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Development of Molecular Probes for Imaging Sigma-2 Receptors In Vitro and In Vivo

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Abstract

The sigma-2 (σ_2) receptor is proving to be an important protein in the field of cancer biology. The observations that σ_2 receptors have a 10-fold higher density in proliferating tumor cells than in quiescent tumor cells, and that σ_2 receptor agonists are capable of killing tumor cells via apoptotic and non-apoptotic mechanisms, indicate that this receptor is an important molecular target for the development of radiotracers for imaging tumors using techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) and for the development of cancer chemotherapeutic agents. In spite of recent promising results towards achieving these goals, research in this field has been hampered by the fact that the molecular identity of the protein sequence of the σ_2 receptor is currently not known. Consequently, most of what is known about this protein has been obtained using either radiolabeled or fluorescent probes for this receptor, or biochemical analysis of the effect of σ_2 selective ligands on cells growing under tissue culture conditions. This article provides a review of the development and use of σ_2 receptor ligands, and how these ligands have been used with a variety of in vitro and in vivo models to gain a greater understanding of the role this receptor plays in cancer.

Keywords

Sigma-2 receptors; cell proliferation; positron emission tomography

INTRODUCTION

Sigma receptors represent a class of proteins that were initially classified as a subtype of the opiate receptors. Subsequent studies revealed that sigma binding sites are a distinct class of receptors that are located in the central nervous system as well as in a variety of tissues and organs.^{1,2} Early studies using radioligand binding methods and biochemical analyses demonstrated that there are at least two types of sigma receptors, termed σ_1 and σ_2 . σ_1 receptors have a molecular weight of ~25 kDa, whereas σ_2 receptors have a molecular weight of ~21.5 kDa. The radioligand [³H](+)-pentazocine has a high (~3 nM) affinity for the σ_1 receptor and a low (>1,000 nM) affinity for the σ_2 receptor, whereas [³H]DTG is equipotent at both σ_1 and σ_2 receptors. An understanding of the functional significance of sigma binding sites has been hampered by the failure to identify an endogenous ligand for these receptors. Recent studies have shown that neuroactive steroids bind with moderate affinity to σ_1 sites, and suggest that σ_1 receptors may modulate the activity of GABA and NMDA receptors in the CNS.³⁻⁵ Whether

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or not neuroactive steroids, or other steroid-based ligands, interact with σ_2 receptors is not known at this time.

The σ_1 receptor has been cloned and displays a 30% sequence homology with the enzyme, yeast C8-C7 sterol isomerase,^{6,7} but this receptor lacks C8-C7 isomerase activity. The σ_2 receptor has not been cloned, and most of what is known regarding the σ_2 receptor has been obtained through the use of in vitro receptor binding studies aimed at the pharmacological characterization of this receptor. The absence of the cloned gene or the purified σ_2 receptor protein has also prevented the generation of antibodies to study the subcellular localization of this receptor using the immunohistochemical techniques previously employed to study the σ_1 receptor.⁸ In spite of this shortcoming, the recent development of highly selective σ_2 receptor radioligands and fluorescent probes has increased our understanding of the subcellular distribution and intracellular trafficking of the σ_2 receptor in vivo.

2. CHARACTERIZATION OF THE σ_2 RECEPTOR AS A BIOMARKER OF THE PROLIFERATIVE STATUS OF SOLID TUMORS

The first report suggesting that there is an overexpression of σ receptors in tumors cells was by Bem et al. in 1991.⁹ A key observation in this study was the lower receptor density obtained with [³H]3-PPP (σ_1 selective ligand) versus [³H]DTG (nonselective for σ_1 and σ_2 receptors) in biopsy samples obtained from patients with renal and colon carcinoma. Although initially interpreted to reflect the disparity between the agonist ([³H]3-PPP) and antagonist ([³H]DTG) properties of these radioligands, the differences in binding properties of the two radioligands in tumor cells could also reflect a higher density of σ_2 versus σ_1 receptors in the tumor samples. This was later confirmed in a report by Vilner et al.¹⁰, who demonstrated that there is a higher density of σ_2 versus σ_1 receptors in a wide variety of human and murine tumor cells growing under cell culture conditions. The additional observation that MCF-7 cells, a human breast adenocarcinoma cell line, possessed a high density of σ_2 receptors as measured by [³H]DTG in the presence of dextrallorphan to mask σ_1 sites, and the absence of specific binding of the σ_1 radiotracer [³H](+)-pentazocine to the MCF-7 cells, suggested that the σ_2 receptor may be a potential biomarker for imaging studies of breast cancer. However, Western blots studies have subsequently shown σ_1 receptor expression in a number of breast tumor cells lines, including MDA-MB-231, MDA-MB-361, MDA-MB-435, MCF-7 and BT20 cells.¹¹

The studies described above clearly indicate that σ_2 receptors may serve as a biomarker for differentiating solid tumors from the surrounding normal tissues. However, these studies did not investigate the relationship between the density of σ_2 receptors and the proliferative status of these tumors. The proliferative status of a solid tumor, which is operationally defined as the ratio of proliferating (P) cells in a solid tumor to those driven into a quiescent (Q) state by nutrient deprivation and/or hypoxia (the P:Q ratio), is an important parameter in determining how to treat a tumor with either radiation or chemotherapy.¹² Tumors having a high proliferative status (i.e., high P:Q ratio) typically respond better to hyperfractionated radiation therapy versus conventional radiation therapy.¹³ Similarly, tumors having a high P:Q ratio will respond better to cell cycle specific agents such as Ara-C and gemcitabine, whereas tumors having a lower proliferative status will respond better to non-cell cycle specific agents such as cis platin and BCNU.¹² Finally, the measurement of the P:Q ratio of a tumor is also expected to be useful in selecting patients that will yield the maximum benefit from “targeted therapies” since many of these newer cancer treatment strategies (e.g., Polo-like kinase inhibitors, Chk-1 inhibitors) are targeting various proteins that are expressed in cycling cells and are not expressed in quiescent tumor cells.^{14,15}

Using the well-established diploid mouse mammary adenocarcinoma cell line 66,^{16,17} Wheeler and colleagues designed a series of experiments aimed at determining if there was a difference

in the density of σ_2 receptors in proliferating (66P) and quiescent (66Q) mouse adenocarcinoma cells growing under cell culture conditions or in solid tumor xenografts. In their initial study, this group demonstrated that the density of σ_2 receptors in 66P cells was about 10 times greater than the density observed in 66Q cells.¹⁸ The density of σ_2 receptors in the 66P cells was found to be quite high, ~900,000 copies/cell versus ~90,000 receptors/cell in the 66Q cells (Figure 1A). Similar results were obtained with the aneuploid mouse mammary adenocarcinoma line, 67.¹⁹ However, the modest σ_2 receptor density in the Q cells suggest that it still may be possible to image solid tumors having a significant Q cell fraction (i.e., low P:Q ratio) because most of the surrounding normal tissues have an even lower σ_2 receptor density.^{9,10}

In a follow-up study, this group reported the upregulation and downregulation of σ_2 receptors follows the transition of mouse mammary cells between the P and Q states.¹⁹ Because the downregulation of σ_2 receptors takes at least three days to complete once the cells make the transition from P to Q (Figure 1B), these data suggest that this receptor is not expressed only in a single phase of the cell cycle such as the cyclin-dependent kinases and other cell-cycle specific proteins. The observation that the P:Q ratio of σ_2 receptor density in solid tumors is identical to that obtained under cell culture conditions confirmed that the results obtained under cell culture conditions can be translated to solid tumors xenografts of these breast cancer cell lines.²⁰ Taken collectively, these data demonstrate that the σ_2 receptor is a receptor-based biomarker of cell proliferation in breast tumors. Therefore, radiotracers having a high affinity and high selectivity for σ_2 receptors have the potential to assess the proliferative status of human breast tumors using noninvasive imaging techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). In addition, it is likely that this approach can be extended to assess the proliferative status of other human tumors, such as head and neck, melanoma, and lung tumors, which are known to express a high density of σ_2 receptors.¹⁰

3. DEVELOPMENT OF σ_2 SELECTIVE LIGANDS

Although many different classes of compounds have been shown to bind to σ_1 and σ_2 receptors, most of these compounds bind selectively to the σ_1 receptor or have similar affinities for both σ_1 and σ_2 receptors.¹ The development of ligands having a high affinity for the σ_2 versus the σ_1 receptor was not straightforward, and in some cases, the identification of σ_2 receptor selective ligands were a serendipitous result when ligands intended for another CNS target were exposed to broad-based screening for cross-reactivity to other receptors (Figure 2).

