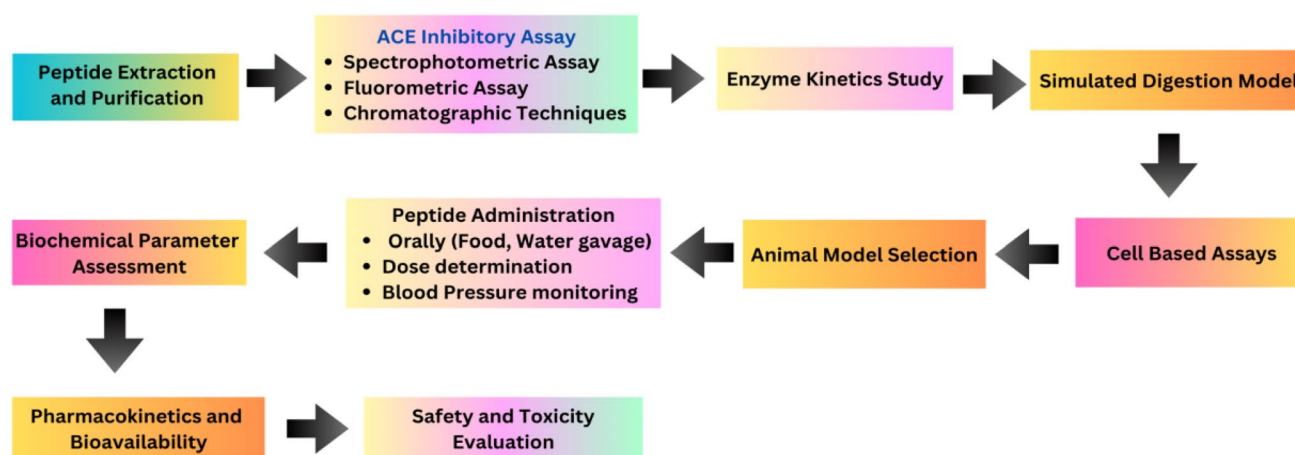


Fig. 2 A stepwise approach to evaluating the effectiveness and therapeutic potential of ACE inhibitory peptides

These peptides are evaluated through *in vitro* biochemical assays and cellular models to understand their interactions with biological targets. *In vivo* studies using animal models help assess their therapeutic effects, particularly on hypertension and neurodegeneration (Ashaolu and Suttikhana 2023). Cereals, legumes and vegetables are rich sources of phytochemicals and bioactive compounds, which are under-explored for their health benefits, including antioxidant, anticancer and antihypertensive properties (Gentile et al. 2025; Aderinola and Duodu 2022). ACE inhibitory peptides derived from sources such as garlic, earthworms, fish, and molluscs have demonstrated strong ACE inhibition both *in vitro* and *in vivo*, modulating the renin-angiotensin system and improving organ function (Xiang et al. 2024; Lin et al. 2023). However, translating *in vitro* findings into *in vivo* applications remains challenging due to variations in experimental methods, species-specific metabolism, and biological complexity, making it difficult to predict real-world efficacy (Cerecedo 2024). Despite advancements in 3D cell cultures and computational models, current *in vitro* methods do not fully replicate human biology (Mattes 2020). This paper aims to explore the methodologies used to assess ACE inhibitory peptides and the challenges in bridging *in vitro* results to *in vivo* outcomes. Figure 2 shows a flow-chart illustrating the sequential steps involved in assessing the ACE inhibitory potential of peptides.

In Vitro Evaluation Techniques

ACE Inhibitory Assays: Techniques, Substrates, and Pros and Cons

The assessment of ACE inhibitory activity plays a crucial role in identifying compounds with potential antihypertensive properties. Several enzymatic assays are employed,

each varying in sensitivity and substrate specificity. Spectrophotometric methods are widely used due to their simplicity and ability to handle multiple samples simultaneously. These assays typically use substrates such as hippuryl-histidyl-leucine (HHL), which ACE cleaves to release hippuric acid, which is detectable by measuring absorbance changes. Despite their utility, these assays generally exhibit lower sensitivity compared to chromatographic techniques (Chen et al. 2013).

Fluorometric assays are preferred when higher sensitivity and precision are required. Substrates such as Abz-Gly-Phe(NO₂)-Pro produce a fluorescent signal upon enzymatic cleavage, making them ideal for rapid screening of ACE inhibitors (Coscueta and Pintado 2022; Tang et al. 2019). Chromatographic techniques, including high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC), offer high precision and reliability in detecting ACE inhibitors. For example, HPLC coupled with mass spectrometry (HPLC-ESI-QqQ-MS) allows for robust detection with excellent reproducibility (Musharraf et al. 2017), while UPLC-DAD is effective for screening plant-derived ACE inhibitors (Tehreem et al. 2021). Innovative methods, such as enzyme immobilization on magnetic beads, enhance throughput and reduce reagent usage (Tang et al. 2019). Colorimetric assays, based on substrate hydrolysis, are also commonly used for preliminary screenings (Tutor and Chichioco-Hernandez 2018). Spectrophotometric assays, often using substrates like furylacryloyl-phenylalanyl-glycyl-glycine (FAPGG), work by measuring changes in absorbance, where a decrease indicates substrate cleavage by ACE. These assays are cost-effective and suitable for routine analysis, providing IC₅₀ values to quantify inhibitor potency. However, their sensitivity is limited, particularly in complex biological samples where interfering substances may impact accuracy (Taibi et al. 2023; Garmidolova and Desseva 2023).

