Opioid agonist – tachykinin antagonist as a new analgesic with adjuvant anticancer properties

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Abstract

Opiate analgesics like morphine or fentanyl are the most widely used medicines for relieving severe acute or chronic pain, including cancer pain. Unfortunately, chronic pain treatment is associated with fast development of tolerance that creates the need to escalate the treatment doses. In addition, opiates may stimulate progression of cancer. Therefore, a new type of effective analgesic especially designed for chronic cancer pain treatment is needed. In this paper, a new opioid peptide analogue has been described as a new analgesic. The compound is characterized by very high agonist affinities to MOR and also high, but ten times lower affinity to DOR. Affinity to hNK1 as an antagonist is on the level of C-terminal hexapeptide fragment analogue of Substance P. The compound expressed reasonable antiproliferative properties toward various cancer cells. Interestingly, the peptide did not interfere with the proliferation of fibroblasts. Therefore, the compound should be considered as a new analgesic for treatment of cancer-related pains with adjuvant anticancer properties which may support cancer treatments.

Key words: opioid peptide analogue, analgesics, cancer, neurokinin antagonist.

Introduction

Opiate analgesics are widely used medicines to relieve severe acute or chronic pain. They are known to exert an antinociceptive effect by interaction with specific opioid receptors. However, the beneficial analgesic effect of opioids is accompanied by side effects such as constipation, vomiting and nausea. Tolerance and physical dependence on opioids are easily developed [9]. In a case of pains related to cancer, stimulation of cancer progression should be also considered [8]. In nociceptive signal induction, transmission and pain perception, different types of endogenous systems are involved. Therefore, development of new analgesics designed to treat cancer pain is an important target of medicinal chemistry [3]. New analgesics that may suppress pain signals in a different manner are recently developed as multitarget medicines, especially to treat chronic pain [6].

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Tachykinins, including undecapeptide substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$) play modulatory roles in the afferent transfer and post-synaptic processing of nociceptive information within the dorsal horn of the spinal cord [2,13]. The superficial dorsal horn of the spinal cord is an area that receives primary synaptic input from sensory fibers originating from dorsal root ganglion (DRG) neurons. This area is a potentially important site for functional regulation (by modulatory opioid peptides) of nociceptive input mediated by substance P and excitatory amino acids released from primary afferent terminals. Anatomical studies indicate a similar distribution of substance P- and opioid-containing neural elements within this area [1,18]. Ligands of the opioid receptors are highly effective in blocking or reducing nociceptive transmission at the spinal level, and this antinociception is thought to be caused at least partially by inhibition of substance P release [5,13]. Therefore, simultaneous partial blocking of presynaptic substance P release and blocking of postsynaptic substance P receptors responsible for signal transmission has been the first multitarget approach. Indeed, co-injection of a weak peptide substance P antagonist together with an opioid peptide strongly enhances opioid analgesia [15]. Consequently, the development of multitarget ligands results in the development of a new type of effective analgesics [6]. Recently it has been published that opioid peptides [8] as well as substance P antagonists [7,16] express significant effects on progression of cancer cells. Therefore, potential reducing of cancer progression should be an additional advantageous effect of substance P-opioid peptide ligands use in the case of cancer-related pains.

Searching for a new type of analgesics we developed compound AA3016 (Fig. 1), analogue of previously synthesized AA501 [14], in which one opioid pharmacophore is hybridized with substance P antagonist motive Z-D-Trp. This paper describes pharmacological binding as well as effect on proliferation of various cancer cells *in vitro*.

