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Report

Expression of sigma 1 receptor in human breast cancer

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Summary

The sigma 1 receptor (S1R) represents a unique drug-binding site that is distinct from any other receptors. We examined S1R expression in human breast cancer and assessed the activity of S1R ligands in breast cancer cell lines. One-hundred nine breast specimens from normal breast, benign breast disease and cancer were examined with immunohistochemistry or RT-PCR and six different cell lines were also evaluated. S1R mRNA overexpression was detected in 64% of breast cancers compared to normal breast tissue. Immunohistochemistry showed positive epithelial cell staining in 60% of invasive and 41% of *in situ* cancers, 75% of ductal hyperplasia and in 33% of normal breast. The pattern of expression was more diffuse in invasive breast carcinoma compared to other conditions ($p = 0.02$). S1R expression was neither a prognostic nor a predictive factor for efficacy of adjuvant chemotherapy but the study only included 58 cancer patients and therefore the statistical power is limited. MDA-MB-361, MDA-MB-435, BT20 and MCF7 cells all expressed S1R protein by Western blot. The non-specific S1R ligands haloperidol, reduced haloperidol and progesterone produced a dose-dependent inhibition of the growth at high (>10 μM) concentrations. Reduced haloperidol also showed additive cytotoxic effects when combined with doxorubicin, vinorelbine, paclitaxel and docetaxel *in vitro*. The S1R-specific ligand, SKF 10047 demonstrated the least growth inhibitory activity and showed no interaction with chemotherapy. These results demonstrate that some normal and most neoplastic breast epithelial cells and cell lines commonly express S1R. High concentrations of haloperidol inhibit the growth of these cells and potentiate the effect of chemotherapy *in vitro*.

Introduction

Sigma receptors are unique drug binding sites of the cell membrane that are unrelated to any other known receptors [1, 2]. Sigma receptors bind a number of structurally unrelated psychoactive compounds such as haloperidol, phencyclidine and benzomorphans [1, 3]. It has been suggested that their endogenous ligand may be progesterone [4, 5]. Based on drug binding affinity, two types of sigma receptors have been described, sigma 1 (S1R) and sigma 2 (S2R). Haloperidol, reduced haloperidol, 1,3-di-*o*-tolylguanidine and (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)-piperidine bind equally to both receptor subtypes. Pentazocine and (+)-SKF 10047 only bind to S1R [3]. S1R was recently cloned from several species including humans [1,6]. The gene encodes a 25 kDa protein with a single putative transmembrane domain and with a cytoplasmic reticulum retention sequence. It is a novel protein with no vertebrate homologues. The only known proteins with some homology are fungal sterol isomerases. S2R has not yet been cloned and is only identified as a drug binding activity on the cell surface.

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Sigma receptor binding activity has been detected on a variety of neuronal tissues and non-neuronal cells by using radiolabeled sigma ligands. Sigma receptor binding activity was also identified on a variety of human tumor cell lines including melanoma, prostate, non-small cell lung and breast cancer cell lines (MCF7 and T47D) [7]. Little is known of the signal transduction mechanisms of sigma receptors. In neuroendocrine cells it may inhibit voltage-activated potassium channel since activation of S1R by SKF-10047 reduced voltage-dependent potassium flow across the plasma membrane [8]. In the brain and heart S1R has been reported to be an intracellular calcium modulatory protein [9, 10]. It has also been reported that S1R ligands inhibit the growth of neoplastic cells including MCF-7 cells, melanoma, colon cancer and glioma cell lines through unknown mechanisms [7, 11, 12]. Sigma receptor agonists can also induce apoptosis in rat cerebellar granular cells and neuroblastoma cells [13]. S1R is an attractive molecule for further investigation because it may represent a potential novel therapeutic target for breast cancer and may also serve as a potential prognostic marker.

