Modulation of expression of somatostatin receptor subtypes in Graves' ophthalmopathy orbits: relevance to novel analogues.

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Running Head: Somatostatin receptors & analogues in GO.

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**Abstract**

Apart from evaluating orbital inflammation in Graves’ ophthalmopathy (GO) somatostatin (SST) analogues have been proposed as a therapy but recent trials were disappointing. We aimed to measure somatostatin receptor (SSTR) expression in orbital tissues *ex vivo* and determine whether the new broad-affinity analogue SOM230 might be of therapeutic use.

Orbital adipose/connective tissues from 29 GO patients and 10 normal individuals were analysed. Transcripts were quantified using SYBR-green and a light cycler. *In vitro* models were used to investigate whether thyrotropin receptor activation (as occurs via thyroid stimulating antibodies) or adipogenesis affected SSTR expression in primary preadipocytes and to compare the biological activity of octreotide and SOM230 in their modulation.

The expression of SSTR1 was significantly higher in GO patients than normal controls (p=0.024). Although differences in the expression of SSTR2 were not significant, 39% of GO samples had levels above the 97th centile of the controls. SSTR3, 4 & 5 were at or below the limit of detection (LOD). The lymphocyte contribution was minimal, since CD3-α transcripts were at the LOD. TSHR activation did not modulate SSTR expression. An *in vitro* model of adipogenesis indicated upregulation of SSTR1 and SSTR2 during differentiation. SOM230 produced significantly greater inhibition of orbital preadipocyte proliferation than octreotide.

*Ex vivo* analysis of orbital tissues reveals upregulation of SSTR1 & 2 in a group of GO patients. Adipogenesis, a process occurring in GO orbits, provides one possible explanation for some of the observed increase.

Key Words: Graves’ ophthalmopathy; somatostatin receptors; somatostatin analogues; adipogenesis; glycosaminoglycans.
Introduction

Somatostatin (SST) analogues such as octreotide (OCT) have a wide application in clinical imaging. Somatostatin receptors (SSTR) are expressed in many tissues, including activated lymphocytes. Consequently OCT scintigraphy was proposed as a method to evaluate orbital inflammation in Graves' ophthalmopathy (GO), with the level of uptake correlating well with the clinical activity score (CAS) (7, 17).

Subsequently it was suggested that SST analogues could provide a treatment for GO, with several studies reporting an improvement in CAS (9, 11). However, in one of two recent double-blind, placebo-controlled trials no significant therapeutic effect of OCT long acting repeatable (LAR) was found in patients with moderate to severe GO (4). In contrast, a second study reported an improvement in CAS (although the clinical benefits were questioned) and eyelid fissure was reduced (21).

A further prospective randomized study (2) showed no difference in the CAS at 12 weeks between patients with active GO and controls treated with slow release Lantreotide at 12 weeks. Although the diplopia in downward gaze improved, this parameter has limited clinical relevance.

The biological effects of SST are mediated via 5 different G protein coupled receptors, SSTR 1-5, with OCT binding with high affinity to SSTRs 2 & 5 and moderate affinity to SSTR3. Two publications have addressed the expression of SSTR in GO orbits. Both have employed a semi-quantitative PCR protocol and have examined tissues following periods of in vitro culture. Normal and GO orbital fibroblasts (14), in common with normal dermal fibroblasts (6) were shown to express SSTRs 2 & 3, only GO orbital fibroblasts express SSTRs 1 & 5. Furthermore, pharmacological doses of OCT inhibited GO orbital fibroblast growth and forskolin-
induced cAMP production (14). Subsequently the same group reported moderate to strong expression of all 5 SSTRs in lymphocytes obtained from GO orbits, in contrast to the equivalent peripheral blood mononuclear cells, which express only SSTRs 2, 3 & 4 (15).

In recent years adipogenesis has been recognised as an important pathogenic mechanism in GO (3, 18). A recent study has demonstrated that SST production is induced and SSTR expression modulated in adipocytes exposed to inflammatory stimuli such as IL-1β (20). However, information regarding SST and SSTR expression during adipogenesis is lacking. Furthermore, SOM230, a synthetic SST analogue with high affinity across a wider range of SSTR subtypes (SSTR1, 2, 3 & 5), has recently been developed (12), making a systematic evaluation of SSTR expression in GO orbits timely.

