Efficacy of a dopamine–somatostatin chimeric molecule, BIM-23A760, in the control of cell growth from primary cultures of human non-functioning pituitary adenomas: a multi-center study

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Abstract

Dopamine D2 and somatostatin receptors (sstrs) were reported to affect non-functioning pituitary adenoma (NFPA) proliferation in vitro. However, the reported results differ according to the experimental conditions used. We established an experimental protocol allowing reproducible evaluation of NFPA cell proliferation in vitro, to test and compare the antiproliferative effects of dopamine and somatostatin analogs (alone or in combination) with the activity of the dopamine–somatostatin chimeric molecule BIM-23A760. The protocol was utilized by four independent laboratories, studying 38 fibroblast-deprived NFPA cell cultures. Cells were characterized for GH, POMC, sstr1–sstr5, total dopamine D2 receptor (D2R) (in all cases), and D2 receptor long and short isoforms (in 15 out of 38 cases) mRNA expression and for α-subunit, LH, and FSH release. D2R, sstr3, and sstr2 mRNAs were consistently observed, with the dominant expression of D2R (2.9 ± 1.0 copy/copy β-glucuronidase; mean ± S.E.M.), when compared with sstr3 and sstr2 (0.6 ± 1.0 and 0.3 ± 0.6 respectively). BIM-23A760, a molecule with high affinity for D2R and sstr2, significantly inhibited [3H]thymidine incorporation in 23 out of 38 (60%) NFPA cultures (EC50 = 1.2 pM and Emax = −33.6 ± 3.7%). BIM-23A760 effects were similar to those induced by the selective D2R agonist cabergoline that showed a statistically significant inhibition in 18 out of 27 tumors (compared with a significant inhibition obtained in 17 out of 27 tumors using BIM-23A760, in the same subgroup of adenomas analyzed), while octreotide was effective in 13 out of 27 cases. In conclusion, superimposable data generated in four independent laboratories using a standardized protocol demonstrate that, in vitro, chimeric dopamine/sstr agonists are effective in inhibiting cell proliferation in two-thirds of NFPA.

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Introduction

Clinically, human non-functioning pituitary adenomas (NFPAs) represent about one-third of pituitary tumors. Such benign tumors grow slowly and are diagnosed late as macroadenomas, producing tumoral symptoms (headaches and visual field defects, due to compression...
of the optic chiasm) and partial or panhypopituitarism. Lateral tumor growth invading the cavernous sinuses occurs frequently as well. By immunohistochemical techniques or by culture studies most of these adenomas were shown to secrete small amounts of either gonadotropins or their subunits. In few rare cases, they may represent silent corticotroph or somatotroph adenomas. Based on ultrastructural characteristics, null cell adenomas and oncocytomas represent the less differentiated tumors containing very few cytoplasmic organelles involved in hormone secretion (see Greenman & Melmed 1996, Chanson & Brochier 2005, Kontogeorgos 2005 for review). Transsphenoidal neurosurgery is the first-line treatment for these tumors, but it is often incomplete in invasive adenomas, particularly in those extending into the cavernous sinuses.

For these reasons adjuvant antitumor pharmacological treatments could be of importance, to prevent the regrowth of tumor remnant, after partial surgical removal of the adenoma. Despite the identification of dopamine (Bevan et al. 1992) and somatostatin receptors (sstrs; Greenman & Melmed 1994a,b), minor tumor shrinkage, if any, was reported in a small series of patients with NFPA treated with either bromocriptine or octreotide (Bevan et al. 1992, de Bruin et al. 1992, Katznelson et al. 1992). A new class of chimeric drugs, combining somatostatin and dopamine receptor binding activity in the same molecule, was recently shown to be more effective than each single agonist in the growth hormone (GH)-secreting adenomas, partially responding to octreotide (Jaquet et al. 2005). Thus, we investigated the efficacy of one of these dopamine–somatostatin chimeric compounds, namely BIM-23A760, in inhibiting [3H]thymidine incorporation in short-term culture studies from a large series of NFPA. The adenomas were characterized quantifying the expression level of the different sstr subtypes (sstr1–sstr5), dopamine D2 receptor (D2R) and its isoforms (D2R_{long} and D2R_{short}) mRNA, using real-time PCR on isolated adenoma cells, in culture. The functional role of these receptors was analyzed using [3H]thymidine incorporation assay in the presence of various concentrations of the somatostatin analog, octreotide, the dopamine agonist, cabergoline, and the somatostatin/dopamine chimeric molecule, BIM-23A760, in cell cultures from 38 consecutive pituitary tumors studied in four different centers.

The main objective was to determine whether the chimeric molecule, BIM-23A760, can achieve a better acute control of cell growth when compared with the individual dopamine and/or somatostatin analogs.

