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Research Article

The Synthesis of 1,3,5-triazine Derivatives and JNJ7777120 Analogues with Histamine H_4 Receptor Affinity and Their Interaction with *PTEN* Promoter

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The involvement of histamine and H_4 receptor (H_4R) in cancer has been investigated recently using the H_4R agonists and antagonists. The scope of the research project was synthesis and exploration of the consequences of a group of compounds with histamine H_4 receptor (H_4R) affinity on the promoter of *PTEN* gene encoding the antitumor PTEN protein. The series of novel compounds based either on H_4R antagonists JNJ7777120 structure or 1,3,5-triazine scaffold were synthesized, evaluated for histamine H_4R affinity and used in this study. Compounds 5 and 7 belonging to the group of JNJ7777120 analogues showed the highest interaction with the promoter of PTEN gene and weak affinity against H_4R with K_i value >100 μM. These compounds showed no significant effect on neuroblastoma IMR-32 cells viability indicating no correlation between PTEN gene promoter affinity and antitumor activity. Compound 6, another JNJ7777120 analogue, showed the highest effect on IMR-32 viability with calculated IC_{50} = 23.27 μM. The 1,3,5-triazine derivatives exhibited generally low or medium interaction with PTEN gene promoter. However, the 1,3,5-triazine derivative 11 with the para-bromo substituent showed the highest affinity against H_4R with K_i value of 520 nM and may be considered as a new lead structure.

Key words: 1,3,5-triazines, histamine H_4 receptor ligands, JNJ7777120 analogues, *PTEN* promoter

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Phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) is one of the most frequently mutated tumor suppressor genes in human cancer. In 1997 *PTEN*, also known as *MMAC1* (mutated in multiple advanced cancers-1) or *TEP1* (tensin-like phosphatase-1), was identified by three independent research groups as a tumor suppressor gene located at the 10q23 region (1–3). The protein encoded by *PTEN* is a phosphatase (PTEN, EC 3.1.3.67), member of the large protein tyrosine phosphatase family. It is a non-redundant, plasma membrane phosphatase with a relative molecular mass of 47 kDa and characterized as a dual specificity phosphatase. Although it can dephosphorylate proteins, its primary targets are highly specialized membrane lipids, mainly facilitating the removal of the phosphate group from the inositol rings. More specifically, PTEN acts on the phosphatidylinositol-3,4,5-triphosphate (PIP3) that is formed through the action of phosphoinositide-3 lipid kinase (PI3K). PIP3 is an important lipid second messenger in tumorigenesis. PTEN reduces the pool of PIP3, inhibiting growth and survival signals and suppressing tumor formation. This way, an 'on-off' switch is evolved that appears to regulate a critical signaling pathway for oncogenesis (4).

Histamine [2-(4-imidazolyl)-ethylamine] (HIS) is one of the most important biogenic amines and it was first identified as a mediator in biological functions in the early 1900s. HIS liberation is a recognized feature of various allergic conditions and for many years its importance was limited to that field. However, HIS is involved in the regulation of numerous physiological and pathophysiological processes including gastric acid secretion, hypersensitivity reactions, bronchial asthma, multiple elements of immune regulation and tumor genesis (5–7). HIS performs its activation via different types of histamine receptors (HR). Four metabotropic HR types have been cloned so far (H_1R, H_2R, H_3R, and H_4R). These receptors belong to the rhodopsin-like family of G protein coupled receptors. Histamine H_4R is

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the latest identified member of the HIS receptor subfamily. The H_4R is widely distributed, especially in organs associated with the immune system. It is preferentially expressed in intestinal tissue, spleen, thymus, medullary cells, bone marrow, and peripheral hematopoietic cells, including eosinophils, basophils, mast cells, T lymphocytes, leukocytes, and dendritic cells. These cell types are primarily involved with the development and continuation of allergic responses. The human H_4R, reported as a sequence of 390 amino acid residues, possesses all of the highly conserved sequence motifs of the class A of the G protein-coupled receptor (GPCR) superfamily. The H_4R is coupled to $G_{i/o}$ proteins and shows higher endogenous ligand affinity than the H_1 and H_2 receptors (8). The *H_4R* gene is located on chromosome 18q11.2. The amino acid sequence of the H_4R shares approximately 26%, 27%, and 58% homology in the transmembrane regions with the H_1, H_2, and H_3 receptors respectively (9).

Although little is known about the physiology of the H_4R, its role in colorectal cancer, melanoma, human breast cancer has been recently explored using the H_4R agonists and antagonists. In several studies, different carcinoma cells showed varying expression of H_4R and this might indicate that H_4R may play a special role in the development of human breast neoplasms. The HIS may regulate through H_4R proliferation of mammary epithelial cells, and a loss of that control may contribute to neoplasia (10,11).

Moreover, an interestingly number of studies have shown that up-regulation of the enzyme L-histidine decarboxylase (HDC; E.C. 4.1.1.22), responsible for HIS synthesis from its precursor histidine, is correlated with growth of several types of human tumors such as: melanoma (12), small cell lung carcinoma (13), breast carcinoma (14), endometrial cancer (15), and colorectal carcinoma (16). The structural analysis revealed that natural compounds might be able to inhibit HIS synthesis by binding to specific regions of HDC (17). All of recent results indicate that HDC can be a potential target for therapeutic intervention of many inflammatory diseases, some neurological and neuroendocrine diseases, osteoporosis and even different carcinoma types and neuroendocrine tumors (18).

