Supporting Information

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SI Text

Cell Culture. ***PC-12 Cells.*** Rat PC-12 cells were cultured in Keighn's modified Ham's F-12 medium (ATCC) with 2 mM L-glutamine and 1,500 mg/l sodium bicarbonate supplemented with 15% horse serum, 2.5% bovine calf serum (FCS), and 20U/ml penicillin-streptomycin (Gibco) in a humidified 5% CO_2/95% O_2 atmosphere at 37 °C. The culture medium was changed every second day. Before the initiation of experiments, cells were grown in this culture medium for 72 h, and then incubated in serum-free medium for the next 12 h (1). Neuropeptide antagonists and agonists were used at a concentration of 10^{-6} mol/l for 6 to 48 h.

SW-13 Cells. SW-13 human adrenal cortical carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated FCS (FCS) and 1% penicillin, streptomycin and amphotericin B (Gibco). When cultures reached ≈20% confluence, they were washed and new medium was added containing the peptides RC-160, MZ-4–71, or Cetrorelix at a concentration of 10^{-6}M or the vehicle (final concentration 0.1% vol/vol DMSO) as control. Cultures were incubated for 6 days under these conditions with fresh peptide (or control vehicle) and medium was replaced every 2 days. The culture percent-confluence was estimated visually at each of these time points.

RNA Isolation and RT-PCR. RNA was isolated using the Macherey-Nagel Nucleospin kit according to the manufacturer's instructions (Macherey-Nagel). For RT-PCR, 500 ng RNA from each sample was reverse transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase using random primers (Promega) in a final volume of 20 μl. One microliter of cDNA was amplified in a 25 μl solution containing 1.5 mM $MgCl_2$, 1xPCR buffer (Invitrogen), 0.2 mM of each deoxynucleotide (Promega), 1 unit of Platinum TaqDNA polymerase (Invitrogen), and 0.25 μM of each of the different primers. Samples were denatured for 5 min at 94 °C, then subjected to 15 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C, and 1 min at 72 °C for 40 cycles altogether (sst2, GHRHR, and LHRH). After agarose gel electrophoresis and ethidium bromide staining, bands were visualized under UV light.

Oligonucleotide Microarrays. Microarray slides were generated from 34,580 longmer oligonucleotide probes obtained from the Human Genome Oligonucleotide Set Version 3.0 from Qiagen Inc. The set represented more than 26,121 unique Refseq genes, more than 24,048 unique Ensembl genes, and more than 25,416 unique Unigene genes. Dried oligonucleotides were resuspended in 3× SSC solution and spotted on epoxy-coated slides. After probe labeling and hybridization, slides were scanned with a laser confocal scanner from Agilent Technologies and the image was analyzed as previously described (2). The final analysis was on the basis of 98 tumors collected from 90 patients (46 female, 42 male, and 2 unknown), including 20 patients with metastatic disease and 70 patients with benign disease. The majority of tumors came from patients with apparently sporadic pheochromocytoma, but the study also includes tumors from 12 VHL and 12 MEN2A (one with metastatic disease) (2).

Immunohistochemical Analysis of Tumor Cells and Human Tumor Sections. Slides ($n = 3$ for patients' specimens and tumor cell lines) were deparaffinized and immunohistochemical staining was performed using an automated immunostainer (Benchmark Ventana) according to the manufacturer's protocols. Primary antibodies were Rabbit Anti-rat-Somatostatin Receptor Type 2 (SSTR2) Polyclonal Antibody (BIOMOL) and Rabbit Anti-human Somatostatin Receptor Polyclonal Antibody (Abcam). The signal was amplified using the VENTANA amplification kit and visualized using avidin-biotin labeling and 3, 3′-diaminobenzidine. Slides were counterstained with H&E. Staining with isotype control antibodies was performed to confirm the staining specificity.

Electron Microscopy. Tissues and cells were fixed in 2.5 M glutaraldehyde in 0.1 M cacodylate buffer and refixed in 1% osmiumtetroxid solution. After dehydration in an ascending ethanol series, specimens were embedded in EPON. Ultrathin sections (60 nm) were made. To obtain a suitable contrast, slices were stained with lead acetate und uranyl acetate and afterward analyzed using an electron microscope.

Cell Count. The cell numbers were determined as previously described (3), using a Coulter Counter (Mölab) after 24 h treatment of PC-12 cells with or without AN-238, AN-162, and RC-160. Forty microliters of cell suspension were diluted in 9,960 μl of the isotonic buffer Celloton (Mölab). The number of cells was determined in Gpt/l. Percent-confluence was estimated optically as previously described (4–7).

Cytotoxicity (LDH Release). The colorimetric CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) was used according to the manufacturer's instructions. The CytoTox 96 Assay quantitatively measures LDH, a stable cytosolic enzyme released upon cell lysis.