**Materials & Methods**

**Patients Studied**

For the QPCR studies adipose/connective tissue samples were obtained from a total of 23 patients with GO. The surgical procedures were orbital decompressions (n=17) and blepharoplasty (n=6). These comprised 21 women and 2 men, with a mean age of 47.2 yrs (range 18-77). They were compared with a group of 8 individuals free of thyroid or other inflammatory eye disease who underwent orbital decompression (n=4) or blepharoplasty (n=4). The controls were 6 women and 2 men with a mean age of 53.1 yrs (range 28-86). The majority of patients were Caucasian except for 2 Asians in each group.

There were 12 non-smokers and 11 smokers in the Graves’ group and 4 non-smokers and 4 smokers in the control group.
All GO patients except for two had inactive GO (CAS below 2) as documented by the examining ophthalmologists (24). All GO patients had received systemic steroid treatment at some stage during their disease.

All samples were obtained with informed consent. The study was performed with appropriate Ethics Committee approval and was conducted in accordance with the principles of ‘The Declaration of Helsinki’.

**RNA extraction, DNase treatment, Reverse Transcription**

Orbital adipose/connective tissues were snap-frozen and stored at -80°C until use. They were pulverized under liquid nitrogen and the RNA extracted using Trizol®, according to the manufacturer's instructions. Following quantification, either 1 µg total RNA or 8 µl (when RNA concentrations were measurable but below 125ng/µl) were treated with DNase (1U; Promega) in a total volume of 10µl. Subsequently, depending on the concentration of the input RNA 1 (>200ng/ul) or 2 µl (<200ng/ul) were reverse transcribed, in a total volume of 20µl, with MMLV reverse transcriptase (0.1U; Promega) using standard protocols (19).

Depending on the amount of RNA extracted, 3 - 6 reverse transcription reactions were performed for each sample; care was taken that individual Q-PCR experiments (please see below) always used cDNA generated in the same batch.

**Preparation of plasmid standards; optimization of standard curves & Q-PCR**

The five SSTR are intronless genes. Consequently we were able to amplify fragments of each subtype from genomic DNA, in a PCR reaction, using standard protocols (19). All primers (designed with Primer 3 software) used are shown in table 1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>SSTR1</td>
<td>TGAGTCAGCTGTCGGTCATC</td>
<td>GCGGTGGCGTAATAGTAAC</td>
</tr>
<tr>
<td>SSTR2</td>
<td>GGCCAATCTTCCCTTTTTCC</td>
<td>TGGTTTGAGGTGTGGTGA</td>
</tr>
<tr>
<td>SSTR3</td>
<td>CTGGGTAACCTCGTGTTGTCAT</td>
<td>CAGGCAGAATATGCTGGTGA</td>
</tr>
<tr>
<td>SSTR4</td>
<td>ACCAGCGTCTTCTGCTCTCA</td>
<td>ACACGCCCAGGTGATGAG</td>
</tr>
<tr>
<td>SSTR5</td>
<td>TCACTGTGCTGTGCTACCTG</td>
<td>GGAGAGGATGACCACGAAGA</td>
</tr>
<tr>
<td>CD3α</td>
<td>CGCCTTCAACAACAGCATTA</td>
<td>GATTAAACCGGGCCACTTTC</td>
</tr>
<tr>
<td>APRT</td>
<td>GCTGCCTGCTCATCCGAAGG</td>
<td>CCTTAAGCGAGTCAGCTCC</td>
</tr>
</tbody>
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**Table 1.** Primers for Somatostatin receptor subtypes; α chain lymphocyte marker (CD3α) and adenosine phosphoribosyl transferase housekeeper gene (APRT).

The PCR fragments were subcloned into pGEM-T according to the manufacturer's instructions and the plasmid identity was confirmed by sequencing. Q-PCR was conducted using SYBR Green incorporation measured on a Stratagene MX 3000. Each reaction comprised 1µl plasmid standard containing 10^6 to 10^2 copies, in a total volume of 25µl. Initially the optimal primer combination, to generate a homogeneous amplification peak in the absence of primer dimers, was determined for each subtype.

