AD_____

AWARD NUMBER DAMD17-97-1-7307

TITLE: Signaling Components of the Anti-Tumor Hormone Somatostatin in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: John J. Tentler, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80262

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-97-1-7307
Organization: University of Colorado Health Sciences Center
Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

8/4/99

_____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (22 Sep 97 - 21 Sep 98)

4. TITLE AND SUBTITLE
Signaling Components of the Anti-Tumor Hormone Somatostatin in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-97-1-7307

6. AUTHOR(S)
John J. Tentler, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80262

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Oct 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The neuropeptide, somatostatin is an important regulatory hormone that is widely distributed throughout the body. Somatostatin's actions are primarily inhibitory, and recently, it has been utilized as an antiproliferative agent against several tumor types, including breast neoplasms. Experimentally and clinically, somatostatin can inhibit breast cancer cell growth, possibly by inhibiting the secretion of growth factors, or by acting directly on the cells themselves to induce programmed cell death, or apoptosis. Despite increased clinical use, the mechanism(s) by which somatostatin acts to control breast cancer cell growth remain largely unknown. In this progress report, I describe studies performed to survey of numerous breast cancer cell lines to predict their usefulness as models to study somatostatin action, which comprised Aim 1 of the proposal. One cell line, MDA231, shows a robust response to somatostatin with regard to growth factor stimulation of the mitogen activated protein kinase, ERK1/2 (or MAPK). Furthermore, I show preliminary data indicating that another signaling pathway, the jun n-terminal kinase (JNK) pathway may be activated in response to somatostatin. (Aim 2). Together, these results begin to explain somatostatin's ability to inhibit cell growth and induce apoptosis in breast cancer. Furthermore, identification of these target pathways defines functional assays by which the efficacy of future anti-cancer drugs can be tested.

14. SUBJECT TERMS
Breast Cancer
Somatostatin, somatostatin receptors, anti-proliferative agents, signal transduction, MAP kinase, c-Jun n-terminal kinase

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

John Tuttle 10/21/98

PI - Signature Date

Table of Contents

Introduction

The neuropeptide, somatostatin is an important regulatory hormone that is widely distributed throughout the body. For years it was recognized as the main negative regulator of growth hormone secretion from the anterior pituitary gland. Recently, however, it has become apparent that somatostatin can act at many tissues and organs, and in addition to its role as an inhibitor of hormone secretion, it is also capable of inhibiting cell growth and proliferation (1,2). As such, somatostatin is currently being investigated for its potential clinical use as an anti-cancer agent, as inhibition of cell growth is an important feature of most anti-cancer therapies. Indeed, stable somatostatin analogs, such as BIM23014 (Lanreotide) and SMS201-995 (Octreotide), are capable of inhibiting the growth of several types of tumors including pituitary adenomas, neuroblastomas, pancreatic cancer and breast cancer (2). At the cellular level, the actions of somatostatin are mediated by a family of G protein-coupled plasma membrane receptors termed ssts. To date, five distinct sst receptor subtypes have been identified, and these are named sst1-5 (3). Subtype 2 (sst2) is the most common somatostatin receptor found on tumor tissue, followed by sst5 (1, 4).

There is currently little information available regarding somatostatin action, especially as it pertains to breast neoplasms, and thus the objective of these studies is to define the functional role of somatostatin in several breast cancer cell lines as it impinges on growth factor-stimulated signaling pathways. Growth factors, such as EGF, FGF, and IGF-1, have been implicated in breast cancer tumorigenesis and progression (5), and therefore I plan to determine if these pathways are affected by somatostatin in a somatostatin-responsive breast cancer cell line. My underlying hypothesis is that somatostatin exerts its antiproliferative effects on a wide variety of tumors by interfering with multiple signaling pathways which lead to cell division.

