Identification of Bioactive Molecules by Adipogenesis Profiling of Organic Compounds

Received for publication, October 8, 2002, and in revised form, December 5, 2002
Published, JBC Papers in Press, December 19, 2002, DOI 10.1074/jbc.M210283200

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An important step in the postgenomic drug discovery is the construction of high quality chemical libraries that generate bioactive molecules at high rates. Here we report a cell-based approach to composing a focused library of biologically active compounds. A collection of bioactive non-cytotoxic chemicals was identified from a divergent library through the effects on the insulin-induced adipogenesis of 3T3-L1 cells, one of the most drastic and sensitive morphological alterations in cultured mammalian cells. The resulting focused library amply contained unique compounds with a broad range of pharmacological effects, including glucose-uptake enhancement, cytokine inhibition, osteogenesis stimulation, and selective suppression of cancer cells. Adipogenesis profiling of organic compounds generates a focused chemical library for multiple biological effects that are seemingly unrelated to adipogenesis, just as genetic screens with the morphology of fly eyes identify oncogenes and neurodegenerative genes.

A complete analysis of human genome is anticipated to produce an unprecedented number of potential drug targets. The development of high throughput assays for these genomic pseudotargets may be a challenging but important step for not limiting drug discovery to the “relatively easy” targets such as G-protein-coupled receptors or particular enzymes. An alternative or complementary effort is the construction of high quality chemical libraries that generate bioactive molecules at higher rates. The small size of the focused libraries would lower the cost of screening processes and enable unique low throughput screens, extending the scope of assays for the genomic targets and for a given therapeutic effect.

Our approach to constructing a focused chemical library is based on the logic of genetics. In genetic screens, clear morphological phenotypes are often used as a sensitive tool for discovering and analyzing genes whose primary functions are seemingly unrelated to the morphological phenotype. A good example is the use of eye morphology in the fruit fly Drosophila melanogaster as a genetic tool for the analysis of genes in disease-linked signaling pathways (1). Although human diseases associated with these pathways, such as cancer and neurodegenerative diseases, are seemingly unrelated to eye development, the use of eye morphology as a sensitive indicator enabled a systematic understanding of the disease-inhibited adipogenesis (2–6). We envisioned that clear morphological phenotypes of cells could similarly be used as a sensitive indicator of the drug effects that are not associated directly with the morphological phenotypes.

The morphological alteration we used is the differentiation of murine 3T3-L1 fibroblasts into adipocytes, one of the most drastic and sensitive morphological alterations in cultured mammalian cells (7). In the presence of insulin, 3T3-L1 cells undergo differentiation into adipocytes, which are visually distinct from the original cells because of the presence of oil droplets in the cytoplasm (Fig. 1). The insulin-induced adipogenesis of 3T3-L1 cells involves a number of disease-linked proteins such as phosphatidylinositol 3-kinase, Ras, peroxisome proliferator-activated receptor γ, p38, or phosphodiesterases, and known drugs for a range of diseases have been reported to have phenotypic effects on the adipogenesis (7–12). A morphology-based adipogenesis screen of a chemical library could identify a pool of biologically active compounds with many distinct pharmacological effects. Here we report a proof-of-principle study using a library of 10,000 divergent compounds.

EXPERIMENTAL PROCEDURES

Adipogenesis Profiling—3T3-L1 fibroblasts were plated in 96-well plates at a density of 5 × 10³ cells/well and allowed to reach maximal confluence. The confluent cells were treated individually with 20 nmol/l of a chemical for 3 days in 100 μl of Dulbecco’s modified Eagle’s medium containing of insulin (5 μg/ml) and 10% fetal bovine serum (FBS). After the removal of insulin and the chemical, the cells were further maintained typically for 8 days with the replacement of media every 3 days. The effects of chemicals on the adipogenesis were evaluated under phase microscope. The control wells with 1% (v/v) Me₂SO had ~5% adipocytes. The compounds that enhanced the adipogenesis >5-folds were scored to be adipogenesis-enhancing chemicals, and the ones that completely suppressed the adipogenesis >5-folds were scored to be adipogenesis-blocking chemicals. The effects of these chemicals were confirmed multiple times by multiple laboratory members. Cell viability was monitored by trypan blue exclusion and by counting cell numbers.

