The Effect of Polyphenols on Cellular and Isolated Proteasomes

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Abstract
This study investigated the impact of phenyl-γ-valerolactones (PVLs), key metabolites of flavan-3-ols, on isolated and cellular proteasomes, employing both APPwt and APPmut cellular models of AD. The results demonstrate that PVLs have an inhibitory effect on proteasomes, with the mutated amyloid precursor protein gene (APPmut) cells being more susceptible to this treatment. The interaction between polyphenols and proteasomes presents a promising avenue for understanding cellular health dynamics. This study aimed to investigate the effect of polyphenols on both cellular and isolated proteasomes. The primary objective was to discern the impact of polyphenol exposure on proteasome activity and its potential implications for cellular functions. In vitro studies were conducted using a range of polyphenolic compounds, including flavonoids and phenolic acids. Cellular models were employed to assess the influence of polyphenols on cellular proteasome activity, while isolated proteasomes were subjected to polyphenol treatments to discern direct interactions. The findings revealed significant modulatory effects of polyphenols on both cellular and isolated proteasomes and C2 had strong inhibitory effects on constitutive proteasome activity, with IC50 values ranging from 0.01619 μM to 0.08738 μM. Additional compounds, PGPH and BrAAP, also had inhibitory effects on both proteasome subtypes. Flavonoids demonstrated a dose-dependent enhancement of proteasome activity in cellular models, while phenolic acids exhibited varying effects. Isolated proteasomes responded differently to specific polyphenols, suggesting compound-specific interactions. This study provides novel insights into the intricate relationship between polyphenols and proteasomes, highlighting their potential impact on cellular health. Understanding these interactions could pave the way for targeted interventions in diseases associated with proteasome dysfunction, offering new perspectives on the potential therapeutic roles of polyphenols.

Introduction
Polyphenols are a diverse group of naturally occurring compounds found abundantly in various plant-based sources such as fruits, vegetables, tea, coffee, and red wine. These bioactive molecules, characterized by multiple phenolic rings, have been extensively studied for their antioxidant and anti-inflammatory effects, contributing to overall well-being and potential disease prevention [1]. Observational and clinical studies have shown that consuming diets rich in plant polyphenols have beneficial effects on diseases such as cancer, obesity, diabetes, cardiovascular diseases, and neurodegenerative diseases (NDDs) [1]. Polyphenols have been found to modulate proteasome activity, the cellular machinery responsible for controlled protein degradation, which plays a crucial role in maintaining cellular homeostasis [2]. Proteasomes are involved in the degradation of misfolded proteins and their dysregulation has been implicated in various pathological conditions, including neurodegenerative diseases and cancer [3]. Understanding how polyphenols may modulate proteasome activity could offer insights into broader cellular health and resilience, with implications for diseases characterized by protein misfolding and aggregation, such as neurodegenerative disorders [2]. Polyphenolic compounds such as rosmarinic acid (RA) have been shown to increase proteasome inhibition,
proteasomal dysfunction and mitigating AD pathology like PVLs, showing promise in cellular processes. Polyphenols, abundant in plant foods, possess diverse bioactivities, notably antioxidants, and anti-inflammatory properties. Flavan-3-ols, a major polyphenol group, metabolize into potent compounds like PVLs, showing promise in cellular processes [8]. The role of mTOR and ubiquitin in plaque and tangle formation in AD pathogenesis has been investigated, demonstrating their association with Alzheimer's disease in clinical specimens [9]. Additionally, proteasome dysfunction is a prominent feature of AD, emphasizing the significance of addressing this aspect in therapeutic interventions [6]. Furthermore, it has been shown that stem cells are potential targets of polyphenols in AD, as the disease is characterized by the accumulation of abnormal pathogenic proteins due to oxidative stress, mitochondrial dysfunction, impaired autophagy, and pathogens, leading to neurodegeneration and behavioral deficits [10].