Thus, my initial goals were to 1) develop a somatostatin-responsive breast cancer cell model system, 2) to determine whether somatostatin inhibits growth factor- or UV light-stimulated Raf kinase, MAP kinase, Jun kinase, Elk and Jun biochemical activities, and 3) to determine the functional consequences of somatostatin action on these same components by determining whether somatostatin inhibits growth factor- or UV light-stimulated Elk-, Jun-, and Ets-mediated transcription.

Elucidation of the mechanisms involved in somatostatin action will not only further our understanding of the regulation of cell growth, and hence tumorigenesis, but may also lead to improved drug design for the treatment of breast cancer.

Body

Aim 1:

Somatostatin receptors are present in very low numbers on most cell types, making analysis of signaling events difficult to measure by current techniques. To overcome this limitation, I initially proposed an approach that has been used by several investigators to elucidate signaling pathways from numerous types of receptors. The strategy was to make stable breast cancer cell lines that would overexpress sst2 and thereby amplify signaling events of this receptor in a physiologically-relevant cell type. I chose to overexpress sst2 as it is believed to be the most prevalent subtype expressed in breast cancer tumors, and it is also the primary target of most of the commercially-available somatostatin analogs. Although I still believe this is a valid approach that could yield valuable information, there are potential problems with such a model system that have been brought to my attention, and furthermore, I have encountered resistance to this approach from reviewers, including one of the reviewers of this proposal, who listed it as one of its weaknesses. For example, there is the possibility that expressing a receptor to higher levels than normally found in the cell could lead to erroneous and nonphysiologically-relevant effects. Additionally, the assumption that sst2 is the only relevant subtype in mediating somatostatin's inhibition of cell proliferation may be erroneous.

For these reasons, I decided to pursue the approach suggested by the reviewers, to identify a breast cancer cell line that shows a robust response to somatostatin via its endogenous somatostatin receptors. Thus far, this is the only major change in the direction of the proposed studies.

The first step towards identifying a somatostatin-responsive cell line was to identify which somatostatin receptor subtypes were expressed in several well-defined breast cancer cell lines, and to what degree. Several cell lines were obtained from the University of Colorado Cell Culture Core Facility, that differed in estrogen receptor (ER) and EGF receptor status (Table 1, Appendix). The literature contains few studies that have assessed sst expression in breast cancer cell lines, not all of the cell lines we have chosen have been examined, and furthermore, variations may exist in the "same" cell lines due to clonal differences. Therefore, I felt the best approach was to perform an analysis of sst expression in these cell lines myself. A reverse transcriptase-polymerase chain reaction (RT-PCR) approach was chosen because, as mentioned above, ssts are present in very low abundance and they cannot be detected by Northern blot or RNAse protection assays. Total RNA prepared from four cell lines was reverse transcribed to cDNA and then amplified by PCR with oligonucleotides specific for each of the five sst subtypes. The quantity of PCR product for each reaction was normalized to an internal control (GAPDH) in an identical reaction mix. The results of the RT-PCR study are depicted in Table 1 (Appendix). The results indicate that each of the four cell lines examined displayed a relatively unique expression pattern of ssts, and some cell lines express certain receptors to a higher degree than others. The information gained from this study is important for two reasons. First, the particular

subtypes expressed in each cell line will aid in the choice of ligands to use, as subtype-selective somatostatin agonists are now becoming available. Second, although RT-PCR is not strictly quantitative, one can gain some information on the level of expression of a particular sst, and thus predict the likelihood of a robust response to somatostatin. For example, the MDA 231 cell line expressed a relatively high amount of sst2 and sst5, and therefore may provide a strong response to somatostatin analogs that target these receptors. Likewise, the ZR 75.1 is likely to be a poor model system to study endogenous sst action, as it did not express any of the ssts to a high degree, yet this cell line may prove an ideal model to study stably transfected sst's.

