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Tyr-c[D-Orn-Tyr(Bzl)-Pro-Gly]: a novel antiproliferative acting somatostatin receptor agonist with μ-opioid receptor-sensitizing properties

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1 Here, we introduce a β-casomorphin-5-derived cyclic pentapeptide, cCD-2 (Tyr-cyclo[D-Orn-Tyr(Bzl)-Pro-Gly]), which inhibits the cell growth of a variety of human cancer cell lines.

2 This opioid-derived peptide possesses only low affinity for μ-receptors, but enhances the agonist binding to μ-receptors *in vitro* and potentiates the analgesic effect of morphin *in vivo*. The molecular mechanism of μ-receptor sensitization by cCD-2 is not yet known.

3 The antiproliferative effect of cCD-2 is independent of μ-, δ-, and κ-receptors.

4 Using SH-SY5Y cells as model, we can demonstrate that cCD-2 specifically binds to somatostatin receptors and stimulates the activity of protein tyrosine phosphatases, which are early downstream targets of SST receptors.

5 In SH-SY5Y cells, cCD-2 specifically increases the activity of the cytosolic PTP SHP-2, stimulates the activity of mitogen-activated protein kinase (MAPK), and elevates the expression of the cyclin-dependent kinase inhibitor p21 (WAF1/Cip1), suggesting the involvement of SSTR1 receptor subtype in cCD-2 action in this cell type.

6 In COS-7 cells, for comparison, we found a stimulation of SHP-2 as well as SHP-1 in response to cCD-2. The activation of SHP-1, which is attributed to the SSTR2 receptor and negatively regulates the EGF receptor, corresponds with the ability of cCD-2 to inhibit the EGF-induced MAPK activation in COS-7 cells.

7 Our results show that in SH-SY5Y cells cCD-2 inhibits cell growth *via* the SSTR1 receptor-signalling pathway but may, in other cells, also use other SSTR subtypes and their signalling mechanisms.

8 cCD-2 represents a novel type of opioid-derived antiproliferative SST receptor agonist, which possesses low μ-receptor affinity but may induce μ-receptor sensitization and is structurally different from the hitherto known SST receptor agonists.

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Keywords: Cyclopeptide; cell growth inhibition; μ-receptor sensitization; SH-SY5Y cells; SSTR1 receptors

Abbreviations: β-CM-5, β-casomorphin-5, Tyr-Pro-Phe-Pro-Gly; BRL 52537, (±)-1-(3,4-dichlorophenyl)-2-(1-pyrrolidinyl) methylpiperidine, κ-receptor agonist; cCD, cyclic casomorphin derivative, Tyr-c[D-Orn-Tyr(Bzl)-Pro-Gly]; COS-7, transformed African green monkey kidney cell line; DADLE, D-Ala^2-D-Leu-enkephalinamid; DAMGO, Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular-signal regulated kinase; GPCR, G protein-coupled receptor; ICI-154,129, *N*,*N*-Diallyl-Tyr-Gyl-Tyr-$^{\psi}$(CH_2)-Phe-Leu-OH, δ-receptor antagonist; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; p21 (WAF1/Cip1), member of the Cip/Kip family of cyclin-dependent kinase (cdk) inhibitors; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase; SH, src homology; SHP-1/2, SH2-domain containing cytoplasmatic protein tyrosine phosphatases; SST, somatostatin; SSTR, somatostatin receptor

Introduction

The majority of peptide hormone and neuropeptide receptors mediates not only short-term regulation of metabolism, secretion or contractility, but also stimulation of cell proliferation. The agonists of these mostly G protein-coupled receptors (GPCRs) may elicit their mitogenic responses by stimulating the mitogen-activated protein kinase (MAPK) cascade (reviewed in Gutkind, 1998). Up to now, there are only few examples suggesting the antiproliferative actions of peptides (reviewed in Liebmann, 2001). Especially, somatostatin (SST) and a great number of SST-derived analogues, structurally mainly based on the octapeptide SMS 201-995

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(Octreotide; D-Phe-cyclo[Cys-Phe-D-Trp-Lys-Thr-Thr-Cys]-Thr), have been demonstrated to induce inhibition of cell growth. A few of these SST analogues are already in clinical studies and/or use for cancer treatment (reviewed in Janeck *et al*., 2001; Scarpignato & Pelosini, 2001). Interestingly, several years ago another family of cyclic SST analogues has been synthesized, possessing relatively low affinity towards SST receptors but high affinity for μ-opioid receptors such as, for example, CTP (D-Phe-cyclo[Cys-Tyr-D-Trp-Lys-Thr-Pen]-Thr-NH_2), CTAP (D-Phe-cyclo[Cys-Tyr-D-Trp-Arg-Thr-Pen]Thr-NH_2), or CTOP (D-Phe-cyclo[Cys-Tyr-D-Trp-Orn-Thr-Pen]Thr-NH_2) (Pelton *et al*., 1986). These cyclic octapeptide SST analogues have been characterized to act as μ-receptor antagonists (Mulder *et al*., 1991). Based on these and additional results, Hruby *et al*. (1998) hypothesized that although SST and opioid receptor ligands have no structural homology, they obviously use similar structural elements for the interaction with the respective receptors. Indeed, even opioid peptides including enkephalin and casomorphin-derived peptides have been shown to inhibit cell growth. These peptides mediate their antiproliferative effects either *via* opioid receptors or SST receptors or *via* both. Thus, in the human breast carcinoma cell line MCF-7, for instance, μ-, δ- and κ-receptor selective agonists were shown to decrease cell proliferation in a naloxone-reversible manner (Maneckjee *et al*., 1990). In another breast carcinoma cell line, T47D, which does not express μ-receptors, α-casomorphins mediate their antiproliferative activity mainly *via* κ-opioid binding sites (Kampa *et al*., 1996), whereas morphine and the μ-receptor preferring casomorphin derivative morphiceptin were found to inhibit cell growth *via* the SST receptor 2 subtype (SSTR2) (Hatzoglou *et al*., 1995). Furthermore, α- and β-casomorphins were reported to decrease cell proliferation of T47D cells (Hatzoglou *et al*., 1996), as well as of prostatic cancer cell lines (Kampa *et al*., 1997) by interaction with both opioid and SST receptors.

In the course of our studies to prepare cyclic β-casomorphin analogues with modified opioid receptor affinity profiles as well as with antiproliferative activity (Schmidt *et al*., 1991), we obtained a cyclic pentapeptide, Tyr-cyclo[D-Orn-Tyr(Bzl)-Pro-Gly] (cCD-2), with unexpected properties. cCD-2 was found to inhibit the cell growth of tumor cell lines, but the antiproliferative effect was clearly independent of opioid receptors. Furthermore, whereas cCD-2 itself has only a very low affinity to μ-receptors, this compound is able to increase the agonist binding to μ-receptors *in vitro* and to potentiate the analgesic effect of the μ-receptor agonist morphine *in vivo*. Using the human neuroblastoma cell line SH-SY5Y as cellular model, we demonstrate that the antiproliferative activity of cCD-2 is exclusively mediated *via* SST receptors and their signalling pathways.