**Patients and methods**

**Patients**

Among the 38 cases, 25 patients were males and 13 females. Their age ranged from 22 to 77 years, with an average of 53.1 ± 13.2 years (mean ± s.d.). All presented with macroadenomas, either non-invasive \( (n=15) \), with suprasellar extensions in all but one case, or with intrasphenoidal or intracavernous extensions \( (n=23) \). In most patients, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and \( \alpha \)-subunit plasma levels were low or in the normal range (data not shown). In all the cases, endocrinological investigations ruled out any GH or adrenocorticotropic hormone (ACTH) hypersecretion. A mild hyperprolactinemia (PRL plasma levels: 24–35 ng/ml) was noticed in four subjects. Analysis of pituitary function, using different provocative tests, revealed normal activity, partial pituitary deficiency, and panhypopituitarism in 20, 10, and 8 patients respectively. Tumor and patient characteristics are described in Table 1. These studies were approved by the Ethics Committee of each individual institution.

**Pharmacological compounds**

The somatostatin analog octreotide was a kind gift of Novartis (Novartis AG). It is essentially directed toward the sstr2 and sstr5 subtypes and, at a lesser degree, the sstr3 subtype \( (\mathrm{IC}_{50}: 0.6, 7.0 \text{ and } 34 \text{ nM}) \) respectively; Jaquet et al. 2005). The D2R agonist cabergoline \( (\mathrm{IC}_{50}: 25 \text{ nM}) \) was a gift from Dr A Harris (Pharmacia Upjohn). The chimeric compound, BIM-23A760, was provided by Biomeasure Inc./IPSEN (Milford, MA, USA). It shows very high affinity to sstr2 \( (\mathrm{IC}_{50}: 0.03 \text{ nM}) \), low affinity to sstr5 \( (\mathrm{IC}_{50}: 42 \text{ nM}) \) and no affinity to sstr3 \( (\mathrm{IC}_{50}: 160 \text{ nM}) \). When compared with somatostatin, and approximately equal affinity to D2R as cabergoline \( (\mathrm{IC}_{50}: 15 \text{ nM}) \); Jaquet et al. 2005). The somatostatin analogs were dissolved as 1 mM solutions in 0.01 M acetic acid containing 0.1% purified BSA (Life Technologies Inc). Cabergoline was prepared as 1 mM solution in 0.01 M acetic acid and 70% ethanol. L-Sulpiride was purchased from Sigma–Aldrich and prepared as a 1 mM solution in ethanol. All compounds were stored at \(-80^\circ\text{C}\). For each experiment, a fresh aliquot of each compound was diluted with PBS supplemented with 1% BSA.

**Primary culture of pituitary adenoma cells and [3H]thymidine incorporation assay**

After surgery, tumor specimens were placed in complete Dulbecco’s minimum Eagle’s culture.
Table 1 Individual clinical and pathological characteristics of the patients and tumors

<table>
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<tr>
<th>Tumor no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Tumor size (mm)</th>
<th>Pituitary functions</th>
<th>Immunohistochemistry</th>
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<td>Normal</td>
<td>FSH: + + + , LH: + + + , α-SU: +</td>
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</table>

*Tumor size was measured on MRI by maximal vertical or horizontal diameters on coronal sections. Tumors were considered invasive when they expanded into cavernous or sphenoidal sinuses or bone.

*Pituitary function: selective hormone deficiency as well as panhypopituitarisms are reported. When hyperprolactinemia was evidenced, actual PRL values are reported.

*Immunohistochemistry: 0, no immunoreactivity detected; +, <20% of immunoreactive cells; ++, >20 <50%; ++++, >50%.
medium (DME medium), supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics, and dissociated by mechanic and enzymatic methods according to the standard protocols. To obtain adenoma cell preparations deprived of rapidly dividing fibroblasts, dispersed cells were filtered through a magnetic bead column coated with antifibroblast antibodies (Miltenyi Biotec, Paris, France), according to the manufacturer’s specifications. The resulting cells were plated in 48-well culture plates at a density of 10^5 cells/well in 0.5 ml of the complete DME medium. Depending on the available tissue, 3–70 × 10^5 isolated cells/adenoma were obtained. After 3 days, the culture medium was collected for hormone determination, and replaced by DME medium containing d-valine (Metachem, London, UK), 10^5 U/l penicillin, 1% FCS. The use of d-valine in the culture medium suppresses the proliferation of any remaining contaminating fibroblasts (Renner et al. 1994). Test substances were added at the appropriate final concentrations for 24 h in the presence of [3H]thymidine (2 μCi/ml; Amersham) and phorbol myristate acetate (PMA, 100 nM; Sigma–Aldrich). At the end of the incubation with [3H]thymidine, cells were washed, harvested, extracted, and counted in a scintillation counter in order to determine [3H]thymidine uptake, according to the standard protocols used by the individual laboratories (Koper et al. 1990, Florio et al. 1992, Renner et al. 1994, Ferone et al. 2005).