AIM 2:

The next goal was to test the cell lines for their ability to mediate somatostatin inhibition of growth-factor regulated signaling pathways (Aim 2). While there are several candidate pathways to examine, we initially focused on the p44/42 MAP kinase (MAPK) pathway. MAPK plays a central role in cell proliferation, and I have shown that this pathway is inhibited by sst2 in pituitary tumor cells (6). Additionally, recent reports have implicated the importance of the MAPK pathway in breast tumorigenesis and it has been shown to be deregulated and MAPK is hyperexpressed in breast carcinomas (7,8). Although the RT-PCR study aided in the choice of cell lines to use for these experiments, I found that expression of somatostatin receptors was not necessarily indicative of a strong response to somatostatin. For example, both the MCF7 and T47D lines showed strong expression of sst2. However when assays were performed on these cell lines, the response to BIM23014, an sst2-selective agonist, very minimal responses were noted. Subsequently, several other cell lines were tested with similar negative results with regards to both MAPK assays and proliferation assays (data not shown). This aspect of the study proved to be very frustrating. However, I continued to test all of the cell lines available to me and fortunately, experiments performed on the MDA 231 cell line successfully demonstrated that the somatostatin analog BIM23014 could completely inhibit EGF-stimulated MAPK phosphorylation (Fig. 1, Appendix).

Figure 1 shows the results of a Western blot analysis of MDA 231 whole cell extracts after various treatments using an antibody that is specific for the active, phosphorylated form of MAPK (phospho-MAPK). Lane 1 is a positive control phosphorylated p44 MAPK protein purchased from New England Biolabs. Lane 2 is a negative control p44 MAPK protein that is not phosphorylated. As expected, the phospho-specific antibody detected the phosphorylated MAPK but did not cross react with a non-phosphorylated MAPK. Lane 3 is extract from MDA 231 cells that were cultured in DMEM plus 10% fetal calf serum (FCS). It is interesting to note that these cells show a very high level of phospho-MAPK. I have observed that this cell line grows very rapidly in culture, and it was derived from an aggressive tumor, therefore, there may be a correlation between it's rapid growth characteristics and the degree of phosphorylation of MAPK (7,8). Since the level of phospho-MAPK is so high in cycling MDA 231cells in complete media, the remaining samples (lanes 4-12) represent MDA 231 cells that were grown in media devoid of FCS. This strategy is commonly employed to increase the signal to noise

ratio, and thus make it easier to detect changes in phospho-MAPK levels in response to somatostatin. Lane 4 represents cells that are cultured in media without FCS. As expected, cells cultured under these conditions have very low levels of phospho-MAPK that are undetectable with the phospho-specific MAPK antibody. However, addition of 25 nM EGF for 10 minutes leads to an approximately 20-fold increase in MAPK phosphorylation (Fig. 1, lanes 5 and 6) when compared to quiescent cells (lane 4). Since MDA 231 cells showed a strong sst5 signal by RT-PCR (Table 1), I used the somatostatin 28 (SRIF 28), which targets this receptor subtype, in lanes 7-9. In lane 7, SRIF 28 alone was added, and this had no further effects on basal phospho-MAPK levels. When SRIF 28 was added 5 minutes prior to EGF, it had no effect on MAPK phosphorylation when compared to EGF alone (lanes 5 and 6). MDA 231 cells also express sst2, and therefore I used the sst2-selective agonist, BIM23014 in lanes 10-12. Again, BIM alone failed to repress steady-state phospho-MAPK (lane 7). However, in contrast to SRIF 28, BIM23014 almost completely abolished the ability of EGF to stimulate MAPK phosphorylation. Therefore, it appears that only sst2 in these cells is coupled to the inhibition of MAPK phosphorylation (lanes 11,12).