Methods

Cell culture and preparation of cell lysates

The cell lines used (either from the German Collection of Microorganisms and Cell Culture, Braunschweig, Germany, or from the American Type Culture Collection, Rockville, U.S.A.) were grown in RPMI 1640 medium supplemented with 10% ($v\,v^{-1}$) fetal calf serum, $100\,U\,ml^{-1}$ penicillin, $10\,\mu g\,ml^{-1}$ streptomycin, and $0.25\,\mu g\,ml^{-1}$ amphotericin B in humified air with 5% CO_2 at 37°C. SH-SY5Y cells (DSMZ, Braunschweig, Germany) were grown in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% calf serum (FCS) and antibiotics. The human lung cancer cell line U1752 was generously provided by Dr J. Bergh, (Uppsala, Sweden). Cells were treated with the different compounds as indicated in the figure legends. For preparation of lysates, cells were washed in cold phosphate-buffered saline (PBS) and lysed at 4°C in a buffer containing 20 mM HEPES, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2 mM sodium vanadate, 1 mM phenylmethylsulfonylfluoride (PMSF), $20\,\mu g\,ml^{-1}$ aprotinin, and $20\,\mu g\,ml^{-1}$ leupeptin. The lysates were centrifuged at $14{,}000 \times g$ for 20 min at 4°C.

Peptide synthesis

The β-casomorphin analogues were synthesized by conventional solution methods or a combination of solid phase and solution synthesis. For example, fragment condensation of Boc-Tyr(tBu)-D-Orn(Fmoc)-OH with H-Tyr(Bzl)-Pro-Gly-ONB (Boc = tert-butyloxycarbonyl; Fmoc = fluorenylmethyorenylmethyloxycarbonyl; Bzl = benzyl; tBu = tert-butyl; ONB = 4-nitrobenzyl ester) resulted in the fully protected linear precursor of the cyclic peptide cCD-2. After deprotection of the side chain ω-amino group of the D-Orn residue and the terminal carboxylic group, the ring closure reaction was carried out using diphenylphosphorylazide as coupling reagent. Flash chromatography of the resulting protected cyclic pentapeptide on silica gel and acidolytic removal of the tyrosine-protecting groups gave the desired peptide. Alternatively, solid-phase syntheses were carried out using the multiple peptide synthesizer Symphony/Multiplex$_{TM}$ (Protein Technologies Inc, U.S.A.). Fmoc-Pro-OH, Fmoc-Tyr(Bzl)-OH, Fmoc-D-Orn(Mtt)-OH and Fmoc-Tyr-OH were successively coupled to a H-Gly-2-ClTrt resin using standard solid-phase protocols. On completion of the synthesis, the peptide resin was treated with 1% trifluoroacetic acid in dichloromethane for cleavage and deprotection of the D-Orn side chain Mtt (4-methyltrityl) group. After cyclization of Fmoc-Tyr-D-Orn-Tyr(Bzl)-Pro-Gly-OH and purification of the resulting cyclopeptide as described above, finally the Fmoc-protecting group was removed.

Determination of analgesic activity

At 1 week prior to the experiments, chronic microcannula were implanted under deep nembutal anesthesia ($40\,mg\,kg^{-1}$) into the lateral ventricle of male Wistar rats (8–9-weeks old). The peptide was dissolved in physiological saline and injected intracerebroventricularly in a volume of 5 μl. The determination of pain threshold was carried out by electrical tail stimulation as described previously (Greksch *et al*., 1981). Briefly, incremental current pulses (rectangular pulses, 50 Hz, 4 ms) were delivered to the tail *via* a stainless-steel electrode subcutaneously implemented in the tail. The minimum current strength (in mA) evoking vocalization was taken as the analgesic threshold. An index of analgesia (I_A) was calculated according to Cox *et al*. (1968).

Binding studies

For opioid receptor binding, crude membranes were prepared from Wistar rat brains (without cerebella and medullae) by homogenization in 50 mM Tris-HCl, pH 7.4. The homogenate was filtered through two layers of medical gauze, centrifuged at $40,000 \times g$ for 30 min, resuspended in buffer to about 5 mg protein ml^{-1}, and stored at $-80°C$. Protein concentration was determined by the method of Lowry *et al*. (1951). Binding affinities were estimated by coincubation of the unlabelled substances with either 0.5 nM [^{3}H]DAMGO for 60 min at 4°C or 1 nM [^{3}H]DPDPE for 180 min at 25°C in 50 mM Tris-HCl, pH 7.4 (displacement studies). The data were analyzed by using a nonlinear regression program (GraphPad Prism).

SST receptor binding studies using whole cells were performed as previously described for the human breast carcinoma cell line T47D (Hatzoglou *et al*., 1995). Briefly, cells (5×10^5 SH-SY5Y cells per well) were washed with 2 ml PBS and then incubated for 45 min at 22°C in a total volume of 0.5 ml PBS containing 10 fmol [^{125}I]Tyr^1-SST and increasing concentrations of SST or cCD-2 as indicated. After incubation, cells were washed twice with 2 ml cold PBS and removed from plates with 0.4 ml 2 N NaOH. The bound radioactivity was estimated using a Gammacounter. The binding data were analyzed by nonlinear regression analysis (GraphPad Prism).

Immunoprecipitation and Western blotting

For immunoprecipitation of tyrosine phosphatases, polyclonal anti-SHP-1 or anti-SHP-2 ($2\,\mu g\,ml^{-1}$) were used. Antigen–antibody complexes were recovered using protein A–sepharose. The immunoprecipitates were washed three times with PBS containing 1% Triton X-100 and 2 mM orthovanadate (omitted in the case of PTP immunoprecipitates). For ERK1 immunoprecipitates (MAPK assay), a separate washing protocol was used. The samples were boiled in 50 μl Laemmli buffer and subjected to SDS–PAGE using 7.5% gels (13% gels for MAPK assay samples). Separated proteins were transferred to PVDF membranes, blocked overnight with 3% nonfat dried milk in TBST (Tris-HCl, 10 mM, pH 7.5/0.5 M NaCl/0.1% Tween 20). For immunoblotting analysis, blots were probed with the respective antibodies. Tyrosine phosphorylation of EGFR was detected with a monoclonal anti-phosphotyrosine antibody 4G10. In this case, the filters were blocked in bovine serum albumin in TBST. For reblotting, a polyclonal anti-EGFR antibody was used. Horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody and detected using a chemiluminescence (ECL) Western blotting detection system by exposure to Biomax films.

MAP kinase assay

MAPK activity was measured using the myelin basic protein (MBP) assay as previously described (Graness *et al*., 2000). Phosphorylated MBP was visualized by autoradiography and quantified with a phosphoimager.

Determination of PTP activation

For substrate labelling, the synthetic peptide Raytide was dissolved in 50 mM HEPES, pH 7.5, containing 0.1 mM EDTA and 0.05% Triton X-100 at a concentration of 1 mg ml^{-1}. The reaction mixture was prepared by mixing 10 μl Raytide solution, 10 μl assay buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.015% Brij 35, 0.1 mg ml^{-1} BSA, and 0.2% β-mercaptoethanol), 10 μl ATP solution (buffer, containing 1 mM ATP and 15 μCi ml^{-1} [γ-^{32}P]ATP), and 2 μl (50 U μl^{-1}) active Src kinase. The reaction mixture was incubated for 3 h at 30°C and then applied to a 0.5 ml Dowex AG 1X8 column. [^{32}P]Raytide was eluted with 30% acetic acid and stored at −80°C not longer than 4 weeks.

