Molecular Heterogeneity of Somatostatin Analogue BIM-23014C Receptors in Human Breast Carcinoma Cells Using the Chemical Cross-Linking Assay

Grégoire Prévost, Monique Lanson, François Thomas, Nathalie Veber, Walter Gonzalez, René Beaupain, Anna Starzec, and Arthur Bogden


ABSTRACT

Distinct proteins complexed with somatostatin and the somatostatin analogue BIM-23014C were revealed in human breast cancer cells using the cross-linking assay. One BIM-23014C-specific complex (M, 57,000) was observed in MCF-7 (monolayer, nodule, and tumor) and T47D. Growth inhibition of MCF-7 tumor xenografts by BIM-23014C was dose related in the 6-day subrenal capsule assay. Three complexes (M, 27,000, 42,000, and 57,000) were detected in MDA-MB-231, and no complex was visible in HBL-100. No correlation was found between receptors for BIM-23014C and epidermal growth factor in these lines. Twenty-seven of 30 human breast tumors (90%) had at least one BIM-23014C receptor. Sixteen had three complexes (M, 27,000, 42,000, and 57,000). Six had the two complexes (M, 27,000 and 57,000), two had M, 42,000 and 57,000 complexes, two had just the M, 27,000 complex, and one had just the M, 42,000 complex. The presence of the three BIM-23014C receptors was positively correlated (P < 0.05) to the low amount of sex steroid receptors (≤20 fmol/mg) [seven of eight (estrogen receptor negative, progesterone receptor positive) versus four of 14 (progesterone receptor positive; Gn-RH, gonadotrophin releasing hormone). This high percentage of BIM-23014C receptor-positive biopsies and its inhibitory activity would support its clinical potential for the treatment of breast cancer.

INTRODUCTION

Growth-inhibitory activity of several somatostatin analogues having a longer half-life than the natural peptide (1) has been demonstrated in vitro with different cell lines of human breast tumors (2, 3). Previous studies have shown specific receptors for these somatostatin analogues in 15% to 47% of human breast tumors (4, 5). In addition to a direct local action in the breast tumor, somatostatin could also act on the pituitary as an inhibitor of the synthesis of lactogenic hormones (GH, PRL) which can stimulate growth of breast carcinoma (6, 7). A decrease of plasma GH level results in an inhibition of the synthesis of IGF-I which could also stimulate mammary cell growth. The plasma concentration of EGF involved in breast tumor growth is also decreased after an in vivo treatment by somatostatin (8). Mammary tumor growth inhibition observed in nude mice with infusion of somatostatin analogues may thus be mediated by direct and/or endocrine/paracrine pathways (9).

Determining the receptor status for EGF, IGF-I, GH, PRL, somatostatin, estrogen, and progesterone in human breast cancer may lead to a more rational endocrine therapy. Contradictory correlations between somatostatin receptor, EGF receptor, and sex steroid receptors were described in human breast tumor samples (10, 11). Concerning the somatostatin receptor, one possible explanation may be the molecular heterogeneity of the somatostatin receptor suggested by its ligand- and tissue-selective binding. Marked variations in binding affinities are noted for several somatostatin analogues in different, normal and cancerous, tissues (12–14).

The somatostatin analogue BIM-23014C (Somatuline) has already been shown to be a potent inhibitor of growth hormone release (15) and of proliferation of malignant tissues such as human breast carcinoma (2), rat prostatic carcinoma (16), and small cell lung carcinoma (17). In the present study, we have analyzed its ability to bind specific proteins in various mammary tumor cells and to inhibit growth of MCF-7 tumor xenografts in mice. Its binding ability was studied in four in vitro maintained human breast cell lines, including BIM-23014C-sensitive cells MCF-7 and T47D as well as BIM-23014C-insensitive cells MDA-MB-231 and HBL-100, in two in vivo maintained mammary solid tumor cell lines (MCF7 and 13762NF), and in 30 surgical tumor samples. Correlation of these findings with both EGF and sex steroid receptors in the cell lines and with the sex steroid receptors in the tumor tissue samples forms the basis of this report.

