Synthesis and biological activity of stable branched neurotensin peptides for tumor targeting

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Abstract

Receptors for endogenous regulatory peptides, like the neuropeptide neurotensin, are overexpressed in several human cancers and can be targets for peptide-mediated tumor-selective therapy. Peptides, however, have the main drawback of an extremely short half-life *in vivo*. We showed that neurotensin and other endogenous peptides, when synthesized as dendrimers, retain biological activity and become resistant to proteolysis. Here, we synthesized the neurotensin functional fragment NT(8-13) in a tetrabranched form linked to different units for tumor therapy or diagnosis. Fluorescent molecules were used to monitor receptor binding and internalization in HT29 human adenocarcinoma cells and receptor binding in HT29 tumor xenografts in nude mice. Linking of chemotherapic molecules like chlorin e6 and methotrexate to dendrimers resulted in a dramatic increase in drug selectivity, uptake of which by target cells became dependent on peptide receptor binding. When nude mice carrying human tumor xenografts were treated with branched NT(8-13)-methotrexate, a 60% reduction in tumor growth was observed with respect to mice treated with the free drug. [Mol Cancer Ther 2007;6(9):2441–8]

Introduction

In the last 20 years, major efforts have been concentrated on the design of new tools for specific tumor targeting, with the aim of overcoming the severe side effects of systemic chemotherapy. Antibodies, especially monoclonal human or humanized antibodies against tumor antigens, were the first to be used as vectors of conjugated moieties for targeted tumor therapy or diagnosis.

Peptides became potential vectors for tumor targeting when receptors for different endogenous peptides were observed to be expressed and overexpressed in different human tumors (1). The finding that somatostatin receptors are expressed on the plasma membrane of several human neuroendocrine tumors opened the way for peptide receptor targeting (2, 3). Somatostatin-derived drugs are now widely used for tumor therapy and diagnosis (4, 5). Nonetheless, the applications are limited to neuroendocrine tumors that express sufficient somatostatin receptors.

Other regulatory peptides are now candidates as tumor-targeting agents (6, 7) and an increasing number of tumors may be addressed by 'peptide-bullet' strategy: peptides can be conjugated with cytotoxic moieties or radionuclides and, provided the receptor-ligand complex is internalized on binding, the functional moiety can be specifically delivered into the tumor cell.

Neurotensin is a 13–amino acid regulatory peptide and has already been studied as a candidate for peptide receptor targeting (8–13). Like other neuropeptides, neurotensin has different functions (14). It is a neurotransmitter and neuromodulator (15) in the central nervous system and a local paracrine hormone in the periphery, particularly in the gastrointestinal tract. Three different receptors for neurotensin (NTS1, NTS2, and NTS3) have been cloned and studied thus far (16, 17). Neurotensin receptors, in particular the high-affinity NTS1, are expressed in several human tumors, such as small cell lung cancer and colon, pancreatic, and prostate carcinomas (2, 6, 14, 17). Neurotensin is considered the best possible candidate for peptide-based therapy of exocrine pancreatic carcinomas (6) due to the high incidence and density of neurotensin receptors in these tumors. Over 75% of all ductal pancreatic carcinomas were reported to overexpress neurotensin receptors, whereas normal pancreas tissue, pancreatitis, and endocrine pancreas do not (18).

The main drawback in the use of neurotensin, or any other endogenous peptide, as a drug is its extremely short half-life due to very rapid cleavage by different peptidases. A truncated COOH-terminal fragment NT(8-13) is slightly more stable while maintaining neurotensin receptor affinity. However, even the half-life of NT(8-13) is too brief for tumor targeting *in vivo* (10).

To circumvent this problem, various neurotensin analogues have been synthesized, including linear peptides (8, 10, 19), cyclic peptides (20), and nonpeptide molecules (21), but chemical modification of the native peptide may radically modify receptor affinity and specificity.

We showed previously that synthesis in branched form increased the biostability of certain peptides (22, 23),

Received 3/7/07; revised 5/24/07; accepted 7/18/07.

Grant support: Associazione Italiana per la Ricerca sul Cancro regional grant 2004-2006, the Italian Ministry of University and Research (PRIN 2005), and the Italian Consortium for Biotechnology and the University of Siena (PAR project).

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doi:10.1158/1535-7163.MCT-07-0164

including neurotensin. In particular, we found that the tetrabranched form of neurotensin and NT(8-13) is stable for 24 h in human plasma and serum and has receptor affinity, which in the case of tetrabranched NT(8-13), is higher than that of the monomeric peptide (22, 23).

In this study, we exploited the affinity and stability of branched NT(8-13) to develop novel peptide molecules for tumor targeting. We synthesized four new neurotensin derivatives: two for *in vitro* cell receptor tracing and two for *in vitro* and *in vivo* specific tumor therapy.