For measuring PTP activity, the reaction mixture contained assay buffer, labelled Raytide (approximately 50,000 cpm per assay), and diluted samples of cell lysates or PTP immunoprecipitates in a total volume of 50 μl. After incubation for 30 min at 37°C, reaction was terminated by adding 750 μl of ice-cold stop solution, containing 0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4, and 4% activated charcoal. After centrifugation for 5 min at $10,000 \times g$, 400 μl of the supernatants were counted for radioactivity.

Measurement of DNA synthesis

Subconfluent cells were deprived of serum for 24 h and then treated with the different compounds as indicated. The cells were incubated for another 24 h, followed by the addition of [^{3}H]thymidine (1 μCi ml^{-1}) for 2 h. Incorporation of [^{3}H]thymidine was measured by washing of cells sequentially twice with ice-cold PBS, 5% trichloracetic acid (TCA), and 5% ethanol. Then, the DNA was extracted with 1 N NaOH and the [^{3}H]thymidine incorporation into the TCA precipitate was assayed by liquid scintillation counting.

Materials

[γ-^{32}P]ATP (3000 Ci $mmol^{-1}$), [^{3}H]DAMGO (50 Ci $mmol^{-1}$), [^{3}H]DPDPE (42 Ci $mmol^{-1}$), and [^{125}I]Tyr^1-SST (2200 Ci $mmol^{-1}$) were obtained from NEN (Perkin-Elmer life sciences, Köln, Germany). [^{3}H]thymidine (2.0 Ci $mmol^{-1}$) and all reagents for SDS—PAGE were purchased from Amersham Biosciences (Europe, Gmbh, Freiburg, Germany). SST, epidermal growth factor (EGF), DAMGO, DADLE, aprotinin, leupeptin, β-glycerophosphate, myelin basic protein, sodium orthovanadate, phenylmethylsulfonyl fluoride, DTT, EGTA, ATP, Triton X-100, protein A–sepharose, HEPES, MOPS, naloxone, and diagnostic film (Biomax, Eastman Kodak Co.) were obtained from Sigma (Deisenhofen, Germany). Raytide was obtained from Oncogene Research Products (Cambridge, U.S.A.). Polyclonal antibodies against p44 MAPK (ERK1), SHP-1, SHP-2, p21 (WAF1/Cip1), and the monoclonal phospho-ERK specific antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The monoclonal anti-phosphotyrosine specific antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, U.S.A.). BRL 52537 hydrochloride, β-funaltrexamine hydrochloride, ICI-154,129, and nor-binaltorphimine dihydrochloride were obtained from Tocris Cookson Ltd, Bristol, U.K.

Results

The β-casomorphin-derived cyclopeptide, cCD-2, inhibits the proliferation of various tumor cell lines

As shown in Figure 1, cCD-2 inhibits the growth of very different tumor cell lines between 20 and 50%. The influence

on DNA synthesis was studied in serum-deprived cells to see not only inhibitory, but also stimulatory effects of cCD-2. In none of the affected cell lines did the opioid receptor antagonist naloxone prevent the inhibitory effect of cCD-2 on cell growth, but rather stabilized the inhibitory effect of cCD-2 in most cell lines. Naloxone alone did not significantly alter DNA synthesis. In Figure 2, it is shown that in different cell lines, cCD-2 may inhibit [^{3}H]thymidine incorporation in a concentration-dependent manner to different degrees. Taken together, these findings suggest a broad but, nevertheless, cell-specific antiproliferative effect of cCD-2, which apparently does not involve its interaction with opioid receptors.

cCD-2 modulates the binding properties of μ*-opioid receptors*

We considered that cCD-2 may exert antiproliferative activity either *via* opioid receptors, SST receptors, or both. We first examined the opioid receptor affinity of cCD-2. Compared with the μ-opioid receptor-specific agonist DAMGO ($IC_{50} = 5$ nM), cCD-2 displayed a weak μ-receptor affinity with an IC_{50} value higher than 1000 nM (Figure 3a). Interestingly, in contrast to other opioids, we found that low concentrations of this cyclopeptide increased the specific binding of [^{3}H]DAMGO nearly up to 150%. The apparent EC_{50} value for elevating the specific binding of [^{3}H]DAMGO was approximately 10 nM. In agreement with the enhanced binding of the μ-receptor agonist [^{3}H]DAMGO, cCD-2 also increased the analgesic potency of the μ-receptor agonist morphine (Figure 3b). cCD-2 alone did not exert any antinociceptive effect. Furthermore, at a concentration of 1.25 mg/kg, morphine itself is unable to induce analgesia. However, this subeffective dose of morphine becomes increasingly analgesic in combination with increasing concentrations of cCD-2.

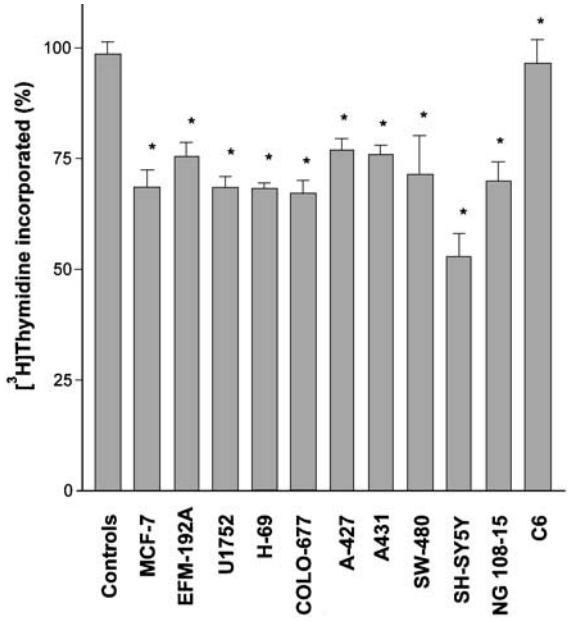

Figure 1 Effects of cCD-2 (1 μM) on DNA synthesis in various cell lines: MCF-7, EFM-192A = human breast carcinoma cell lines; U1752 = human squamous lung cell carcinoma; H-69 and COLO-677 = human small cell lung carcinoma; A-427 = human lung carcinoma; A431 = human epidermoid carcinoma cells; SW-480 = human colon carcinoma cells; SH-SY5Y = human neuroblastoma cells; NG 108-15 = mouse neuroblastoma × rat glioma hybrid cells; C6 = rat glioma cells. DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation using the TCA precipitation technique. Each value is the mean ± s.e.m. of three to six independent experiments in six-fold determinations. *Significantly lower compared with the respective basal [^{3}H]thymidine incorporation (controls) of each cell line (Student's t-test; $P < 0.05$).

