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Antiproliferative effects of [D-Pro^2, D-Trp^{7,9}]-Substance P and aprepitant on several cancer cell lines and their selectivity in comparison to normal cells

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Abstract

A neuropeptide, Substance P (SP), has mitogenic action in many types of cancer cells mediated via the neurokinin-1 receptor (NK1R). Small molecular NK1R antagonists have been frequently shown to possess anticancer activity both in vivo and in vitro, but there are only a few papers on such activity regarding peptide antagonists. In order to extend the data on this class of compounds, we have compared the effects of a peptide antagonist, [D-Pro^2, D-Trp^{7,9}]-Substance P, and a small molecular antagonist, aprepitant on the proliferation of five cancer and three normal cell lines. The comparison was based on three assays: cell proliferation test, MTT test and assay for colony formation. Consistently with earlier reports, aprepitant potently reduced cell proliferation in cancer cell lines in all assays, but in contrast to previous works, the compound was not selective and it affected normal cell lines to a similar degree. The studied peptide antagonist, [D-Pro^2, D-Trp^{7,9}]-Substance P, was able to decrease proliferation only in a few cell lines, and only in the highest concentration (100 μM). In a lower concentration, a slight pro-proliferative effect was observed in a few cell lines. No statistically significant effects on colony formation were found for this compound.

Key words: *Substance P, aprepitant, [D-Pro^2, D-Trp^{7,9}]-Substance P, neurokinin-1 receptor, antiproliferative effect.*

Introduction

A neuropeptide, Substance P (SP, sequence: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), works as a mitogen in many kinds of tumour cells [11,28]. The compound increases tumour cell proliferation, stimulates migration of cancer cells and angiogenesis [17]. These actions are dependent on the binding of SP to the neurokinin-1 receptor (NK1R) which is overexpressed by numerous types of cancer cells. Thus, it had been proposed that blocking this receptor could be a therapeutic strategy for anti-cancer drugs [15].

Following this proposal, several NK1R antagonists were validated to possess anticancer, cytotoxic action *in vitro* on many cell lines and *in vivo* in animals xenografted with tumours [17,19,22]. The tested antagonists included mainly small organic molecules, like aprepitant, L732,138 or L733,060. Studies devoted to this type of compounds are numerous, while relatively little is known on anticancer action of peptide NK1R antagonists [10,24,25,27]. The latter

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were the first NK1R antagonists [23], but with small molecular NK1R antagonists having been developed, peptides were largely abandoned since until recently a prevalent opinion was that peptides were not good candidates for drugs [7].

The opinion mentioned above was based on the fact that in general peptides have low stability in plasma (high susceptibility to proteolysis) and poorly cross biological membranes. This results in low oral bioavailability. Nowadays the view has been changed. Peptides receive more and more attention in drug discovery efforts [5,7]. This is inter alia due to the fact that peptides are particularly well suited for use in the design of multitarget compounds [3] as they can be easily hybridized by simple formation of a peptide bond between two (or more) functionalities.

With this in mind, when looking for a potential anticancer pharmacophore of a peptide character, suitable for further hybridization, we have turned to peptide NK1R antagonists [12]. Among these compounds, our attention was attracted by [D-Pro^2, D-Trp^{7,9}]-Substance P, which was one of the early potent antagonists. It was synthesized by Folkers *et al.* and found to be the most potent antagonist out of a considered group of 16 SP analogues [4]. In another study, the compound antagonized effects of exogenous SP in a competitive manner with $pA_2 = 6.1$ [4]. The peptide was found to have a moderate binding affinity, with inhibition constant/half-maximal inhibitory concentration values ranging from 0.4 µM to 5 µM, depending on the species being the source of the tissue preparation, type of the preparation and radioligand used [2,8,26]. Some authors characterized it as a partial agonist of Substance P rather than a typical antagonist [14,26].

No data on anticancer activity of this NK1R peptide antagonist have been ever reported to our knowledge. Therefore, we have decided to fill this gap by comparing the effects [D-Pro^2, D-Trp^{7,9}]-Substance P and aprepitant have on cells of five cancer and three normal cell lines.