Material and methods

Compounds

Substance P and Lys-Phe-Phe-Gly-Leu-MetNH$_2$ (AA2077) have been synthesized by solid phase synthesis using Fmoc-strategy, followed by preparative HPLC purification. AA3266 has been synthesized in solution methodology by an already patented method [10]. Aprepitant, 5-[[(2R, 3S)-2-[(1R)-1-[3,5-bis (trifluoromethyl) phenyl] ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3 H-1,2,4-triazol-3-one has been isolated from commercially available pills (Merck). The structure and purity of compounds were confirmed by HPLC-MS analysis. The compounds, opi-
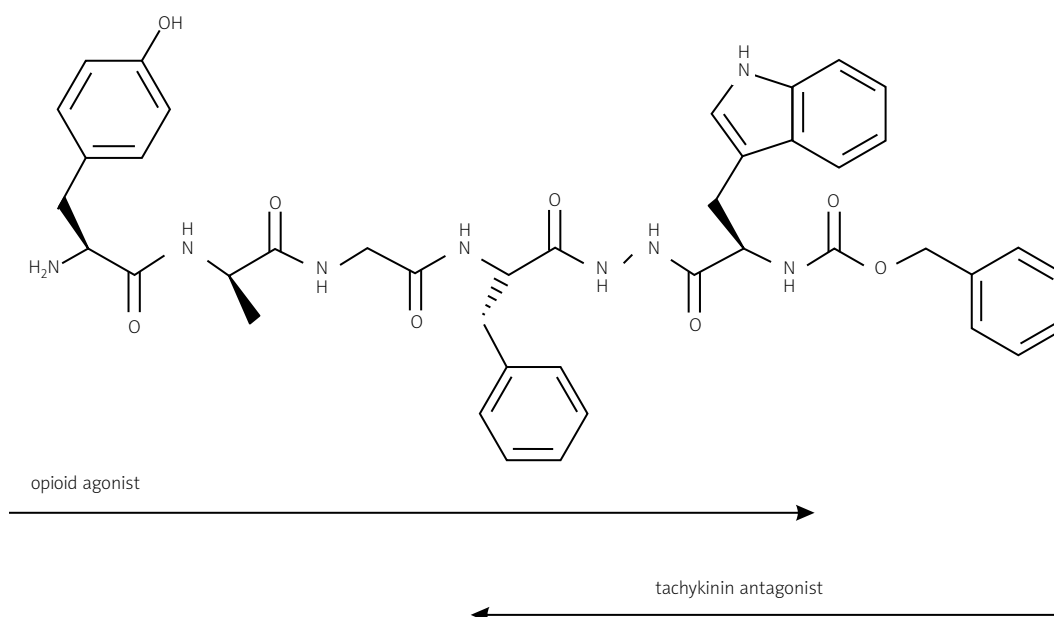

opioid agonist

tachykinin antagonist

Fig. 1. Chemical structure of AA3266.

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oid antagonist naltrexone (NTX) and NK-1 antagonist – L703,606 have been purchased from Tocris.

Tachykinin hNK1 receptor binding

Neurokinin 1 receptor binding assays were performed as previously described [19]. Briefly, recombinant hNK1/CHO cells were grown to 90% confluence in a humidified atmosphere (95% air and 5% CO_2) at 37°C, in Ham's F12 medium supplemented with 10% foetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 500 μg/mL of Geneticin (G 418). The cell monolayers were then washed with calcium and magnesium deficient phosphate-buffered saline (PD buffer) and harvested in the same buffer containing 0.025% EDTA. After centrifugation at 2700 rpm for 12 minutes, the cells were homogenized in ice cold 10 mM Tris-HCl and 1 mM EDTA (pH = 7.4) buffer. The crude membrane fraction was collected by centrifugation at 18,000 rpm for 12 minutes at 4°C, the pellet was suspended in 50 mM Tris-Mg buffer, and the protein concentration of the membrane preparation was determined using the Bradford assay. Six different concentrations of the test compound were each incubated, in duplicates, with 20 μg of membrane homogenate and 0.5 nM [3H] Substance P (44.1 Ci/mmol, Perkin-Elmer, United States) in 1 mL final volume of assay buffer (50 mM Tris-HCl pH 7.4, containing 5 mM of Magnesium chloride, 50 μg/mL of bacitracin, 30 μM of bestatin, 10 μM of captopril and 100 μM of Phenylmethylsulfonylfluoride).

Substance P (10 μM) was used to define the non-specific binding. The samples were incubated in a shaking water bath at 25°C for 20 minutes. The reaction was terminated by rapid filtration through Whatman GF/B filter (Gaithersburg, MD) presoaked in 1% polyethyleneimine, washed three times with 2 mL of cold saline, and filter-bound radioactivity was determined by liquid scintillation counting (Beckman LS5000TD).

Data analysis was performed from three independent experiments by using the GraphPad Prizm4 software (GraphPad, San Diego, CA).

Log IC 50 values for each compound were determined from linear regression. The inhibition constant (Ki) was calculated using the Cheng-Prusoff equation [4].