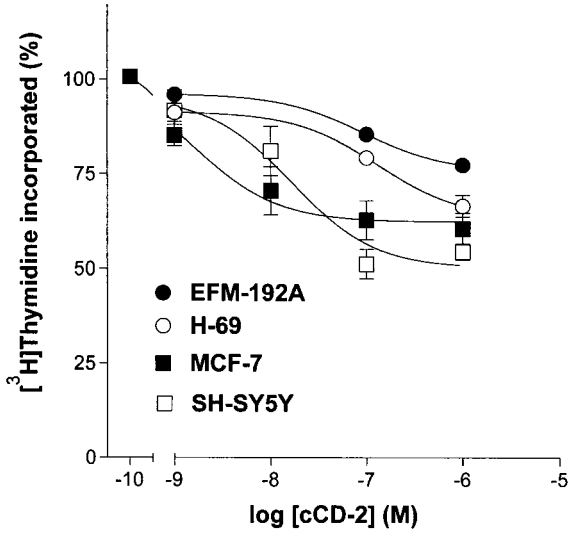

Figure 2 Effects of cCD-2 on DNA synthesis in different cell lines in dependency on peptide concentration. Different serum-deprived tumor cells were treated with increasing concentrations of cCD-2 and assayed for [^{3}H]thymidine incorporation as described under Methods. The results shown represent the mean ± s.e.m. of 12 wells in three independent experiments.

The inhibitory effect of cCD-2 on cell proliferation is not mediated via *opioid receptors*

Using the human neuroblastoma cell line SH-SY5Y as cellular model, we evaluated several derivatives of cCD-2 for their ability to inhibit [^{3}H]thymidine incorporation. Figure 4 shows that only analogues containing either Tyr(Bzl) or Tyr in position 3 may act antiproliferative, whereas other modifications in position 3 or the linearization of the cyclopeptide resulted in the loss of the antimitogenic potency. These findings indicate a structure–activity relationship which additionally demonstrates the specificity in the antiproliferative action of cCD-2. To clarify whether opioid receptors might play a role in the antimitogenic signalling pathway of cCD-2, we measured its effect on [^{3}H]thymidine incorporation in the absence and presence of opioid receptor subtype-specific antagonists. In Figure 5, it is shown that these antagonists did not significantly block the antiproliferative effect of cCD-2 in SH-SY5Y cells. In contrast, the μ-receptor-selective agonist DAMGO, the δ-receptor-selective agonist DADLE as well as the κ-receptor-selective agonist BRL 52537 significantly decreased DNA synthesis, suggesting that in SH-SY5Y cells all opioid receptor subtypes may mediate the inhibition of cell growth. The cell growth-inhibiting effects of the respective receptor subtype-specific agonists were significantly attenuated by the respective antagonists. These results confirm our assumption that in SH-SY5Y cells the antiproliferative action of cCD-2 is independent of μ-, δ-, and κ-receptors.

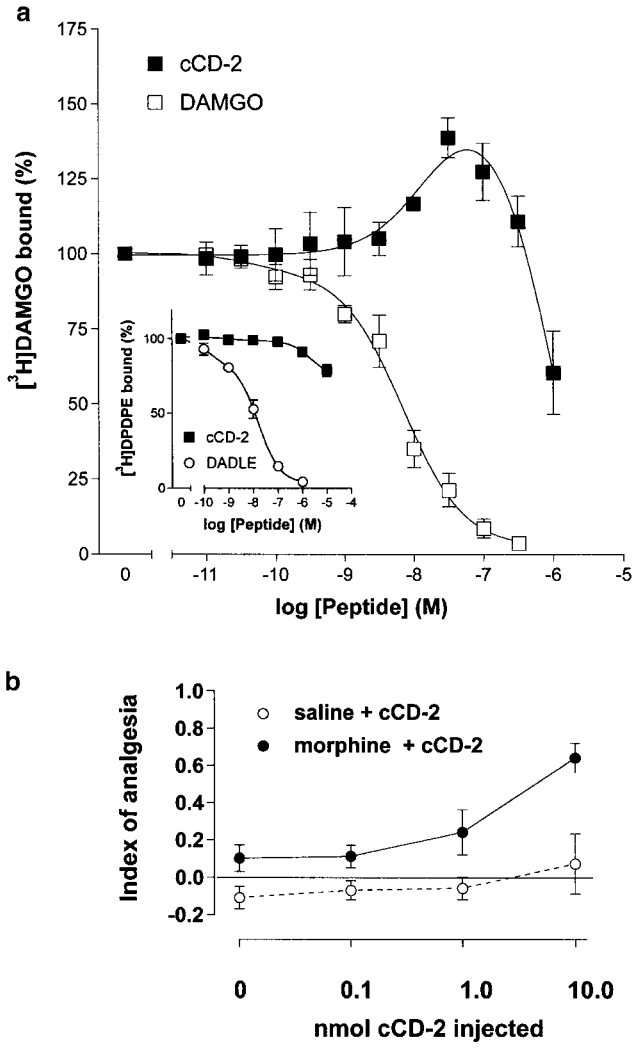

Figure 3 The cyclopeptide cCD-2 is able to increase agonist binding to μ-opioid receptors. (a) Binding of the μ-receptor agonist [^{3}H]DAMGO (0.5 nM) to rat brain membranes was measured in the presence of increasing concentrations of cCD-2. For comparison, the inhibition curves of [^{3}H]DAMGO binding by unlabelled DAMGO is shown. Inset: cCD-2 does not modulate the binding of the δ-receptor agonist [^{3}H]DPDPE. Data for cCD-2 are mean $\pm$ s.e.m. of six independent experiments in duplicate determinations. (b) cCD-2 is capable of increasing the analgesic potency of morphine *in vivo*. Male Wistar rats were pretreated with either saline (open symbols) or 1.25 mg kg^{-1} morphine (s.c.) (filled symbols) 20 min prior to intracerebroventricular injection of the peptide. The effect of cCD-2 was determined 40 min after peptide application as described under Methods. The mean index of analgesia $\pm$ s.e.m. calculated for the different peptide concentrations used in two independent series of experiments is shown. Statistical analysis was performed using the two-way ANOVA program.

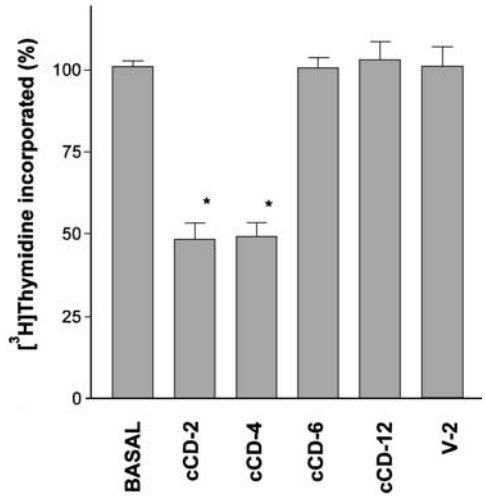

Figure 4 Influence of different analogues of cCD-2 (cCD-4 = H-Tyr-c[D-Orn-Tyr-Pro-Gly]; cCD-6 = H-Tyr-c[D-Orn-Phe-Pro-Gly]; cCD-12 = H-Tyr-c[D-Orn-1-Nal-Pro-Gly]; V-2 = H-Tyr-D-Orn-Tyr (Bzl)-Pro-Gly) on the rate of DNA synthesis in the human neuroblastoma cell line SH-SY5Y. Serum-deprived cells were treated with the compounds, as indicated, in a concentration of 1 μM for 24 h and then with [^{3}H]thymidine (1 μCi ml^{-1}) for 4 h of incubation. The results are expressed as the mean $\pm$ s.e.m. from 12 wells in three independent experiments. *Significantly different compared with the basal value ($P<0.05$; Student's *t*-test).

