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FULL PAPER

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Phosphonic Analogs of Alanine as Acylpeptide Hydrolase Inhibitors

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Acylpeptide hydrolase is a serine protease, which, together with prolyl oligopeptidase, dipeptidyl peptidase IV and oligopeptidase B, belongs to the prolyl oligopeptidase family. Its primary function is associated with the removal of N-acetylated amino acid residues from proteins and peptides. Although the N-acylation occurs in 50–90% of eukaryotic proteins, the precise functions of this modification remains unclear. Recent findings have indicated that acylpeptide hydrolase participates in various events including oxidized proteins degradation, amyloid β-peptide cleavage, and response to DNA damage. Considering the protein degradation cycle cross-talk between acylpeptide hydrolase and proteasome, inhibition of the first enzyme resulted in down-regulation of the ubiquitin-proteasome system and induction of cancer cell apoptosis. Acylpeptide hydrolase has been proposed as an interesting target for the development of new potential anticancer agents. Here, we present the synthesis of simple derivatives of (1-aminoethyl)phosphonic acid diaryl esters, phosphonic analogs of alanine diversified at the N-terminus and ester rings, as inhibitors of acylpeptide hydrolase and discuss the ability of the title compounds to induce apoptosis of U937 and MV-4-11 tumor cell lines.

Keywords: acylpeptide hydrolase, protease inhibitors, apoptosis, 1-aminoalkylphosphonates, anticancer agents..

Introduction

Acylpeptide hydrolase (APH; EC 3.4.19.1), also known as acylaminoacyl-peptidase (AAP), is a serine protease, which, together with prolyl oligopeptidase (POP), dipeptidyl peptidase IV (DPPIV) and oligopeptidase B (OB), belongs to the prolyl oligopeptidase family (clan SC, family S9).[1–3] APH primarily removes N-acetylated amino acids residues and thus regulates the cellular level of N-terminal acetylation of proteins.[4] Although this post-translational modification occurs in 50–90% of eukaryotic proteins and N-acetylated proteins show higher stability *in vivo* as compared to their non-acetylated counterparts,[5–7] the precise function of this process has not been fully understood. APH also displays a peptidase activity on oxidized and glycated proteins,[8,9] it participates in the cellular response to DNA damage,[10] cleaves amyloid β-peptide,[11,12] while its overexpression results in crystallin degradation facilitating cataract development and it regulates the activity of proteasome.[13,14] Since APH is involved in the protein degradation cycle, its cellular communication with proteasome allows the cell to discard cytotoxic denatured proteins.[15] Interestingly, APH was found to be more sensitive than acetylcholinesterase to some organophosphorus compounds, which suggests its potential role in cognitive functions of the brain.[16–19] Some researchers have demonstrated that the deficiency of human APH is associated with various carcinomas malignancies.[20,21] On the other hand, it has been shown that inhibition of APH induces apoptosis.[22,23] Recent findings have demonstrated that APH inhibition induces down-regulation of the ubiquitin-proteasome system (UPS).[14,24] Although the molecular relationship between APH and UPS is yet to be discovered, APH has been considered

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as an interesting target for new anticancer molecules development.

