Multifunctional thiosemicarbazones and deconstructed analogues as a strategy to study the involvement of metal chelation, Sigma-2 (Î²) receptor and P-gp protein in the cytotoxic action: In vitro and in vivo activity in pancreatic tumors

This is a pre print version of the following article:

Original Citation:
Multifunctional thiosemicarbazones and deconstructed analogues as a strategy to study the involvement of metal chelation, Sigma-2 (Î²) receptor and P-gp protein in the cytotoxic action: In vitro and in vivo activity in pancreatic tumors / Pati, Maria Laura; Niso, Mauro; Spitzer, Dirk; Berardi, Francesco; Contino, Marialessandra; Riganti, Chiara; Hawkins, William G.; Abate, Carmen. - In: EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY. - ISSN 0223-5234. - 144(2018), pp. 359-371.

Availability:
This version is available http://hdl.handle.net/2318/1657298 since 2018-01-12T16:56:38Z

Published version:
DOI:10.1016/j.ejmech.2017.12.024

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)
Title: Multifunctional Thiosemicarbazones and Deconstructed Analogues as a Strategy to Study the Involvement of Metal Chelation, Sigma-2 (σ2) Receptor and P-gp Protein in the Cytotoxic Action: in vitro and in vivo Activity in Pancreatic Tumors

Article Type: Research Paper

Keywords: sigma-2 receptors; pancreatic cancer; multifunctional compounds; thiosemicarbazones; mitochondrial superoxide

Abstract: The aggressiveness of pancreatic cancer urgently requires more efficient treatment options. Because the sigma-2 (σ2) receptor was recently proposed as a promising target for pancreatic cancer therapy, we explored our previously developed multifunctional thiosemicarbazones, designed to synergistically impair cell energy levels, by targeting σ2 and P-gp proteins and chelating Iron. A deconstruction approach was herein applied by removing one function at a time from the potent multifunctional thiosemicarbazones 1 and 2, to investigate the contribution to cytotoxicity of each target involved. The results from in vitro (panel of pancreatic tumor cells) and in vivo experiments (C57BL/6 bearing KP02 tumor), suggest that while the multifunctional activity was not required for the antitumor activity of these thiosemicarbazones, σ2-targeting appeared to allow alternative tumor cell death mechanisms, leading to potent and less toxic off-targets toxicities compared to other thiosemicarbazones devoid of σ2-targeting.
Dear Editor,

After a thorough revision, I would like to submit the revised version of the manuscript entitled:

“Multifunctional Thiosemicarbazones and Deconstructed Analogues as a Strategy to Study the Involvement of Metal Chelation, Sigma-2 (σ2) Receptor and P-gp Protein in the Cytotoxic Action: in vitro and in vivo Activity in Pancreatic Tumors”

Maria Laura Pati, Mauro Niso, Dirk Spitzer, Francesco Berardi, Marialessandra Contino, Chiara Riganti, William G Hawkins, and Carmen Abate

We thank the reviewers for their careful analysis of the manuscript and incorporated most of the suggestions and changes required. Because of an additional experiment performed in order to respond to one of the reviewers, another co-author was added to the list (Prof. Chiara Riganti). We revised and corrected the manuscript for the syntax errors as suggested. We dealt with the comments as reported in the ‘Response to reviewers’ and we think that through these changes the quality of the manuscript improved.

With my best regards
Carmen Abate
Reviewer 1
We thank this reviewer for the careful reading of the manuscript and the changes suggested that we dealt with as follows:
1. The sentence '...worthy of note is that sigma-2-targeting appears to allow a more specific targeted delivery to tumors...' was removed from the abstract as suggested.
2. Dosages of the compounds. In Figure 3A, 3B and TOC were adjusted according with the reviewer.
3. The syntax errors pointed out were fixed (e.g. 'ovexepressing' and 'filed').

Reviewer #2:
We took into account this reviewer's suggestions and determined the sigma-1 binding of the three best compounds 1, 2 and 7 using the flow cytometry approach that we lately set up and published [42] on MCF7 cells overexpressing sigma-1. The experimental is reported in the main body of the manuscript and a sentence was inserted in the Thiosemicarbazones reduces tumor volume in preclinical model of pancreatic cancer. Flow cytometry curves are reported in the Supporting Information. As expected from their structures, the three compounds do not show any binding with sigma-1 receptor (No displacement of the fluorescent tracer with sigma-1 reference ligands for compounds 1 and 7 and just an insignificant shift of the curves for compound 2). Therefore, binding of the three compounds with sigma-1 receptor has been ruled out and their activity as antiproliferative agents cannot be related to the interaction with the sigma-1 subtype.

