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1,4-dihydropyridine derivatives increase mRNA expression of *Psma3*, *Psmb5*, and *Psmc6* in rats

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The ubiquitin-proteasome system modifies different cellular and protein functions. Its dysregulation may lead to disrupted proteostasis associated with multiple pathologies and aging. Pharmacological regulation of proteasome functions is already an important part of the treatment of several diseases. 1,4-dihydropyridine (1,4-DHP) derivatives possess different pharmacological activities, including antiaging and neuroprotective. The aim of this study was to investigate the effects of several 1,4-DHP derivatives on mRNA expression levels of proteasomal genes *Psma3, Psmb5*, and *Psmc6* in several organs of rats. Rats were treated with metcarbatone, etcarbatone, glutapyrone, styrylcarbatone, AV-153-Na, or AV-153-Ca *per os* for three days. mRNA expression levels were determined with real-time polymerase chain reaction (PCR). For AV-153-Na and AV-153-Ca, we also determined the expression of the *Psma6* gene. In the kidney, metcarbatone, etcarbatone, styrylcarbatone, and AV-153-Na increased the expression of all analysed genes. Glutapyrone increased the expression of *Psmb5* and *Psmc6* but did not affect the expression of *Psma3*. In the blood, glutapyrone increased *Psmb5* expression. In the liver, AV-153-Na increased the expression of *Psma6* and *Psmc6* but lowered the expression of *Psmb5*, while AV-153-Ca only increased *Psma6* expression. The ability of 1,4-DHP derivatives to increase the expression of proteasome subunit genes might hold a therapeutic potential in conditions associated with impaired proteasomal functions, but further research is needed.

KEY WORDS: AV-153-Ca; AV-153-Na; etcarbatone; gene expression; glutapyrone; impaired proteasomal functions; metcarbatone; pharmacological activities; proteasome subunits; styrylcarbatone; ubiquitin-proteasome system

Proteostasis, or cellular protein homeostasis, relies on the regulation of protein synthesis, folding, conformational maintenance, and degradation (1). Deviations from optimal proteostasis can result in serious pathologies and accelerate the aging of an organism. Proteostasis is maintained by several control systems, all of them of equal importance (1). Protein degradation is partly regulated by the ubiquitin-proteasome system (UPS), which ensures rapid and specific turnover of proteins. UPS modifies cellular and protein functions, including cell cycle, cell signalling, DNA repair, chromatin modifications, and protein trafficking (2). These are mediated by ATPase (ubiquitination enzymes encoded by the *Psmc* genes in rodents) and the 20S core particle. The latter has a cylinder-like structure consisting of 28 proteins arranged in four heptameric rings. The outer ring is formed by alpha subunits encoded by the *Psma* genes in rodents (PSMA in humans). The inner, beta subunits are encoded by the *Psmb* genes (PSMB in humans) (2). Proteasome gene expression is triggered by the nuclear respiratory factor 1 (Nrf1). This transcription factor is, in turn, regulated by the mammalian target of rapamycin complex 1 (mTORC1) (3) and a feedback mechanism compensating proteasome dysfunction (4). Nrf1-dependent transcription of proteasomal genes is also increased by pharmacological inhibition of proteasomes (2). Pharmacological inhibition of proteasome function is important for the treatment of several diseases such as cancer (5), whereas stimulators of proteasome activity are being researched (6) as potential remedies against neurodegenerative diseases and as antiaging agents. It was shown that proteasome activity in long-living mammal species is higher than in short-living animals (7). Several natural substances were found to stimulate proteasome activity (8), synthetic molecules are also intensively studied (9).

In our opinion, testing of the impact of 1,4-dihydropyridine (1,4-DHP) derivatives, a vast group of compounds with different pharmacological activities, on the UPS could be a prospective research branch. A big group of these compounds has been synthesised in the Latvian Institute of Organic Synthesis over the last few years. Some of them manifest interesting effects besides antioxidant activity (9), as they can modify cell proliferation (10), bind DNA and proteins, or stimulate DNA repair by activating DNA repair

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enzymes (11, 12). These novel 1,4-DHP derivatives have a weak Ca^{2+} channel blocker activity and are water-soluble unlike "classical" Ca^{2+} channel blockers, which are hydrophobic. Yet they can modify the expression of several genes and proteins (13, 14), including the proteasome gene *Psma6* (15). The present work aimed to expand our previous research by studying the effects of several 1,4-DHP derivatives on mRNA expression levels of proteasomal genes *Psma3, Psmb5,* and *Psmc6* in several organs of rats to see if they have pharmacological potential as UPS modulators.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia) and was carried out according to the guidelines of the 1986 European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (16). Male Wistar rats (215.0±5.6 g) were purchased from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. Animals were kept at 22±0.5 °C with a 12 h light/dark cycle and fed standard laboratory diet.

