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The sigma-2 receptor as a therapeutic target for drug delivery in triple negative breast cancer

Mehran Makvandi, **Estifanos D. Tilahun**, **Brian P. Lieberman**, **Redmond-Craig Anderson**, **Chenbo Zeng**, **Kuiying Xu**, **Catherine Hou**, **Elizabeth S. McDonald**, **Daniel A. Pryma**, and **Robert H. Mach**

University of Pennsylvania, Perelman School of Medicine, Department of Radiology and Division of Nuclear Medicine and Clinical Molecular Imaging, Philadelphia, PA 19104, USA

Abstract

Background—Triple-negative breast cancer (TNBC) is associated with high relapse rates and increased mortality when compared with other breast cancer subtypes. In contrast to receptor positive breast cancers, there are no approved targeted therapies for TNBC. Identifying biomarkers for TNBC is of high importance for the advancement of patient care. The sigma-2 receptor has been shown to be overexpressed in triple negative breast cancer *in vivo* and has been characterized as a marker of proliferation. The aim of the present study was to define the sigma-2 receptor as a target for therapeutic drug delivery and biomarker in TNBC.

Methods—Three TNBC cell lines were evaluated: MDA-MB-231, HCC1937 and HCC1806. Sigma-2 compounds were tested for pharmacological properties specific to the sigma-2 receptor through competitive inhibition assays. Sigma-2 receptor expression was measured through radioligand receptor saturation studies. Drug sensitivity for taxol was compared to a sigma-2 targeting compound conjugated to a cytotoxic payload, **SW IV-134**. Cell viability was assessed after treatments for 2 or 48 hours. Sigma-2 blockade was assessed to define sigma-2 mediated cytotoxicity of **SW IV-134**. Caspase 3/7 activation induced by **SW IV-134** was measured at corresponding treatment time points.

Results: SW IV-134—was the most potent compound tested in two of the three cell lines and was similarly effective in all three. MDA-MB-231 displayed a statistically significant higher sigma-2 receptor expression and also was the most sensitive cell line evaluated to **SW IV-134**.

Conclusion—Targeting the sigma-2 receptor with a cytotoxic payload was effective in all the three cell lines evaluated and provides the proof of concept for future development of a therapeutic platform for the treatment of TNBC.

Corresponding Author: Robert H. Mach, PhD, Address: 231 S. 34 Street Philadelphia, PA 19104, Telephone: 215-746-8233, Fax: 215-746-0002, rmach@mail.med.upenn.edu.

Mehran Makvandi, PharmD, Postdoctoral Research Fellow, Address: 231 S. 34 Street Philadelphia, PA 19104, Telephone: (215) 746-8755, Fax: (215)349-5843, makvandi@mail.med.upenn.edu

Keywords

Triple-negative breast cancer; sigma-2; targeted therapy; drug delivery

Introduction

Triple-negative breast cancer (TNBC) is an aggressive cancer phenotype that is associated with high relapse rates and poor patient prognosis compared to receptor positive breast cancers.[1–5] Approximately 15–20% of breast cancers fall into this category without expression of the estrogen receptor (ER), progesterone receptor (PR) or amplification of the human epidermal growth factor receptor 2 (HER2) gene.[6, 7] Therapies targeted to these receptors are highly effective, leading to improved patient response and survival. There are no approved targeted therapies for TNBC and, the best options for treatment are chemotherapeutic regimens that have limited long-term success rates and significant morbidity.[8, 9] As a result, scientists and clinicians have focused on discovering integral biomarkers to allow specific targeting of this heterogeneous and treatment refractory disease.[3]

The sigma-2 receptor was identified as the progesterone receptor membrane component 1 (PGRMC1), and is overexpressed in multiple nonneural tumors, including TNBC. [10, 11] PGRMC1 expression detected by IHC in 60 surgical specimens was also an independent prognostic factor in multivariate survival analysis of breast cancer patients.[12]