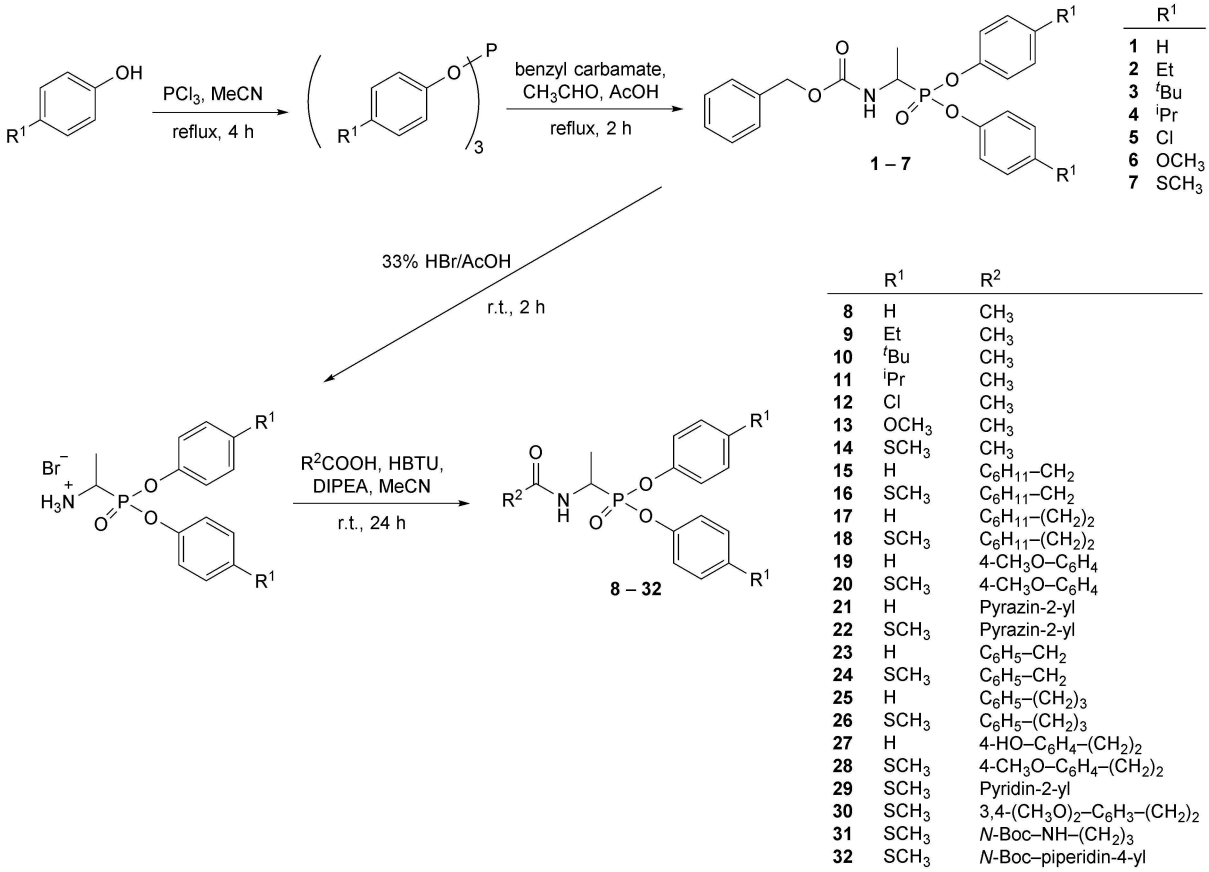
Since diaryl esters of 1-aminoalkylphosphonates (1-AAPs) were obtained about 40 years ago,[25] which was followed by the discovery of their ability to inhibit serine proteases,[26,27] they have been widely used to target many human (e.g., DPPIV,[28] thrombin,[29] proteinase 3,[30] neutrophil elastase,[31] cathepsin G,[32] matriptase-2[33]), bacterial (e.g., SufA,[34] SplA,[35] GluC[36]), and viral proteases (e.g., WNV,[37] HCV,[38] ZIKV[39]). The most essential attribute of diaryl esters of 1-aminoalkylphosphonates as inhibitors is their complete lack of reactivity with cysteine, threonine, aspartyl and metalloproteases.[40] The selectivity and reactivity of these compounds with the target protease can be increased by introducing a peptide chain or by modifying the ester ring structures to change the electrophilicity of the phosphorus atom.[41] They also have a few disadvantages, e.g., relatively low solubility, cell membrane permeability or bioavailability.[42] Despite these limitations these compounds are very stable in plasma and buffers. They were found useful in catalytic antibodies development.[43] Due to such functional and structural diversity, these compounds can serve as the activity-based probes for an *in vivo* study of protease function or for cellular localization of proteases in living cells.[44,45]

The method for the 1-APPs synthesis originally developed by Oleksyszyn *et al.* relies on the α-amidoalkylation reaction of triphenyl phosphite with benzyl carbamate and appropriate aldehyde.[25] Since then, several new synthetic methods – among them the use of Lewis or Brønsted acids – have been developed.[46,47,48] The Cbz group can be easily removed either with HBr/AcOH or *via* hydrogenolysis (Pd/C), allowing for further coupling with amino acids, peptides, or proteins. Although the application of these methods leads to 1-APP obtained as racemic mixtures, various chromatographic techniques have been found useful in separation of single diastereoisomers of 1-APP peptidyl derivatives.[29,31]