MATERIALS AND METHODS

Materials. BIM-23014C (Somatuline) was initially synthesized by D. Coy (Tulane University, New Orleans, LA) and was provided by Ipsen-Biotech, Paris, France: d-β-Nal–Cos–Tyr–d-Trp–Lys–Val–Cy3–Thr–NH3.

The labeled analogue (2000 Ci/mmol) was obtained from F. Dray and C. Rougeot (Institut Pasteur, Paris, France). EGF was labeled by the chloramine T method, and its specific activity was 106 dpm/μg. EGS, EGF, bovine insulin, Gn-RH, and d-6-TRP-Gn-RH were obtained from Sigma (France).

Cell Lines. T47D, MCF-7, and MDA-MB-231 were established from pleural effusions of human breast cancers, and HBL-100 was established from milk of a nursing mother without breast disease (18–21). NCI-H69 was established from a small cell lung carcinoma (17). All cells were cultured following American Type Culture Collection instructions. MCF-7 cell nodules were cultured in serum-free medium without additional steroid. Multiplication was done by cutting the nodules with irrirectomy scissors without any enzymatic dissociation, as described (22, 23). The monolayer MCF-7 variant growing in serum-free medium was obtained from A. Valette (Toulouse, France) (2). FCS (0.5%) must be added for 16 to 20 h in the medium to allow cell adhesion after seeding. Steroid-depleted FCS obtained with charcoal-dextran treatment could be used to grow these monolayer cells without any steroids (24). The rat mammary solid tumor 13762NF was obtained from J. P. Moreau (Biomeasure, Boston, MA) (25).

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The 6-Day SRCA with the Transplantable MCF-7 Tumor. This assay was performed as described (26). A MCF-7 tumor is carried in serial transplantations in exogenously estrogenized (17β-estradiol pellet implanted) athymic nude females. Tumor donors for 6-day SRCA are selected on the basis of bearing a s.c. tumor which has increased at least 3-fold in volume in 30 to 40 days postimplantation. When a tumor is the optimal size for transplantation (500 to 800 mg), the donor animal is given an i.m. injection (hind leg) with 10 µg of 17β-estradiol in 0.1 ml of sesame oil. Exactly 2 days later, the animal is sacrificed, and the tumor tissue for assay implantation is excised. It is important to note that implanted assay control and test groups are not administered exogenous estrogen during the assay time period. Each group was initially composed of 10 animals. Vehicle ovariectomized controls as well as BIM-23014C groups received administrations s.c. on schedule (every day for Days 0 to 5, twice daily). BIM23014-C was administered at 2 µg, 10 µg, and 50 µg per injection. Tumor size was measured in ocular micrometer units, and the data were reported as change (Δ) in tumor size from day of implantation. The significance of the difference was controlled by using the Student t test.

Tumor Tissue Samples. Tumor specimens were obtained from 30 patients undergoing surgery for breast cancer in Gynecology, Hospital Bretonneau, Tours, France. Only two patients (Cases 16 and 29) have received prior treatments. Frozen tissues were pulverized and homogenized in 10 ml Tris (pH 7.8):1.5 mM EDTA:10 mM ammonium molybdate:1.2 mM mononothioglycerol:10% glycerol. The cytosolic fraction was separated from the membrane-enriched pellet by centrifugation (60,000 × g for 1 h at 4°C). Estrogen and progesterone receptor assays were performed on the cytosolic fraction, and Somatuline receptors, on the pellet. The significance of the correlations was controlled by the χ2 test.