The expression of S1R in breast cancer and its biological relevance is not well understood. Our hypothesis was that S1R is expressed in a subset of human breast cancers and its expression may contribute to the biology of the disease. One goal of this research was to evaluate the expression of the S1R in human breast cancer and normal breast tissues by immunohistochemistry. The other goal was to examine the effect of S1R ligands on cell growth and sensitivity to chemotherapy of breast cancer cell lines *in vitro*.

Materials and methods

Patient samples and cell lines

Patient samples used in this study were retrieved from the breast cancer tumor bank of the University of Texas MD, Anderson Cancer Center (UT MDACC). Fourteen frozen invasive breast cancers and eight frozen normal breast reduction mammoplasty specimens were used for the RT-PCR analysis and an additional 95 formaldehyde fixed paraffin embedded tumors were used for the immunohistochemical analysis (IHC). The IHC specimens included 58 invasive, stage I–III breast cancer specimens and 37 samples from individuals with no invasive breast cancer (reduction mammoplasty specimens and ductal carcinoma *in situ*). All individuals were treated at UT MDACC between 1990 and 2002. Thirty-eight patients underwent a radical mastectomy and 20 a segmental mastectomy. Thirty-nine patients received neoadjuvant or adjuvant anthracyclin-based chemotherapy and 27 received adjuvant endocrine therapy only. The Institutional Review Board of UT MDACC approved this research on human tissues.

Human breast tumor cell lines MCF-7, MDA-MB-231, MDA-MB-361, MDA-435, T47D and BT20 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in 1:1 Ham's F12 Medium and Dulbecco's Modified Eagle's Medium with 2 mM L-glutamine. The medium was supplemented with 5% calf serum (MCF-7), 10% calf serum (MDA-MB-231) or 10% fetal bovine serum (T47D), and 10 μg/ml insulin and 1.2 mg/ml sodium bicarbonate. All culture media components were purchased from the MD Anderson Tissue Culture Core Facility (Houston, TX). All the media were supplemented with 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin. Cells were monitored routinely for mycoplasma contamination by using a detection kit (Boehringer Mannheim, Indianapolis, IN).

RNA isolation and RT-PCR

Frozen tumor specimens with >60% carcinoma tissue were used for total RNA isolation using a Qiagen Kit. For the cell lines, total RNA was extracted from the cells using TRIZOL reagent (Invitrogen) following the manufacturer's protocol. RNA yields were measured by spectrophotometer and RNA integrity was analyzed by agarose gel electrophoresis. Prior to reverse transcription, RNA was treated with DNAse I for 30 min at 37 °C using a DNA-free kit (Ambion, Inc., Austin, TX). Four μg of total RNA was reverse transcribed and amplified using the reagents and protocol of the GeneAmp RNA PCR Core Kit (Perkin Elmer, Norwalk, CT). S1R mRNA expression was detected by real-time RT-PCR using TGGATGGGCGCCATGT as forward primer and AAGGCGGTGC CGAAGAG as reverse primer. PCR conditions were as follows:

2 min 50 °C, 10 min 95 °C, 40 cycles of 15 second 95 °C and 1 min 60 °C.

Immunohistochemical analysis of sigma receptor in breast tumors

Paraffin-embedded tissues were sectioned in 4–6-μm slices. Sections were deparaffinized in xylene, and rehydrated by treatment with a graded series of alcohol washes. Antigen retrieval was performed by heating the slides in antigen retrieval type II solution (Vel-Lab Research, Houston, TX) for 20 min in a steamer. Slides were subsequently incubated for 15 min in 3% EtOH:water to quench endogenous peroxidase activity and incubated with blocking serum for 30 min. The slides were incubated overnight at 4 °C with affinity purified goat polyclonal anti-human S1R primary antibody (Sig-1R, L-20, Santa Cruz Biotechnology, Inc.). Positive reactions were visualized by using the Vectastain ABC Elite staining kit (Vector Laboratories). The sections were rinsed with distilled water, and counterstained with hematoxylin for 1 min. The number of S1R positive epithelial cells was determined (at ×100 magnification), and the staining intensity was recorded. Staining was scored as negative, 1+ or 2+. A 2+ tumor specimen was used as positive control with each batch of staining. Specificity of the staining was shown by pre-incubation with Sig-1R blocking peptide (Santa Cruz Biotechnology, Inc.) that abolished signal completely.