Much of the drug research in H_4R field is focused on the search for H_4R antagonists because of possibilities for the treatment of different inflammatory conditions, e.g. skin diseases, airway diseases or bowel diseases (19–21). However, preclinical results show also utility of H_4R ligands in cancer, neuropathic pain or vestibular disorder (20). For years JNJ7777120 (1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine; Table 1), the first potent and selective H_4R ligand, has been used as a reference antagonist in *in vitro* and *in vivo* studies (22). Recently, JNJ7777120 has been identified as partial agonist in a β-arrestin recruitment assay (23). On the basis of JNJ7777120 structure varieties of structurally diverse classes of compounds have been developed (20,24). Among them azine derivatives, especially with pyrimidine moiety are important group (25). Recently, some derivatives of 1,3,5-triazine showed H_4R nanomolar affinities (25–27).

The scope of the research was the synthesis and exploration of the consequences of a group of compounds obtained as ligands of histamine H_4R modifying JNJ7777120 structure or based on 1,3,5-triazine scaffold, on the promoter of *PTEN* gene encoding the antitumor PTEN protein. Next, the antiproliferative assay was applied to determine the influence of the selected compounds on a human embryonic kidney HEK-293 and neuroblastoma IMR-32 cell lines viability.

Methods and Materials

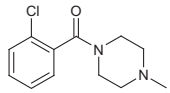
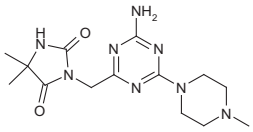
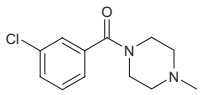
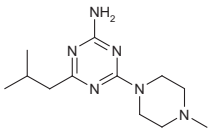
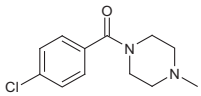
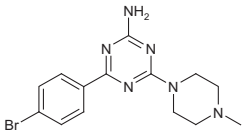
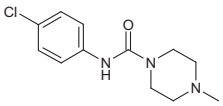
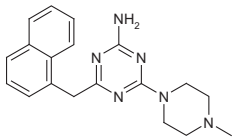
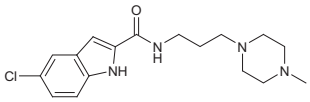
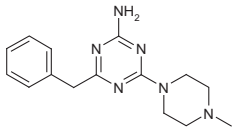
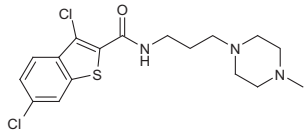
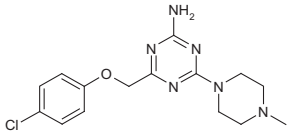
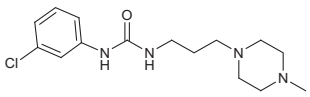
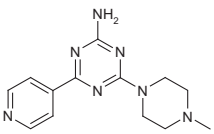
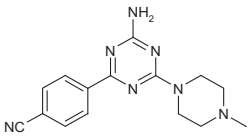
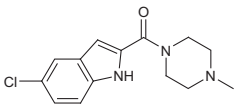
Chemistry

Melting points were determined on MEL-TEMP II apparatus and are uncorrected. IR spectra were measured as KBr pellets on FT Jasco IR spectrometer (JASCO, Easton, MD, USA). Mass spectra (LC/MS) were performed on Waters TQ Detector mass spectrometer (Waters, Milford, MA, USA). ^{1}H-NMR and ^{13}C NMR spectra were recorded on Varian-Mercury 300 MHz spectrometer in DMSO-d_6. Chemical shifts in ^{1}H-NMR spectra are expressed in ppm downfield from deuterated solvent signal treated as reference. Data are reported in the following order: multiplicity (br, broad; def, deformed; s, singlet; d, doublet; t, triplet; m, multiplet; Im, imidazole, Bzt, benzothiophene Naph, naphthalene, Ph, phenyl; Pp, piperazine; Py, pyridine, Tr, triazine), approximate coupling constants J in Hertz (Hz), number of protons. LC- MS were carried out on a system consisting of a Waters Acquity UPLC, coupled to a Waters TQD mass spectrometer. Retention times (t_R) are given in minutes. The UPLC/MS purity of all final compounds was determined (%). Elemental analyses (C, H, N) were measured on Elemental Vario-EL III instrument and are within ±0.5% of the theoretical values. TLC was carried out using silica gel F254 plates (Merck, Darmstadt, Germany). The spots were visualized with Dragendorff's reagent or by UV absorption at 254 nm. Abbreviations: AcOEt (ethyl acetate), CDI (carbonyldiimidazole), CH_3CN (acetonitrile), Et_2O (diethyl ether).

Amide and urea derivatives

(2-Chlorophenyl)(4-methylpiperazin-1-yl)methanone (1). To the solution of 2-chlorobenzoyl chloride (1.75 g, 10 mmol) in 30 mL of benzene 4-methylpiperazine (1.00 g, 10 mmol), natrium carbonate (6.36 g, 60 mmol) and 20 mL of water was added and stirred in the room temperature for 12 h. The solid was filtered off, separated and the organic phase was washed with 30 mL of 0.5% NaOH and water. After drying with Na_2SO_4, the filter was evaporated to dryness. The product was crystallized from Et_2O. White solid. Mp. 60–63 °C. Yield: 29%. $C_{12}H_{15}N_2OCl$ (MW = 238.71) (lit. (28)) $C_{12}H_{15}N_2OCl$ x HCl

Table 1: Structures and histamine H_4R affinities of synthesized compounds

Compound		Histamine H_4R $K_i \pm$ SE [μM]	Compound		Histamine H_4R $K_i \pm$ SE [μM]
1		>100	**9**		14.61 ± 1.78
2		3.88[a]	**10**		8.26 ± 1.50
3		7.55[a]	**11**		0.52 ± 0.16[b]
4		3.52[a]	**12**		5.60 ± 1.32
5		>100	**13**		7.50 ± 1.37
6		>100	**14**		10.61 ± 1.33
7		>100	**15**		3.19 ± 0.02
8		2.36 ± 0.90[b]	**JNJ7777120**		0.0045[c]

[a]K_i values were determined in duplicates in one experiment;
[b]Data from (27);
[c]Data from (22).