Fluorometric assays offer greater sensitivity and specificity, using substrates that emit fluorescence upon cleavage. The Abz-Gly-Phe (NO₂)-Pro substrate, for example, generates a fluorescent signal proportional to ACE activity, making it useful for detecting low enzyme activity and assessing the inhibition potential of compounds such as flavonoids and captopril (Coscueta and Pintado 2022; Zhan et al. 2023).

Hippuryl-histidyl-leucine (HHL) is a commonly used substrate in ACE inhibitory assays due to its structural similarity to natural ACE substrates. When ACE hydrolyzes HHL, hippuric acid is released, which can be quantitatively measured using spectrophotometry. This method is simple and reliable for determining the inhibitory potential of compounds (Antony et al. 2024; Alkathiri 2024). HHL-based assays offer a significant advantage by providing standardized comparisons across studies, facilitating the consistent evaluation of antihypertensive agents (Wang et al. 2024a, 2024b; Dai et al. 2023). These assays are widely used for screening natural peptides derived from various protein sources, such as marine organisms, dairy products, and plant-based proteins, to identify ACE inhibitory peptides (Jo et al. 2024; Zheng et al. 2023). Additionally, synthetic compounds are also tested to identify new antihypertensive candidates (Lin et al. 2023). Furthermore, HHL assays complement *in silico* analyses, providing experimental data to validate computational predictions and enhance the reliability of inhibitor screening (Dai et al. 2023; Munawaroh et al. 2024).

ACE inhibitory assays are essential in identifying compounds that inhibit ACE, a key enzyme in controlling hypertension. These assays offer various advantages, such as high sensitivity and specificity. For instance, UPLC-MS techniques can detect and quantify ACE inhibitors even at very low concentrations, making them particularly effective for analyzing complex mixtures (Geng et al. 2010). Fluorescence-based assays are also highly sensitive, allowing for continuous monitoring of enzymatic reactions and studying enzyme kinetics (Ángel Sentandreu and Toldrá 2006; Alves et al. 2005). Additionally, these assays are rapid and efficient, with methods like UPLC-MS reducing analysis time to as little as 3.5 min per run (Geng et al. 2010), while fluorescence assays typically complete analysis in around 1.5 h, enabling the testing of multiple samples quickly (Ángel Sentandreu and Toldrá 2006). Another key advantage of ACE inhibitory assays is their ease of use. Fluorescence assays, for instance, are user-friendly and often involve a simple one-step reagent process, with commercially available kits streamlining integration into standard laboratory workflows (Ángel Sentandreu and Toldrá 2006). Moreover, these assays can be adapted to measure ACE activity in various biological fluids and tissues, providing insights into the enzyme's role in different physiological contexts (Carmona

et al. 2006). Furthermore, *in vitro* assays help reduce the need for animal testing, supporting ethical considerations in drug development (Radheshyam et al. 2024). However, ACE inhibitory assays do have limitations. One of the main drawbacks is their complexity and cost. For example, UPLC-MS requires sophisticated instruments and expertise, making it less accessible for laboratories with limited resources (Geng et al. 2010). Fluorescence assays also require specific substrates and reagents, which can add to the cost and setup complexity (Carmona et al. 2006). Additionally, *in vitro* assays are conducted in controlled environments that do not fully replicate the complexity of biological systems. As a result, there may be discrepancies between *in vitro* enzyme activity measurements and *in vivo* outcomes, where enzyme behavior is influenced by metabolic interactions and tissue-specific factors (Reneland and Lithell 1994; Vilvanathan 2022). Furthermore, *in vitro* models have limited predictive power regarding pharmacokinetics, as they do not account for how a compound is absorbed, distributed, metabolized, or excreted in the human body (Radheshyam et al. 2024). *In vitro* models also fail to account for factors like enzyme isoforms and the presence of other biological molecules that can affect ACE inhibitor activity (Endo et al. 2022).

Enzyme Inhibition Kinetics

Enzyme inhibition kinetics is central to understanding the mechanism of action of ACE inhibitors and their effects on hypertension management. The study of enzyme inhibition, particularly through *in vitro* evaluation techniques, provides insights into how inhibitors interact with ACE at the molecular level, offering valuable information about their therapeutic potential (Khan and Kumar 2019). One of the most well-known forms of enzyme inhibition is competitive inhibition, where inhibitors such as captopril and lisinopril bind directly to the active site of ACE. These inhibitors compete with the natural substrate, angiotensin I, for access to the enzyme's active site, effectively preventing the conversion of angiotensin I to angiotensin II. The inhibition of this conversion reduces the production of angiotensin II, a potent vasoconstrictor, thereby promoting vasodilation and lowering blood pressure (Grönhagen-Riska et al. 1986; Kumar et al. 2024). *In vitro*, competitive inhibition can be studied using the Competitive Inhibitor Binding Assay (CIBA), which measures the concentration of ACE inhibitors in blood and demonstrates how these inhibitors compete with labeled analogues for binding to ACE. This assay highlights the competitive nature of these inhibitors, providing essential data on their pharmacological effects (Grönhagen-Riska et al. 1986).

