Role of NK-1 and NK-2 tachykinin receptor antagonism on the growth of human breast carcinoma cell line MDA-MB-231

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We demonstrate that neurokinin A (NKA) and substance P (SP) play a role in the proliferation of the estrogen receptor-negative (ER–) cell line MDA-MB-231, a human breast carcinoma expressing both NK-1 and NK-2 receptors. *In vitro* experiments showed that the specific receptor antagonists MEN 11467 (NK-1) and nepadutant (MEN 11420; NK-2) inhibited tumor cell proliferation, and blocked the stimulatory effect of SP and NKA. Anti-tumoral activity of NK-1 and NK-2 receptor antagonists was demonstrated in nude mice, measuring growth inhibition of MDA-MB-231 tumor cells xenografted s.c. and by using the hollow-fiber assay. In both systems a significant inhibition was found when compounds were administered at 5 mg/kg i.v. every day for 2 weeks. Results obtained from both these models suggest that the *in vivo* activity of NK-1 and NK-2 antagonists may be a result of a cytostatic effect rather than a cytotoxic effect. Our results suggest that the control of breast carcinoma (ER–) growth by tachykinin receptor antagonists may become a new form of targeted therapy for these human tumors. *Anti-Cancer Drugs* 16:1083–1089

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Introduction

Neuropeptides, including substance P (SP) and neurokinin A (NKA), are increasingly recognized as potent cellular growth factors, and a role for these molecules in the control of normal and abnormal cell proliferation is now well established [1].

Both SP and NKA belong to the tachykinin family, a group of peptides sharing the common C-terminal sequence Phe–X–Gly–Leu–Met-NH_2 and are encoded by mRNAs resulting from pre-pro-tachykinin I gene transcription [2]. A number of physiological actions, including exocrine and endocrine secretion, smooth muscle contraction, pain transmission, fluid homeostasis, blood pressure regulation, and inflammation, are mediated by the interaction of these peptides with at least three different transmembrane G-protein-coupled receptors, i.e. NK-1, NK-2 and NK-3 receptors [2]. Recent evidence suggested that inappropriate expression or regulation of these receptors on tumor cells as a result of oncogene amplification may contribute to tumor growth and invasion [1], thus suggesting that once specific receptors are identified, specific neuropeptide antagonists may inhibit the paracrine or autocrine loops; they could therefore be used as potential treatment in neuropeptide-secreting tumors [3]. A role for SP and NKA as autocrine/paracrine growth factors for human cancer has been demonstrated, notably, but not exclusively, for small cell lung cancer (SCLC) [3,4]. Emerging findings suggest that the growth of SCLC may be inhibited by SP antagonists although with a lower order of magnitude than other peptide antagonists as well as to the bombesin receptor [3]. The presence of tachykinin NK-1 receptors, the subtype with the highest activity for SP, has been established in human astrocytoma and glioma primary tumors [5]. A direct role of SP in supporting human glioma development and the inhibition of human glioma xenograft growth by using specific and selective NK-1 antagonist has been well demonstrated [6,7]. Recent findings indicated that neuroendocrine functions are also implicated in the development of breast cancer [8]. In these tumors, expression of NK receptors has been associated with neoangiogenesis [8], a hallmark of tumor development, and the presence of the pre-pro-tachykinin gene has been demonstrated to be involved in the metastasis of breast cancer cells to the bone marrow [9,10]. In recent decades a wide number of antagonists highly specific and selective for human tachykinin NK-1 and NK-2 receptors have been described with heterogeneous chemical structures and pharmacological properties. MEN 11467 and nepadutant (MEN 11420) are potent human tachykinin receptor antagonists that showed high affinity for the respective receptors in terms of K_i values [11,12]. Previous data demonstrated that NK-1 receptor blockade by MEN 11467 resulted in the control of human glioma U373 MG tumor xenotransplanted in nude mice for at least 6 weeks [7].