Cross-Linking Assays of Receptor for Somatostatin, Somatuline and Epidermal Growth Factor. A cross-linking assay with iodinated BIM23014C was performed as previously described (2). Cells were rinsed with cold PBS, scrapped, and centrifuged (800 × g, 10 min, 4°C). The pellet was resuspended at 1 g per ml of cold buffer [20% glycerol:20 mM Tris-HCl (pH 7.4):1 mM PMSF] and stored at −20°C. The protein content of the cell homogenate was determined by a colorimetric method (Sigma kit). Cellular proteins were diluted at 1 mg per ml in binding buffer [20 mM Tris-HCl (pH 7.4):5 mM MgCl2:1 mM PMSF]. A total of 100 µl of the cellular protein solution were incubated at 22°C for 90 min with 100 µl of 125I-BIM23014 (106 dpm) solubilized in the binding buffer and 100 µl of the same buffer containing the competitor. For the determination of the nonspecific binding, an excess of cold BIM-23014C was added to the mixture. Samples were centrifuged at 12,000 × g for 15 min at 4°C. Pellets were resuspended in 80 µl of the binding buffer containing 10−4 M ethylene glycol-bis(succinimidyl succinate) at 4°C for 15 min. The cross-linking step was stopped by addition of 20 µl of loading buffer [125 mM Tris-HCl (pH 7.4):10% SDS:50% glycerol:5% β-mercaptoethanol:0.025% bromophenol blue]. Samples were heated at 95°C for 2 min, and proteins were separated on 12.5% SDS-polyacrylamide gel. The gel was dried and autoradiographed at −80°C. The specific activity of iodinated BIM-23014C was 2000 Ci/mmol. The same procedure was used to cross-link 125I-EGF. These two labeled ligands could be used in this assay separately or simultaneously, because required conditions (buffer, temperature, time) are similar and because the molecular weights of their respective receptors are really distinct.

The procedure was modified for the cross-linking assay with the iodinated somatostatin-14. The incubation was for 30 min at 4°C, and the centrifugation step was suppressed. The experiments comparing Somatuline and somatostatin-14 binding data (Table 1; Fig. 1) followed this last protocol.

125I-BIM-23014C Binding Assay. Competitive binding experiments were performed with intact attached cells. MDAMB231 cells were seeded in 6-well plates (3.105 cells/well). After 48 h, the culture medium was removed, and cells were rinsed twice with 1 ml of ice-cold PBS. Cells were incubated for 2 h at 4°C in a final volume of 1 ml of binding buffer [20 mM Tris-HCl (pH 7.4):5 mM MgCl2:1 mM PMSF] containing a constant amount of labeled BIM-23014C (2.5 × 106 dpm, 5.5 × 10−11 M and increasing amounts of cold BIM-23014C. After the incubation, binding buffer was aspirated, and each well was rinsed twice with 1.25 ml of binding buffer without any BIM-23014C. Cells were dissolved in 1 ml of 1 N NaOH for 30 min at 60°C. Acetic acid (100 µl) was added to each sample to prevent chemiluminescence, and radioactivity was measured in a liquid scintillation beta counter.

Estrogen and Progesterone Receptor Assays. Estrogen and progesterone receptors were measured on the cytosolic fractions using enzyme immunoassay kits (Abbott Laboratories, Abbott Park, IL). Values are expressed in fmol/mg of cytosolic protein.

RESULTS

Receptors for Somatostatin-14 and BIM-23014C in MCF-7 and 13762NF Cells. Determination of polypeptides bound to the natural somatostatin-14 and BIM-23014C was performed with MCF-7 monolayer cells and both MCF-7 and 13762NF solid tumor tissues by the cross-linking assay with the EGS linkage agent. An autoradiogram obtained with the labeled somatostatin-14 is presented in Fig. 1. Autoradiographic signals completely competed by the addition of cold somatostatin-14 or cold BIM-23014C (3.3 × 10−5 M) were considered as specific complexes. Determination of the molecular weights of these complexes and their relative intensity are presented in

Fig. 1. Autoradiogram of 125I-labeled somatostatin-14 cross-linked to MCF7 monolayer cells and both MCF7 and 13762NF solid tumor tissue. Binding for each sample was assayed with 125I-labeled somatostatin (106 dpm) and with 200 µg of total proteins from MCF7 monolayer cells (MCF7-MNL), MCF7 solid tumor tissue (MCF7-SLDT), and 13762NF solid tumor tissue (13762) in the absence or presence of unlabeled competitor products. The presence (3.10−10 M) of cold competitors BIM-23014C or somatostatin-14 is indicated below each lane. Lane MW indicates the molecular weight of complexes compared with the migration of molecular weight marker proteins.
Table 1. Results obtained with $^{125}$I-BIM-23014C and these different cells are included in Table 1. $^{125}$I-somatostatin-14 with MCF-7 monolayer cells labeled two major proteins (M, 42,000 and 57,000). The observed pattern with MCF-7 solid tumor was slightly different with one major band (M, 57,000). Three complexes were observed with the rat mammary tumor 13762NF with two major polypeptides with molecular weights of 52,000 and 56,000. These molecular weights are slightly different compared with those obtained with MCF-7 cells.