Opioid MOR and DOR receptor binding

Opioid receptor binding assays were performed as previously described [12]. The brain homogenates of Wistar rats (250-300 g body weight) were used for further binding experiments. Animals were housed in the Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, in groups of four, allowed free access to standard food and tap water, and maintained on a 12 : 12 h light/dark cycle until the time of sacrifice. Animals were handled according to the Directives of the Council of European Communities (86/609/EEC) and the permission of the local Animal Ethical Commission.

All binding assays were performed at 25°C for 30 minutes in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 ml, containing 1 mg of BSA and 0.2-0.4 mg/ml of membrane protein. The samples were prepared in disposable plastic assay tubes (Sarstedt Co., Nümbrecht, Germany). Rat brain membranes were incubated with the selective MOP receptor agonist [3H]DAMGO (0.9-1.2 nM) and the DOP receptor selective agonist ([3H]Tyr1,Ile5,6deltorphin-2; 0.8-1.3 nM) in the presence of unlabeled test ligands with concentrations ranging from 10^{-5} to 10^{-11} M. Non-specific binding was determined in the presence of 10 μM naloxone. Three peptidase inhibitors (1 μM captopril, 1 μM bestatin and 1 μM phosphoramidon) were included in the assay buffer to prevent metabolic inactivation of the peptides. The experiment was terminated and both bound and free radioligands were separated by rapid filtration under vacuum through Whatman GF/C (radiolabeled peptides) glass fibre filters by using Brandel M24R Cell Harvester. Subsequently, the filters were washed three times with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4) buffer. After completion of the filtration and separation procedure, all fibre-disks were dried under an infrared lamp and removed from the filter-sheet by use of tweezers. Each disk was inserted into UltimaGold™ environment friendly, non-volatile, toluene-free scintillation cocktail and placed into individual sample vials (transparent glass, Packard). The bound radioactivity was determined in a Packard Tricarb 2300TR liquid scintillation analyzer. Receptor binding experiments were performed in duplicate and repeated at least three times.

[35S]GTPγS binding assay

Crude rat membrane preparation (10-15 μg protein/ 1 ml) was incubated for 60 min at 30°C in a Tris-EDTA (pH 7.4) buffer containing 50 mM of Tris-HCl, 1 mM of EDTA, 3 mM of $MgCl_2$ and 100 μM of GDP in the presence of 0.05 nM [35S]GTPγS and increasing concentrations (10^{-10}– 10^{-5}M) of the opioid-SP hybrid coded "3266" in a total volume of 1 ml. All experiments were performed in triplicate. Non-specific binding was determined by using 10 μM of unlabelled GTPγS. The 3266 binding was reversed with an addition of either 10^{-5} M naloxone or

a NK-1 antagonist – L703,606. Membrane-bound and free radioligand were separated by rapid triple filtration with Tris-EDTA (pH 7.4) buffer on Tris-EDTA-soaked glass microfiber GF/B Whatman filters using a M-24 Cell Harvester apparatus (Brandel, USA). Then, filter discs were soaked in an Ultima Gold MV scintillation fluid (Perkin Elmer, USA) and placed in glass tubes. Radioactivity was determined in a TRI-CARB 2100TR (Canberra-Packard, Perkin-Elmer Life Science, USA) scintillation counter. Binding curves were fitted using nonlinear regression and were depicted as a percentage of specific compound-stimulated [^{35}S]GTPγS binding to a log of compound concentration. Efficacy was presented as the maximum possible effect (Emax) value.

Cell proliferation assays

Human melanoma cell lines MeW164, MeW155, MeW151, human lung cancer E14, urinary bladder carcinoma cell line T24, and also normal adult human fibroblast cell lines Fib9 and FlW180 and human foetal fibroblast cell line FlWp95 and FIW p98 were cultured in Eagle's 1959 MEM (Biomed-Lublin) supplemented with 10% foetal calf serum (Invitrogen).

Adherent cell cultures were plated in 24-well plates and harvested at sub-confluency using 0.25% trypsin with EDTA (200 mg/L). The cells collected from the cultures were counted directly under a microscope. Cell viability was evaluated according to trypan blue staining and visualisation under an inverted phase-contrast microscope.

Results

Opioid receptor affinity of AA3266 in radioligand displacement binding assay

The radioligand displacement binding studies were performed with receptor-specific probes [^{3}H]DAMGO and [^{3}H]DELT II in rat membrane preparations. The opioid peptide codenamed AA3266 effectively displaced the binding of both specific radiolabeled agonists with an MOR IC$_{50}$ 0.06 nM ± 0.01 and DOR IC$_{50}$ 0.6 nM ± 0.24. The AA3266 peptide showed ten times higher affinity at the MOR opioid receptor than at DOR receptors.