Sigma-2 receptor density is a biomarker of proliferative status both in mouse mammary adenocarcinoma cells in vitro and in vivo solid tumor xenographs.[13,14] The novel imaging probe [^{18}F]ISO-1 targets the sigma-2 receptor and is the only validated PET method to measure tumor proliferation in vivo.[15]

Another valuable application of the sigma-2 receptor is its use as a site for therapeutic drug delivery, which has been validated in preclinical models of ovarian and pancreatic cancer. [16–18] Multiple classes of sigma-2 ligands have been pharmacologically characterized as either agonists, partial agonists or antagonists.[19–21] Sigma-2 agonists have been shown to undergo receptor-mediated endocytosis providing a method to deliver therapeutics into cancer cells.[16–18] Subcellular localization studies have shown sigma-2 agonists are trafficked to the mitochondria, a common site of therapeutic target due to the regulatory processes of cell homeostasis and apoptosis.[22] Sigma-2 agonists are capable of activating caspase 3, 8 and 9 and inducing cell death through multiple pathways, and there are established methods of increasing the potency through conjugation with cytotoxic payloads. [16–18, 21, 23, 24] Non-targeted chemotherapeutics rely on high concentrations to penetrate tissues, which can result in detrimental systemic effects. By harnessing the pharmacological properties of sigma-2 agonists and the selective overexpression of sigma-2 receptors in highly proliferative tumor cells, we provide a mechanism to deliver cytotoxic payloads with the benefit of greatly improving the therapeutic potential of conventional chemotherapeutics.

Second mitochondria-derived activator of caspase (SMAC) is a pro- apoptotic protein that is released from the mitochondria into the cytosol during apoptosis.[25] SMAC antagonizes

the anti-apoptotic proteins XIAP, cIAP1 and cIAP2; compounds that are responsible for inhibiting caspases, an executionary protein family that proteolytically digests enzymes in preparation for apoptosis. Small-molecule SMAC mimetic compounds (SMCs) have been developed and are capable of inducing apoptosis in cancer cells.[26] Through conjugation of SMCs with sigma-2 targeting compounds, there is potential to deliver SMCs to cancer cells enhancing the drug delivery and minimizing potential side effects.[16–18]

The primary aim of the present study was to evaluate the sigma-2 targeted therapeutic **SW IV-134** in TNBC cell lines in comparison with taxol, a current first line breast cancer therapy. **SW IV-134** is comprised of a sigma-2 receptor targeting moiety (**SW43**) and SMC (**SW IV-52s**), shown in Figure 1. The secondary aim of this study was to measure the sigma-2 receptor expression in TNBC cell lines to explore the potential use of the sigma-2 receptor as a biomarker in TNBC.[27]

Methods

Cell Culture

Triple negative breast cancer cell lines MDA-MB-231, HCC1937 and HCC1806 were cultured in either MEM with added nonessential amino acids, sodium pyruvate 10% FBS and 1% Pen/Strep (MDA-MB-231), or RPMI with 10% FBS and 1% Pen/Strep (HCC1937 and HCC1806) at 37° C with 5% CO_2. Cells were cultured for up to 20 passages over the course of 10 weeks.

Drug Solutions

Taxol was purchased from Fisher Scientific and the remaining drug compounds were synthesized as previously reported.[16, 21]

Competitive Inhibition

Liver homogenates and [^{125}I]-RHM-4 were prepared as previously described, see Figure 1 for the molecular structure of RHM4.[27, 28] [^{125}I]-RHM-4 was used in competitive inhibition assays at a concentration of 1 nM. Non-radioactive SW43 or SW IV-134 were added at varying concentrations from 1 nM – 1 μM and reaction mixtures were allowed to equilibrate at room temperature for 1 hr. Next, samples were filtered on a Brandel Harvester, washed three times with ice-cold buffer and filter papers were removed. Radioactivity was assayed on a Perkin Elmer Wizard Gamma Counter (Waltham MA). Data was fitted using GraphPad Prism version 6.0 and non-linear fit one site Ki and competitive inhibition (Ki) values for SW43 and SW IV-134 were calculated.