One of the first σ_2 selective ligands reported was the benzomorphan-7-one analog, **CB-64D**.²¹ This compound was identified as part of a structure-activity relationship (SAR) study aimed at refining the affinity of (-)-2-methyl-5-(3-hydroxyphenyl)morphan-7-one for μ versus κ opioid receptors.²² Incorporation of an (E)-benzylidene moiety into the 8-position of the ring system increased affinity for σ receptors, with the (-)-1S,5R isomer (**CB-64L**) having a high affinity for σ_1 versus σ_2 receptors, whereas the (+)-1R,5R isomer (**CB-64D**) had a 185-fold higher selectivity for σ_2 versus σ_1 receptors (Figure 2). The corresponding 3,4-dichloro analog (**CB-184**) had an even higher affinity and selectivity for σ_2 versus σ_1 receptors (Figure 2). A second series of compounds having a high affinity for σ_2 receptors are the 3-(ω -aminoalkyl)-1H-indole analogs.^{23, 24} SAR studies with compounds that were originally designed to be serotonin 5-HT_{1A} agonists, resulted in the synthesis of **Lu 28-179**,²⁵ which has a subnanomolar affinity for σ_2 receptors and a 140-fold selectivity for σ_2 versus σ_1 receptors (Figure 2). **Lu 28-179**, also known as siramesine, was initially evaluated as a drug to treat anxiety and depression, but subsequent studies have shown that this compound kills tumor cells via a caspase-independent method of cell death.²⁶ Similar results have been reported with **CB-64D**, which has been shown to result in an intracellular rise in Ca²⁺ levels via the release of a thapsigargin-sensitive store in the endoplasmic reticulum.²⁷ Siramesine has been suggested

to induce cell death via a lysosomal leakage pathway resulting in the formation of reactive oxygen species²⁶ This suggests that σ_2 receptors are localized in the lysosomes in addition to the plasma membrane of cells.

Other compounds that were found to have a higher affinity for σ_2 versus σ_1 receptors are: 1) the hallucinogen, ibogaine;²⁸⁻²⁹ 2) the mixed serotonin 5-HT₃ antagonist/5-HT₄ agonist **BIMU-1**;³⁰ 3) the tropane analog **SM-21**, an acetylcholine releaser that has been utilized as an antinociceptive agent;^{31,32} 4) the trishomocubane analog **ANSTO-19**,³³ which has a modest affinity for σ_2 receptors; and 5) the piperazine analog **PB28** which has a subnanomolar affinity for σ_2 receptors, but high affinity for σ_1 receptors resulting in only a moderate selectivity for σ_2 versus σ_1 receptors ($\sigma_1 : \sigma_2$ ratio = 29, Figure 2).³⁴ The neurotoxic effects of ibogaine have been linked to the affinity of this indole alkaloid for σ_2 receptors.²⁸

A number of SAR studies using **BIMU-1** as the lead compound have identified high affinity, high selectivity σ_2 receptor ligands.^{35,36,37} **BIMU-1** is an ideal lead compound for SAR studies since it provides a variety of regions where structural modifications can be made to optimize the σ_2 receptor affinity and reduce the affinity for serotonin 5-HT₃ and 5-HT₄ receptors (Figure 3). Addition of a benzene ring to the N-methyl of the bridgehead nitrogen to give the corresponding N-benzyl group and replacement of the urea linkage of **BIMU-1** resulted in a dramatic increase in affinity for both σ_2 and σ_1 receptors, a loss of affinity for 5-HT₃ receptors, but moderate affinity for 5-HT₄ receptors.³⁵ Expansion of the 8-azabicyclo[3.2.1]octan-3 β -yl ring system (i.e., tropane ring) to the corresponding 9-azabicyclo[3.3.1]nonan-3 β -yl ring system (i.e., granatane ring) did not alter the affinity for σ_1 and σ_2 receptors relative to the tropane analogs, but the ring expansion eliminated affinity for the 5-HT₄ receptor. The most interesting analog from this initial SAR study was the compound **1**, which had a σ_2 receptor affinity of ~3 nM and a $\sigma_2 : \sigma_1$ selectivity of ~30 (Figure 3).³⁵

Compound **1** was used as a secondary lead compound for a series of subsequent SAR studies aimed at producing second-generation granatane analogs having an improved σ_2 receptor affinity and high $\sigma_2 : \sigma_1$ selectivity ratio. Homologation of the benzyl group by one carbon atom to give the corresponding 2-phenethyl analog, **2**, resulted in a slight improvement in σ_2 receptor affinity and increased the $\sigma_1 : \sigma_2$ selectivity ratio to ~50 (Figure 3).³⁶ Substitution of the para position of **1** with a dimethylamino group and the para position of **2** with an amino group resulted in a further increase in the selectivity for σ_2 receptors, largely by a reduction in affinity for σ_1 receptors.³⁶ The amino group appears to be a preferred substituent for assuring a high affinity for σ_2 receptors and high $\sigma_1 : \sigma_2$ selectivity ratio based on the in vitro binding properties of the aminoalkyl analogs **5** and **6**.³⁷ The high affinity of compound **6** was used in the design of the fluorescent probes **SW107** and **K05-138** (Figure 3), which have been useful in two photon and confocal microscopy studies of σ_2 receptors in tumor cells growing under cell culture conditions.³⁸

A second class of compounds having a high affinity for σ_2 receptors and excellent $\sigma_1 : \sigma_2$ selectivity ratios are the conformationally-flexible benzamide analogs shown in Figure 4. These compounds were identified in an SAR study of the benzamide analog, **7** that was developed as a dopamine D₃-selective ligand.³⁹ The relatively high lipophilicity of **7** (log P = 5.76) indicates that the compound would not readily cross the blood-brain barrier by a passive diffusion mechanism and be active in behavioral studies. The strategy used to reduce the lipophilicity of **7** involved replacing the 4-(2,3-dichlorophenyl)piperazine ring with other aromatic amine groups that were intended to reduce the overall log P of the compound without the altering affinity for dopamine D₃ receptors.³⁹ Although this strategy resulted in a number of useful dopamine D₃ receptor ligands,⁴⁰ it was also observed that replacement of the 4-phenylpiperazine moiety with a 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline ring resulted in

compounds having a high affinity and excellent selectivity for σ_2 versus σ_1 receptors, and a dramatic reduction in affinity for dopamine receptors (Figure 4).³⁹

Additional SAR studies within this class revealed that shortening the length of the spacer group between the amide nitrogen and the nitrogen atom of the 1,2,3,4-tetrahydroisoquinoline moiety from 4 carbons to 2 carbons (i.e., compound **9**) did not alter binding to the σ_2 receptor, but resulted in an increase in affinity for dopamine D₃ receptors.³⁹ Removal of the 6,7-dimethoxy groups from **9** to give **10** resulted in a dramatic reduction in affinity for σ_2 receptors, but no change in affinity for D₃ receptors; this demonstrated the importance of the 6,7-dimethoxy groups in the 1,2,3,4-tetrahydroisoquinoline moiety for maintaining a high affinity for the σ_2 receptor.³⁹ In contrast, the 3-methoxy group in the benzamide aromatic ring was not critical for σ_2 receptor affinity, but was critical for maintaining a high affinity for dopamine D₃ receptors (compare **9** versus **11**). Replacement of the 5-bromo group of **11** with a methyl group resulted in a further reduction in D₃ affinity and no change in affinity for the σ_2 receptor. As expected, extension of the 2 carbon spacer of **12** to the corresponding 4 carbon spacer, **13**, also retained a high affinity for σ_2 receptors. As discussed in greater detail below, the conformationally-flexible benzamide analogs with the 4 carbon spacer group have proven to be an important class of σ_2 -selective compounds for the preparation of radiolabeled probes to image this receptor both in vitro and in vivo.