3266-induced G-protein stimulation

The hybrid opioid-SP antagonist, AA3266 effectively stimulated G-protein activation in the rat membrane homogenate with efficacy characteristic of full agonists (Emax = 215.1 ± 5.7%) (Fig. 2). The extra sum of squares

F test revealed that the addition of naloxone considerably decreased AA3266-induced G protein stimulation (F1,34 = 13.61; p < 0.001) but failed to abolish it fully (F1,34 = 27.54; p < 0.001), leaving the Emax value of 124 ± 3.4%. Whereas the NK-1 antagonist – L703,606 did not alter 3266-induced G-protein activity (F1,34 = 1.28; p = 0.26) as the Emax value equalled 220 ± 3.6% but in turn shifted the curve to the right affecting AA3266 potency (F1,34 = 5.91; p < 0.05). As a result, the potency of AA3266 was reduced by 0.3 log units (AA3266: logED$_{50}$ = −6.7 ± 0.1; 3266 + L703,606: logED$_{50}$ = −6.4 ± 0.06).

Receptor binding to tachykinin receptor hNK-1 is K$_i$ = 180.0 + 13.5 nM, that is similar to reference compound of C-terminal hexapeptide analogue (AA2077), K$_i$ = 262.1 ± 14.3 nM [Tomczyszyn, in preparation].

Effects of the compounds on the proliferation of normal and cancer cells

The compound AA3266 exerted a strong inhibitory effect on the proliferation of human melanoma cell lines (Fig. 3), and also inhibited proliferation of lung cancer and urinary bladder cancer cells (Figs. 4 and 5).
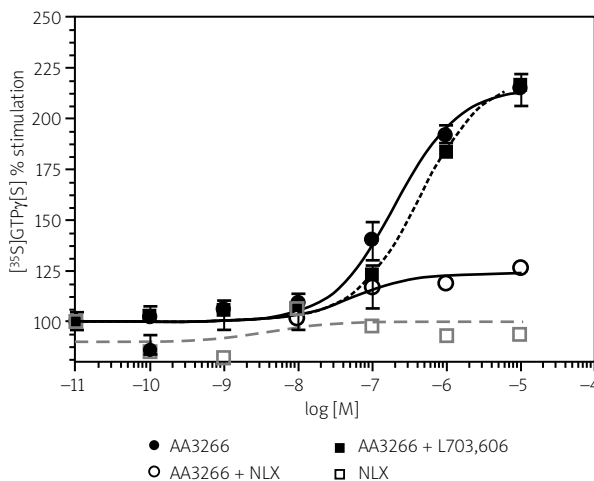

Fig. 2. Specific stimulation of [35S]GTP S binding by AA3266. Crude rat membrane preparation was incubated with 0.05 nM of radioligand in the presence of 10^{-11}–10^{-5} M of either AA3266 (•) or naloxone (□). The AA3266-induced [35S]GTP S specific stimulation was assessed in the presence of 10^{-5} M naloxone (○) or a NK-1 antagonist – L703,606 (■). Specific binding was defined as 100%. Results are expressed as means ± SEM.
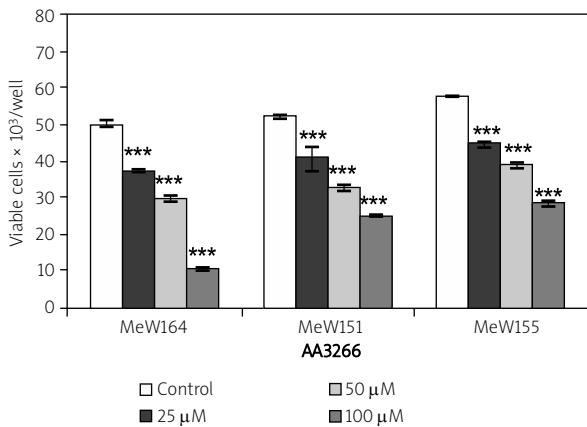
Fig. 3. Inhibitory effect of AA3266 on the proliferation of human melanoma cells. Melanoma cells were plated at 5×10^3 cells/well and cultured for 4 days. The groups of cultures with AA3266 were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; ***$P < 0.001$.
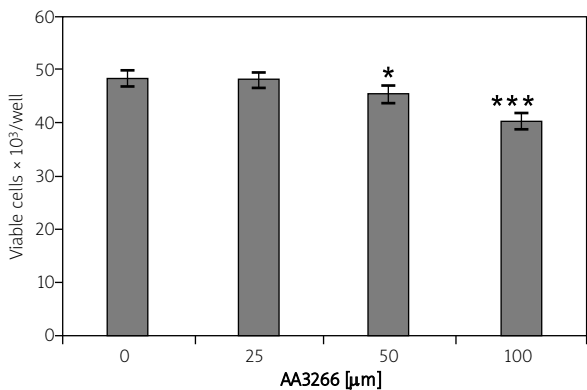