Sigma-2 Saturation and B_{max} Determination

MDA-MB-231, HCC1937 or HCC1806 tumor cell homogenates were prepared as previously described.[28] [^{125}I]-RHM-4 was used to measure sigma-2 binding sites through saturation experiments with increasing concentrations of radioligand. Concentrations ranged from 10 pM – 10 nM and reactions were allowed to equilibrate at room temperature for 1 hr. Next, solutions were harvested and assayed for radioactivity as described in the section above. Radioactivity measured was converted to molar concentrations using a specific

activity of 2200 Ci/mmol and data was plotted using GraphPad Prism version 6.0 non-linear fit one-site binding hyperbola. The maximum number of binding sites (B_{max}) and dissociation constant (K_d) were calculated for each tumor cell line. Statistical analysis of B_{max} was performed with an ordinary one-way ANOVA test using Prism version 6.0.

Cell Viability

MDA-MB-231, HCC1937 and HCC1806 were plated in black wall clear bottom 96-well plates at 10,000 cells/well and 5,000 cells/well 24 hrs before treatment, for 2 hr and 48 hr treatment, respectively. On the day of drug treatment, of **SW43**, **SW IV-52s**, Taxol or **SW IV-134** were added at concentrations from 10 pM – 100 μM. Cells with appropriate treatments were incubated for either 2 or 48 hrs. For 2 hr treatments, drug solution was removed and cells were replenished with fresh media, and then allowed to regrow for 48 hrs. Viability was determined using commercially available CellTiter Glo® (Promega, Madison WI) chemiluminescent assay that measures ATP. For 48 hr treatment, the media was removed and CellTiter Glo® was added, then plates were immediately read on a Perkin Elmer Enspire® Multimode Plate Reader. Luminescence detected for each well was normalized to the untreated control and data was calculated as percent viability. All experiments were completed in triplicate at three independent times. Data was plotted using GraphPad Prism version 6.0 non-linear fit sigmoidal dose response variable slope and effective concentrations for 50% maximal reduction in cell viability (EC_{50}) were calculated.

Caspase 3/7 Activation

MDA-MB-231, HCC1937 and HCC1806 were plated in a black wall clear bottom 96 well plate at 10,000 cells/well 24 hrs before the addition of **SW IV-134** drug treatment. Caspase 3/7 activation was measured using Caspase 3/7 Glo® assay (Promega) either 2 hrs or 48 hrs post treatment addition. Once Caspase 3/7 Glo® was added and plates were read on a Perkin Elmer Enspire® Multimode Plate Reader. Data was normalized to untreated controls and plotted as fold increase in capsase 3/7 activities.

Sigma-2 Blockade

MDA-MB-231 cells were incubated with **SW IV-134** at 1 or 0.5 μM in the presence of 10 μM RHM1 (a non-radioactive analogue of RHM4) for 2 hrs. Media containing the treatment was then removed and cells were allowed to regrow for 48 hrs in fresh media. After 48 hrs of regrowth cell viability was assayed as previously described. RHM1 is considered a non-toxic (<100μM) sigma-2 antagonist, because it does not undergo receptor-mediated internalization or induce caspase-3 activation. By incubating MDA-MB-231 cells with **SW IV-134** in the presence of RHM1 we challenged whether the selective mechanism in which **SW IV-134** exerts its cytotoxic effects is sigma-2 dependent. Statistical analysis was performed using a t-test in Prism version 6.0.

Results

Competitive Inhibition

Ki values for **SW43** and **SW IV-134** are presented in Table 1 and competitive inhibition curves are plotted in Figure 2 C. Experimental data is in accordance with previously published results.[16, 21]

Sigma-2 Receptor B_{max} Determination

Sigma-2 receptor expression was quantified through saturation experiments and revealed that MDA-MB-231 had a statistically significant higher maximum number of binding sites defined as B_{max} (ordinary one-way ANOVA, P-Value =0.0001). Saturation curves and B_{max} values are represented in Figures 2 A–B and Table 1.