Western blot

Cell lysates from 1×10^6 cells were prepared by scraping cells into ice-cold harvesting buffer (PBS pH = 7.4 with 0.5% hSDS, 1% Nonidet P-40, proteinase inhibitor PMSF 100 μg/ml aprotinin 1 μg/ml). The lysates were boiled for 5 min and supernatants were collected after centrifugation in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4 °C. The protein concentration of the supernatant was determined by BCA protein assay (Bio-Rad Laboratories). Equal amounts of protein were loaded onto 10% SDS-polyacrylamide mini-gels and after gel electrophoresis proteins were transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA) and blocked overnight at 4 °C using 3% non-fat milk blocking buffer (3 g non-fat milk powder per 100 ml of TBS (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl) and 0.05% (v/v) Tween 20). Membranes were incubated for 3 h at room temperature with goat polyclonal anti-S1R (Sig-1R), IL-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibody. The primary antibody was diluted 1:250 in 1% non-fat milk in TBS and 0.05% Tween 20. After washing three times with TTBS (TBS with 0.05% Tween 20), bovine anti-goat-IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1500 in TTBS, was added and incubated for 2 h at room temperature. After washing three times with TTBS, S1R protein was visualized using ECL reagent (Amersham Bioscience, Buckinghamshire, England) following the manufacturer's instructions. Images were obtained with autoradiography.

Assessment of cell growth

Cells were seeded at 1000 cells/well density into 96-well microplates and cultured for 12 h before adding various test substances to the medium. After a further 72 h exposure, cell growth was determined by CellTiter 96 AqueousOne® cell proliferation assay (Promega, Madison, WI) following the manufacturer's instructions. The results were expressed as percent inhibition defined as the ratio Treated/Control (T/C), where T = optical density (OD) of treated cells and C = OD of control cultures. The concentration of each agent that inhibited cell growth by 50% (IC50) was determined using nonlinear regression analysis to fit the inhibition data (Prism 3; GraphPad Software, Inc., San Diego, CA). The effect of adding a sigma ligands to cells treated with a cytotoxic agent was tested for doses of chemotherapy less than IC50 to detect potential synergy.

Chemotherapy drugs, progesterone and sigma ligands SKF10047, reduced haloperidol and haloperidol were purchased from Sigma (St. Louis, MO). SKF10047 was dissolved in culture medium and reduced haloperidol in dimethyl sulfoxide (DMSO) (2% W/V). Before adding to medium, all chemical solutions were sterile filtered.

Statistical analysis

Proportions of S1R staining in normal breast and pathologic tissue were compared using non-parametric Kruskall–Wallis test and χ^2 test.

Associations between sigma receptor expression and various clinical and pathological parameters of cancer were evaluated with a χ^2 test or Fisher exact test if necessary. Disease-free (DFS) survival was dated from the date of diagnosis. DFS was estimated using the Kaplan–Meier product-limit method and a two-sided log-rank test was used to test the association between sigma receptor and DFS. Results of the cell growth assay were expressed as mean ± stan- dard deviation (SD) and the differences between treatment groups were compared by one-way ANOVA followed by Dunnet's test. *p*-Values less than 0.05 were considered significant.