Chemicals

All drugs used in the study – metcarbatone, etcarbatone, glutapyrone, styrylcarbatone (J-9-125), and AV-153 Na and Ca salts (Figure 1) were synthesised at the Latvian Institute of Organic Synthesis. Other chemicals were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany).

Experimental design

Rats were divided into control and treatment groups. The latter received 0.05 mg/kg or 0.5 mg/kg of metcarbatone,

etcarbatone, glutapyrone, styrylcarbatone, AV-153-Na, or AV-153-Ca *per os* by gavage for three days. The rats were then euthanised and their organ samples (kidneys, blood, and liver) taken and frozen in liquid nitrogen until analysis. There were two sets of rats. Kidneys and blood were taken from the control group (11 animals) and groups treated with metcarbatone, etcarbatone, glutapyrone, and styrylcarbatone (3–4 animals per group). Kidneys and liver were taken from the control group (10 animals) and groups treated with AV-153-Na and AV-153-Ca (3–5 animals per group).

RNA extraction and cDNA synthesis

Total RNA was isolated from the kidneys, blood, and liver with a TRI reagent (Sigma Aldrich, Taufkirchen, Germany). RNA was purified from DNA with a DNA-free kit (Ambion, Austin, TX, USA) and its quantity and purity determined with a NanoPhotometer® NP80 spectrophotometer (ImplenGMBH, Munich, Germany).

The quality of RNA was analysed with gel electrophoresis. cDNA was synthesised from the obtained RNA (5 μg from kidneys and liver, and 2 μg from blood) with random hexamer primers (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas, Vilnius, Lithuania).

Real-time reverse-transcription polymerase chain reaction tests

mRNA expression of *Psma3*, *Psmb5* and *Psmc6*, and a reference gene (*RNA-polymerase II*) in the kidney, blood, and liver was determined using the SYBR® Green PCR Master Mix (Applied Biosystems**,** Foster City, CA, USA) according to the instructions provided by the manufacturer. For the AV-153-Na and AV-153-Ca treatment groups, we also determined the expression of the *Psma6* gene.

Primers were designed using Primer-BLAST software (17). Primer sequences were: *Psma3* – 5'-CACCATCCTCTGGTGTCCATT-3' (forward) and
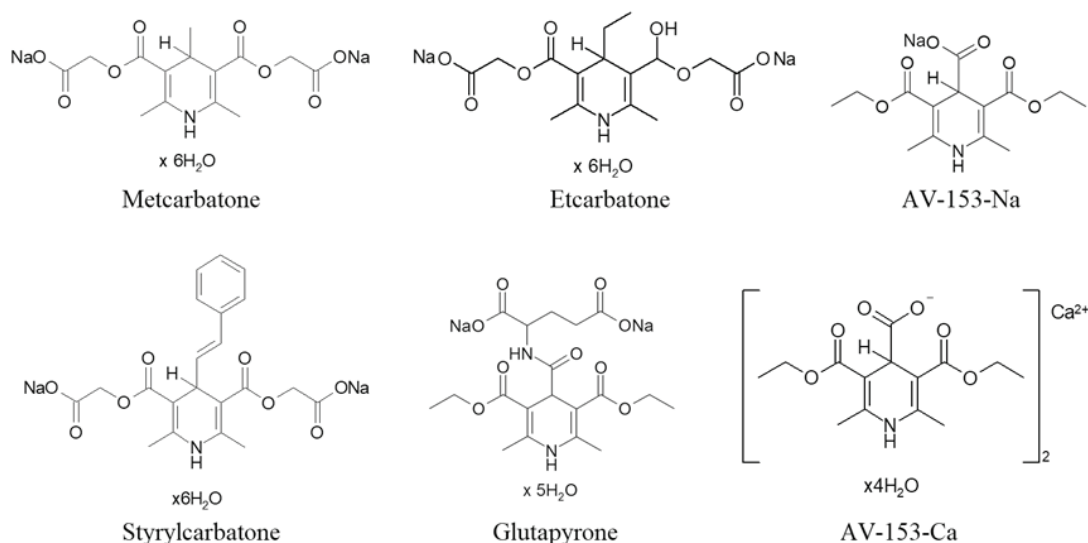
Figure 1 Formulas of 1,4-dihydropyridine derivatives used in the study