Reverse Transcription (RT)-PCR—Total RNA was isolated with TRI-reagent (Molecular Research Center) at day 7 (aP2) or day 3 (osteocalcin). 5 μg of total RNA was reverse-transcribed to cDNA by using oligo(dT) primer with avian myeloblastosis virus reverse transcriptase for 60 min at 42 °C. The cDNA was then amplified by using ExTaq

The abbreviations used are: FBS, fetal bovine serum; RT, reverse transcription; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; IGF, insulin-like growth factor; SEAP, secreted alkaline phosphatase; AP, activating protein; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

This paper is available on line at http://www.jbc.org

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 9, Issue of February 28, pp. 7320–7324, 2003
Printed in U.S.A.
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(Takara) with following primer pairs: 5'-AACACCGGAGATTTCC- TCAAA-3' and 5'-TACGGCTTTGATAAACATG-3' forAFP; 5'-TCTGAA CAAACCCTCAGTCC-3' and 5'-AAATAGTTGATACCGTAGTGGG-3' for osteocalcin. The amplification conditions are as follows: 95°C (30 s), 60°C (30 s), 72°C (30 s) for 23 cycles (aP2) or 30 cycles (osteocalcin).

Glucose Uptake Study—3T3-L1 fibroblasts were induced to differentiate into adipocytes by incubation in a medium containing 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, and 1.7 μM insulin. After 2 days, the medium was switched to the one containing 10% FBS and 1.7 μM insulin for 2 days and then to a normal 10% FBS medium for 3 days. After the total of 7 days, almost 100% 3T3-L1 cells were differentiated into adipocytes. These fully differentiated cells were treated on 24-well plates with varied concentrations of chemicals (0.1% Me2SO for 24 h and then incubated with 100 nM insulin and 2-14C2-deoxyglucose. The cells were extensively washed, and their radioactivity was measured by scintillation counting. All of the samples were tested in duplicate.

Cytokine Production Assay—For the analysis of IL-6 and TNF-α, the mouse macrophage cell line RAW264.7 was used. Cells were seeded onto 96-well plates, and the cytokine production was induced by adding 10 μg/ml lipopolysaccharide. Upon stimulation, each one of the adipogenesis-enhancing chemicals was also added to the culture at varied concentrations. After incubating for 48 h at 37°C, the cytokine concentrations in the culture supernatants were measured by ELISA. For the analysis of IL-2, the mouse thymoma cell line EL-4 was used, and the IL-2 production was induced by adding phorbol ester and ionophore. The effects of chemicals on the IL-2 production were similarly examined by ELISA. All of the samples were tested in duplicate.

Mineralization Assay—The clonal osteoblastic cell line MC3T3-E1, clone 14, was grown in α-minimum Eagle’s medium supplemented with 10% FBS until confluent in 96-well plates. For induction of mineralization, the cells were further incubated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate in the presence or absence of chemicals. On day 14, the cells were washed with phosphate-buffered saline, fixed in 10% formalin, and washed with distilled water. Bonelike mineral formation was evaluated by examining the area stained by 2% (w/v) Alizarin Red S (pH 4.2).

