Role of nerve growth factor and its receptors in non-nervous cancer growth: efficacy of a tyrosine kinase inhibitor (AG879) and neutralizing antibodies antityrosine kinase receptor A and antinerve growth factor: an in-vitro and in-vivo study

Mario Rende[a], Alessandra Pistilli[a], Anna Maria Stabile[a], Adelmo Terenzi[b], Antonino Cattaneo[c], Gabriele Ugolini[c] and Pietro Sanna[d]

Neurotrophins, originally identified as neuronal survival and differentiation factors, exert their actions through tyrosine kinase receptors such as TrKA, in the case of the nerve growth factor. Neurotrophins also interact with p75, a common receptor devoid of kinase activity and connected to apoptosis. Here we show that nerve growth factor, TrKA and p75 are expressed in cell lines of human cancers of various non-neuronal lineages, including a panel of muscular sarcomas, and we show that all cell lines investigated actively release nerve growth factor into the medium. Treatment by AG879 (a tyrosine kinase inhibitor that inhibits TrKA phosphorylation, but not TrKB and TrKC) or by neutralizing antibodies anti-nerve growth factor and anti-TrKA dramatically decreases their proliferation with a variable increase in apoptosis. Similarly, p75 transfection induced a significant increase in apoptosis. Furthermore, for the first time we have determined by high-performance liquid chromatography the pharmacokinetic profile of a novel preparation of AG879 and we have established an optimal plasmatic concentration for in-vivo administration. Treatment with AG879 in immunodepressed mice grafted with leiomyosarcoma or promyelocytic leukemia cells resulted in dramatic reductions in tumor sizes. In conclusion, our data have a novel preclinical potential for revealing a possible therapeutical utility in targeting in-vivo nerve growth factor/TrKA by AG879 or neutralizing antibody anti-TrKA in cancer proliferation and in muscle sarcomas, in particular. *Anti-Cancer Drugs* 17:929–941

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[a]Section of Anatomy, Department of Experimental Medicine and [b]Section of Haematology, Department of Clinical and Experimental Medicine, University of Perugia School of Medicine, Perugia, Italy, [c]Lay Line Genomics SpA, Rome, Italy and [d]Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California, USA.

Correspondence to M. Rende, Section of Anatomy, Department of Experimental Medicine, University of Perugia School of Medicine, Via del Giochetto, 06122 Perugia, Italy.
Tel/fax: +39 075 585 7454;
e-mail: rende@unipg.it

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Introduction

Neurotrophins (NTs) [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT3 and NT4/5] are regulators of development, survival and plasticity of specific populations of neurons and non-neural cells. They act through three specific high-affinity receptors with intrinsic tyrosine kinase activity (TrKA, TrKB and TrKC), and by a common non-transducing low-affinity receptor (p75) that regulates cell survival and apoptosis [1–5]. In addition to their classical role as target-derived trophic factors for developing neurons, NTs have shown pleyotropic actions on various populations of non-neuronal cells in an autocrine/paracrine mode [6–8]. Furthermore, mounting evidence from several groups, including our own, supports a role for NTs and their receptors in cancer growth both in nervous system-derived and in non-nervous system-derived tumors [1,2,9–13]. In the present report, we show that targeting NGF/TrKA signalling in cell lines of human cancers of several non-neuronal lineages results in reduction in tumor growth both *in vitro* and *in vivo*.

These data support the role of NGF in cancer growth and suggest that inhibition of NGF signalling could be a valuable therapeutic target for a broad range of non-neuronal cancers.

Methods

Materials

Recombinant human β-NGF (Alomone Lab, Israel; no. N-235), tyrphostin AG879 (Calbiochem-Novabiochem, La Jolla, California, USA; no. 658460), also kindly prepared and provided for the in-vivo studies by Professor A. Levitzki (Hebrew University of Jerusalem, Israel); tyrphostin A1 (Calbiochem-Novabiochem; no. 658390); acetonitrile (no. 412412) and ammonium acetate (no. 418777) [high-performance liquid chromatography (HPLC) grade, Carlo Erba, Italy]; dimethylsulfoxide (DMSO) (Sigma, Milan,

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Italy; no. D 2650); chloroform (Merck PDH, Milan, Italy; no. 10077); dipalmitoylphosphatidylcholine (DPPC, Sigma; no. P0763); and Trizol (Invitrogen, Milan, Italy; no. 15596–026) were used. Media used included minimum essential medium (MEM) (Gibco-BRL, Milan, Italy; no. 21090-022) containing 1 g/l glucose and supplemented with antibiotic/antimycotic (Gibco-BRL; no. 15240-062), L-glutamine 2 mmol/l, MEM non-essential amino acid solutions (100 ×) (Gibco-BRL; no. 11140-035), sodium pyruvate (Gibco-BRL; no. 11840-048) and 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL; no. 10106-169); McCoy medium (Gibco-BRL; no. 26600-023) with 10% heat-inactivated FBS; and Dulbecco modified Eagle medium (DMEM) (Gibco-BRL; no. 31885-023) supplemented with antibiotic/antimycotic, L-glutamine 2 mmol/l and 5% heat-inactivated FBS; RPMI 1640 (EuroClone EC B9006L) supplemented with antibiotic/antimycotic and 10% heat-inactivated FBS; and D-PBS (Gibco-BRL; no. 1490-094).

Statistical analysis

GraphPad software Prism 2 (San Diego, California, USA) was used for statistical analyses.

In-vitro studies

Cell cultures

Cell lines from human leiomyosarcoma (HTB-114, HTB-115, HTB-88), rhabdomyosarcoma (HTB-82, TE-671), prostatic adenocarcinoma (PC-3), acute promyelocytic leukemia (HL-60) and histiocytic lymphoma (U-937) were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC protocols. Briefly, HTB-114, HTB-115, HTB-88 and PC-3 cells were grown in MEM; HTB-82 cells were grown in McCoy medium; TE-671 cells were grown in DMEM; HL-60 and U-937 cells were grown in RPMI 1640.

For the in-vitro experiments, cells were plated in polystyrene 24-well culture plates (Corning; no. 3524) at a density of 50 000 cells/well and maintained at 37°C in humidified air with 5% CO_2.

Reverse-transcriptase polymerase chain reaction, immunocytochemistry and Western blotting analysis of nerve growth factor, tyrosine kinase receptor A and p75 expression

Expression of NGF and its receptors was analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) in all cell lines used. In some cell lines, expression of NGF, TrKA and p75 was also demonstrated by immunocytochemistry (ICC) and Western blotting (WB). For RT-PCR, cells (2.5 million) were obtained from each cell line at different time points from seeding (6, 12, 24 h and 3, 10 days) and extracted by the Trizol method. Total mRNA was transcribed into cDNA with 200 U of RT (Invitrogen; no. 28025-013). cDNA was amplified with AmpliTaq Gold (Invitrogen; no. 10966-026) and 10 pmol/μl from the specific primers (see below) with a thermal cycler (Hybaid, LabX Midland, Ontario, Canada). An initial denaturation step at 95°C for 10 min was used, followed by denaturation at 94°C for 30 s, an annealing phase at 61°C for 45 s and a synthesis phase at 72°C for 1 min. Forty-five cycles were performed, ending with an extension phase at 72°C for 10 min.