Table 1 Kidney and blood real-time PCR cycle threshold (C$_t$) values

	Kidneys				Blood			
	RNSpolII	*Psma3*	*Psmb5*	*Psmc6*	*RNSpolII*	*Psma3*	*Psmb5*	*Psmc6*
Control	20,6336	21,2849	20,2966	18,5916	19,3183	24,7317	23,9346	23,4343
	19,9632	21,0402	20,1468	18,5301	19,1140	24,9544	23,9330	23,6485
	20,5162	20,6843	19,9793	18,4140	19,5568	23,5918	23,6477	22,6171
	19,7661	20,8542	20,0899	18,4168	19,5473	25,3724	23,5371	23,2719
	20,4493	21,1992	20,3281	18,7329	19,2752	24,2432	23,6730	22,2758
	20,0575	20,7519	19,8123	18,2283	18,0247	24,1311	23,7417	21,8996
	19,8994	20,8551	20,1670	18,5733	19,6721	24,1230	22,6278	22,9333
	19,9792	21,2709	20,5719	18,8297	18,5737	23,8446	23,0603	22,4729
	20,3273	21,4559	20,4872	18,9060	18,9473	24,8475	24,5772	23,0626
	20,7163	21,1733	20,4132	18,8730	19,7215	24,5549	23,4031	23,6338
	20,1879	21,1945	20,4594	18,7461	18,7025	24,7850	24,1693	
					18,7703	24,3940	24,0506	
Metcarbatone 0.05 mg/kg	21,1421	21,1700	20,2690	18,7102	19,1992	24,9793	24,9819	23,4209
	20,6063	20,9449	20,0149	18,6103	18,2122	25,0053	24,6967	22,8411
	20,9352	21,4263	20,5656	18,9078	19,6951	24,3028	23,8090	22,9598
	20,5415	20,9494	20,0938	18,4946	18,7135	24,5634	24,1763	22,6806
Metcarbatone 0.5 mg/kg	20,8324	20,9915	20,1140	18,8390	18,9062	25,2567	23,8337	23,6736
	21,1187	21,0831	20,1682	18,7917	19,2715	25,2724	24,5450	23,6550
	21,0912	21,1248	20,3896	18,8515	18,5728	24,6956	23,9355	22,8742
	20,6640	21,1151	20,0875	18,7441	18,6701	25,2925	24,5882	23,2121
Etcarbatone 0.05 mg/kg	20,6521	21,3114	20,2236	18,6247	18,8542	24,8812	24,1604	22,9606
	21,2283	21,3560	20,3069	18,6993	19,1319	24,8758	24,7965	22,9433
	21,3387	21,4437	20,1647	18,7293	18,1798	24,6700	24,4486	22,5183
Etcarbatone 0.5 mg/kg	20,6957	21,2241	20,1316	18,4727	19,2888	24,9479	24,4943	23,1595
	21,4283	21,6113	20,5876	18,8926	19,2577	24,8016	24,0260	23,0911
	21,0995	21,5415	20,3180	18,8051	18,9519	24,5479	24,2079	22,4058
	20,9716	21,4134	20,4994	18,8108	19,0836	24,6532	24,6596	22,5140
Styrylcarbatone 0.05 mg/kg	21,4476	21,4452	20,5627	18,6984	19,5796	23,9558	23,0535	22,6217
	20,9010	21,0415	20,1628	18,3986	19,0160	24,8889	24,1739	23,2842
	21,3434	21,2509	20,2865	18,6662	18,1215	23,5157	23,3238	21,4245
	21,6191	22,0462	21,5846	18,9777	19,5722	25,4267	24,9225	23,4448
Styrylcarbatone 0.5 mg/kg	21,5660	21,5113	21,2236	18,9375	19,1745	23,8688	23,2048	22,2596
	21,2131	21,5601	20,7720	18,7713	19,5076	24,7026	23,6100	23,0168
	21,0897	21,4685	20,4881	18,7617	18,9420	24,6156	23,8653	23,2664
	21,4455	21,6779	20,9215	18,9282				
Glutapyrone 0.05 mg/kg	22,0678	22,8544	21,6248	19,8827	18,9078	23,7505	22,4539	22,0790
	21,0281	22,1696	20,8820	19,3440	19,1537	23,5877	22,7654	22,3149
	21,1787	21,9149	20,8096	19,0287	20,1436	25,5499	23,8626	23,8251
	21,3287	21,5062	20,4549	18,7512	19,6614	24,1258	23,0498	23,1506
Glutapyrone 0.5 mg/kg	21,0105	21,8076	20,5749	18,8806	19,3639	24,6586	23,1130	22,9958
	20,6910	21,6908	20,2656	18,6775	19,6876	25,6195	23,7417	24,0393
	21,1142	21,4909	20,2270	18,7062	19,4588	24,3839	23,1421	22,8727
	21,3715	21,4707	20,0467	18,8292	19,4118	24,7691	23,3703	23,2199