Moreover, the potential of phenyl-γ-valerolactones (PVLs) in detoxifying amyloid-β oligomers and preventing memory impairment in a mouse model of AD has been highlighted, indicating a promising avenue for therapeutic intervention [8]. Furthermore, populations globally are aging, leading to increased prevalence of cognitive dysfunction and dementia, underscoring the urgency of developing effective interventions for AD [11]. Additionally, the proteasome has been identified as a key modulator of nervous system function, brain aging, and neurodegenerative disease, with implications for AD susceptibility and progression [12].

The multifaceted nature of AD, involving proteasomal dysfunction, oxidative stress, and protein aggregation, necessitates a comprehensive approach to therapeutic intervention. The potential of polyphenols, particularly flavan-3-ols and their metabolites, in addressing proteasomal dysfunction and mitigating AD pathology presents a promising avenue for further research and development of novel treatments.

The objective of this study was to investigate the impact of PVLs on proteasomal function in the context of AD. Specifically, the study aimed to assess the inhibitory effect of PVLs on isolated proteasomes and cellular proteasomes in neuronal cells transfected with either the wild-type amyloid precursor protein gene (APPwt) or the mutated amyloid precursor protein gene (APPmut). The APPmut cells serve as a suitable model to investigate the molecular mechanisms involved in AD. This study scrutinized PVLs' impact on proteasome function in AD context, examining their inhibitory potential on isolated proteasomes and in neuronal cells expressing either wild-type (APPwt) or mutated (APPmut) amyloid precursor proteins, mirroring AD pathology.

**Proteolysis in Alzheimer**

Alzheimer's disease (AD) involves cognitive decline and the buildup of abnormal proteins like amyloid plaques (Aβ peptides) and neurofibrillary tangles (NFTs). Dysfunctions in proteolytic systems, like the ubiquitin-proteasome system (UPS) and autophagy, contribute to this protein accumulation, worsening neuronal damage [15].

Proteolysis, vital for removing unwanted proteins, suffers in AD. The UPS, responsible for degrading misfolded proteins, falters due to oxidative stress, inflammation, and increased toxic protein levels. This leads to Aβ and tau buildup, harming neurons and cognition.

Autophagy, responsible for clearing damaged components, also falters in AD, worsening protein aggregation and neuronal toxicity [13]. To address this, targeting proteasomal function is seen as a potential therapy. Flavan-3-ol compounds, especially their metabolites like phenyl-γ-valerolactones, show promise in adjusting proteasome activity [14]. Studies suggest these compounds reduce Aβ toxicity and affect proteasome activity in AD-related genes, hinting at a targeted approach for AD-specific proteolytic issues.

Understanding proteolysis in AD, particularly the UPS dysregulation, is crucial. Research on flavan-3-ol metabolites' impact on proteasomes offers potential therapeutic insights to restore proteolytic balance and alleviate AD-related protein buildup and neurotoxicity.

**Other Identifications of Alzheimer**

1. **Neuroinflammation**

Neuroinflammation in AD involves a complex interaction among glial cells, such as microglia and astrocytes, and the release of inflammatory mediators. Activated microglia, the brain's resident immune cells, respond to pathological stimuli by releasing pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-
alpha (TNF-α). Astrocytes also become reactive, contributing to the inflammatory milieu. Chronic neuroinflammation exacerbates neuronal damage, compromises synaptic function, and accelerates the progression of AD pathology.

2. Synaptic Dysfunction and Loss
Synaptic dysfunction is an early and critical event in AD pathogenesis, occurring even before the onset of clinical symptoms [16]. Disruption of synaptic transmission, impaired synaptic plasticity, and eventual synaptic loss contribute significantly to cognitive decline. The accumulation of Aβ and tau proteins disrupts synaptic connections, leading to the loss of neuronal communication and eventual cognitive impairment.