Mp. 292–294 °C). Anal. Calcd for $C_{12}H_{15}N_2OCl$: C60.37; H6.33; N11.73; Found: C60.14; H6.64; N11.62.

(3-Chlorophenyl)(4-methylpiperazin-1-yl)methanone (2). Synthesized as **1** from 3-chlorobenzoyl chloride (1.75 g, 10 mmol). White solid. Mp. 75–77 °C. Yield: 49%. $C_{12}H_{15}N_2OCl$ (MW = 238.71) ^{1}H-NMR [DMSO-d_6] δ (ppm): 7.39–7.53 (m, 3H, Ph-2,4,6-*H*), 7.32 (def dt, 1H, *J* = 7.2 Hz; *J* = 1.29 (1.79) Hz, Ph-5-*H*), 3.50–3.70 (br s, 2H, Pp-2,6-H_e), 3.18–3.40 (br s, 2H, Pp-2,6-H_a), 2.20–2.40 (br s, 4H, Pp-3,5-*H*), 2.17 (s, 3H, Pp-CH_3). Anal. Calcd for $C_{12}H_{15}N_2OCl$: C60.37; H6.33; N11.73; Found: C60.15; H6.38; N11,65.

(4-Chlorophenyl)(4-methylpiperazin-1-yl)methanone (3). Synthesized as **1** from 4-chlorobenzoyl chloride

(1.75 g, 10 mmol). White solid. Mp. 70–72 °C. Yield: 10%. $C_{12}H_{15}N_2OCl$ (MW = 238.71) (lit. (28) $C_{12}H_{15}N_2OCl$ x HCl Mp. 279–281 °C). ^{1}H-NMR [DMSO-d_6] δ (ppm): 7.46–7.51 (m, 2H, Ph-2,6-*H*), 7.36–7.42 (m, 2H, Ph-3.5-*H*), 3.50–3.70 (br s, 2H, Pp-2,6-H_e), 3.20–3.40 (br s, 2H, Pp-2,6-H_a), 2.20–2.40 (br s, 4H, Pp-3,5-*H*), 2.17 (s, 3H, Pp-CH_3). Anal. Calcd for $C_{12}H_{15}N_2OCl$: C60.37; H6.33; N11.73; Found: C60.47; H6.44; N11,75.

N-(4-Chlorophenyl)-4-methylpiperazine-1-carboxamide (4). To 4-methylpiperazine (0.25 g, 2.5 mmole) in 15 mL of CH_3CN was added 1-chloro-4-isocyanatobenzene (0.38 g, 2.5 mmole) and refluxed for 8 h. The white precipitate was filtered, dissolved in acetone and two drops of conc. hydrochloric acid was added. The solid was filtered. White crystals. Mp. 252 °C (dec). Yield: 40%. $C_{12}H_{16}N_3OCl$ x HCl × 0.25H_2O (MW = 294.69). ^{1}H-NMR [DMSO- d_6]: δ (ppm) 8.98 (s, 1H, CO-N*H*), 7.52 (d, *J* = 8.7 Hz, 2H, Ph-3,5-*H*), 7.30 (d, *J* = 9.0 Hz, 2H, Ph-2,6-*H*), 3.32 (br s, 4H, Pp-2,6-*H*), 2.76 (s, 3H, Pp-CH_3), 2.50 (m, 4H, DMSO-d_6 + Pp-3,5-*H*). ^{13}C-NMR [DMSO-d_6]: δ (ppm) 154.8, 139.7, 128.6, 126.0, 121.5, 52.5, 42.4, 41.5. LC/MS$^{\pm}$: purity (100%); t_R = 3.04, (ESI) *m/z* $[M+H]^+$ = 254.24.