One complex (M, 57,000) is detected with the ligand $^{125}$I-BIM-23014C in both monolayer and solid tumor MCF-7 cells, but its amount in solid tumor was very low compared with the one observed in monolayer cells (data not shown). Just one complex (M, 52,000) is also detected with the ligand $^{125}$I-BIM-23014C in 13762NF tumor tissue.

The specificity of the M, 57,000 complex obtained with $^{125}$I-BIM-23014C was controlled with MCF-7 cell variant growing without PCS (Fig. 2). This complex (Lane 1) completely disappears with an addition of cold Somatuline (Lane 2). The presence of different amounts of EGF (Lanes 3 and 4) or of bovine insulin (Lanes 9 and 10) does not affect this complex. Only large excesses of cold Gn-RH analogue (d-Trp-6-GnRH) (Lane 5) or of Gn-RH (Lane 6) seem to slightly affect its binding.

Although the cross-linking assay is considered to be qualitative, it could be considered as semiquantitative when data from the same experiment are compared (the chemical linkage is just a partial reaction). It is interesting to note in Fig. 3 that this M, 57,000 complex was largely reduced when MCF-7 cells were cultured as nodules (Lanes 1 and 2) instead of monolayer (Lanes 3 to 6). Addition of steroid-depleted serum instead of complete serum for cell attachment did not significantly modulate the receptor content in the monolayer cells (Lanes 3 and 5).

Dose-related Inhibition of MCF-7 Tumor Growth by BIM-23014C. MCF-7 cells growing with or without FCS are inhibited in vitro by BIM-23014C in the absence of estradiol (2, 3). Low amounts of BIM-23014C receptors could be detected in MFC-7 tumor tissue implanted in nude mice. These data prompt us to study the activity of BIM-23014C treatment in the transplantation-established MCF-7 tumor in the 6-day subrenal capsule assay. With this method, implanted assay control or test groups are not administered estradiol to get tumor growth. The BIM-23014C treatment resulted in significant inhibition of MCF-7 tumor growth after 6 days (Table 2). The tumor-inhibitory effect is dose related, with a maximum efficiency (90%) at the highest dose (50 µg/injection). The average tumor size in ovariectomized mice is very close to the average tumor sizes obtained from the two groups receiving the lower doses of BIM-23014C. Treatment with this analogue (from 2 to 50 µg per injection) did not modify the body weight of the

Table 1 Somatostatin-14 and BIM-23014C receptors in MCF7 monolayer cells and in both MCF-7 and 13762NF solid tumor tissue

Each complex obtained by cross-linking assay was characterized by its molecular weight, and its specificity was controlled by addition of cold BIM-23014C (3.10$^{-5}$ M) and cold somatostatin-14 (3.10$^{-5}$ M. The relative proportions (percentage) were obtained by densitometric analyses of the autoradiogram.