In these experiments, octreotide, cabergoline, the combination of the two and the chimeric molecule, BIM-23A760, were tested at predetermined maximal concentrations (10^-9 M), or at concentrations ranging from 10^-12 to 10^-9 M, in order to provide dose–response relationship and to determine the EC50 for each compound with regard to the inhibition of [3H]thymidine incorporation. In selected experiments, the D2R antagonist, l-sulpiride (10^-5 M), was added in combination with either cabergoline, octreotide or BIM-23A760, in order to dissect the specific role of D2R and sstr subtypes in BIM-23A760 effects. Each measurement was performed in quadruplicate. In addition, the pituitary fibroblasts, trapped by the antifibroblast antibody-coated beads were eluted from the column and expanded by multiple passages in 10% FCS–DME medium, without d-valine, in culture dishes, in order to obtain purified fibroblasts originating from the tumoral tissue. In such preparations, no LH, FSH, or PRL could be measured in the culture medium. These fibroblasts were used for both mRNA quantification and [3H]thymidine incorporation studies.

**mRNA analysis**

mRNA analysis was performed at University of Mediterranean (Marseilles, France) on cell samples derived from all the 38 tumors. After adenoma cell isolation, two 2.5 × 10^5 cell aliquots were resuspended in RLT lysis buffer (Quiagen) and stored at −80 °C until quantitative PCR characterization. One aliquot was used to quantify mRNA expression of D2R and sstr1–sstr5. In 15 tumors, the detection of the D2R isoforms, D2R<sub>long</sub> and D2R<sub>short</sub>, was also assessed. The second aliquot was used for quantification of FSH, LH, α-subunit, GH, and pro-opiomelanocortin (POMC) mRNA expression, in order to characterize the different NFPAs and to screen for potential contamination of the tumor cells by adjacent pituitary non-tumoral cells. From each cell sample, 1–1.5 μg RNA was obtained, of which 1 μg of total RNA was used for cDNA synthesis, as previously described (Saveanu et al. 2002). Expression of D2R and the sstr subtype mRNA was determined by real-time quantitative PCR, using primer and probes as previously described (Saveanu et al. 2002). Primers and probe for D2R<sub>long</sub> (TaqMan Gene Expression Assay Hs01024460_m1) were purchased from Applied Biosystems (Foster City, CA, USA). Primers and probe for D2R<sub>short</sub> were the following: forward, 5'-TTGTCAACCTCTGCTGCTAC-3'; reverse, 5'-GGAGAGCATCTCCATCTCCA-3'; LNA probe: 5'-HEX-CTCCACTA68GA8GGCTGCCC-TAMRA-3'. TaqMan Gold nuclease assay was used (Perkin–Elmer, Foster City, CA, USA). The amplification reactions were carried out on an ABI PRISM 7700 sequence Detection System (Perkin–Elmer), according to the manufacturer’s protocol. For quantification of the results, the D2R and sstr subtype mRNA levels were normalized in the same reaction to the β-glucuronidase (β-Gus) mRNA level. For each measurement, two independent RT-PCR analyses were performed.

**Immunohistochemical methods**

Immunohistochemical staining for LH, FSH, and α-subunit was performed on formalin-fixed paraffin embedded sections in the individual centers according to the standard protocols. The results of immunohistochemical analysis were obtained by counting the percentage of positive cells for the respective hormones by two persons and scored as: 0, no immunoreactivity detected; +, <20% of the cells; + +, >20 <50%; + + +, >50%.
Hormone assays
Hormone content was analyzed in 3-day culture medium from 13 out of 38 cell cultures and performed at Erasmus Medical Center (Rotterdam, The Netherlands) for all the samples analyzed. The following assays were used: α-subunit by IRMA from Immuno-tech SAS (Marseille, France), intra-assay coefficient of variation (CV) 5.4%, inter-assay CV 8.8%, results in IU/l; ACTH by solid phase, two-site sequential chemiluminescent immunometric assay (ICMA, Diagnostic Products Corporation, DPC, Los Angeles, CA, USA), intra-assay CV 7.5%, inter-assay CV 8.4%, results in pg/ml; LH by ICMA from DPC, intra-assay CV 10.7%, results in IU/l; FSH by ICMA from DPC, intra-assay CV 3.4%, inter-assay CV 5.4%, results in IU/l; PRL by ICMA from DPC, intra-assay CV 6.2%, inter-assay CV 8.5%, results in IU/l; and GH by ICMA from DPC, intra-assay CV 5.7%, results in μg/l.

Statistical analysis
The results are expressed as mean ± s.e.m. Statistical significance was determined by ANOVA followed by Newman–Keuls test. P < 0.05 was considered statistically significant.