Material and methods

Chemicals

[D-Pro^2, D-Trp^{7,9}]-Substance P (Arg-D-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Met-NH_2) was resynthesized in our laboratory by solid phase peptide synthesis (SPPS). Aprepitant was isolated from commercially available tablets (Merck). Both compounds were purified by HPLC and their identity was confirmed by the mass spectrometry method.

Cell culture

Five cancer cell lines (human melanoma: MeW151, MeW155, MeW164; human lung cancer: E14 and human urinary bladder carcinoma: T24) and three normal cell lines (human adult fibroblast lines: Fib9 and FlW180; and human foetal fibroblast line: FlWp95) were used in the study. They were obtained from the institutional cell bank at the Maria Sklodowska-Curie Memorial Institute and Oncology Centre in Warsaw.

The cells were cultured in Eagle's 1959 MEM medium (Biomed, Lublin, Poland), supplemented with 10% foetal calf serum (Invitrogen), 50 µg/ml penicillin G, 50 µg/ml streptomycin, and 0.1% glutamine. The cells were kept at 37°C, in humidified atmosphere (5% CO_2).

Assessment of cell proliferation

The effects [D-Pro^2, D-Trp^{7,9}]-Substance P and aprepitant have on the cell lines were assessed with respect to:

– influence on the number of cells after 4 or 7 days of incubation (8 cell lines),

– influence on cell viability as measured by the MTT assay (8 cell lines),

– influence on the ability to form colonies (5 cancer cell lines).

In all three types of assays we have followed the previously described procedures [12]. In brief, the cells were incubated with the compounds in three concentrations (25 µM, 50 µM and 100 µM, in separate wells), for 4 or 7 days (test a), 24 h (test b) or 7 days (test c). The number of the cells seeded were either 5000 cells per well (tests a and b) or 100 cells per dish (test c). After the incubation and additional steps if necessary, the readout followed. The result of the assay was the number of cells as counted in a haemocytometer (test a), or optical density read at 570 nm by using HR 7000 spectrophotometer (test b), or number of colonies counted under a microscope. As a control, cultures growing without tested compounds were used for each assay.

All determinations were done in two independent experiments with three repetitions per each data point. The results were normalized so that

the control value was 100%. The data are given as means with standard errors of the mean. They were analysed with the one-way ANOVA test with post-hoc Dunnett's test at significance level $\alpha = 0.05$. The results of the cell proliferation (a) test for aprepitant were partially presented previously in ref. [13].

Results

The effect that both considered compounds ([D-Pro^2, D-Trp^{7,9}]-Substance P and aprepitant) have on cells was evaluated in three tests, on eight cell lines (5 cancer and 3 normal lines). We considered direct influence on the number of cells following a few days of incubation (cell proliferation test), effects on the cell viability (MTT test) and effects on the extent of colony formation (colony formation test). The compounds were tested in three concentrations (25 µM, 50 µM and 100 µM). The results of the assays are presented graphically in Figures 1-3. Some of the results presented for aprepitant (cell proliferation test) were taken from ref. [13].

Antiproliferative effects of aprepitant are clearly visible in the cell proliferation test (Fig. 1A). For all considered cell lines, there is a statistically significant decrease in the number of cells incubated with 100 µM or 50 µM aprepitant. The effect is also seen for incubations with 25 µM aprepitant for all cells with the exception of Fib9 and FlWp95. The most sensitive is urinary bladder carcinoma T24 cell line (52 ±2% of the control value at 100 µM), while the least affected cells are melanoma MeW151 and fibroblasts FlWp95 (72 ±7% and 73 ±1% of the control value at 100 µM, respectively). It is worth noting that, on average, proliferation of cancer cells and normal cells is equally