Q-PCR measurement of SSTR transcripts in the samples used 1µl cDNA in the reaction. Comparison with standard curves for each subtype (included in each experiment) permitted calculation of absolute values for each sample (transcripts/µg input RNA). In addition, transcripts for a housekeeping gene, APRT, were measured (as previously described) so that values could be expressed relative to this (transcripts/APRT). In a single Q-PCR experiment all measurements were made in triplicate; each subtype in all samples was evaluated on at least two different batches of cDNA. The standard curve was also run in at least duplicate in each reaction.

To assess the contribution of lymphocytes to the SSTR transcript measurements, Q-PCR for the T cell receptor constant region (CD3-α) was undertaken on all 31 samples analysed ex vivo.
Preadipocyte culture, adipogenesis protocol, evaluation of proliferation

Orbital preadipocytes (<5 passages) were obtained from adipose tissue explants from an additional six patients with GO; 1 M, 5 F with a mean age of 52.1 yrs (range 40-61) and two women (aged 52 and 70) free of inflammatory eye disease. They were cultured in 6cm dishes in DMEM/F12 10% FCS (complete medium). Adipogenesis was induced in confluent cells by replacing with medium having reduced FCS and containing a range of hormones and PPARγ agonists (differentiation medium), for 10-12 days, as previously described (22). To determine whether adipogenesis had an effect on SSTR expression, RNA was extracted at various time points during the differentiation protocol, for measurement of SSTR transcripts as described above. Total protein lysates were extracted at the same times, in Laemmli buffer containing 1mM sodium orthovanadate and 1mM PMSF. Samples were separated by SDS-PAGE and the gels then electroblotted onto PVDF membranes. The blots were probed with the following goat polyclonal antibodies (Santa Cruz):- anti SSTR1, (1:500 overnight; 4°C) anti actin, (1:1000, overnight; 4°C) followed by an anti-goat IgG-HRP conjugate (1:5000, room temperature for 1 hour Amersham Biosciences) and visualised by enhanced chemiluminescence (ECL Plus, Amersham Biosciences). Films were analysed using the Alpha Imager 1200 digital imaging system (Alpha Innotech Corp, San Leandro, CA). The blots were initially probed with the SSTR1 antibody, they were then stripped and reprobed with antibodies that recognise actin.

Preadipocytes were cultured in 12 well plates to investigate the effects of varying concentrations of OCT and SOM230 on proliferation and adipogenesis (spontaneous and induced). Proliferation was assessed by direct cell counting of preadipocytes, 2 and 5 days after plating in 12 well plates, in complete medium, using a Coulter particle counter. Results are expressed as a percentage of the mean of time fold
increase \([(\text{Count d5}-\text{Count d2})/\text{Count d2}]\) of the cells in the absence of any analogue.

At each time point an aliquot of cells was stained with an equal volume of 0.1% trypan blue and the percentage of blue and white cells counted using a haemocytometer.

Microscopic examination provided a means of determining whether morphological changes which accompany adipogenesis, e.g. rounding-up of cells and/or acquisition of lipid filled droplets, had occurred. The degree of adipogenesis was also evaluated by measuring transcripts for the adipocyte differentiation markers C/EBPβ, PPARγ, and LPL as previously described (25). Briefly the various cell populations were plated in 6 well plates in complete or differentiation medium, supplemented or not with analogue. Fourteen days later, RNA was extracted, reverse transcribed and transcript copy numbers measured using SYBR Green and a Stratagene MX3000 light cycler. Standard curves (the PCR amplicon subcloned into pGEM-T at \(10^6\) to \(10^2\) copies) were included for each gene and results are expressed as an absolute value, either per microgram of input mRNA or relative to the housekeeping gene APRT.

A possible effect of TSHR activation on SSTR expression was examined. Activating mutant TSHR, L629F and M453T, and the WT TSHR were introduced into the orbital preadipocytes (2 of the GO and 1 normal) using retroviral vectors, previously produced in our laboratory (5). Geneticin selection resulted in mixed populations stably expressing the various TSHR, which exhibit increased basal levels of cAMP compared with the equivalent non-modified cell population, all as previously described (25). RNA was extracted from the non-modified and mutant TSHR expressing cells for measurement of SSTR transcripts.
Statistics

The Mann-Whitney Rank Sum Test was used for assessing non-parametric data (Q-PCR measurements) and Student’s t test for parametric data (differentiation effects, evaluation of adipogenesis). The effects of SST analogues on proliferation were compared using one way ANOVA and Tukey’s post hoc test (SPSS version 10.0 for Windows, SPSS, Inc., Chicago, IL). All results are expressed as the mean ± SEM unless otherwise stated. A p-value of less than 0.05 was considered significant.