Reviewer #3:
We thank this reviewer for the careful reading of the manuscript. His/her comments were addressed as follows:
1. p.4, "I would say chelating metal ion is an independant action rather than hitting a target, as this action may involve many targets (e.g. metalloenzymes)."
   This referee is completely right: hitting a target was more a lexical 'strategy' in order to involve in one sentence all the actions of the compounds. Where possible, we changed the verb 'hit' with 'involve'.
2. p.4, "The statement "sigma2 receptor affinity and/or P-gp modulation is not required for the cytotoxic activity" and the reasoning by the authors may not convince the readers like me."
   We believe that the 'conclusion' is straightforward: Compound 7 only differs from 1 and 2 for the absence of the sigma-2 targeting moiety, as also confirmed by the binding. If compound 7 is endowed with cytotoxic activity, it is not for the interaction with sigma-2 receptors, which is absent in this compound. The same applies to P-gp modulation.
3. p.9, "Cell pathways activated" section, I can not see the results anywhere. Or, it should be integrated with the "Caspase-3 activation" section?"
   This paragraph was a sort of introduction to the rest of the assays. We rearranged the content in order to make it clearer, and changed the title as "Studies on cell mechanisms involved in the activity".
4. "Although demonstrating their efficacy in in vitro and in vivo assays, 1 and 2 may exhibit less ideal ligand efficiency, compared with 7 as well as other reported sigma2 agonists. It is suggested to make a comparison and analyze the result and its impact on the value of their compounds."

Some of the concepts pointed out were further extended, though we believe that the binding affinity at sigma receptors as well as the impact of each structure to the overall effect and in each pathways studied of these ligands were already extensively treated (both in tables figures and body of text). We now think that further repetitions/extension of these concepts could be redundant.

5. Syntax errors as well as the English language were revised.

6. HR-MS was provided for all new compounds.

7. Highlight was inserted.

Reviewer #4:
We thank this reviewer for his/her comments and pointing out some errors. We took into account most of the suggestions as follows:

1. "13 C NMR and IR spectra data was needed for the characterization of the new synthesized compounds."

13 C NMR were inserted for novel compounds as well as HRMS spectra data. IR spectra were not recorded for a problem with the IR spectrometer. However, we feel that 1H-NMR, LC-MS, HR-MS and 13 C NMR spectra represent abundant criteria for the identification of the new substances.

2. "In figure 1 and table 1, "Values represent the mean of n ≥ 2 separate experiments in duplicate ± SEM". However, the mean should always be n ≥ 3."

The experiments were actually repeated three times, a 'copy and paste' procedure was responsible for the mistake.

3. Dosages and formal errors were fixed in the revised version.

4. References style was fixed in the revised version.
Less toxicity of $\sigma_2$ target compounds 1 and 2 versus metal chelator 7
Highlights

- Metal Chelation, $\sigma_2$ and P-gp proteins interaction in multifunctional thiosemicarbazones.
- Deconstruction of potent multifunctional thiosemicarbazones.
- Multifunctional ligands reduce pancreatic cancer growth in mouse stroma dense model.
- Diverse death pathways from compounds sharing $N,N$-dimethyl-thiosemicarbazone.
- Different sensitization of cells useful for developing tailored treatments.
Multifunctional Thiosemicarbazones and Deconstructed Analogues as a Strategy to Study the Involvement of Metal Chelation, Sigma-2 (σ2) Receptor and P-gp Protein in the Cytotoxic Action: in vitro and in vivo Activity in Pancreatic Tumors

Maria Laura Pati,a,b Mauro Niso,a Dirk Spitzer,b Francesco Berardi,a Marialessandra Contino,a Chiara Riganti,c William G Hawkins,b,d and Carmen Abatea,d*

aDipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari ALDO MORO, Via Orabona 4, I-70125 Bari, Italy.
bDepartment of Surgery, Division of Hepatobiliary, Pancreatic, and Gastrointestinal Surgery, Washington University School of Medicine, St. Louis, MO, USA.
cDipartimento di Oncologia, Università degli Studi di Torino, via Santena 5/bis, I-10153, Torino, Italy.
dCo-last authors

*To whom correspondence should be addressed. Tel.: +39-080-5442231. E-mail: carmen.abate@uniba.it

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; Cr, Creatinine; i.p, intraperitoneal; ROS, reactive oxygen species;

Supplementary Information: Synthesis of intermediate compound 12 and final compounds 13 and 14. Western blot for P-gp detection in KP02 cell. ROS involvement, caspase-3 activity and
mitochondrial superoxide detection in AsPC1 and Panc02 cell lines. Flow cytometry curves for $\sigma_1$ binding of references compounds and 1, 2 and 7.