The following primers were used. NGF: 5′-CTTCAGCA TTCCCTTGACAC-3′ (sense), 5′-AGCCTTCCTGCT GAGCACAC-3′ (antisense) (localized at nt 262–281 and 836–855 of the β-polypeptide, respectively, amplify a fragment of 594 bp). TrKA: 5′-CCATCGTGAAGAG TGGTCTC-3′ (sense) 5′-CCAGCTCTGTGAGGATCC AG-3′ (antisense) (localized at nt 290–309 and 678–697, respectively, amplify a fragment of 401 bp). p75: 5′-CC CTGGCCGTTGGATTACAC-3′ (sense) 5′-GAGATGC CACTGTCGCTGTG-3′ (antisense) (localized at nt 686–695 and 1017–1036, respectively, amplify a fragment of 351 bp). Amplified fragments were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide.

ICC and WB procedures for NGF and its receptors were carried out with the avidin–biotin complex method (Vector ABC Elite, Burlingame, California, USA) according to the manufacturer's instructions. The primary antibodies used were anti-TrKA [14], anti-NGF [15], anti-p75 human (ME 20-4, Amersham, Milan, Italy; no. RPN.514) and anti-p75 rat (192-IgG, Boehringer Mannheim, Milan, Italy; no. 198645). For the WB, cellular extracts were obtained after washing the cells with a solution of Tris–HCl containing 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol and bromophenol blue. Proteins from the extracts were electrophoretically separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels containing 15 or 7.5% acrylamide for NGF and TrKA, respectively.

Enzyme-linked immunosorbent assay determination for nerve growth factor

Conditioned medium from each cell line was collected at 24 h and 3 days after seeding. NGF concentration into the medium was detected by enzyme-linked immunosorbent assay (ELISA) according to the procedure described in the EmaxTM ImmunoAssay System (Promega; no. G7630) (not described here for the sake of brevity). The amount of NGF present in a control, unconditioned medium was always subtracted from the total concentration of NGF determined in the conditioned medium.

Cell proliferation: [^{3}H]thymidine incorporation assay and cell counting

Proliferation activity was analyzed both by [^{3}H]thymidine assay (Amersham) and by direct cellular counts. To compare all data, cells were contemporaneously seeded in three plates: two plates were used for the [^{3}H]thymidine assay and total protein assay (Bradford method), and the

third plate was used to count and assess the viability of the cells in a Bürker chamber after trypan blue staining.

Briefly, cells were seeded in 24-well plates at a density of 50 000 cells/well. After 36 h of culture, 1 μCi/ml of [^{3}H]thymidine was added into the wells and the incubation was continued for an additional 12 h. Afterwards, cells were washed in D-PBS and digested for 3 h at 37°C with 0.5 N NaOH. Then, 500 μl of the digested cell mixture was transferred into 15-ml tubes containing 2.5 ml of 10% trichloroacetic acid. This mixture was incubated in the dark at 4°C for 30 min and then filtered on cellulose acetate filters to isolate the intact DNA. The filters were dried and placed into scintillation tubes containing 10 ml of scintillation fluid and left for 2 h at 4°C. The samples were analyzed in a counter. Radioactivity (c.p.m.) was compared with the corresponding protein concentration that was determined in parallel for each condition and treatment of the cells. In specific experiments, the cells of some wells were treated with 20 ng/ml of β-NGF at day 1 and proliferation was determined as described above.

Analysis of apoptosis

Apoptosis was detected by cytofluorimetric detection of fluorescent annexin V. Cells were seeded in six-well plates at a density of 100 000 cells/well and analyzed according to the manufacturer's protocol of annexin-V-FLUOS (Roche, Milan, Italy; no. 1858777).

Some wells were treated with 20 ng/ml of β-NGF at day 1 and apoptosis was determined as described above, for the purpose of control.

For the analysis of the results, in consideration of the very different basal apoptotic values, we normalized the data among the cell lines.

Effects of tyrosine kinase inhibitor tyrphostin AG879 on cell proliferation

Each NGF/TrKA positive cell line was treated with serial concentrations (0.5, 5, 20 and 50 μmol/l) of tyrphostin AG879, a tyrosine kinase inhibitor that inhibits TrKA phosphorylation, but not TrKB and TrKC [10–12]. Doses were based on our previous experience with TE-671 rhabdomyosarcoma cells [13]. AG879 was dissolved in DMSO and added into the culture medium 24 h after plating followed by incubated for an additional 48 h before analysis for proliferation and apoptosis (as described above). Untreated cells of each lineage were used as control. Morphological modifications of the cells were checked by histochemical analysis.

p75 transfection

Selected lineages were transiently transfected with rat p75 using LipofectAMINE reagent (Life Technology, Milan, Italy; no. 18324-012) according to the manufacturer's instructions. Each well containing human cancer cells received 2 μg of pFEV expression vectors containing the coding region for rat p75 receptor (pFp75EV no. 5) (day 1). A pFEV antisense for rat p75 was used as control. Transfection was detected (day 4) by ICC for rat p75 using the 192 IgG antibody that is specific for rat p75 and does not recognize the endogenous human p75. Apoptosis in p75-transfected cells was analyzed as described above (annexin V or FACS assay) at the same time point.

Effects of neutralizing antinerve growth factor or anti-tyrosine kinase receptor A antibodies on cancer cell number

As AG879 also acts on HER2-ErbB2 and FLK-1, consequently, its effects cannot be related only to the inhibition of TrKA. For this reason, we verified the data obtained with AG879 testing the effects of neutralizing antibodies anti-NGF and anti-TrKA on the proliferation of selected neoplastic cell lines.

Neutralizing rat antihuman NGF IgG (clone α-D11; Lay Line Genomics, Italy) [15–17] and neutralizing mouse antihuman TrKA IgG (clone MNAC 13; Lay Line Genomics) [14,18,19] were used. Anti-NGF IgG (0.5, 1, 2.5, 5 or 10 μg/ml) was added in each well three times: at the time of the seeding (day 0), at day 1 and at day 2. At day 3, the cells were trypsinized and counted using the Bürker chamber after trypan blue staining. Anti-TrKA IgG (24, 40 or 80 μg/ml) was added in each well as described for anti-NGF IgG. At day 3, the cells were similarly trypsinized and counted. The same amount of non-immune rat or mouse IgG was used as controls.

In-vivo studies

Animals

Athymic, immunodepressed NOD/SCID female mice (20 g body weight) were purchased from Charles Rivers (Como, Italy). After arrival, they were given a minimum acclimation period of 1 week. They received autoclaved food and water.

AG879 incorporation in small unilamellar liposomes

Under sterile conditions, a mixture of DPPC and AG879 (2:1) was dissolved in chloroform (700 μl) and then evaporated to dryness under a stream of nitrogen, to form a thin film on the glass tube. The thin lipid film was hydrated by adding 450 μl of D-PBS at 45°C (above the main phase transition temperature of DPPC) and then vortexed for 15 min at 45°C. Small unilamellar liposomes were formed via sonication of the sample (5 × 60 s) with a titanium probe. HTB-114 and HL-60 were treated with AG879 encapsulated in liposomes to determine efficacy for in-vivo use (see below). Briefly, 24 h after plating, cells were divided into two sets: in the first set, cells were treated with AG879 dissolved in DMSO; in the second set, cells were treated with AG879 encapsulated in liposomes (same concentrations of AG879 as above). These two sets of cells were tested in parallel for

proliferation to determine whether each single concentration of the two preparations of AG879 has similar efficacy and biological effects. All experiments were run in triplicate.

Drug administration and blood sample collection

AG879 encapsulated in liposomes (1 or 2 mg) was subcutaneously injected into 15 NOD/SCID mice. At 2, 4, 8, 18, 24 and 48 h after dosing, three mice for each time point were killed and blood samples (500 µl) were obtained. All collected heparinized blood samples were centrifuged at 4000 *g* for 15 min to obtain plasma. Aliquots of plasma were stored at −80°C until analysis of AG879 levels.