5-Chloro-*N*-(3-(4-methylpiperazin-1-yl)propyl)-1H-indole-2-carboxamide (5). 5-Chloroindole-2-carboxylic acid (0.49 g, 2.5 mmol) and CDI (0.41 g, 2.5 mmol) in 3 mL of absolute CH_3CN was stirred at r.t. for 3 h. Then the mixture was cooled to 0^0 C and 1-(3-Aminopropyl)-4-methylpiperazine (0.39 g, 2.5 mmol) in 2 mL of absolute CH_3CN was added dropwise and stirred at this temperature for 0.5 h and at r.t. for 72 h. The solid was filtered and crystallized from CH_3CN (with carbon). Light yellowish crystals. Mp. 182–185 °C. Yield: 44%. $C_{17}H_{23}N_4OCl$ (MW = 334.85).^{1}H-NMR [DMSO-d_6] δ (ppm): 11.75 (br s, 1H, indole-1-*H*), 8.54 (t, 1H, *J* = 5.6 Hz, CO-N*H*), 7.67 (d, 1H, *J* = 2.1 Hz, indole-4-*H*), 7.40 (d, 1H, *J* = 8.7 Hz, indole-7-*H*), 7.15 (dd, 1H, *J* = 8.7 Hz, *J* = 2.1 Hz indole-6-*H*), 7.06 (s, 1H, indole-3-*H*), 3.31 (m, 2H, CONH-CH_2), 2.50–2.20 (m, 10H, Pp-CH_2 + Pp-2,3,5,6-*H*), 2.12 (s, 3H, Pp-CH_3), 1.66 (qu, 2H, *J* = 6.9 Hz, Pp-CH$_2$-CH_2). ^{13}C NMR (DMSO-d_6): δ (ppm) 161.1, 135.2, 133.9, 128.6, 124.6, 123.7, 120.9, 114.3, 102.2, 56.1, 55.3, 37.9, 26.9. IR (KBr) [cm^{-1}] *v* = 3222 (N-H), 1645 (C=O, amide), 1550 (N-H, amide). LC/MS$^{\pm}$: purity (100%); t_R = 3.42, (ESI) *m/z* $[M+H]^+$ = 335.30. Anal. Calcd for $C_{17}H_{23}N_4OCl$: C60.98; H6.92; N16.73; Found: C61.23; H7.02; N16.90.

3,6-Dichloro-N-(3-(4-methylpiperazin-1-yl)propyl)benzo[b]thiophene-2-carboxamide (6). 3,6-Dichlorobenzo[b]thiophene-2-carbonyl chloride (6a)—Compound was obtained according to the method described by Kaizerman *et al.* (29).

To 4-chlorocinnamic acid (2.56 g, 14 mmol) in 15 mL of chlorobenzene, thionyl chloride (8.63 g, 720 mmol) and slowly pyridine (0.14 g, 2 mmol) were added. The solution was refluxed for 95 h, and n-hexane was added. The white solid (1.52 g, 41%; Mp. 133–136 °C) was filtered, washed with n-hexane and used for further reaction with any more purification.

3,6-Dichloro-*N*-(3-(4-methylpiperazin-1-yl)propyl)benzo[b]thiophene-2-carboxamide (6). 3,6-dichlorobenzo[b]thiophene-2-carbonyl chloride (0.40 g, 1.5 mmol) and 1-(3-aminopropyl)-4-methylpiperazine (0.24 g, 1.5 mmol) in 10 mL of absolute CH_3CN was stirring in r.t. for 4 h and heated to reflux for 20 h. The solvent was evaporated, and to the residue 35 mL AcOEt was added and washed with 35 mL of 5% HCl. The acidic layer was alkalized (10% $NaHCO_3$) and extracted with CH_2Cl_2. The organic layer was then dried over anhydrous Na_2SO_4, filtered and after concentration converted into hydrochloride (3 mL of acetone and concentrated HCl). Cream solid. Mp. 254 °C (dec). Yield: 41%. $C_{17}H_{21}N_3SOCl_2$ × 2HCl x 2H_2O (MW = 493.30). ^{1}H-NMR [DMSO-d_6] δ (ppm): 11.73 (s, 1H, Pp-H^+), 8.60 (t, 1H, *J* = 5.4 Hz, -CON*H*), 8,31 (s, 1H, Bzt-7-*H*), 7.79 (d, 1H, *J* = 8.7 Hz, Bzt-5-*H*), 7.61 (dd, 1H, *J* = 8.7 Hz *J* = 1.8 Hz, Bzt-4-*H*), 4.10-3.10 (m, 12 H, Pp-2,3,5,6-*H* + Pp-CH_2 + -CONH-CH_2), 2.80 (s, 3H, CH_3), 1.99 (br s, 2H, Pp-CH$_2$-CH_2). ^{13}C NMR (DMSO-d_6): δ (ppm) 160.5, 138.3, 135.3, 133.6, 132.8, 127.1, 124.5, 123.5, 119.1, 50.1, 48.6, 37.3, 23.8. IR (KBr) [cm^{-1}] *v* = 3502, 3455 (N-H), 1624 (C=O, amide), 1541 (N-H, amide). LC/MS$^{\pm}$: purity (100%); t_R = 4.03, (ESI) *m/z* $[M]^+$ = 386.33.

1-(3-Chlorophenyl)-3-(3-(4-methylpiperazin-1-yl)propyl) urea (7). 1-chloro-3-isocyanatobenzene (0.38 g, 2.5 mmol) and 1-(3-aminopropyl)-4-methylpiperazine (0.40 g, 2.5 mmol) in 5 mL of absolute CH_3CN was heated to reflux for 6 h. After evaporation to dryness the residue was dissolved in CH_2Cl_2, purified by CC (eluent CH_2Cl_2:MeOH:NH_3 – 90:9:1) and after concentration crystallized as salt of oxalic acid from EtOH/Et_2O. White solid. Mp. 165–168 °C. Yield: 46%. $C_{15}H_{23}N_4OCl$ × 0.8$C_2H_2O_4$ (MW = 382.86). ^{1}H-NMR [DMSO-d_6] δ (ppm): 9.04 (s, 1H, Ph-CO-N*H*), 7.66 (s, 1H, Ph-2-*H*), 7.20 (d, 2H, *J* = 3.6 Hz, Ph-5,6-*H*), 6.85–6.92 (m, 1H, Ph-4-*H*), 6.65 (t, 1H, *J* = 5.4 Hz, NH-CO-N*H*), 3.08 (q, 2H, *J* = 6.4 Hz, CO-NH-CH_2), 2.70–2.80 (br s, 4H, Pp-2,6-*H*), 2.60–2.70 (br s, 4H, Pp-3,5-*H*), 2.49 (def t, 2H, Pp-CH_2-CH$_2$), 2.44 (s, 3H, Pp-CH_3), 1,60 (qt, 2H, *J* = 6.9 Hz, Pp-CH$_2$-CH_2-CH$_2$). ^{13}C NMR (DMSO-d_6): δ (ppm) 165.6, 155.6, 142.8, 133.5, 130.6, 117.3, 116.4, 55.0, 53.5, 51.2, 44.2, 37.6, 26.9. IR (KBr) [cm^{-1}] *v* = 3288 (N-H), 1695 (C=O, amide), 1592 (N-H, amide). LC/MS: *m/z* (%) = 310.99 ([M·]$^+$,100). Anal. Calcd for $C_{15}H_{23}N_4OCl$ × 0.8$C_2H_2O_4$: C52.08; H6.48; N14.63; Found: C52.02; H6.53, N14.76.