The development of APH inhibitors is not very advanced, which is quite surprising considering a broad spectrum of APH's biological function. Only few APH inhibitors have been described so far including chloromethylketones, peptides and linoleic acid isomers.[14,22–24] Although a number of 1-APPs have been designed toward many serine proteases including DPPIV of the prolyl oligopeptidase family, there was no account on their application to block the activity of APH. Here we report the inhibitory activity of simple phosphonic analogs of alanine as APH inhibitors. Considering the highest activity of APH toward substrates containing N-acetylated terminal alanine residue,[49,50] we have synthesized and evaluated the inhibitory potential of phosphonic alanine derivatives containing structurally diverse N-terminal acyl functions and different phenyl ester rings against APH isolated from bovine liver (APH^{PL}) and U937 cell lines-derived APH (APH^{U937}) and suggested their potential to induce the apoptosis in selected cancer cell lines.

Results and Discussion

Considering the substrate specificity of APH toward *N*-acetylalanine, we have synthesized a series of diaryl esters of 1-aminophosphonic acid derivatives (*Scheme 1*; **1**–**32**), analogs of alanine having different N-acyl groups and phenyl ester rings (*Table 1*). Target phosphonates were synthesized *via* an amidoalkylation reaction applying the method originally developed by Oleksyszyn *et al.*[25] Hydrobromide salts of the obtained 1-aminoalkylphosphonate diaryl esters were next acylated with different carboxylic acids using HOBT as the coupling agent in the presence of DIPEA. All of the compounds were further analyzed in order to evaluate their inhibitory potential toward APH. APH of two origin was used (APH^{PL}, isolated from porcine liver and APH^{U937}, form U937 cell lines lysate). Most of synthesized compounds displayed low potency of action toward both APHs. The highest activity was observed for a series of N-acetylated phosphonic alanines, where Ac-$Ala^{P}(OC_6H_4\text{-}4\text{-}SCH_3)_2$ (**14**) displayed the lowest IC_{50} values of 26 μM and 20 μM toward APH^{PL} and APH^{U937}, respectively. Replacing the methylthio group in **14** by a chlorine atom resulted in an approximately ten-fold decrease in the inhibitor's potency of action (**12**). From the group of Cbz-protected alanine derivatives, Cbz-$Ala^{P}(OC_6H_4\text{-}4\text{-}OCH_3)_2$ ($IC_{50} = 250$ μM) and Cbz-$Ala^{P}(OC_6H_4\text{-}4\text{-}Cl)_2$ ($IC_{50} = 190$ μM) showed the highest activity toward APH^{U937} while none of Cbz-analogs showed any activity toward APH^{PL}. As the highest activity was observed for 4-methylthio-substituted esters, this group was selected for further derivatives synthesis. APH from porcine liver was significantly (with minor exceptions) less susceptible to inhibition by synthesized inhibitors than APH^{U937}. Since the crystal structures of both, porcine and human APHs have not been solved yet, it could only be speculated that the structural differences between two APHs tested here result in different inhibitors accessibility. Although synthesized compounds display relatively low potency