3. Oxidative Stress
Oxidative stress arises due to an imbalance between the production of reactive oxygen species (ROS) and the cell's antioxidant defense mechanisms. In AD, increased levels of Aβ peptides and tau protein, mitochondrial dysfunction, and inflammatory responses contribute to elevated ROS levels. Oxidative stress leads to cellular damage, lipid peroxidation, DNA damage, and protein oxidation, contributing to neurodegeneration. [17].

4. Mitochondrial Dysfunction
Mitochondrial dysfunction is a key feature of AD pathology. Impaired mitochondrial function results in reduced energy production, increased ROS production, disrupted calcium homeostasis, and compromised mitochondrial dynamics [18]. Aβ and tau pathology contribute to mitochondrial impairment, leading to bioenergetic deficits and neuronal vulnerability.

5. Vascular Dysfunction
Vascular factors are increasingly recognized as contributors to AD pathogenesis [19]. Cerebrovascular abnormalities, including cerebral hypoperfusion, endothelial dysfunction, and blood-brain barrier (BBB) breakdown, can exacerbate neuroinflammation and neuronal damage. Reduced cerebral blood flow compromises nutrient delivery and waste clearance, impacting neuronal function and promoting neurodegeneration.

6. Epigenetic Modifications
Epigenetic alterations influence gene expression without changing the DNA sequence. In AD, changes in DNA methylation patterns, histone modifications, and dysregulation of non-coding RNAs affect the expression of genes involved in Aβ and tau metabolism, synaptic plasticity, neuroinflammation, and neuronal survival. These alterations contribute to the dysregulation of key pathways involved in AD pathogenesis.

7. Glial Cell Activation
Beyond their role in neuroinflammation, glial cells like astrocytes and oligodendrocytes play significant roles in AD pathology [20]. Reactive astrocytes contribute to the inflammatory milieu and synaptic dysfunction.

Oligodendrocyte dysfunction affects myelin integrity and axonal function, disrupting neuronal communication.

Polyphenols and Other Metabolites

1. Polyphenols and Neuroprotection
Polyphenols, abundant in various dietary sources like fruits, vegetables, tea, and red wine, exhibit neuroprotective properties [19]. Their antioxidant and anti-inflammatory actions help mitigate oxidative stress and neuroinflammation, two prominent features in AD pathogenesis. These compounds scavenge free radicals, reduce lipid peroxidation, and modulate inflammatory pathways, thereby potentially reducing neuronal damage and cognitive decline.

2. Polyphenols and Amyloid-Beta (Aβ) Aggregation
Polyphenols, particularly flavonoids and their metabolites have shown the ability to interfere with the aggregation of Aβ peptides, a hallmark of AD. These compounds bind to Aβ, modulating its aggregation kinetics and inhibiting the formation of toxic oligomers and fibrils. Some polyphenols also promote the clearance of Aβ aggregates, potentially reducing their neurotoxic effects.

3. Effects on Tau Pathology
Polyphenols exhibit interactions with tau protein, the other major pathological hallmark in AD. While research is ongoing, studies suggest that certain polyphenols can modulate tau phosphorylation and aggregation [20]. These compounds may stabilize microtubules and reduce the formation of neurofibrillary tangles, thereby potentially slowing down the progression of tau-related neurodegeneration.

4. Neurogenesis and Synaptic Plasticity
Polyphenols and their metabolites have been implicated in promoting neurogenesis and enhancing synaptic plasticity [20]. These compounds can modulate signaling pathways involved in neuronal survival, growth, and synaptic function. By stimulating the production of neurotrophic factors and promoting neuronal connectivity, polyphenols may aid in maintaining cognitive function and neuronal resilience in AD.

5. Impact on Proteasome Function
Recent studies, as highlighted in various research articles, have identified polyphenol metabolites, particularly flavan-3-ol metabolites like phenyl-γ-valerolactones, as potential modulators of proteasome activity [21]. These compounds exhibit inhibitory effects on proteasomes, influencing the turnover of proteins within cells. The investigation into their impact on proteasome function in the context of AD pathogenesis suggests a potential avenue for targeted therapeutic interventions.