4. IN VITRO BINDINGS STUDIES OF RADIOLABELED σ_2 RECEPTOR LIGANDS

Historically, the radioligand that has proven to be most useful in the study of the σ_2 receptor is [³H]di-o-tolylguanidine ([³H]DTG).^{1,2} Although this ligand binds with equal affinity to both σ_1 and σ_2 receptors, it was the difference in binding properties between [³H]DTG and radiolabeled benzomorphan analogs such as [³H](+)-SKF 10,047 and [³H](+)-pentazocine that first led to the pharmacological characterization of the σ_2 receptor.^{1,2} The photoaffinity analog, [³H]azido-DTG was also instrumental in the characterization of the molecular weight of σ_1 and σ_2 receptors.^{1,2} [³H]Azido-DTG was found to label two distinct proteins in liver membranes; these proteins had molecular weights of ~25 kDa and ~21.5 kDa. Blocking studies that used 100 nM dextrallorphan to mask σ_1 binding sites indicated that the 25 kDa protein was the σ_1 receptor, whereas the 21.5 kDa was the σ_2 receptor. The 25 kDa and 21.5 kDa proteins labeled by [³H]azido-DTG have also been identified in other tissues, including guinea pig brain and rat phenochromocytoma (PC12) cells.² The accepted radioligand binding techniques for Scatchard and competition studies involve [³H](+)-pentazocine to label and quantify σ_1 receptors, and [³H]DTG in the presence of 100 nM of unlabeled (+)-pentazocine to mask σ_1 receptors and quantify σ_2 receptors.² Autoradiography studies measuring the density of σ_1 and σ_2 receptors in rat and guinea pig brain have also been conducted using in vitro techniques.^{41,42} It was the use of radioligand binding techniques that resulted in the identification of the high σ_2 receptor density in a wide panel of murine and human tumor cells growing under cell culture conditions,¹⁰ and the elucidation of the relationship between the σ_2 receptor density and the proliferative status of mouse mammary tumor cells.¹⁸⁻²⁰

An observation made during an attempt to purify the sigma receptor proteins was the low recovery of the solubilized σ_2 receptor protein compared to σ_1 receptor protein.⁴³ A potential explanation for the low σ_2 protein recovery is that the receptor protein is localized in lipid rafts of the plasma membrane. Lipid rafts are microdomains in the cell membrane and are believed to play a key role in the secretory and endocytotic pathways of eukaryotic cells. Lipid rafts are enriched with cholesterol, sphingolipids, and glycosylphosphatidylinositol-linked proteins that form specialized structures termed caveolin upon the incorporation of a cholesterol binding protein. Using sucrose density centrifugation of CHAPS extracts of rat liver P2 membranes, Gebreselassie and Bowen⁴⁴ were able to demonstrate a [³H]DTG-binding site in protein

fractions containing flotillin-2, a molecular marker of lipid rafts. Subsequent in vitro binding studies revealed that [³H]DTG binding was blocked by the σ_2 selective ligand **CB-64D** and not by the σ_1 receptor ligand (+)-pentazocine, confirming that σ_2 receptors are colocalized in lipid rafts. Other in vitro binding studies using [³H]DTG in the presence of (+)-pentazocine have shown that σ_2 receptor binding sites are located in the mitochondria.[†] The diverse distribution of the σ_2 receptor between the plasma membrane and cell organelles such as the mitochondria and endoplasmic reticulum further emphasized the need for the development of antibodies or fluorescent probes for the σ_2 receptor so that the subcellular localization of this receptor can be studied using imaging techniques such as confocal and two photon microscopy.

Although [³H]DTG has been a very useful ligand for characterizing the σ_2 receptor, and for identifying σ_2 selective ligands, its rapid dissociation rate (i.e. k_{off}) is not ideal for in vitro binding studies. The identification of the σ_2 selective ligands described above has resulted in the preparation of a number of tritiated compounds having improved σ_2 receptor binding properties relative to [³H]DTG. The first σ_2 selective ligand labeled with tritium was **Lu 28-179** (siramesine). Scatchard studies in rat and human brain samples revealed that [³H]**Lu 28-179** has a K_d value of 1.1 nM.²⁵ Autoradiography studies of [³H]**Lu 28-179** revealed a high density of σ_2 receptors in the motor cortex, hippocampus, and hind brain nuclei, which is consistent with previous autoradiography studies using [³H]DTG in the presence of 1 μ M dextralorphan to mask σ_1 binding sites. Other σ_2 selective ligands that have been labeled with tritium include [³H] **13**, also known as [³H]**RHM-1**,⁴⁵ and [³H]**PB28**.⁴⁶ Of the three tritiated σ_2 selective ligands reported to date, [³H]**13** has the highest selectivity for σ_2 versus σ_1 receptors; a key property for conducting Scatchard studies of σ_2 receptors in tumors and normal tissues.

A number of sigma receptor ligands have also been radiolabeled with iodine-125, another useful radionuclide for in vitro binding studies of σ_2 receptors. However, most ¹²⁵I-labeled ligands developed to date have a high affinity for the σ_1 and either low or marginal affinity for the σ_2 receptor.⁴⁷⁻⁴⁹ The only σ_2 selective ligand labeled with iodine-125 reported to date is the conformationally-flexible benzamide analog, [¹²⁵I]**14** (Figure 5).⁵⁰

5. IN VIVO IMAGING STUDIES OF σ_2 RECEPTORS

5.1. The importance of σ_2 selectivity in imaging solid tumors with PET

Over the past decade, a number of structurally-diverse compounds having a high affinity for σ receptors have been radiolabeled and evaluated as potential radiotracers for imaging solid tumors with PET or SPECT. Table 1 gives a list of the different isotopes used in these imaging techniques. These radioisotopes have been incorporated into many of the σ receptor ligands shown in Figures 2-5. Most of the radiolabeled compounds evaluated to date display high affinity for σ_1 receptors, or relatively equal affinity for both σ_1 and σ_2 receptors.¹ Since σ_1 receptors are expressed in many normal tissues,^{1,2} it is important to determine the selectivity of a σ receptor-based radiotracer before it is used in solid tumor imaging studies. This was addressed in a series of in vivo studies in tumor-bearing rodents using the radiotracer, [¹⁸F]N-(4'-fluorobenzyl)-4-(3-bromophenyl)acetamide, **16** (Figure 6).⁵¹

Synthesis of the [¹⁸F]**16** was achieved via N-alkylation of the corresponding *des*-benzyl precursor with [¹⁸F]4'-fluorobenzyl iodide. In vivo studies were conducted in nude mice implanted with mouse mammary adenocarcinoma cells, line 66, a murine breast tumor cell line that has a modest σ_1 receptor density and a high σ_2 receptor density.⁵¹ Mice were injected with ~120 μ Ci of [¹⁸F]**16**, and the uptake of the radiotracer in the organs of interest was measured

[†]Wang L-M, Bowen WD, Childers SR, Mach RH, Wheeler KT, Williams W, Morton K.. Sigma-2 receptor binding activity in rat liver mitochondria. Proc. Am. Assoc. Cancer Res. 44: 1154; 2003 (abstract).