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5'-CGCAGATATCCTCAATTACCCAAC-3' (reverse) (fragment size 128 bp); *Psmb5* – 5'-AGGTGCCTACATTGCTTCCC-3' (forward) and 5'-GAGATGCGTTCCTTGTTGCG-3' (reverse) (fragment size 159 bp); *Psmc6* – 5'-TACATTGGGGAAAGCGCTCG-3' (forward) and 5'-TCAGAAAACCGACGACCACC-3' (reverse) (fragment size 116 bp); and *Psma6* – 5'-GTGTGCGCTACGGGGTGTA-3' (forward) and 5'-AGTCACGGTGCTGGAATCCA-3' (reverse) (fragment size 247 bp). The choice of the reference *RNA-polymerase II* gene was described earlier (18). Primer sequences for this gene were 5'-GCCAGAGTCTCCCATGTGTT-3' (forward) and 5'-GTCGGTGGGACTCTGTTTGT-3' (reverse) (amplified fragment size 135 bp).

Oligonucleotides were supplied by Metabion International AG (Martinsried, Germany). qPCR reactions were performed using a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: one cycle at 95 ºC for 10 min, 40 cycles at 95 ºC for 15 sec, and one cycle at 60 ºC for

1 min (Applied Biosystems StepOne software, version 2.1). The specificity of amplification products was verified by dissociation curve: one cycle at 95 ºC for 15 sec, one at 60 ºC for 1 min, and one at 95 ºC for 15 sec. The cycle threshold (C_t) values are presented in Tables 1 and 2.

Statistics

Reference gene stability was analysed with BestKeeper provided in RefFinder (https://www.heartcure.com). Among studied organs, standard deviation (SD) values ranged from 0.22 to 0.54, and the coefficient of variance (CV) ranged from 1.13 to 2.63. These values are consistent with those reported for stable housekeeping genes (19). Gene expression data were expressed using the $2^{-\Delta\Delta Ct}$ method – mean fold difference with standard error of the mean (SEM) (20). The P values were calculated from delta values using one-way ANOVA followed by Dunnett's test for multiple comparisons between the groups. In all tests, the P value of <0.05 was considered statistically significant. All

Table 2 Liver real-time PCR cycle threshold (C_t) values

	Kidneys					Liver				
	RNSpolII	*Psma3*	*Psmb5*	*Psmc6*	*Psma6*	*RNSpolII*	*Psma3*	*Psmb5*	*Psmc6*	*Psma6*
Control	20,5970	22,5839	19,2551	19,1623	19,9986	19,8560	23,0557	21,1670	18,6385	19,8489
	20,4384	22,5746	19,0794	18,8645	19,5620	19,5794	22,9181	21,1984	18,6208	19,6455
	20,4358	22,5837	19,1140	18,8865	19,7310	19,9334	23,3011	21,1796	19,6168	20,4226
	20,5461	22,7381	19,0685	19,1585	19,7124	19,7203	23,1264	21,1636	19,4941	20,2836
	20,3772	22,3517	19,0689	19,2026	20,2427	19,1888	22,9224	21,0717	19,1761	19,8373
	20,3640	22,2591	18,9613	18,9177	19,7695	19,7114	23,1850	21,1812	19,1708	20,0905
	20,4210	22,1444	19,0667	18,8171	19,3981	19,8140	23,5071	21,3909	19,3937	20,2497
	20,5429	22,3808		19,2618	20,5558	19,7631	23,3798	21,3486	19,0927	20,2443
	20,4095	22,3303		18,9823	20,3727	19,6435	23,5475	21,2771	19,3322	20,4155
	19,9648	22,0525		18,7116	19,2846					
AV-153-Na 0.05 mg/kg	21,1756	22,9314	19,3199	19,5803	20,0066	19,3193	22,6985	21,0440	18,3758	18,9400
	22,0513	23,4752	19,6423	19,9317	20,6172	18,8415	22,8245	20,9705	18,3795	19,0355
	21,4856	23,2240	19,4647	19,6623	19,8643	19,5196	22,7070	21,0585	18,3857	19,0187
	21,6234	23,2381	19,5993	19,5928	19,6469	18,8631	22,7883	20,9608	18,3031	18,9503
AV-153-Na 0.5 mg/kg	21,9367	23,2775	19,4898	19,8077	20,0014	19,6059	22,7776	20,9764	18,4171	19,0567
	21,6564	23,2385	19,5634	19,4900	19,8338	19,6035	22,8215	21,0093	18,4078	19,0701
	21,2077	23,2004	19,4074	19,5859	19,9741	19,5527	22,8589	20,9877	18,3254	19,1177
AV-153-Ca 0.05 mg/kg	19,7019	22,0370	19,1800	18,7166	19,3175	19,3393	22,9415	20,8086	19,1408	19,8715
	19,8564	22,2813	19,3695	18,7706	19,2143	19,6280	23,3113	21,0154	19,1477	20,3142
	20,1111	21,8349	19,0583	18,6593	19,3757	19,3697	22,8512	20,9235	18,9642	20,2187
						19,6506	23,1935	21,0658	19,3068	20,0824
AV-153-Ca 0.5 mg/kg	20,3086	22,0432	19,5138	18,8374	19,3591	19,4528	22,9892	20,7577	19,0212	19,3202
	20,5201	22,7760	18,8733	19,2125	20,2666	19,5880	23,2170	21,2606	19,0527	19,6300
	19,5801	21,8729	18,8382	18,7051	19,3437	18,8662	23,0295	21,3512	18,8270	19,3601
	20,1326	22,0602	18,9902	18,8147	19,6876	19,5368	23,2560	21,5740	19,0952	19,4394
	20,1226	21,8402	18,8235	18,8101	19,4492	19,5666	23,0126	21,1141	18,7163	19,2355