In competitive inhibition, the inhibitor mimics the natural substrate, competing for binding at the active site, which

results in a higher Michaelis constant (K_m) while leaving the maximum reaction velocity (V_{max}) unchanged. This behavior can be visualized in a Lineweaver–Burk plot, where the slope increases due to the rise in K_m , but the y-intercept, which corresponds to $1/V_{max}$, remains constant (Baici et al. 2013; Bachurin et al. 1993). This kind of kinetic behavior is also observed in other enzyme systems, such as in studies with carboxypeptidase A, where inhibitors like N-benzoyl-L-phenylalanine compete with peptide substrates at the enzyme's active site (Rahmo and Fife 2000). Competitive inhibition is considered a valuable model in ACE inhibition, where inhibitors like captopril and lisinopril block ACE activity by competing with the enzyme's natural substrates without altering the enzyme's overall catalytic capacity (Rahmo and Fife 2000). Competitive inhibition of ACE plays a significant role in the management of hypertension, and its clinical relevance extends beyond blood pressure control. By decreasing vascular resistance, these inhibitors improve cardiac output and reduce mortality in heart failure (Kumar et al. 2024). Additionally, the reversible nature of competitive inhibitors allows for dynamic regulation based on substrate concentration, enabling better control over enzyme inhibition. This reversibility is beneficial in minimizing side effects, such as cough and hyperkalemia, making these drugs safer for patients (Borghi et al. 2020).

On the other hand, non-competitive inhibition occurs when an inhibitor binds to a site on the enzyme other than the active site, often leading to a conformational change that reduces catalytic activity without affecting substrate binding (Delaune and Alsayouri 2019). In the case of ACE, non-competitive inhibitors interact with zinc ions near the active site, as seen with thiol-containing inhibitors such as captopril (Wang et al. 2011). Unlike competitive inhibitors, non-competitive inhibitors can bind to both the free enzyme and the enzyme–substrate complex, ensuring a consistent reduction in enzyme activity regardless of substrate concentration (Delaune and Alsayouri 2019). This makes non-competitive inhibition a valuable mechanism for ACE inhibitors that do not require high substrate concentrations to be effective. In vitro studies with flavonoids like myricetin and chrysoeriol, which inhibit β -glucuronidase, demonstrate non-competitive inhibition by decreasing enzyme activity without affecting substrate binding (Kamel et al. 2024).

Moreover, discrepancies between in vitro and in vivo inhibition kinetics have been observed, particularly with enalaprilat, a derivative of enalapril. While enalaprilat exhibits competitive inhibition in vitro, it shows non-competitive behavior in vivo. This contrast underscores the influence of experimental conditions on observed inhibition kinetics, emphasizing the importance of considering both in vitro and in vivo environments when evaluating ACE

inhibitors (Weisser and Schloos 1993). In vivo, these inhibitors may interact with additional physiological factors that influence their effectiveness.

Lastly, mixed-type inhibition involves inhibitors that bind to both the active site and an allosteric site on the enzyme. This type of inhibition affects both the enzyme's affinity for its substrate (K_m) and the maximum reaction velocity (V_{max}). Mixed-type inhibitors, which can bind to both the free enzyme and the enzyme–substrate complex, alter both K_m and V_{max} , providing a unique mechanism to regulate enzyme activity (Pesaresi 2022; Walsh et al. 2011). Peptides capable of binding to allosteric sites, affecting enzyme conformation and activity, exemplify mixed-type inhibitors and are essential for studying complex regulatory mechanisms in metabolic pathways, such as ACE inhibition (Lai and Wu 2003).

Simulated Digestion Models

Simulated digestion models play a vital role in examining the bioavailability and stability of peptides following digestion. Designed to replicate the human gastrointestinal system, these models provide valuable insights into how protein-derived peptides behave and retain their bioactivity throughout the digestion process. The use of simulated gastrointestinal digestion (SGD) models has gained popularity in bioavailability research due to their ability to closely mimic real physiological conditions (Dupont et al. 2019; Sensoy 2021). These models are particularly useful for studying the stability and functionality of peptides under conditions that resemble the human gastrointestinal tract. Among these, in vitro models are commonly employed because they replicate digestive processes without the ethical and logistical constraints of in vivo studies. By utilizing simulated gastric and intestinal fluids (SGF and SIF), these models assess peptide stability under various digestive phases, with the INFOGEST harmonized static model being highly regarded for its physiological relevance (Luz et al. 2024). Another widely used approach is the two-phase digestion model, which simulates both gastric and intestinal phases with the help of digestive enzymes such as pepsin, trypsin, and chymotrypsin. These enzymes facilitate the investigation of structural changes and the stability of peptides before and after digestion, providing critical insights into their bioactive potential (Ahmed et al. 2022; Toomer et al. 2013; Zhang et al. 2023). Additionally, simplified digestion models, though less physiologically accurate, offer a less complex setup by employing peptic-tryptic or chymotryptic treatments. These models are valuable for preliminary screenings and comparative studies of peptide stability across various food matrices (Prandi et al. 2014).