Materials and Methods

Peptide Synthesis

Protected L-amino acid, coupling reagents, and resins were purchased from Novabiochem. Tetrabranched NT(8-13)-PEG-Biotin (NT4-Bio) and tetrabranched NT(8-13)-PEG-tetramethyl rhodamine (NT4-TMR) were synthesized using Fmoc strategy with Fmoc-Lys(Biotin)-OH and Fmoc-Lys(TMR)-OH, respectively, as first coupling step and Fmoc-PEG-OH as second coupling step. Fmoc-Lys(Fmoc)-OH was used to build the three-lysine branched core and NH_2-terminal stepwise automated elongation was carried out by the classic HBTU/DIPEA method with a multiple peptide synthesizer (MultiSynTech). The final products were cleaved from the solid support with 95% TFA. A similar approach was used to synthesize tetrabranched NT(8-13)-PEG-chlorin e6 (NT4-Che6) and tetrabranched NT(8-13)-PEG-methotrexate (NT4-MTX), but the side chain amine group for functional unit coupling was rendered more accessible by stretching with Fmoc-PEG-OH. Thus, NT4-Che6 and NT4-MTX were synthesized using Fmoc-Lys(Dde)-OH as first and β-Ala as second amino acid on Novasyn TGR resin. The tetramer was then built as above but with Boc-Arg(Pbf)-OH instead of Fmoc-Arg(Pbf)-OH as last amino acid of the neurotensin sequence, so that the last two coupling steps occurred selectively on the side chain arm. Fmoc-PEG-OH was then introduced as side chain linker on the first lysine amino group, after Dde removal. Finally, APA-Glu(tBut)-OH and Che6 were coupled. Scramble tetrabranched peptides (amino acid sequence LRIPRY) were synthesized using the same procedures used for corresponding neurotensin peptides.

All products were characterized by matrix-assisted laser desorption/ionization-time-of flight mass spectrometry and purified by reversed-phase high-performance liquid chromatography.

Peptide Processing in Human Plasma

Peptide stability in human serum and plasma was assayed as described previously (22, 23).

Gene Expression

Reverse transcription-PCR was used for analysis of mRNA. Total RNA was isolated using an RNeasy kit (Qiagen) with 3×10^6 cells, as suggested by the manufacturer. One-step reverse transcription-PCR (Qiagen) was applied for retrostranscription and cDNA amplification of NTS1. The following oligonucleotides were used as primers: NTS1 5′-TCATCGCCTTTGTGGTCTGCT-3′ (sense) and 5′-TGGTTGCTGGACACGCTGTCG-3′ (antisense) and β_2-microglobulin 5′-ACCCCCACTGAAAAAGATGA-3′ (sense) and 5′-ATCTTCAAACCTCCATGATG-3′ (antisense).

Peptide Binding Kinetics

Peptide receptor binding assays were done using cell membranes prepared from HT29 human adenocarcinoma cell line (Istituto Zooprofilattico Sperimentale). Cells were grown as a monolayer in McCoy's 5A medium supplemented with 10% fetal bovine serum, 200 μg/mL glutamine, 60 μg/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C in an atmosphere with 5% CO_2. Cell membranes were prepared as described previously (22).

HPA sensorchips (BIAcore AB) for membrane immobilization were washed and prepared following the manufacturer's instructions. HT29 cell membranes (200 μg in PBS) were injected at a flow rate of 2 μL/min at 25°C, obtaining a final response of about 2,000 to 3,000 resonance units. Peptides diluted in PBS at concentrations ranging from 1 to 100 μg/mL were injected at a flow rate of 20 μL/min. Regeneration of the matrix was obtained by a 30-s pulse of glycine pH 2.5. K_D and kinetic rates were calculated using the BIAevaluation 4.1 software (BIAcore AB).

Cell Localization of Peptide

HT29 cells (10^6) were saturated for 30 min at 37°C with PBS-1% bovine serum albumin (BSA) and incubated with NT4-TMR (5 μg/mL in PBS-1% BSA) for 1 h at 4°C to enable peptide binding. Cells were washed with ice-cold medium to remove free peptide and then incubated at 37°C with prewarmed medium for 0, 2, 4, and 18 h for peptide internalization. After fixing with 4% formalin, the plasma membrane was stained by incubation with concanavalin A-biotin (10 μg/mL in PBS-1% BSA; Sigma-Aldrich) at 4°C for 15 min and avidin-FITC (0.5 μg/mL in PBS-1% BSA; Sigma-Aldrich) for 10 min at 4°C. Subcellular localization was analyzed by confocal laser microscope (Bio-Rad MRC600) with a 564 nm and 488 nm laser and 600 and 550 nm emission filters for rhodamine and FITC, respectively. Subcellular localization of NT4-TMR was examined using costaining with LysoTracker Green (Molecular Probes), a lysosomal organelle probe. HT29 cells (10^6) were saturated and incubated with NT4-TMR as described above and then kept with prewarmed medium for 4 h at 37°C for complete internalization. After centrifuging, the cells were incubated for 10 min in 50 nmol/L PBS solution of LysoTracker Green.

Receptor Regulation

HT29 cells (10^6) were incubated with NT4-TMR (100 μg/mL in PBS-1% BSA) for 1 h at 4°C to allow peptide binding and receptor saturation. After washing, the cells were divided into two aliquots: one was immediately incubated with NT4-Bio (0.5 μg/mL in PBS-1% BSA) and avidin-FITC (2.5 μg/mL in PBS-1% BSA) to assess receptor saturation and the other was kept at 37°C in culture medium for 18 h and then treated with NT4-Bio and avidin-FITC as above.