Cell Viability

2 hr treatment experiments showed a sigma-2 targeting effect with compounds **SW43** and **SW IV-134** where non-targeted compounds **SW IV-52s** and taxol were not as potent, except in HCC1806 which was sensitive to taxol. In MDA-MB-231 and HCC1937 cell lines SW IV-134 was the most potent compound for the 48 hr treatment. Dose response curves are shown in Figure 3 and EC_{50} values are presented in Table 2.

Caspase 3/7 Activation

Caspase 3/7 activation was detected in all three cell lines at 48 hrs at low concentrations of **SW IV-134** treatment (<25μM). The MDA-MB-231 showed the highest fold increase from untreated control at two hours and corresponds with the data obtained through cell viability experiments. Also, the two hour treatment of HCC1937 resulted in a medium level of caspase activation while HCC1806 remained unaffected. Caspase activation is presented in Figure 4.

Sigma-2 Blockade

Saturating sigma-2 binding sites with 10 μM RHM1 showed a significant inhibitory effect of SW IV-134 cytotoxicity at concentrations of 1 and 0.5 μM. See Figure 4 C. Sigma-2 inhibition with RHM1 was able to block the loss of approximately 30% of cell viability in the MDA-MB-231. This evidence supports the proposed mechanism of action of SW IV-134 is mediated by the sigma-2 receptor.

Discussion

We demonstrate that selectively targeting the sigma-2 receptor with SMC in TNBC results in cytotoxicity that exceeds the most commonly used breast cancer therapy. This raises the possibility of the sigma-2 receptor as a viable TNBC target, utilizing the highly proliferative nature of this disease and the relatively higher expression of the sigma receptor in this cancer subtype. The sigma-2 receptor expression was highest in one of the three TNBC cell lines evaluated (MDA-MB-231). This line also demonstrated the highest sensitivity to sigma-2 SMC targeted therapy and was relatively taxol resistant. This observation may provide a window into TNBC heterogeneity and a biomarker for sensitivity to targeted

therapy. Response to treatment with the sigma-2 receptor targeted SMC corresponded to receptor expression in all three TNBC cell lines and provides the proof of concept for defining sigma-2 receptor expression in TNBC.

The sigma-2 targeted SMC, **SW IV-134**, was more effective in reducing cell viability in 2 out of the 3 TNBC cell lines evaluated. While taxol was potent in HCC1806, the other two cell lines (MDA-MB-231 and HCC-1937) were largely resistant to treatment. These findings are consistent with what has been reported for cell lines HCC1806 and MDA-MB-231.[29] Also, there is evidence that taxol treatment in MDA-MB-231 leads to the upregulation of anti-apoptotic proteins and confers resistance through activation of the toll-like 4 receptor (TLR4).[29] Interestingly, MDA-MB-231 cells were responsive to the SMC **SW IV-52s** and highly sensitive to the sigma-2 receptor targeted conjugate **SW IV-134** providing a potential novel mechanism to overcome chemoresistant TNBCs that overexpress TLR4. Mechanistically the TLR4 increases anti-apoptotic proteins that can be inhibited by SMCs blocking the resistance pathway. Future studies are needed to examine the relationship of TLR4 and sigma-2 receptor expression.

The main limitation of this study is that in vitro results may not translate into in vivo models. Thus, we are currently evaluating this potential therapy in mouse models of TNBC.

The sigma-2 targeted SMC conjugate **SW IV-134** was the most potent treatment tested in 2 out of 3 TNBC cell lines. Thus, further study into this potential TNBC biomarker and therapeutic target is warranted.