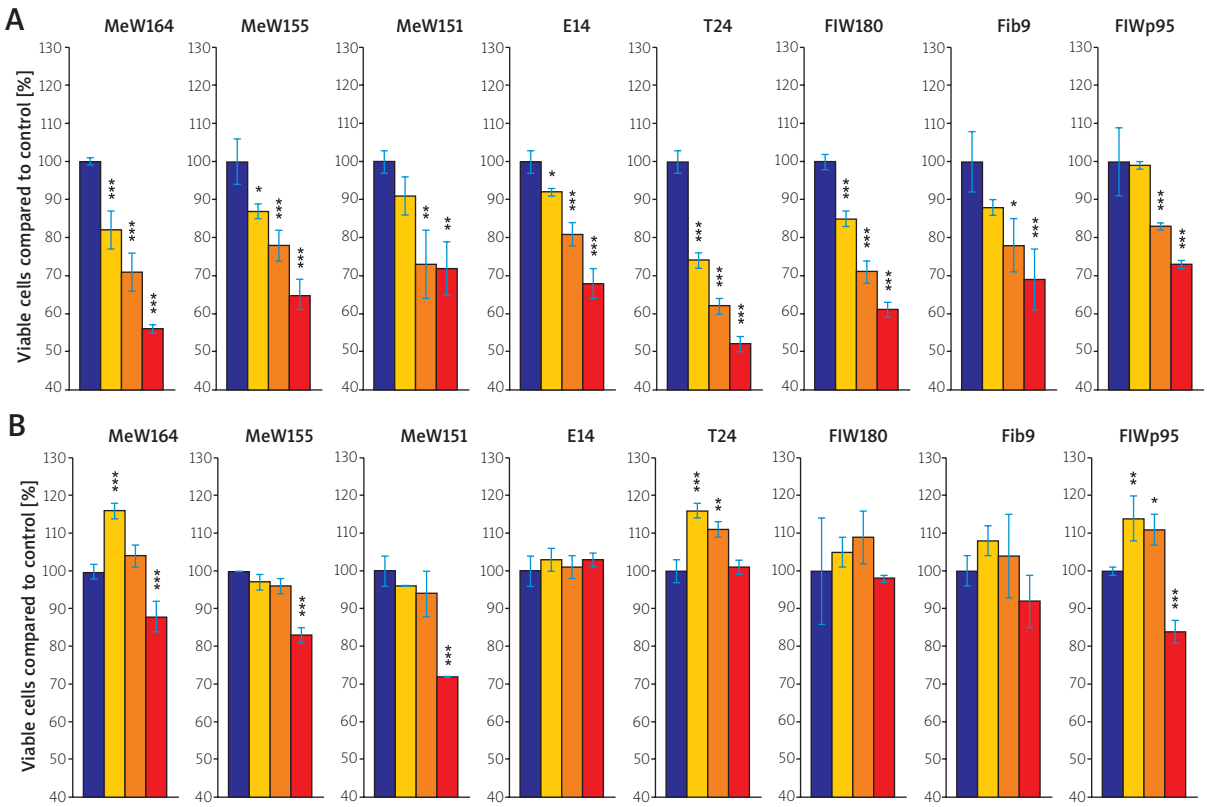

Fig. 1. The effects of the tested compounds on the cell number of different cell lines following a few days of incubation, **A)** aprepitant, **B)** [D-Pro^2, D-Trp^{7,9}]-Substance P. The results are expressed as a percent of the control value. Bar colouring corresponds to the concentration of the compounds: blue – control, yellow – 25 µM, orange – 50 µM, red – 100 µM. Cell lines designations given in the text. The blue thin bar shows standard error of the mean. The asterisks denote the statistical significance of the difference between the given value found for the given concentration and the control (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). The statistical analysis used is the one-way ANOVA with post-hoc Dunnett's test at significance level $\alpha = 0.05$.