Abstract
The aggressiveness of pancreatic cancer urgently requires more efficient treatment options. Because the sigma-2 ($\sigma_2$) receptor was recently proposed as a promising target for pancreatic cancer therapy, we explored our previously developed multifunctional thiosemicarbazones, designed to synergistically impair cell energy levels, by targeting $\sigma_2$ and P-gp proteins and chelating Iron. A deconstruction approach was herein applied by removing one function at a time from the potent multifunctional thiosemicarbazones 1 and 2, to investigate the contribution to cytotoxicity of each target involved. The results from in vitro (panel of pancreatic tumor cells) and in vivo experiments (C57BL/6 bearing KP02 tumor), suggest that while the multifunctional activity was not required for the antitumor activity of these thiosemicarbazones, $\sigma_2$-targeting appeared to allow alternative tumor cell death mechanisms, leading to potent and less toxic off-targets toxicities compared to other thiosemicarbazones devoid of $\sigma_2$-targeting.
1. Introduction

According to the latest Cancer Statistics’ report [1], cancer death rates have been decreasing by 23% since 1991. Despite that, cancer overtook cardiovascular diseases becoming the first cause of death in 21 states within US, while death rates are increasing for cancers of the liver and pancreas. Pancreatic cancer is one of the most aggressive diseases characterized by a rapid progression, high probability of local recurrence and occurrence of early liver metastases. [2] It represents the fourth cause of death for cancer related mortalities with a very poor prognosis [3], and less than 8% five years survival rate. Currently, for patients that are diagnosed early enough, surgery represents the preferred treatment option, further supported by radiation- and gemcitabine-based chemotherapy, but all of these treatment regimens generally result in heterogeneous response rates with a high probability of developing recurrent disease [4-6]. Pancreatic cancer ultimately develops resistance to gemcitabine and thus requires an urgent need of identifying more efficient treatment options as well as better understanding the deranged molecular pathways characteristic for this debilitating malignancy.

In recent years, many attempts to harness the $\sigma_2$ receptor as a tumor-specific molecular target for pancreatic cancer therapy have emerged. The $\sigma_2$ receptor belongs to the $\sigma$ receptors family, divided in $\sigma_1$ and $\sigma_2$ proteins. After being proposed as a histone [7,8], the $\sigma_2$ receptor was later identified as the progesterone receptor membrane component 1 (PGRMC1) protein complex [9]. However, mounting evidence suggested that the $\sigma_2$ receptor might actually not be related to PGRMC1 [10-12] and has thus been recently proposed to be the endoplasmic reticulum ER-resident membrane protein TMEM97 [13]. Despite such a controversial identification, the $\sigma_2$ receptor has been increasingly studied due to its overexpression and activity in a number of human tumors. $\sigma_2$ Ligands are able to selectively induce tumor cells death through mechanisms that may involve caspase-dependent and -independent apoptosis, lysosomal membrane permeabilization, generation of reactive oxygen species (ROS), and autophagy [14-20].
Recently, the mitochondrial superoxide pathway has been recognized as $\sigma_2$ receptor-activated process in pancreatic cancer cells [21]. Importantly, certain $\sigma_2$ receptor agonists have shown efficacy in preclinical tumor models of pancreatic cancer [22-25] highlighting the notion that this receptor is a promising target for cancer therapy. In an effort to develop new drugs that target multiple biologic functions with a single molecule (i.e. multi-target approach), we recently developed a novel class of $\sigma_2$ receptor ligands, capable of chelating metal ions through their thiosemicarbazone moiety [20]. These molecules were also able to interact with the P-glycoprotein (P-gp) efflux pump, i.e. the most well-described ABC ‘polyspecific’ transporter, whose overexpression is associated with poor prognosis and poor quality of life in cancer patients. Our choice to focus on these particular cellular targets was made based on recent evidence that cancer cells are exquisitely vulnerable to changes in energy levels due to their increased metabolism to sustain rapid cell proliferation. Accordingly, a simultaneous action on the three targets would synergistically compromise cells energy levels. Accordingly, engaging these three drug targets simultaneously was projected to compromise the cellular energy levels in a synergistic manner, because i) $\sigma_2$ receptor ligand cytotoxicity is correlated with lysosomal membrane permeabilization and an increase in ROS production [18,25]; ii) metal chelators that complex endogenous ironII/III are known as redox cycling agents that also result in increased production of ROS; and iii) P-gp modulation with activation of the futile ATP-cycle would lead to increases in oxidative phosphorylation, subsequently increasing the level of ROS production [26]. Therefore, our multifunctional thiosemicarbazones were anticipated to combine the cytotoxic properties of $\sigma_2$ agonists with the ironII/III chelating and potentially P-gp modulating capacity within a single drug conjugate, thereby creating a highly lethal and cancer-directed therapy option.