Results

*SSTR1 is over expressed in all & SSTR2 increased in a proportion of GO orbits*

SSTR1 was detected and quantified in all samples; 144.6 transcripts/µg input RNA [29.4-976] in GO orbits (n=23) and 79.9 transcripts/µg input RNA [11.9-130] in normal controls (n=8) (median and [range]). The difference was statistically significant (p=0.024) (figure 1A).

SSTR2 was detected in all samples and was measurable in GO (n=13), 2.3 transcripts/µg input RNA [0.1-520.8] and also in normal controls (n=4), 0.2 transcripts/µg input RNA [0.1-1.9]. We assigned an arbitrary value of 0.1 transcripts/µg input RNA to samples at the LOD to facilitate statistical comparisons. The difference was not statistically significant (p=0.092) however 39% of the GO samples had levels above the 97th centile of normals. Results are shown in figure 1A.

Similar results (i.e. a significant p=0.009 difference in the level of SSTR1 expression between GO and controls and 26% of GO above the 97th centile for SSTR2) were obtained when comparing absolute transcript values of individual SSTR relative to the APRT housekeeper (figure 1B).
Plotting the expression of SSTR1 against SSTR2 results showed that the expression of SSTR1 in GO samples and controls does not parallel the expression of SSTR2 (data not shown). There was no statistically significant difference in the expression of SSTR1 and 2 between smokers and non-smokers.

SSTR3, 4 & 5 are at the limit of detection

Transcripts for SSTR 3, 4 and 5 were undetectable in the majority of samples and even when they were present, were at the limit of detection. SSTR3 was detected in 10/23 GO and 6/8 normal orbital samples; SSTR4 in 2/23 and 0/9 and SSTR5 in 3/23 and 0/9 GO and normal orbital samples respectively.

Are SSTR transcripts derived from infiltrating lymphocytes or resident orbital cells?

Since lymphocytes have been reported to express SSTR (15), and as lymphocytic infiltration is a feature of GO (13), we measured transcripts for the T cell receptor constant region CD3-α to assess the lymphocyte contribution to SSTR expression. The lymphocyte contribution was minimal, since transcripts for CD3-α were at the limit of detection (LOD) in all 31 orbital samples tested ex vivo. This contrasted with Jurkatt lymphocyte cDNA, used as a positive control, in which >10^4 transcripts/µg input RNA were measured (data not shown).

Does TSHR activation influence SSTR expression?

Having established that the SSTR were expressed by orbital cells, we sought an explanation for the increased levels of SSTR1 and SSTR2 in some GO samples. The most severe GO correlates with Graves' patients having the highest titres of thyroid stimulating antibodies (8, 1). We have previously reported that a small proportion of
fibroblast-like cells in GO orbits express the TSHR (3). Thus it seemed reasonable to investigate the effects of TSHR activation. We have used a previously described in vitro model in which TSHR activation is achieved by stable incorporation of gain of function mutant TSHR (25). Examination of preadipocytes from two GO and one normal patient sample in this protocol revealed no consistent or significant change in the expression of any of the 5 SSTR subtypes.

*Does adipogenesis provide an explanation for the increased expression of SSTR1 &2?*

We and others have reported an increase in adipogenesis in GO orbits (23, 10). We thus investigated whether this process affects SSTR expression. As shown in figure 2A, transcripts for SSTR1 and SSTR2 are increased in the process. The experiment was repeated, with or without DNAse treatment, with five different populations of GO preadipocytes and one normal control, all with similar results. We were also able to demonstrate that the transcript levels for SSTR1 reflect those of the protein, in a parallel western blot performed on samples taken at the same time points, as shown in figure 2B. Transcripts for SSTR 3, 4 & 5 were all at or below the limit of detection and were unchanged by adipogenesis.