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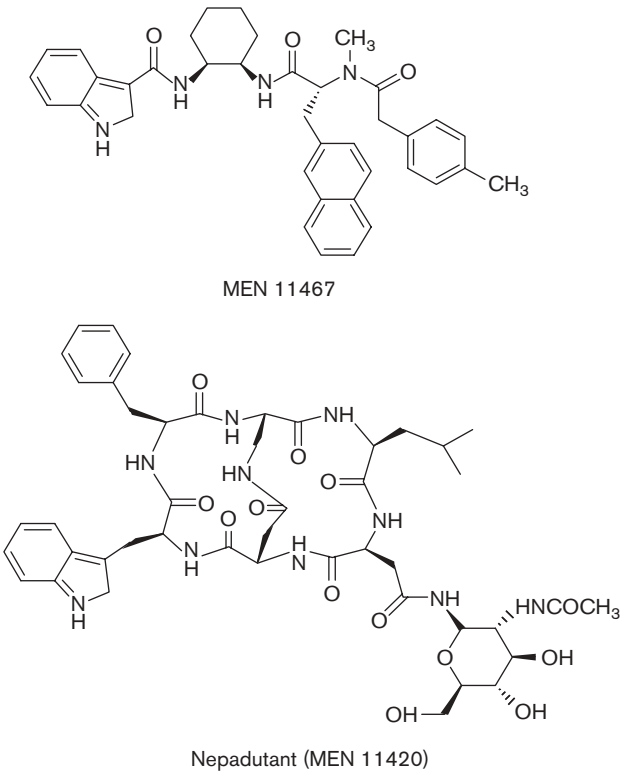
In this study, we evaluated the possible role of NK-1 and NK-2 receptors in the modulation of growth or development of MDA-MB-231, an estrogen-independent human breast carcinoma, by using the NK-1 and NK-2 receptor antagonists MEN 11467 and nepadutant. The expression of mRNAs for NK-1 and NK-2 receptors was determined, and the effect of tachykinin receptor blockade on MDA-MB-231 tumor growth was evaluated *in vitro* and *in vivo* by using the conventional xenograft model and the hollow-fiber (HF) assay.

Materials and methods

Chemicals

NK-1 tachykinin receptor antagonist, MEN 11467 ((1*R*,2*S*)-2*N*[1(H)indol-3-yl-carbonyl)]-1-*N*-{*N*α(*p*-tolylacetyl)-*N*α(methyl)-D-3-(2-naphthyl) alanyl diaminocyclohexane; molecular weight = 600.8) and NK-2 tachykinin receptor antagonist, nepadutant (MEN 11420) (N^4-(2-acetylamino-2-deoxy-β-D-glucopyranosyl)-(L-asparaginyl-L-aspartyl-L-tryptophyl-L-phenylalanyl-L-2,3-diaminopro-pionyl-L-leucyl)-C-4.2–N-3.5-lactam-C-1.6–N-2.1-lactam; molecular weight = 947), were synthesized at the Chemistry Department of Menarini Ricerche (Pomezia, Italy) (Fig. 1). For the purposes of this study, these antagonists were dissolved in DMSO and diluted in saline containing 6% Tween-80. SP, NKA and doxorubicin were purchased from Sigma (St Louis, Missouri, USA), dissolved in water and stored at −20°C. Aliquots of peptide solution were diluted in saline immediately before use.

Fig. 1



Chemical structures of MEN 11467 and nepadutant (MEN 11420).

Cell cultures

Human breast carcinoma MDA-MB-231 (HTB-26) and human astrocytoma U373 MG (HTB-17), naturally expressing NK-1 receptor, were obtained from ATCC (Manassas, Virginia). Cells were maintained in RPMI 1640 (Gibco/BRL, Grand Island, New York, USA) supplemented with 10% FBS, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2/95% air humidified incubator. CHO/NK_2 cells stably expressing the human NK-2 receptor were obtained from Menarini Biotech as already described [13]. Cells were cultured in α-MEM supplemented with 10% FBS and 2 mmol/l L-glutamine at 37°C in a 5% CO_2/95% air humidified incubator.