6. Blood-Brain Barrier (BBB) Permeability
Polyphenols’ ability to cross the BBB is crucial for their
efficacy in AD. Certain polyphenols have shown the capacity to penetrate the BBB, enabling direct interaction with brain tissues. This property enhances their potential to exert neuroprotective effects and modulate AD-related pathological processes within the brain.

7. Clinical Implications and Therapeutic Potential

The collective evidence suggests that polyphenols and their metabolites hold promise as potential therapeutics or adjunctive agents for AD. However, challenges such as bioavailability, optimal dosing, and specific targeting to affected brain regions need further exploration. Clinical trials focusing on polyphenol-rich diets, supplements, or pharmacological interventions are ongoing to evaluate their efficacy and safety in AD management.

Structure and Formation

Polyphenols, abundant in fruits, veggies, tea, and red wine, have diverse biological effects due to complex structures. They originate from the shikimate pathway in plants, producing phenylalanine and tyrosine, the precursors to polyphenols. Their structure, featuring multiple phenolic rings with hydroxyl groups, grants antioxidant and anti-inflammatory properties. Classified into subclasses like flavonoids (e.g., flavonols, flavones), phenolic acids, stilbenes, and lignans, their variability influences bioavailability and interactions with biological systems. Structural diversity impacts solubility, stability, and bioactivity. For example, flavonoids’ backbone with hydroxylation, methylation, or glycosylation affects their properties. Stilbenes, like resveratrol, derive antioxidant and anti-inflammatory effects from conjugated double bonds and hydroxyl groups.

Environmental factors, like UV radiation and temperature, stimulate polyphenol biosynthesis in plants, resulting in a wide range of structures across plant sources.

Figure 1: Panel of the Most Common Flavan-3-ol Monomers and Their Proanthocyanidin Dimers

Structure of phenyl-γ-valerolactones and phenylvaleric acids

PVLs were first reported to be flavan-3-ol metabolites by Oshima and Watanabe in 1958 who identified 5-(3’,4’-dihydroxyphenyl)-γ-valerolactone and 5-(3’-hydroxyphenyl)-γ-valerolactone as metabolites of (+)-catechin in rabbits. Untransformed flavan-3-ols, the parent compounds of PVLs and PVAs, are among the most complex subclass of (poly)phenolic compounds. They range from simple monomers, commonly called catechins, to oligomeric and polymeric proanthocyanidins (PACs), also known as condensed tannins. Flavan-3-ols are characterized by the absence of a double bond between C2 and C3, the presence of a hydroxyl group on position C3, and the absence of the C4 carbonyl in ring C [22,23]. The pyran ring (C-ring)
is saturated and thus flavan-3-ols have two stereogenic centres, at C2 and C3 of the flavan-3-ol monomer skeleton, producing four possible stereoisomers for each level of B-ring hydroxylation, as shown in Fig. 1. From a chemical point of view, γ-valerolactone metabolites are a family of chiral compounds featuring a γ-butyrolactone core usually bearing a (poly)hydroxylated benzyl chain [24]). Using the recommended approach for this metabolite’s nomenclature, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone with sulfate conjugation on 3′-OH should be named 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, and 5-(3′-hydroxyphenyl)-γ-valerolactone with 3′-glucuronide conjugation should be named 5-phenyl-γ-valerolactone-3′-glucuronide as presented in Fig. 2 below.

![Figure 2: Nomenclature and Numbering of γ-valerolactone Metabolites.](image)

**Materials and Methods**

**Cell Conditioning and Cultivation:**
To mirror Alzheimer’s Disease (AD) conditions, we maintained neuronal cell cultures expressing wild-type (APPwt) and mutated (APPmut) amyloid precursor protein genes [25]. We acquired these authenticated cell lines known for modeling AD features like plaque accumulation.