NT4-Che6 Cytotoxicity

HT29 cells (2×10^4 per well) were plated in 96-well microplates. After growing for 2 days, the cells were incubated at 37°C in the dark with different concentrations

of NT4-Che6 (100–0.1 μmol/L in RPMI 1640 with 10% FCS) for 4 h. The cells were then washed and irradiated (except dark controls) with a Zeiss KL1500 tungsten halogen lamp equipped with a 600 to 800 nm band pass filter and with two optical fibers placed 1 cm from the well. The plate was placed back in the incubator overnight and the phototoxicity of the conjugate was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Similar experiments were done with scramble NT4(8-13)-Che6 and with free Che6. Further controls included HT29 cells, (*a*) incubated with the conjugate and not irradiated, (*b*) incubated without the conjugate and irradiated, and (*c*) untreated.

NT4-MTX Cytotoxicity

HT29 cells were plated at a density of 5×10^3 per well in a 96-well microplate. Different concentrations of NT4-MTX, from 0.05 to 30 μmol/L, were added 24 h after plating. Growth inhibition was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 6 days later. The same experiments were carried out with the scramble analogue and with free MTX.

Ex vivo Peptide Binding

Frozen sections of tumors were removed from nude mice transplanted with HT29 cells. CD-1 female nude mice (Charles River Laboratories, Inc.), 6 to 7 weeks of age (mean weight of 25 g), were injected s.c. in the right flank with 10^6 HT29 cells. When tumors reached a diameter of 5 to 6 mm, mice were anesthetized with ketamine and sacrificed. Tumors were removed, embedded in Tissue-Tek (Sakura Finetechnical), and frozen. Cryostat sections (15 μmol/L) were fixed in 4% paraformaldehyde for 15 min at room temperature and treated with 0.1 mol/L glycine for 15 min at 37°C. After saturation with FCS for 30 min at room temperature, the sections were incubated with NT4-Bio or scramble NT4-Bio (5 μg/mL in PBS-1% BSA) for 1 h at room temperature followed by incubation with avidin-FITC (1 μg/mL in PBS-1% BSA) for 45 min at 37°C.

Inhibition of Tumor Growth

HT29 tumors were induced in nude mice as described above. When tumors reached a diameter of 4 to 5 mm, the mice were randomly divided into groups (four mice per group) and repeatedly injected in the tail vein (on days 0, 5, 10 and 15) with 300 μL of the following solutions in 0.9% NaCl: (*a*) 1 mg/mL NT4-MTX (2.45 μmol/kg); (*b*) 1 mg/mL scramble NT4(8-13)-MTX (2.45 μmol/kg); (*c*) 94 μg/mL MTX (2.45 μmol/kg); and (*d*) 0.9% NaCl.

Tumor volumes were measured daily with a caliper using the following formula: volume = length × width^2 × π / 6. At day 20, the mice were sacrificed. Experiments were done according to Italian regulations and under a project license ''Dendrimeric peptides for tumor targeting'' granted by the Associazione Italiana per la Ricerca sul Cancro.

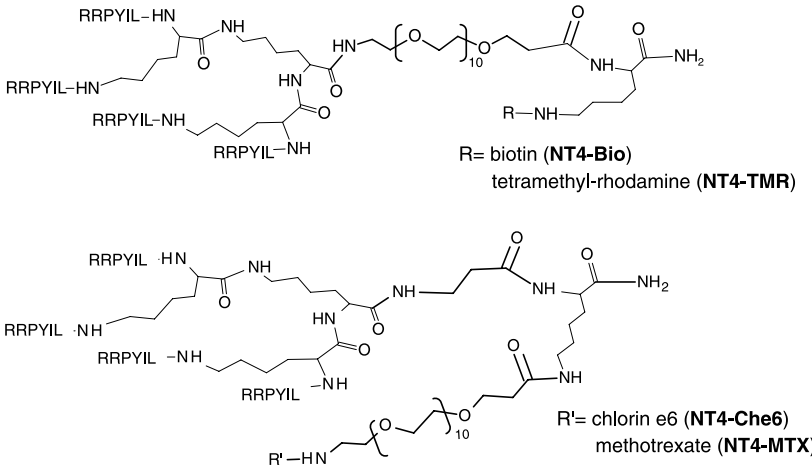
Statistical analysis was done using Student's *t* test.

Results and Discussion

Peptides Synthesis

NT4-Bio, NT4-TMR, NT4-Che6, and NT4-MTX were synthesized on a three-lysine core and spaced from the functional moiety by a suitable linker molecule (Fig. 1A). They all carry the functional unit on the side chain ε-amino

A



B **KD and kinetic rates of tetrabranched peptides as calculated from membrane binding in BIACORE.**

	NT4	NT4-Bio	NT4-TMR	NT4-MTX	NT4-Che6
KD (nM)	0.6	0.9	2.1	0.8	11
Kon (1/Ms)	1.5×10^5	2×10^5	2.0×10^5	1.8×10^5	1.1×10^5
Koff (1/s)	8.6×10^{-5}	1.8×10^{-4}	4.2×10^{-4}	1.4×10^{-4}	1.3×10^{-3}

Figure 1. **A,** structures of NT4-Bio, NT4-TMR, NT4-Che6, and NT4-MTX. **B,** K_D and kinetic rates of tetrabranched peptides as calculated from membrane binding in BIAcore.