Determination of AG879 pharmacokinetics in the mouse

Plasma samples obtained *in vivo* at different time points following AG879 administration (see below) were analyzed as follows to establish an AG879 kinetic in blood. HPLC analysis of AG879 was carried out according to the procedure of Ellis *et al.* [20] with minimal changes. A spectroscopic analysis of AG879 was first carried out to determine its maximum absorption peak. Plasma samples were extracted by adding two volumes of acetonitrile (containing 10 µmol/l of A1, an inactive structural analog of AG879, as an internal standard) to one volume of mouse plasma with or without AG879. The samples were vortexed and centrifuged (10 000 *g* for 5 min). The supernatants were transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The resulting film in each tube was reconstituted in 90 µl of acetonitrile/ultrapure water (1:1 v/v). After further centrifugation (10 000 *g* for 5 min) to obtain pellet undissolved debris, 20 µl of the clear supernatants was injected into the HPLC column.

The HPLC system consisted of a JASCO PU-980 pump (LabX) equipped with an UV/Vis detector (model UV 975). Chromatographic data were assessed using the software Jasco-Borwin 1.50 (Omnilab, Mettmenstetten, Switzerland).

Separations were performed at room temperature using a Waters Spherisorb 5 µmol/l ODS2 column (150 × 4.6 mm inner diameter) protected with a guard column (10 × 4.6 mm inner diameter) filled with the same stationary phase. The mobile phase was composed of ammonium acetate buffer (0.1 mol/l, pH 6.0) and acetonitrile (45:55, v/v), and the flow rate was 1 ml/min. A standard curve of AG879 (from 0 to 10 µmol/l) was obtained by dilution into mouse plasma from a 10 µmol/l stock in acetonitrile. The ratio between the peak areas of the AG879 and A1 was used as a correction factor for the assay.

Cancer grafting and AG879 treatment

NOD/SCID mice were grafted with HTB-114 and HL-60 cells according to the following procedure. Cells were cultivated and collected under sterile conditions into serum-free media, and injected subcutaneously in the back of the mice in a volume of 500 µl: 10×10^6 cells/mice for HTB-114 or 50×10^6 cells/mice for HL-60. At this time (day 1), grafted mice were assigned to two groups of 10–15 mice each: the first group was injected subcutaneously, close to the cancer site grafting, with 2 mg (100 mg/kg) of AG879 encapsulated in liposomes and the second group received the same amount of liposomes without the chemical. Altogether, AG879 was administered 10 times in 19 days, divided into two periods of five administrations each: daily administration from day 1 to day 5 and from day 15 to day 19, with an interruption from day 6 to day 14. All mice were killed at day 21. For HPLC analysis, blood samples were obtained from AG879-treated mice 48 h after each period of treatment (at days 7 and 21, respectively). When the tumor mass became visible, its size was measured with a caliper at least twice a week. After the mice were killed, cancer masses in control and AG879-treated mice were explanted, measured, photographed and histologically examined. The size, volume and percentage of tumor growth inhibition were calculated according to Miknyoczki *et al.* [21] Some samples of explanted cancers were homogenized and analyzed by RT-PCR to determine the presence of mRNA for NGF, TrKA and p75. Samples of explanted AG879-treated and control cancer masses were also dissociated and cultivated *in vitro* to compare their basal proliferation and responses to AG879 with the original in-vitro maintained cells lines.

Results

Non-neuronal cancer cell lines express nerve growth factor and its receptors, and release nerve growth factor into the medium

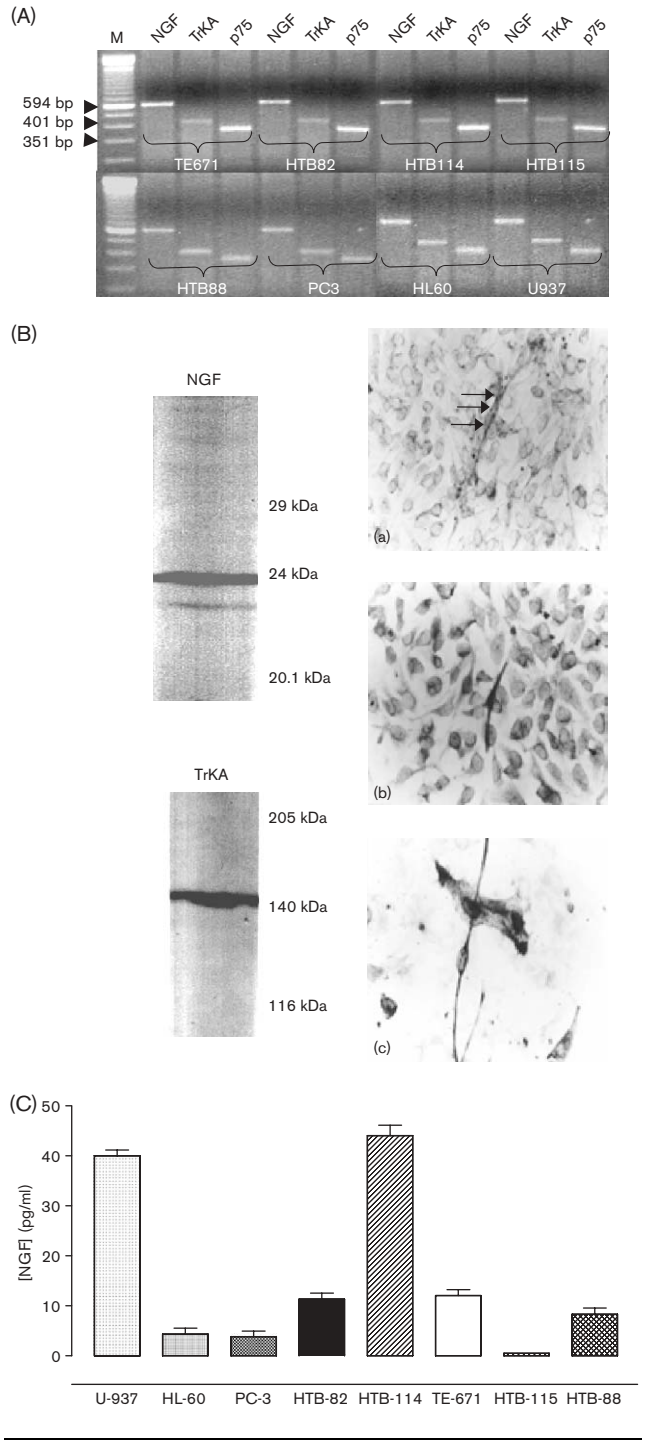
RT-PCR analysis demonstrated the presence of mRNA for NGF, TrKA and p75 in all the cell lines tested in this study (Fig. 1A). Consistently, NGF and its receptors were also detected by ICC and WB in all the lines (Fig. 1B). ELISA analysis of the conditioned media showed variable levels of NGF released by the lines. The highest concentration of NGF (44 ± 3.6 pg/ml) was found in cultures of HTB-114 (leiomyosarcoma), while the lowest concentration of 0.5 ± 0.3 pg/ml was found in another leiomyosarcoma cell line, HTB-115 (Fig. 1C). These concentrations did not vary between 24 h and 3 days after seeding.

Tyrosine kinase inhibitor AG879 decreases proliferation in non-neuronal cancer cell lines and induces apoptosis

Tyrphostin AG879 significantly and dose dependently decreased cell proliferation in all the cell lines, as revealed by thymidine incorporation (Fig. 2a). Significant reductions were seen in some lines at a concentration of 5 µmol/l and in all lines at a concentration of 20 µmol/l. At 50 µmol/l, proliferation was inhibited by over 90% in all the lines: from 90.9% inhibition in the promyelocytic

leukemia line HL-60 ($P < 0.002$) to 99.7% in the rhabdomyosarcoma line HTB-82 ($P < 0.002$) (Fig. 2a). At 50 µmol/l of AG879, total cell numbers obtained by direct cell counts showed reductions ranging from 62.0% for leiomyosarcoma line HTB-88 ($P < 0.002$) to 94.0% for the histiocytic lymphoma line U-937 ($P < 0.002$) (Fig. 2b).