Adipogenesis-blocking chemicals were assessed for their ability to inhibit the growth of IGF-activated cancer cells. For the discovery of inhibitors of IGF2, we used five distinct human hepatocellular carcinoma cell lines, Hep-G2, SK-Hep-1, and three lines that we recently characterized. Three of them produce IGF2 at high levels, whereas two express ~10 times less amounts of IGF2 as measured by ELISA, RT-PCR, and DNA microarray experiments. Treatment with a neutralizing antibody against IGF2 selectively inhibited the growth of the IGF2-overexpressing cell lines but had little effects on that of the cell lines with low levels of IGF2. Thus, these cell lines served as an excellent system for discovering the chemicals that selectively impair the growth of IGF2-overexpressing hepatocellular carcinoma cells. For cell viability assays, IGF2-expressing cells were plated at a density of 4 × 103 onto 96-well plates. After a 24-h incubation, the cells were treated with varied amounts of chemicals for 72 h. The effects of chemicals were evaluated by microscopic observation and MTT assay. All of the samples were tested at least three times. For reporter gene assays, IGF2-expressing cells were transfected with a reporter construct in which a gene encoding secreted alkaline phosphatase (SEAP) is controlled by the IGF2 promoter, AP-1 sites, NFkB sites, or the SV40 promoter. After chemical treatment in the presence of insulin, the cell morphology was examined under microscope. The control wells that are treated with 1% (v/v) Me2SO (DMSO) have ~5% adipocytes. The compounds that enhanced adipogenesis >6-folds were scored to be adipogenesis-enhancing chemicals (C), and the compounds that completely inhibited adipogenesis without detectable cytotoxicity were scored to be adipogenesis-blocking chemicals (D). E, RT-PCR analysis of adipocyte-specific aP2. 3T3-L1 cells were treated with chemicals for 3 days, and total RNA was isolated at day 7. Typical results of four representative compounds are shown along with the positive control of 1 μM dexamethasone (DEX) and 0.5 mM methylisobutylxanthine (MIX).

Collection 2000 Format Q (ChemBridge). In this format, 10,000 druglike molecules are rationally preselected to form a library that covers the maximum pharmacore diversity with the minimum number of compounds. Two academic groups (14, 15) have reported successful isolations of unique compounds from a similar chemical library, indicating that this type of chemical library contains a diverse set of compounds that are suited for a proof-of-principle study. Our cell morphology profiling of the 10,000-compound library identified 188 chemicals that clearly modulated the insulin-induced differentiation of 3T3-L1 cells at 20 ng/μl (Fig. 1): 81 compounds potentiated the adipogenesis; 87 compounds completely blocked the differentiation; and

RESULTS

Adipogenesis Profiling of 10,000 Divergent Druglike Compounds—The divergent chemical library used for our case study was a Prime druglike compounds. A, 3T3-L1 cells have a morphology characteristic of fibroblasts. After chemical treatment in the presence of insulin, the cell morphology was examined under microscope. B, the control wells that are treated with 1% (v/v) Me2SO (DMSO) have ~5% adipocytes. The compounds that enhanced adipogenesis >6-folds were scored to be adipogenesis-enhancing chemicals (C), and the compounds that completely inhibited adipogenesis without detectable cytotoxicity were scored to be adipogenesis-blocking chemicals (D). E, RT-PCR analysis of adipocyte-specific aP2. 3T3-L1 cells were treated with chemicals for 3 days, and total RNA was isolated at day 7. Typical results of four representative compounds are shown along with the positive control of 1 μM dexamethasone (DEX) and 0.5 mM methylisobutylxanthine (MIX).

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13 compounds induced other morphological phenotypes such as adipocyte-like cells without oil droplets. Thus, the screen reduced a pool of chemicals by 53-fold. The adipogenesis-modulating activity of selected compounds was confirmed by RT-PCR analysis of aP2, an adipocyte-specific fatty acid-binding protein (an example is shown in Fig. 1E). The 188 adipogenesis-modulating chemicals that we found are apparently non-toxic for confluent 3T3-L1 cells and almost certainly modulate particular biologic responses in mammalian cells. The chemical structures of adipogenesis-enhancing and -blocking compounds are disclosed in supplementary Figs. 1 and 2.

Glucose-uptaking Insulin Sensitizers—We first focused on the 81 chemicals that potentiated the insulin-induced adipogenesis. Their insulin-sensitizing activity in the adipocyte differentiation suggests that some of them enhance the insulin-induced glucose uptake with anti-diabetic properties. This prediction was supported by the fact that the thiazolidinedione family of anti-diabetic drugs enhances the adipogenesis of 3T3-L1 cells through the activation of peroxisome proliferator-activated receptor γ, a nuclear receptor that plays an important role in adipocyte differentiation (16). In fact, among the adipogenesis-enhancing compounds, nine had a structural element chemically equivalent to thiazolidinedione. These known chemicals were eliminated, and the remaining 72 chemicals were assayed for their ability to potentiate insulin-induced glucose uptake in cultured adipocytes. The 72 compounds contained as many as 11 molecules that enhanced the glucose uptake at comparable levels with that of pioglitazone, a clinically used anti-diabetic drug, demonstrating the validity of our approach. Four of them exhibited insulin-sensitizing activity stronger than pioglitazone at 10 μM, and the most potent one was 124D8 (Fig. 2). Its kinase-inhibitor-like structure is novel as an insulin sensitizer and appears to modulate the function of insulin independently from the major insulin pathways because 124D8 had no effects on the phosphorylation of Akt and MAPK in 3T3-L1 cells. Adipogenesis profiling of a larger chemical library is likely to generate a number of glucose-uptaking compounds with a novel mechanism of action.