cCD-2 interacts with SST receptors of the SSTR1 subtype

The proliferation of SH-SY5Y cells is not only inhibited by opioid peptides (Figure 5) but also by SST (Figure 6a). To investigate a putative affinity of cCD-2 towards SST receptors, SH-SY5Y cells were labelled with [^{125}I]Tyr^1-SST and assayed for the competition of the bound radioligand by increasing concentrations of cCD-2 or of SST. In Figure 6b, the respective displacement curves are demonstrated. SST displayed an IC_{50} value of 20 nM towards the binding sites of [^{125}I]Tyr^1-SST. The affinity of cCD-2 with an IC_{50} of approximately 250 nM is about one order of magnitude lower than that of SST. Thus, our results suggest a specific binding of cCD-2 to SST receptors in SH-SY5Y cells. To obtain indications for the SSTR subtype involved in the cCD-2 actions, we analysed the effect of cCD-2 on MAPK activity. MAPK is differentially affected by SSTR receptor subtypes. Among the five SST receptor subtypes known, only SSTR1 has been demonstrated to mediate a stimulation of ERK activity (Florio *et al*., 1999; 2000). Indeed, a small amount of endogenously expressed SSTR1 was immunochemically detectable in SH-SY5Y cells (Figure 7a), and we observed a stimulation of MAPK activity by SST as well as cCD-2 (Figure 7b). When both peptides were added together no additive effect was observed, suggesting that the peptides act *via* the same receptor. For comparison, the levels of SSTR1 in COS-7 cells were measured, which are highly expressing SSTR1 (Stetak *et al*., 2001). Activation of SSTR1 receptors has also been shown to result in G1 arrest, which is specifically induced by the cdk/cyclinD complex inhibitor p21 (WAF1/Cip1) (Florio *et al*., 1999; 2000). When SH-SY5Y cells were stimulated by SST or cCD-2, we detected in both cases a higher amount of the mitotic inhibitor p21 than in non-stimulated cells (Figure 7c). Taken together, these findings suggest that in SH-SY5Y cells the SSTR1 receptor mediates the cell growth-inhibiting effect of cCD-2.

cCD-2 stimulates protein tyrosine phosphatase (PTP) activity

PTPs have been reported to be essentially involved in the SST receptor signalling pathways (Lopez *et al*., 1997; Florio *et al*., 1999; 2000; Pages *et al*., 1999). When SH-SY5Y cells were

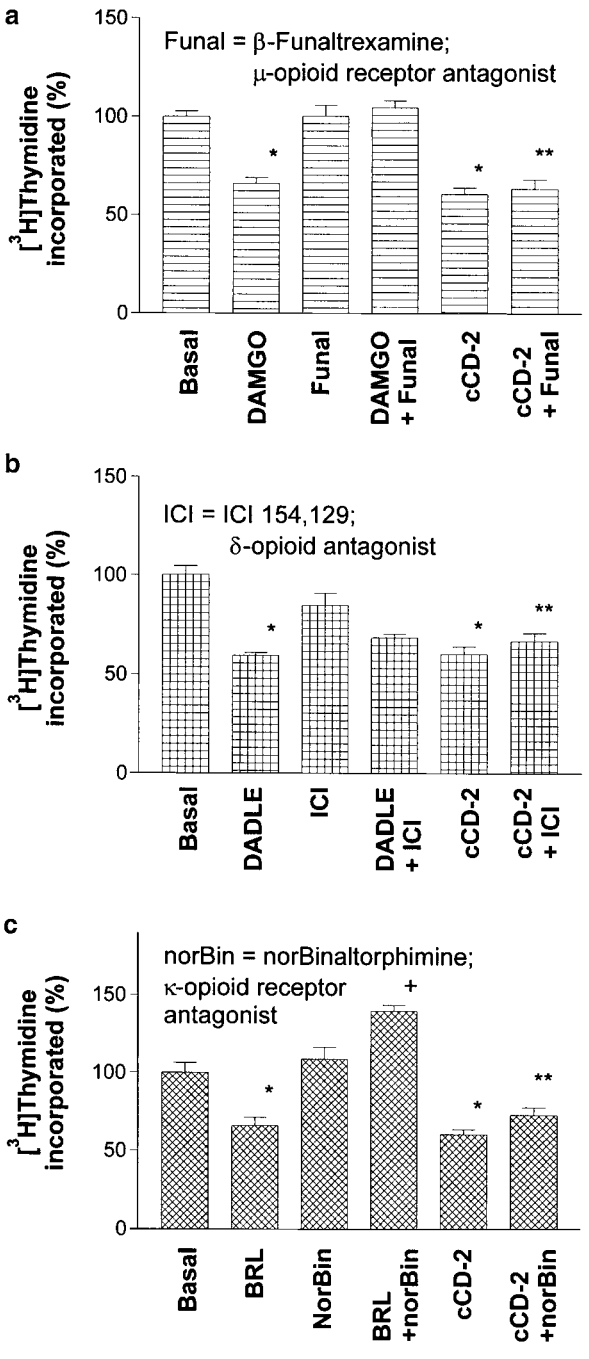

Figure 5 Effects of opioid receptor subtype-specific antagonists on cCD-2-induced inhibition of DNA synthesis in SH-SY5Y cells. Serum-starved cells were pretreated for 30 min with 1 μM of the μ-receptor-selective antagonist β-funaltrexamine hydrochloride (a), the δ-receptor-selective antagonist ICI-154,129 (b), or the κ-receptor-selective antagonist nor-binaltorphimine dihydrochloride (c). Then, cCD-2 (100 nM) or, for comparison, 100 nM of the respective receptor subtype-specific agonists DAMGO (μ-receptors), DADLE (δ-receptors), or BRL 52537 (κ-receptors) were added. The incorporation of [^{3}H]thymidine was measured as described under Methods. The results are expressed as the mean $\pm$ s.e.m. of 12 wells representative of two independent experiments. *Significantly lower compared with the basal values; $^+$significantly higher compared with the basal value; **no significant difference compared with the effect of cCD-2 ($P<0.05$; Student's t-test).