Proliferation studies

Breast carcinoma MDA-MB-231 cells were plated in 12-well tissue culture plates (2×10^4 cells/well in 1 ml of medium). At 24 h after seeding, the cells were exposed to drugs at the appropriate dilutions in RPMI 1640 medium supplemented with 0.8% FBS until the end of the experimental period, as indicated in the figures. Cell viability was measured by Trypan blue exclusion and the surviving cells were reported as a function of the drug concentration.

RNA isolation and RT-PCR

Total RNA was isolated from cell cultures by Trizol methods (Gibco/BRL). RNA (1 µg) was reverse transcribed in 20 µl for 15 min at 42°C and then stopped at 99°C for 5 min. The reverse transcription mixture contained 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 50 U MuLV-RT (Applied Biosystems, Foster City, California, USA), 20 U RNase inhibitor (Perkin-Elmer Cetus, Norwalk, Connecticut, USA), 2.5 mmol/l oligo-d$(T)_{16}$ (Perkin-Elmer Cetus) and 1.0 mmol/l dNTP (Perkin–Elmer Cetus). The reverse transcription reaction was performed at 42°C for 15 min and stopped at 99°C for 5 min. Samples were overlaid with oil and then amplified for 30 cycles in a DNA thermal cycler (Perkin-Elmer Cetus). The profile for each cycle was 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. Reactions were subjected to a final extension at 72°C for 7 min. PCR products (20 µl) were separated by electrophoresis on 2% agarose containing ethidium bromide. NK-2 primers, 5′-AGT CTC CTT AGT GTG ACA CC-3′ (sense) and 5′-CTA CCA CCT CTA CTT CAT CC-3′ (antisense), span 274 bp. NK-1 primers, 5′-CTG CTG GAT AAA CTT CTT CAG GTA G-3′ (sense) and 5′-AGG ACA GTG ACG AAC TAT TTT CTG G-3′ (antisense), span 665 bp. Primers for human G3PDH were purchased from Clontech (Milan, Italy).

Human tumor xenograft

Female athymic nude mice (6–8 weeks old), were purchased from Harlan (Udine, Italy), maintained in microisolator cages and supplied with sterile materials under standard conditions, according to UKCCCR guidelines [14].

Human tumor MDA-MB-231 originated from s.c. *in vivo* injection of tumor cell (20×106 cells/flank/0.2 ml) in the right flank of adult athymic female nude mice. Tumor growth was followed by caliber measurement of length and width twice weekly. Tumor Volume (TV) was calculated by using the formula: volume (mm^3) = width^2 × length/2 [15].

MEN 11467 and nepadutant were administered i.v. at a dose of 5 mg/kg (dose volume of 10 ml/kg body weight) every day for 2 weeks starting when tumors were approximately 50 mm^3 in volume. Doxorubicin was administered i.v. at 7 mg/kg (dose volume of 10 ml/kg body weight) with the schedule q.7d. × 3 starting when tumors were approximately 50 mm^3 in volume. The effect achieved by the drug treatment was evaluated as tumor volume inhibition percent (TVI%) in treated versus control mice, determined at the nadir of tumor volume in the treated group as follows [16]: $[1 - (TV_{treated}/TV_{control\ group}) \times 100]$.

HF assay

PVDF HFs (M_r 500 000 molecular weight cutoff; 1 mm internal diameter) were purchased from Spectrum (Breda, The Netherlands). Before being filled with tumor cell suspensions, the fibers were individually flushed with 70% ethanol and soaked in 70% ethanol for 4 days. After rinsing and autoclaving in deionized water, the fibers were filled with RPMI 1640, supplemented with 20% FBS and incubated at 37°C for 24 h. Exponential growing cells cultures of MDA-MB-231 were trypsinized, pelleted by centrifugation, resuspended in RPMI plus 20% FBS and loaded into the fibers with the aid of a 20-gauge needle. A correlation between initial cell number and optical density measurement by the MTT viability assay was established by implanting different initial cell numbers into the fibers. The optimal window of cell growth was defined by percent net growth from day 0 to 7, which occurred at the lower cell densities tested. The initial cell density selected for MDA-MB-231 tumor cells was 2×10^7 cells/ml. Single implants were formed by crimping the fiber at 2-cm sections and applying a heat-seat at the crimp sites. Implants were transferred into six-well plates containing cold (4°C) complete medium and then incubated at 37°C overnight in 5% CO_2 prior to implantation into mice. The fibers were implanted s.c. using aseptic surgical procedures and the animals were dosed. Nepadutant and MEN 11467 (5 mg/kg) were administered i.v. for 6 consecutive days starting 1 day after fiber implantation. Doxorubicin was administered as a single dose of 7 mg/kg i.v. 1 day after fiber implantation. The studies were terminated on day 7, the fibers removed from the mice and cell viability evaluated by the MTT assay. The percent of net growth for each fiber was determined from the MTT assay as follows [17]: percent net growth = {[sample OD – day 0 (*in vitro*) sample OD]/day 0 (*in vitro*) sample OD × 100. The percent net growth obtained from drug-treated mice was compared to the percent net growth obtained in control mice as an assessment of anti-tumor activity.