at 1 and 2 hr post-i.v. injection. There was a high uptake of [^{18}F]**16** in tissues known to express a high density of σ receptors (e.g., brain, lung, liver). There was also a high tumor uptake (% I.D./c.c. = 2.5%) and a high tumor : blood ratio. In vivo blocking studies were conducted by co-injecting compound **17** (2 mg/kg, i.v) with [^{18}F]**16** to determine the effect of σ_1 receptor blockade on both the tumor uptake and the signal : noise ratio (Figure 6). The results of these biodistribution studies were also compared to biodistribution studies with [^{18}F]FDG, an anatomical imaging agent, and [^{125}I]IUdR, a DNA precursor which potentially images proliferation. The σ_1 blocking study reduced the tumor uptake of [^{18}F]**16** to ~1.8 %I.D./c.c. of tumor, and lowered the tumor : blood ratio from 24.5 in the no-carrier-added study to ~15, which is similar to the tumor : blood ratio obtained with [^{18}F]FDG. However, blocking σ_1 receptors resulted in a dramatic increase in the tumor : lung and tumor : muscle ratios of [^{18}F]**16** relative to the no-carrier-added conditions (Figure 6); the improved signal : normal tissue ratios were obtained primarily by blocking the σ_1 receptors expressed in normal tissues. These data suggest that a radiotracer possessing a higher affinity for σ_2 versus σ_1 receptors would yield a higher signal : normal tissue ratio for tumor imaging studies than that obtained with a radiotracer possessing a high affinity for both σ_1 and σ_2 receptors.

This suggestion was confirmed with microPET imaging studies using [^{18}F]**16** under no-carrier-added and σ_1 blocking conditions (Figure 7). Note the lower uptake in the normal tissues when σ_1 receptors were blocked. These data indicate that a σ_2 -selective radiotracer will work better than a nonselective σ_1/σ_2 radiotracer for clinical imaging studies. These blocking studies also demonstrate that a σ_2 selective radiotracer should give higher signal : normal tissue ratios than [^{18}F]FDG or an ^{18}F -labeled DNA precursor like [^{125}I]IUdR (Figure 6).

5.2. In Vivo Tumor imaging studies with σ_2 receptor radiotracers

The high correlation between the density of σ_2 receptors and the proliferative status of solid tumors indicates that σ_2 selective radiotracers are likely to be a better alternative to the thymidine analogs, [^{11}C]thymidine, [^{18}F]FLT, and [^{18}F]FMAU, for imaging the proliferative status of solid tumors with PET and SPECT.⁵² As mentioned above, the two classes of compounds developed to date that have a high affinity and selectivity for σ_2 versus σ_1 receptors are the 9-azabicyclo[3.3.1]nonane (granatane) analogs and the conformationally-flexible benzamides. Of the two classes, the conformationally-flexible benzamide analogs have proven to be the most useful for the development of PET radiotracers for imaging the σ_2 receptor status of solid tumors.⁵³ The presence of the 2-methoxy group in compounds **8**, **11**, **12**, and **13** (Figure 4) facilitated the preparation of the corresponding ^{11}C -labeled derivatives via the alkylation of the corresponding 2-hydroxy precursors with [^{11}C]methyl iodide.⁵⁴ MicroPET and tumor uptake studies were conducted with [^{11}C]**8**, [^{11}C]**11**, [^{11}C]**12**, and [^{11}C]**13**; the most promising analog proved to be [^{11}C]**13**. Although all four analogs had a high affinity for σ_2 receptors, the optimal lipophilicity of [^{11}C]**13** was responsible for the high tumor uptake and suitable signal : normal tissue ratios for imaging (Figure 8). These data indicate that, in addition to receptor affinity, lipophilicity is an important property that must be considered in the design of receptor-based tumor imaging agents. MicroPET and MicroCT imaging studies in a murine solid breast tumor EMT-6 (Figure 8) clearly show the potential of [^{11}C]**13** as a radiotracer for imaging the σ_2 receptor status of breast tumors with PET.

Although [^{11}C]**13** provides a clear image of breast tumors in microPET imaging studies, the short half life of carbon 11 ($t_{1/2} = 20.4$ min) is not ideal for the development of radiotracers which can be used in clinical PET imaging studies. The longer half-life of ^{18}F ($t_{1/2} = 109.8$ min) places fewer time constraints on tracer synthesis and allows imaging studies to be conducted up to 2 h after injection of the radiotracers. This usually results in higher tumor : normal tissue ratios for the ^{18}F -labeled radiotracers relative to their ^{11}C -congeners. The benzamide analogs shown in Figure 4 have also served as lead compounds in the development of ^{18}F -labeled σ_2

selective radiotracers. The strategy chosen involved replacement of the 2-methoxy group in the benzamide ring with a 2-fluoroethoxy group (Figure 9).⁵⁵ The 2-fluoroethoxy- for methoxy-substitution is a common strategy used in the development of ¹⁸F-labeled radiotracers. MicroPET imaging studies indicate that the corresponding ¹⁸F-labeled congener of [¹¹C]**13**, [¹⁸F]**18**, is a suitable probe for imaging the σ_2 receptor status of solid tumors with PET (Figure 10).⁵⁶ Clinical studies of [¹⁸F]**18** are currently in progress in the US.

Two studies have been published comparing the σ_2 receptor imaging approach to [¹⁸F]FLT, a thymidine analog that is not incorporated into DNA.^{54,56} Studies with the ¹¹C-labeled radiotracer, [¹¹C]**13**, displayed higher tumor : blood and tumor : muscle ratios for the σ_2 receptor-based imaging agent compared to [¹⁸F]FLT; the tumor : fat ratios of the two different radiotracers were similar.⁵⁴ The presence of the bromine atom in the benzamide ring of compound **8** (Figure 4) also led to the preparation of a Br-76 labeled radiotracer, [⁷⁶Br]**8**.⁵⁶ Although the limited availability and high positron energy of Br-76 which degrades image resolution limits the utility of [⁷⁶Br]**8** as a potential radiotracer in clinical PET studies, the microPET imaging study comparing [⁷⁶Br]**8** and [¹⁸F]FLT clearly showed a much greater visualization of the tumor and higher tumor : normal tissue ratios for the σ_2 receptor imaging agent (Figure 11).⁵⁶ It will be of interest to see how the σ_2 receptor-based radiotracer, [¹⁸F]**18**, compares with [¹⁸F]FLT in clinical imaging studies in breast cancer patients.

A limited number of ¹²³I-labeled radiotracers have been prepared and SPECT imaging studies have been conducted in breast and melanoma patients. However, since these radiotracers are selective for the σ_1 receptor and have a low affinity for σ_2 receptors, they will not be discussed here. Three ^{99m}Tc- labeled sigma receptors ligands have been reported in the literature (Figure 12) [^{99m}Tc]**BAT-EN6**, which is an analog of 4-PEMP, has been shown to have a high level of binding in breast tumor membrane homogenates, but its affinity for σ_1 and σ_2 receptors has not been reported.⁵⁷ [^{99m}Tc]**22** and its 1-carbon homologue, [^{99m}Tc]**23**, have been prepared and evaluated both in vitro and in vivo.⁵⁸ The relatively low uptake of [^{99m}Tc]**22** and [^{99m}Tc]**23** in MCF-7 breast tumor cells that have a high density of σ_2 receptors, and the high uptake of these radiotracers in B16/F0 melanoma cells that have a moderate density of both σ_1 and σ_2 receptors, suggests that the uptake of these radiotracers by melanomas may not be related to their σ_2 receptor binding, rather, it may be related to their melanin production.⁵⁹ This rationale has been used to explain the high uptake of structurally-related radioiodinated benzamide analogs that have a modest affinity for the σ_1 and σ_2 receptors in melanoma cells.^{59,60} In vivo studies with [^{99m}Tc]**24**, which has a high affinity for σ_2 versus σ_1 receptors,⁶¹ demonstrated a high uptake and clear visualization of 66 murine breast tumor xenografts in nude mice.⁶² These data suggest that [^{99m}Tc]**24** may be a useful radiotracer for SPECT imaging studies of breast cancer patients.