Fig. 4. Inhibitory effect of AA3266 on the proliferation of human lung cancer cells E-14 plated at 5×10^3 cells/well and cultured for 4 days. The groups of cultures with AA3266 were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; ***$P < 0.001$.

Fig. 5. Inhibitory effect of AA3266 on the proliferation of human urinary bladder cancer cells T24 plated at 5×10^3 cells/well and cultured for 4 days. The groups of cultures with AA3266 were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; *$P < 0.05$, ***$P < 0.001$.
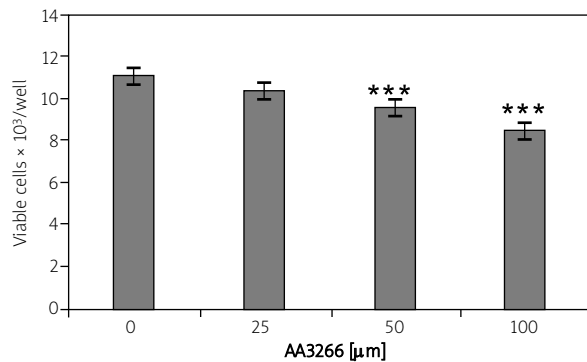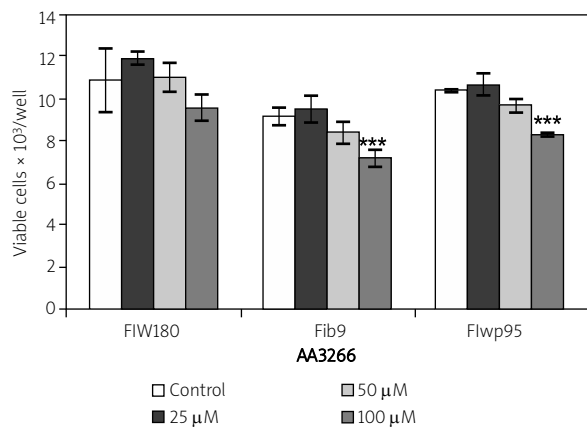

Fig. 6. Effect of AA3266 on the proliferation of normal adult human fibroblast cell lines FlW180 and Fib9, and on the proliferation of human fetal fibroblast cell line FlWp95. Fibroblasts were plated at 8×10^3 cells/well and cultured for 4 days. The groups of cultures with AA3266 were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; ***$P < 0.001$.