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Highlights

- TNBC cells are sensitive to sigma-2 receptor targeted drug conjugate SW IV-134
- MDA-MB-231, a chemo-resistant TNBC cell line, displayed the highest amount of sigma-2 receptors and this corresponded to sensitivity with treatment of SW IV-134.
- The sigma-2 receptor is a potential biomarker in TNBC for prognosis and therapy

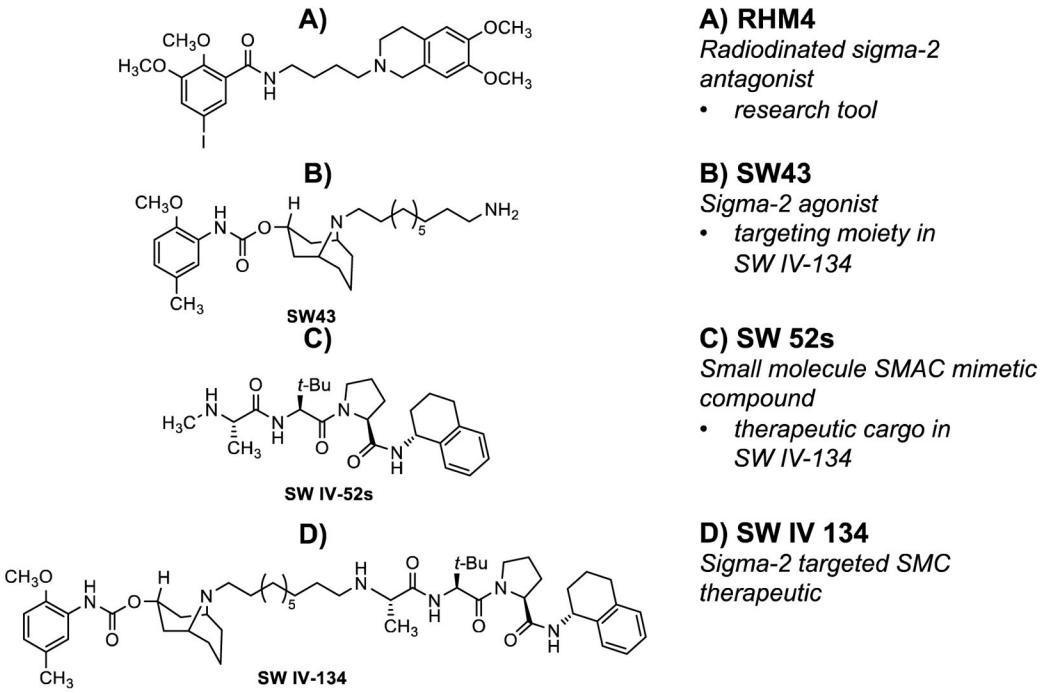
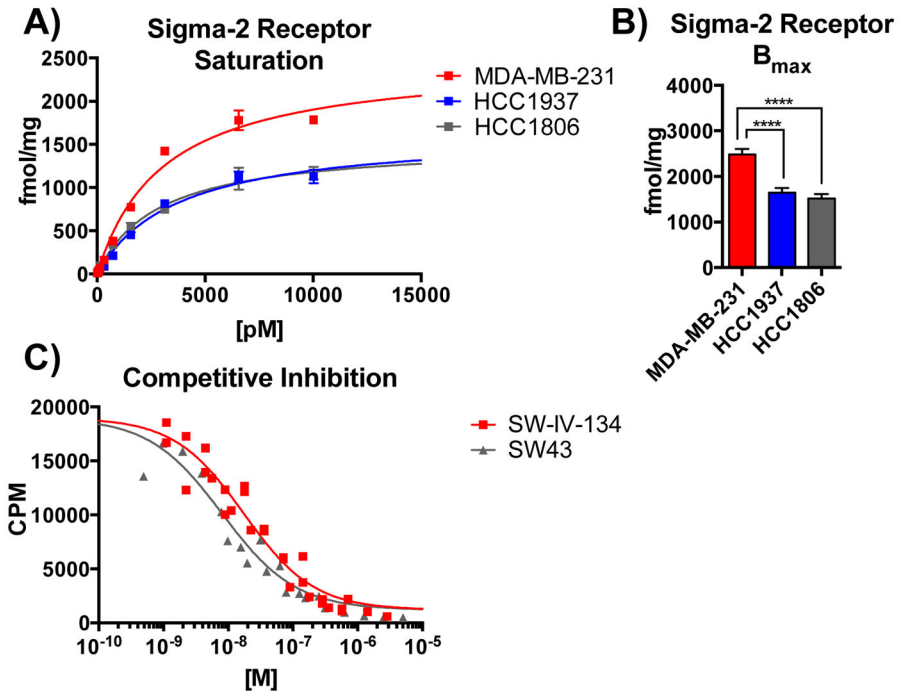