6. CONFOCAL AND TWO PHOTON MICROSCOPY STUDIES OF σ_2 RECEPTORS IN TUMOR CELLS

Although the above data demonstrate that the σ_2 receptor is a useful biomarker for imaging the proliferative status of solid tumors, the σ_2 receptor cannot be classified unequivocally as a *molecular marker of proliferation* until its amino acid sequence is known, and the role the σ_2 receptor plays in cell proliferation is completely understood. Since the σ_2 receptor protein has not been purified and sequenced nor has the gene been cloned, current knowledge of this receptor is based predominantly on receptor binding studies with radiolabeled probes such as [³H]DTG. The function and localization of the σ_2 receptors has been investigated by studying, 1) the binding of σ_2 ligands to P and Q cells, and 2) their effects on the biochemical and physiological properties of tumor cells. These properties include, 1) calcium release from the endoplasmic reticulum and mitochondria,²⁷ 2) incorporation of [³H]palmitic acid to form [³H]ceramide,⁶³ 3) the release of cathepsins B and L from lysosomal stores,²⁶ and 4) the

generation of reactive oxygen species.²⁶ However, these studies only provide an indirect measure of potential sites of localization of σ_2 receptors within the cell. In vitro receptor binding studies on subcellular fractions of brain and liver tissue have suggested that σ_2 receptors are localized on the endoplasmic reticulum, mitochondria, and plasma membrane.^{2,45}

Using the fluorescent probes shown in Figure 3, Zeng and colleagues⁶⁴ recently conducted a series of confocal and two photon microscopy studies that have provided a clearer picture of the localization of σ_2 receptors in breast tumor cells. These studies were conducted using a panel of “Tracker” dyes in order to determine which organelles and subcellular compartments express σ_2 receptors. EMT-6 (mouse breast tumor) or MDA-MB-435 (metastatic human tumor) cells were incubated with 200 nM **SW107** and either 50 nM MitoTracker®, 500 nM ER-Tracker®, or 75 nM LysoTracker® for 2 h at 37°C. MDA-MB-435 cells were also incubated with 200 nM **SW107** and 5 µg/mL of the membrane tracker, FM®1-43FX, for 5 min at 0°C. After the incubation period, imaging of the live cells by two-photon microscopy revealed that **SW107** was distributed throughout the cytoplasm of the cells, but not in the nucleus (Figure 13). The staining of **SW107** was highly punctate, suggesting that the label has been sequestered in different membrane-bound compartments. Tracker co-localization studies demonstrated that **SW107** co-localizes with MitoTracker, LysoTracker, ER Tracker and the membrane marker. This indicates that σ_2 receptors are localized in the mitochondria, lysosomes, endoplasmic reticulum and cytoplasmic membrane (Figure 13). Similar results were obtained with confocal imaging studies using the fluorescent probe, **K05-138**.

Time-lapsed confocal microscopy studies revealed a rapid uptake of **K05-138** ($t_{1/2}$ = 16 sec), suggesting that the uptake of the fluorescent probe into the tumor cells occurred by receptor-mediated endocytosis. This was confirmed with the use of phenylarsine oxide (PAO), a well-characterized endocytosis inhibitor followed by the σ_2 selective ligand, **K05-138**. Pretreatment of MDA-MB-435 cells with either 5 µM or 10 µM PAO for 30 min reduced the uptake of **K05-138** by 31% and 38%, respectively (Figure 14).⁶⁴ These data demonstrate that ~40% of the σ_2 receptors were internalized by receptor-mediated endocytosis, while the remaining ~60% was internalized by other mechanisms such as passive diffusion. The rapid internalization of σ_2 receptors via endocytosis suggests that σ_2 selective ligands may potentially serve as receptor-mediated probes for delivering cytotoxic agents to solid tumors.

The two photon and confocal microscopy studies conducted by Zeng et al.⁶⁴ have provided useful information for the interpretation of studies demonstrating that σ_2 receptor ligands may have a role as cancer chemotherapeutic agents. For example, cell culture studies have revealed that σ_2 receptor ligands induce apoptosis by caspase-dependent and/or caspase-independent pathways.^{26,65} These studies also suggest that subcellular organelles, such as mitochondria and lysosomes, are involved in this σ_2 ligand-induced cell death. Although the exact mechanism(s) by which σ_2 ligands induce cell death are still unclear, these confocal and two photon microscopy studies provide valuable insights into the mechanisms and functions of σ_2 selective ligands and σ_2 receptors.

Mitochondria are a key organelle with respect to mediating cell death and regulating the intrinsic apoptotic pathway. Apoptotic signals after UV irradiation or treatment with chemotherapeutic agents cause the release of cytochrome C from the mitochondria and the subsequent activation of caspase-3 leading to an apoptotic cell death. The observation that σ_2 ligands reduce mitochondrial membrane potential and cause caspase-dependent apoptosis in SK-N-SH neuroblastoma cells suggests that σ_2 receptors play a role in the intrinsic apoptotic pathway.²⁷ The subcellular localization of σ_2 ligands in mitochondria is consistent with the observation that σ_2 ligands may induce, either directly or indirectly, the intrinsic apoptotic pathway.^{26,27}

Additional evidence suggests that lysosomal proteases, such as cathepsins, calpains and granzymes, may also contribute to σ_2 ligand-induced apoptosis.⁶⁶ Under physiological conditions, these proteases are found within the lysosomes but are released into the cytoplasm after exposure to cell damaging agents, thereby triggering a cascade of intracellular events leading to cell death. The σ_2 selective ligand siramesine has been reported to cause lysosomal leakage and induce cell death by caspase-independent mechanisms, and siramesine-induced cell death was partially blocked by an inhibitor of the lysosome protease, Cathepsin B.²⁶ The localization of fluorescent σ_2 receptor probes in the lysosomes is consistent with the hypothesis that siramesine induces cell death partially by targeting lysosomes to cause lysosomal damage, the release of proteases, and eventually cell death.²⁶ Alternatively, the lysosomal degradation of σ_2 receptors may be responsible for the presence of the fluorescent σ_2 probes in the lysosomes. Although the confocal and two photon microscopy studies are consistent with the expression of σ_2 receptors in the lysosomal membrane, the lysosomal degradation pathway cannot be ruled out at this time.⁶⁴

Evidence has also been reported that σ_2 receptors exist in lipid rafts which are mainly found in the plasma membrane.⁴⁴ Lipid rafts play an important role in the signaling associated with a variety of cellular events including adhesion, motility, and membrane trafficking.^{67,68} The observation that σ_2 fluorescent ligands are co-localized with cytoplasmic membrane markers, and undergo receptor mediated endocytosis, is consistent with their localization in lipid rafts. Thus, the fluorescent probes **SW107** and **K05-138** may prove to be useful tools for studying σ_2 receptors in lipid rafts using two-photon and confocal microscopy.

7. CONCLUSION

The σ_2 receptor continues to be an important molecular target in the field of tumor biology. The high expression of this receptor in proliferating versus quiescent breast tumors indicates that the σ_2 receptor is an important clinical biomarker for determining the proliferative status of solid tumors using the functional imaging techniques PET and SPECT. The ability of σ_2 selective ligands to kill tumors by apoptotic and non-apoptotic pathways also suggests that this receptor is a potential target for the development of cancer chemotherapeutic agents. However, the full utility of the σ_2 receptor in the diagnosis and treatment of cancer will not likely be achieved until, 1) the gene is cloned, and 2) the protein is purified and sequenced so that its functional role in normal and tumor cell biology can be understood. Until this occurs, our knowledge of this receptor will continue to rely on the use of σ_2 selective ligands, radioligands, and fluorescent probes to elucidate the σ_2 receptor properties of in vitro and in vivo models of normal tissues and tumors.