Among all the novel $\sigma_2$ thiosemicarbazone ligands, two compounds, i.e. (Z)-2-(1-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2-oxoindolin-3-ylidene)-N,N-dimethylhydrazinecarbothioamide 1 and (Z)-2-[1-[4-(4-Cyclohexylpiperazin-1-yl)butyl]-2-
oxoindolin-3-ylidene]-N,N-dimethylhydrazinecarbothioamide 2 (Figure 1), stood out due to their promising cytotoxic activity in two cell line pairs: MCF7 breast cancer cells, A549 lung cancer cells and their corresponding doxorubicin-resistant cell lines, namely MCF7dx and A549dx, both overexpressing P-gp (EC_{50}s, Figure 1) [20]. With the aim of contributing to the proposal of alternative strategies for treating pancreatic cancer, herein we studied the effects of 1 and 2 in diverse pancreatic cancer cells. Because of the multifunctional nature of these molecules, we aimed to study the impact of each target involved to the overall effect, as well as on the respective mechanism leading to cell death. In order to do that, we applied a structural deconstruction approach on thiosemicarbazones 1 and 2. Structural modifications were carried out so that the metal chelating moiety or the interaction with σ_2 receptor and/or with P-gp were alternatively removed. Besides their affinity to the σ_2 receptor, all ‘deconstructed’ analogues were evaluated for their interaction capacity with P-gp, primarily because it has been recently shown that P-gp could play a critical role in the acquired resistance of pancreatic cancer to gemcitabine [27]. For all of the compounds, cytotoxic activity and pathways activated (e.g. caspase-3 activity, ROS and mitochondrial superoxide generation) were studied in a panel of pancreatic cancer cells, with the attempt to shed new light on the contribution of the targets involved in the mechanisms of cells death which also depend on the different sensitivities of diverse cells. The most promising compounds were tested in a pre-clinical tumor model of syngeneic KP02 pancreatic cancer cells, which are known for closely mimicking the human disease with regard to a highly fibrotic tumor stroma, providing valuable hints about the beneficial effects of a multifunctional, σ_2 ligand-based cancer therapeutics.

2. Results and Discussion

2.1. Chemistry

The synthetic scheme for the synthesis of isatin-β-thiosemicarbazones 7, 8 and 9 is depicted in Scheme 1. 3,4-Dihydroisoquinolin-1(2H)-one 3 [28] was alkylated with 1-bromo-4-chlorobutane
using NaH as a base to afford 2-(3-chlorobutyl)-3,4-dihydroisoquinolin-1(2H)-one 4 which was used to alkylate isatin 5 providing intermediate 6. Isatins 5 or 6, were dissolved in hot ethanol and treated with thiosemicarbazide or N,N-dimethyl-3-thiosemicarbazide to provide the final thiosemicarbazones 7, 8 and 9 respectively. In Scheme 2 the synthesis of the final compounds 10, 11, 13 and 14 is illustrated. The already known compounds 13 and 14 [29] were obtained through a slightly modified procedure. Treatment of indole with 1-bromo-4-chlorobutane in the presence of KOH gave the intermediate 12. Final compounds 10-14 were obtained upon alkylation of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline or 1-cyclohexylpiperazine with 1-bromo-butane or with intermediate 12 in the presence of K$_2$CO$_3$.

2.2. $\sigma_2$ Receptor Binding

For all the compounds, $\sigma_2$ receptor affinity was evaluated by radioligand binding assays and the results are reported as inhibition constants ($K_i$ values) in Table 1. Removal of the N-butyl linked basic moieties (7 and 8), as well as elimination of the basic properties of the N-atom (9), dramatically reduced the $\sigma_2$ receptor affinity (7, 8 and 9, $K_i > 10000$). This outcome was anticipated because of the lack of structural molecule domains that are essential for an interaction with $\sigma_2$ receptor (i.e. basic moieties). On the other hand, butyl-6,7-dimethoxytetrahydroisoquinoline and butyl-cyclohexylpiperazine showed appreciable $\sigma_2$ affinity values (10 and 11, $K_i = 15.0$ nM and 65.2 nM respectively) despite the lack of an important hydrophobic portion required for the optimal interaction with the $\sigma_2$ receptors, according to the $\sigma_2$ receptors pharmacophoric models [30-32]. The presence of the indole hydrophobic portion in the 6,7-dimethoxytetrahydroisoquinoline 13 and cyclohexylpiperazine 14 derivatives, that mimicks the isatin ring in 1 and 2, led a 3- to 30-fold increase in the $\sigma_2$ affinity ($K_i = 3.66$ nM and 1.90 nM respectively) compared to 10 and 11, in agreement with the $\sigma_2$ receptor’s pharmacophore.