Triazine derivatives

All compounds were obtained as described previously by Łażewska *et al.* (27). Compounds **8** and **11** are described in lit. (27). Physical properties and spectrum data of all triazine derivatives are included in Appendix S1.

In vitro [^{3}H]histamine binding assay on human H_4R

Prior to the experiments, cell membranes were sedimented by a 10 min centrifugation at 4 °C and 16 000× *g* and re-suspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4). Competition binding experiments were carried out by incubating membranes, 35 μg/well (prepared from Sf9 cells transiently expressing human H_4R, co-expressed with G proteins $G_{\alpha i2}$ and $G_{\beta 1\gamma 2}$ subunits) in a final volume of 0.2 mL containing binding buffer and [^{3}H]histamine 2× HCl (10 nM, 15.3 Ci/mmol, PerkinElmer, Waltham, MA, USA) in a 96 well microtitre plate. Assays were run in duplicates or triplicates with four or seven appropriate concentrations between 100 nM or 0.1 nM and 100 μM of the test compounds. Amount of experiments was at least 2. Only exceptions were for substance **2**, **3,** and **4** where only one experiment in duplicates was performed. Incubations were performed for 60 min at 25 °C and shaking at 250 rpm. Non-specific binding was determined in the presence of 100 μM unlabeled JNJ7777120. Bound radioligand was separated from free radioligand by filtration through GF/B filters pretreated with 0.3% (mass/vol) polyethyleneimine and washed three times with 5 mL of ice-cold binding buffer (4 °C). The filters were soaked in a sample bag with 9 mL scintillator liquid. The amount of radioactivity collected on the filter was determined by liquid scintillation counting. Competition binding data were analysed by the software GRAPHPAD PRISM 3.02 (San Diego, CA, USA) using non-linear least squares fit. K_i values were calculated from the IC_{50} values according to the Cheng-Prusoff equation (30). Mean values of three independent experiments in triplicate are stated in the Table 1, with the exception for compounds **2**, **3** and **4**.

Electrophoretic mobility shift assay

DNA extraction

Isolation of genomic DNA from whole human blood was performed using the Kit NucleoSpin Blood XL (MACHEREY-NAGEL, Düren, Germany).

Promoter's amplification

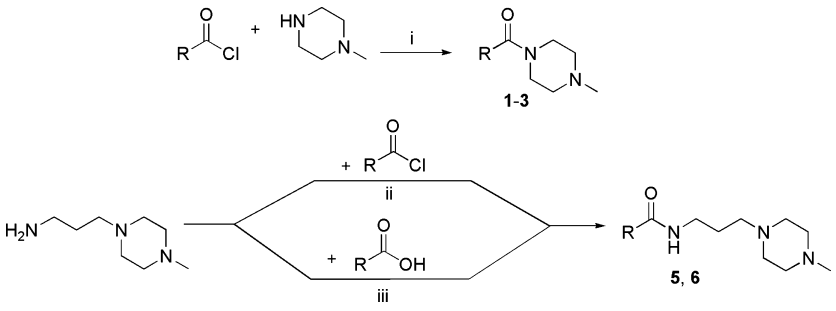
Specific primers were designed to amplify *PTEN* promoter. The primers that were used for the amplification of each promoter were as follows:

5′-CAGAAAGACTTGAAGCGTAT-3′ forward,

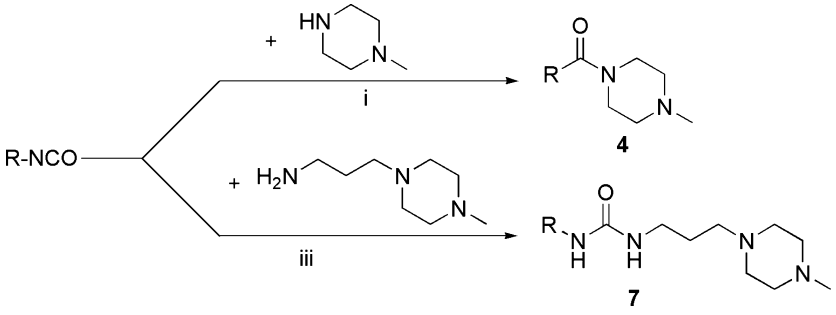
5′-AACGGCTGAGGGAACCTC-3′ reverse

primer for *PTEN* promoter. PCR reactions using genomic DNA were performed on a MJ Research P200 thermal cycler (MJ Research Inc., Waltham, MA, USA).