Fig. 1



As shown in Fig. 3, AG879 treatment also induced a dose-dependent increase in apoptosis as revealed by annexin V assay. Such an increase was seen in all cell lines with the exception of the lines TE-671 (rhabdomyosarcoma) and HTB-88 (leiomyosarcoma), despite considerable inhibition of proliferation in these lines (see data in Fig. 2a and b). Beside these exceptions, in the other cell lines, at a concentration of 50 µmol/l (Table 1), AG879 increased the absolute percentage of apoptotic cells from 30.4% (HTB-82) to 97.6% (HL-60). These data were normalized among the cell lines (Fig. 3), showing that at 50 µmol/l of AG879 the increase in apoptotic cells ranges from 69.3% (U-937) up to 98.9% (PC-3). Furthermore, it shows that the AG879-induced increase in apoptosis was quite dramatic in the leiomyosarcoma cell line HTB-114 and the prostatic adenocarcinoma cell line PC-3. FACS analysis confirmed these results and failed to reveal a statistically significant increase in necrosis even in the cell lines displaying the greatest degree of inhibition (i.e. HTB-114 and HTB-82) (Fig. 4).

Addition of 20 ng/ml of β-NGF into the medium of the cell lines did not modify their proliferation or apoptosis (not shown).

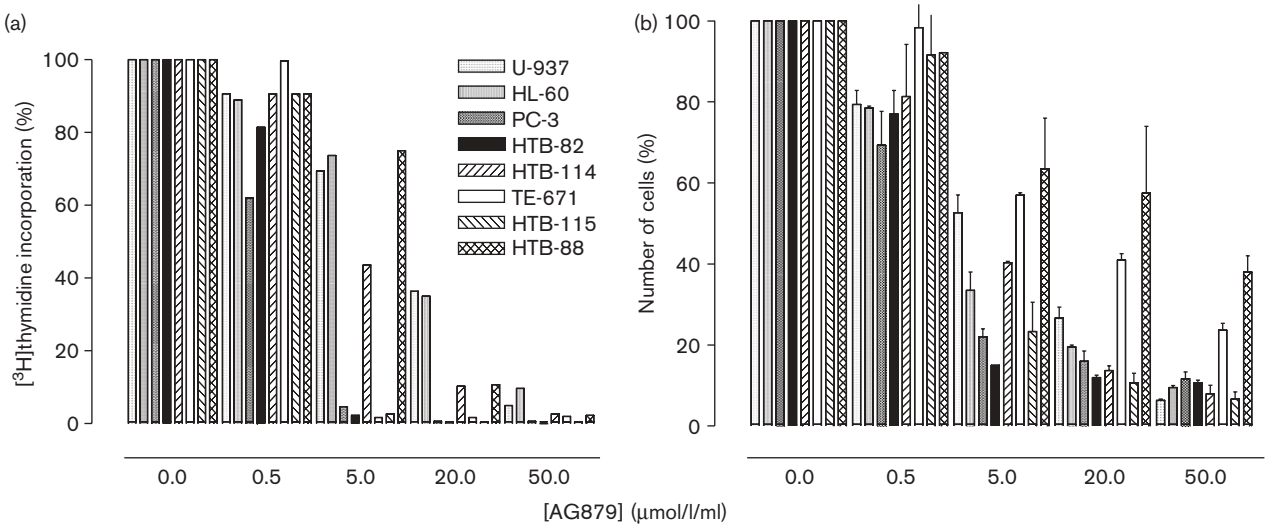
Overexpression of p75 by cDNA transfection induces increased apoptosis

For this experiment, we selected three cell lines differing in their basal apoptosis levels and sensitivity to AG870-induced apoptosis: TE-671 (low basal apoptosis level not modified by AG879), HTB-82 and HTB-114 (low basal apoptosis level, but a high AG879-induced increases in apoptosis). Successful transfection of rat p75 in these human cell lines was confirmed by ICC detection of rat p75 by the 192 IgG antibody that does not recognize endogenous human p75. Overexpression of p75 greatly increased the percentage of basal apoptosis in all cell lines: TE-671 from 2.1 to 41.9%, HTB-82 from 3.7

Fig. 1

(A) Reverse-transcriptase polymerase chain reaction analysis shows the presence of mRNA for NGF, TrKA and p75 in all the cell lines tested in this study: HTB-114, HTB-115, HTB-88 (leiomyosarcoma), HTB-82, TE-671 (rhabdomyosarcoma), PC-3 (prostatic adenocarcinoma), HL-60 (acute promyelocytic leukemia) and U-937 (histiocytic lymphoma). The amplification bands are shown at 594 bp for NGF, 401 bp for TrKA and 351 bp for p75. (B) ICC and WB analysis for the presence of NGF, TrKA and p75 proteins. For the sake of brevity, this figure shows only the case of TE-671 cells. WB analysis is shown in the left part of the figure, ICC in the right part. (a) NGF-immunoreactive (IR) cells (arrows); (b) TrKA-IR cells; (c) p75-IR cells. (C) Enzyme-linked immunosorbent assay analysis for NGF release into the culture medium at 3 days after plating. The data, subtracted from the concentration of NGF present into the unconditioned medium, show that all the cell lines released NGF even if with different amounts that do not depend from the histological type of cancer: a leiomyosarcoma cell line presents the highest (HTB-114) and the lowest (HTB-115) amount. NGF, nerve growth factor; TrKA, tyrosine kinase receptor A; ICC, immunocytochemistry; WB, Western blotting.

Fig. 2



(a) Dose-dependent effects on cell proliferation revealed by thymidine incorporation at basal condition and following AG879 treatment. Note the decrease in cell proliferation especially after a concentration of 20 μmol/l. At 50 μmol/l, proliferation was almost inhibited by over 90% in all cell lines. For graphical reasons, the basal proliferation values among the cell lines were normalized in order to compare the antiproliferative effects of AG879 among the lineages. (b) Dose-dependent effects on cell number revealed by direct cell counts at basal condition and following AG879 treatment. Note the decrease in cell number after treatment. These data are in agreement with those obtained counting thymidine incorporation [see (a)]. The basal cell number value was normalized among the lineages as described in (a).