Results
Characteristics of the tumors
The immunohistochemical analysis of sections from all the tumors studied is reported in Table 1. Null cell tumors, not showing granules positive for α-subunit, FSH, or LH, represented 18% of the cases, while adenoma cells were positively stained by anti-FSH, anti-LH, and anti-α-subunit antibodies in 66, 43, and 38% of the tumors respectively (Table 1). None of these adenomas exhibited ACTH- or GH-positive cells as observed in silent somatotroph or corticotroph adenomas. In six tumor fragments, foci of normal pituitary tissue were noted within the adenomatous fragments (Table 1).

Characterization of the NFPA cell cultures
Hormone release in the medium was monitored for a 72 h period under basal conditions in 13 out of 38 cell cultures (Table 2). In 10 out of 13 cases, adenoma cells released FSH and LH in variable amounts. In 4 out of 13 tumor cell cultures, the release of FSH and LH was low (< 1 IU/medium) and three tumors (no. 2, 14, and 17) secreted higher amounts of all the hormones tested (LH, FSH, and α-subunit). The α-subunit was detectable in five cases (39%). In three tumors no hormone release was detected.

In order to check the possible contamination of dispersed adenoma cells by normal anterior pituitary tissue, the transcripts for GH, PRL, and POMC were quantified in all the adenoma cell samples. The GH, PRL, and POMC mRNA expression levels were < 1% in 35 out of 38 measurements when compared with the mean mRNA levels measured in series of ACTH-, PRL-, and GH-secreting adenomas. In the remaining three tumor cell cultures, the level of GH (n = 2) or POMC (n = 2) mRNA expression varied from 1.2 to 6.3% indicating a slight contamination with somatotroph or corticotroph cells, most likely originating from normal pituitary, as already evidenced by immunohistochemical analysis of sections from the same tumors (Table 1).

D2R and sstr subtype mRNA expression in NFPA cell cultures
D2R, sstr2, or sstr3 mRNAs were detected in cells isolated from most of the tumors, but at very different levels of expression (Fig. 1a). D2R, sstr2, and sstr3 mean mRNA levels were 2.9 ± 2.6, 0.3 ± 0.6, and 0.6 ± 1.0 copy/copy β-Gus respectively. With the exception of only three cases, D2R mRNA expression was predominant over sstr. Under individual analysis, 7 out of 38 tumors lacked sstr2 mRNA, 8 out of 38 tumors lacked sstr3 mRNA, and all the tumors expressed D2R.

In this series, only one NFPA showed low level expression of sstr1 mRNA (0.06 ± 0.02 copy/copy β-Gus), and only four tumors showed a weak sstr5 expression (0.03 ± 0.14 copy/copy β-Gus). Individual

<table>
<thead>
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<th>Tumor no.</th>
<th>FSH (IU/l)</th>
<th>LH (IU/l)</th>
<th>α-SU (IU/l)</th>
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<tr>
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<td>2.9</td>
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Values represent hormone release per 72 h from dispersed NFPA cells, in basal conditions. ND, not detectable.
values of mRNA expression for each tumor are presented in Table 3.

In 15 NFPAs, mRNAs for the two predominant D2R isoforms (long and short) were quantified. In two cases (NFPa no. 7 and 38), D2R_short isoform was not detected. In the other 13 cases, mRNAs for both variants were identified although D2R_long was the predominant isoform (95.4 ± 4.7% of total D2R mRNA), as shown in all the individual cases in Table 3.

The sstr subtype and D2R mRNA expression was also measured in two subpopulations of purified human pituitary-derived fibroblasts. As shown in Fig. 1b, only sstr1 mRNA was detected (0.4 ± 0.1 copy/copy β-Gus) in these cell cultures. According to the tumors, 12–38% of the dispersed adenoma cells were trapped by the antifibroblast antibodies coated on the magnetic beads, which retain fibroblasts and non-viable cells.

Maximal effects of octreotide, cabergoline, and BIM-23A760 on [3H]thymidine incorporation

Prior to the primary studies, we compared the yield of [3H]thymidine incorporation in the absence or the presence of PMA (100 nM). As shown in four different experiments (Table 4), in the absence of PMA, BIM-23A760 was unable to affect [3H]thymidine incorporation. When the DNA synthesis was activated by PMA, a −20 to −33% inhibition of [3H]thymidine incorporation was observed in BIM-23A760-treated cells derived from the same tumors. Consequently, we performed all the following experiments in the presence of PMA stimulation.

Preliminary experiments were performed to evaluate the efficacy of different BIM-23A760 concentrations. The dose–response curve of BIM-23A760, used at concentrations up to 100 nM, demonstrated that its maximal inhibitory effect on [3H]thymidine incorporation was obtained at 1 nM (data not shown). Thus, this concentration was selected as the highest BIM-23A760 concentration in the following experiments.