Apoptosis (Caspase 3/7 Activation). The Caspase-Glo 3/7 Assay (Promega) was used, which provides a homogeneous luminescent assay that measures caspase-3/7 activities. The assay was performed according to the guidelines of the manufacturer.

Neuropeptides. In this study we used somatostatin octapeptide analogue RC-160, targeted cytotoxic somatostatin analogues AN-162, AN-238, LHRH antagonist Cetrorelix, and the GHRH antagonist MZ-4–71. Doxorubicin (DOX) and Dox hydrochloride were obtained from Chemex Export-Import. The somatostatin analogue RC-121 was coupled to 1 molecule of 2-pyrrolinodoxorubicin-14–0-hemiglutarate (AN-201) to form AN-238 (8, 9). GHRH antagonists MZ-4–71 and MZ-5–156 are based on the N-terminal sequence of 29-aa residues of hGHRH, but contain D-Arg-2, Nle-27, and other modifications. All analogues were synthesized in the laboratories of one of us (A.V.S.) (10, 11).

Primer Sequences (5′-3′). We used the sstr2 primers CGGAGTGACAGTAAGCAGGA (forward primer) and GGAAGCCAGTGTGGGTAGG (reverse primer), the GHRH pituitary type primers CACGTCTTCTGCGTGTTGAG (forward primer) and GCATCTCCTCTGCTGCTTGT (reverse primer), the GHRH SV1 primers TGGGGAGAGGGAAGGAGTTGT (forward primer) and GCGAGAACCAGCCACCAGAA (reverse primer), as well as the LHRHR primers ACCGCTCCCTGGCTATCAC (forward primer) and TGTATAACTGTGGTCCTGCAAA (reverse primer).

Statistical Analyses. In all experiments, statistical differences between experimental groups relative to appropriate controls were determined by ANOVA. Data are presented as means ± SEM. Significance of differences was tested by analysis of variance with Bonferroni's or Bennett's as a secondary test. Differences were considered significant at values of $P < 0.05$. Cells from at least 2 different passages were used for each experimental series; n represents the number of cells or tissue culture dishes investigated.

1. Ziegler CG, et al. (2006) DHEA reduces NGF-mediated cell survival in serum-deprived PC12 cells. *Ann N Y Acad Sci* 1073:306–311.
2. Brouwers FM, et al. (2006) Gene expression profiling of benign and malignant pheochromocytoma. *Ann N Y Acad Sci* 1073:541–556.
3. Ziegler CG, et al. (2008) Dehydroepiandrosterone induces a neuroendocrine phenotype in nerve growth factor-stimulated chromaffin pheochromocytoma PC-12 cells. *Endocrinology* 149:320–328.
4. Brown JW, Kesler CT, Neary JT, Fishman LM (2001) Effects of marine sponge extracts on mitogen-activated protein kinase (MAPK/ERK(1,2) activity in SW-13 human adrenal carcinoma cells. *Toxicon* 39:1835–1839.
5. Brown JW, Kesler CT, Neary T, Fishman LM (2001) Effects of androgens and estrogens and catechol and methoxy-estrogen derivatives on mitogen-activated protein kinase (ERK(1,2)) activity in SW-13 human adrenal carcinoma cells. *Horm Metab Res* 33:127–130.
6. Montoya M, Brown JW, Fishman LM (2008) Comparative effects of chemotherapeutic agents on the growth and survival of human adrenal carcinoma cells in culture. *Horm Metab Res* 40:302–305.
7. Brown JW, et al. (2008) Estrogen and progesterone lower cyclin B1 and D1 expression, block cell cycle in G2/M, and trigger apoptosis in human adrenal carcinoma cell cultures. *Horm Metab Res* May 40:306–310.
8. Nagy A, Armatis P, Schally AV (1996) High yield conversion of doxorubicin to 2-pyrrolinodoxorubicin, an analog 500–1000 times more potent: structure-activity relationship of daunosamine-modified derivatives of doxorubicin. *Proc Natl Acad Sci USA* 93:2464–2469.
9. Szepeshazi K, Schally AV, Nagy A, Halmos G, Groot K (1997) Targeted cytotoxic luteinizing hormone releasing hormone (LH-RH) anlalogs inhibit growth of estrogen independent MXT mouse mammary cancers in vivo by decreasing cell proliferation and inducing apoptosis. *Anticancer Drug* 8:974–987.
10. Zarandi M, et al. (1994) Synthesis and biological activities of highly potent antagonists of growth hormone-releasing hormone. *Proc Natl Acad Sci USA* 91:12298–12302.
11. Zarandi M, et al. (1997) Synthesis and in vitro evaluation of new potent antagonists of growth hormone-releasing hormone (GH-RH). *Peptides* 18:423–430.