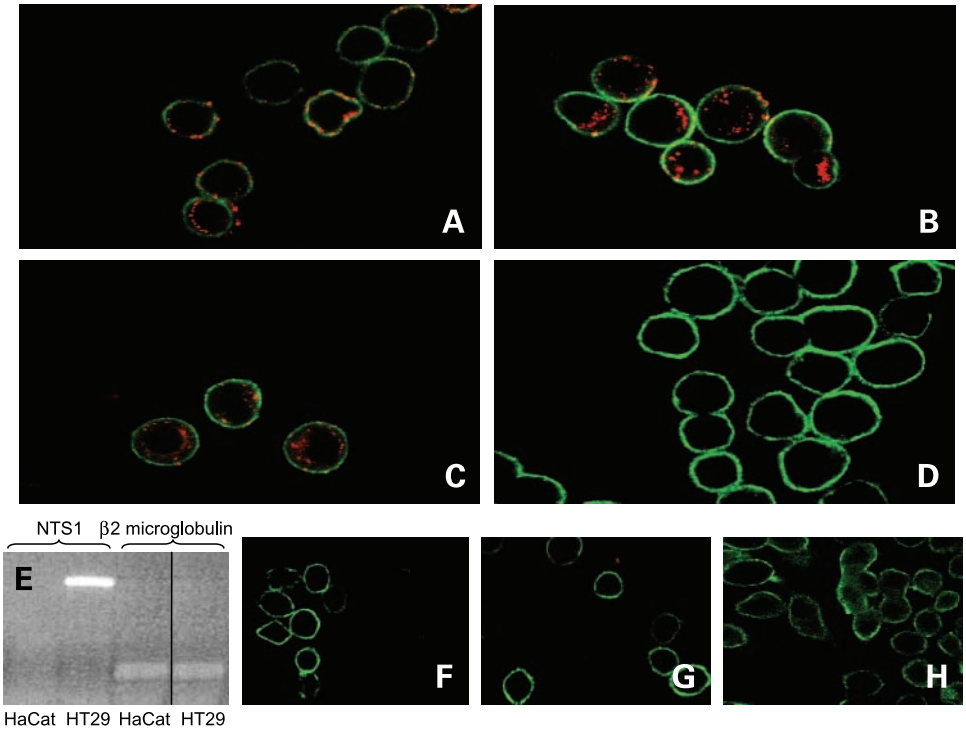


Figure 2. **A** to **D,** peptide binding and cell internalization. HT29 cells were incubated with NT4-TMR for 1 h at 4°C to allow peptide binding, washed, and then incubated at 37°C with previously warmed medium for 0, 2, 4, and 18 h (**A – D**, respectively). After fixing with 4% formalin, the plasma membrane was stained (*green*) with concanavalin A-biotin and avidin-FITC. **E,** reverse transcription-PCR bands of RNA extracted from cells. *Lane 1,* signal from reverse transcription-PCR of RNA extracted from HacaT cells and amplified with NTS1 primers; *lane 2,* RNA extracted from HT29 cells and amplified with NTS1 primers; *lane 3,* RNA extracted from HacaT cells and amplified with β_2-microglobulin primers; *lane 4,* RNA extracted from HT29 cells and amplified with β_2-microglobulin primers. **F,** HaCaT cells were incubated with NT4-TMR for 1 h at 4°C. **G,** HT29 cells were incubated with scramble NT4-TMR for 1 h at 4°C. **H,** competition test was done incubating HT29 cells with NT4-TMR in the presence of unlabeled NT4; no red signal is visible.

group of a COOH-terminal lysine residue. The tetramer was built automatically on solid phase. All products were characterized univocally by mass spectrometry: NT4-Bio *m/z* $[M]^+$ found 4,553.90 (calculated 4,551.63); NT4-TMR *m/z* $[M]^+$ found 4,741.06 (calculated 4,738.78); NT4-Che6 *m/z* $[M]^+$ found 4,976.10 (calculated 4,976.05); and NT4-MTX *m/z* $[M]^+$ found 4,835.24 (calculated 4,833.82).

Peptide Characterization

Peptide Stability. All the tetrabranched peptides were tested for stability in human plasma. The presence of uncleaved molecules or fragments after incubation at 37°C for 2, 5, and 24 h was monitored by high-performance liquid chromatography and mass spectrometry. All the conjugated molecules were detected as uncleaved molecules after 24 h of incubation in human plasma, whereas linear neurotensin and NT(8-13) in the same conditions were degraded within 2 h.