The efficacy of BIM-23A760 to inhibit PMA-stimulated DNA synthesis was analyzed in 38 individual NFPa cultures. In 23 out of 38 (60%) cell cultures, BIM-23A760 (1 nM) produced a significant (P < 0.05) inhibition of [3H]thymidine incorporation (−33.6 ± 3.7%). In the other 15 cases, non-statistically significant variations of the percentage of [3H]thymidine incorporation versus respective controls occurred (ranging from −7.8 to +17.4%), as shown in Table 3.

To correlate the responsiveness of the NFPAs studied with their receptor expression pattern, we analyzed whether differences in receptor mRNA content occurred in responsive tumors versus the non-responsive ones. In the 23 tumors sensitive to BIM-23A760 (Table 3, tumors no. 1–23), the mean levels of D2R, sstr2, and sstr3 mRNA expression were 2.19 ± 0.49, 0.36 ± 0.15, and 0.54 ± 0.25 copy/copy β-Gus respectively, when compared with those measured in tumors resistant to BIM-23A760 (Table 3, tumors no. 24–38), in which the following mean values were detected: 3.92 ± 0.69, 0.26 ± 0.10, and 0.61 ± 0.21 copy/copy β-Gus.

The sstr2 and sstr3 expression was similar in both groups (P < 0.4 and < 0.3 respectively). Surprisingly, D2R mRNA expression was significantly higher in the group of tumors resistant to BIM-23A760 (P < 0.02). However, no differences in the expression level of the D2R_long and D2R_short isoforms was observed in a subgroup of NFPAs responsive (n = 7) and non-responsive (n = 8) to BIM-23A760 (Table 3).

We also compared the biological and clinical characteristics of BIM-23A760 responsive versus non-responsive NFPAs. However, as far as differentiation status (i.e., percentage of null cell tumors), aggressive behavior in vivo (invasive versus enclosed tumors), or tumor-dependent panhypopituitarism (Table 1) are concerned, we did not observe statistically significant differences between the two groups of tumors.

BIM-23A760 maximal effects were also compared with those induced by the selective D2R and sstr2 agonists cabergoline and octreotide (1 nM), in a subgroup (n = 27) of these tumors. In these NFPAs, BIM-23A760 caused a statistically significant
inhibition of \[^{3}H\]thymidine incorporation in 17 out of
27 tumors (63%), when compared with cabergoline
that was effective in 18 out of 27 tumors (66%).
Octreotide caused a comparable effect in 13 out of 27
tumors (48%) and the combined treatment octreotide +
cabergoline was effective in 12 out of 22 tumors (54%).
In the responsive NFPAs of this subgroup, octreotide,
cabergoline, and the combination of the two, produced
a mean maximal inhibition of \[^{3}H\]thymidine incorpor-
ation, similar to that achieved with BIM-23A760
(\(-22, \ -27\) and \(-22\)% respectively, versus \(-28\)%
with BIM-23A760, Fig. 2b).

Dose–response of BIM-23A760, octreotide, and
cabergoline on \[^{3}H\]thymidine incorporation
The dose–response of BIM-23A760 (1 pM–1 nM) on
[^{3}H]thymidine incorporation was tested in all the
38 NFPAs analyzed. In 15 out of 38 tumor cultures,
BIM-23A760 had no effect on \[^{3}H\]thymidine

<table>
<thead>
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<th>Tumor no.</th>
<th>sstr2</th>
<th>sstr3</th>
<th>D2R</th>
<th>Percentage of D2R\textsubscript{long}</th>
<th>[^{3}H]thymidine incorporation (percentage of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.77</td>
<td>0.00</td>
<td>0.59</td>
<td>\n</td>
<td>2</td>
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</table>

Mean ± S.E.M. values for tumors sensitive (1–23) and resistant (24–38) to BIM-23A760 are reported.

Endocrine-Related Cancer (2008) 15 583–596
www.endocrinology-journals.org
incorporation at any concentration. In 23 out of 38 NFPA cell cultures, a significant ($P<0.05$) dose-dependent inhibition of [³H]thymidine incorporation was observed with an EC₅₀ of 1.2 pM (Fig. 2a). The majority of the NFPA responsive to BIM-23A760 (61%) displayed a statistically significant inhibition at low concentrations (1 and 10 pM), although proliferation of a number of tumors (9 out of 23, 39%) was significantly inhibited only at the highest BIM-23A760 concentration tested (1 nM). In control experiments using pituitary fibroblasts, BIM-23A760 (Fig. 2a), as well as cabergoline (data not shown), had no effect on [³H]thymidine uptake.

When the cell cultures derived from a group of NFPA responsive to BIM-23A760 were treated with 1 pM–1 nM concentrations of octreotide, cabergoline, the combination of the two, and BIM-23A760, a similar dose–response curve for inhibition of [³H]thymidine uptake was obtained ($n=4$, Fig. 2c). The EC₅₀ for these compounds ranged from 10 to 50 pM.