2.3. Calcein-AM assay
For all the novel deconstructed analogues of thiosemicarbazones 1 and 2, interaction with P-gp was investigated employing the Calcein-AM assay. Results as EC₅₀ values are reported in Table 1. Lead compounds 1 and 2 moderately inhibited P-gp (EC₅₀ = 3.04 μM and 2.83 μM, respectively) [20], whereas the absence of the N-butyl-linked basic moiety at the isatin nucleus (7 and 8) completely abolished the interaction with the P-gp, demonstrating how the sole isatin-thiosemicarbazone portion is unable to interact with the efflux pump (Table 1). In accordance with previous SAfiR analyses [33], removal of the basic character of the N-atom in the tetrahydroisoquinoline ring through the replacement of 6,7-dimethoxytetrahydroisoquinoline with the corresponding amide 3,4-dihydrisoquinolin-(2H)-1-one (9), kept a moderate interaction with the efflux pump (EC₅₀ = 11.0 μM) [33]. On the other hand, 2-butyl-6,7-dimethoxytetrahydroisoquinoline (10) and 1-butyl-cyclohexylpiperazine (11) respectively showed modest (EC₅₀ = 76.4 μM) or a complete loss of interaction with P-gp. Conversely, the corresponding indole derivatives 13 and 14 modulated the efflux pump in the low micromolar or submicromolar range (EC₅₀ = 6.09 μM and 0.46 μM, respectively), indicative of a key importance of the the hydrophobic portion within these derivatives for the interaction with P-gp.

2.4. Cytotoxic activity

The cytotoxic activities of 1 and 2 and their deconstructed analogues were in vitro measured in a panel of human (MIAPaCa-2, BxPC3, AsPC1 and Panc-1) and mouse (Panc02, KP02 and KCKO) pancreatic cancer cell lines, which were anticipated to display differential sensitivities to structurally diverse σ₂ receptor ligands, as already shown [21]. Cytotoxic activities of the ligands are expressed as EC₅₀ values in Table 2. Thiosemicarbazones 1 and 2 generated potent cytotoxic effects in all the pancreatic cells studied, in line with the potent efficacy showed in the MCF7/MCF7dx and A549/A549dx cell lines pairs (Figure 1) [20]. These compounds displayed a potent activity even in the human pancreatic cancer cell line PANC-1 for which other σ₂ ligands previously developed, exhibited no or only minimal cytotoxic activity [21]. Therefore, combination
of the \( \sigma_2/P\)-gp targeting moieties with ironII/III chelator properties of the thiosemicarbazone group in a single structure (1 and 2) resulted in cytotoxic agents with potent activity in pancreatic tumors (EC\(_{50}\) values ranging from 1.17 \( \mu \)M to 14.66 \( \mu \)M). In order to evaluate the contribution of the metal chelator properties alone on the overall activity profile of 1 and 2, the \( \sigma_2/P\)-gp targeting moiety (i.e. N-butyl-linked portions) was removed from the isatin-N-atom, and the thiosemicarbazone structure was kept, in the \( N,N \)-dimethylated (7), and non-dimethylated forms (8), both inactive at \( \sigma_2 \) receptor and at P-gp. Compound 7 determined a strong cytotoxic activity in most of the cell lines studied (EC\(_{50}\) values ranging from 1.21 \( \mu \)M to 3.14 \( \mu \)M) except for MIAPaCa-2 (EC\(_{50}>18.3 \mu M\)) and Panc-1, where curiously no cytotoxic activity was detected (EC\(_{50}>100 \mu M\)). In contrast, the non-dimethylated compound 8 did not show cytotoxic activity in the cell lines tested (EC\(_{50}>100 \mu M\)). This result is consistent with our previous data, in which \( N \)-terminal-dimethylation in thiosemicarbazones resulted in enhanced antitumor activity in comparison to the un-substituted and mono-substituted variants, similarly to what has been reported for other thiosemicarbazones [34-36]. Therefore, independently from the presence of the \( \sigma_2 \) targeting moiety, the absence of the dimethyl substitution at the thiosemicarbazone moiety abolished the cytotoxic activity also in pancreatic cancer cells. Removal of the sole \( \sigma_2 \)-mediated activity from the overall action of 1, was obtained in compound 9, in which the metal chelator and P-gp targeting moieties were retained. Thiosemicarbazone 9 displayed good efficacy in all the pancreatic cancer cell lines tested (EC\(_{50}\) values ranging from 2.18 \( \mu \)M to 10.30 \( \mu \)M). MIAPaCa-2 and Panc-1 responded to this compound the least, which was not unexpected given the lack of sensitivity to compounds 1 and 2 and suggests that these cell lines are less sensitive to thiosemicarbazones in general. The sole contribution of the \( \sigma_2 \) targeting moieties with respect to the overall activity profile of 1 and 2 was studied through the \( N \)-butyl-6,7-dimethoxytetrahydroisoquinoline and 1-butyl-4-cyclohexylpiperazine (10 and 11), and more reliably through their indole-bearing analogues (13 and 14), which are characterized by
higher $\sigma_2$ receptor affinities. While 13 and 14 displayed only a weak activity profile in KP02, KCKO and MIAPaCa-2 cells (EC$_{50}$ values ranging from 40 $\mu$M to 79 $\mu$M), and even further reduced activity in the other pancreatic cancer cell lines studied (EC$_{50}$ > 100 $\mu$M), 10 and 11 were inactive in all the cell lines tested in our current study (EC$_{50}$ > 100 $\mu$M). Since a number of other $\sigma_2$ ligands devoid of metal chelator moieties show cytotoxic activity in pancreatic cancer cells [21,23], the lack of cytotoxic effects in these $\sigma_2$ ligands may suggest an antagonist activity at the $\sigma_2$ receptor (generally referred as lack of activity).