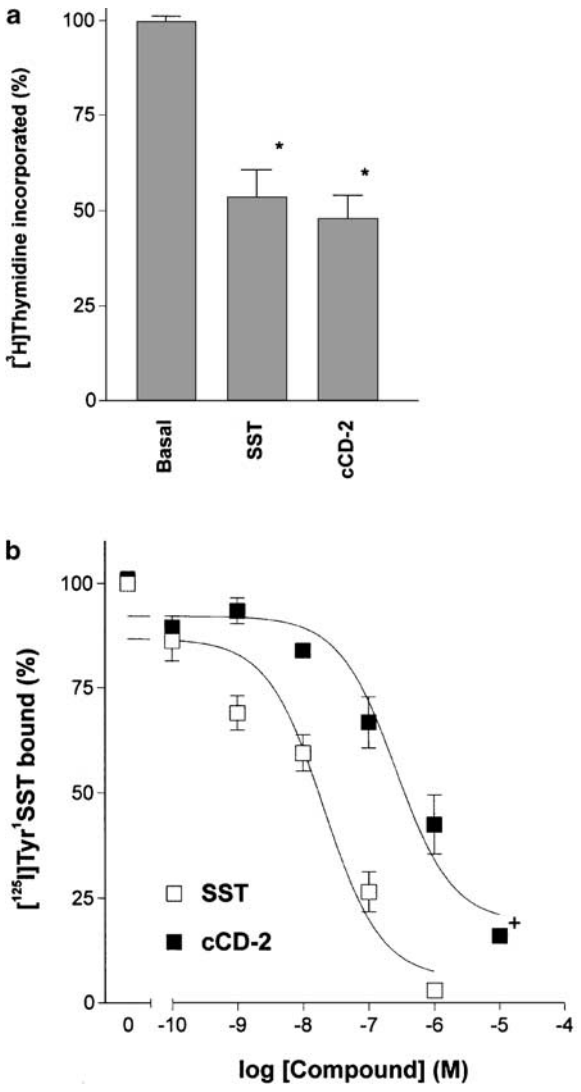

Figure 6 In SH-SY5Y cells both SST and cCD-2 inhibit cell growth and compete for [^{125}I]Tyr^1-SST binding. (a) Serum-deprived SH-SY5Y cells were incubated with 1 μM SST or cCD-2 for 24 h, followed by treatment with [^{3}H]thymidine (1 μCi ml^{-1}) for 4 h. The results are expressed as the mean $\pm$ s.e.m. from 12 wells in three independent experiments. *Significantly different compared with the basal value ($P<0.05$; Student's t-test). (b) Displacement of [^{125}I]Tyr^1-SST binding to SH-SY5Y cells by SST and cCD-2. Cells were labelled with approximately 50,000 cpm [^{125}I]Tyr^1-SST and incubated with increasing concentrations of either SST or cCD-2 for 45 min at 22°C. The specific binding was determined as described under Methods. The results are the mean $\pm$ s.e.m. from two separate experiments in quadruplicate determinations. $^+$Value from a single experiment.

treated with cCD-2, the cyclopeptide moderately but significantly increased PTP activity in total cell lysates in a concentration-dependent manner, as measured by the dephosphorylation of [^{32}P]Raytide (Figure 8a). Corresponding with the lower affinity of cCD-2 towards [^{125}I]Tyr^1-SST binding sites, the total increase in PTP activity in response to cCD-2 was lower than that induced by SST. In Figure 8b, the effects of several cCD-2 analogues on PTP activity are demonstrated. Among them, only the cyclic peptides with Tyr(Bzl) or Tyr in position 3 are capable of stimulating PTP activity. These are the same derivatives that have antiproliferative activity. This

finding suggests that the effect of cCD-2 on PTP might be involved in its antimitogenic action.

We next investigated whether SHP-1 and/or SHP-2, which are well-known targets of SST receptors, might be differentially activated by cCD-2. For that purpose, lysates from SH-SY5Y cells triggered with either SST or cCD-2 were subjected to immunoprecipitation with the respective antibodies. As shown in Figure 9a, increased PTP activity was detectable only in immunoprecipitates of SHP-2 but not in those of SHP-1. The expression patterns of the types of PTP may vary in different cells. Therefore, the endogenous expression levels of SHP-1 and SHP-2 were immunochemically detected in SH-SY5Y cells and, for comparison, in COS-7 cells. In both cell lines high levels of SHP-2 could be detected, whereas SHP-1 could only be detected in COS-7 cells but not in SH-SY5Y cells (Figure 9c). These findings might explain why in SH-SY5Y cells SHP-2 was selectively activated by cCD-2 or SST. Indeed, in COS-7 cells both SHP-1 and SHP-2 were significantly stimulated in response to SST or cCD-2 (Figure 9b). In addition, in COS-7 cells cCD-2 is able to inhibit the EGF-induced activation of ERKs (Figure 9d). The EGF receptor is known to be a target of SHP-1 but not of SHP-2. These results suggest that in SH-SY5Y cells cCD-2 stimulates SHP-2, which is selectively coupled to the SSTR1 subtype. In dependency on their expression patterns, cCD-2 appears to activate even other PTPs preferentially *via* other SST receptor subtypes such as SHP-1 *via* SSTR2 receptors.