	R^1
1	H
2	Et
3	^{t}Bu
4	^{i}Pr
5	Cl
6	OCH_3
7	SCH_3

	R^1	R^2
8	H	CH_3
9	Et	CH_3
10	^{t}Bu	CH_3
11	^{i}Pr	CH_3
12	Cl	CH_3
13	OCH_3	CH_3
14	SCH_3	CH_3
15	H	C_6H_{11}–CH_2
16	SCH_3	C_6H_{11}–CH_2
17	H	C_6H_{11}–$(CH_2)_2$
18	SCH_3	C_6H_{11}–$(CH_2)_2$
19	H	4-CH_3O–C_6H_4
20	SCH_3	4-CH_3O–C_6H_4
21	H	Pyrazin-2-yl
22	SCH_3	Pyrazin-2-yl
23	H	C_6H_5–CH_2
24	SCH_3	C_6H_5–CH_2
25	H	C_6H_5–$(CH_2)_3$
26	SCH_3	C_6H_5–$(CH_2)_3$
27	H	4-HO–C_6H_4–$(CH_2)_2$
28	SCH_3	4-CH_3O–C_6H_4–$(CH_2)_2$
29	SCH_3	Pyridin-2-yl
30	SCH_3	3,4-$(CH_3O)_2$–C_6H_3–$(CH_2)_2$
31	SCH_3	*N*-Boc–NH–$(CH_2)_3$
32	SCH_3	*N*-Boc–piperidin-4-yl

Scheme 1. General synthesis of 1-aminophosphonic acid derivatives (**1**–**32**).

of action, their activity is comparable to that of the most potent APH inhibitors reported up to date such as chloromethyl ketones (Ac-L-Leu-CMK; IC_{50} (APH^{U937}) = 15 μM),[23] peptides (SsCEI 4; IC_{50} (APH^{PL}) = 84 ± 16 μM)[14] or linoleic acid isomers (t10,c12-CLA; IC_{50} (APH^{PL}) = 105 ± 23 μM).[14]

The selected compounds most active in enzymatic assays were further evaluated for their antiproliferative activity *in vitro* using two cancer cell lines – U937 and MV-4-11 (*Table 2*). In comparison to cisplatin and two known inhibitors of proteasome – MG-132 (Z-Leu-Leu-Leu-CHO) and Bortezomib (Pyz-Phe-boroLeu) the compounds exhibited weak (**12**) to moderate (**7**, **14**) antiproliferative activity. The most pronounced differences between cell lines were observed for compounds **6** for which the IC_{50} value on MV-4-11 was over two times higher than for U937.

Further studies showed that compounds **7** (IC_{50} values of 26.8 ± 7.4 μM and 23.1 ± 1.9 μM toward U937 and MV-4-11, respectively) and **14** (IC_{50} = 20.1 ± 8.6 μM for U937 and 51.5 ± 6.7 μM for MV-4-11) are relatively potent inducers of caspase-3 activity, which is a direct indicator of apoptosis induction (*Figure 1*). Both compounds induced caspase-3 activity in a dose-dependent manner and the observed activity was statistically higher in comparison to control starting from 50 μM concentrations. Compound **14** showed significantly higher activity in U937 than in MV-4-11, which corresponds to the results of their antiproliferative activity. Compounds **5** and **12** showed limited influence on caspase-3 activity. The observed discrepancies in compounds' activity as APH inhibitors and antiproliferative agents might indicate that APH might not be their major intracellular target.

Conclusions

The reported compounds which belong to a class of serine protease inhibitors showed ability to inhibit APH activity. Although the inhibitory potency of synthesized compounds is relatively low (IC_{50} values in a micromolar range), they were able to induce apoptosis in U937 and MV-4-11 tumor cell lines. This

Table 1. Inhibitory activity of obtained (1-aminoethyl) phosphonic acid diaryl esters toward APH^{PL} and APH^{U937}.

Compound	APH^{PL} inhibition [%][a]	IC_{50} [μM]	APH^{U937} inhibition [%][a]	IC_{50} [μM]
1	$<5^{\#}$	–	29	–
2	$<5^{\#}$	–	34	–
3	$<5^{\#}$	–	30	–
4	$<5^{\#}$	–	23	–
5	$<5^{\#}$	–	50	250
6	5	–	65	190
7	<5	–	47	–
8	6.5	–	21	–
9	12	–	22	–
10	$<5^{\#}$	–	33	–
11	9	–	37	–
12	48	–	48	–
13	16	–	34	–
14	**99**	**26**	**99**	**20**
15	17	–	36	–
16	<5	–	39	–
17	18	–	57	219
18	<5	–	36	–
19	13	–	43	–
20	<5	–	49	–
21	<5	–	48	–
22	<5	–	49	–
23	9	–	40	–
24	<5	–	46	–
25	6	–	34	–
26	<5	–	39	–
27	17	–	69	180
28	<5	–	46	–
29	<5	–	40	–
30	<5	–	37	–
31	<5	–	32	–
32	<5	–	44	–

[a] Percentage of inhibition after 15 min incubation of APH with tested inhibitor (250 or 50 μM[#]).

class of inhibitors has never been examined toward their ability to block APH's activity thus the presented preliminary data might induce further, more advanced research on the development of novel specific APH inhibitors of this class with potential anticancer properties.