Table 1 Average percentage of apoptotic cells at basal condition and after AG879 treatment (annexin V assay)

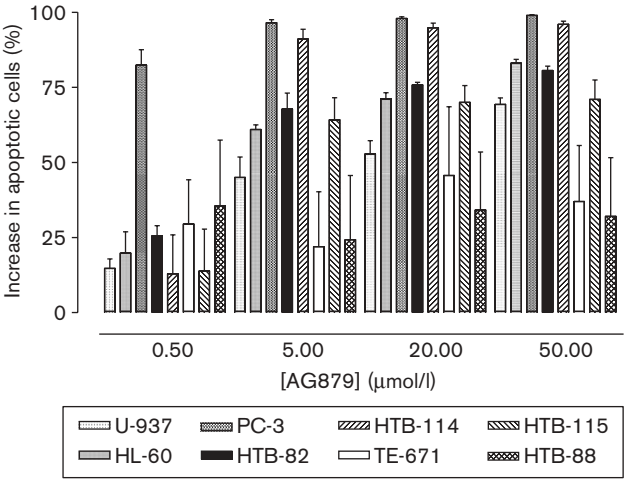
AG879 (μmol/l)	Apoptotic cells (%)			
	U-937	HL-60	PC-3	HTB-82
0	18.5 ± 2.5	16.5 ± 1.8	0.4 ± 0.2	5.8 ± 0.5
0.5	21.7 ± 2.3	20.7 ± 1.2	2.4 ± 0.3	7.8 ± 0.6
5	34.0 ± 2.6	42.2 ± 2.6	11.7 ± 1.2	19.1 ± 5.6
20	39.3 ± 1.2	57.3 ± 1.5	20.3 ± 2.3	24.1 ± 1.5
50	60.3 ± 4.5	97.6 ± 2.2	38.7 ± 3.5	30.4 ± 4.2
AG879	HTB-114	TE-671	HTB-115	HTB-88
0	1.2 ± 0.39	2.03 ± 0.4	20.9 ± 8.4	1.38 ± 0.6
0.5	2.4 ± 2.3	2.69 ± 1.6	22.1 ± 11.5	2.66 ± 1.9
5	21.4 ± 3.5	2.48 ± 2.2	58.6 ± 8.3	1.90 ± 1.5
20	36.2 ± 5.0	4.88 ± 2.7	69.5 ± 11.3	2.20 ± 1.8
50	42.9 ± 7.3	3.63 ± 1.7	73 ± 10.5	2.21 ± 1.6

to 21.6% and HTB-114 from 0.7 to 37.7% (Fig. 5 and Table 2).

Neutralizing antinerve growth factor or antityrosine kinase receptor A antibodies decrease cancer cell number

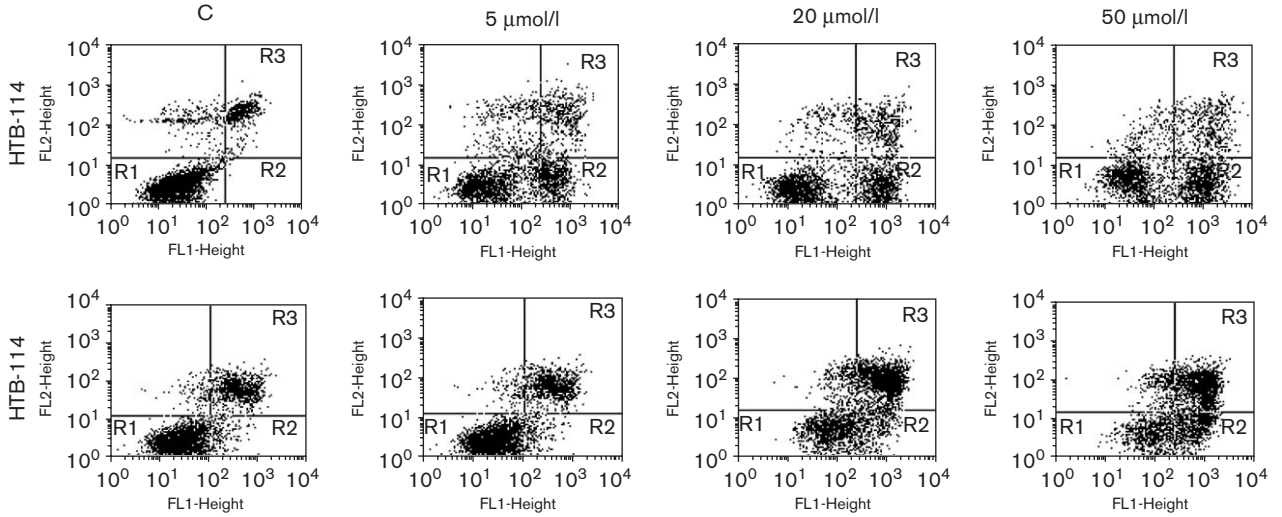
Addition of monoclonal neutralizing antibodies to either NGF or TrKA induced a significant dose-dependent decrease in cell number as determined by direct counts (Fig. 6). Addition into the medium of the highest concentration of a neutralizing anti-NGF IgG (10 μg/ml) induced a significant decrease in the percentage of cell number by 23.5 ± 0.7 and 22.4 ± 0.8% in HTB-114 (leiomyosarcoma) and PC3 (prostatic adenocarcinoma) cell lines, respectively ($P < 0.002$). A significant reduction was also seen at 5 μg/ml in HTB-114 (9.5 ± 0.5, $P < 0.002$), but not in PC3.

Fig. 3



Representation of the percentage in the increase in apoptosis after AG879 treatment determined by annexin V assay. HTB-88 and TE-671 are not shown as they did not show significant increases in apoptosis (see Table 1). In order to compare the data among the lines, the basal apoptotic value of each line was compared versus the values obtained after administration of the different concentrations of AG879 and expressed as percentage.

Fig. 4



FACS analysis of HTB-114 and HTB-82 lines after AG879 treatment. These lines showed a high increase in apoptosis by annexin V assay. FACS analysis confirmed that the decrease in cell number after AG879 administration depends by the increase in apoptosis (R2 section) and not by an increase in necrosis (R3 section).

A neutralizing anti-TrKA IgG (80 µg/ml) also induced a decrease in percentage of cell number by 36.6 ± 0.5 and 34.0 ± 0.1% for HTB-114 and PC-3 cells, respectively ($P < 0.001$). At 40 µg/ml, the anti-TrKA IgG decreased the percentage of cell number by 10.3 ± 1.2 and 25.0 ± 0.3% for HTB-114 and PC-3 cell lines, respectively ($P < 0.001$). At the lowest concentration tested (24 µg/ml), a significant decrease of 10.3 ± 0.6 ($P < 0.001$) was seen in PC-3 cell, but not in HTB-114 cells (Fig. 6). Non-immune control IgG had no effects.

In-vitro potency of liposome-encapsulated AG879 is comparable to AG879 dissolved in dimethylsulfoxide

For in-vivo administration, AG789 was encapsulated in liposomes to prevent toxic effects of DMSO *in vivo*. As shown in Fig. 7, reductions of [^{3}H]thymidine incorporation in cell lines treated with identical concentrations of AG879 dissolved in DMSO or encapsulated in liposomes were similar at all doses tested.

High-performance liquid chromatography determination of AG879 pharmacokinetics in mouse plasma