<table>
<thead>
<tr>
<th>Binding sites (M,)</th>
<th>$^{125}$I-Somatostatin-14</th>
<th>$^{125}$I-BIM-23014C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 Monolayer</td>
<td>90,000 (5.4), 70,000 (8.2), 57,000 (54.7), 42,000 (37.7)</td>
<td>57,000 (100)</td>
</tr>
<tr>
<td>Solid tumor</td>
<td>90,000 (5.9), 70,000 (19.2), 57,000 (58.7), 42,000 (11.3), 37,000 (4.8)</td>
<td>57,000 (100)</td>
</tr>
<tr>
<td>13762NF Solid tumor</td>
<td>70,000 (16), 56,000 (33), 52,000 (51)</td>
<td>52,000 (100)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Fig. 2. Autoradiogram of $^{125}$I-labeled BIM-23014C cross-linked to MCF7 cell proteins. Binding for each sample was assessed with 200 µg of total proteins from the monolayer MCF-7 variant growing without FCS and $^{125}$I-labeled BIM-23014C (10$^6$ dpm) in the absence or presence of unlabeled competitor products. Binding without competitor is presented in Lane 1. Cold competitors are BIM-23014C (6.10$^{-5}$ M) (Lane 2), insulin [3.3 µg/ml (Lane 3) and 0.33 µg/ml (Lane 4)], d-Trp-6-Gn-RH [3.33 µg/ml (Lane 5) and 0.33 µg/ml (Lane 6)], Gn-RH [1 µg/ml (Lane 7) and 0.1 µg/ml (Lane 8)], and EGF [3.33 µg/ml (Lane 9) and 0.33 µg/ml (Lane 10)]. Lane MW indicates molecular weight of this complex compared with the migration of molecular weight marker proteins.
animals. No toxicity was observed in mice treated with 500 μm per injection twice a day (25). Treatment with BIM-23014C slow-release formulation was well tolerated in patients with metastatic breast cancer (27).

Simultaneous Characterizations of Receptors for BIM-23014C and EGF in Human Breast Cell Lines. Receptors for BIM-23014C and EGF were analyzed with the cross-linking assay in five in vitro maintained cell lines, including three BIM-23014C-sensitive cells MCF-7, T47D, and NCI-H69 as well as BIM-23014C-insensitive cells MDA-MB-231 and HBL-100. Fig. 4 shows just one M, 57,000 Somatuline-specific complex in MCF-7, T47D, and NCI-H69 (Lanes 1, 3, and 9). A very long gel exposure could reveal two EGF-specific complexes (M, 182,000 and 165,000) in T47D (data not shown). MCF7 cells have no measurable amount of EGF receptors with this assay, but a very low amount could be detected with an assay using polyethylene glycol precipitation (data not shown). Three Somatuline-specific complexes (M, 27,000, 42,000, and 57,000) and two EGF-specific complexes (M, 182,000 and 165,000) were detected in MDA-MB-231. The intensity of autoradiographic signals was quantified by densitometry in the MDA-MB-231 cells. The relative proportions of the three Somatuline binding proteins (M, 57,000, 42,000, and 27,000) were 26.6%, 54.8%, and 18.5%, respectively. Only the EGF-specific complexes were visible in the HBL-100 cell line. No major complexes in the five cell lines were still observed in the presence of cold EGF and cold BIM-23014C, demonstrating specificity of the different complexes (Lanes 2, 4, 6, 8, and 10). The background observed in Lanes 5 and 7 is due to the presence of degraded EGF. Table 3 summarizes these data and includes published data corresponding to the sex steroid receptors with these cell lines (28, 29). Determination of these different complexes in these cell lines was confirmed by experiments using just one iodinated ligand, EGF or BIM-23014C (data not shown).

MDA-MB-231 compared with MCF7 exhibited different complexes formed with labeled BIM-23014C. In competitive binding experiments, the concentration of cold BIM-23014C that blocks 50% of the specific 125I-BIM-23014C binding on attached MDA-MB-231 (Fig. 5) was 1.25 × 10⁻⁷ M. This value was in the same range as the one previously published with MCF7 cells (8 × 10⁻⁷ M) (2). Receptors for BIM-23014C and Sex Steroids in Human Breast Carcinomas. The existence of these different BIM-23014C receptor patterns detected in the cells cultured in vitro prompted us to analyze human breast biopsies with this following assay. Biopsies were homogenized, membrane fractions were used for the determination of BIM-23014C receptors, and cytosolic fractions were used for determination of both estrogen and progesterone receptors. Table 4 shows the complete results of receptor determinations and available clinical data including age, N-stage, differentiation, tumor type, and clinical T-stage. Samples in Table 4 were ranked in accordance with increasing amounts of estrogen receptors. Biopsies with estrogen receptors greater than 20 fmol/mg of cytosolic protein were present in the highest proportion (73.3%), and binding sites for progesterone were found in about 50% of samples investigated (<20 fmol/mg of cytosolic protein). Eight of 30 biopsies were negative for both estrogen and progesterone receptors (26.6%). Twenty-seven of the 30 samples (90%) had at least one Somatuline receptor. Sixteen had three complexes (M, 27,000,