Experimental Section

Chemical Synthesis

All reagents were purchased from Sigma-Aldrich (Poznan, Poland). The solvents were from POCh (Gliwice, Poland). Triaryl phosphites were synthesized as described previously.[41] Briefly, phosphorus trichloride (10 mmol) was added to a solution of substituted phenol (30 mmol) in acetonitrile (50 mL) and the mixture was refluxed for 8 h. The volatile elements of the mixture were removed in vacuum and the resulting crude product was used directly in the next step for the synthesis of Cbz-protected 1-aminoalkylphosphonate diaryl esters. The crude triaryl phosphite was dissolved in glacial acetic acid (25 mL) followed by the addition of an aldehyde (11 mmol) and benzyl carbamate (11 mmol). The mixture was heated at 80–90 °C for 2 h. Volatile elements were removed under reduced pressure, the resulting oil was dissolved in methanol and left at −20 °C for crystallization.[25] Next, the Cbz-group was removed with 33% HBr in acetic acid solution. Cbz-protected 1-aminoalkylphosphonate diaryl ester (10 mmol) was dissolved in a 33% HBr/AcOH solution (4 mL). The reaction was performed at room temperature for 2 h. The volatile components of the mixture were removed under reduced pressure and the product was crystallized from diethyl ether. For the preparation of N-acyl derivatives of 1-aminoalkylphosphonate diaryl esters, HBTU was used as a coupling agent. The hydrobromide salt of 1-aminoalkylphosphonate diaryl ester (10 mmol) was suspended in acetonitrile (25 mL) followed by the addition of *N*,*N*′-diisopropylethylamine (25 mmol), ap-

Table 2. Antiproliferative activity of most active compounds assessed *in vitro* on two cancer cell lines after 72 h of incubation.

Compound	$IC_{50} \pm SD$ [μM], $n=3$ U937		MV-4-11	
12	[32]%[a]	±3	**88.7**	±5.5
14	**20.1**	±8.6	**51.5**	±6.7
7	**26.8**	±7.4	**23.1**	±1.9
5	**42.1**	±9.8	**41.4**	±2.4
MG-132	**0.49**	±0.18	**0.13**	±0.07
Bortezomib	**0.031**	±0.008	**0.006**	±0.001
Cisplatin	**1.1**	±0.2	**1.4**	±0.3

[a] Reported values are mean cell proliferation inhibition ±SD at highest concentration used [100 μM].

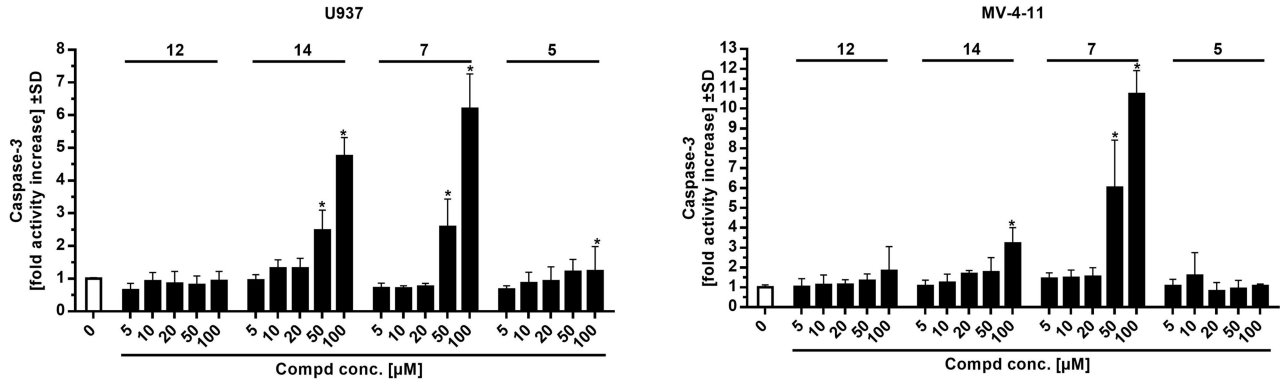


Figure 1. Caspase-3 activity assessed *in vitro* on two cancer cell lines after 24 h of incubation with most active compounds and are presented as fold change in caspase-3 activity ±SD; * – $p < 0.05$, one-way ANOVA with Dunnett's post-test to untreated control, $n = 4$.