In summary, the results obtained in pancreatic cancer with our lead thiosemicarbazones 1 and 2 and their deconstructed analogues show that while different pancreatic cell lines respond with differential sensitivity to structurally diverse $\sigma_2$ ligands, their $\sigma_2$ receptor affinity and/or their capacity to modulate P-gp does not seem to be required for their cytotoxic activity profiles. In these molecules, the $N,N$-dimethyl-substituted-thiosemicarbazone appears to play a dominant role for their augmented cytotoxic action, likely because of a good compromise in metal chelation and cell permeation. In any case, it appears that a multitarget approach represents a beneficial constellation, as the $\sigma_2$ receptor is overexpressed in a number of diverse cancers and, in addition to the direct cytotoxic effects of its ligands toward the cancer cells, the $\sigma_2$ receptor could be harnessed for the targeted delivery of drugs to tumor foci. In this regard, the combination of metal chelation and $\sigma_2$-targeting may be a promising strategy for the treatment of human malignancies.

2.5. Studies on cell mechanisms involved in the activity

We next investigated the contribution of the diverse targets hit by the thiosemicarbazones 1 and 2 to the pathways activated that eventually lead to tumor cell death. Therefore, thiosemicarbazones 1 and 2 and their analogues displaying relevant cytotoxic activity (7 and 9) were evaluated using mouse KP02 pancreatic cancer cells, characterized by forming a stroma-rich tumor
microenvironment *in vivo* and thus more closely mimic the human disease.

2.5.1. *P*-gp expression in KP02 tumor cells

In this cell line, where σ₂ ligands previously displayed promising *in vitro* and *in vivo* efficacy [37], we assessed the expression level of P-gp as a mean to determine what role, if any, this efflux pump plays in the context of the cytotoxicity of our drugs. Western blot analysis clearly showed no expression of the efflux pump in KP02 cells, so that activity at the P-gp of these compounds did not seem to be involved in their action in this cell line (Figure S1, Supporting Information). Nevertheless, knowledge about the interaction of these compounds with P-gp may provide useful information in case of their administration in P-gp overexpressing tumor cells.

2.5.2 ROS involvement in the antiproliferative activity

We next explored ROS involvement in KP02 tumor cells employing lipid antioxidant α-tocopherol and the hydrophilic precursor of glutathione N-acetyl-L-cysteine (NAC). The effect of antioxidants was evaluated 48 hours after treatment with 4 μM of 1, 2, 7 and 9 (Figure 2, panel A). Addition of 100 μM α-tocopherol 1 hour prior to drug treatment, rescued the pancreatic cancer cells, thus effectively reducing cell death caused by 1, 2 and 9. By contrast, no cells were rescued by α-tocopherol upon treatment with 7, with no differences between the α-tocopherol treated and untreated cells. The same results were also obtained in AsPC1 and Panc02 tumor cells upon treatment with the same compounds (Figure S2, Supporting Information). Interestingly, ROS production was reduced following treatment with 1, 2 and 9 in the presence of α-tocopherol but not in the presence of NAC. Cell death caused by the four compounds administered at a 4 μM concentration was not rescued by NAC in the KP02 cell line, and even higher levels of cell death were recorded when cells were treated with NAC and 7 or 9 (Figure 2, panel A). These results are in agreement with data obtained upon treatment of pancreatic cancer cells with σ₂ ligands and NAC [21]. We used two different antioxidants on the basis of their different mechanisms: while NAC is a
hydrophilic antioxidant, precursor of glutathione that provides SH-groups to prevent oxidation, \( \alpha \)-tocopherol is a chain-breaking lipophilic antioxidant localized in cellular membranes responsible of their integrity through inhibition of lipid peroxidation. Taken together, these results support the hypothesis that generation of ROS is at least partially responsible for the mechanism of action of these compounds in all the cell lines studied, except for 7. Therefore, the strong cytotoxic activity of 7 can only be explained with a mechanism of action that does not seem to involve ROS production. These data also suggest that, despite of sharing the same thiosemicarbazone-\( \beta \)-isatin structure, ROS generating pathways do not depend on it, as the presence of the N-butyl-linked moieties in thiosemicarbazones 1, 2 and 9 triggers a pathway that is different from that of compound 7.