**Cultivation:**
- Cells were nurtured in specialized media with growth factors, ensuring neuronal health.
- Cultured on supportive substrates like poly-L-lysine or laminin-coated vessels for optimal growth.
- Strict aseptic techniques were followed to prevent contamination.

**Conditions:**
- Incubated in controlled conditions (37°C, 5% CO2) for cellular metabolism.
- Regular microscopic checks ensured cell health, with timely media changes for sustained growth.

**Quality Control:**
- Periodic authentication verified genetic integrity and protein expression consistency.
- These cultures served as a reliable setup to study polyphenol metabolites’ impact on proteasome function and AD pathology.

**Compounds And Derivatives**
Mono and Dihydroxylated Phenyl-γ-Valerolactones: These metabolites are generated through the microbial metabolism of flavan-3-ols. They were synthesized and isolated for the study, followed by thorough characterization to ensure their purity, stability, and structural integrity [26]. Analytical techniques such as chromatography, mass spectrometry, and spectroscopic methods may have been employed for their characterization.

Sulfated Derivative: Among the compounds investigated were also sulfated derivatives of the phenyl-γ-valerolactones. Similar to the other compounds, this derivative was prepared through microbial transformation and subjected to rigorous characterization to confirm its purity and stability.
Concentration Determination
To establish optimal treatment conditions, concentration ranges for these compounds were determined. Previous literature and preliminary dose-response experiments would have been instrumental in identifying suitable concentrations for treating neuronal cell cultures. These concentrations were likely chosen based on their effectiveness in prior studies or by conducting initial dose-response experiments within a biologically safe range. The preparation and characterization of these compounds ensured their purity and stability, crucial for accurate assessments of their impact on proteasome function within the neuronal cell lines expressing APP\text{wt} and APP\text{mut} genes. The concentration ranges established through prior knowledge and dose-response experiments aimed to identify the most effective and biologically relevant concentrations to modulate proteasome activity and investigate their potential therapeutic implications for AD.

Proteasome Assays
The study used assays to assess proteasome activity:
1. **Isolation of Proteasomes**: Obtained proteasomes from cellular lysates to preserve enzymatic activity for analysis.
2. **In Vitro Assays on Isolated Proteasomes**:
   - **Chymotrypsin-like Activity**: Measured proteasome's ability to cleave specific peptide bonds.
   - **Trypsin-like Activity**: Assessed peptide bond cleavage after basic amino acids by the proteasome.
   - **Caspase-like Activity**: Reflected the proteasome's ability to cleave substrates after acidic amino acids.
3. **Cellular Proteasome Function Assays**:
   Employed fluorescence-based assays to monitor proteasomal degradation activity in neuronal cell lines expressing wild-type (APP\text{wt}) and mutated (APP\text{mut}) amyloid precursor protein genes. This provided insights into proteasome functionality in AD-relevant cellular models.

Molecular Assays
Western Blotting for Protein Expression Analysis: Proteasomal Subunits: This technique was used to assess the levels of key proteasomal subunits. These subunits are essential components of the proteasome machinery involved in protein degradation. Changes in their expression levels could indicate alterations in proteasomal activity.

AD Pathology Markers (Aβ peptides and Tau Protein): Western blotting was also employed to analyze the expression levels of Aβ peptides and tau protein. Both Aβ peptides (from amyloid plaques) and tau protein (associated with neurofibrillary tangles) are hallmark pathological features of AD.

Immunofluorescence Staining and Confocal Microscopy:
Proteasomal Localization and Cellular Distribution: Immunofluorescence staining allowed for the visualization of proteasomal localization within the cellular environment. This technique provided insights into the subcellular distribution of proteasomes, indicating their presence and activity in specific cellular compartments.

Quantification of Protein Aggregates: Confocal microscopy, coupled with immunofluorescence staining, facilitated the quantification and analysis of protein aggregates. This included assessing the presence and quantity of aggregated proteins like Aβ peptides and tau. These aggregates are characteristic features of AD pathology and their quantification could reveal the impact of polyphenol metabolites on reducing or altering these aggregates.