AIM 3:

Finally, I performed experiments designed to determine if BIM23014 activates the Jun n-terminal kinase (JNK) pathway. Previously, our lab and others have shown that somatostatin can induce apoptosis in breast cancer cells (9). The JNK pathway is one of several potential routes in the apoptotic process, and in conjunction with an inhibition of MAPK provides an attractive model for somatostatin's inhibition of cell growth. In this experiment, T47D breast cancer cells were treated with and subjected to Western blot analysis and probed with an antibody specific for phospho-JNK. The results are shown in Fig. 2. A time course treatment shows a modest (approximately 2-fold) and transient increase in JNK phosphorylation, peaking at 5 minutes (Fig 2, lane 3), and then rapidly diminishing to control levels by 15 minutes. It is unclear at this point whether there is any significance to such a modest and rapid activation of JNK. Further experiments will be needed to determine whether this effect is meaningful. Since the MDA 231 cells are very somatostatin responsive, I plan to do similar experiments in these cells to see if there is a more robust and prolonged JNK activation, which in other cell types is the typical pattern.

Conclusions

In this report, I describe the progress made thus far on the elucidation of the signaling components mediating the anti-proliferative effects of somatostatin and its analogs in breast cancer cells. In an attempt to find an appropriate model system, my first goal was to define the somatostatin receptors (ssts) that are expressed on various breast cancer cell lines. Using an RT-PCR approach, I have determined expression levels of the all five ssts, in four cell lines and the results are compiled in Table 1. For some of these cell lines, this is the first description of somatostatin receptors in these cells. Despite apparently high expression of certain ssts, I found that several breast cancer cell lines are still unresponsive with respect to the growth regulatory pathways that I have proposed to investigate, specifically, the MAPK pathway. However, I am very excited with the recent progress of this project, and the development of a somatostatin-responsive cell system (Aim 1). This was a major step in completing the remaining aims of this grant proposal. This appears to have been accomplished with the finding that the MAPK pathway is dramatically inhibited by the somatostatin analog BIM23014 in MDA 231 cells (Fig. 1). I have shown that in these cells, activation of sst2, but not sst5, leads to inhibitory signals that block the EGF stimulation of the MAPK pathway. Since EGF and the MAPK pathway have been shown to be critical in breast cancer tumorigenesis, these findings offer significant insights into the antiproliferative actions of somatostatin. Additionally, some preliminary data in T47D cells indicates a possible role of the JNK pathway in the control of cell proliferation as well. These studies need to be repeated, perhaps in the more responsive MDA231 cells to assess the significance of this activation. Further studies will be performed shortly that will attempt to identify the precise point(s) in the MAPK pathway where sst2 signals act (Aim 2). I will be investigating the potential role of phosphatases, including MAPK phosphatase in somatostatin's inhibition of MAPK. Recently, our laboratory has acquired an antibody directed towards the activated EGF receptor, which I will use to determine if somatostatin acts directly at this receptor by dephosphorylating it. Next, I will assess the significance of the inhibition of this pathway by performing cell proliferation assays and measuring the effects of somatostatin on downstream target promoters known to regulated by MAPK (Aim 3). Finally, I also plan to assess the effects of somatostatin on various cell cycle regulating factors, which may be critical effectors of the somatostatin inhibitory signal.

References

1. Lamberts SWJ, Krennig EP, Reubi J-C. The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocrine Reviews 1991; 12: 450-482.

2. Reubi J-C and Laissue JA. Multiple actions of somatostatin in neoplastic disease. Trends Pharm Sci 1995; 16:110-115.

3. Bell GI, Reisine T. Molecular biology of somatostatin receptors. Trends in Neurosciences 1993; 16:120-131.

4. Xu Y, Song J, Berelowitz M, Bruno JF. Estrogen regulates somatostatin receptor subtype 2 messenger ribonucleic acid expression in human breast cancer cells. Endocrinology 1996; 137:5634-5640.

5. McGuire WL, Dickson RB, Osborne CK, Salomon D. The role of growth factors in breast cancer. Breast Cancer Research and Treatment 1988, 12:159-166.