Three drug effect categories were considered: (i) a positive net cell growth less than seen in the vehicle control samples can be considered as a growth inhibitory effect, (ii) a value of moderate to total net cell growth inhibition indicates a cytostatic effect and (iii) a negative net cell growth indicates cytocidal activity of the tested compound [17].

Statistical analysis

InStat 2.0 software was used for statistical evaluation. The comparison was made using Kruskall–Wallis nonparametric and Student–Newman–Keuls ANOVA tests. A significant level was considered at $P < 0.05$.

Results

Expression of mRNA for NK-1 and NK-2 in MDA-MB-231 human breast carcinoma

RT-PCR analyses demonstrated that MDA-MB-231 tumor cells expressed mRNA for tachykinin NK-1 and NK-2 receptors (Fig. 2). U373 MG human astrocytes and CHO/NK_2 cells were used as positive control for NK-1 and NK-2 receptors, respectively.

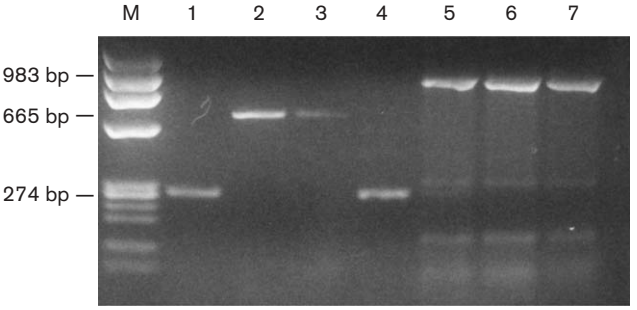
NK-1 mRNA expression was detectable in MDA-MB-231 cells as a product of the expected size of 665 bp, as well as in U373 MG tumor cells. NK-2 mRNA expression was detectable in MDA-MB-231 as a 274-pb product corresponding to the expected RT-PCR product in CHO/NK_2 cells, although with a lower expression (Fig. 2).

As control, Fig. 2 (lanes 5–7) shows the RT-PCR products of 983 bp corresponding to the G3PDH human gene.

Effects of the receptor antagonists MEN 11467 (NK-1) and nepadutant (NK-2) on the growth of MDA-MB-231 tumor cells *in vitro*