Figure 1.

Chemical structures of sigma-2 compounds and small molecule SMAC mimetic compound (SMC) used in experiments. The name of compound and its use are listed to the right of the chemical structure with corresponding letters.



**** Denotes statistical significance, ordinary one-way ANOVA, p-value=0.0001

Figure 2.

A) Graphical representation of sigma-2 receptor saturation experiment performed using [^{125}I]**RHM-4. B)** Maximum number of sigma-2 binding sites presented as a bar graph. **C)** Competitive inhibition curves for **SW43** and **SW IV-134**.

**** Denotes statistical significance, ordinary one-way ANOVA, p-value=0.0001

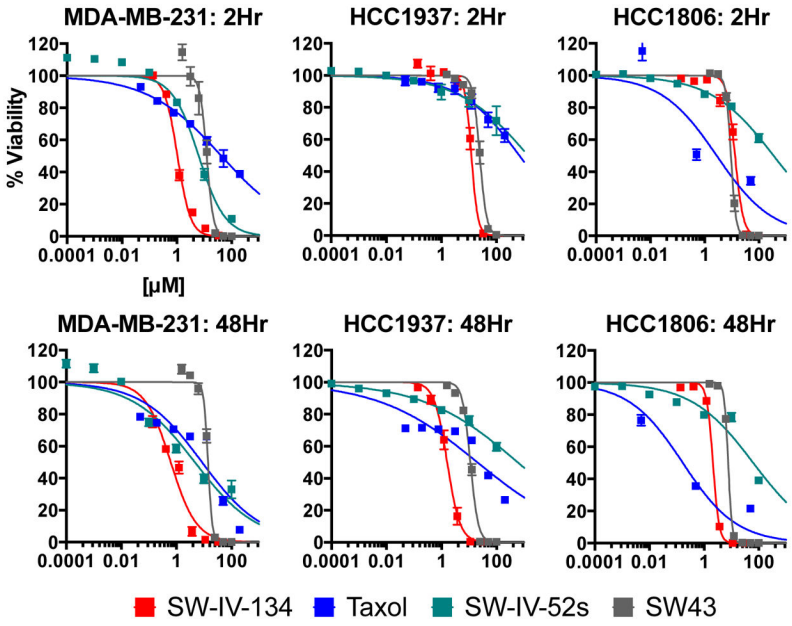


Figure 3.

Cell viability dose response curves are presented for each cell line treated for either 2 or 48 hrs.