Binding Kinetics. Peptide receptor binding was analyzed using membrane preparations from HT29 human adenocarcinoma cells that overexpress neurotensin receptors. To evaluate possible hindrance of the conjugated molecules that might affect peptide binding kinetics, the tetrabranched

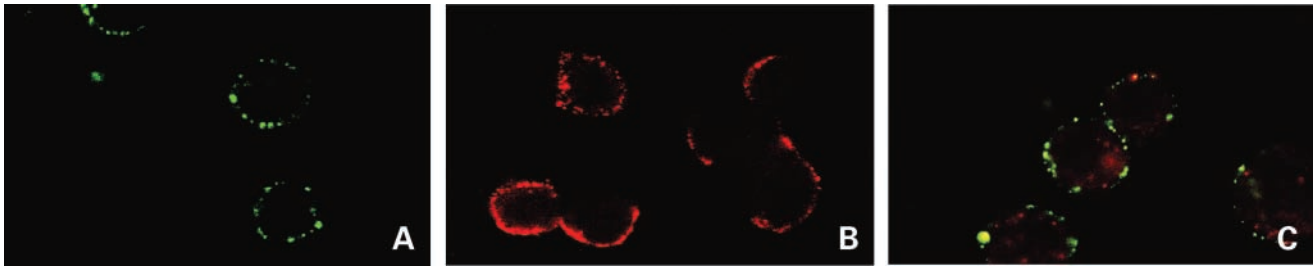


Figure 3. NTR regeneration. **A,** HT29 cells were incubated for 15 min at 4°C with NT4-Bio and avidin-FITC. **B,** HT29 cells were first incubated with excess NT4-TMR for 1 h at 4°C and then, after washing, with NT4-Bio followed by avidin-FITC as in **A. C,** HT29 cells were incubated with NT4-TMR as in **B**, washed, kept for 18 h at 37°C in culture medium, and treated with NT4-Bio and avidin-FITC as in **A** and **B**. *Red,* NT4-TMR; *green,* NT4-Bio.

peptides were compared with each other for K_A and kinetic binding rates by BIAcore, using an HPA hydrophobic sensor chip prepared with HT29 cell membranes.

The K_D of the tetrabranched NT(8-13) (NT4) as calculated from membrane binding in BIAcore was 6×10^{-10} mol/L (Fig. 1B), which is compatible with the IC_{50} of the same peptide as measured in competition RIA with ^{3}H-neurotensin on the same membrane preparation (2×10^{-10} mol/L; ref. 22). NT4-Bio, NT4-TMR, and NT4-MTX essentially maintained NT4 binding variables, with a slight increase in K_{off} of NT4-TMR with respect of NT4, whereas binding of NT4-Che6 decreased by more than one log, due to increased K_{off}. No binding was detected in BIAcore using the scramble tetrabranched peptide (scramble NT4).

The protocol for receptor binding experiments is crucial when dealing with multivalent binding peptides. In fact, neurotensin receptor expression may vary substantially in different cell samples and membrane preparations and we found that receptor concentration with respect to total protein concentration in the sample greatly affected measurement of IC_{50} of branched peptides, whereas it scarcely affected that of monomeric ligands. Moreover, measurement of peptide IC_{50} on whole cells can be additionally affected by internalization of bound ligands, which could be different for branched and monomeric peptides (cross binding by ligands may enhance receptor internalization). This may explain discrepancies in IC_{50} of tetrabranched NT(8-13) measured by us here and in previous studies (22), with respect to the higher values reported by others (24).

Peptide Activity *In vitro*

Peptide Receptor Binding and Internalization in Whole Cells. HT29 cells were incubated for 1 h at 4°C with NT4-TMR. The red fluorescent signal on the plasma membrane showed binding of the peptide to receptors (Fig. 2A).

Cell internalization was analyzed monitoring the localization of NT4-TMR in HT29 cells over time. After 1 h of incubation at 4°C to enable peptide binding, the cells were washed to remove unbound peptide and kept at 37°C for different time intervals. At time 0, peptide fluorescence was localized exclusively on the plasma membrane (Fig. 2A). After 2 h of incubation, the peptide was present on the plasma membrane and in the cytoplasm (Fig. 2B), whereas after 4 h, it was completely internalized (Fig. 2C). Finally, no signal was detectable after 18 h of incubation at 37°C (Fig. 2D). NT4-TMR was completely localized in lysosomes after 4 h of incubation, as shown by colocalization with the lysosome-specific marker LysoTracker Green (data not shown).

Cells that do not express NTS1 were used to assess peptide binding specificity. Gene expression of the high-affinity NTS1 was analyzed in HT29 human adenocarcinoma cell line and in human HaCaT keratinocytes by reverse transcription-PCR. A strong amplification signal of NTS1 cDNA was evident in HT29 and absent in HaCaT (Fig. 2E). Binding specificity was confirmed by the absence of red fluorescence in controls obtained by incubating HaCaT cells with NT4-TMR (Fig. 2F) and HT29 with scramble NT4-TMR (Fig. 2G). A competition test was done incubating HT29 cells with NT4-TMR in the presence of unlabeled NT4 ensuring that the red signal in Fig. 1A belongs to intact peptide (Fig. 2H).