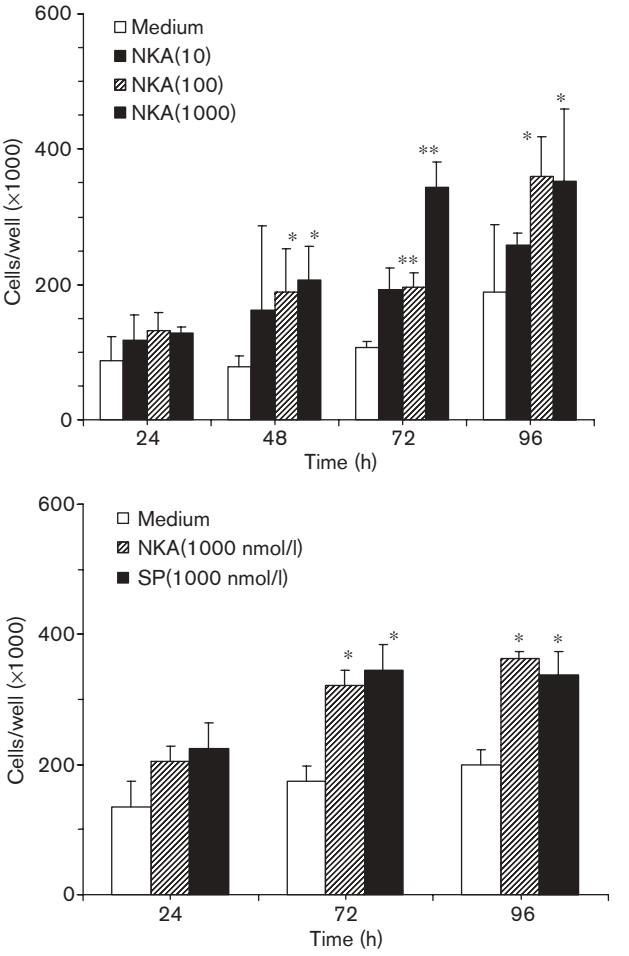
The aim of these experiments was to verify the ability of SP and NKA to stimulate the growth of MDA-MB-231 tumor cells *in vitro*, and to measure the effect of NK-1 and NK-2 receptor antagonists MEN 11467 and nepadutant on tumor cells growth in the presence and absence of specific agonist receptor stimulation.

Fig. 2



Expression of mRNA for NK-1 and NK-2 receptors in MDA-MB-231 human breast carcinoma. Total RNA (1 μg) was reverse transcribed and cDNA was amplified with specific primers. The PCR products were resolved on a 2% agarose ethidium bromide gel. The sizes of expected products are shown. Lane M, molecular markers; lane 1, PCR product of 274 bp from CHO/NK_2; lane 2, PCR product of 665 bp from U373 MG; lanes 3 and 4, PCR products for NK-1 and NK-2 receptors obtained from MDA-MB-231 tumor cells; lanes 5, 6 and 7 mRNA expression for human G3PDH (PCR product of 983 bp), used as internal control from MDA-MB-231, CHO/NK_2 and U373 MG, respectively.

Fig. 3



In vitro stimulation of MBA-MB-231 tumor cell growth by NKA and SP. (Upper panel) Effect of different NKA concentrations; *$P<0.05$ NKA-treated versus medium; **$P<0.01$ NKA-treated versus medium. (Lower panel) Effect of 1000 nmol/l SP and NKA; *$P<0.05$ NKA and SP-treated versus medium.

In a first series of experiments, MDA-MB-231 tumor cells were cultured in a low serum concentration (0.8%) medium and cell growth was evaluated at different time points in the presence of exogenous SP or NKA (10–1000 nmol/l) (Fig. 3). SP and NKA significantly stimulated tumor cell proliferation *in vitro* with a similar order of magnitude. Indeed, both substances were able to increase MDA-MB-231 cell growth, with a maximum stimulation effect observed at 1000 nmol/l after 96 h of incubation. A second series of experiments showed that the NK-1 and NK-2 receptor antagonists MEN 11467 and nepadutant were able to reduce MDA-MB-231 cell proliferation induced *in vitro* by 100 nmol/l exogenous NKA (Fig. 4). The effect was already statistically significant after 72 h for both compounds and still present after 96 h of incubation (Fig. 4).