6. Tentler J, Gutierrez-Hartmann A. Signaling via the type 2A somatostatin receptor (sst2A) inhibits growth factor stimulated MAP kinase activity in GH4 pituitary cells. J Endocrinol Invest. 1997; 20:77.

7. Coutts AS, Murphy LC. Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res 1998; 58(18):4071-4.

8. Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest 1997; 99(7):1478-83.

9. Candi E, Melino G, DeLaurenzi V, Piacentini M, Guerrieri P, Spinedi A, Knight R. Tamoxifen and somatostatin affect tumours by inducing apoptosis. Cancer Letters 1995; 96:141-145.

Table 1

		Somatostatin Receptors				
Cell Line	**Background**	**sst1**	**sst2**	**sst3**	**sst4**	**sst5**
MCF-7	Human tumorigenic adenocarcinoma ER+, EGFR-	-	+ +	-	+ +	-
MDA 231	Human tumorigenic carcinoma ER-, EGFR+	-	+ +	-	-	+++
ZR 75.1	Human tumorigenic carcinoma ER+, EGFR-	-	+	-	-	-
T47D	Human tumorigenic carcinoma ER+, EGFR+	-	+	-	+ +	-

Table 1: Results of RT-PCR study of various breast cancer cell lines. ER= estrogen receptor, EGFR= estrogen receptor, sst1-5 =somatostatin receptor subtypes 1-5. - = no receptor detected. + =sst receptor positive.

Figure 1.

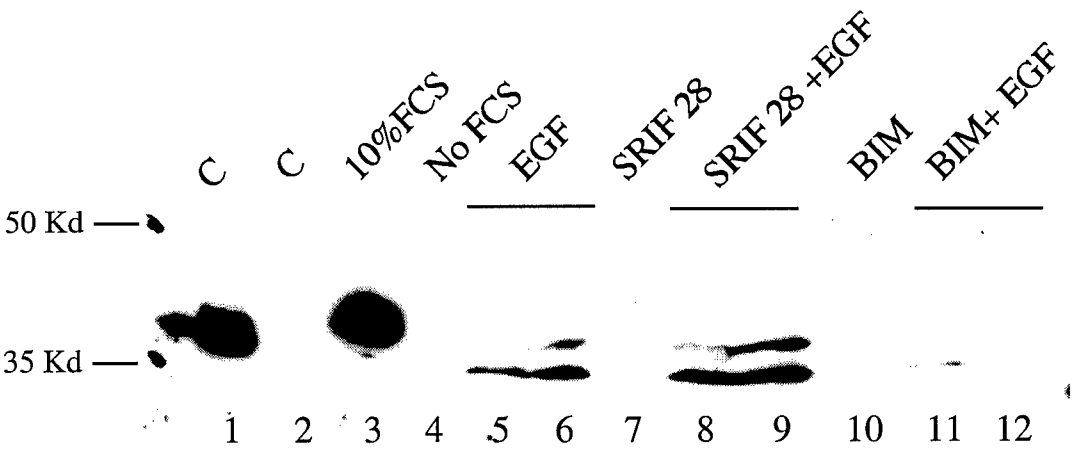

Figure 1: Western blot analysis of MDA231 breast cancer cell extracts treated as shown above and as described in Body text. Equal proteins amounts (50 μg) were separated on 12% SDS-PAGE, blotted onto Immobilon and probed with an antibody specific for the phosphorylated form of MAPK.

Figure 2.