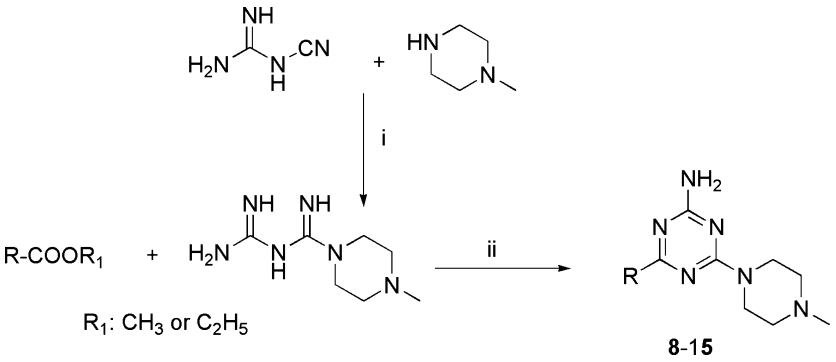
The PCR reactions for *PTEN* promoter were performed in a mix containing 1.5 mM $MgCl_2$, 1 unit per reaction of DyNAzyme *EXT* DNA polymerase, 0.5 mM of each primer, 0.2 mM of each dNTP and approximately 30 pg of template DNA in a total reaction volume of 20 μL. After an initial denaturing step for 3 min at 98 °C, amplification comprised of 35 cycles of 30 seconds at 98 °C, 1, 30 min at 50 °C and 5 min at 72 °C. Final elongation was for 7 min at 72 °C. *PTEN* promoter was amplified using human genomic DNA as template that was isolated from whole blood.



Scheme 1: Synthetic route of amides (**1-3**, **5**, **6**). Reagents and conditions: (i) benzene/water/Na_2CO_3, r. t. 12 h; (ii) CH_3CN, r.t 4 h, reflux 20 h; (iii) carboxylic acid, CH_3CN, CDI, r.t. 3 h; amine added at 0 °C, 0.5 h at 0 °C, r.t. 72 h.



Scheme 2: Synthetic route of ureas (**4**, **7**). Reagents and conditions: (i) CH_3CN, reflux 6–8 h.



Scheme 3: Synthetic route of 2,4,6-trisubstituted 1,3,5-triazines (**8**-**15**). Reagents and conditions: (i) BuOH, temperature gradually increased from 50 to 90 °C during 1 h, 5 h reflux; (ii) MeONa, reflux from 15 to 30 h.

Band shift assay

Each compound, at a final concentration of 2 μM, was incubated with an equal amount of PCR product at 24 °C for 30 min. Subsequently, the mixtures were electrophoresed on 2% w/v agarose gel.

Antiproliferative assay

Human embryonic kidney HEK-293 cell line (ATCC CRL−1573) was kindly donated by Prof. Dr. Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn). Neuroblastoma IMR-32 cell line was provided by Department of Oncogenomics, Academisch Medisch Centrum, Amsterdam, Holland (31,32). The cell lines were seeded in 96-well flat-bottomed microtitre plates at a concentration of 1.5×10^4 cells/well (HEK-293) and 2×10^4 cells/well (IMR-32) and cultured in 200 μL of Dulbecco's Modified Eagle's Medium – DMEM (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere (5% CO_2, 95% air) at 37 °C for 24 h to reach 60% confluence. The stock solution of H_4R ligand in DMSO (25 mM) was diluted into the fresh growth medium and added to the wells at final concentrations 0.01–250 μM. Final DMSO concentration did not exceed 1%. After 48 h of incubation, the EZ4U (EZ4U Non-radioactive cell proliferation and cytotoxicity assay, Biomedica, Vienna, Austria) labeling mixture (20 μL) was added to each well and the cells were incubated under the same conditions for 5 h. The absorbance of the samples was measured using a microplate reader (EnSpire, PerkinElmer, Waltham, MA, USA) at 492 nm. The IC_{50} values of the reference compound doxorubicin (DX), used as a standard during this study, against HEK-293 and IMR-32 cell lines were obtained and calculated as we described previously (33).

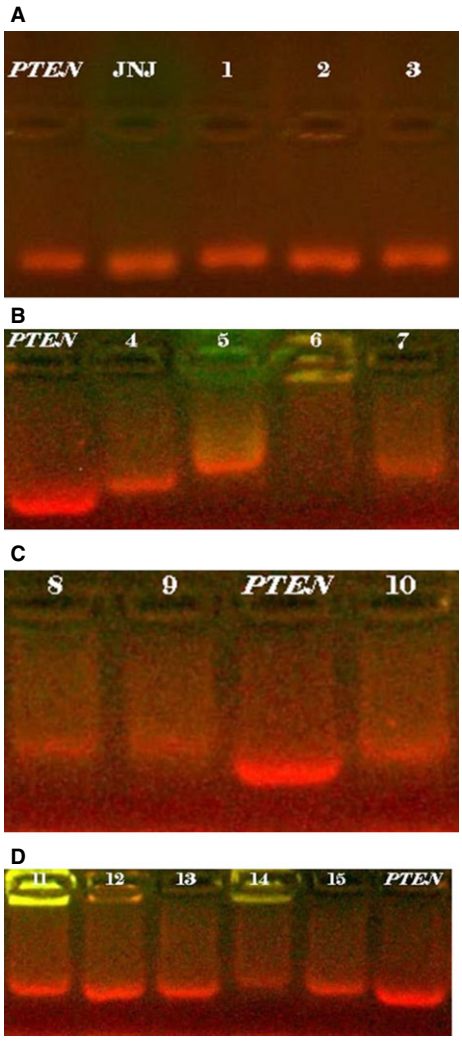


Figure 1: Agarose gel electrophoresis (2% w/v) of *PTEN* promoter, JNJ7777120, and compounds **1**, **2**, **3** (A), *PTEN* promoter and compounds **4**, **5**, **6**, **7** (B), *PTEN* promoter and compounds **8**, **9**,**10** (C), *PTEN* promoter and compounds **11**, **12**, **13**, **14**, **15** (D).