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analyses were run on the GraphPad Prism 6 version 6.01 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Metcarbatone

In the kidney, metcarbatone at both doses significantly increased the expression of the *Psma3, Psmb5*, and *Psmc6* genes (Table 3). The higher dose produced a more pronounced effect for *Psma3* and *Psmb5*. The increase ranged from 1.43-fold with *Psmc6* (0.05 mg/kg: P=0.010; 0.5 mg/kg: P=0.015) to 1.69-fold with *Psmb5* (0.5 mg/kg: P=0.001). In the blood, metcarbatone significantly decreased the *Psmc6* gene expression by 0.60 (0.5 mg/kg: P=0.032).

Etcarbatone

The *Psma3, Psmb5*, and *Psmc6* gene expression significantly increased in the kidney (Table 4), from 1.36-fold at the higher dose for *Psma3* (P=0.046) to 1.82-fold at the lower dose for *Psmb5* (P=0.002). No significant differences were detected in the blood.

Styrylcarbatone

Styrylcarbatone significantly increased the expression of the *Psma3, Psmb5* and *Psmc6* genes (Table 5). It was the most pronounced for *Psmc6* – up to 2.05-fold at the lower dose (P<0.0001). No significant differences were detected in the blood.

Glutapyrone

In the kidney, the higher dose of glutapyrone significantly increased *Psmb5* expression 1.73-fold (P=0.003; Table 6) and *Psmc6* expression up to 1.59-fold (P=0.004). In the blood, the lower dose of glutapyrone increased *Psmb5* expression 2.04-fold (P=0.036).

AV-153-Na

AV-153-Na significantly increased the expression of *Psma3, Psmb5, Psmc6*, and *Psma6* at both doses (Table 7) in the kidney. The higher dose resulted in the highest (2.17-fold) increase in *Psma6* (P=0.0007), while the lower dose increased it 1.91-fold (P=0.0002). *Psmc6* gene expression increased 1.61-fold at the higher dose (P=0.017), but the lower dose decreased the *Psmb5* gene expression by 0.79 (P=0.029).

AV-153-Ca

In the kidney, AV-153-Ca significantly affected only the *Psmb5* gene expression, which decreased by 0.62 at the lower dose (P=0.0043; Table 8). In the blood, *Psma6* gene expression increased 1.35-fold at the lower dose. Other tested genes were not affected significantly.

DISCUSSION

Most of the tested 1,4-DHP derivatives increased gene expression levels in the kidney but were mainly without a significant effect in the blood and liver. The general sensitivity of the kidney cells to 1,4-DHP could simply be explained by accumulation of the compounds in the kidney before excretion, but there are no data to support it.

Comparing the effects of 1,4-DHP derivatives on different proteasome subunit genes, we noticed that subunit mRNA expression did not follow a uniform pattern. Other authors have also reported divergent effects of drugs on different proteasome subunit gene expression. For example, cocaine mainly upregulated the *PSMB1* and *PSMA5* subunits and downregulated the *PSMA6* subunit but did not affect the *PSMB2* and *PSMB5* subunits (21).