### Discussion

NFPA are slow growing tumors diagnosed late in the course of the disease by compressive symptoms caused by the macroadenomas. Surgery is the first-line treatment, as current medical therapies are mostly unsuccessful in shrinking NFPA mass. Due to the often incomplete surgical removal of these adenomas, tumor regrowth occurs in 40–52% of non-irradiated patients, according to long-term retrospective or prospective studies (Comtois et al. 1991, Gittoes et al. 1998, Greenman et al. 2003, Ferrante et al. 2006). In one prospective study, in the subgroup of patients that...
In this study, we investigated the antiproliferative effect of a novel chimeric compound, BIM-23A760, on human NFPA cell short-term culture experiments, under PMA-stimulated conditions. Human pituitary adenoma cells, in primary in vitro cultures, have almost lost their capability of duplication, and, even in vivo, NFPA are considered as slow growing tumors (Dekkers et al. 2007). Indeed, unlike previous papers, where in vitro proliferation of human pituitary adenoma cells was reported (up to 3 days in Zatelli et al. (2004) and Batista et al. (2006)), no significant cell growth was observed in our experiments. In previous studies from our group (Florio et al. 1999) basal [3H]thymidine uptake was relatively low in most adenomas and, on the average, somatostatin treatment did not result in a significant reduction of basal DNA synthesis in 9 out of 11 non-functioning adenomas. Similarly, in another study (Renner et al. 1994), we observed suppression of basal cell proliferation by bromocriptine in only three out of nine adenoma cell cultures. For these reasons, in the present protocol, we triggered the activation of the PKC pathway by PMA previously shown to induce a significant increase in DNA synthesis in most of the tumors and to restore the inhibitory effect of somatostatin on DNA synthesis (Renner et al. 1994, Florio et al. 1999). Indeed, although very few NFPA cells actually divide in vitro, they still possess the needed machinery to initiate DNA replication, an event easily detectable in the [3H]thymidine uptake assay. PMA is a known tumor promoter that directly activates PKC and, indirectly, the ERK1/2 MAP kinase cascade (Kolch et al. 1993). ERK1/2 is one of the main regulators of cell proliferation being responsible, among other effects for the synthesis of cyclin D (Cheng et al. 1998), a prerequisite for cells to enter the S-phase of the cell cycle. Importantly, a PKC-dependent ERK1/2 activation was reported to increase mitogenic activity (i.e., cyclin D1 expression) but not hormonal secretion from in vitro cultures of both human GH-secreting pituitary adenomas and NFPA (Lania et al. 2003). On the other hand, in NFPA cell cultures, PKC inhibition by calphostin C is the main intracellular signal to downregulate ERK1/2 activation (Mantovani et al. 2005). Thus, we propose that, in vitro, the presence of PMA can surrogate growth factors likely present in vivo to support NFPA cell duplication. More importantly, in these experimental conditions, the effects of both somatostatin and dopamine analogs can be more clearly detected, since the PMA-activated tentative of entering the cell cycle can effectively be inhibited by blocking ERK1/2 through the activation of both D2R and sstr. In fact, it is now well accepted that

![Figure 3 Effect of D2R blockade by L-sulpiride on [3H]thymidine uptake inhibition, in the presence of PMA, in five NFPA cultures equally responsive to octreotide and cabergoline.](image-url)

(a) Dose–response of BIM-23A760 (10 pM–1 nM) in the absence or presence of L-sulpiride (10 μM). (b) L-Sulpiride (10 μM) reversal of cabergoline (1 nM), but not octreotide (1 nM), inhibition of [3H]thymidine uptake. *P<0.05 versus respective control values.

presented after surgery with cavernous sinus invasion or large suprasellar tumor remnants, a 70% further tumor extension was observed by repetitive magnetic resonance imaging over the subsequent 5 years (Greenman et al. 2003). In such a subgroup of invasive adenomas, medical therapy, able to prevent or slow down tumor regrowth, would be beneficial to avoid the long-term complications produced by radiotherapy. Nevertheless, the overall efficacy of the currently used somatostatin analogs to limit NFPA growth is poor. In vitro, they produce a 28–34% inhibition of FSH, LH, or α-subunit release in 28% of cases (Klibanski et al. 1991, de Bruin et al. 1992, Hofland et al. 1997) and the efficacy of octreotide in inducing tumor shrinkage in vivo is even less impressive and poorly conclusive. Among 48 patients with NFPA, a >20% reduction in the maximal tumor diameter was reported in seven cases (Plockinger et al. 1994, Warnet et al. 1997). Medical treatment, using the dopamine agonist bromocriptine, also shows efficacy in reducing tumor mass in few patients with NFPA (van Schaardenburg et al. 1989). More recently, treatment with the dopamine D2R superagonist cabergoline provided variable results (Colao et al. 2000, Lohmann et al. 2001, Pivonello et al. 2004) that were eventually linked to differential expression of the dopamine D2R short isoform. Up to now, there has been only one convincing report that showed prospectively a decrease or a stabilization of tumor mass in 18 out of 20 patients presenting with a tumor remnant after surgery, followed by treatment with bromocriptine over a mean follow-up period of 41 months (Greenman et al. 2005).
specific phosphotyrosine phosphatases, activated by both somatostatin (Pan et al. 1992, Lopez et al. 1997) and dopamine (Florio et al. 1992) receptors, can interfere with ERK1/2 activation to prevent cell proliferation (Florio et al. 2001, Massa et al. 2004).