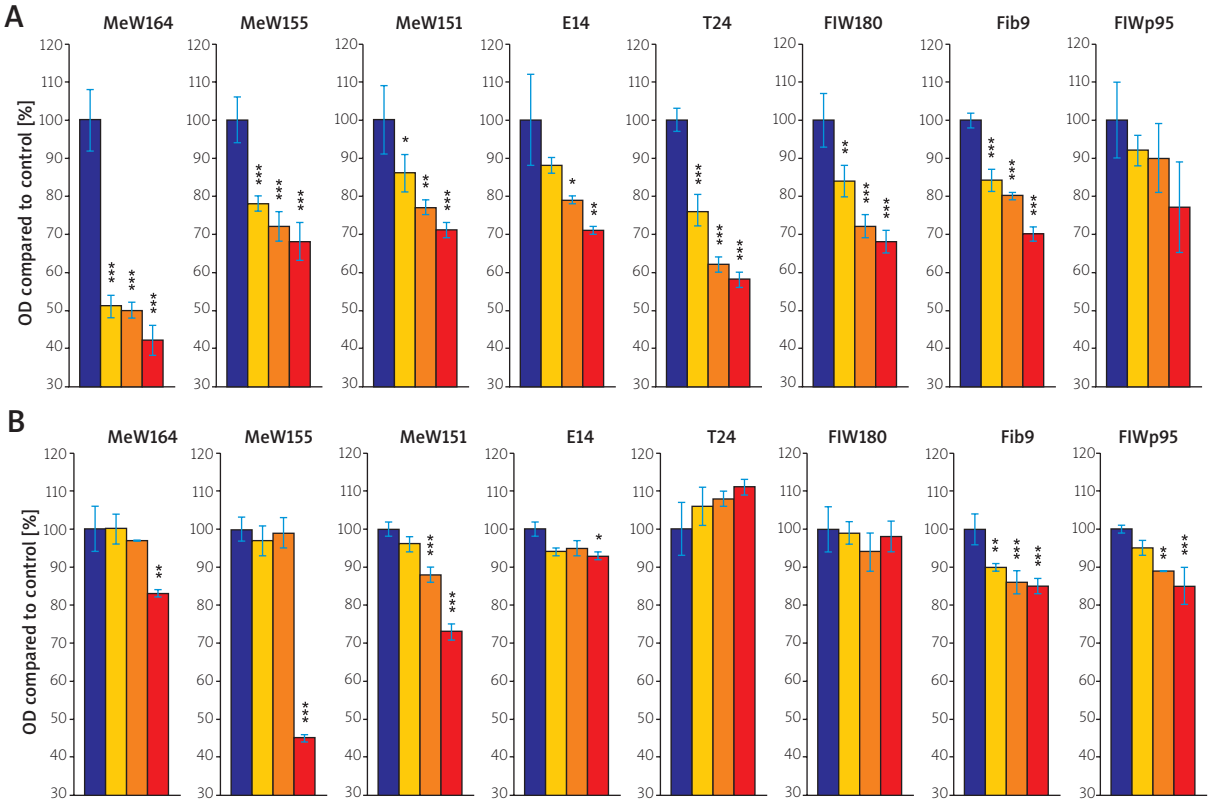

Fig. 2. The effects of the tested compounds on cell viability (MTT test) of different cell lines, **A)** aprepitant, **B)** [D-Pro^2, D-Trp^{7,9}]-Substance P. The results are expressed as a percent of the control value. Bar colouring corresponds to the concentration of the compounds: blue – control, yellow – 25 μM, orange – 50 μM, red – 100 μM. Cell lines designations given in text. The blue thin bar shows standard error of the mean. The asterisks denote the statistical significance of the difference between the given value found for the given concentration and the control (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). The statistical analysis used is the one-way ANOVA with post-hoc Dunnett's test at significance level $\alpha = 0.05$.

reduced by aprepitant (63 ±8% and 68 ±5% of the control value at 100 μM, respectively).

[D-Pro^2, D-Trp^{7,9}]-Substance P does not reduce the number of cells in majority of cases (Fig. 1B), exceptions being MeW164, MeW155, MeW151 and FlWp95 cell lines incubated with 100 μM of the peptide. The strongest reduction among these is found for MeW151 (72 ±1% of the control value at 100 μM). Surprisingly, 25 μM [D-Pro^2, D-Trp^{7,9}]-Substance P stimulates proliferation of MeW164, T24 and FlWp95 cells (116 ±2%, 116 ±2% and 114 ±6% of the control, respectively). Some slight stimulation is also observed for incubating 50 μM of the peptide with T24 and FlWp95 (111 ±2% and 111 ±4% of the control value, respectively).

A similar picture is yielded in the MTT test. Here, aprepitant reduces cell viability in all pairs cell line/concentration (Fig. 2A) with the exception of FlWp95 (all concentrations). The effect is most pronounced in the MeW164 cell line (42 ±4% of the control value at 100 μM). For the rest of the affected cell lines, the values are more or less similar, and no selectivity (cancer vs. normal cells) can be found.