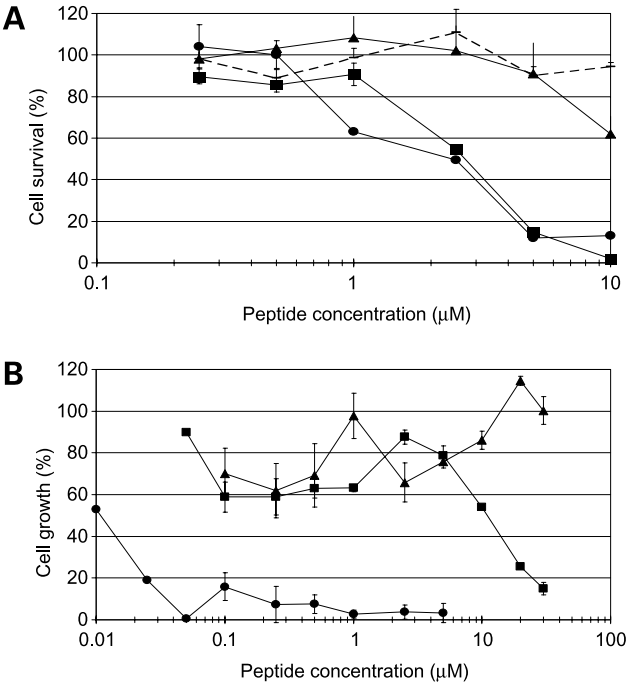


Figure 4. **A,** NT4-Che6 cytotoxicity. HT29 cells were plated with different concentrations of NT4-Che6 (■), scramble NT4-Che6 (▲), and free Che6 (●). The cells were then washed to remove unbound conjugate and irradiated. Drug phototoxicity was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h after irradiation. Negative control (−) is obtained with HT29 cells incubated with the conjugate and not irradiated. **B,** NT4-MTX cytotoxicity. HT29 cells were treated with NT4-MTX (■), scramble NT4(8-13)-MTX (▲), and MTX (●). Inhibition of cell growth was tested by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 72 h of incubation. *Points,* mean of three independent experiments done in triplicate; *bars,* SD.

The ability of the tetrabranched peptide conjugated with a functional unit to be rapidly and selectively internalized in cells is an important feature for possible therapeutic applications of the molecules.

Receptor Regulation. Receptor regulation following peptide binding was studied by confocal laser microscopy. Receptors were first saturated, incubating HT29 cells with a large excess of NT4-TMR, to exclude the subsequent binding of NT4-Bio detectable with avidin-FITC (Fig. 3A and B). Membrane receptors were actually saturated with NT4-TMR because subsequent incubation with NT4-Bio and avidin-FITC did not produce any detectable green signal (Fig. 3B). When the same cells, treated with NT4-TMR, were washed, kept at 37°C in culture medium overnight, and newly treated with NT4-Bio and avidin-FITC, a green signal similar to that obtained on untreated cells was detected, indicating that the membrane receptor was again available for ligand binding (Fig. 3C). The intracellular red signal of NT4-TMR was still detectable

after 18 h of incubation (Fig. 3C), unlike in the above described binding experiment (Fig. 2D). This might be due to the large excess of peptide (100 versus 5 μg/mL) needed for the competition experiment.

Peptide receptors, which are largely G protein–coupled receptors, have the important ability to be internalized on ligand binding. This characteristic is one of the features that have made the peptide-bullet strategy a promising approach for tumor therapy (2, 25–28). Nonetheless, down-regulation of receptors and their depletion from the membrane may dramatically impair the efficiency of peptide-based therapy, particularly in the case of branched peptides, the multivalent binding of which may improve receptor down-regulation. From our experiments, we did not detect any receptor depletion following saturating binding of the branched ligand.

We cannot assess whether the membrane receptors are actually recycled receptors or *de novo* synthesized proteins based on IF localization by means of peptide binding. Nonetheless, the immediate availability of membrane peptide receptors that remain prone to ligand binding is a very promising feature of neurotensin-based tumor targeting, in view of the possible use of neurotensin branched peptide as specific bullets for selective tumor killing, which should occur before the onset of long-term desensitization.

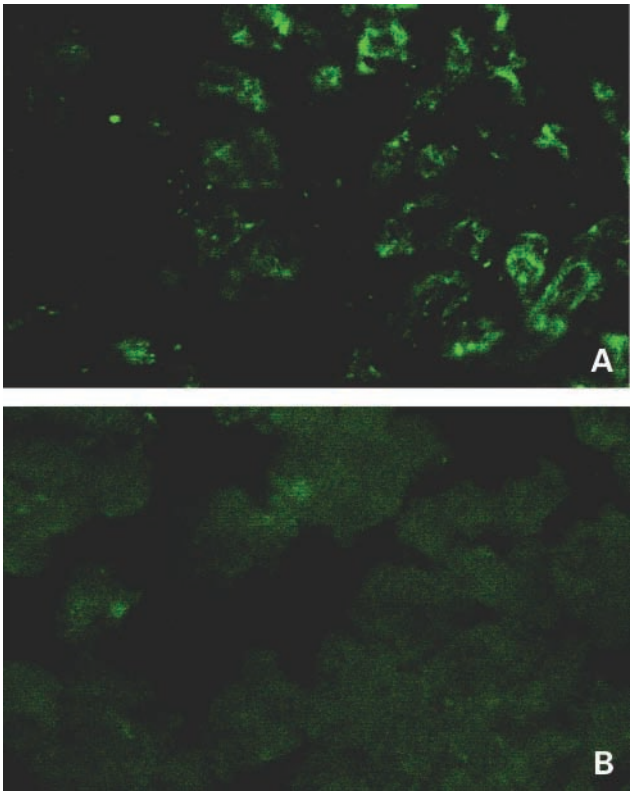


Figure 5. *Ex vivo* immunofluorescence. An HT29 solid tumor was removed from transplanted nude mice, frozen in Tissue-Tek, and sectioned with a cryostat (15-μm sections). The sections were incubated for 1 h at 37°C with NT4-Bio (**A**) or scramble NT4(8-13)-Bio (**B**), followed by 45 min of incubation with avidin-FITC.