Simulated digestion models include static, dynamic, and advanced microfluidic systems, each offering unique advantages for studying peptide digestion and bioavailability. The INFOGEST static model, one of the most widely accepted methods, simulates human gastrointestinal processes using enzymes like amylase, pepsin, and pancreatin under defined pH conditions for different digestive phases. This model is appreciated for its simplicity, reproducibility, and effectiveness in replicating physiological digestion (Luz et al. 2024). In contrast, dynamic digestion models provide a more comprehensive simulation by incorporating continuous changes in pH, enzyme secretions, and mechanical forces that mimic in vivo conditions. Although these models better replicate human digestion, their complexity, high costs, and lack of standardization limit their widespread use (Álvarez-Olguín et al. 2022). Emerging as cutting-edge technology, microfluidic platforms integrate miniaturized systems that simulate the entire gastrointestinal tract. These platforms enable real-time analysis of digestion, intestinal permeability, and peptide transport, making them invaluable for pharmacokinetics and bioavailability studies. Despite their promise, microfluidic systems are still in developmental stages and require further refinement before becoming mainstream tools (Xavier et al. 2023).

Cellular Models

Cell-based systems are crucial for studying peptide bioactivity, absorption, and mechanisms of action in a controlled environment, offering reproducible results. They help uncover peptide interactions with cellular receptors, pathways, and therapeutic targets using advanced tools like cDNA arrays and MALDI-TOF. These models also simulate intestinal absorption, providing insights into stability and transport across barriers. Additionally, they serve as ethical alternatives to animal testing, offering more human-relevant insights (Kues 2000). Cellular models like HUVECs and Caco-2 cells are key for studying the effects of ACE inhibitory peptides and their potential in treating hypertension. HUVECs are used to investigate the protective effects of peptides, such as those from *Takifugu bimaculatus* and yeast hydrolysates, which regulate key signaling pathways and increase nitric oxide levels (Cai et al. 2021; Huang et al. 2021). The peptide VIPVPFF from yeast hydrolysates also shows promise by upregulating GUCY1A1 gene expression, a marker in hypertension-related pathways (Huang et al. 2021). Caco-2 cells simulate the intestinal absorption of ACE inhibitory peptides, especially from milk, shedding light on their bioavailability and metabolism (Guo et al. 2019). In addition, synthetic peptide studies and molecular docking approaches help uncover structure–activity relationships and predict ACE inhibitory activity, with peptides

from sources like sesame protein and marine peptides being tested in cellular models (Yakimova et al. 2021; Wang et al. 2020; Jo et al. 2024). Human Umbilical Vein Endothelial Cells (HUVECs) are employed to study the cellular effects of ACE inhibitory peptides, particularly their influence on nitric oxide (NO) production and oxidative stress markers. For instance, peanut-derived peptides have been found to enhance NO and superoxide dismutase (SOD) production while lowering reactive oxygen species (ROS) and malondialdehyde (MDA) levels in Ang II-injured HUVECs (Liu et al. 2024).

Vascular Smooth Muscle Cells (VSMCs) play a crucial role in vascular remodeling and hypertension, making them a valuable model for studying ACE inhibitory peptides (Feng et al. 2024; San Sebastian-Jaraba et al. 2024). These cells regulate blood pressure and vascular tone, directly impacted by ACE activity. VSMCs allow researchers to explore peptide effects on gene expression, protein synthesis, and cellular signaling (Li et al. 2024; Yu 2023). Marine-derived ACE inhibitory peptides, for example, reduce ACE activity through competitive binding and structural interference, with their binding affinity enhanced by amino acids like tryptophan, which can be studied in VSMC models (Jo et al. 2024; Ma et al., 2023). Therapeutically, VSMCs are used to assess how ACE inhibitory peptides might reduce hypertension and prevent vascular diseases, such as by modulating VSMC behavior, reducing vascular calcification, and testing sustained-release peptide formulations (Miyazaki 2023; Cai et al. 2023). However, the complexity of in vivo systems, including the interplay between endothelial cells and VSMCs, should be considered when interpreting ACE inhibition study outcomes (San Sebastian-Jaraba et al. 2024; Yurtseven et al. 2023). An endothelial model to explore the effects of ACE inhibitory peptides from Muga and Eri pupae, focusing on their ACE inhibition and anti-inflammatory properties. It assessed the peptides' impact on the expression of COX-2, iNOS, and pro-inflammatory cytokines like IL-6, IL-1 β , and TNF- α , highlighting their potential therapeutic role in modulating inflammation (Sarkar et al. 2023).

Limitations of In Vitro Methods

In vitro methods face several limitations that hinder their ability to accurately simulate physiological conditions. These methods often fail to replicate the absorption, distribution, metabolism, and excretion (ADME) processes essential for understanding peptide bioavailability, as peptides are prone to enzymatic degradation, significantly reducing their effectiveness before reaching systemic circulation (Foltz et al. 2010). In addition, in vitro systems oversimplify biological environments, disregarding the intricate architecture,

cellular interactions, and compensatory mechanisms present in vivo, such as those involved in liver detoxification and drug metabolism (Ghallab 2013; Yakimova et al. 2021). The use of isolated enzymes or cells neglects the influence of complex physiological interactions, including enzyme activity and inhibition, which can affect the activity and efficacy of peptides (Yakimova et al. 2021; Greim et al. 1986). Non-physiological experimental conditions, such as unrealistic pH levels, temperatures, or peptide concentrations, often lead to exaggerated or misleading results, overestimating peptide effectiveness (Amigo-Benavent et al. 2021). Furthermore, challenges in translating in vitro findings to in vivo applications arise due to the absence of systemic factors like blood flow, immune responses, and feedback mechanisms, which are critical for understanding systemic effects (Ghallab 2013; Markowitz and Zhu 2012). Establishing a reliable correlation between in vitro and in vivo outcomes remains problematic, particularly in multifactorial conditions such as drug-induced liver injury (Funk and Roth 2017). Despite these challenges, integrating in vitro methods with in vivo experiments and computational approaches holds promise for improving the evaluation of ACE inhibitory peptides. Techniques such as co-culture systems, three-dimensional setups, and predictive computational models can enhance the physiological relevance of in vitro studies, providing a more comprehensive understanding of peptide bioavailability and activity (Funk and Roth 2017; Dai et al. 2023; Zhao et al. 2019; Cerecedo 2024).