Again, the tested SP analogue is less efficient in reducing the cell viability in the MTT test. Here however the situation is more diversified than in the case of the cell proliferation test. T24 and FlW180 lines are not affected at all at any concentration. Yet Fib9 cell line is sensitive to all used concentrations, though, not to a great extent (85 ±2% of the control value at 100 μM). The viability of FlWp95 and MeW151 lines is reduced by 50 and 100 μM of the peptide. For the remaining lines (MeW164, MeW155 and E14), it is only the largest concentration (100 μM) that

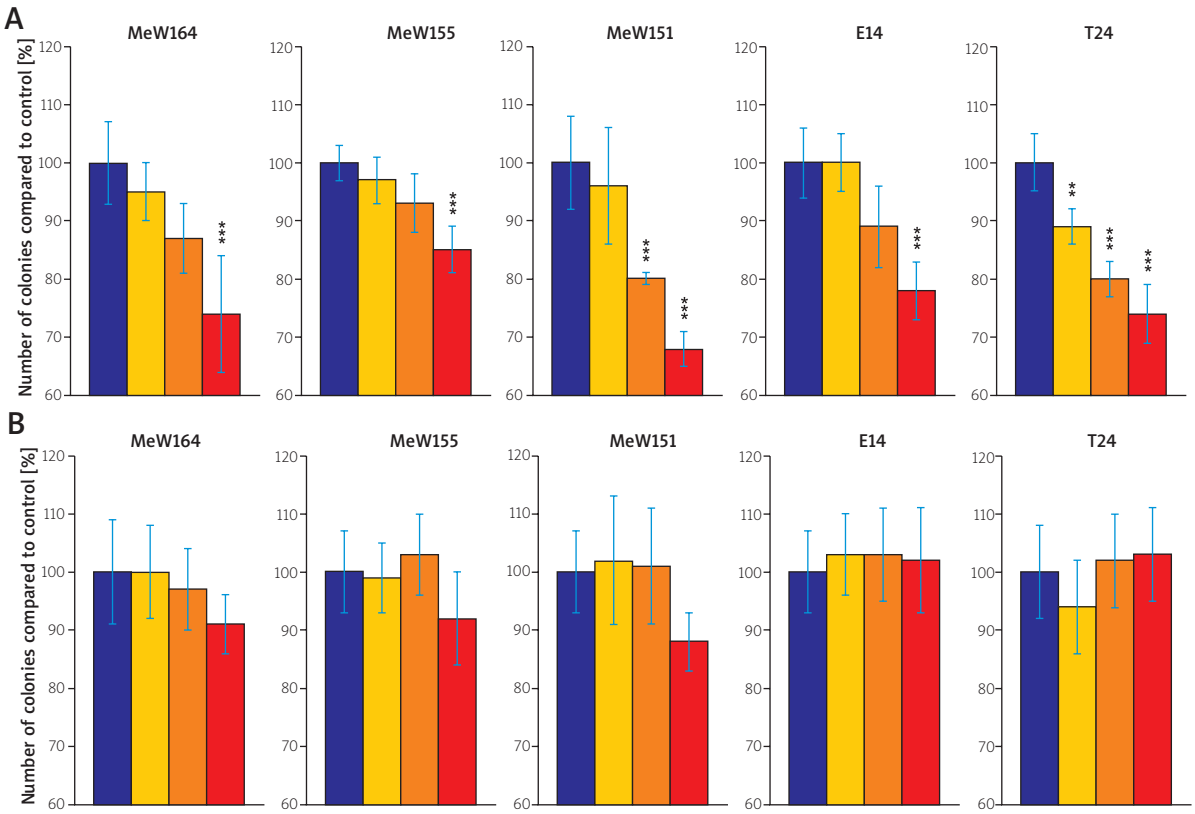

Fig. 3. The effects of the tested compounds on colony formation in different cell lines, **A)** aprepitant, **B)** [D-Pro^2, D-Trp^{7,9}]-Substance P. The results are expressed as a percent of the control value. Bar colouring corresponds to the concentration of the compounds: blue – control, yellow – 25 μM, orange – 50 μM, red – 100 μM. Cell lines designations given in text. The blue thin bar shows standard error of the mean. The asterisks denote the statistical significance of the difference between the given value found for the given concentration and the control (**$p \leq 0.01$, ***$p \leq 0.001$). The statistical analysis used is the one-way ANOVA with post-hoc Dunnett's test at significance level $\alpha = 0.05$.