Results

Expression of S1R in human breast cancer

Nine of 14 invasive breast cancers (57%) overexpressed S1R mRNA greater than twofold compared to a reference pool of normal breast tissues measured by TaqMan-PCR (Table 1). Next, we examined the expression and cellular localization of the protein by immunohistochemistry in 95 separate cases. These included 12 normal breast tissues from reduction mammoplasty, 8 cases of ductal hyperplasia, 17 ductal carcinoma *in situ* (DCIS) and 58 invasive breast cancers. Variable cytoplasmic expression was detected in normal and neoplastic epithelial cells (Figure 1). The proportion of positive cases defined as any S1R expression was the highest in invasive cancer (60%) and lowest in normal breast epithelium (33%) but this difference did not reach statistical significance (non-parametric Kruskall–Wallis test, $p = 0.13$) (Table 2). As reported in Table 3, the pattern of expression was significantly more likely to be diffuse (all cells positive) in invasive breast carcinoma (26/58) than in other conditions (8/37) ($p = 0.02$, χ^2 test) where it was more likely to be focal (few positive cells).

Table 1. Relative expression of the S1R mRNA in breast cancer samples compared to normal breast tissue by real-time RT-PCR

Sample #:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Ratio:	0.22	0.02	0.11	**24.0**	**11.5**	**7.0**	**2.0**	0.03	**3.2**	**2.7**	0.8	**4.0**	**3.0**	**37**

Expression ratios of S1R normalized to beta-actin are presented (ratio = normalized cancer sigma mRNA/normalized normal breast tissue sigma RNA).

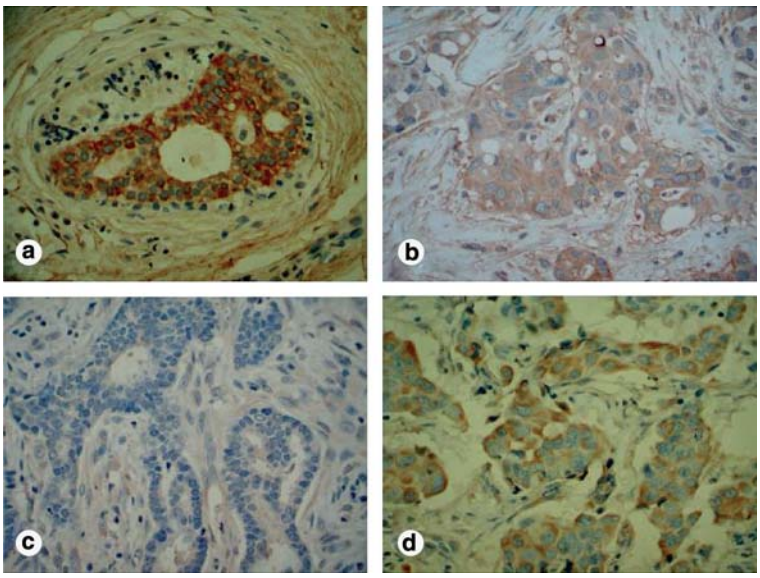

Figure 1. Immunohistochemical staining for S1R in formaldehyde fixed paraffin embedded tissues: (a) ductal hyperplasia scored 3+, (b) DCIS scored 1+, (c) invasive ductal carcinoma with no staining and (d) invasive ductal carcinoma scored 2+ (magnification ×40).

Relationship between S1R expression by immunochemistry and various clinicopathologic parameters of invasive breast carcinoma

No statistically significant associations were found between S1R expression and age ($p = 0.79$), tumor size ($p = 0.63$), pathological nodal status ($p = 0.21$), histological grade ($p = 0.93$), estrogen receptor ($p = 0.18$), progesterone receptor ($p = 0.30$) and HER2 status ($p = 0.31$). We also performed an exploratory analysis to estimate the potential prognostic value of S1R expression. The median follow-up of the population was 36 months (range: 1–162). S1R expression was not associated with better or worse distant DFS ($p = 0.60$) nor local relapse-free survival ($p = 0.26$) (Figure 2). In order to assess a potential interaction between S1R expression and the effect of adjuvant anthracyclin-based chemotherapy, we stratified outcome results according to the systemic treatment received. S1R expression was not a significant predictive factor of survival either in patients treated with chemotherapy or without chemotherapy (Figure 2c and 2d).