Table 2: Dose response of the transplantation-established MCF7 human breast tumor with BIM-23014C in the 6-day subrenal capsule assay

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Treatment</th>
<th>Av. Δ tumor size</th>
<th>% of test/control</th>
<th>FBW/IBW* ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Intact control, 0.2 ml/injection</td>
<td>3.65 ± 2.06⁶</td>
<td>40</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>Ovariectomized (Day 0) control, 0.2 ml/injection</td>
<td>1.44 ± 0.55⁵</td>
<td>99</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Intact: BIM-23014C, 50 μg/injection</td>
<td>0.35 ± 0.78⁴</td>
<td>10</td>
<td>0.98</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>Intact: BIM-23014C, 10 μg/injection</td>
<td>1.15 ± 0.95⁵</td>
<td>32</td>
<td>0.99</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>Intact: BIM-23014C, 2 μg/injection</td>
<td>1.65 ± 1.00⁴</td>
<td>45</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*FBW, final body weight; IBW, initial body weight.
⁶ Mean ± SD compared with the control value.
⁵ P < 0.01 (significance of difference from control).
⁴ P < 0.001.
³ P < 0.05.

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**Fig. 4.** Simultaneous analysis of EGF receptors and BIM-23014C receptors in different cell lines. $^{125}$I-labeled EGF (2.4 x 10^7 dpm) and $^{125}$I-labeled BIM-23014C (10^7 dpm) were simultaneously cross-linked to 100 µg of proteins from five cell lines: MCF-7; T47D; MDA-MB-231; HBL-100; and NCI-H69. Total binding (Lanes 1, 3, 5, 7, and 9) and nonspecific binding (Lanes 2, 4, 6, 8, and 10 with cold EGF, 1.25 µg/ml, and cold BIM-23014C, 6.10^{-5} M) for each cell line are presented in this autoradiogram. Molecular weights of each complex are indicated in Lane MW. 182kD and 165kD (EGF-R) were two complexes formed with $^{125}$I-labeled EGF; 57kD, 42kD, and 27kD (BIM-23014-R) were three complexes obtained with $^{125}$I-labeled BIM-23014C. Molecular weights of these complexes are compared with the migration of molecular weight markers.

**Table 3** Summary of the receptors for estradiol, progesterone, EGF, and BIM-23014C in five human cell lines

<table>
<thead>
<tr>
<th>Receptors for</th>
<th>Estradiol</th>
<th>Progesterone</th>
<th>EGF</th>
<th>BIM-23014C (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7 pleural effusion</td>
<td>+++*</td>
<td>+</td>
<td>+</td>
<td>57,000</td>
</tr>
<tr>
<td>T47D pleural effusion</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>57,000</td>
</tr>
<tr>
<td>MDA-MB-231 pleural effusion</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>57,000, 42,000, 27,000</td>
</tr>
<tr>
<td>HBL100 from milk of disease-free nursing mother</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>NCI-H69 from small cell lung carcinoma</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>57,000</td>
</tr>
</tbody>
</table>

* ++++, large amount; +, low amount; -, no detectable amount; ND, not determined.

42,000, and 57,000); six had M, 27,000 and 57,000 complexes; two had M, 42,000 and 57,000 complexes; two had just the M, 27,000 complex; and one, just the M, 42,000 complex.