The inhibitory effect of AA3266 on human fibroblast lines was absent or found only at a dose of 100 μM (Fig. 6). Aprepitant inhibited proliferation of human cancer cell lines similarly as the compound AA3266 (Figs. 7-9). In contrast to AA3266, aprepitant efficiently inhibited proliferation of normal human fibroblast lines (Fig. 10). SP did not substantially inhibit proliferation of human normal or cancer cell lines (Figs. 11-14).
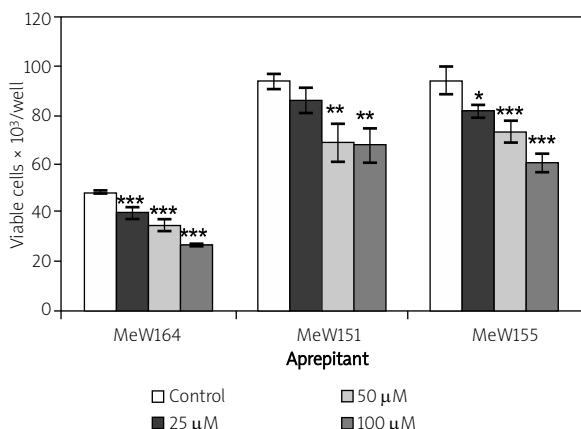
Fig. 7. Inhibitory effect of aprepitant on the proliferation of human melanoma cells. Melanoma cells were plated at 5 × 10³ cells/well and cultured for 4 days. The groups of cultures with aprepitant were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
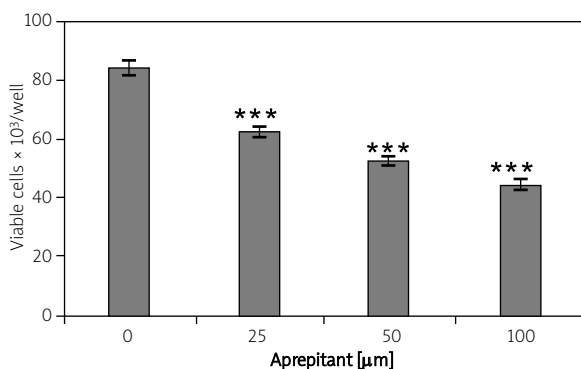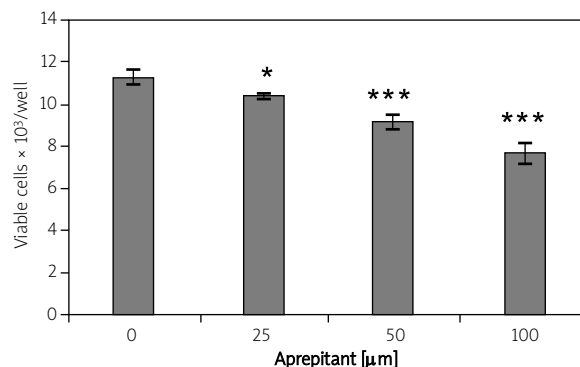

Fig. 8. Inhibitory effect of aprepitant on the proliferation of human lung cancer cells E-14 plated at 5 × 10³ cells/well and cultured for 4 days. The groups of cultures with aprepitant were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; *$P < 0.05$, ***$P < 0.001$.

Fig. 9. Inhibitory effect of aprepitant on the proliferation of human urinary bladder cancer cells T24 plated at 5 × 10³ cells/well and cultured for 4 days. The groups of cultures with aprepitant were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; ***$P < 0.001$.
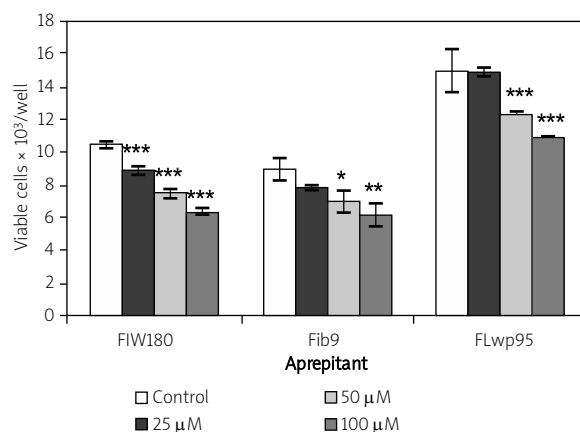

Fig. 10. Inhibitory effect of aprepitant on the proliferation of normal adult human fibroblast cell lines FlW180 and Fib9, and on the proliferation of human fetal fibroblast cell line FlWp95. Fibroblasts were plated at 5 × 10³ cells/well and cultured for 4 days. The groups of cultures with aprepitant were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Discussion