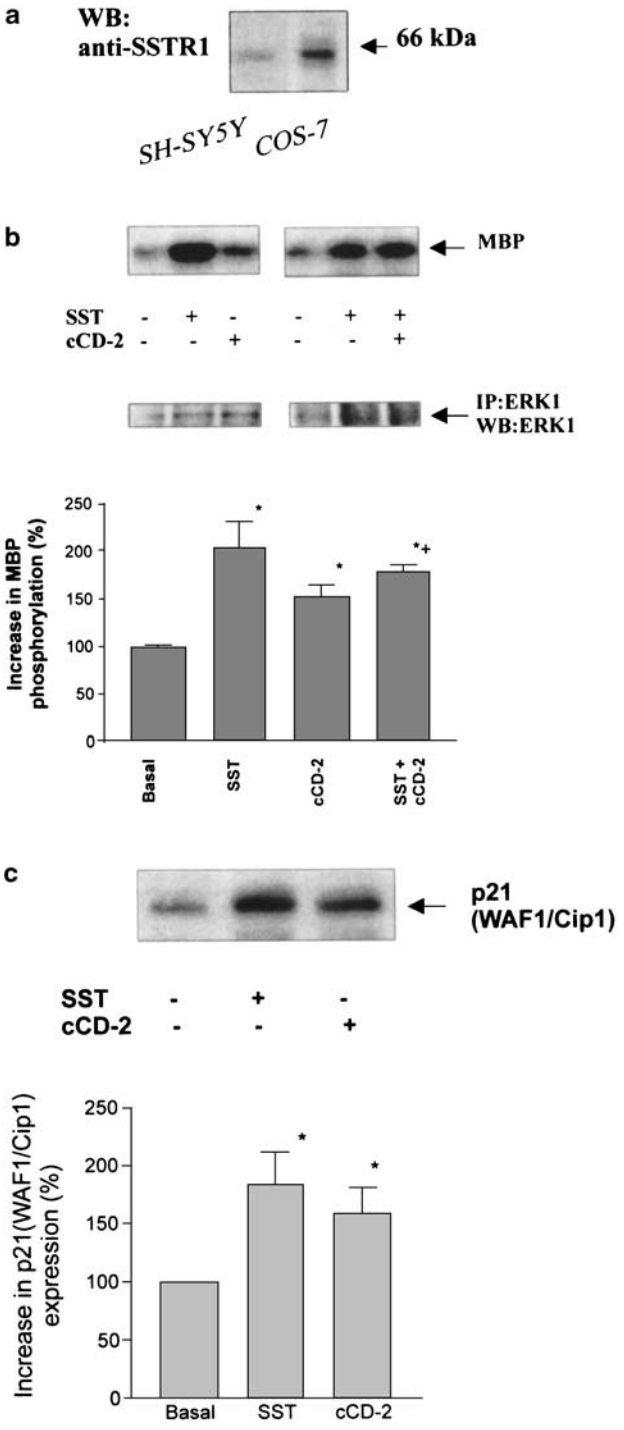

Figure 7 In SH-SY5Y cells, cCD-2 activates the SSTR1 receptor signalling pathway. (a) Western blots from SH-SY5Y cell lysates or, for comparison, COS-7 cell lysates using anti-SSTR-1 receptor antibodies. (b) Effects of cCD-2 and SST on MAPK activity in SH-SY5Y cells. Lysates from SH-SY5Y cells differently stimulated with SST (10 nM), cCD-2 (100 nM), or both together were analysed for MAPK (ERK1) activity using the myelin basic protein (MBP) assay as described under Methods. The representative autoradiograms of four independent experiments are shown. Control blots of immunoprecipitated ERK1 after Western blotting with anti-ERK1 are presented in the panel below the MBP blot. (c) Influence of SST and cCD-2 on the expression of the cdk inhibitor p21 (WAF1/Cip1) in SH-SY5Y cells. Cells were treated with either SST (10 nM) or cCD-2 (100 nM) for 4 h. Then, lysates were prepared and analysed using anti-p21 (WAF1/Cip1). A representative Western blot from three separate experiments is shown. (b, c). *Significantly higher compared with the basal values; $^{+}$no significant difference compared with the single effects ($P<0.05$; Student's *t*-test).

Discussion

Opioids including morphine, enkephaline-like peptides as well as casomorphins have been demonstrated to inhibit cell growth *via* opioid receptors, SST receptors or both (Maneckjee *et al*., 1990; Hatzoglou *et al*., 1995; 1996; Kampa *et al*., 1996; 1997). Here, we introduce a β-casomorphin-5-derived cyclic pentapeptide, cCD-2, which inhibits the DNA synthesis in a variety of tumor cell lines *via* SST receptors. In contrast to morphin or antimitogenic acting opioid peptides, cCD-2 possesses only weak affinity towards μ-receptors and no affinity at δ-receptors. Most interestingly, in the presence of cCD-2, μ-receptors were sensitized towards their agonists *in vitro* and *in vivo*. The potency of cCD-2 to induce μ-receptor sensitization strongly depends on Tyr(Bzl) in position 3, whereas its antimitogenic activity is dependent on either Tyr(Bzl) or Tyr in position 3 within the cyclopeptide-ring structure. Therefore, μ-opioid receptor sensitization and cell growth inhibition in response to cCD-2 appear to be mediated by different mechanisms.

We focussed our further investigations on the molecular mechanism of the antimitogenic activity of cCD-2. We have chosen the human neuroblastoma cell line SH-SY5Y as cellular model, because these cells endogenously express opioid receptors of the μ-, δ- and κ-subtype (Cheng *et al*., 1995; Toll *et al*., 1997; Wang & Sadee, 2000) as well as SST receptors

(Connor *et al*., 1997). Furthermore, SH-SY5Y cells were most sensitive to growth inhibition among a panel of cell lines.

The principle finding of the present study is that cCD-2 acts *via* SST receptors but not *via* opioid receptors. This is supported by two lines of evidence: (i) specific antagonists of μ-, δ-, and κ-receptors did not significantly attenuate the effect of cCD-2 in SH-SY5Y cells, and (ii) cCD-2 specifically binds to SST receptors in these cells. Binding studies with [^{125}I]Tyr^{1}-SST reveal a single IC_{50} value of 20 nM for SST-14 and an IC_{50} value of approximately 250 nM for cCD-2. In contrast, in T47D cells the presence of multiple SST binding sites with IC_{50} values of 0.8 and 22.6 nM has been reported (Hatzoglou *et al*., 1995). The discrepancy might be explained by the use of a different cell line and/or the use of different labels. In addition,