In Vivo Evaluation Techniques

In vivo evaluation techniques for ACE inhibitory peptides are essential for assessing their therapeutic potential in hypertension treatment. Animal models, particularly spontaneously hypertensive rats (SHRs), are commonly used to simulate human hypertension. Various peptides from natural sources, including garlic proteins and Pacific saury, have shown significant blood pressure reductions in SHRs (Wang et al. 2024b; Xiang et al. 2024). Blood pressure is typically measured by monitoring systolic and diastolic values, with peptides such as WGAP from rabbit meat displaying notable antihypertensive effects (Chen et al. 2021). Peptides must remain stable in the gastrointestinal tract to be effective, with the peptide LVLPG showing high resistance to enzymatic breakdown (Pei et al. 2021). Additionally, understanding peptide absorption through intestinal barriers is crucial, as demonstrated by LEPWR from Pacific saury, which utilizes paracellular transport (Wang et al. 2024b). Many peptides, such as MGR and HDCF from garlic, exert their antihypertensive effects by modulating the renin-angiotensin system and improving endothelial function (Xiang et

al. 2024). Furthermore, pharmacokinetic studies reveal that dosage optimization is key for maximizing efficacy, with peptides like LVLPG showing a stronger antihypertensive effect at a specific dose (Pei et al. 2021). In vivo studies with hypertensive rat models have shown promising results for ACE inhibitory peptides. Oyster-derived AEYLCEAC, rabbit meat-derived WGAP, and *Gracilariopsis lemaneiformis* peptides all significantly lowered blood pressure in hypertensive rats, indicating their potential for hypertension management (Chen et al. 2021, 2022; Su et al. 2022). Beyond SHRs, other models, such as mice, are also employed to evaluate ACE inhibitory peptides. For instance, cashew-derived peptides were studied in mice for their ability to protect against alcohol-induced acute hepatitis, highlighting the broad applicability of animal models in exploring various physiological effects of bioactive peptides (Yang et al. 2019).

Gastrointestinal Stability, Bioavailability, and Pharmacokinetics of ACE Inhibitory Peptides

The in vivo evaluation of ACE inhibitory peptides examines their gastrointestinal stability, antihypertensive effects, dose optimization, and pharmacokinetics. The peptide LVLPG showed 97.8% stability against gastrointestinal enzymes, ensuring its bioavailability, and was absorbed via the PepT1 transporter and passive mechanisms (Pei et al. 2021). It significantly reduced systolic blood pressure by 39 mmHg at 10 mg/kg, demonstrating its antihypertensive potential (Pei et al. 2021). Dose optimization revealed a more pronounced effect at 30 mg/kg than 50 mg/kg, emphasizing the need for precise dosing. Rapid absorption, with a C_{max} of 4.65 ng/mL at 2 min, allows for quick therapeutic action (Pei et al. 2021). Additionally, the ADME characteristics of these peptides depend on hydrophobicity and molecular size, influencing their pharmacological behavior (Yadav and Mohite 2020; Klepach et al. 2022). Spontaneously Hypertensive Rats (SHRs) are widely used to evaluate the antihypertensive effects of ACE inhibitory peptides due to their similarity to human hypertension. Studies on the peptide IRW demonstrated a bioavailability of 11.7% and highlighted the significant role of metabolites in its antihypertensive activity. The delayed reduction in blood pressure observed after IRW administration suggests that metabolites, such as tryptophan-derived kynurenine, contribute to its mechanism by enhancing ACE2 levels (Wang et al. 2024a, 2024b). Comparisons of administration routes, such as intragastric and intravenous, have further underscored the impact of these factors on peptide bioavailability and therapeutic efficacy (Wang et al. 2024a, 2024b).

The structural characteristics of peptides, including their amino acid sequence and three-dimensional conformation,

are critical for ACE inhibitory activity. Marine-derived peptides, for instance, exhibit unique structural features that enhance ACE binding and result in low IC₅₀ values and potent inhibition (Jo et al. 2024). Stability against gastrointestinal digestion and efficient absorption into the bloodstream also depend on peptide structure. For example, the peptide FPHPP, derived from peanut proteins, demonstrated high stability and bioavailability, reinforcing its potential as a therapeutic agent (Liu et al. 2024). Molecular docking and dynamics simulations have provided insights into how specific amino acids and structural features improve binding affinity and inhibitory potency (Liu et al. 2024; Zheng et al. 2023). *In silico* techniques, such as molecular docking and ADMET predictions, are commonly used to identify peptides with favorable bioavailability characteristics. These methods provide valuable insights into the absorption, distribution, metabolism, and excretion (ADME) properties of potential ACE inhibitors (Komari et al. 2024). Additionally, hydrothermal processing methods have been shown to retain the bioactivity of ACE inhibitory peptides, such as those derived from legumes, highlighting the role of processing in maintaining peptide functionality (Ojeda-Ojeda et al. 2024). The importance of metabolites in peptide efficacy was further demonstrated in studies on IRW, where tryptophan-derived metabolites significantly contributed to the peptide's antihypertensive effects (Wang et al. 2024a, 2024b). These findings underscore the importance of peptide structure, bioavailability, and metabolite activity in the development of effective antihypertensive agents.