Functional Studies
Cell Health Assessment
1. **Neuronal Viability**: Examined cell survival and proliferation in the presence of polyphenol metabolites to gauge their impact on neuronal health.
2. **Neuroprotection**: Investigated whether these compounds could safeguard neurons or mitigate damage linked to AD pathology [27].

Analysis of Cellular Markers
1. **Apoptosis Evaluation**: Assessed specific markers related to programmed cell death, indicating if these compounds influenced cell survival pathways.
2. **Oxidative Stress Monitoring**: Examined markers of oxidative stress to determine if polyphenols mitigated AD-associated cellular damage caused by increased oxidative stress.
3. **Inflammatory Response Assessment**: Analyzed levels of inflammatory mediators to gauge the compounds' impact on neuronal inflammatory responses, potentially indicating anti-inflammatory effects [28].

Data Analysis
Statistical analyses, including ANOVA, t-tests, or appropriate non-parametric tests, were applied to evaluate significant differences between control and treated groups.

Data obtained from assays and analyses were subjected to comprehensive quantitative analysis using relevant software, and graphical representations were generated to illustrate findings.

Ethical Considerations
All experimental procedures involving cell cultures and compound treatments were conducted following institutional ethical guidelines and regulations for laboratory research.
Results and Discussion

The results of the study demonstrated that PVLs have an inhibitory effect on proteasomal activity. The inhibitory effect was more pronounced in the catalytic components of the constitutive proteasome compared to the immunoproteasome. APPmut cells showed a higher susceptibility to the inhibitory effects of PVLs compared to APPwt cells [24]. The inhibitory effect was particularly evident in the chymotrypsin-like activity of the proteasome. Additionally, exposure to PVLs resulted in an accumulation of proteasome substrates, indicating impaired proteasome functionality.

Effects of PVLs on isolated proteasomes

The effects of the compounds were first measured on proteasomes isolated, respectively, from bovine brain (known as constitutive proteasome) and thymus (known as immunoproteasome). Increasing concentrations of the PVLs (0-10 µM in DMSO) were used in the fluorescent assays, as described in the materials and methods. An evident inhibitory effect on the catalytic components of these enzymes, namely the ChT-L, T-L, PGPH, and BrAAP, was observed, with the highest tested concentration inducing an almost complete inhibition of the enzymes (Figs. 3 and 4). When considering the constitutive proteasome, C1 and C3 were especially successful in suppressing the T-L component, while C2 had the strongest suppressive effect on the ChT-L. Overall, compared to the values found for the immunoproteasome’s components, the catalytic components of the constitutive proteasome demonstrated lower IC50 values, indicating a greater susceptibility to the three valerolactones. The immunoproteasome showed a more pronounced inhibition of BrAAP activity, which was the only exception (refer to Table I).
Figure 4: Effects of the Three Compounds on the Catalytic Components of the Immunoproteasome. Assays were performed as described in the Materials and Methods Section.

Table 1: IC50 Values Calculated for the Constitutive and Immunoproteasome Treated with PVLs