Prior to the pharmacology studies, we measured the expression of both sstr and D2R mRNAs in purified cultured tumor cells. Previous qualitative analyses about sstr subtypes expressed by tumor specimens from non-functioning and gonadotroph human pituitary adenomas revealed mainly sstr3 and sstr2 expression (Greenman & Melmed 1994a,b, Nielsen et al. 2001, Reubi et al. 2001). In a recent quantitative analysis of sstr gene expression, sstr3 mRNA was identified at the highest level, followed by sstr2. The sstr1, sstr4, and sstr5 mRNAs were detected, at low levels, only in few cases (Taboada et al. 2007). In this study, using isolated NFPA cells, a predominant sstr3/sstr2 pattern of expression in NFPA was also observed. This contrasts with previous reports in which sstr1, sstr2, sstr3, and sstr5 are equally expressed in the majority of the tumors analyzed by either PCR (Florio et al. 1999) or immunohistochemistry (Pawlikowski et al. 2003). In another study, a selected subgroup of 12 out of 71 NFPA (16.9%) expressing α-subunit mRNA and secreting α-subunit in vitro, was reported to express mRNA for sstr1, sstr2, and sstr5, as determined by RT-PCR (Zatelli et al. 2004). Interestingly, in these adenoma cells, α-subunit secretion was inhibited by sstr1- and sstr2-specific agonists, while the cell viability was reduced by an sstr1-selective compound. Such discrepancies between the different studies can be, at least partly, explained by the heterogeneity of the NFPA populations analyzed. For example, silent somatotroph or corticotroph adenomas, not observed in our series although included in the NFPA classification, express sstr1, sstr2, and/or sstr5 (Jaquet et al. 2000, Hofland et al. 2005). Furthermore, plurihormonal gonadotroph–lactotroph tumors suppress their hormone secretion when exposed to dopamine or somatostatin analogs in vitro and in cell culture studies, in accordance with the high expression of sstr2, sstr3, and sstr5 (Saveanu et al. 2001). This study also differs from previous reports because adenoma cells were stripped of fibroblasts, which, as previously shown in sarcoma fibroblasts (Reubi et al. 2001) and confirmed in fibroblasts isolated from pituitary adenomas in our study, only expressed sstr1 transcripts. The sstr1 was faintly expressed in only 1 out of 38 fibroblast-free NFPA cell culture. The sstr5 mRNA was detected at very low levels (0.03 ± 0.14 copy/copy β-Gus) in 4 out of 38 tumors, which also expressed POMC and GH mRNAs, suggesting the likely contamination by pituitary cells of non-tumoral origin.

This study compared, for the first time, the level of expression of both sstr and D2R mRNAs in NFPA. All the tumors expressed D2R mRNA at a mean level of expression higher than those observed for sstr3 or sstr2 in most of the individual adenoma cell cultures. Nevertheless, D2R expression observed in NFPA was 30-fold less than the D2R mRNA level measured in a series of PRL-secreting adenomas (Jaquet et al. 1999).

Importantly, we show that the chimeric somatostatin/dopamine agonist BIM-23A760 significantly inhibited [3H]thymidine uptake in ~60% of the NFPA treated. A significant inhibition only at the highest concentration tested (1 nM) was reached in 39% of the responsive tumors while, in the other cases, 1–10 pM BIM-23A760 was sufficient to induce a statistically significant inhibition of DNA synthesis. In agreement with the highest expression of D2R, this effect was mainly dependent on D2R activation since it was greatly reduced (although not completely abolished) by the D2R antagonist, l-sulpiride. Similar results were reported by Gruszka et al. (2006) which showed comparable antiproliferative effects in six out of ten NFPA cultures using either the D2R agonist bromocriptine, sstr1, sstr2, and sstr5 selective agonists or another dopamine/somatostatin chimeric compound, BIM-23A387, although the most effective was bromocriptine.