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REFERENCES

1. Walker JM, Bowen WD, Walker FO, Matsumoto RR, De Costa B, Rice KC. Sigma receptors: biology and function. *Pharmacol. Rev* 1990;42:355–402. [PubMed: 1964225]
2. Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. Rat liver and kidney contain high densities of sigma-1 and sigma-2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur. J. Pharmacol. Mol. Pharmacol. Sec* 1994;268:9–18.

3. Maurice T, Roman FJ, Privat A. Modulation by neurosteroids of the in vivo (+)-[³H]SKF-10,047 binding to sigma-1 receptors in the mouse forebrain. *J. Neurosci. Res* 1996;46:734–743. [PubMed: 8978508]
4. Maurice T, Junien J-L, Privat A. Dehydroepiandrosterone sulfate attenuates dizocilpine-induced learning impairment in mice via sigma-1-receptors. *Behavioural Brain Research* 1997;83:159–164. [PubMed: 9062676]
5. Romieu P, Martin-Fardon R, Bowen WD, Maurice T. Sigma-1 receptor-related neuroactive steroids modulate cocaine-induced reward. *J. Neurosci* 2003;23:3572–3576. [PubMed: 12736327]
6. Hanner M, Moebius FF, Flandorfer A, Knaus H-G, Striessnig J, Kempner E, Glossmann H. Purification, molecular cloning, and expression of the mammalian sigma-1 binding site. *Proc. Natl. Acad. Sci. USA* 1996;93:8072–8077. [PubMed: 8755605]
7. Seth P, Leibach FH, Ganapathy V. Cloning and structural analysis of the cDNA and the gene encoding the murine type 1 sigma receptor. *Biochem. Biophys. Res. Commun* 1997;24:535–540. [PubMed: 9425306]
8. Alonso G, Phan V-L, Guillemain I, Saunier M, Legrand A, Anol M, Maurice T. Immunocytochemical localization of the sigma-1 receptor in the adult rat central nervous system. *Neuroscience* 2000;97:155–170. [PubMed: 10771347]
9. Bem WT, Thomas GE, Mamone JY, Homan SM, Levy BK, Johnson FE, Coscia CJ. Overexpression of sigma receptors in nonneural human tumors. *Cancer Research* 1991;51:6558–6562. [PubMed: 1660342]
10. Vilner BJ, John CS, Bowen WD. Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. *Cancer Research* 1995;55:408–413. [PubMed: 7812973]
11. Wang B, Rouzier R, Albarracin CT, Sahin A, Wagner P, Yang Y, Smith TL, Meric-Bernstam F, Aldez C, Marcelo, Hortobagyi GN, Pusztai L. Expression of sigma-1 receptor in human breast cancer. *Breast Cancer Res. Treat* 2004;87:205–214. [PubMed: 15528963]
12. Mach RH, Wheeler KT. Imaging the proliferative status of tumors with PET. *Journal of Labelled Compounds and Radiopharmaceuticals* 2007;50:366–369.
13. Fornace, AJ.; Fuks, Z.; Weichselbaum, RR.; Milas, L. *The Molecular Basis of Cancer*. Vol. Second Edition. Mendelsohn, J.; Howley, PM.; Israel, MA.; Liotta, LA., editors. W. B. Saunders Co.; Philadelphia: 2001. p. 423–466.
14. Collins I, Garrett MD. Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. *Current Opinion in Pharmacology* 2005;5:366–373. [PubMed: 15964238]
15. Strebhardt K, Ulrich A. Targeting polo-like kinase 1 for cancer therapy. *Nature Reviews Cancer* 2006;6:321–330.
16. Wallen CA, Higashikubo R, Dethlefsen LA. Murine mammary tumour cells in vitro. I. The development of a quiescent state. *Cell Tissue Kinet* 1984;17:65–77. [PubMed: 6198084]
17. Wallen CA, Higashikubo R, Dethlefsen LA. Murine mammary tumour cells in vitro. II. Recruitment of quiescent cells. *Cell Tissue Kinet* 1984;17:79–89. [PubMed: 6198085]
18. Mach RH, Smith CR, Al-Nabulsi I, Whirrett BR, Childers SR, Wheeler KT. Sigma-2 receptors as potential biomarkers of proliferation in breast cancer. *Cancer Research* 1997;57:156–161. [PubMed: 8988058]
19. Al-Nabulsi I, Mach RH, Sten K, Childers SR, Wheeler KT. Effect of ploidy, recruitment, environmental factors, and tamoxifen treatment on the expression of sigma-2 receptors in proliferating and quiescent tumor cells. *British Journal of Cancer* 1999;81:925–933. [PubMed: 10576647]
20. Wheeler KT, Wang LM, Wallen CA, Childers SR, Cline JM, Keng PC, Mach RH. Sigma-2 receptors as a biomarker of proliferation in solid tumours. *British Journal of Cancer* 2000;82:1223–1232. [PubMed: 10735510]
21. Bowen WD, Bertha CM, Vilner BJ, Rice KC. CB-64D and CB-184: ligands with high sigma-2 receptor affinity and subtype selectivity. *Eur. J. Pharmacol* 1995;278:257–260. [PubMed: 7589164]
22. Bertha CM, Mattson MV, Flippen-Anderson JL, Rothman RB, Xu H, Cha X-Y, Becketts K, Rice KC. A marked change of receptor affinity of the 2-methyl-5-(3-hydroxyphenyl)morphans upon attachment of an (E)-8-benzylidene moiety: synthesis and evaluation of a new class of σ receptor ligands. *J. Med. Chem* 1994;37:3163–3167. [PubMed: 7932540]