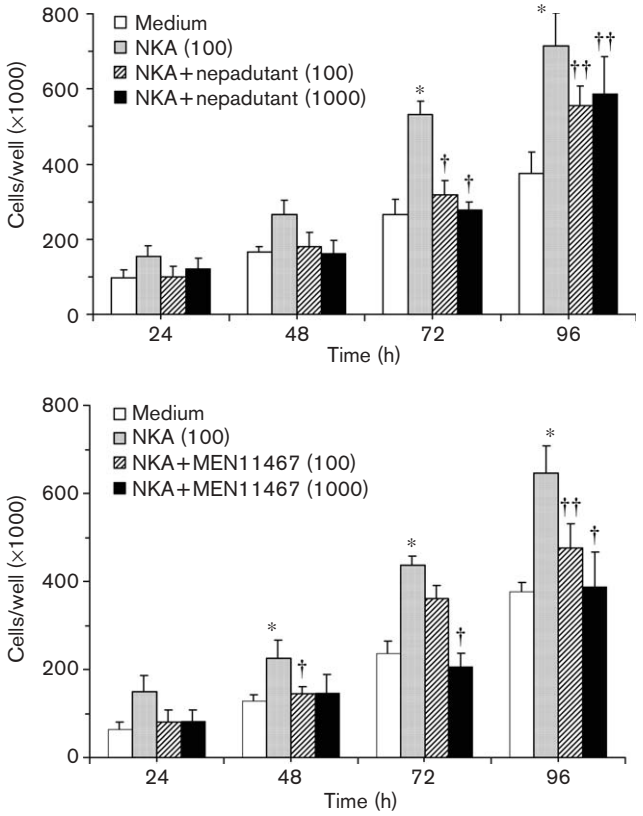
MEN 11467 was slightly more efficient than nepadutant, being already active after 48 h and inducing a more pronounced effect at 96 h (Fig. 4, lower panel).

In the absence of exogenous NKA stimulation, only a marginal tumor cells growth inhibition was observed when both receptor antagonists were used (Fig. 5).

Activity of MEN 11467 (NK-1) and nepadutant (NK-2) on the growth of MDA-MB-231 tumor cells in the HF and xenograft model

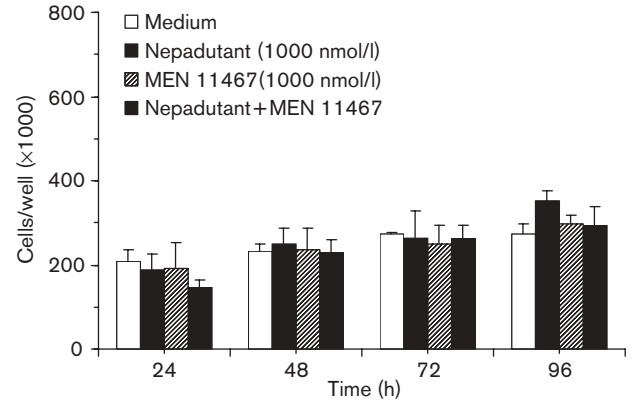
The two compounds MEN 11467 and nepadutant were evaluated for their anti-proliferative property by using the HF assay and xenograft model (Table 1 and Fig. 6). Both antagonists were administered i.v. at 5 mg/kg, a dose that has been previously shown to produce a complete blockade of the tachykinin-mediated response elicited by systemic administration [11,12]. For the HF assay, animals were treated daily for 6 days by i.v. bolus. Doxorubicin, used as standard cytotoxic reference, was administered as a single dose of 7 mg/kg – its optimal dose as already described [18]. Treatment with both MEN 11467 and nepadutant exhibited an inhibitory effect on MDA-MB-231 tumor cell growth in the HF assay (Fig. 5, upper panel), with a value of net growth ranging from 43.5 to 51%, whereas the combination of both was cytostatic with a net growth of 22.7% (Table 1). Doxorubicin confirmed its cytotoxic property with a negative value of net growth percent (Table 1). Similar results were achieved in the tumor xenograft model, where the combination of both antagonists was active in reducing the growth of MDA-MB-231 tumor (Fig. 5, lower panel). It should be noted that treatment with both

Fig. 4



Effect of NK-2 receptor antagonist nepadutant (upper panel) and NK-1 receptor antagonist MEN 11467 (lower panel) on *in vitro* MDA-MB-231 tumor cell growth stimulated by 100 n mol/l of NKA in the presence of 0.8% FBS. (Upper panel) $^{*}P<0.01$ NKA versus medium; $^{\dagger}P<0.01$ nepadutant versus NKA-treated cells; $^{\dagger\dagger}P<0.05$ nepadutant versus NKA-treated cells. (Lower panel) $^{*}P<0.01$, NKA versus medium; $^{\dagger}P<0.01$ MEN 11467 versus NKA-stimulated cells. $^{\dagger\dagger}P<0.05$ MEN 11467 versus NKA-stimulated cells.