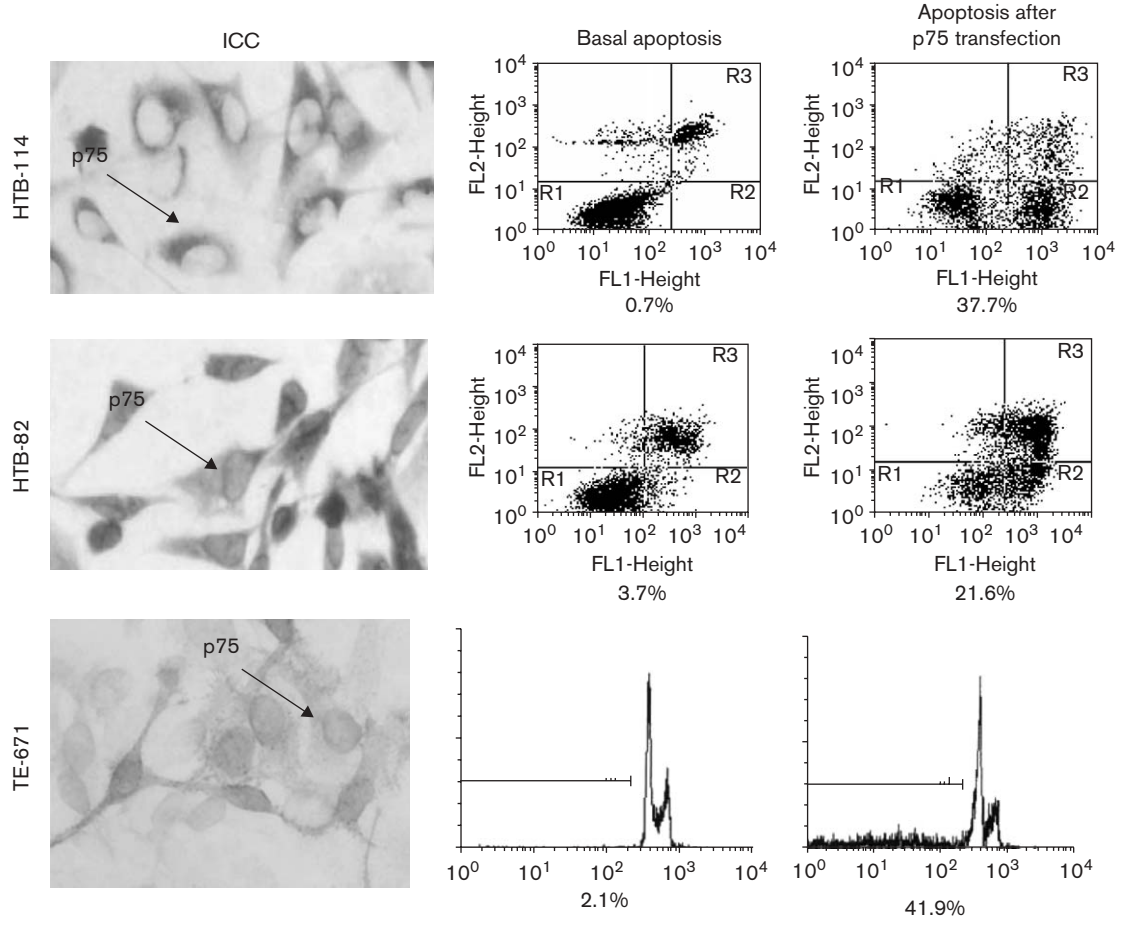
The spectroscopic profile of AG879 (0.01 mg/ml in DMSO) displayed a maximum absorption peak at 353 nm. In mouse plasma, AG879 and its inactive internal standard A1 were eluted as single symmetrical peaks with retention times of 7.45 and 4.45 min, respectively (Fig. 8a). No interference from mouse endogenous plasma elements was seen. HPLC analysis of AG879 kinetic in mouse plasma was determined after either single or multiple administrations. After a single administration of 1 or 2 mg of AG879 in liposomes, HPLC analysis of mouse plasma peaked 4 h after subcutaneous injection, with concentrations of about 0.35 or 0.83 µmol/l after 1 or 2 mg of AG879, respectively. These peaks were followed by rapid declines and at 48 h the drug was almost undetectable (Fig. 8b). The plasma half-life of AG879 was approximately 2.6 h. HPLC analysis in mouse plasma after five or 10 daily subcutaneous administrations of 2 mg (100 mg/kg body weight) of AG879 clearly indicated that the concentration of the drug with this dosing regimen remained above the IC_{50} determined for inhibition of cell proliferation for HL-60 and HTB-114 of about 1 mg/l of AG789 or 3.3 µmol/l (Fig. 8b). In fact, at 48 h after the fifth (day 7) and the 10th (day 21) injection, the concentrations of AG879 were 0.996 and 1.520 µmol/l, respectively.

AG879 induces *in vivo* a decrease in cancer growth in grafted athymic NOD/SCID mice

Athymic NOD/SCID mice were grafted with HTB-114 (leiomyosarcoma) or HL-60 (acute promyelocytic leukemia) and treated over 21 days with a total of 10 subcutaneous injections of 2 mg of AG879 in liposomes. Control mice were similarly injected but only with liposomes. No apparent or relevant side-effects were noticed in AG879-treated mice.

In untreated control mice, cancer masses become evident between day 7 and 10 and in AG879-treated mice between day 9 and 12. Figure 9 shows an example of comparison of control and AG879-treated HTB-114 (Fig. 9a) and HL-60 (Fig. 9b) tumor masses explanted at day 21.

Fig. 5



ICC, annexin V or FACS analyses at 4 days after rat p75 transfection in HTB-114, HTB-82 and TE 671 lines. ICC shows an evident positivity for rat p75 (on the left, arrows). Annexin V (HTB-114 and HTB-82) or FACS (TE-671) analysis show the increase in apoptosis after p75 transfection. ICC, immunocytochemistry.

Table 2 Comparison of the values of apoptosis from HTB-114, HTB-82 and TE-671 lines after AG879 exposure or after p75 overexpression by rat p75 transfection

Cell lines	Apoptotic cell (%)	
	+ AG879 (50 μmol/l)	+ p75 Transfection
HTB-114	42.9 ± 7.3	37.7 ± 6.3
HTB-82	30.4 ± 4.2	21.6 ± 3.5
TE-671	3.63 ± 1.7	49.9 ± 5.6

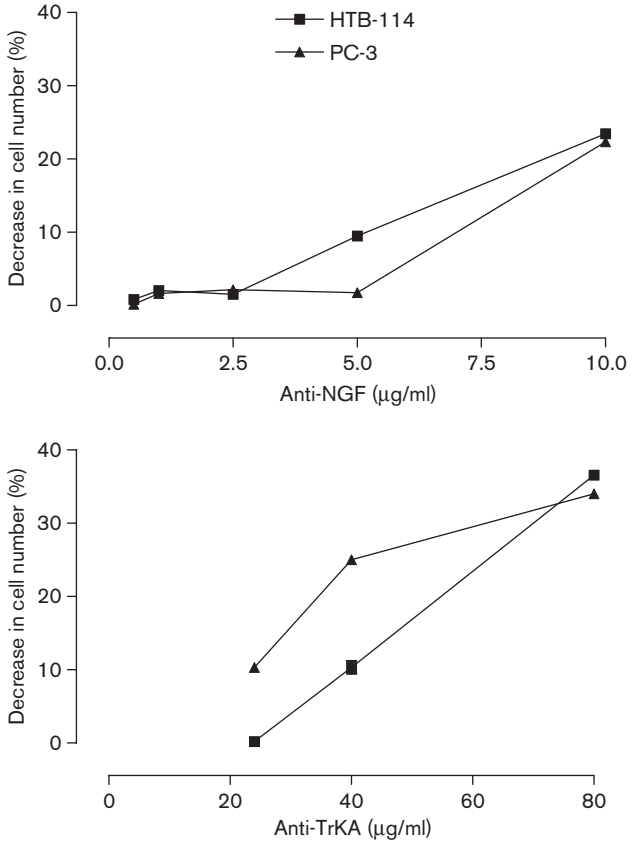
Note the agreement between the two methods to bring an increase in apoptosis levels for HTB-114 and HTB-82, but an important increase in apoptosis level in TE-671 only after p75 transfection and not after AG879 exposure.

In HTB-114-grafted mice, AG879 treatment induced an average reduction of 81.5% of tumor mass *in vivo* and 89.4% in volume as compared with control untreated mice. The final average weight of the tumor mass was 1.35 ± 0.4 and 0.25 ± 0.05 g in control and AG879-treated mice, respectively. The average volumes were 1.13 ± 0.04 and $0.12 \pm 0.05\,cm^3$ in control and AG879-treated mice, respectively ($P < 0.02$). In the HTB-114-grafted mice group, all AG879-treated mice displayed tumors, except for one mouse in which the macroscopic post-mortem inspection did not show a tumor mass.