Newly synthesized opioid agonist-substance P antagonist AA3266 expressed high affinity to both, MOR and DOR opioid receptors, with preference to MOR opioid receptors. The level of affinities is similar to biphalin that confirms the previous observation that one opioid pharmacophore with an additional lipophilic group on the other side of hydrazide bridge determines 'biphalin like' properties [11,12]. The affinity to human NK₁ receptors is similar to C-terminal hexapeptide fragment of substance P. Compound AA3266 in an *in vitro* cell breading test showed antiproliferative properties against sever-
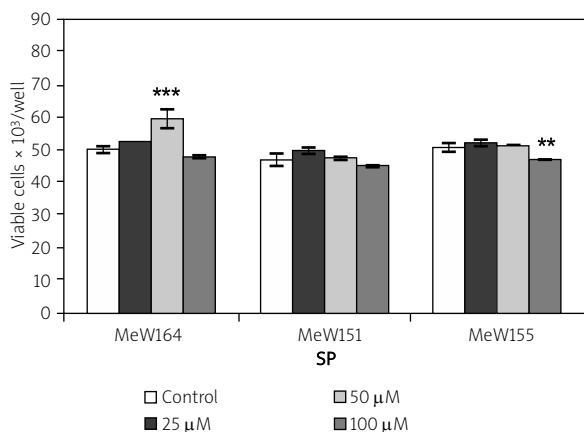
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Fig. 11. Effect of SP on the proliferation of human melanoma cells. Melanoma cells were plated at 5×10^3 cells/well and cultured for 4 days. The groups of cultures with SP were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures. **$P < 0.01$; ***$P < 0.001$.
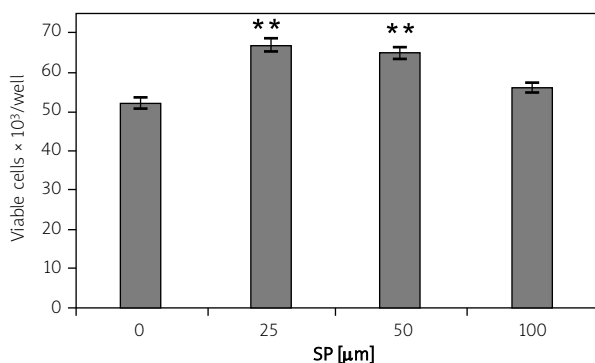

Fig. 12. Effect of SP on the proliferation of human lung cancer cells E-14 plated at 5×10^3 cells/well and cultured for 4 days. The groups of cultures with SP were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; *$P < 0.05$.

Fig. 13. Effect of SP on the proliferation of human urinary bladder cancer cells T24 plated at 5×10^3 cells/well and cultured for 4 days. The groups of cultures with SP were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; **$P < 0.01$.
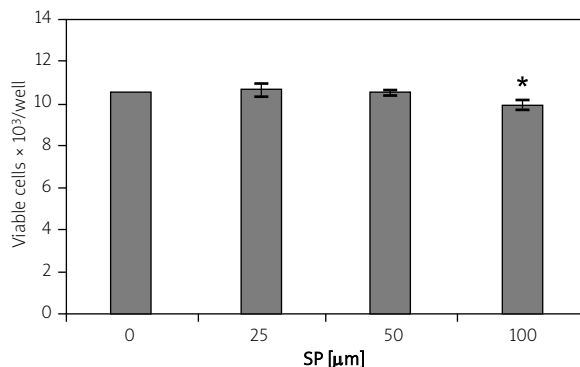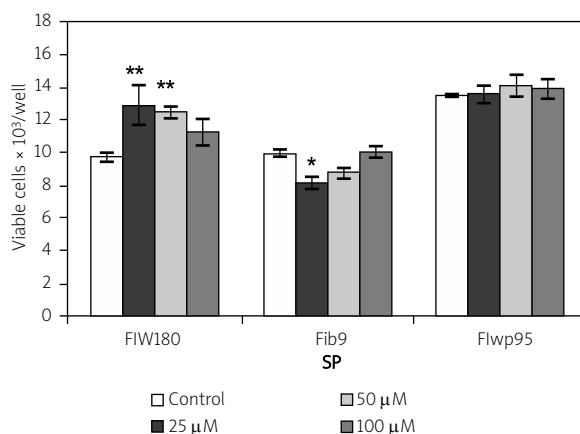

Fig. 14. Effect of SP on the proliferation of normal adult human fibroblast cell lines FlW180 and Fib9, and on the proliferation of human fetal fibroblast cell line FlWp95. Fibroblasts were plated at 8×10^3 cells/well and cultured for 4 days. The groups of cultures with SP were compared to the control cultures using Dunnett's test. The bars represent the mean values ± s.d. of the viable cell count in triplicate cultures; *$P < 0.05$, **$P < 0.01$

al types of cancer cells. As previously described selective tachykinin antagonist aprepitant [17], the highest antiproliferative effect was exerted on melanoma cells. Interestingly, the antiproliferative properties against normal cells, i.e. fibroblasts were visibly lower.

To summarize, newly synthesized opioid agonist-tachykinin antagonist AA3266 expressed high affinity to opioid MOR and DOR receptors, and significant antagonist affinity to NK_1 receptors. The compound expressed antiproliferative properties against various cancer cells. Therefore, the compounds applied in the treatment of chronic cancer-related pain may additionally enhance cancer directed therapies.

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