Fig. 5



Effect of both antagonists MEN 11467 and nepadutant on MBA-MB-231 tumor cell growth. $^{*}P<0.05$ nepadutant plus MEN 11467 versus medium; $^{**}P<0.01$ nepadutant plus MEN 11467 versus medium.

antagonists resulted in inhibition of tumor growth until the last administration; thereafter, the tumor started to re-grow. After 12 days of treatment the final tumor volume at the end of the experiment was decreased by 60% (Table 1) with respect to vehicle-treated animals. As a control, we used doxorubicin (7 mg/kg) with a weekly schedule (q.7d. × 3).

Discussion

Recently published data have addressed the influence of neurotransmitters on cancer cell proliferation, suggesting that SP and NKA binding to their receptors can result in multifunctional activities that may differ according to the histological type of the tumor [1]. Thus, the specific biologic function of tachykinin receptors in human tumors, especially in solid tumors, is not well understood and needs further clarification. Our results, obtained using both *in vitro* and *in vivo* tumor models, demonstrate a role for NKA, SP and their receptors, NK-1 and NK-2, in the proliferation of the estrogen receptor-negative human breast MDA-MB-231 carcinoma cells, addressing the finding that neurokinin receptors and their modulation by specific antagonists may play an important role in the regulation of breast cancer growth and development. The MDA-MB-231 cell line expresses both tachykinin receptors, NK-1 and NK-2, as demonstrated by RT-PCR experiments (Fig. 2). The presence of these receptors has already been described for breast cancer cells, and a close correlation has been found between neurokinin receptor presence and tumor progression [9,10].

We observed that NK-1 and NK-2 receptor stimulation by SP and NKA supports the growth of MDA-MB-231 cells when cultured in a low serum condition, indicating that SP and NKA may cooperate with other growth factors in the regulation of tumor growth proliferation. A similar effect has been already described for other tumor types, including SCLC, glioblastoma and pancreatic carcinoma [3,4,7].

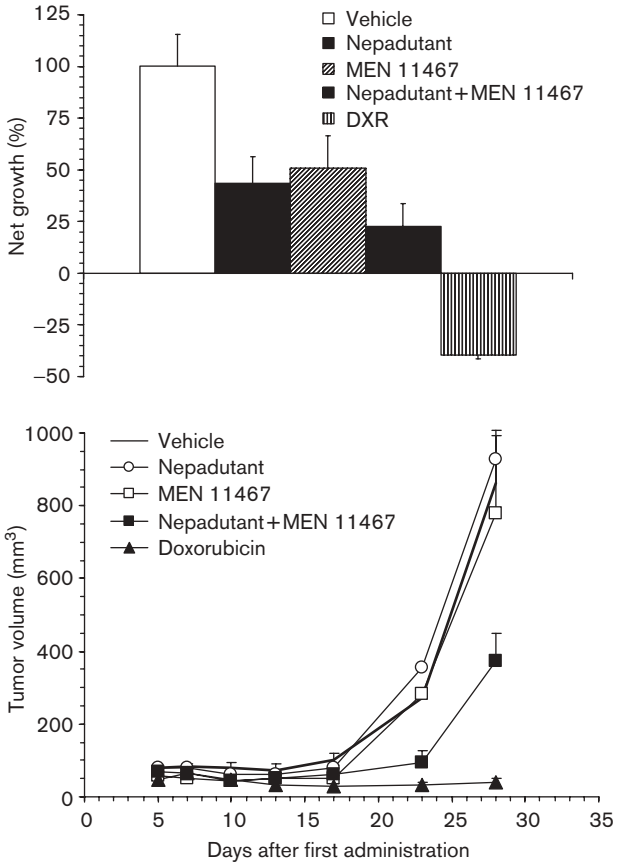
Further experiments demonstrated that both MEN 11467 and nepadutant were able to significantly reduce the stimulatory effect of NKA. The observed inhibition of tumor cells proliferation seem to be exclusively dependent on the NK-1 and NK-2 tachykinin receptors blockade nepadutant and MEN 11467 being devoid of cytotoxic property *per se* (Fig. 5). In this breast cancer cell line, the functions of the two receptors seem to overlap with respect to tumor growth. Although other studies showed that NK-2 has to be downregulated to allow NK-1 induced proliferation [9], in MDA-MB-231 cells the NK-2 receptor is expressed at a level similar to NK-1 (Fig. 2) and the NK-2 antagonist nepadutant is able to reduce tumor cell growth when also used at low concentrations. Interestingly, *in vivo* data further confirm the *in vitro* observations regarding the anti-proliferative