In HL-60-grafted mice, AG879 treatment induced an average reduction of 80.0% of the tumor mass and 88.1% in volume from untreated controls. The final average weight of the tumor masses was 1.60 ± 0.3 and 0.32 ± 0.14 g in control and AG879-treated mice, respectively. The average volumes were 1.34 ± 0.08 and $0.16 \pm 0.05\,cm^3$ in control and AG879-treated mice, respectively ($P < 0.02$). In the HL-60-grafted mice group, all AG879-treated mice displayed tumor masses.

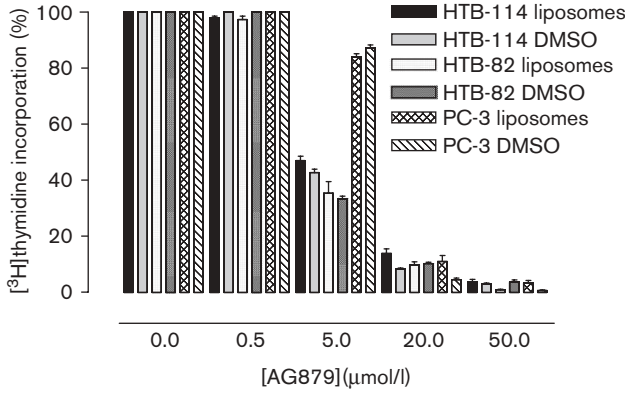
Cancers from control and AG879-treated mice were also analyzed for histopathology. In control untreated tumors, the morphology of the tissue was uniform and the cell morphology was similar to the original cell line *in vitro*

Fig. 6



Graphical representation of the decrease in cell number after treating HTB-114 and PC-3 cells with several concentrations of neutralizing anti-NGF or anti-TrKA IgG. The same amounts of non-immune rat or mouse IgG were used as controls. Note the progressive decrease in cell number following the increase of IgG concentration. NGF, nerve growth factor; TrKA, tyrosine kinase receptor A.

Fig. 7



Comparison of the percentage of [^{3}H]thymidine incorporation between selected cell lines treated with identical concentrations of AG879 dissolved in dimethylsulfoxide (DMSO) or encapsulated in liposomes. Note a comparable biological effect between the two preparations of AG879. For graphical reasons, the basal [^{3}H]thymidine incorporation of each cell line was normalized to 100%.

(Fig. 9c), while cancer cells from AG879-treated mice showed picnotic nuclei with deep eosinophilic cytoplasmatic staining and the tissue always showed large acellular areas (Fig. 9d). RT-PCR analysis confirmed the presence of mRNA for NGF, TrKA and p75 in the explanted AG879-treated masses (not shown). Proliferation of trypsin-dissociated cells from HTB-114-derived tumors ([^{3}H]thymidine incorporation) showed that ex-vivo-derived cells retained the same sensitivity to AG879 as to the original cell line (Fig. 10).

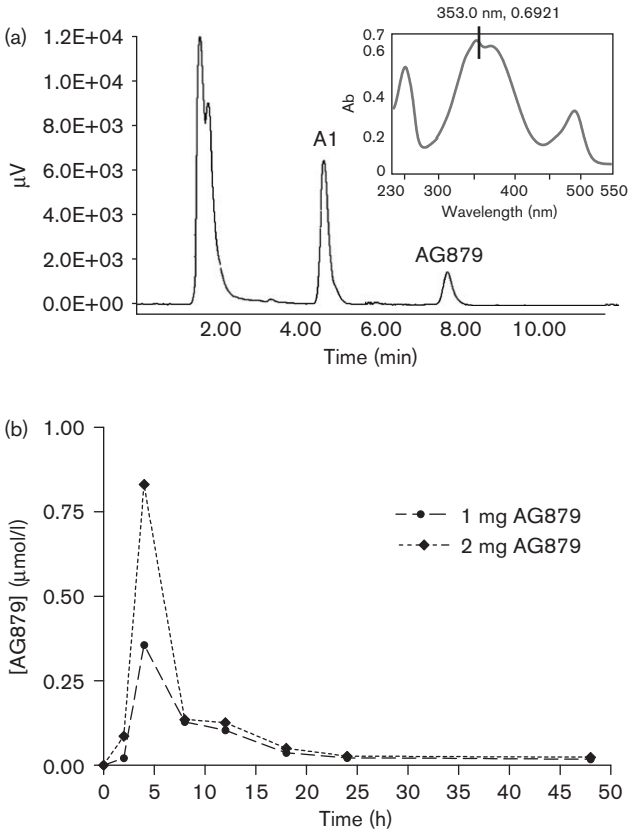
Discussion

Several studies have demonstrated a role of the NGF/TrKA/p75 in nervous and non-nervous neoplasms including rhabdomyosarcoma [13], melanoma [22], prolactinoma [23], neuroblastoma [24–26], and cancers from the breast [27–31], prostate [32–36], lung [37], bladder [38,39], ovary [40], pancreas [35,41–43] and thyroid [44]. In particular, NT transducing receptors, such as the NGF receptor TrKA, are promising therapeutic targets [9]. Despite the relevant number of studies in this field, however, data are often fragmentary and sometimes contradictory. In some cases [38] the presence of NGF/TrKA appears to promote differentiation, while in others [43] it seems to promote cancer proliferation. Contradictory results have also been reported on the expression of NGF receptors TrKA and p75 in neoplastic cells (i.e. in the case of prostate cancer [30,34]). These apparent discrepancies may derive from methodological and biological differences. In this study, we used a multidisciplinary in-vivo and in-vitro approach to study the antiproliferative effects of TrKA inhibition.

Altogether, the present work consisted of three parts. (1) We have investigated expression of NGF, TrKA and p75 in several cell lines from human non-nervous tumors, their release of NGF into the medium, and the biological effect of tyrphostin AG879 on their in-vitro proliferation and apoptosis. (2) We have investigated the antiproliferative efficacy of neutralizing antibodies anti-NGF and anti-TrKA, and of the overexpression of p75 *in vitro*. (3) We have preliminarily determined the pharmacokinetics of AG879 *in vivo* and then we have treated athymic, immunodepressed mice grafted with selected tumoral cell lines with AG879.

ICC, RT-PCR, WB and ELISA indicated that all the cell lines examined in this study expressed NGF and its receptors, and actively released NGF into the culture medium. The amount of NGF released into the medium was very similar among the cell lines, with the exception of the very high release of HTB-114 and U-937 lines (a leiomyosarcoma and a histiocytic lymphoma, respectively),

Fig. 8



(a) Spectroscopic and HPLC analysis of AG879. The spectroscopic profile shows a maximum absorption peak at 353 nm (insert). AG879 and its internal standard A1 dissolved in mouse plasma were eluted as single symmetrical peaks with retention times of 7.45 and 4.45 min, respectively. No interferences from mouse plasma were seen. (b) HPLC analysis of AG879 kinetic in mouse plasma after a single subcutaneous administration of 1 or 2 mg of AG879 in liposomes. Note the peaks at 4 h after administration (0.35 or 0.83 µmol/l after 1 or 2 mg of AG879, respectively). These peaks were followed by a rapid decline and at 48 h the drug was undetectable. HPLC, high-performance liquid chromatography.