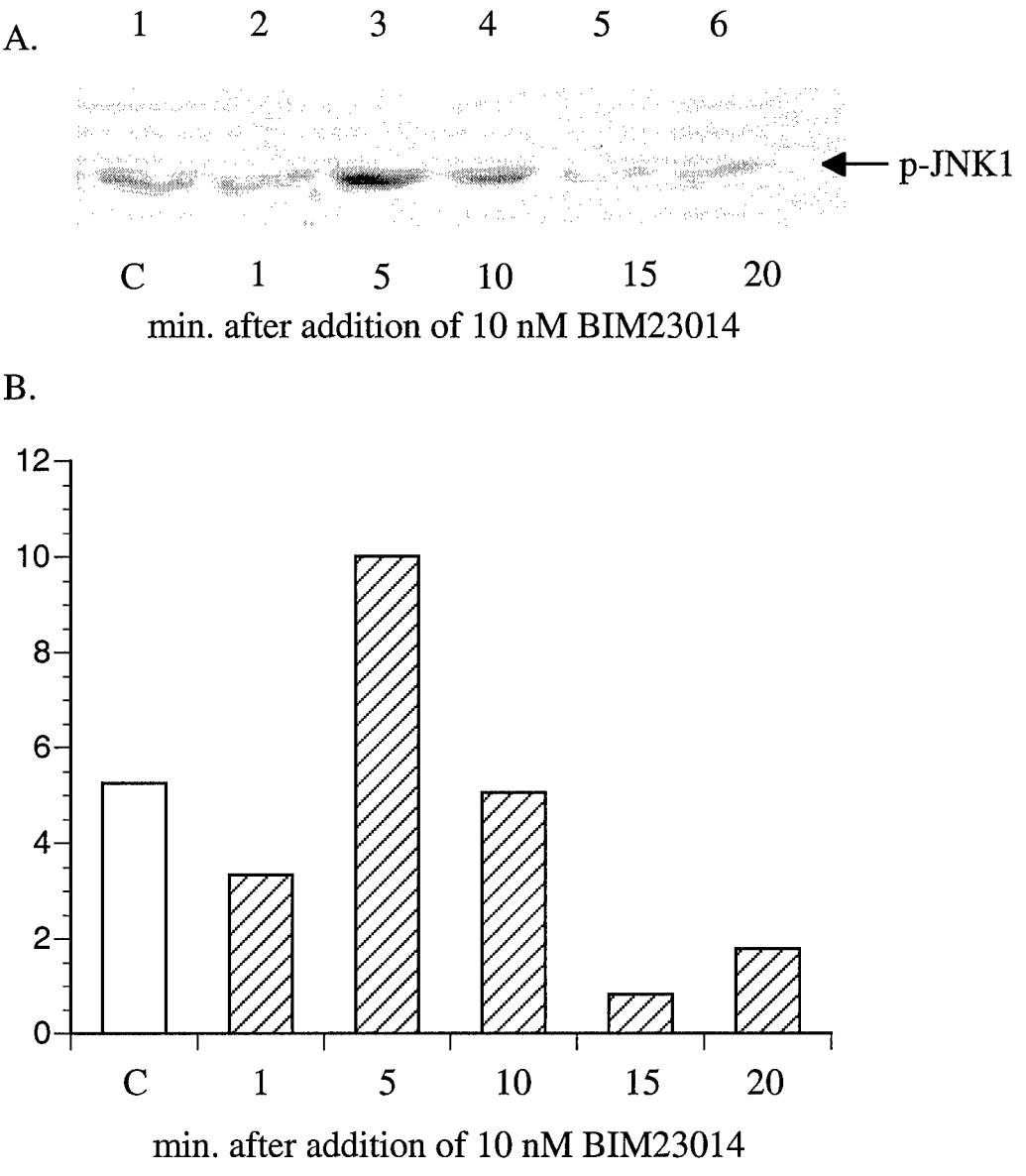

Figure 2. The somatostatin analog BIM23014 induces a transient increase in JNK phosphorylation. **A**. Western blot analysis of T47D breast cancer cells treated with vehicle (C) or 10nM BIM23014 for the indicated time points, and as described in the Body text. Extracts were prepared, and equal protein amounts (50 μg) were separated on a 12%SDS-PAGE, transferred to Immobilon and probed with anatibody specific for phosphorylated JNK. **B**. Densitometric analysis of the blot shown in **A**. Values are corrected for loading differences.