In the kidney, metcarbatone, etcarbatone, styrylcarbatone, and AV-153-Na increased the expression of all analysed genes, but glutapyrone and AV-153-Ca showed varying effects. Glutapyrone did not affect the expression of *Psma3*, which codes for the outer ring subunit of the 20S

Table 3 The effect of metcarbatone on *Psma3*, *Psmb5* and *Psmc6* gene expression in the kidney and blood

	Metcarbatone (mg/kg)	Kidney fold difference (SEM range)	Blood fold difference (SEM range)
Psma3	Control	1.00 (0.93–1.07)	1.00 (0.87–1.14)
	0.05	1.44 (1.34–1.54)*	0.76 (0.56–1.04)
	0.50	1.61 (1.50–1.74)**	0.53 (0.48–0.58)
Psmb5	Control	1.00 (0.93–1.08)	1.00 (0.84–1.18)
	0.05	1.51 (1.40–1.63)**	0.55 (0.39–0.77)
	0.50	1.69 (1.60–1.79)**	0.58 (0.51–0.67)
Psmc6	Control	1.00 (0.94–1.07)	1.00 (0.90–1.12)
	0.05	1.43 (1.34–1.54)*	0.83 (0.68–1.01)
	0.50	1.43 (1.34–1.53)*	0.60 (0.55–0.64)*

*P<0.05 and **P<0.01 compared to control

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Table 4 The effect of etcarbatone on *Psma3*, *Psmb5* and *Psmc6* gene expression in the kidney and blood

	Etcarbatone (mg/kg)	Kidney fold difference (SEM range)	Blood fold difference (SEM range)
Psma3	Control	1.00 (0.93–1.07)	1.00 (0.87–1.14)
	0.05	1.46 (1.29–1.65)*	0.61 (0.52–0.70)
	0.50	1.36 (1.29–1.43)*	0.85 (0.84–0.87)
Psmb5	Control	1.00 (0.93–1.08)	1.00 (0.84–1.18)
	0.05	1.82 (1.56–2.12)**	0.45 (0.37–0.55)
	0.50	1.61 (1.52–1.71)**	0.66 (0.59–0.74)
Psmc6	Control	1.00 (0.94–1.07)	1.00 (0.90–1.12)
	0.05	1.72 (1.52–1.95)**	0.79 (0.71–0.88)
	0.50	1.62 (1.53–1.72)**	1.07 (0.99–1.17)

*$P<0.05$ and **$P<0.01$ compared to control

Table 5 The effect of styrylcarbatone on *Psma3*, *Psmb5* and *Psmc6* gene expression in the kidney and blood

	Styrylcarbatone (mg/kg)	Kidney fold difference (SEM range)	Blood fold difference (SEM range)
Psma3	Control	1.00 (0.93–1.07)	1.00 (0.87–1.14)
	0.05	1.65 (1.53–1.79)**	0.99 (0.78–1.26)
	0.50	1.53 (1.43–1.64)**	1.13 (0.93–1.37)
Psmb5	Control	1.00 (0.93–1.08)	1.00 (0.84–1.18)
	0.05	1.63 (1.39–1.90)**	0.87 (0.64–1.18)
	0.50	1.41 (1.36–1.47)*	1.18 (0.97–1.44)
Psmc6	Control	1.00 (0.94–1.07)	1.00 (0.90–1.12)
	0.05	2.05 (1.98–2.13)***	1.09 (0.90–1.32)
	0.50	1.83 (1.75–1.91)***	1.08 (0.84–1.39)

*$P<0.05$, **$P<0.01$, and ***$P<0.0001$ compared to control

proteasome, but did increase the expression of *Psmc6*, which encodes for the subunit of the 19S regulatory complex. At the higher dose glutapyrone also increased the expression of *Psmb5*, which codes for the inner ring subunit of the 20S proteasome. Interestingly, while AV-153-Na increased *Psmb5* expression in the kidney, AV-153-Ca decreased it. Furthermore, AV-153-Ca was the only compound that decreased the expression of any of the tested genes in the kidney.

In the blood, glutapyrone increased only *Psmb5* expression. An earlier study (15) reported that glutapyrone increased the mRNA expression of *Psma6*, another subunit of the outer ring of the 20S proteasome, both in the kidney and blood. Metcarbatone at the higher dose decreased *Psmc6* expression in blood.

In the liver, AV-153-Na upregulated the expression of *Psma6* and also of *Psmc6* at the higher dose but downregulated *Psmb5* at the lower dose. The higher dose of AV-153-Ca increased only *Psma6* expression.

1,4-DHP derivatives as prospective drugs have already shown antioxidant activities and a wide range of antiaging, antibacterial, anticancer, and neuroprotective actions (10). Glutapyrone, a representative of the novel group of 1,4-DHP derivatives with weak Ca^{2+} channel blocker activity has very low toxicity and multiple pharmacological properties, including concomitant effects on multiple neurotransmitter systems and antioxidant activities (22). Carbatone, another compound of this group, administered orally, showed fast absorption in the gastrointestinal tract and 62 % bioavailability. It quickly spreads across tissues and is excreted mostly through urine and faeces. This group of 1,4-DHP derivatives seems to have a very low cytotoxicity at the tested doses (unpublished data).