23. Perregaard J, Moltzen EK, Meier E, Sanchez C. σ Ligands with subnanomolar affinity and preference for the sigma-2 binding site. 1. 3-(ω -aminoalkyl)-1H-indoles. *J. Med. Chem* 1995;38:1998–2008. [PubMed: 7783131]
24. Moltzen EK, Perregaard J, Meier E. σ Ligands with subnanomolar affinity and preference for the σ_2 binding site. 2. Spiro-joined benzofuran, isobenzofuran, and benzopyran piperidines. *J. Med. Chem* 1995;38:2009–2017. [PubMed: 7783132]
25. Soby K, Mikkelsen JD, Meier E, Thomsen C. Lu 28-179 labels a σ_2 -site in rat and human brain. *Neuropharmacology* 2002;43:95–100. [PubMed: 12213263]
26. Ostenfeld MS, Fehrenbacher N, Høyer-Hansen M, Thomsen C, Farkas T, Jäättelä M. Effective tumor cell death by sigma-2 receptor ligand siramesine involves lysosomal leakage and oxidative stress. *Cancer Research* 2005;65:8975–8983. [PubMed: 16204071]
27. Vilner BJ, Bowen WD. Modulation of cellular calcium by sigma-2 receptors: release from intracellular stores in human SK-N-SH neuroblastoma cells. *J. Pharmacol. Exp. Therap* 2000;292:900–911. [PubMed: 10688603]
28. Bowen WD, Vilner BJ, Williams W, Bertha CM, Kuehne ME, Jacobson AE. Ibogaine and its congeners are σ_2 receptor-selective ligands with moderate affinity. *Eur. J. Pharmacol* 1995;279:R1–R3. [PubMed: 7556375]
29. Mach RH, Smith CR, Childers SR. Ibogaine possesses a selective affinity for σ_2 receptors. *Life Sciences* 1995;57:PL 57–62.
30. Bonhaus DW, Loury DN, Jakeman LB, To Z, DeSouza A, Eglen RM, Wong EH. [3 H]BIMU-1, a 5-hydroxytryptamine $_3$ receptor ligand in NG-108 cells, selectively labels sigma-2 binding sites in guinea pig hippocampus. *J. Pharmacol. Exp. Therap* 1993;267:961–970. [PubMed: 8246171]
31. Mach RH, Wu L, West T, Whirrett BR, Childers SR. The analgesic tropane analogue ($\pm$)-SM 21 has a high affinity for σ_2 receptors. *Life Sciences* 1999;64:PL131–137. [PubMed: 10096443]
32. Ghelardini C, Galeotti N, Bartolini A. Pharmacological identification of SM-21, the novel σ_2 antagonist. *Pharm. Biochem. Behav* 2000;67:659–662.
33. Nguyen VH, Kassiou M, Johnston GA, Christie MJ. Comparison of binding parameters of sigma 1 and sigma 2 binding sites in rat and guinea pig brain membranes: novel subtype-selective trishomocubanes. *Eur J Pharmacol* 1996;311:233–240. [PubMed: 8891604]
34. Azzariti A, Colabufo NA, Berardi F, Porcelli L, Niso M, Simone GM, Perrone R, Paradiso A. Cyclohexylpiperazine derivative PB28, a σ_2 agonist and σ_1 antagonist receptor, inhibits cell growth, modulates P-glycoprotein, and synergizes with anthracyclines in breast cancer. *Mol. Cancer Therap* 2006;5:1807–1816. [PubMed: 16891467]
35. Mach RH, Yang B, Wu L, Kuhner RJ, Whirrett BR, West T. Synthesis and sigma receptor binding affinities of 8-azabicyclo[3.2.1]octan-3 α -yl and 9-azabicyclo[3.3.1]nonan-3 α -yl phenylcarbamates. *Med. Chem. Res* 2001;10:339–355.
36. Mach RH, Vangveravong S, Huang Y, Yang B, Blair JB, Wu L. Synthesis of N-substituted 9-azabicyclo[3.3.1]nonan-3 α -yl phenylcarbamate analogs as sigma-2 receptor ligands. *Med. Chem. Res* 2003;11:380–398.
37. Vangveravong S, Xu J, Zeng C, Mach RH. Synthesis of N-substituted 9-azabicyclo[3.3.1]nonan-3 α -yl phenylcarbamate analogs as σ_2 receptor ligands. *Bioorg. Med. Chem* 2006;14:6988–6897. [PubMed: 16837201]
38. Zeng C, Vangveravong S, Xu J, Chang KC, Hotchkiss RS, Wheeler KT, Shen D, Zhuang Z-P, Kung HF, Mach RH. Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy. *Cancer Research* 2007;67:6708–6716. [PubMed: 17638881]
39. Mach RH, Huang Y, Freeman RA, Wu L, Vangveravong S, Luedtke RR. Conformationally-flexible benzamide analogues as dopamine D $_3$ and σ_2 receptor ligands. *Bioorg. Med. Chem. Lett* 2004;14:195–202. [PubMed: 14684327]
40. Chu W, Tu Z, McElveen E, Xu J, Taylor M, Luedtke RR, Mach RH. Synthesis and in vitro binding of N-phenyl piperazine analogs as potential dopamine D $_3$ receptor ligands. *Bioorg. Med. Chem* 2005;13:77–87. [PubMed: 15582454]
41. Walker JM, Bowen WD, Goldstein SR, Roberts AH, Patrick SL, Hohmann AG, DeCosta B. Autoradiographic distribution of [3 H](+)-pentazocine and [3 H]1,3-dio-tolylguanidine (DTG)

- binding sites in guinea pig brain: a comparative study. *Brain Research* 1992;581:33–38. [PubMed: 1323368]
42. Bouchard P, Quirion R. [³H]1,3-di-o-tolylguanidine and [³H](+)-pentazocine binding sites in rat brain: autoradiographic visualization of the putative sigma-1 and sigma-2 receptor subtypes. *Neuroscience* 1997;76:467–477. [PubMed: 9015331]
43. Torrence-Campbell C, Bowen WD. Differential solubilization of rat sigma-1 and sigma-2 receptors: retention of sigma-2 sites in particulate fractions. *Eur. J. Pharmacol* 1996;304:201–210. [PubMed: 8813603]
44. Gebreselassie D, Bowen WD. Sigma-2 receptors are specifically localized to lipid rafts in rat liver membranes. *Eur. J. Pharmacol* 2004;493:19–28. [PubMed: 15189760]
45. Xu J, Tu Z, Jones LA, Wheeler KT, Mach RH. [³H]N-[4-(3,4-dihydro-6,7-dimethoxy-isoquinolin-2(1H)-yl)butyl]-2-methoxy-5-methylbenzamide: a Novel Sigma-2 Receptor Probe. *Eur. J. Pharmacol* 2005;525:8–17. [PubMed: 16289030]
46. Colabufo NA, Abate C, Contino M, Inglese C, Ferorelli S, Berardi F, Perrone R. Tritium radiolabeling of PB28, a potent sigma-2 receptor ligand: pharmacokinetic and pharmacodynamic characterization. *Bioorg. Med. Chem. Lett* 2008;18:1484–1488. [PubMed: 18248813]
47. John CS, Gulden ME, Vilner BJ, Bowen WD. Synthesis, in vitro validation and in vivo pharmacokinetics of [¹²⁵I]N-[2-(4-iodophenyl)ethyl]-N-methyl-2-(1-piperidinyl)ethylamine: a high affinity ligand for imaging sigma receptor positive tumors. *Nucl. Med. Biol* 1996;23:761–766. [PubMed: 8940718]
48. Waterhouse RN, Chaption J, Izard B, Donald A, Belbin K, O'Brien JC, Collier TL. Examination of four ¹²³I-labeled piperidine-based sigma receptor ligands as potential melanoma imaging agents: initial studies in mouse tumor models. *Nucl. Med. Biol* 1997;24:587–593. [PubMed: 9316089]
49. John CS, Bowen WD, Fisher SJ, Lim BB, Geyer BC, Vilner BJ, Wahl RL. Synthesis, in vitro pharmacologic characterization, and preclinical evaluation of N-[2-(1'-piperidinyl)ethyl]-3-[¹²⁵I] iodo-4-methoxybenzamide (P[¹²⁵I]MBA) for imaging breast cancer. *Nucl. Med. Biol* 1999;26:377–382. [PubMed: 10382840]
50. Hou C, Tu Z, Mach RH, Kung HK, Kung M-P. Characterization of a novel radioiodinated sigma-2 receptor ligand as a cell proliferation marker. *Nucl. Med. Biol* 2006;33:203–209. [PubMed: 16546674]
51. Mach RH, Huang Y, Buchheimer N, Kuhner R, Wu L, Morton TE, Wang L-M, Ehrenkauser RL, Wallen CA, Wheeler KT. [¹⁸F]N-4'-Fluorobenzyl-4-(3-bromophenyl)acetamide for imaging the sigma receptor status of tumors: comparison with [¹⁸F]FDG and [¹²⁵I]IUDR. *Nucl. Med Biol* 2001;28:451–458. [PubMed: 11395319]
52. Schwartz JL, Tamura Y, Jordan R, Grierson JR, Krohn KA. Monitoring tumor cell proliferation by targeting DNA synthetic processes with thymidine and thymidine analogs. *J. Nucl. Med* 2003;44:2027–2032. [PubMed: 14660729]
53. Chu W, Xu J, Zhou D, Zhang F, Jones LA, Wheeler KT, Mach RH. New N-substituted 9-azabicyclo [3.3.1]nonan-3α-yl phenylcarbamate analogs as σ₂ receptor ligands: synthesis, in vitro characterization, and evaluation as PET imaging and chemosensitization agents. *Bioorg. Med. Chem.* in press.
54. Tu Z, Dence CS, Ponde DE, Jones L, Wheeler KT, Welch MJ, Mach RH. Carbon-11 labeled sigma-2 receptor ligands for imaging breast cancer. *Nucl. Med. Biol* 2005;32:423–430. [PubMed: 15982571]
55. Tu Z, Xu J, Jones LA, Li S, Dumsdorf C, Vangveravong S, Chen D, Wheeler KT, Welch MJ, Mach RH. Fluorine-18 labeled benzamide analogs for imaging the sigma-2 receptor status of solid tumors with positron emission tomography. *J. Med. Chem* 2007;50:3194–204. [PubMed: 17579383]
56. Rowland DJ, Tu Z, Xu J, Ponde D, Mach RH, Welch MJ. Synthesis and in vivo evaluation of 2 high-affinity ⁷⁶Br-labeled sigma2-receptor ligands. *J. Nucl. Med* 2006;47:1041–1048. [PubMed: 16741315]
57. John CS, Lim BB, Geyer BC, Vilner BJ, Bowen WD. ^{99m}Tc-Labeled sigma-binding complex: synthesis, characterization, and specific binding to human ductal breast carcinoma (T47D) cells. *Bioconjug. Chem* 1997;8:304–309. [PubMed: 9177835]
58. Friebe M, Mahmood A, Bolzati C, Drews A, Johannsen B, Eisenhut M, Kraemer D, Davison A, Jones AG. [^{99m}Tc]Oxotechnetium (V) complexes of amine-amide-dithiol chelates with dialkylaminoalkyl