2.5.3. Caspase-3 activation

\( \sigma_2 \) Ligands have been reported to activate mechanisms that are cell and ligand specific and can cause in some cases caspase-dependent and in others caspase-independent apoptosis [21,25,38-40]. Therefore, activation of caspase-3 was evaluated for thiosemicarbazones 1, 2, 7 and 9 in mouse KP02 tumor cells [41]. Pancreatic mouse adenocarcinoma cells were treated with the ligands (25 \( \mu \)M) for 5 hours and assayed for cleavage of the proluminescent caspase-3 substrate and subsequent generation of a glow-type luminescent signal. Compound 2 induced a good activation of caspase-3, increasing caspase-3 activity by 6-fold \( (p < 0.001) \) while compounds 1, 7, and 9 did not activate the caspase-3 at all (Figure 2, panel B). Despite targeting the same biological targets, thiosemicarbazones 1 and 2, which both increase ROS, did not share caspase-3 activation, in accordance with previously reported \( \sigma_2 \) ligands, whose activation of caspase-3 appeared to be both cell dependent and molecule dependent. Similar results were obtained in AsPC1 and Panc02 tumor cells upon the treatment with the same compounds (Figure S3, Supporting Information).

2.5.4. Superoxide radical detection in the mitochondria of KP02
Since mitochondrial superoxide production was recently identified as a novel mechanism of \( \sigma_2 \) receptor-mediated cell death [21], we wished to study superoxide radical production in mitochondria of KP02 pancreatic cancer cell lines. MitoSOX™ Red reagent is a novel fluorogenic dye specifically targeted to mitochondria in live cells, which is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species (RNS)-generating systems (e.g. such as peroxides, hydroxyl radical, singlet oxygen, nitric oxide and peroxynitrite). KP02 cells were treated with 50 \( \mu \text{M} \) of 1, 2, 7 and 9 for 2 hours (Figure 2, panel C). While \( \sigma_2 \) ligands 1 and 2 induced a strong mitochondrial ROS production with the mean fluorescence intensities increasing by 6-fold and 20-fold respectively, thiosemicarbazones 7 and 9 were not capable of superoxide radical production in the mitochondria of KP02 tumor cells. With the aim of obtaining a more complete picture, we assessed the mitochondrial superoxide production in the presence of the lipid antioxidant \( \alpha \)-tocopherol, and found that it was strongly reduced when cells were treated with \( \sigma_2 \) ligands in the presence of \( \alpha \)-tocopherol, according to the rescue of cell viability displayed in the MTT assay (Figure 2, panel A). Using the same compounds, similar results were obtained in Panc02 and AsPC1 cells (Figure S4, Supporting Information). Taken together, these results demonstrate that the cytotoxic activity of thiosemicarbazones 7 and 9, devoid of \( \sigma_2 \) affinity, does not rely on superoxide radical production in the mitochondria. On the other hand, we have confirmed that the mitochondrial superoxide production is as a common pathway shared by the two \( \sigma_2 \)-targeting thiosemicarbazones 1 and 2 in pancreatic cancer cells, in accordance with the results shown by other \( \sigma_2 \) ligands in the same cells [21].

2.6. Thiosemicarbazones reduces tumor volume in preclinical model of pancreatic cancer

Based on the strong \textit{in vitro} antitumor activity and diversity of death pathways activation patterns induced by the most promising thiosemicarbazones 1, 2 and 7, we next investigated these promising drug candidates for efficacy in a syngeneic, genetically-engineered murine stroma-dense cancer model.
model, closely mimicking the human disease. Using this configuration, the in vivo efficacy of the two σ2/P-gp mixed ligands and iron chelators 1 and 2 (that share ROS and mitochondrial superoxide production capacity, but not caspase-3 activation), could be compared with that of the iron chelator 7, that does not act through ROS increase. For these three compounds, interaction with the σ1 receptor, which is also endowed with antitumor properties, was ruled out through a flow cytometry experiment previously set up (Figure S6, Supporting Information) [42].

C57BL/6 female mice were inoculated subcutaneously with 2.5 × 10^5 KP02 cells and a week later, when tumors reached ~ 5-6 mm in diameter, mice were randomized into control and treatments groups of (n = 7-10). Vehicle consisting of 25% Cremophor in water, multifunctional compounds 1 and 2 (750 nmol/100μL vehicle), or 7 (750 nmol/100μL vehicle) were given by i.p. injection daily for two weeks. After conclusion of treatment, tumors were smaller for mice treated with iron chelator 7 (mean = 328 mm^3), compared to vehicle (mean = 596 mm^3) (Figure 3 panel A; p < 0.0001). Mice treated with compounds 1 and 2 had both tumor volumes that were statistically similar to vehicle, without experiencing any treatment-related deaths. Therefore, we repeated the experiment by employing higher concentrations of compounds 1 and 2 (1500 nmol/100μL vehicle).

At this concentration, both multifunctional compounds were capable of reducing the mean tumor volume (mean = 196 mm^3 for compound 2 and mean = 205 mm^3 for compound 1) compared to vehicle treated group (mean = 592 mm^3) (Figure 3, panel B; p > 0.001). Moreover, compounds 1 and 2 turned out to be more effective in reducing tumor burden than gemcitabine when compared to mice bearing a KP02 orthotopic tumor and treated twice weekly by i.p. injections with 20 mg/kg gemcitabine [37].