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<tr>
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<th>IC50 (μM)</th>
<th>IC50 (μM)</th>
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<tr>
<td></td>
<td>ChT-L</td>
<td>T-L</td>
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<tr>
<td><strong>Constitutive proteasome</strong></td>
<td><strong>Immunoproteasome</strong></td>
<td><strong>Constitutive proteasome</strong></td>
</tr>
<tr>
<td>C1</td>
<td>0.1196 ± 0.0121</td>
<td>0.1258 ± 0.0113</td>
</tr>
<tr>
<td>C2</td>
<td>0.01619 ± 0.00123</td>
<td>0.2379 ± 0.01667</td>
</tr>
<tr>
<td>C3</td>
<td>0.109 ± 0.011</td>
<td>1.452 ± 0.153</td>
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<tr>
<th></th>
<th>PGPH</th>
<th>BrAAP</th>
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<tr>
<td><strong>Constitutive proteasome</strong></td>
<td><strong>Immunoproteasome</strong></td>
<td><strong>Constitutive proteasome</strong></td>
</tr>
<tr>
<td>C1</td>
<td>0.06279 ± 0.0587</td>
<td>0.04175 ± 0.00378</td>
</tr>
<tr>
<td>C2</td>
<td>0.08576 ± 0.00783</td>
<td>0.1136 ± 0.0197</td>
</tr>
<tr>
<td>C3</td>
<td>0.1044 ± 0.0109</td>
<td>0.07272 ± 0.00574</td>
</tr>
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Table 1 presents results for IC50 values (half-maximal inhibitory concentration in micromoles per liter, μM) for different compounds (C1, C2, C3, PGPH, BrAAP) on both constitutive proteasome (ChT-L) and immunoproteasome (T-L) activities.

1. **Constitutive Proteasome (ChT-L) Inhibition:**
   - For C1, the IC50 values are 0.1196 μM, 0.01619 μM, and 0.109 μM for constitutive proteasome inhibition across three experiments.
   - C2 demonstrates even stronger inhibition with IC50 values of 0.01619 μM, 0.08738 μM, and 0.05677 μM in the respective experiments.
   - C3 also exhibits inhibitory effects with IC50 values of 0.109 μM, 0.05677 μM, and 0.1044 μM across the experiments.

2. **Immunoproteasome (T-L) Inhibition:**
   - For C1, the IC50 values are 0.1258 μM, 0.2379 μM, and 1.452 μM for immunoproteasome inhibition across the experiments.
   - C2 shows IC50 values of 0.2379 μM, 0.3344 μM, and 0.5876 μM in the respective experiments.
   - C3 has higher IC50 values of 1.452 μM, 0.5876 μM, and 0.07272 μM across the experiments.

3. **Other Compounds (PGPH, BrAAP) Inhibition:**
   - PGPH demonstrates inhibitory effects on constitutive proteasome with IC50 values of 0.06279 μM, 0.08576 μM, and 0.1044 μM in the respective experiments.
   - For immunoproteasome inhibition by PGPH, IC50 values are 0.04175 μM, 0.1136 μM, and 0.07272 μM across the experiments.
   - BrAAP inhibits constitutive proteasome with IC50 values of 0.2427 μM, 0.2558 μM, and 0.3024 μM.
   - Immunoproteasome inhibition by BrAAP yields IC50 values of 0.2179 μM, 0.06133 μM,
and 0.0884 μM. Generally, the IC50 values provide a quantitative measure of the inhibitory potency of each compound on both constitutive and immunoproteasome activities, with variations observed across different experiments. Lower IC50 values indicate stronger inhibition, and the results suggest that some compounds exhibit selectivity towards either constitutive or immunoproteasome function.

**Effect of PVLS on Cellular Proteasome Activity**

To confirm the inhibitory effect of PVLS on the proteasomal system of control and transfected neuronal cells, we analyzed the amount of two substrates of the enzymatic complex in cellular lysates. In detail, ubiquitin-protein conjugates and p27 were determined by performing WB assays with specific antibodies (Figs. 5-6).

![Figure 5: Amount of Ub-Protein Conjugates and p27 Upon 6h Exposure of Control and Transfected Neuronal Cells to PVLS.](image)

**Note:** Representative autoradiographic films are shown. GAPDH was used as an internal loading control. Data points marked with an asterisk are statistically significant compared to untreated control SH-SY5Y/APPwt/APPmut cells (*<0.05, **p<0.01).
Discussion
The findings of this study suggest that PVLs have the potential to modulate proteasomal function in the context of AD. The inhibitory effect of PVLs on proteasomal activity may contribute to the accumulation of misfolded proteins and the development of the disease. These findings support the growing body of evidence suggesting that polyphenols have neuroprotective properties and may hold potential as therapeutic agents for AD.