- substituents as potential diagnostic probes for malignant melanoma. *J. Med. Chem* 2001;44:3132–3140. [PubMed: 11543682]
59. Pham TQ, Berghofer P, Liu X, Greguric I, Dikic B, Ballantyne P, Mattner F, Nguyen V, Loc'h C, Katsifis A. Preparation and biologic evaluation of a novel radioiodinated benzylpiperazine, ¹²³I-MEL037, for malignant melanoma. *J. Nucl. Med* 2007;48:1348–1356. [PubMed: 17631542]
60. Eisenhut M, Hull WE, Mohammed A, Mier W, Lay D, Just W, Gorgas K, Lehmann WD, Haberkorn U. Radioiodinated N-(2-diethylaminoethyl)benzamide derivatives with high melanoma uptake: structure-affinity relationships, metabolic fate, and intracellular localization. *J. Med. Chem* 2000;43:3913–3922. [PubMed: 11052796]
61. Mach RH, Wheeler KT, Blair S, Yang B, Day CS, Blair JB, Choi SR, Kung KF. Preparation of a technetium-99m SPECT agent for imaging the sigma-2 receptor status of solid tumors. *J. Labelled. Cpd. Radiopharm* 2001;44:899–908.
62. Choi S-R, Yang B, Plossl K, Chumpradit S, Wey S-P, Acton PD, Wheeler KT, Mach RH, Kung HF. Development of a Tc-99m labeled sigma-2 receptor-specific ligand as potential breast tumor imaging agent. *Nucl. Med. Biol* 2001;28:657–666. [PubMed: 11518647]
63. Crawford KW, Coop A, Bowen WD. Sigma-2 Receptors regulate changes in sphingolipid levels in breast tumor cells. *Eur. J. Pharmacol* 2002;443:207–209. [PubMed: 12044812]
64. Zeng C, Vangveravong S, Xu J, Chang KC, Hotchkiss RS, Wheeler KT, Shen D, Zhuang Z-P, Kung HF, Mach RH. Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy. *Cancer Research* 2007;67:6708–6716. [PubMed: 17638881]
65. Crawford KW, Bowen WD. Sigma-2 receptor agonists activate a novel apoptotic pathway and potentiate antineoplastic drugs in breast tumor cell lines. *Cancer Research* 2002;62:313–322. [PubMed: 11782394]
66. Chwieralski CE, Welte T, Buhling F. Cathepsin-regulated apoptosis. *Apoptosis* 2006;11:143–149. [PubMed: 16502253]
67. Brown DA, London E. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol* 1998;14:111–136. [PubMed: 9891780]
68. Simons K, Toomre D. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol* 2000;1:31–39. [PubMed: 11413487]

Figure 1. Differences in the density of the σ_2 receptors in 66P and 66Q cells (A) and the upregulation kinetics of the σ_2 receptors in 66 cells during the Q to P transition and the downregulation kinetics during the P to Q transition (B).

Figure 2.
Structures of ligands that are selective for σ_2 versus σ_1 receptors (Ki values).

Figure 3.
 σ_2 receptor ligands based on the granatane analog, **1** (Ki values).

Figure 4.

Structures of the Conformationally-flexible benzamide analogs. Receptor affinity measurements are K_i values for the respective receptors.

Figure 5.
Radiolabeled σ_2 receptor ligands for in vitro binding studies.

Figure 6.

Tumor uptake and biodistribution studies of $[^{18}\text{F}]\mathbf{16}$ under no-carrier added and σ_1 blocking conditions. Data for the metabolic tracer, $[^{18}\text{F}]\text{FDG}$, and the thymidine analog, $[^{125}\text{I}]\text{IUDR}$, are included for comparison.

Figure 7. MicroPET imaging studies with [^{18}F]**16** under no-carrier-added (nonselective) and σ_1 blocking (σ_2 selective) conditions. Note the higher background in normal tissues under the nonselective condition.

Figure 8.
In vivo studies with ^{11}C -labeled conformationally-flexible benzamide analogs

Figure 9. Structures and in vitro binding affinities (K_i values) of the ortho-2-fluoroethoxy conformationally-flexible benzamide analogs

MicroPET/MicroCT

Figure 10.

MicroPET/MicroCT imaging study of $[^{18}\text{F}]\mathbf{18}$ at 2 h post-i.v. injection of the radiotracer. Note the high uptake of the radiotracer in the EMT6 mammary adenocarcinoma and low uptake in the surrounding normal tissues.

Figure 11.

Comparison of the σ_2 receptor imaging agent, [⁷⁶Br]8 (right) with the thymidine analog, [¹⁸F]FLT (left) in EMT-6 tumors. Note the high signal : noise ratio of [⁷⁶Br]8, relative to [¹⁸F]FLT.

Figure 12. Structures of ^{99m}Tc -labeled agents having a moderate to high affinity for σ_2 receptors. Affinity measurements are K_i values.

Figure 13.

Two-photon microscopy studies with **SW107**. These “Tracker” co-localization studies reveal that σ_2 receptors are found in the mitochondria (A), lysosomes (B), endoplasmic reticulum (C), and plasma membrane (D), but not in the nucleus.

Figure 14.

A: Time-lapsed confocal microscopy studies with K05-138 showing that the σ_2 receptors are located in the cytoplasm, but not in the nucleus. B: The rapid uptake of the fluorescent probe in MDA-MB-435 cells suggested that internalization of the probe may occur by receptor-mediated endocytosis. C: Blocking studies with PAO, a known inhibitor of endocytosis, confirm that the internalization of the σ_2 receptor probe is, in part, by receptor-mediated endocytosis.

Table 1

Radionuclidic properties of radioisotopes used in PET, SPECT and autoradiography.

Radionuclide	Half-Life	Nuclear Reaction	Maximum Specific Activity (Ci/mmol)*	Use
H-3	12.35 years	-	29	Scatchard studies and autoradiography
I-125	59.7 days	Xe-114(n, γ)Xe-125→I-125	2.18×10^3	Scatchard studies and autoradiography
C-11	20.4 min.	N-14(p, α)C-11	9.18×10^6	PET
F-18	109.8 min.	O-18(p,n)F-18	1.70×10^6	PET
Br-76	16.2 hr.	Se-76(p,n)Br-76	1.93×10^5	PET
I-123	13.2 hrs.	Te-124(p,2n)I-123	2.36×10^5	SPECT
Tc-99m	6.02 hrs.	Mo-99/Tc-99m generator	5.38×10^5	SPECT

* 100% isotopic abundance