significantly reduces the MTT readout when compared to control. The melanoma MeW155 cell line is the most sensitive of all lines at 100 μM (45 ±1% of the control value at 100 μM), but strangely this is not paralleled by proportionally significant toxicities with 25 and 50 μM concentrations. Except for this MeW155/100 μM combination, no other achieves values lower than about 70% of the control value. Contrary to what was found in the cell proliferation test, no stimulatory effects were present in the MTT assay with [D-Pro^2, D-Trp^{7,9}]-Substance P.

The effect on colony formation was tested only for cancer cells. The normal cells in the particular testing conditions (seeding density, time etc.) do not form colonies. Aprepitant affects this property in all cancer cell lines at 100 μM (Fig. 3A). The extent of the effect is similar for all lines, being on average 76 ±6% of the control value at 100 μM. A statistically significant reduction in colony formation is also found for MeW151 at 50 μM and for T24 at 25 and 50 μM.

On the contrary, in the case of [D-Pro^2, D-Trp^{7,9}]-Substance P, none of the tested concentrations was able to influence the colony formation in any of the lines (Fig. 3B).

Discussion

Small molecules antagonising the action of a neuropeptide, Substance P, were many times shown to possess anticancer activity [17,19]. On the other hand, only several studies on cytotoxicity were devoted to peptide NK1R antagonists [10,24,25,27]. In order to extend these scarce findings we set out to compare

antiproliferative action of a small molecular NK1R antagonist, aprepitant, and a peptide NK1R antagonist, [D-Pro^2, D-Trp^{7,9}]-Substance P. The comparison was based on three tests performed on a set of 5 cancer and 3 normal cell lines. The tests showed the influence the compounds have on a number of cells (cell proliferation test), their viability (MTT assay) and their ability to form colonies.

Consistently with the literature data, aprepitant showed significant cytotoxicity in almost all tested conditions. The compound reduced the number of cells even in the lowest of the tested concentrations (25 μM). With a few exceptions, this concentration was also sufficient to decrease viability as found in the MTT test. Higher concentrations were able to significantly suppress the colony formation of cancer cells.

Let us note here that the cell lines tested herein seem more resistant to aprepitant than the lines tested by other authors. In majority of cases, earlier data pointed to IC_{50} values of around 20-40 μM and IC_{100} of 40-80 μM in assays similar to the MTT assay [17,20]. For example, Muñoz *et al*. investigated the effect of aprepitant on human melanoma cell lines MEL HO, COLO 858 and COLO 679, for which they found growth inhibition with IC_{50} values of 29.6 μM, 24.3 μM and 32.1 μM, respectively [21]. In MG-63 osteosarcoma cell line, aprepitant influenced the cells with IC_{50} = 30 μM [16]. Another study considered the effect of aprepitant on GAMG glioma cell line (IC_{50} = 32 μM) [9]. With regard to breast cancer cell lines, Muñoz *et al*. reported that aprepitant inhibits the growth of a number of these, with IC_{50} values reading 31.4 μM for BT-474, 35.6 μM for MCF-7, 29.5 μM for MDA-MB-468 and 40.8 μM for MT-3 [18]. In contrast to these and other findings [17,20], in our study, aprepitant even at concentrations as high as 100 μM at best approached a 50% reduction of a given assay readout.

Regarding the aprepitant's selectivity, this NK1R antagonist has been usually considered to selectively affect cancer cells and to have little effect on normal cells. For example, Muñoz and Rosso reported that aprepitant influenced human embryonic kidney 293 (HEK293) cells with IC_{50} values more than three times higher than in cancer cell lines [20]. A yet better example of NK1R antagonists' selectivity was provided by Ge *et al*. [6] who found that aprepitant (in concentrations up to 30 μM) and another small molecular NK1R antagonist, SR140333 (in concentrations up to 60 μM), had no effect on viability of human normal CD34$^+$ hematopoietic cells. Neither had they shown any haemolytic toxicity in human red blood cells.