propriate carboxylic acid (10 mmol) and HBTU (12 mmol). The reaction was performed at room temperature for 24 h. Next, the solvent was evaporated and the resulting oil was dissolved in ethyl acetate (100 mL) and washed with 5% $NaHCO_3$, 5% $KHSO_4$ and brine. The final compounds were purified by HPLC (Varian ProStar HPLC System, Palo Alto, California, USA) equipped with C8 column 250× 21.2 mm, 10 μm (Supelco, Bellefonte, USA) using 5–100% in 20 min gradient of A in B, where A: H_2O with 0.05% TFA, B: CH_3CN with 0.05% TFA. The purity and mass of compounds was confirmed using UltiMate 3000 HPLC System with ISQ EC Mass Spectrometer (Thermo Scientific, Waltham, Massachusetts, USA) equipped with Uptisphere Strategy C18HQ column 250×4.6 mm, 5 μm (Interchim, Montluçon, France) using 5–95% in 20 min gradient of A in B, where A : H_2O with 0.1% HCOOH, B: CH_3CN with 0.1% HCOOH (Supporting Material).

Cell Lines

The MV-4-11 (human biphenotypic B myelomonocytic leukemia) and U937 (human histiocytic lymphoma) were purchased from the American Type Culture Collection (ATCC; Rockville, USA) and are maintained at Hirszfeld Institute of Immunology and Experimental Oncology (HIIET, Wroclaw, Poland). The MV-4-11 and U937 cell lines were cultured in RPMI-1640 medium w/ GlutaMAX® (Thermo Fisher Scientific, Warsaw, Poland) supplemented with 10% fetal bovine serum (FBS; GE Healthcare HyClone, Logan, USA), additionally supplemented with 1 mM sodium pyruvate (Sigma-Aldrich, Poznań, Poland) for MV-4-11. All culture media contained antibiotics – 100 U/mL penicillin and 100 μg/mL streptomycin (both Polfa-Tarchomin, Warsaw, Poland). All cell lines were cultured in a humidified atmosphere at 37 °C with 5% CO_2 and passaged twice a week.

Enzymatic Studies

The APH (APH^{PL}) was isolated in house from porcine liver based on the method described by Kiss *et al.*[51] Fresh porcine liver obtained from a local butcher (2 kg) was homogenized in 50 mM phosphate buffer, pH 7.2, containing EDTA (1 mM), 2-mercaptoethanol (5 mM) and Na_2SO_3 (5 mM). Next, the homogenate was heated to 64 °C for 7 min, cooled on ice and centrifuged (4750 *g*, 1 h, 4 °C). The obtained supernatant was supplemented with ammonium sulfate up to 20% and stirred for 30 min at 4 °C. The precipitate was centrifuged (4750 *g*, 1 h, 4 °C) and dissolved in 50 mM phosphate buffer, pH 7.2, containing EDTA (1 mM) and 2-mercaptoethanol (5 mM). The purification was performed on DEAE-Sepharose column (CL6B; AKTAprime plus, Pittsburgh, USA) equilibrated with 50 mM phosphate buffer, pH 7.2, containing EDTA (1 mM) and 2-mercaptoethanol (5 mM). The enzyme was eluted with the linear salt gradient (0–1 M NaCl supplemented with 0.1 M ammonium acetate). The enzymatic activity at each step of isolation was evaluated in 10 mM PBS buffer (pH 7.4) using Ac-Ala-pNA as the substrate (synthesized in house). Fractions displaying enzyme activity were combined, dialyzed against 20 mM phosphate buffer, pH 7.2. and concentrated in Amicon Ultra-15 unit (MWCO 30 kDa; Merck Millipore, Darmstadt, Germany). The enzymatic activity was assayed with Ac-Ala-pNA (4 mM) using 5 μg/mL of APH in 10 mM PBS buffer (pH 7.4).