NT4-Che6 Cytotoxicity. Photodynamic therapy is a promising therapeutic approach to cancer, based on selective killing of malignant cells by singlet oxygen, $^1O_2$, and other reactive products generated by photoactivated photosensitizers that accumulate in tumor tissue (29). Che6 and its derivatives are promising sensitizers for photodynamic therapy, although their selectivity for cancer cells needs to be potentiated (30). To assess the toxicity of NT4-Che6, photokilling experiments were carried out on HT29 cells. The phototoxicity of NT4-Che6 was compared with that of the free photosensitizer and a scramble NT4-Che6 conjugate.

As shown in Fig. 4A, Che6 not only maintained its cytotoxicity after conjugation with branched NT(8-13) but also acquired selectivity with respect to free Che6, shown by the lack of activity of scramble NT4-Che6. Che6 is cytotoxic for most cells when they are irradiated at the appropriate wavelength (29). Its hydrophobicity enables nonspecific internalization in the cell, causing cell toxicity. Conjugation with branched peptides prevents diffusion of Che6 through cell membranes, only allowing cell internalization through receptor-mediated endocytosis, which may be induced by binding neurotensin peptides to their receptors. Based on our data, we cannot assess whether Che6 cytotoxicity is mediated by conjugated Che6 or by free Che6 that may be released when the peptide breaks down inside the cells.

NT4-MTX Cytotoxicity. MTX is effective in many malignancies: as a folate antagonist, it inhibits dihydrofolate reductase. This causes a lack of reduced folate substrates, therefore impairing synthesis of purine nucleotides, thymidylate, and certain amino acids, which can ultimately lead to cell death. Clinical use of this drug is limited by its toxic dose-related side effects. Moreover, resistance to MTX often develops by various mechanisms, including decreased folate carrier-mediated membrane transport (31). New antifolate drugs are currently investigated with the aim of avoiding the systemic toxicity of MTX and to overcome drug resistance (32).

To evaluate the cytostatic activity of NT4-MTX, HT29 cells were treated with different concentrations of labeled peptide. After 6 days, cell growth was tested by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. The same experiments were done with the scramble analogue and with unconjugated MTX.

Figure 4B shows that peptide-conjugated MTX was only active when bound to the branched neurotensin peptide, whereas the MTX-conjugated scramble analogue did not have cytostatic activity. This result clearly showed that the targeting ability of neurotensin peptide is necessary to achieve the therapeutic efficacy of conjugated MTX. Hence, the same conclusions may be drawn for the acquired specificity of MTX toxicity, once linked to a specific peptide carrier, as discussed above for Che6 cytotoxicity. Conjugation of these toxic moieties with branched peptide molecules makes them a sort of prodrug, which can no longer be transported across plasma membranes by the mechanisms of the corresponding free drugs and can only be 'activated' via peptide receptor binding that mediates their specific

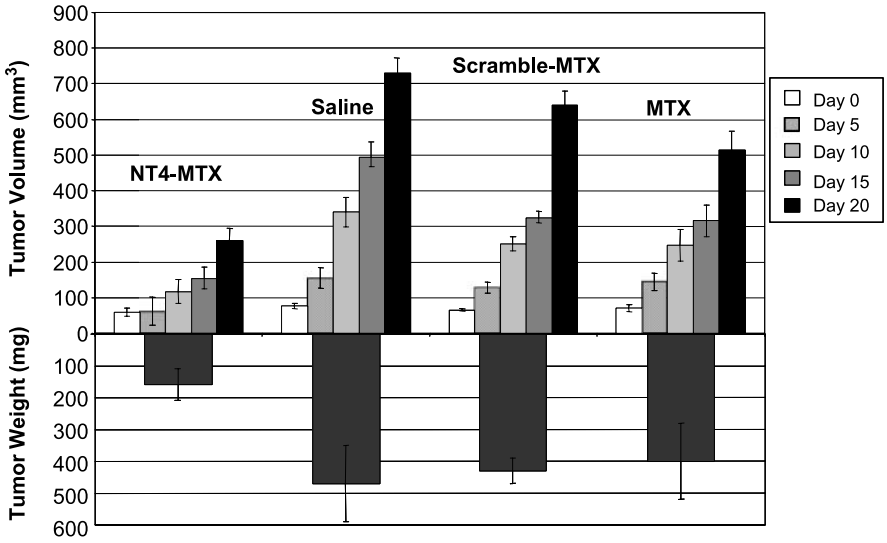


Figure 6. *In vivo* tumor growth retardation mediated by NT4-MTX. Mice bearing HT29 tumors were randomly divided into four groups and injected i.v. with NT4-MTX, scramble NT4(8-13)-MTX, free MTX, and saline on days 0, 5, 10 and 15. *Columns,* mean; *bars,* SD.

translocation inside the cell. This feature profoundly decreases the nonspecific toxicity of the drug. Moreover, receptor-mediated transport of the peptide across the target cell membrane may help by-pass drug resistance mechanisms based on specific membrane transporters, like that of MTX.