Clinical Trials and Human Studies

The design of clinical trials for ACE inhibitory peptides involves several key stages to ensure their safety, efficacy, and therapeutic potential. The first step is identifying peptides with strong ACE inhibitory properties, which is commonly done through computational methods like molecular docking. These techniques screen large peptide libraries to identify sequences that bind effectively to the ACE enzyme, with specific amino acids like Tyr, Phe, and Trp playing a crucial role in this binding process (Ma et al., 2023). After identifying promising peptides, the next focus is on enhancing their stability and bioavailability. Peptides are often susceptible to degradation and poor absorption, which can be mitigated using methods like solid-phase peptide synthesis (SPPS) and chemical modifications such as DSPE-PEG to improve stability and ensure prolonged therapeutic effects (Singh et al. 2023; Cai et al. 2023). Additionally, advanced delivery systems, including nanoparticles and liposomes, are utilized to improve targeted delivery and controlled release of peptides, maximizing their therapeutic impact (Singh et al. 2023; Mahto et al. 2023). Once these optimized

peptides are ready, they undergo preclinical evaluation, which includes both *in vitro* and *in vivo* testing to assess their ACE inhibitory activity, stability, bioavailability, and potential toxicity (Jo et al. 2024; Balgir and Sharma 2017). These tests help identify the most promising candidates for further clinical investigation. Clinical trials are generally conducted in phases, beginning with Phase I trials focused on safety and dosage in small groups. Following this, Phase II and III trials evaluate the efficacy and safety in larger populations, often using randomized, placebo-controlled designs to ensure robust and reliable results (Cicero et al. 2010; Leo et al. 2009). The primary outcome of these trials is usually the reduction in systolic and diastolic blood pressure, while secondary outcomes assess cardiovascular biomarkers and overall safety profiles (Cicero et al. 2010; Leo et al. 2009).

Successful human studies on ACE inhibitory peptides have demonstrated their potential in managing hypertension. For example, marine-derived peptides, particularly those from fish and mollusks, have shown potent ACE inhibitory activity, with clinical trials indicating promising results in lowering blood pressure and improving cardiovascular health markers (Jo et al. 2024; Lin et al. 2013). Food-derived peptides, such as those from peanut yogurt, have also displayed antihypertensive effects, suggesting their potential for incorporation into functional foods aimed at blood pressure regulation (Wang et al. 2024b). In addition, sustained-release formulations of peptides, such as those modified with DSPE-PEG, have proven effective in maintaining prolonged ACE inhibition, an important aspect for long-term hypertension management (Cai et al. 2023). These successful human studies underline the therapeutic promise of ACE inhibitory peptides, with some studies also suggesting ethnic variations in response to certain peptides, such as the lactotripeptides VPP and IPP, which have shown stronger effects in Asian populations (Cicero et al. 2010). Despite these successes, several challenges remain in translating ACE inhibitory peptides into practical clinical applications. Issues such as peptide stability, efficient delivery systems, and regulatory approval processes must be addressed to fully realize their therapeutic potential. Furthermore, the integration of personalized medicine, which tailors treatments based on individual genetic profiles, could further enhance the effectiveness of peptide-based interventions (Slater 2024; Singh et al. 2023). Additionally, functional foods like Calpis, a fermented dairy product rich in ACE inhibitory peptides, have been validated in clinical trials for their efficacy in managing blood pressure (Das and Hati 2022; Leo et al. 2009).

Limitations of In Vivo Studies

In vivo studies, particularly in clinical trials for ACE inhibitory peptides, face several limitations arising from ethical concerns, cost considerations, and variability in human responses. Ethical issues are central to these challenges, especially when human participants or animal models are involved. In studies with vulnerable populations, such as Ebola survivors in Liberia, ethical dilemmas regarding informed consent, participant rights, and the risk of exploitation are prevalent. Instances of misinformation and mistreatment have highlighted the complexities of conducting research ethically in such contexts (Hanson-DeFusco et al. 2024). In animal research, ethical concerns revolve around ensuring humane treatment and minimizing pain and distress, as outlined in frameworks like the 3 R's (Replacement, Reduction, Refinement). Approval from ethical review boards is often required, underscoring the need for adherence to strict ethical guidelines (Noviana et al. 2016; Mehamrita 2024; Wargent 2020). Additionally, there is a growing interest in alternative approaches such as in silico models, which aim to address ethical concerns while providing cost-effective and efficient methods for drug development (Verma and Namdeo 2024).

Cost considerations also pose significant challenges in in vivo studies. These studies are resource-intensive, requiring substantial financial investments in animal care, housing, specialized personnel, and regulatory approvals. For instance, maintaining animal models or using human skin explants for *Mycobacterium leprae* research demands significant resources, making such studies prohibitively expensive for smaller institutions or projects with limited funding (Paula et al. 2024; Brake et al. 2017). Furthermore, the need for advanced infrastructure and technology, coupled with extended timelines, adds to the financial burden, limiting the scope and scale of many research endeavors (Negi et al. 2023; Verma and Namdeo 2024).