Effect of sigma receptor ligands on the growth of breast cancer cell lines in vitro

Cell lines MDA-MB-231,-361,-435, MCF 7 and BT20 all expressed S1R protein by Western blot (Figure 3) and also by Northern blot analysis (data not shown). T47D cells did not express this receptor in our laboratory. The non-specific S1R ligands haloperidol, reduced haloperidol and progesterone showed a dose-dependent inhibition of the growth of essentially all cell lines at high concentrations (10–100 μM) after 72 h of exposure (Figure 4). Low doses (<10 μM) of any of these ligands had no effect on cell growth (Figure 5). Of the three drugs tested, reduced haloperidol had the most pronounced effect. The S1R-specific SKF 10,047 demonstrated a growth inhibitory activity only in MDA 435 cells.

Effect of sigma receptor ligands on chemotherapy sensitivity of breast cancer cell lines in vitro

We also examined whether sigma ligands altered the sensitivity of cells to various chemotherapy drugs. We tested the effect of adding 20 μM SKF 10,047

Table 2. S1R protein expression assessed by immunohistochemistry in normal breast, benign epithelial hyperplasia, ductal carcinoma *in situ* (DCIS) and invasive carcinoma. Number of patient within each category is presented in the table

	Sigma 1 receptor expression							
	Intensity			*p*	Pattern			*p*
	0	1+	2+		0	Focal	Diffuse	
Normal	8 (67%)	3 (25%)	1 (8%)		8 (67%)	3 (25%)	1 (8%)	
Ductal hyperplasia	2 (25%)	4 (50%)	2 (25%)		2 (25%)	4 (50%)	2 (25%)	
DCIS	10 (59%)	6 (35%)	1 (6%)		10 (59%)	1 (6%)	6 (35%)	
Invasive carcinoma	23 (40%)	29 (50%)	6 (10 %)	0.13[a]	23 (40%)	9 (16%)	26 (44%)	0.08[a]

[a]Non-parametric Kruskall-Wallis test.

Table 3. S1R protein expression in invasive carcinoma compared with other conditions (normal breast epithelium, benign epithelial hyperplasia and ductal carcinoma *in situ*): χ^2 test

	Sigma 1 receptor expression					
	Intensity		*p*	Pattern		*p*
	0	1+/2+		0/focal	Diffuse	
Invasive carcinoma	23 (40%)	35 (60%)		32 (55%)	26 (45%)	
Other conditions	20 (54%)	17 (46%)	0.17	29 (78%)	8 (22%)	0.02