In contrary to these reports, in our study aprepitant did not exhibit selectivity and it had similar antiproliferative action on both cancer and normal cell lines. This is the first time such a lack of selectivity (cancer vs. normal cells) has been shown for aprepitant regarding the effect on cellular proliferation. This finding calls for further inquiry as to its potential implications.

[D-Pro^2, D-Trp^{7,9}]-Substance P showed a significantly weaker antiproliferative action than aprepitant. It is only the highest concentrations that decreased the cell numbers in the multi day tests (but not in all cell lines). In the MTT assay, the cytotoxicity was present only in a few combinations of concentration/cell line. None of the tested concentrations influenced colony formation in cancer cells. What is interesting, lower concentrations of [D-Pro^2, D-Trp^{7,9}]-Substance P had a positive effect on a number of cells in MeW164 and T24 cancers and FlWp95 normal cells. In the case of these lines, it is possible to draw a nonlinear dose-response curve with an inverted U/J-shape. In such a relationship, low doses of the tested substance appear to stimulate the cell proliferation, while the higher doses have a negative impact thereon. However, for scarcity of the points in the plots, the proposition that the tested SP analogue affects the cells in a biphasic manner is only of a tentative character. Furthermore, this type of relationship does not appear to be present in the results of the MTT assay. Thus the question whether the observed effects do in fact have a biphasic character (of hormetic or non-hormetic type [1]) requires further investigation, including more data- and perhaps time-points. It is equally hard to speculate on what could constitute the mechanistic basis behind such a potential biphasic response. In theory, this could be associated with partial agonism, involvement of more than one molecular target or compensatory mechanisms of the cells. We are not aware of any observation of U/J-shaped dose-response curves for NK1R antagonists with respect to effects on cell proliferation, so our report seems to be the first of this kind in the literature.

The Substance P analogue tested herein is an SP antagonist of peptide character. So far, there has been no report on the effects it has on cancer

cells. With respect to other peptide antagonists, a few authors considered their anticancer action *in vitro* and *in vivo*. Woll *et al*. investigated the effects [D-Arg^1, D-Phe^5, Trp^{7,9}, Leu^{11}]-SP has on human small lung cancer cells (SLCC) *in vitro* [27]. They found it inhibits this cancer in a concentration-dependent manner. Seckl *et al*. analysed the effects of [D-Arg^1, D-Trp^{5,7,9}, Leu^{11}]-SP on small cell lung cancer cells [25]. This analogue turned out to inhibit not only H-510 and H-69 SLCC cells in culture but also the growth of H-69 xenograft in nude mice. In the liquid culture, 25 µM concentration of this compound was able to supress as much as 92% of cancer growth. Langdon *et al*. considered [Arg^6, D-Trp^{7,9}, MePhe^8]-SP(6-11) and [D-Arg^1, D-Phe^5, Trp^{7,9}, Leu^{11}]-SP with respect to their action in SLCC and found similar results [10]. Here it is to be noted that these compounds were considered broad-spectrum neuropeptide antagonists, and their action was also associated with binding to the bombesin or vasopressin receptors. For this and other reasons (differences in both cell lines and the methods used), it is hard to directly compare the effects these peptides have on the cancers with the data we have presented in this paper.

Conclusions

In conclusion, the peptide NK1R antagonist, [D-Pro^2, D-Trp^{7,9}]-Substance P displays antiproliferative action on cancer or normal cell lines, which is however much weaker than the action exerted by aprepitant. In some cell lines, the compound shows a slight pro-proliferative effect at lower concentrations. This suggests that there exists a U/J-shaped dose-response relationship for these cell lines which however requires further studies to confirm it.

It is surprising that aprepitant is not selective. The substance affects both the tested cancer and normal cells to a similar degree. This finding seems important since previous papers indicated that this compound is selective in this respect.

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Disclosure

The authors declare no conflict of interest.

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