The switch to a different mechanism of drug internalization, once either Che6 or MTX is coupled to branched neurotensin, is further shown by the similar concentration-activity curve obtained with the two conjugated molecules. Despite the clear difference in activity of free MTX with respect to free Che6, which can be explained by the different mechanism of cell internalization and toxicity, and despite the different activity measured in the two sets of experiments (cell death and inhibition of cell growth, for Che6 and MTX, respectively), the efficiency of the two drugs becomes very similar once they are conjugated to branched neurotensin.

Ex vivo Peptide Binding

Immunofluorescence experiments were carried out to check the presence of neurotensin receptors in solid tumor xenografts.

Binding of NT4-Bio was evident throughout the tumor section (Fig. 5A), whereas no signal was detected with the scramble peptide (Fig. 5B), indicating that neurotensin receptors were expressed in the solid tumors and could be suitable targets for neurotensin-mediated treatment of HT29 tumor xenografts. The same experiments were also encouraging for *ex vivo* or *in vivo* diagnostic use of dendrimeric neurotensin peptides conjugated to fluorescent or radiodiagnostic probes.

In vivo Inhibition of Tumor Growth

Nude mice bearing HT29 tumor xenografts were randomly divided into groups and injected with NT4-MTX or scramble NT4-MTX or an equimolar amount of free MTX. A further control group was injected with saline. Tumor growth was clearly less in mice treated with NT4-MTX than in those injected with saline. The reduction was evident soon after the first injection and, at the end of treatment, the mean volume of tumors from animals treated with NT4-MTX was substantially lower (260.5 mm^3) than that of tumors from mice injected with saline (730 mm^3; >99.9% confidence level) or with scramble NT4-MTX (640 mm^3; >99.9% confidence level; Fig. 6). A slight decrease in tumor growth was found in animals treated with MTX alone (512 mm^3). It should be noted that the MTX dose was about one tenth of the dose commonly used for the treatment of tumor xenografts in nude mice (33). Five days after the last injection, animals were weighed and sacrificed and tumors were removed and weighed. Tumor weights at the end of treatment clearly confirmed reduction in tumor growth induced by NT4-MTX (Fig. 6). The mean weight of tumors treated with NT4-MTX (0.159 g) was about one third (>99.9% confidence level) that of the scramble NT4-MTX (0.433 g), MTX (0.407 g), and saline group (0.471 g).

Results of the experiments with mice indicated higher *in vivo* activity of NT4-MTX than the free drug, unlike in *in vitro* experiments with HT29 cells. In fact, in the *in vitro* cytotoxicity experiments, free MTX was several log more active than the MTX-conjugated peptide. Free MTX at the dose used *in vivo* produced about a 10% reduction in tumor weight compared with injection of saline, whereas the same dose of MTX coupled to NT4 produced a >60% reduction in tumor weight.

The increase in activity of the cytotoxic drug *in vivo*, induced by conjugation with NT4, may be related to peptide selectivity toward cells that express specific receptors, which provides an advantage with respect to the free drug, the active concentration of which is decreased *in vivo* by nonselective uptake by tumor and nontumor cells. Branched neurotensin peptide selectivity and stability may be responsible for the good *in vivo* activity of drug-conjugated molecules, which is encouraging for an efficient targeted therapy mediated by neurotensin branched peptides.

Taken together, *in vitro* and *in vivo* experiments suggest that coupling of toxic moieties to NT4 not only produces

specific receptor-mediated activity that may decrease nonspecific toxicity but also increases *in vivo* efficiency of the drug for tumor therapy.

In conclusion, we showed that use of neurotensin tetrabranched peptides as tumor targeting agents enables conjugation of active peptide sequences with functional moieties for tumor tracing or therapy and the construction of promising new peptide-based molecules for tumor targeting. We also showed that tetrabranched neurotensin molecules offer the added advantages of (*a*) increased metabolic stability, (*b*) multimericity with defined and unambiguous chemical structure, (*c*) the ability to convey drugs across cell plasma membranes by receptor-specific binding, and (*d*) intracellular localization of the conjugated toxic molecule. At the same time, we showed that although neurotensin receptors efficiently mediate cell internalization of drug-conjugated peptides, they are reexposed on the plasma membrane, enabling intracellular accumulation of the cytoxic molecule.

We also showed that conjugation of NT4(8-13) with either the photosensitizer Che6 or the chemotherapic molecule MTX dramatically decreased the nonspecific toxicity of the drugs by inducing receptor-specific uptake of the toxic moieties by target cells.

The promising features of branched NT(8-13) conjugated with chemotherapic molecules were confirmed by *in vivo* experiments using human tumor xenografts in nude mice. Animals treated with NT4(8-13) conjugated with MTX showed a clear decrease in tumor growth with respect to untreated mice and mice treated with equimolar amounts of MTX.

We showed previously that synthesis in tetrabranched form makes it possible to maintain (or even increase) the biological activity of very different peptide sequences, including endogenous peptides and *de novo* generated bioactive peptides, and can be a general method to dramatically increase peptide half-life. The branched peptide strategy we tested here for neurotensin can therefore also be seen as a general method to construct different peptide-based molecules for tumor targeting and can be applied with other peptides, the receptors of which are expressed or overexpressed in tumors.

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