Results and Discussion

Chemistry

Two different strategies for the synthesis of the novel amides (**1**, **2**, **3**, **5**, **6**) were employed. In the first aminolysis approach: acid chlorides reacted with the appropriate amines (Scheme 1) giving amides **1-3**, **6**, whereas in the second one amide (**5**) was produced from the carboxylic acid and amine in the presence of CDI as an activator

Table 2: The antiproliferative effect and the interaction of compounds with *PTEN* promoter: not examined (NE), no (–), low (+), medium (++), high (+++) effect

Comp.	Effect		
	PTEN promoter interaction	Anti-proliferative effect	
		IMR-32	*HEK-293*
1	–	NE	NE
2	–	NE	NE
3	–	NE	NE
4	++	–	–
5	+++	+	+
6	–[a]	++	++
7	**+++**	–	–
8	+	NE	NE
9	+	NE	NE
10	+	NE	NE
11	++	–	+
12	+	NE	NE
13	+	NE	NE
14	++	+	+
15	+	NE	NE
JNJ7777120	–	–	+

[a]DNA and **6** probably precipitated during the incubation process.

(Scheme 1). Non-commercially available acid chloride **6a** was synthesized according to the procedure described by Kaizerman *et al.* (29).

Commercially available isocyanates were refluxed in CH_3CN with the appropriate amines to give ureas **4** and **7** (Scheme 2).

2,4,6-Trisubstituted 1,3,5-triazines (**8-15**) were obtained as the result of cyclization of appropriate esters with 4-methylpiperazin-1-yl biguanide dihydrochloride as described previously (Scheme 3) (27). Biguanide dihydrochloride intermediate was obtained by the heating of cyanoguanidine and 4-methylpiperazine dihydrochloride in 1-butanol (34). All acid methyl or ethyl esters were commercially available (except **9E** and **14E** that were synthesized according to described procedures (35,36). The structures of all synthesized final compounds are shown in Table 1 with their affinities at human H_4R.

Histamine H_4R screening

As depicted in Table 1, all compounds showed moderate to low human H_4R affinities, except compound **1**, **5**, **6** and **7**. In JNJ7777120 analogues series (**1**-**7**) the most potent were compounds **2** (K_i = 3.88 μM) and **4** (K_i = 3.52 μM). The introduction of a carbon chain (propyl linker) between amide/urea moiety (**5**-**7**) caused the strongest decrease of H_4R affinities (K_i > 100 μM). In the triazine series (**8**-**15**) the most potent was phenyl derivative with the *para*-bromo substituent **11** with K_i of 0.52 μM. The change of the phenyl moiety for a pyridine ring **15** or an aliphatic chain **10** as