It also seems that the protective antioxidant activities of 1,4-DHP derivatives are achieved by targeting the mitochondria. They might be working through direct scavenging of reactive oxygen species and decomposition of hydrogen peroxide. Furthermore, they stimulate cell growth and differentiation (23). An *in vitro* study in human osteoblast-like cells treated with 1,4-DHP derivatives (23) demonstrated *de novo* glutathione synthesis, indicating the involvement of the NRF2 signalling pathway in the action of these compounds. It also suggested that the bioactivity of 1,4-DHP derivatives is associated with 4-hydroxynonenal and related second messengers of free radicals, but precise bioactivity mechanisms remain to be elucidated. The increase in proteasomal gene expression by 1,4-DHP derivatives may have similar beneficial mechanisms as those reported for antioxidants, which elevate transcription levels of 26S proteasome subunits responsible for removal of damaged proteins and attenuating the progression of human diseases related to oxidative stress (24).

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Table 6 The effect of glutapyrone on *Psma3*, *Psmb5* and *Psmc6* gene expression in the kidney and blood

	Glutapyrone (mg/kg)	Kidney fold difference (SEM range)	Blood fold difference (SEM range)
Psma3	Control	1.00 (0.93–1.07)	1.00 (0.87–1.14)
	0.05	1.10 (0.95–1.26)	1.49 (1.27–1.74)
	0.50	1.21 (1.05–1.39)	0.99 (0.86–1.14)
Psmb5	Control	1.00 (0.93–1.08)	1.00 (0.84–1.18)
	0.05	1.40 (1.26–1.55)	2.04 (1.94–2.14)*
	0.50	1.73 (1.49–2.01)**	1.66 (1.56–1.77)
Psmc6	Control	1.00 (0.94–1.07)	1.00 (0.90–1.12)
	0.05	1.46 (1.28–1.66)*	1.30 (1.19–1.42)
	0.50	1.59 (1.46–1.73)**	0.96 (0.84–1.11)

*P<0.05 and **P<0.01 compared to control

Table 7 The effect of AV-153-Na on *Psma3*, *Psmb5*, *Psmc6* and *Psma6* gene expression in the kidney and liver

	AV-153-Na (mg/kg)	Kidneys fold difference (SEM range)	Liver fold difference (SEM range)
Psma3	Control	1.00 (0.97–1.03)	1.00 (0.95–1.05)
	0.05	1.28 (1.21–1.35)*	0.94 (0.82–1.08)
	0.50	1.28 (1.12–1.46)*	1.23 (1.19–1.26)
Psmb5	Control	1.00 (0.99–1.02)	1.00 (0.94–1.04)
	0.05	1.64 (1.51–1.78)***	0.79 (0.71–0.87)*
	0.50	1.68 (1.47–1.91)***	1.09 (1.08–1.11)
Psmc6	Control	1.00 (0.97–1.03)	1.00 (0.92–1.09)
	0.05	1.39 (1.29–1.51)**	1.19 (1.07–1.33)
	0.50	1.47 (1.30–1.66)**	1.61 (1.59–1.62)*
Psma6	Control	1.00 (0.92–1.08)	1.00 (0.94–1.06)
	0.05	2.00 (1.78–2.25)***	1.49 (1.32–1.68)**
	0.50	2.17 (1.87–2.52)***	1.91 (1.86–1.95)***

*P<0.05, **P<0.01, and ***P<0.001 compared to control

The downregulation of UPS genes in the liver seems to correspond with age. In old mice, this downregulation was reported to lead to the accumulation of IκBα in the cytoplasm, which prevented the activation of the NF-κB protein, which is important for hepatocyte survival and liver health (25). Older age also seems to be associated with lower mRNA levels of both proteasome beta subunits, which are directly involved in the proteolytic function of the proteasome and antioxidant activity (26), but some healthy centenarians were reported to have proteasome subunit mRNA levels close to young donors. Furthermore, one study showed that a stable transfection of either *PSMB1* or *PSMB5* enhanced proteasome function and resistance to oxidative stress (27). This is why our findings of increased proteasome subunit gene expression by 1,4-DHP derivatives seem promising in terms of pharmacotherapy.