*SOM 230 produces significantly greater inhibition of orbital pre-adipocyte proliferation than Octreotide.*

We compared the effects of SOM 230 and OCT on orbital preadipocyte proliferation in vitro. As shown in figure 3, neither SOM230 nor OCT at 10^{-8}M had any effect on proliferation. The effect of analogue at 10^{-6} was significant (p ≤ 0.001) for proliferation measured by Coulter counting on day 2 and 5 compared to control: SOM vs control p ≤ 0.001, OCT vs control, p=0.019 and the two analogues differed
significantly from one another: SOM vs OCT, \( p=0.017 \). Proliferation inhibition by analogue at \( 10^{-7} \) concentration was significant (\( p \leq 0.001 \)). The individual effects were SOM vs control \( p \leq 0.001 \), OCT vs control, \( p \) not significant and the two analogues differed significantly from one another: SOM vs OCT, \( p \leq 0.001 \).

In neither case was the reduced proliferation due to increased cell death, since the percentage of trypan blue stained cells was <3% in all culture conditions.

*Do somatostatin analogues have any effect on adipogenesis?*

In complete medium, even when the cells had been confluent for up to 14 days, there was no obvious difference in the morphology of cells (e.g. rounding up and accumulation of lipid filled droplets which would be consistent with adipogenesis) in the presence and absence of \( 10^{-6} \) or \( 10^{-7} \) SOM230 or OCT indicating that neither analogue induced spontaneous adipogenesis.

PPAR\( \gamma \) agonist induced differentiation of the cells produced the expected change in morphology. Similar signs of adipogenesis were present in cells incubated in differentiation medium supplemented with SOM230 or OCT, although the process appeared to be delayed, with foci being smaller and containing fewer lipid droplets.

To investigate further, we measured transcripts of gene markers of early (CEBP/\( \beta \)), mid (PPAR\( \gamma \)) and late (LPL) adipogenesis. Since the RNA was collected on day 14, results for the late marker are presented (CEBP/\( \beta \) and PPAR\( \gamma \) transcript numbers were unaffected by analogue). In complete medium transcripts for LPL were undetectable and increased to \( 2.0 \times 10^5 \) \( [1.18] \) copies /\( \mu \)g input RNA after 14 days in differentiation medium. In the presence of \( 10^{-6} \) SOM230 or OCT the increase was slightly, but not significantly reduced, reaching levels of \( 1.6 \times 10^5 \) \( [0.99] \) and \( 1.6 \times 10^5 \) \( [1.06] \) copies /\( \mu \)g input RNA respectively. The analogues at a concentration of \( 10^{-7} \) M did not
reduce LPL transcript copy numbers induced by adipogenesis. Results are expressed as the mean [S.D.] of 5 independent experiments performed on preadipocytes from 1 normal individual and 4 GO patients.

**Discussion**

To our knowledge, this is the first report of SSTR expression in GO orbits analysed *ex vivo* and compared with tissue from normal controls. We find an upregulation in SSTR1 in the GO group as a whole and in SSTR2 in a proportion of the patients. Our observations of SSTR1 overexpression in GO orbits are consistent with, and extend those of Pasquali *et al* who found expression of SSTR 1 in 5 of 10 GO primary orbital fibroblast cultures compared with none of 10 controls (14). Our findings of measurable SSTR 2 expression in all samples examined are also similar. Pasquali and colleagues were also able to demonstrate expression of SSTR 3 and 5 in a significant number of samples (50 and 80% of GO samples respectively). This contrasts with our observations where transcripts for the SSTR 3, 4 and 5 were at or below the LOD. However, since all of the receptors have a single exon, we were obliged to treat our samples with DNase to avoid quantifying copies amplified from genomic DNA. Thus we may have missed differences in the expression levels of SSTR 3, 4 and 5. Since we did not detect transcripts for CD3-α, which we have used as a marker of lymphocytic infiltration in the ex vivo samples, we assume that the SSTRs are expressed on the orbital preadipocyte/fibroblast population. The absence of lymphocytes may be expected in orbital tissue taken from individuals with inactive disease (as in 21/23 in the present study) and is probably the result of previous steroid therapy for immunosuppression.
Our samples were derived from within the orbit and also from the eyelid, but no differences were found in the expression levels. Similarly when analyzing the data, taking into account the smoking status of the donor, no differences were apparent. Thus smoking does not seem to modify the expression of SSTR.