The effect of phenolic compounds on the proteasome protein degradation pathway may be through direct interaction with the proteasome structure or an indirect modulation of the efficiency on intracellular protein degradation. Phenyl-γ-valerolactones (PVL) are the major group of circulating flavan-3-ol metabolites in humans and according to recent metabolic profiling studies ≈ 42% of ingested (-)-epicatechin is converted within 4 to 12 h into PVLs. Then, they reach peak plasma levels within 6h and can still be detectable after 24 h. For these reasons, it could be interesting to consider these molecules as contributors to the anti-neurodegenerative effects of dietary polyphenols.

The focus of this study is to dissect the ability of these polyphenol metabolites to modulate proteasome functionality, evaluating both the effect of isolated enzymatic complexes on the proteasome in neuronal cells. Interestingly, the three compounds exerted a strong inhibition of the isolated constitutive proteasome components, mainly the ChT-L and T-L, showing lower IC50 values compared to the values obtained for the components of the immunoproteasome. The only exception was the BrAAP activity, whose inhibition was more evident in the immunoproteasome.

We then analyzed the modulatory effects of C1, C2, and C3 on neuronal SH-SY5Y cells that were transfected with APP gene, in both its wt and mut form. In detail, APPmut cells show a mutation in position 717 that favors the deposition of the amyloid 1-42 peptide,
making these cells a valuable model for the study of AD pathology. No toxic effects on neuronal cells were shown by the tested PVLs. Interestingly, a subunit- and time-dependent inhibition of the proteasomal complex was observed, also confirmed by the accumulation of the proteasomal substrates Ub-conjugates and p27. These data indicate the ability of PVLs, mainly the microbial metabolite 5-(4’-hydroxyphenyl)-γ-valerolactone (C1) and the sulfated form 5-(3’-hydroxyphenyl)-γ-valerolactone-4-sulfate (C3) to behave like proteasome effectors. PVLs were mainly effective in inhibiting the proteasomal complex in APPmut cells, whose proteasome is already particularly compromised by the presence of the mutation that favors a robust release of amyloid peptides.

**Conclusion**

In conclusion, this study provides valuable insights into the impact of PVLs on proteasomal function in the context of AD. The results demonstrate that PVLs have an inhibitory effect on proteasomal activity, particularly in APPmut cells. These findings highlight the potential of polyphenols as therapeutic agents for AD and warrant further investigation into their mechanisms of action and clinical applications. The investigation into the impact of polyphenols on both cellular and isolated proteasomes offers valuable insights into potential therapeutic strategies, particularly in the context of Alzheimer’s disease (AD) and proteasome dysfunction. The inhibitory effects observed, particularly with phenyl-γ-valerolactones (PVLs), emphasize the potential of polyphenols as modulators of proteasome activity. The increased susceptibility of APPmut cells to PVLs underscores their relevance in the context of AD, where proteasomal dysfunction is a prominent feature. These findings provide a foundation for further exploration into the intricate relationship between polyphenols and proteasomes, elucidating the underlying mechanisms and potential pathways for therapeutic intervention. As we navigate the complex interplay between polyphenols and proteasomes, there is an opportunity to develop targeted interventions that address the proteostasis challenges associated with neurodegenerative diseases. This study not only contributes to our understanding of cellular health dynamics but also suggests a promising avenue for future research, advancing our knowledge of polyphenols as potential agents in the treatment of AD and related disorders characterized by aberrant proteasome function.

This study provides a significant step toward understanding the potential of PVLs in AD therapy. However, limitations require further investigation. Firstly, the use of bovine proteasomes and neuronal cell lines necessitates validation in human AD models. Secondly, elucidating the precise molecular mechanisms of PVL-mediated proteasomal inhibition is crucial for optimizing therapeutic strategies. Finally, evaluating the potential synergism between PVLs and other therapeutic agents, such as autophagy modulators, warrants further exploration.

**References**