Inhibitors of Inflammatory Cytokine Production—Recent studies (7, 12) suggest a cross-talk between insulin-induced adipogenesis and inflammatory responses. Anti-inflammatory drugs including glucocorticoid, phosphodiesterase inhibitors, and salicylates stimulate insulin-induced adipogenesis of 3T3-L1 cells, and molecular targets for anti-inflammatory drugs such as p38, TNF-α, and IL-1 are involved in adipogenesis or insulin resistance of somatic cells (11, 17–19). Although the molecular mechanism of the cross-talk remains unclear, these lines of evidence implicate the presence of anti-inflammatory compounds in the pool of the adipogenesis-enhancing chemicals. We assayed the 72 adipogenesis-enhancing chemicals for their ability to reduce the production of three inflammatory cytokines, IL-6, IL-2, and TNF-α. Eighteen compounds inhibited the production of a cytokine >50% at 10 μM without notable cytotoxicity, suggesting a high density of cytokine production inhibitors in the adipogenesis-enhancing chemicals. Among those, the compound that we call 69A10 inhibited the TNF-α production in macrophage RAW cells with a IC50 of 0.3 μM (Fig. 3). A focused library of adipogenesis-enhancing chemicals may be useful for identifying anti-TNF-α compounds, and their mechanistic studies would clarify the interesting cross-talk between adipogenesis and inflammatory responses.

Osteogenesis Stimulators—Insulin shares sequence homology and biological activity with IGFs. Deficiency in IGF1, a prominent member of IGFs, is suggested to be a cause of decrease in bone density with aging (20, 21), and administration of IGF1 prevents the decrease of bone density in osteoporosis patients in part by stimulating osteogenesis (22–24). The high homology between IGF1 and insulin suggested that the osteogenesis-enhancing activity of IGF1 may be mimicked by the chemicals that potentiated the insulin-induced adipogenesis. As a quick test, the adipogenesis-enhancing chemicals were assayed for their ability to stimulate the formation of bonelike mineral deposition in MC3T3-E1 cells. We found three compounds that increased the mineralization at 5 μM as much as IGF1 or ipriflavone, a clinically used anti-osteoporosis drug (Fig. 4A). Their osteogenesis-stimulating activity was confirmed by RT-PCR analysis of osteocalcin, a marker gene of osteoblastic differentiation. The three compounds exhibited an
increased induction of osteocalcin after 3 days of incubation (Fig. 4B). These compounds may serve as a small molecule tool for the mechanistic analysis of osteogenesis, and such studies could lead to the development of pharmaceuticals for osteoporosis, one of the most underdiagnosed and undertreated disorders in medicine.

Suppressors of IGF-activated Cancer Cells—We next turned our attention to the 87 compounds that blocked the insulin-induced adipogenesis. Both insulin and IGFs stimulate oncogenic signaling pathways including those of Ras-MAPK and phosphatidylinositol 3-kinase-Akt, and overexpression of IGFs is often associated with cancer malignancy (25). Patients with IGF-overexpressing tumors tend to have severe hypoglycemia despite low levels of serum insulin (known as non-islet cell tumor hypoglycemia) (26), demonstrating a functional overlap between oncogenic IGFs and insulin in vivo. These considerations led to the hypothesis that the pool of the adipogenesis-blocking chemicals contains anti-cancer compounds that suppress the IGF-stimulated survival and proliferation of malignant tumor cells. We first examined whether the adipogenesis-blocking chemicals impair the viability of human hepatocellular carcinoma cells that overexpress IGF2, a member of IGFs that is often produced at high levels in liver tumors (27).