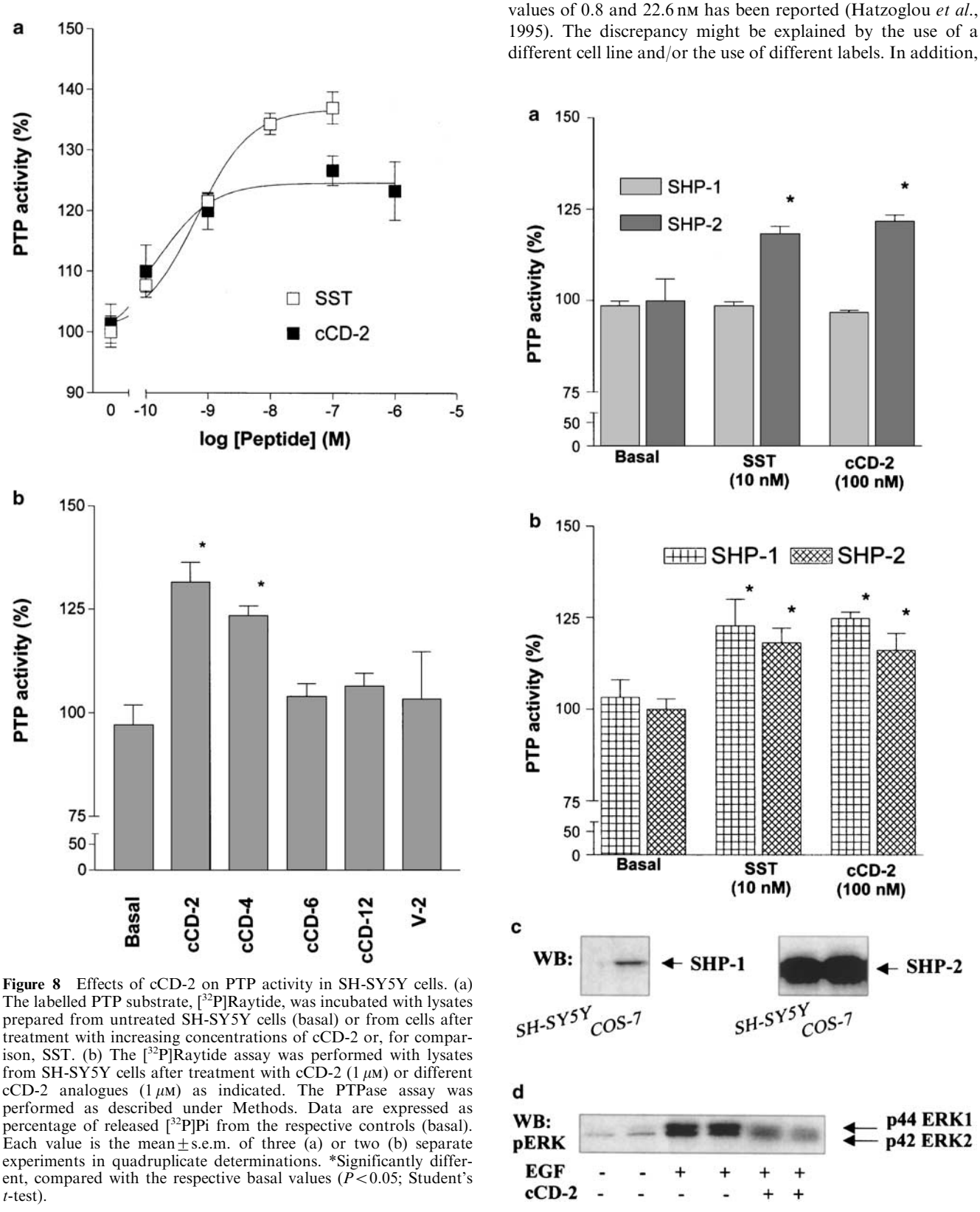

Figure 8 Effects of cCD-2 on PTP activity in SH-SY5Y cells. (a) The labelled PTP substrate, [^{32}P]Raytide, was incubated with lysates prepared from untreated SH-SY5Y cells (basal) or from cells after treatment with increasing concentrations of cCD-2 or, for comparison, SST. (b) The [^{32}P]Raytide assay was performed with lysates from SH-SY5Y cells after treatment with cCD-2 (1 μM) or different cCD-2 analogues (1 μM) as indicated. The PTPase assay was performed as described under Methods. Data are expressed as percentage of released [^{32}P]Pi from the respective controls (basal). Each value is the mean $\pm$ s.e.m. of three (a) or two (b) separate experiments in quadruplicate determinations. *Significantly different, compared with the respective basal values ($P<0.05$; Student's *t*-test).

cCD-2 is able to induce a main downstream signalling event of SST receptors, such as the stimulation of PTP activity. As we demonstrated in cell lysates, the dephosphorylation of the specific PTP substrate [^{32}P]Raytide was increased by cCD-2 in a concentration-dependent manner and with a structure–activity relationship that corresponds with the effects of cCD-2 analogues on DNA synthesis.

The identification of the SSTR1 subtype to mediate the antimitogenic effect of cCD-2 in SH-SY5Y cells was based on three lines of evidence. First, treatment of SH-SY5Y cells with both SST and cCD-2 specifically increased the activity of SHP-2, but not of SHP-1. Secondly, SST as well as cCD-2 stimulated the activity of MAPK (ERK1) in SH-SY5Y cells. The effects are not additive, suggesting that both peptides act *via* the same type of receptor. Thirdly, both SST and cCD-2 increased the expression level of the cyclin-dependent kinase (cdk) inhibitor p21 (WAF1/Cip1). All of them indicate signalling mechanisms which have been specifically attributed to the SSTR1 subtype (Florio *et al*., 1999; 2000). Indeed, SH-SY5Y cells express SSTR1 receptors endogenously, but it cannot be excluded that other SSTR subtypes may be also expressed. Thus, even the existence of a SSTR2-like receptor has been postulated in SH-SY5Y cells (Connor *et al*., 1997). It may be assumed that the specific stimulation of SHP-2 by SST as well as cCD-2 depends on the high expression level of SHP-2 and the absence of SHP-1 in SH-SY5Y cells. This hypothesis is supported by our finding that in COS-7 cells expressing comparable amounts of SHP-1 as well as SHP-2, both PTPs were equally activated by SST and cCD-2. SHP-1 belongs to the few PTP isoforms that are able to negatively modulate EGF receptor (reviewed in Östman & Böhmer, 2001) and has been attributed to the SSTR2 subtype (Lopez *et al*., 1997; Pages *et al*., 1999). We found, indeed, that cCD-2 inhibits the EGF-induced MAPK activation in COS-7 cells. These findings suggest that in dependency on the cell type, cCD-2 is able to interact with different SSTR subtypes and, subsequently, different types of PTP. One could speculate that SSTRs may modulate PTP activity by some general mechanism as for example, local modulation of redox conditions (Östman & Böhmer, 2001). This might lead to activation of different available PTPs in a given cell type. However, SSTR1 and SSTR2 elicit cell cycle arrest (Lopez *et al*., 1997; Florio *et al*., 1999; 2000; Pages *et al*., 1999). Their identification as targets of cCD-2 suggests a cytostatic rather than a cytotoxic effect of cCD-2. When we studied the effect of cCD-2 in an apoptosis assay, no cleavage of poly-ADP-ribose-polymerase (PARP) was found. In contrast, in a positive control with cytosine β-D-arabino-furanoside, the 89 kDa PARP fragment as marker of apoptosis was detectable (not shown). Therefore, at least in SH-SY5Y cells, a possible apoptotic effect of cCD-2 may be excluded.

Taken together, in this study we present a β-casomorphin-derived cyclopeptide as a novel type of compound with two interesting properties: inhibition of tumor cell growth and sensitization of μ-opioid receptors towards agonists such as morphine. Both effects are independent of each other. Whereas the molecular mechanism of μ-receptor sensitization is not yet understood, we demonstrate that the antiproliferative effect of cCD-2 is selectively mediated *via* SST receptors and their signalling pathways. In the human neuroblastoma cell line SH-SY5Y, the molecular mechanisms of cCD-2 action can be related to the signalling pathway of SSTR1 subtype. In other cells, such as COS-7, signalling events of cCD-2 were identified, which are mediated *via* SSTR2 receptors. Therefore, the signalling of cCD-2, like that of SST, appears to depend on the cell-specific expression patterns of SSTR subtypes, of different PTPs, and of other putative effector molecules. Thus, cCD-2 represents a novel type of opioid peptide-derived SST receptor agonist with low affinity towards μ-receptors, but μ-opioid receptor-modulating properties that are structurally different compared with the hitherto existing peptide SST receptor ligands. The combination of two therapeutically interesting properties, such as inhibition of tumor cell growth and stimulation of the analgesic potency of morphine within the structure of a single pentapeptide may be useful in the development of new anticancer drugs.

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Figure 9 Effects of SST and cCD-2 on the PTPs SHP-1 and SHP-2. Lysates from untreated cells (basal) and SH-SY5Y cells (a) or COS-7 cells (b) stimulated for 5 min with either 10 nM SST or 100 nM cCD-2 were immunoprecipitated with anti-SHP-1 or anti-SHP-2 antibodies (2 μg ml^{-1}). The immunoprecipitates were then subjected to the [^{32}P]Raytide assay as described under Methods. The results are expressed as a percentage of released [^{32}P]Pi obtained with the respective immunoprecipitates from untreated cells (basal). For control, immunoprecipitation was performed with nonimmune serum and assayed for dephosphorylation of [^{32}P]Raytide. The nonspecific release of [^{32}P]Pi varied between 0.5 and 1.0% of the basal values and was neglected. The values shown are the mean$\pm$s.e.m. of six (a) or four (b) separate experiments in duplicate determinations. *Significantly different from the respective control values ($P<0.05$; Student's *t*-test).). (c) Western blots from SH-SY5Y cell lysates as well as COS-7 cell lysates using polyclonal antibodies against SHP-1 and SHP-2. (d) COS-7 cells were stimulated with EGF (10 nM) or costimulated with EGF (10 nM) and cCD-2 (100 nM). Lysates were assayed for MAPK activity using a phospho-ERK specific antibody. A Western blot from a representative experiment in duplicate determinations, which was repeated twice with similar results, is shown.

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