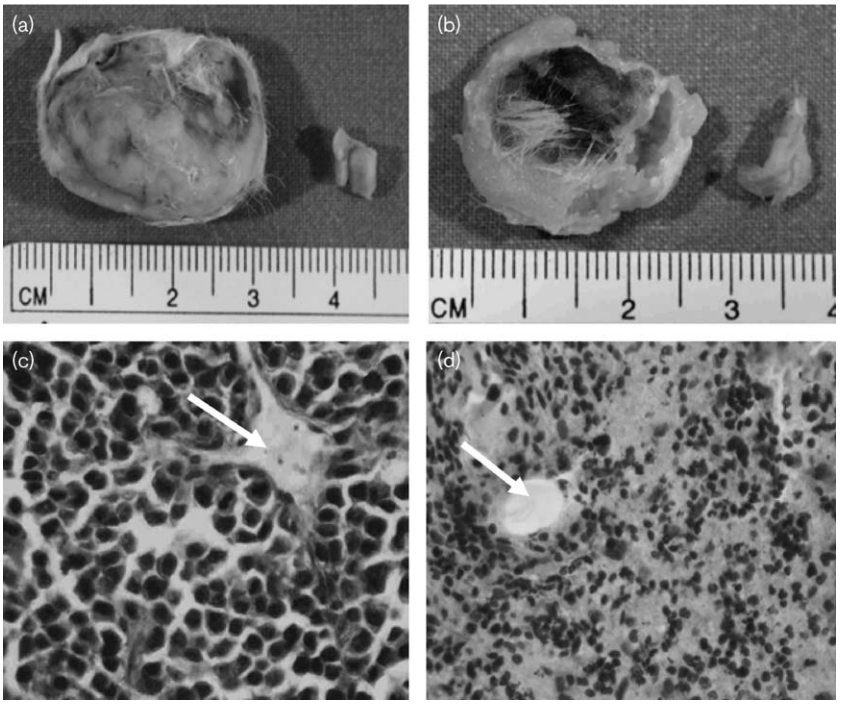
and the low release in HTB-115 (also a leiomyosarcoma). These data expand our knowledge on the presence of NGF in cancer cells showing that different cancer cell lines of the same histological type (i.e. leiomyosarcoma) may release very different amounts of NGF. This is a point of potential importance as many previous reports have drawn conclusions analyzing just a single cell line. Furthermore, future investigations should analyze more elements, such as the amount of TrKA receptors in the different cancer cell lines. In fact, although the two lines with the highest levels of NGF released into the medium (HTB-114 and U-937) also showed a very rapid proliferation, however, the cell line HTB-115, which showed a very low level of NGF release, also presented a rapid proliferation.

The demonstration of the widespread presence of NGF and its receptors in tumors of various lineages is in agreement with previous studies and supports the notion that neoplastic cells that express NGF and its receptors may use it in autocrine/paracrine modality to promote proliferation. Consistent with this hypothesis, we showed that a tyrosine kinase inhibitor, tyrphostin AG879, capable of blocking TrKA autophosphorylation [12] inhibited proliferation and induced apoptosis in a dose-dependent manner in all the cell lines tested. While AG879 also acts on other kinases [12], the role of TrKA in the proliferation of the cancer cell lines investigated is supported by the inhibitory action exerted by neutralizing anti-TrKA and anti-NGF antibodies, and by the observation of the effects of the transfection with p75, the low-affinity NGF receptor. In fact, TrKA and p75 have been shown to exert opposite actions on cell proliferation and apoptosis [45]. In fact, transfection of human cancer cell lines with rat p75 induced considerable apoptosis, even in cell lines with low basal rate of apoptosis, such as TE 671 (from 2.1 to 41.9%). This suggests that inhibition of TrKA transduction due to AG879 may tilt the balance between the two receptors toward the p75 pathway leading to apoptosis. Previous studies suggested that anti-NGF antibodies can be effective in reducing tumor cell proliferation [46]. The present results on the antiproliferative action of the anti-TrKA IgG, however, are a novelty of our investigation. The inhibitory activity of a neutralizing anti-TrkA antibody on cell growth, taken together with the effects of AG879 and p75 transfection, supports the antiproliferative action of tilting the TrkA/p75 balance toward p75.

For the design of an appropriate in-vivo regimen, we established a method to monitor the pharmacokinetics of AG879 in plasma and devised a new formulation of AG879 in liposomes. The antineoplastic efficacy of this preparation was tested in immunodepressed mice grafted with representative tumoral lines previously characterized *in vitro* for their sensitivity to AG879 and neutralizing anti-TrKA IgG. Previously, AG879 has been administered *in vivo* either topically [47] or systemically but at low dosages and using DMSO as a vehicle [48]. The improved in-vivo efficacy of AG879 in liposomes showing a significant decrease of cancer mass after treatment with AG879 is consistent with the in-vitro data and its new preparation. The in-vivo determination of AG879 pharmacokinetics and its new formulation in liposomes are also a novelty of our investigation.

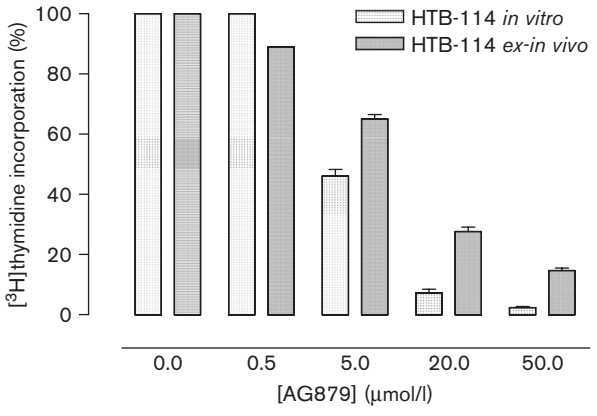
The present data and the aforementioned observations from other groups also support a very old hypothesis on the role of NGF in cancer that dates back to the very beginning of the NGF saga. In fact, NGF discovery stemmed from on observation made in 1948 by a student of Victor Hamburger who reported that after grafting a murine sarcoma into a chick embryo a robust axonal elongation of sensory neurons was seen extending toward the sarcoma [49]. He concluded that the sarcoma

Fig. 9



(a and b) Comparison of control and AG879-treated HTB-114 (a) and HL-60 (b) tumor masses explanted at day 21. Control tumors are on the left, AG879-treated masses are on the right. (c and d) Comparison of the morphological aspect of a control and AG879-treated HTB-114 cancer mass. In control untreated tumors (c), the morphology of the tissue was uniform and the cell morphology was similar to the original cell line *in vitro*, while in the AG879-treated cancer tissue (d) there were large zones of necrosis and the cells showed picnotic nuclei (H.H. staining). Arrows point a vessel of similar diameter ($\times$ 50).

Fig. 10



Comparison of the percentage of [^{3}H]thymidine incorporation (cpm) following AG879 treatment between HTB-114 cell line versus an 'ex-in vivo' AG879-treated HTB-114 mass that was explanted, dissociated and re-cultivated *in vitro*. The data clearly show that the 'ex-in vivo' cells still maintain their ability to respond to AG879 similarly to the original cell line.

released substances that were attractive for sensory ganglia [49]. Rita Levi-Montalcini and Stanley Cohen later identified that substance as NGF. Since that initial observation, the oncological origin and role of NGF was largely neglected until recently in favor of its physiological and developmental actions in the nervous system. These data and mounting evidence in the field indicate that the involvement of NGF in tumor biology is not less important than its effects on nervous system development. Furthermore, a better definition of the role of NGF in oncology may contribute to clear some aspects of cancer pain. In consideration of the powerful propriety of NGF to attract sensitive fibers (as target-derived factor) and its ability to induce sensitization in a variety of chronic pain states, it is possible that the release of NGF from the cancer cells not only contributes to their proliferation, but is in part also responsible for the oncological pain induced by the sensitive fibers around the cancer mass [50]. It is possible that inhibition of the activity of NGF released from cancer cells may act both to decrease cancer proliferation and to counter the cancer pain.

In conclusion the present data strongly support the hypothesis that NGF and its receptors TrKA and p75 are involved in regulating cancer proliferation, and that targeting the TrKA NGF receptors in cancer is a promising therapeutic approach.

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