On the basis of these Somatuline receptor profiles, only the largest group of samples (samples with the three BIM-23014C receptors) can be compared with sex steroid binding data (Fig. 6). A positive correlation ($P < 0.05$) was shown between the presence of the three Somatuline receptors and the absence of sex steroid receptors (cut off value, sex steroid receptor < 20 fmol/mg) [seven of eight ER−, PR− versus four of 14 (ER+, PR+)]. Another positive correlation was established between the presence of three BIM-23014C complexes and the absence of progesterone receptors [12 of 16 (PR−) versus four of 14 (PR+)]. Both correlations are not significant if the cut off value for the sex steroid receptors is considered as 10 fmol/mg.

**DISCUSSION**

The chemical cross-linkage between both iodinated somatostatin-14 and the somatostatin analogue BIM-23014C and their receptors associated with an electrophoresis in SDS-polyacrylamide gel were used as a qualitative assay to determine the simultaneous presence of distinct receptors in MCF-7 and 13762NF cells. One major M, 57,000 complex was obtained with both somatostatin and Somatuline. The specificity of this binding was confirmed by addition of cold somatostatin or Somatuline. The molecular weight of this complex is similar to that of one of the three complexes M, 57,000, 42,000, and 27,000 detected with labeled somatostatin-14 or -18 by cross-linking analysis in the two rat pituitary cell lines AT-20 and GH3 (14). It is interesting to note that the amount of this...
complex could be modulated by culture conditions (monolayer, nodule, and solid tumor). MCF7 cells cultured in nodules present several aspects of differentiated cells, including glandular pseudoacini formation with polarized epithelial cells (23). This modulation observed between monolayer and nodule may be related to the state of differentiation. The absence of steroids could be involved, but this absence has no effect in the monolayer system.

The in vivo inhibitory activity of the somatostatin analogue BIM-23014C in MCF7 tumor growth appears to be dose related and very effective compared with data obtained with other somatostatin analogues and the same MCF-7 tumor (9). Priming of the athymic MCF-7 tumor donor animal with exogenous estradiol just prior to tumor excision for assay implantation permits positive tumor growth in the graft recipient during the 6-day assay period. Therefore the addition of exogenous estradiol is unnecessary to obtain MCF-7 tumor growth in this system and could be one major reason for the high activity, since it was shown in vitro that addition of estradiol was sufficient to block the inhibitory activity of the somatostatin analogues (2, 3).

The existence of three different Somatuline receptors and the high frequency of Somatuline receptor-positive cells were observed in the in vitro cultured epithelial cells as well as in human breast biopsies. The highest percentage of somatostatin receptor-positive breast biopsies previously known was around 47%, determined by autoradiography on cryostat sections with the iodinated somatostatin analogue 204-090 (30). This very high percentage (90%) obtained with the cross-linkage method is probably due to several reasons, including: (a) the absence of
problems to determine the background level, (b) the possibility to observe several and distinct receptors at the same time with this ligand, and (c) the absence of problems with the heterogeneous distribution of receptor in the tissue sample that is particularly problematic with cryostat sections (31).

Somatuline receptors were found in three of the four tested human breast cell lines (MCF-7, T47D, and MDA-MB-231). However, just MCF-7 and T47D were inhibited by Somatuline in vitro (2), indicating that the presence of Somatuline receptors was not sufficient to get an in vitro inhibition with this peptide. It is interesting to note that the same pattern of Somatuline receptor was observed in cell lines which had only one Somatuline receptor and both sex steroid receptors.

Patients with a somatostatin receptor-positive breast tumor have a relatively good prognosis, compared with patients with a somatostatin receptor-negative tumor (31). It will be interesting to know within a large-scale clinical study if this favorable prognosis is related to one specific type of somatostatin receptor and to its concentration. The cross-linkage method for somatostatin receptor measurement appears suitable for large-scale clinical studies, using the membrane fraction of a sample which is not used for either estrogen or progesterone receptor measurement. This membrane fraction could also be used to determine other membrane receptors related to the prognosis (e.g., EGF receptor) (32, 33).

The high percentage of Somatuline receptor-positive breast biopsies would support the utilization of this peptide to block cell growth by its own antitumor action and also by affecting growth factor-promoting effects in these human breast cancer cells (34–36). This heterogeneity of the binding sites may lead to the development of more selective somatostatin analogues.

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