Downregulated proteasomal gene expression is also associated with several pathologies. For instance, in patients with schizophrenia, dentate granule neurons showed decreased expression of several proteasome subunit and other genes involved in protein processing by proteasomes and ubiquitin, resulting in a deficient ubiquitin-proteasome function that can lead to reduced neuron responsiveness (28). In patients with Parkinson's disease, both catalytic and regulatory subunits of the UPS, including the *PSMA3* gene analysed in this study, showed decreased gene expression in substantia nigra (29). This might lead to decreased levels of the 26S proteasome complex, insufficient degradation of short-lived proteins such as cyclins, and accumulation of ubiquitinated proteins, which can eventually result in dopaminergic neuronal damage. In this kind of pathologies, the potential of 1,4-DHP derivatives to increase proteasomal gene expression might lead to restored proteasome function and therapeutic effect. However, additional research is needed to determine whether 1,4-DHP derivatives increase proteasomal protein expression in the same way as they increase gene expression.

To sum up, our research has confirmed the ability of several 1,4-DHP derivatives to increase the expression of proteasome subunit genes. This might be a promising property for the development of drugs for conditions associated with impaired proteasomal functions and low mRNA levels of proteasome subunits.

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Table 8 The effect of AV-153-Ca on *Psma3, Psmb5, Psmc6* and *Psma6* gene expression in the kidney and liver

	AV-153-Ca (mg/kg)	Kidney fold difference (SEM range)	Liver fold difference (SEM range)
Psma3	Control	1.00 (0.97–1.03)	1.00 (0.95–1.05)
	0.05	0.89 (0.76–1.03)	0.96 (0.94–0.99)
	0.50	1.00 (0.92–1.09)	0.89 (0.81–0.97)
Psmb5	Control	1.00 (0.99–1.02)	1.00 (0.94–1.04)
	0.05	0.62 (0.55–0.71)**	1.05 (1.03–1.08)
	0.50	0.85 (0.75–0.95)	0.82 (0.71–0.95)
Psmc6	Control	1.00 (0.97–1.03)	1.00 (0.92–1.09)
	0.05	0.85 (0.77–0.93)	0.89 (0.86–0.93)
	0.50	0.90 (0.84–0.96)	0.96 (0.88–1.05)
Psma6	Control	1.00 (0.92–1.08)	1.00 (0.94–1.06)
	0.05	1.03 (0.96–1.11)	0.87 (0.82–0.93)
	0.50	0.98 (0.89–1.07)	1.35 (1.22–1.48)*

*$P<0.05$ and **$P<0.01$ compared to control

Conflicts of interest

The authors declare that they have no conflict of interest.

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1,4 dihidropiridinski derivati povećavaju ekspresiju gena *Psma3*, *Psmb5* i *Psmc6* u glasničkoj RNA štakora

Ubikvitin-proteasomski sustav utječe na različite funkcije bjelančevina i stanica. Poremećaji u njegovoj regulaciji mogu dovesti do poremećaja u proteostazi koji su povezani s nastankom različitih bolesti i sa starenjem, ali se sustav može regulirati u liječenju pojedinih bolesti lijekovima poput 1,4 dihidropiridinskih (1,4 DHP) derivata, koji štite živčani sustav i usporavaju starenje. Cilj ovoga istraživanja bio je utvrditi djelovanje nekoliko 1,4 DHP derivata na ekspresiju glasničke RNA (mRNA) u proteasomskim genima *Psma3*, *Psmb5* i *Psmc6* u više štakorskih organa. Štakori su tri dana dobivali oralne doze metkarbatona, etkarbatona, glutapirona, stirilkarbatona, AV-153-Na ili AV-153-Ca, a genska se ekspresija u mRNA utvrdila polimeraznom lančanom reakcijom u stvarnom vremenu (engl. *real-time polymerase chain reaction*, krat. PCR). Osim toga, utvrdili smo djelovanje derivata AV-153-Na i AV-153-Ca na ekspresiju gena *Psma6*. U bubrezima su metkarbaton, etkarbaton, stirilkarbatoni AV-153-Na povećali ekspresiju svih analiziranih gena. Glutapiron je povećao ekspresiju *Psmb5* i *Psmc6*, ali ne i *Psma3*. U krvi je glutapiron povećao gensku ekspresiju *Psmb5*. U jetrima je AV-153-Na povećao ekspresiju *Psma6* i *Psmc6,* istodobno smanjivši ekspresiju *Psmb5*. AV-153-Ca utjecao je samo na *Psma6*, povećavši mu ekspresiju. Sposobnost 1,4-DHP derivata da povećaju gensku ekspresiju proteasomskih podjediničnih proteina obećava u smislu mogućnosti liječenja bolesti povezanih s poremećenom proteasomskom funkcijom, no nužna su daljnja istraživanja.

KLJUČNE RIJEČI: AV-153-Ca; AV-153-Na; etkarbaton; glutapiron; metkarbaton; stirilkarbaton; poremećena proteasomska funkcija; proteasomske podjedinice; ubikvitin-proteasomski sustav