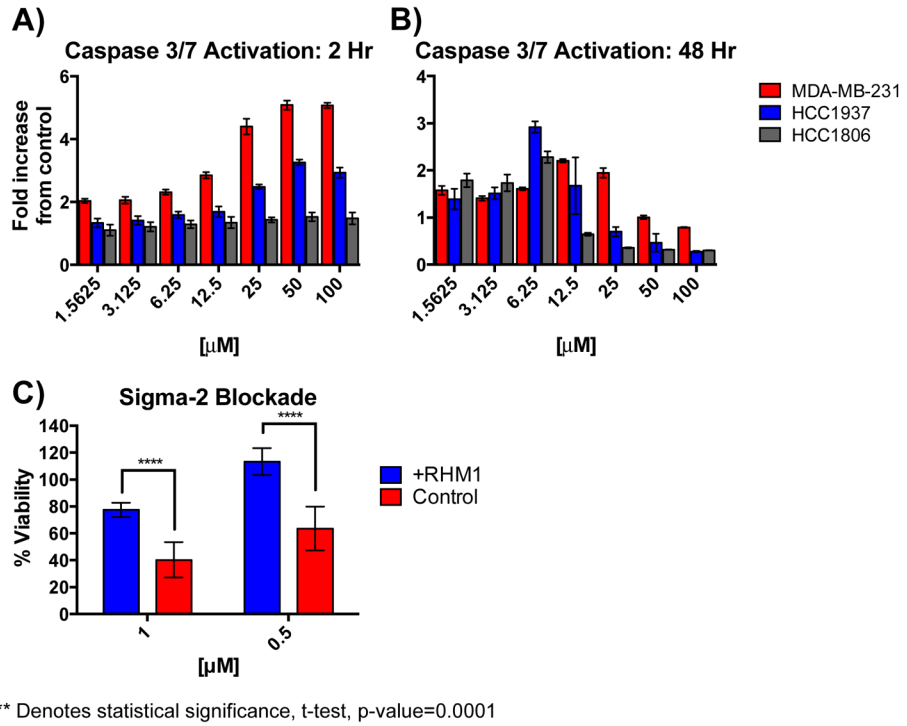


Figure 4.

A) Caspase 3/7 activation induced by **SW IV-134** at 2 hr. MDA-MB-231 showed a 5-fold increase from healthy control at higher concentrations of treatment. **B)** Caspase 3/7 activation at 48 hrs. Lower concentrations are activating caspase 3/7. The absence of signal in higher concentrations at 48 hrs is due to cell death. C) Sigma-2 blockade with RHM1 reduces cytotoxic effects of **SW IV-134** In Vitro.

**** Denotes statistical significance, t-test, p-value=0.0001

Table 1

Competitive inhibition (K_i) for sigma-2 compounds, **SW43** and **SW IV-134**, are presented in part A) and maximum number of sigma-2 receptor binding sites are listed in part B). Error presented as standard error of measurement.

Sigma-2 Pharmacology	
A) Compounds	**K_i (nM)**
SW43	8.1 ± 1.8
SW IV-134	17.5 ± 3.7
B) Cell Line	**B_{max} (fmol/mg)**
MDA-MB-231	2485 ± 119 ****
HCC1937	1645 ± 99
HCC1806	1517 ± 93

**** Denotes statistical significance, ordinary one-way ANOVA, p-value=0.0001

Table 2

Effective concentrations for 50% reduction in cell viability from healthy controls of each treatment tested in respective cell lines are presented below. Error is represented as standard error of measurement

EC_{50}(μM)				
MDA-MB-231				
	2 Hr EC_{50}	**SEM**	**48 Hr EC_{50}**	**SEM**
SW43	12.23	1.07	14.07	1.03
SW-52s	6.280	1.14	4.636	1.47
Taxol	42.34	1.18	8.447	1.40
SW-IV-134	1.055*	1.04	0.623*	1.13
HCC1937				
	2 Hr EC_{50}	**SEM**	**48 Hr EC_{50}**	**SEM**
SW43	24.88	1.01	11.11	1.02
SW-52s	1412	3.07	575.7	1.47
Taxol	679.1	1.60	19.79	1.26
SW-IV-134	12.42*	1.05	1.602*	1.06
HCC1806				
	2 Hr EC_{50}	**SEM**	**48 Hr EC_{50}**	**SEM**
SW43	9.345	1.02	7.622	1.01
SW-52s	344.9	1.29	62.45	1.25
Taxol	2.770	2.42	0.165	2.07
SW-IV-134	13.16	1.50	2.105	1.02

*Denotes lowest EC_{50} that is statistically significant, ordinary one-way ANOVA, p-value=0.05