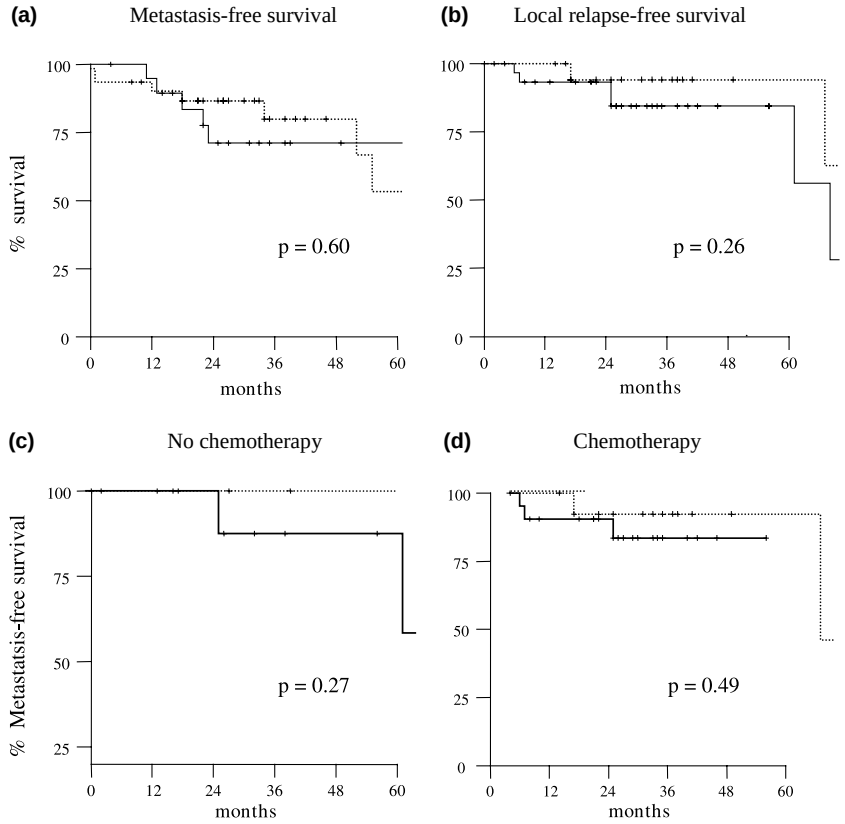

Figure 2. Distant and local relapse-free survival according to S1R expression and distant disease-free survival in patients with and without adjuvant chemotherapy. Patients with S1R positive tumors are represented by the dotted line.

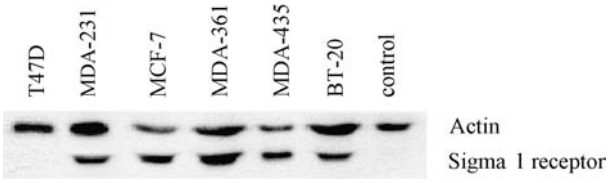

Figure 3. Sigma 1 Receptor expression in six breast cancer cell lines by Western blot analysis.

(S1R specific ligand) and 20 μM reduced haloperidol (non-specific ligand) to doxorubicin, paclitaxel, docetaxel and vinorelbine, respectively. Figure 6 shows that reduced haloperidol enhanced the effect of the drugs tested. However, no additive or antagonistic effect was seen when (+)-SKF 10,047 was combined with any of the cytotoxic agents.

Discussion

Sigma receptors were identified as high-affinity binding sites for a variety of psychoactive drugs on neurons [2, 14]. Two subtypes, S1R and S2R are distinguishable pharmacologically but only S1R has been cloned. Although initially described in neuronal tissues, these receptors are also expressed in other organs including the liver, kidneys, lungs and the gonads [15, 16] and are also present in various cancer cell lines [7, 11, 13, 17, 18]. This broad pattern of expression suggests that they may play an important but yet to be elucidated physiologic role in a variety of cell types. In this study we examined the expression of S1R at the mRNA and protein levels in normal and neoplastic breast tissues and assessed the effect of S1R ligands on

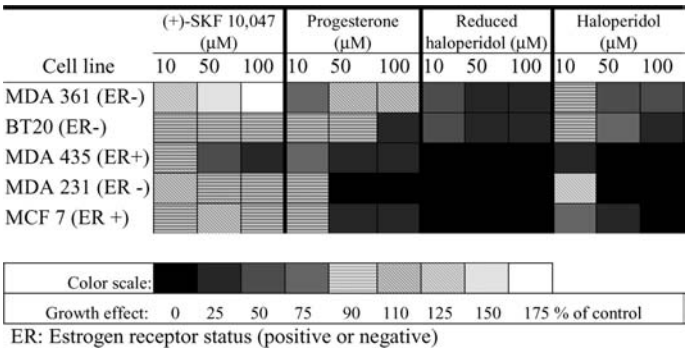

Figure 4. Effects of various concentrations of sigma 1 receptor ligands (+)-SKF 10,047, progesterone, reduced haloperidol and haloperidol on cell growth in five different Sigma 1 receptor positive cell lines. Growth inhibitory effect is expressed as % of medium/diluent treated control and results are presented as a gray scale heat map with 25% increments.