Human response variability is another critical limitation in in vivo studies. Differences in genetic and physiological characteristics between humans and animal models can result in discrepancies between preclinical findings and human outcomes (Negi et al. 2023; Brake et al. 2017). For example, species-specific differences in ACE inhibitory peptides' efficacy and safety profiles may affect the translation of animal model results to humans (Lal et al. 2016). Even within human populations, genetic diversity, environmental influences, and individual factors such as age, sex, and health conditions contribute to variability in responses (Wu et al. 2024; Wang et al. 2024b). These differences complicate the extrapolation of in vivo findings to the broader population. Challenges also arise in studying bioavailability; for instance, polyphenols show inconsistent absorption

among individuals, with encapsulation techniques yielding variable results (Redha et al. 2024). Similarly, transcriptional responses in human tissues to infections can differ, complicating the interpretation of disease-specific research findings (Sohail et al. 2024).

Methodological and technological limitations further constrain in vivo studies. Common issues include inadequate randomization, lack of blinding, and insufficient sample sizes, which introduce bias and reduce result validity. Many studies fail to adhere to established reporting guidelines, emphasizing the need for improved methodological rigor (Gupta 2019). Technological constraints, such as limited access to advanced imaging and monitoring tools, hinder the accurate collection of pharmacokinetic and physiological data, reducing the reliability of findings (Pendry et al. 2023; Wilson et al. 2023). Additionally, the development of real-time monitoring systems for peptides is challenged by the lack of suitable biosensing technologies, limiting the ability to track pharmacokinetics effectively (Wilson et al. 2023). Despite these limitations, in vivo studies remain invaluable for providing insights unattainable through in vitro or in-silico methods alone. Efforts to address these challenges include the adoption of alternative models and advanced methodologies, which aim to enhance the ethical, financial, and scientific aspects of research, ultimately improving the translatability of findings to human populations (McNally and Loizou 2015).

Innovative Approaches for Advancing ACE Inhibitory Peptide Research

Biomedical research and drug development are rapidly evolving with innovations like predictive modeling techniques, organ-on-chip (OoC) technology, and biomarkers. QSAR models and molecular docking are key for understanding ACE-peptide interactions and designing hypertension inhibitors (Roy et al. 2015). Organ-on-chip systems replicate human physiology, enhancing drug testing and disease modeling (Boyle 2023; Sadeghzade et al. 2024). Biomarkers aid in validating treatment efficacy and refining therapies for conditions like hypertension (Sunildutt et al. 2023; Qiao et al. 2024). Predictive modeling techniques, especially QSAR models and molecular docking, have become crucial tools in understanding ACE (angiotensin-converting enzyme)-peptide interactions, providing valuable insights for drug discovery, particularly in cardiovascular disease management. QSAR models, such as 3D-QSAR and 2D-QSAR, are extensively used to predict the bioactivity of peptides based on their structural characteristics. For example, a 3D-QSAR model developed for ACE inhibitory dipeptides achieved high predictive accuracy, with Q² and

R² values of 0.840 and 0.998, respectively, demonstrating its potential for accurate prediction of ACE inhibition (Liu et al. 2023). In contrast, 2D-QSAR models, employing techniques like genetic algorithm-multiple linear regression (GA-MLR), are valuable in identifying novel ACE inhibitors, with robust R² values between 0.70 and 0.75 (Shah et al. 2023). Additionally, machine learning approaches have been integrated with chemical descriptors to enhance the predictive power of QSAR models, enabling the discovery of new dipeptides with promising ACE inhibitory activity (Wang et al. 2020). Molecular docking complements QSAR by simulating the binding interactions of peptides with ACE, revealing binding modes and interaction profiles. Docking studies, such as those involving chickpea-derived peptides, have shown promising ACE-peptide interactions through hydrogen bonds and hydrophobic forces (Arámburo-Gálvez et al. 2022). The integration of QSAR and molecular docking has proven effective in predicting and validating ACE-peptide interactions, supporting the development of natural ACE inhibitors (Wang and Zhou 2020). However, challenges remain, including variability in peptide activity data and the necessity of experimental validation. Future directions involve refining these predictive techniques and exploring ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties, which are essential for designing potent and safe ACE inhibitors (Arámburo-Gálvez et al. 2022; Wang et al. 2020). Organ-on-chip (OoC) systems are innovative platforms that replicate the microenvironment of human tissues, enabling precise modeling of physiological and pathological conditions. These systems are crucial for studying ACE inhibitory peptides, as they simulate their interactions with the renin-angiotensin system to regulate blood pressure and fluid balance (Harriot et al. 2024; Zhou et al. 2024). (Verma and Namdeo 2024; Paula et al. 2024). OoC devices, incorporating patient-derived cells, account for genetic variability and individual responses, advancing personalized medicine (Harriot et al. 2024). They also support high-throughput screening for evaluating peptide efficacy and pharmacokinetics in a controlled environment (Fernández-Costa et al. 2024; Sunildutt et al. 2023). Furthermore, OoC models provide mechanistic insights into diseases like hypertension, with integrated biosensors offering real-time data (Ahmed 2022; Ingber 2022). However, challenges such as technological complexity and regulatory barriers remain to be addressed for broader implementation (Ingber 2022; Syama and Mohanan 2021). Biomarkers aid in diagnosing, predicting, and managing hypertension by reflecting physiological processes like RAAS and sympathetic activity. Key biomarkers in hypertension include components of the renin-angiotensin-aldosterone system (RAAS), such as plasma renin activity and aldosterone, which regulate blood pressure by influencing fluid and