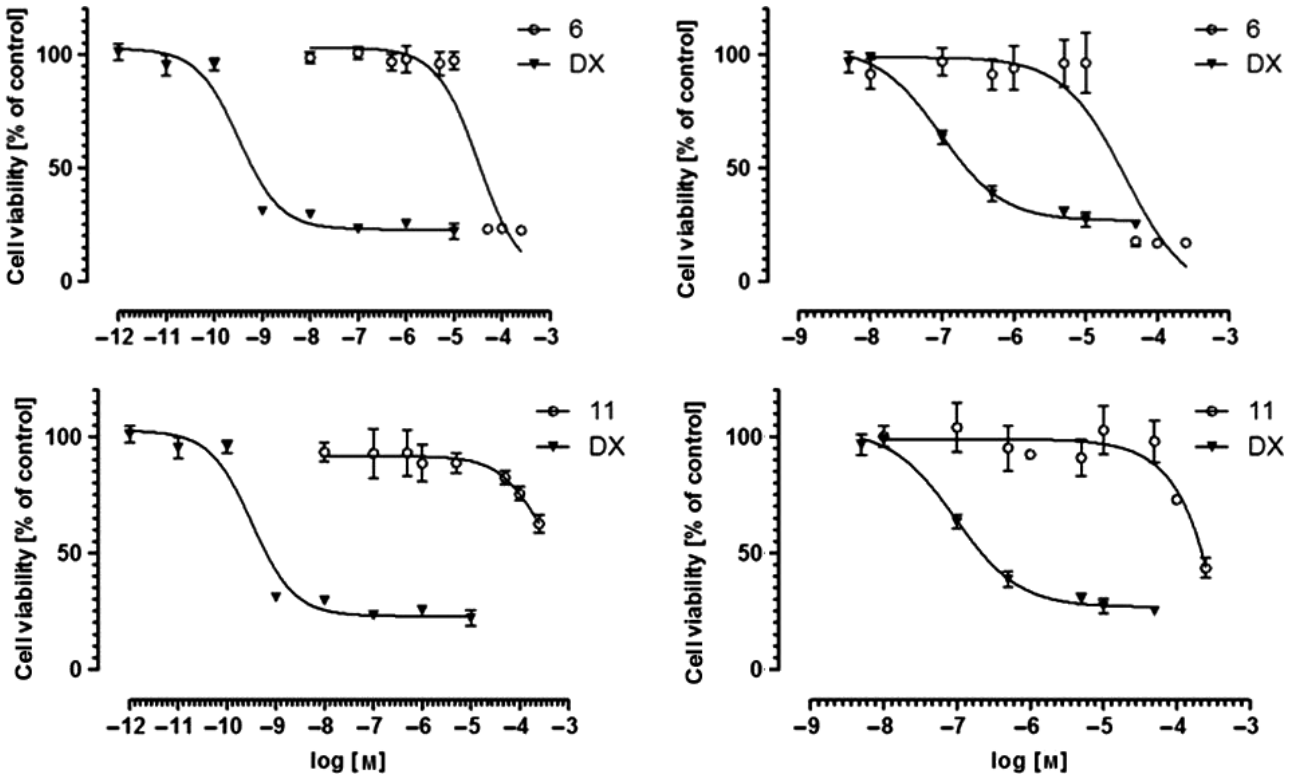


Figure 2: The effect of compounds **6** and **11** on IMR-32 (left) and HEK-293 (right) cell lines viability. All data are included in Appendix S1.

well as the introduction of the methylene linker between triazine moiety and the substitutent (hydantoin **9**, naphthyl **12**, phenyl **13**, or phenoxy **14**) decreased the affinity (K_i > 3 μM).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA), also known as band shift assay was applied to study potential promoter's PCR products – substances interactions. This procedure determines if proteins or compounds are capable of binding to a given DNA or RNA sequence (37). Regarding *PTEN* promoter, among the group of JNJ7777120 analogues, no effect for **1**-**3** was observed, as well as JNJ7777120 also did not reduce *PTEN* promoter mobility (Figure 1A). On the other hand, the highest interaction among all tested compounds was observed for other JNJ7777120 analogues **5** and **7** followed by **4**, while **6** had no effect. However, the effect of **6** on *PTEN* promoter was ambiguous due to its potential ability to induce DNA precipitation during the incubation process (Figure 1B). Among 1,3,5-triazine derivatives the agarose gel electrophoretic separation showed, that all compounds exhibited the effect on *PTEN* promoter mobility, where the **11** and **14** derivatives reduced the mobility of the *PTEN* PCR product to a greater extent than **8, 9, 10, 12, 13** and **15** (Figure 1C,D). All data were summarized in Table 2.

The antiproliferative assay

The antiproliferative colorimetric assay was applied to determine potentially correlation between interaction of compounds with *PTEN* gene promoter and tumor activity in reference to the cytostatic effect of doxorubicine (DX). The influence of synthesized molecules on the viability of normal HEK-293 cell line was also examined. To this study were chosen compounds which reduced the mobility of *PTEN* promoter with either medium (**4**, **11**, **14)** or strong (**5**, **7**) manner (Table 2). Additionally, the antiproliferative effect of the compound **6** and JNJ7777120 was determined. The obtained data showed, that **6** reduced most significantly cells viability among all compounds with calculated IC_{50} = 23.27 μM against IMR-32 and IC_{50} = 36.61 μM against HEK-293. However, **6** did not possess potent antitumor activity compare with the reference drug DX (calculated DX IC_{50} = 0.000325 μM against IMR-32, DX IC_{50} = 9.5 μM against HEK-293) (Figure 2). Moreover, comparable IC_{50} values of **6** against normal as well as tumor cells indicated no specific antitumor mechanism of action. Interestingly, despite of their high interaction with *PTEN* promoter the other examined JNJ7777120 analogues showed very low (**5**) or no significant (**4**, **7**) antitumor and antiproliferative activity. The examined compounds from the triazine series, **11** and **14**, showed comparable, very low cytotoxic effect ($IC_{50} \geq 100$ μM). However, regarding **11**, the derivative with the highest affinity against H_4R among all compounds presented in that study, as the new lead structure, its low cytotoxic effect on normal HEK-293 cell line is an additional benefit in terms of preliminary ADME-Tox parameters determination (Figure 2).

Conclusions

The high and medium interactions with *PTEN* gene promoter were determined for five synthesized compounds – three JNJ7777120 analogues (**4**, **5** and **7**) and two 1,3,5-triazine derivatives (**11** and **14**). The JNJ7777120 analogues (**5** and **7**), with the weakest potency against H_4R among all synthesized compounds, showed the highest interaction with the *PTEN* gene promoter. However, no significant effect of these compounds on neuroblastoma IMR-32 cell line proliferation and no correlation between *PTEN* promoter interaction and antitumor activity were shown. Compound **6** showed the highest antiproliferative effect against tumor IMR-32 as well as normal HEK-293 cell lines. On the other hand, the similar IC_{50} values of **6** against tumor and normal cell lines excluded its potential specific antitumor activity. Though, we conclude that further study should be conducted to determine potential inhibition or activation effect of the most interacting with *PTEN* promoter JNJ7777120 analogues on PTEN protein expression in several tumor cell lines. Almost all synthesized and examined compounds showed moderate to low human H_4R affinities. Generally, the group of triazines showed higher potency as H_4R ligands in compare to the urea/amide series designed as JNJ7777120 analogues. Moreover, compound **11**, the 1,3,5-triazine phenyl derivative with the *para* bromine substituent, showed the highest affinity against H_4R among all compounds and no cytotoxicity, therefore may be considered as a promising lead structure for further investigations.

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Conflict of Interest

Declared none.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Physical properties and spectrum data of triazine derivatives.

Figure S2. The effect of compounds on cell lines viability.