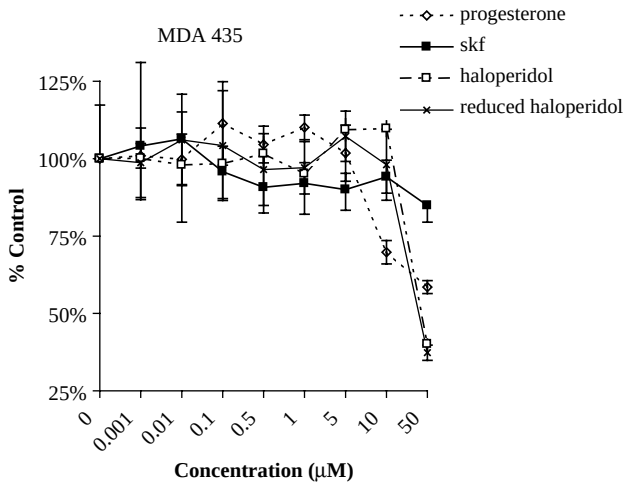

Figure 5. Growth inhibitory effects of four different S1R ligands at low concentrations on MDA 435 cells.

the growth and chemotherapy sensitivity of breast cancer cells *in vitro*.

Fifty seven percent of invasive breast cancers overexpressed S1R mRNA greater than twofold compared to a reference pool of normal breast tissues measured by TaqMan-PCR. Immunohistochemical analysis confirmed the frequent expression of S1R protein in breast cancer. The proportion of S1R positive cases was highest among invasive cancers (60%) and lowest in normal breast specimens (33%) but this difference did not reach statistical significance. However, the pattern of expression was significantly more likely to be diffuse in invasive breast carcinoma (45%) compared to all other conditions including normal or benign proliferative epithelium (22%) ($p = 0.02$). We did not observe any statistically significant association between S1R expression and patient's age, tumor size, nodal status, nuclear grade or estrogen progesterone and HER-2 receptor status. S1R also failed to show any prognostic value in this small study. However, this study was not powered to formally evaluate the prognostic and predictive value of S1R expression, therefore our results can only be considered exploratory. A larger study could detect a modest prognostic or predictive value.

There is one previous study that assessed S1R expression in breast cancer using immunohistochemistry, with an antibody that was different from ours. The investigators reported expression of S1R in 76% of stage I–III breast cancers [19].