Table 1 Comparison of MEN 11467 (NK-1) and MEN 11420 (NK-2) receptor antagonists effect on the growth of MDA-MB-231 tumor cells in the HF and xenograft model

Compounds	Dose (mg/kg)	HF		Xenograft	
		Schedule	Net growth (%)	Schedule	TVI%
Vehicle	–	–	100.3	–	–
MEN 11467	5	q.d. × 6	51.0	q.d. × 12	16
MEN 11420	5	q.d. × 6	43.5[a]	q.d. × 12	7
MEN 11467 + MEN 11420	5 + 5	q.d. × 6	22.7[a]	q.d. × 12	60[a]
Doxorubicin	7	single	−39.5	q.7d. × 3	96

[a]$P < 0.05$ when compared with treated mice versus vehicle.

Fig. 6



In vivo effect of MEN 11467 (5 mg/kg, i.v.) and nepadutant (5 mg/kg, i.v.), alone or in combination, on MBA-MB-231 tumor cell growth in HFs or implanted s.c. in nude mice. (Upper panel) Tumor cells were cultured *in vitro* in HF and then implanted s.c. in nude mice. Doxorubicin administered i.v. at 7 mg/kg was used as a standard cytotoxic drug. The treatment with doxorubicin, MEN 11467 and nepadutant was administered 1 day after HF implantation. *$P < 0.05$ treated versus control. (Lower panel) Tumor cells (20×10^6) were implanted s.c. in nude mice and, when tumor volume was about 50 mm^3, tumor-bearing mice were treated i.v. from day 0 to day 12 with receptor antagonists. Doxorubicin was administered at 7 mg/kg according to a q.7d. × 3 schedule. Each point on the graph indicates the average volume of six tumors. Statistical analysis was performed by ANOVA test. *$P < 0.05$ combination nepadutant plus MEN 11467 versus medium.

property of these tachykinin receptor antagonists. Both in the HF assay and in a conventional xenograft model, tumor growth inhibition was obtained following treatment with one or both antagonists. The results obtained suggest that nepadutant and MEN 11467 *in vitro* and *in vivo* operate in a similar manner. As shown in the Fig. 6, the anti-tumor effect of both antagonists lasted as long as the time of administration. Treatment resulted in a growth arrest of the MDA-MB-231 xenograft until the last administration on day 15; thereafter, the tumor started to re-grow. The data of net growth (Table 1) obtained in the HF assay confirm the results obtained in the xenograft model. The negative value of percent net growth, characteristic of cytotoxic drugs, is found only for doxorubicin, while nepadutant and MEN 11467 possess a characteristic cytostatic property against this tumor line.

Recently published data indicate a role of neuropeptides in promoting the bone marrow metastasis of breast cancer cells in early cancer progression through a modulation of the interaction between breast cancer cells and stromal compartments [10], suggesting a more complex role for these molecules than direct tumor stimulation. However, our data demonstrated a role for both the endogenous neuropeptides both using HFs and tumor xenografts, in spite of the differences mainly in the complex interactions between tumor cells and the host microenvironment in the two models. Although further studies are necessary to fully elucidate these complex interactions, the results of anti-tumor efficacy obtained against MDA-MB-231 tumors by using these different in-vivo models produced evidence that tachykinin SP and NKA, through their receptor activation, may participate in breast cancer growth and development, confirming a possible link between the neuroendocrine system and cancer proliferation.

The results obtained strongly support the use of tachykinin antagonists as an innovative approach to anti-tumor therapy and confirm the potential use of these antagonists as therapeutic tools.

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