electrolyte balance. Norepinephrine, a sympathetic nervous system component, plays a role in stress responses affecting blood pressure. Markers like urinary albumin/creatinine ratio (UACR) and C-reactive protein (CRP) reflect kidney function and inflammation, respectively, while pro-brain natriuretic peptide (Pro-BNP) is associated with cardiac function and fluid homeostasis. Emerging biomarkers like adrenomedullin show potential in identifying individuals at risk of hypertension and related complications (Mujadzic et al. 2022). Recent advancements, such as metabolomics combined with explainable AI, have identified novel biomarkers like Vanillylmandelic acid (VMA) and Glycero-phosphorylcholine (GPC), which are linked to hypertension and offer potential for early diagnosis and treatment (Sekar and Zayed 2024). Proteomic studies have further highlighted proteins involved in inflammation and cholesterol regulation, uncovering new therapeutic targets (Aggarwal et al. 2024). Clinically, biomarkers like troponin and B-type Natriuretic Peptide (BNP) are utilized for risk stratification and monitoring, enabling targeted interventions for high-risk individuals (Jena 2023; Kotta and Nambi 2023). Additionally, they provide insights into therapy selection and optimization. By supporting personalized medicine, biomarkers improve treatment outcomes, reduce complications, and facilitate the early detection of hypertension and associated conditions, ultimately enhancing patient quality of life (Sireesha et al. 2024; Ribeiro et al. 2022).

Challenges and Future Directions

The development of ACE inhibitory peptides faces challenges in bridging the gap between in vitro and in vivo results, establishing regulatory guidelines for functional claims, and integrating multi-omics approaches for comprehensive bioactivity evaluation. While in vitro studies provide critical insights into peptide bioactivity, translating these findings to in vivo systems is hindered by factors such as peptide stability during digestion, absorption efficiency, and bioavailability. The gastrointestinal environment, with enzymatic degradation and low permeability, poses significant barriers, although advancements in encapsulation technologies like liposomes and microcapsules show promise in enhancing stability and efficacy (Pei et al. 2022; Wang et al. 2024b). Regulatory challenges further complicate progress, as the current frameworks lack harmonized guidelines for validating functional claims of ACE inhibitory peptides. Ensuring product quality, controlling impurities, and generating robust clinical data are essential to gaining regulatory approval and consumer trust (Wolf and Ricci 2024). Additionally, the integration of multi-omics approaches, including genomics, proteomics, and metabolomics, offers

a holistic evaluation of peptide bioactivity. Multi-omics data facilitates the discovery of novel peptides and understanding their mechanisms of action, but challenges in computational prediction and data standardization remain (Coelho et al. 2024; Rivero-Pino et al. 2023). Future efforts should focus on developing advanced computational models, standardized methods, and clear regulatory frameworks to optimize the therapeutic potential of ACE inhibitory peptides.

Conclusion

The evaluation of ACE inhibitory peptides is important for effective hypertension management whether they provide similar efficacy without inducing kallikrein–kinin–related side effects such as dry cough and angioedema. These side effects arise due to the accumulation of bradykinin and substance P, which are normally degraded by ACE. When ACE is inhibited, these peptides build up in the respiratory tract, stimulating airway nerves and the alveoli, resulting in a persistent dry cough (Manoharan 2023). In vitro methods, including ACE inhibition assays and enzyme kinetics, provide valuable insights into peptide bioactivity but are limited by the inability to fully replicate the complexities of the human body. On the other hand, in vivo studies, despite their crucial role in assessing the physiological impact of peptides, face challenges such as ethical concerns, high costs, variability in human responses, and technological limitations. These limitations highlight the need for continuous innovation in evaluation techniques. Recent advancements, such as predictive modeling, organ-on-chip technologies, and the identification of novel biomarkers, show promise in enhancing the accuracy and predictive power of ACE inhibitory peptide research. Predictive models, including QSAR and molecular docking, have proven effective in predicting ACE-peptide interactions, while organ-on-chip systems allow for more personalized and accurate representations of human physiology. Biomarkers are emerging as essential tools for validating the efficacy of ACE inhibitory peptides and monitoring their impact in real-time. Moving forward, addressing the challenges associated with bioavailability, stability, and regulatory approval will be crucial for the successful integration of ACE inhibitory peptides into hypertension therapies. Future research should focus on refining both experimental methods and computational approaches, while also developing standardized guidelines for regulatory approval and clinical implementation. The ongoing advancements in technology and interdisciplinary research provide a hopeful outlook for ACE inhibitory peptides, with the potential to become an integral component of future hypertension management strategies.

Acknowledgements The author (Sonia) is thankful to the University Grants Commission (UGC) and the Ministry of Social Justice and Empowerment, Government of India, for awarding the National Fellowship for Scheduled Castes (NFSC) [No.NSFDC/E-71131]. The authors are sincerely grateful to Central University of Haryana for infrastructural support.

Author Contribution S.B.: Writing—Original draft preparation, Conceptualization, Formal analysis, Writing—Review and Editing J.N.: Writing—Review and Editing T. D.: Supervision, Validation. All authors reviewed the manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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