A crucial issue in the analysis of these results was the identification of possible molecular determinants of NFPA sensitivity to sstr/D2R chimeric agonist. To this end, we compared the biological characteristics of BIM-23A760 responsive versus non-responsive NFPA. However, no statistically significant differences were found between the two groups considering all the parameters tested (differentiation status defined by the percentage of cell null tumors, in vivo aggressive behavior including invasive versus enclosed tumors or tumor-dependent panhypopituitarism, etc).

In our study, the levels of expression of sstr was not correlated with the pharmacological responses to either octreotide, cabergoline, or BIM-23A760, while non-responsive NFPA expressed higher D2R mRNA level than the responsive ones (P < 0.02). Such an absence of correlation is in contrast with the significant relationship previously reported between the expression of sstr2 and the degree of suppression of GH secretion, observed in GH-secreting tumors (Jaquet et al. 2000, Hofland et al. 2004, Taboada et al. 2007) or D2R mRNA level and the response to dopamine analogs in
prolactinomas (Pellegrini et al. 1989). The lack of correlation between D2R mRNA expression and control of [3H]thymidine incorporation is surprising. Previous studies associated the D2Rshort isoform expression with a better response to long-term cabergoline treatment, in terms of inhibition of α-subunit secretion and tumor shrinkage (Renner et al. 1998, Pivonello et al. 2004). Indeed, from a biochemical point of view, the differences in the antiproliferative effects elicited by the two D2R isoforms are related to the capability to inhibit the ERK1/2 pathway, an effect that was exerted only by the short variant (Iaccarino et al. 2002, Van Ham et al. 2007). We analyzed 15 NFPAs for the expression of the two D2R variants. The D2Rlong isoform was largely predominant in all analyzed cases, as previously outlined (Renner et al. 1998, Pivonello et al. 2007), but again, no correlation between the D2Rshort isoform mRNA expression levels and sensitivity to BIM-23A760 was found (Table 3). In agreement with our in vitro data, in vivo observations looking for a predictive role of [123I]epidepride D2R imaging showed the absence of correlation between the receptor-binding levels and the long-term efficacy of dopamine agonist treatments in terms of tumor mass stabilization or shrinkage (de Herder et al. 2006). Consequently, we cannot provide an answer to the question why approximately one-third of the NFPAs studied are insensitive to the chimeric compound. However, this phenomenon, rather than to be dependent on BIM-23A760 characteristics, seems to be related to the biological features of individual NFPAs, since similar results were observed using the selective D2R and sstr agonists, cabergoline, and octreotide.

Our results did not show any statistically significant difference between the various treatments with regard to maximal inhibition, EC50 or percentage of responsive tumors. Again, such an absence of synergistic effects seems to be an event related to individual characteristics of the NFPAs analyzed. Indeed, while no synergism was recently observed in GH and prolactin-secreting rat pituitary cell lines (Gruszka et al. 2007), a different response was observed in human GH-secreting adenomas in which the simultaneous activation of sstr and D2R significantly enhanced their antisecretory and antiproliferative responses (Jaquet et al. 2005). Interestingly, a different sstr expression pattern was observed in tumors from acromegalic patients that show significantly higher sstr5 mRNA levels than NFPAs, which, instead, display higher levels of sstr3.

As far as the molecular mechanism of BIM-23A760 effects is concerned, it could differ according to cell types. In this respect, it is relevant to discuss the recent demonstration that a different pattern of sstr2–D2R heterodimerization occurs in cultured striatal neurons versus Chinese hamster ovary (CHO) or human embryonic kidney (HEK)293 cells transfected with both receptors (Baragli et al. 2007). In these neurons, naturally co-expressing sstr2 and D2R, constitutive sstr2–D2R heterodimers were detected and heterodimerization was further promoted only by dopamine treatment. Conversely, in CHO- or HEK293-transfected cells, dopamine and somatostatin agonists synergistically promoted receptor heterodimerization, which was absent in basal conditions. These observations suggest that the different pattern of receptor association is the constitutive key regulator of cell responses to multiple receptor ligands, like BIM-23A760. Thus, although it was proposed that heterodimerization of dopamine and sstrs may result in an increased biological response in some cellular environments (Rocheville et al. 2000), this event does not seem to participate in the antiproliferative activity of BIM-23A760 in NFPAs cell cultures. Thus, as far as NFPAs are concerned, the main interest of an sstr2/D2R molecule could lie in its ability to elicit responses in those tumors lacking or expressing very low levels of one of these receptors.

In conclusion, our short-term in vitro study shows that BIM-23A760 is able to inhibit [3H]thymidine incorporation in similar dose-related manner than octreotide and cabergoline. Despite the absence of synergism between the combination of both drugs or activating sstr and D2R using the somatostatin/dopamine chimeric molecule, BIM-23A760, the latter compound was able to inhibit cell proliferation in ~60% of the tumors in culture. A long-term clinical trial, as defined previously (Greenman et al. 2005), will be necessary to confirm the possible therapeutic utility of such a compound.

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References