The activity of the obtained inhibitors toward APH^{U937} was performed according to the procedure described by Yamaguchi *et al.*[23] U937 cells were solubilized with PBS supplemented 0.05% Triton X-100 followed by centrifugation (13,000 *g*, 5 min. The obtained supernatant was assayed for APH^{U937} activity with Ac-Ala-pNA (4 mM, synthesized in house) and 50 μg/mL of cell lysate (calculated for the total protein content; protein content was determined by the micro BCA™ assay (Pierce, Gdańsk, Poland) containing U937-derived APH in 10 mM PBS buffer (pH 7.4) at room temperature.

Inhibition Studies

The inhibitory potency of synthesized compounds toward APHs was evaluated in PBS buffer, using Ac-Ala-pNA (4 mM) as a substrate and 5 μg/mL of APH (isolated from porcine liver) or 50 μg/mL (calculated for the total protein content) of U937-derived APH. All tested inhibitors were dissolved in DMSO (10 mM). Final DMSO concentration in the assay did not exceed 2.5%. The progress of the enzymatic reaction was monitored at 405 nm at room temperature for 60 min. The IC_{50} values were calculated from the plot of the percent of inhibition vs. inhibitor concentration. For inhibitors of low potency of action, the percent of APH inhibition at 50 μM or 250 μM inhibitor concentration was calculated in relation to control sample without inhibitor.

Compound's antiproliferative activity by MTT assay was performed as previously described.[52] 24 h after seeding the cells (10^4 cells/well) in 96-well plates (Sarstedt, Nümbrecht, Germany), tested compounds at concentrations ranging from 100 to 1 μM were added. Cisplatin (Ebewe, Unterach am Attersee, Austria) as well as two proteasome inhibitors – MG-132 and Bortezomib (Sigma-Aldrich, Poznan, Poland) were used as the reference compounds. After 72 h treatment, plates underwent MTT protocol and the absorbance was recorded at 570 nm with a Biotek Hybrid H4 Reader (Biotek Instruments, Bad Friedrichshall, Germany).[53] Compounds at each concentration were tested in triplicate in a single experiment, and each experiment was repeated at least three times independently. The results are presented as the mean cell proliferation inhibition or IC_{50} (half-maximum inhibitory concentration) ± standard deviation (SD) calculated by means of the Prolab-3 system based on the Cheburator 0.4 software.[54]

Apoptosis evaluation by caspase3/7 activity assay was performed as previously described.[52] 24 h after seeding the cells (10^5 cells/well) on 24-well plates (Sarstedt, Nümbrecht, Germany), the tested compounds at various concentrations were applied for 24 h. Then, cells were lysed applying ice-cold lysis buffer (50 mM HEPES, 10% (*w/v*) sucrose, 150 mM NaCl, 2 mM EDTA, 1% (*v/v*) Triton X-100, pH 7.3). Next, 25 μL of each sample was transferred to an opaque 384-well plate (Corning, NY, USA) containing 75 μL of the reaction buffer (10 μM Ac-DEVD-AMC, 20 mM HEPES, 10% (*w/v*) sucrose, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, pH 7.3) and the fluorescence was continuously recorded at 37 °C for 2 h with a Biotek Synergy H4 Hybrid Reader (λ_{ex} 350 nm, λ_{em} 460 nm) and used to calculate the maximal velocity of the reaction. Compounds at each concentration were tested in duplicates in a single experiment and each experiment was repeated at least three times. Results were normalized by means of the MTT method and are reported as mean relative caspase-3/7 activity compared to the untreated control.

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Author Contribution Statement

All the authors have contributed significantly to the submitted work. J.O. and M.S. conceived and designed the experiments. M.W., A.C., S.O., M.P., Ł.W., and K.T. performed the experiments and analyzed the data. M.W. and M.S. analyzed the data and wrote the article.

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