We identified three chemically analogous compounds that killed IGF2-overexpressing hepatocellular carcinoma cells (Hep-G2) but had milder effects on the cell line with low levels of IGF2 (SK-Hep-1) (28). Repeated experiments with three additional human hepatocellular carcinoma cell lines that we recently characterized indicated that one of the three chemicals, 94G6, exhibited the highest cytotoxicity to IGF2-producing hepatocellular carcinoma cells with selectivity similar to that of a neutralizing antibody against IGF2 (Fig. 5A). This
benzochromene derivative killed the IGF2-producing cells at an I$_{50}$ of 29 nM but had –33 times weaker effects on the hepatocellular carcinoma cells with low level of IGF2. Reporter gene transcription assays showed that 94G6 selectively inhibits the promoter of IGF2 in the hepatocellular carcinoma cells, suggesting that 94G6 blocks the autocrine loop of IGF2 (Fig. 5D). Although 94G6 may target multiple cellular events for causing cell death, the selective inhibition of the IGF2 autocrine loop provides a reasonable explanation for its inhibitory effects on adipogenesis and cancer cell survival.

Another type of IGF-associated tumors is prostate cancer, one of the most common malignant tumors in Western countries. Elevated levels of circulating IGF1 are strongly associated with the risk of developing prostate cancer, and modulation of IGF1 functions by small molecules is an attractive therapeutic approach when combined with androgen-targeting therapies (29). For a chemical screen, we used DU-145 androgen-independent prostate cancer cells whose growth can be stimulated by IGF1 by as much as 2% serum. The pool of the adipogenesis-blocking chemicals contained two analogous chemicals that specifically inhibited the IGF1-induced growth of DU-145 cells but had little effect on their serum-induced growth. One of them, 125B11, had the greatest differential activity in which the simple druglike thiazole derivative impaired the IGF1-induced growth at an I$_{50}$ of 0.1 μM but had little effects on the serum-dependent growth (Fig. 5C). IGF1-induced phosphorylation of Akt and MAPK in DU-145 cells was unaffected by 125B11, suggesting that 125B11 inhibits the cell-proliferative function of IGF1 in a way independent of the known IGF1-signaling pathway. Deregulation of the IGF axis is associated with the initiation and progression of many types of human carcinoma including breast (30) and colorectal cancers (31). A focused library of adipogenesis-blocking chemicals may serve as a source of anti-proliferative agents against the IGF-linked cancers.

**DISCUSSION**

Fat cell differentiation per se has no direct link to glucose uptake, cytokine inhibition, osteogenesis, and selective suppression of cancer cells. Nevertheless, our proof-of-principle study using a 10,000-compound library successfully identified non-cytotoxic bioactive compounds for these seemingly disparate pharmacological effects, just as genetics has identified non-lethal disease-linked genes by examining the eye morphology of fruit flies. We randomly picked up 70 compounds that had no detectable phenotypes in the adipogenesis profiling and assayed for their ability to modulate glucose uptake, cytokine production, IGF-selective cytotoxicity, and osteogenesis. As expected, no significant hits were found in each assay, indicating that the adipogenesis profiling with 3T3-L1 cells is a good filter for lead-like bioactive molecules that can be used for further biological, chemical genetic, and medicinal chemical studies.

Adipogenesis-based profiling of more chemical compounds including clinically proven drugs would catalog the biological activities of small organic molecules and help to design a focused chemical library that is small enough to be screened with unique low throughput assays yet generates drug seeds for a broad range of disease conditions. Systematic chemical genetic studies on morphological changes of cells could provide small molecule tools for biological studies of human diseases as found in the role of developmental biology in the analysis of disease-linked genes.

**Acknowledgments**—We thank M. Nakatsuka, M. Taji, F. Nishikaku, and A. Tsuchida for assistance in assays and J. W. Harper for comments on the paper.