Application of *in vitro* models to investigate the effects of TSHR activation and adipogenesis on SSTR expression levels produced opposing results. We did not find a consistent or significant change in the level of SSTR 1 and 2 expression in non-modified orbital preadipocytes when compared with the same population expressing an activating mutant TSHR, thus we can eliminate a role for thyroid stimulating antibodies (which also result in TSHR activation) in this aspect of orbital biology. In contrast, we observed upregulation of SSTR1 and 2 during differentiation to mature adipocytes, suggesting that adipogenesis provides one explanation for the increased expression measured in the GO samples *ex vivo*. Furthermore, we were able to demonstrate that the transcript and protein levels of SSTR1 correlate and our subsequent proliferation studies using somatostatin analogues validate the functional relevance of these receptors.

We have previously reported (23) increased adipogenesis in GO orbits (compared with normal) in samples obtained from patients with inactive disease, (as in the current study) and this has been confirmed by others (10). Our findings suggest that even in apparently inactive disease, as defined by CAS, pathological mechanisms may still be in operation. The expression of SSTR1 did not correlate with that of SSTR2 in either GO patients or controls analysed *ex vivo*. Since both receptor subtypes are upregulated during adipogenesis, this indicates that additional mechanisms must be contributing to the increased expression, including the possibility that a fundamental difference exists in SSTR expression between GO preadipocytes and controls.
We then determined whether established and novel somatostatin analogues exerted an effect on the biological activity of the orbital preadipocyte/fibroblasts. As expected, the effects of OCT and SOM230 were very similar, with both inhibiting proliferation, although SOM230 had a significantly greater impact on proliferation than OCT. Our attempts to define whether the reduced proliferation was caused by increased apoptosis or a block/prolongation of the cell cycle were not successful due to the combination of slow growth, large size of cells and limited availability. OCT has previously been shown to induce apoptosis in GO fibroblasts, accompanied by reduced Bcl-2 expression (15). Preliminary reports suggest that these findings are also replicable in GO fibroblasts treated with SOM230 (16).

In conclusion, we have examined expression of SSTR subtypes in GO orbital tissues ex vivo and find evidence of upregulation of SSTR 1 for which the novel somatostatin analogue SOM230 has high affinity. Initial experiments indicate that SOM230 may be beneficial for GO, by reducing preadipocyte proliferation. These observations could thus form the basis for a re-evaluation of the role of newer somatostatin analogues in the management of patients with Graves’ Ophthalmopathy.

Acknowledgements

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Disclosures
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References


Figure 1. Box and whisker plots of SSTR1 and 2 expression in orbital fat/connective tissue samples from GO and normal controls. A. The results are expressed as transcript/µg input RNA. B. The results are expressed as transcript/APRT (adenosinephosphoribosyltransferase) housekeeper gene. One outlier (GO sample) has been omitted (values for SSTR1 = 976/µg input RNA and SSTR2 = 520/µg input RNA).

A.
B.

![Box plots for SSTR 1 and SSTR 2](image)

- **SSTR 1**
  - **Graves'**
  - **Controls**

- **SSTR 2**
  - **Graves'**
  - **Controls**
**Figure 2.** Expression of SSTR1 and SSTR2 during adipogenesis. A. Transcript copy numbers; Data are presented as mean and SEM. Preadipocytes were plated in 6-cm dishes and differentiation medium was added at confluence. RNA was extracted at the indicated time points (D4=day 4 etc). The experiment was repeated five times with similar results (preadipocytes from four GO and one normal control). B. Western blot analysis performed at the same time points; the upper panel is SSTR1 (Mr ~60kD) and the lower panel actin (Mr ~40 kD).
Figure 3. Effects of SOM230 and OCT on orbital preadipocyte proliferation. Cells were counted at days 2 and 5 after plating $5 \times 10^4$ cells/well. Results are expressed as a percentage of the mean of time fold increase $[(\text{Count d5} - \text{Count d2})/\text{Count d2}]$ of the cells in the absence of any analogue. The mean numbers are the results of triplicates (which agree within 2%) for one representative of four independent experiments.