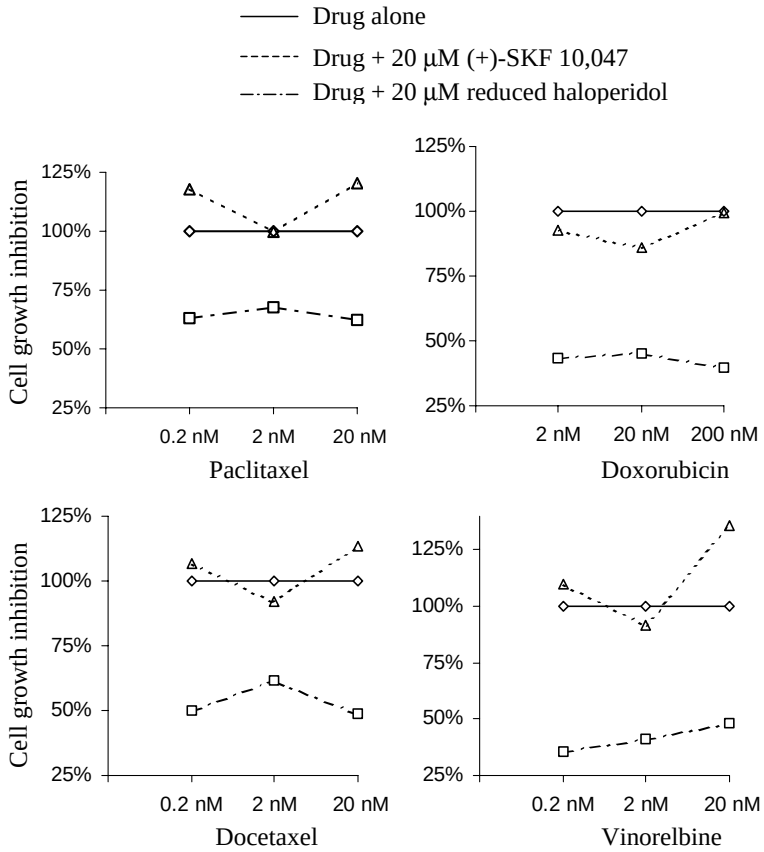

Figure 6. Association between four different cytotoxic drugs and 20 μM of (+)-SKF 10,047 or reduced haloperidol in MDA 361 cells. At each dose level of cytotoxic agent, the inhibitory effect of adding a sigma receptor ligand to chemotherapy is expressed as % of cell growth inhibition compared to the cytotoxic drug alone.

Similar to our study there was no correlation with age, tumor size, nodal status, histological grade or proliferative activity of the tumor. However, a significant positive correlation with progesterone receptor positive status and also a poorer disease-free survival in patients with S1R-negative tumours were noted ($p = 0.045$). The differences between the findings of these two studies may be due to the different antibodies and antigen retrieval methods used or to the different patient populations that were included (proportion of patients with adjuvant chemotherapy 19% versus 67% in the MDACC study). Nevertheless, both studies confirmed that S1R is frequently and variably expressed in invasive breast cancer.

Next, we examined the effects of S1R ligands on the growth and chemotherapy sensitivity of breast cancer cell lines *in vitro*. Almost all cell lines expressed S1R protein by Western blot. Haloperidol, reduced haloperidol and progesterone all showed a dose-dependent inhibition of the growth of these cells at concentrations ≥10 μM. Reduced haloperidol (non-specific ligand) produced the greatest inhibition whereas SKF 10,047 (S1R-specific ligand) showed the least growth inhibitory activity. Reduced haloperidol also demonstrated a significant additive effect in combination with doxorubicin, paclitaxel, docetaxel and vinorelbine. However, no similar additive (or antagonistic) effect was seen with (+)-SKF 10,047. Because of the weak effect of this ligand, no combination indexes could be calculated to formally test synergy as described by Chou et al. [20]. Since haloperidol only exerted these effects at high doses, and this drug binds to several receptors including S1R, S2R as well as to D2 dopamine receptors its growth inhibitory and chemotherapy sensitizing activity may not be mediated directly by S1R. Indeed, the

relatively weak effects of the more specific (+)-SKF 10,047 suggest that S1R itself may have a limited influence on the proliferation or chemotherapy response of breast cancer cells.

The lack of highly specific S1R ligands and poor understanding of the signaling mechanisms of this receptor hinder the true determination of the contribution of S1R to neoplastic cell growth. However, several other investigators observed that ligands that bind sigma receptors can inhibit cell growth. Berthois et al. [21] reported that SR31747A, a ligand which binds to S1R and S2R and also to the human sterol isomerase, inhibits cell proliferation. Haloperidol also inhibits tumor growth *in vitro* [11, 12, 22, 23]. Increasing evidence suggests that S2R may play a more important role in cell proliferation and cell death than S1R [11, 12, 18]. Unfortunately, S2R cannot be directly studied since the molecule has no been cloned.

The widespread expression of S1R in invasive and *in situ* breast cancers, ductal hyperplasia and its focal expression in some normal breast epithelial cells is an intriguing observation. Because of its frequent expression, it is likely to play some biological role in breast epithelial cells. The natural ligand of sigma receptors, their signal transduction mechanisms and biological function in non-neural tissues remain to be elucidated. Forced expression of S1R in sigma-negative cell lines and down regulation of S1R expression with small interfering RNAs in sigma-positive cells may help evaluating further the biology of this unique receptor.

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