**REFERENCES**

1. Thomas, B. J., and Wassarman, D. A. (1999) Trends Endocrin. Metab. 15, 184–190
2. Wassarman, D. A., Therrien, M., and Rubin, G. M. (1995) Curr. Opin. Endocrinol. 3, 44–50
3. Luo, H., and Deardoff, C. R. (2001) Bioessays 23, 1138–1147
4. McCall, K., and Steller, H. (1997) Trends Genet. 13, 222–226
5. Burke, R., and Basler, K. (1997) Curr. Opin. Neurobiol. 7, 55–61
6. Min, K. T., and Benzer, S. (1999) Science 284, 1985–1988
7. Rosen, E. D., and Spiegelman, B. M. (2000) Annu. Rev. Cell Dev. Biol. 16, 145–171
8. Klemm, D. J., Leitner, J. W., Watson, P., Nesterova, A., Reusch, J. E., Goaslone, M. L., and Dravem, B. (2001) J. Biol. Chem. 276, 28430–28435
9. Ho, I. C., Kim, J. H., Roney, J. W., Spiegelman, B. M., and Gimlicher, L. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15537–15541
10. Dowell, P., Plesner, C., Krivitrovich, P. O., and Lane, M. D. (2000) J. Biol. Chem. 275, 43125–43132
11. Engelman, J. A., Lisanti, M. P., and Scherer, P. E. (1998) J. Biol. Chem. 273, 32111–32120
12. Engelman, J. A., Berg, A. H., Lewis, R. Y., Lin, A., Lisanti, M. P., and Scherer, P. E. (1999) J. Biol. Chem. 274, 35630–35638
13. Ishinaga, M., Sluss, P. M., Casamento, J. B., and Cockett, A. T. (1993) Prostate 24, 243–252
14. Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Fujiwara, T., Horikoshi, H., and Serizawa, N. (1994) J. Biol. Chem. 269, 2282–2290
15. Mayer, T. U., Kapoor, T. M., Haggarty, S. J., King, R. W., Schreiber, S. L., and Kliewer, S. A. (1995) J. Biol. Chem. 270, 12953–12958
16. Ohsumi, J., Sakakibara, S., Yamaguchi, J., Miyadai, K., Yoshioka, S., Fujisawa, T., Horikoshi, H., and Serizawa, N. (1994) Endocrinology 135, 2279–2282
17. Peterschke, T., and Hauner, H. (1993) J. Clin. Endocrinol. Metab. 76, 742–747
18. Zick, Y. (2001) Trends Cell Biol. 11, 437–441
19. Rosen, C. J., and Donahue, L. R. (1998) Proc. Soc. Exp. Biol. Med. 219, 1–7
20. Baker, J., Liu, J. P., Robertson, E. J., and Elfratiadis, A. (1988) Cell 57, 73–82
21. Bianda, T., Hussain, M. A., Glazt, Y., Bouillon, R., Frosch, E. R., and Schmid, C. (1997) J. Intern. Med. 241, 143–150
22. Eidelberg, P. R., Jones, D. J., O’Fallon, W. M., Jones, C. H., and Riggs, B. L. (1993) J. Clin. Endocrinol. Metab. 77, 1384–1387
23. Grinspon, S., Bauman, H., Lee, K., Anderson, E., Herzog, D., and Klibanski, A. (1996) J. Clin. Endocrinol. Metab. 81, 3864–3870
24. Yu, H., and Rohan, T. (2000) J. Natl. Cancer. Inst. 92, 1472–1489
25. Daughaday, W. H. (1995) Diabetes Rev. 3, 62–72
26. Surf, J. G., Dombrowski, F., and Ramadori, G. (2001) Mol. Pathol. 54, 138–144
27. Zvivel, I., Halay, E., and Reid, L. M. (1991) Mol. Cell. Biol. 11, 108–116
28. Djavan, B., Waldert, M., Seitz, C., and Marberger, M. (2001) World J. Urol. 19, 225–229
29. Sachdev, D., and Yee, D. (2001) Endocr. Relat. Cancer 8, 197–209
30. Hassan, A. B., and Macaulay, V. M. (2002) Ann. Oncol. 13, 349–356
31. Deleted in proof
32. Balasubramaniam, A. (2002) Am. J. Surg. 183, 430–434
33. Dere, S., Kar, S., and Quirion, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4772–4777