Importantly, we did not observe treatment-related deaths or gross abnormalities in mouse behavior. In order to assess for more subtle toxicities, serum chemistries (AST, ALT, BUN, total protein, glucose, and Cr) and complete blood counts were analyzed with no significant differences noted when compared to the control group (Table 3). Necropsy revealed no difference in mouse weights
(vehicle: 19.5 ± 0.5 grams; 7: 19.5 ± 0.5 grams; 1: 17.5 ± 0.5 grams; 2: 19 ± 1 grams; Figure 4; \( p > 0.05 \)). There were no significant lesions in the brain, heart, alimentary tract, kidneys, liver, or pancreas. Only mild peritonitis was identified at the site of repeated drug injections. However, the compound 7 treated group revealed foci of pulmonary metastases of the implanted tumor and a mild chronic progressive nephropathy that were not noticed in mice treated with compounds 1 and 2.

3. Conclusion

Based on the encouraging activity profiles of the multifunctional thiosemicarbazones 1 and 2 that target the \( \sigma_2 \) receptor and the P-gp efflux pump and have Iron chelating properties, a deconstruction approach was applied to investigate the contribution of each target hit to the overall action of these molecules. Because of the urgent need of novel treatments for pancreatic cancers, lead compounds 1 and 2 and the deconstructed analogues (7-11, 13 and 14) were investigated in a panel of pancreatic tumor cells. Among the compounds, \( N,N \)-dimethyl-thiosemicarbazone-bearing derivatives (1, 2, 7, 9) showed potent activity in most of the pancreatic cell lines studied, also in the absence of the \( \sigma_2 \)-targeting moiety (7 and 9), so that the iron chelating structural portion (\( N,N \)-dimethyl-thiosemicarbazone) appeared to be mainly responsible for the cytotoxic activity in these cells (likely because it confers a good balance between activity and cell permeability). Nevertheless, we showed that the death pathways engaged by these compounds (1, 2, 7 and 9) are not governed by the common \( N,N \)-dimethyl-thiosemicarbazones portion since: i) ROS increase was shown by 1, 2, and 9 but not by 7; ii) caspase-3 activity was only increased by \( \sigma_2 \)-targeting thiosemicarbazone 2; iii) mitochondrial superoxide production was only detected with the two \( \sigma_2 \)-targeted thiosemicarbazones 1 and 2. Our deconstructive approach showed that while \( \sigma_2 \) receptor targeting is not necessary for the in vitro cytotoxicity of these multifunctional thiosemicarbazones (except for Panc-1 cells), the presence of the N-butyl linked basic moieties triggers alternative death pathways in comparison to those compounds that only contain metal chelating features, thus allowing for differential sensitization levels of cell death, which we believe could be useful for developing
tailored treatments. Importantly, besides a contribution to the cytotoxic activity (as in Panc-1 cells), \( \sigma_2 \) receptor binding may be beneficial for the targeted delivery to \( \sigma_2 \) receptors overexpressing tumors, according to a promising recent approach [39]. *In vivo* administration of the multifunctional \( \sigma_2 \) ligands 1, 2 or the metal chelator 7 markedly slowed the growth rate in a mouse stroma dense model of pancreatic cancer (C57BL/6 bearing KP02 tumors), demonstrating that these compounds reach their target and exert antitumor activity. While mice treated with the iron chelator 7 revealed foci of pulmonary metastases of the implanted tumor and a mild chronic nephropathy, mice treated with multifunctional compounds 1 and 2 better tolerated the treatment without showing signs of off-target toxicity.

Despite the suggested role that P-gp may play in the resistance of pancreatic cancer to gemcitabine, the absence of the efflux pump in KP02 cells did not allow us to evaluate our compounds in the context of P-gp expression.

Overall, the results from our deconstructive approach from *in vitro* and *in vivo* experiments suggest that while the multifunctional drug composition is not necessary for the antitumor activity of these thiosemicarbazones, \( \sigma_2 \)-targeting may allow for a more specific targeted delivery to tumor foci *in vivo* \( \sigma_2 \) overexpressing tumors) with less toxic off-targets effects, together with the activation of alternative tumor cell death mechanisms. Such multifunctional molecules endowed with potent *in vivo* antitumor activity and reduced off-site toxicity are certainly worth studying further in the oncology field.

**Acknowledgements**

This work was funded in part by a National Institute of Health R01 grant (US NIH 5R01CA16376402) (W.G. Hawkins). This work was conducted under the Agreement regulating cultural and scientific cooperation between the University of Bari ALDO MORO (Italy) and Washington University School of Medicine in St. Louis (USA).
4. Experimental Section

4.1. Chemistry

Both column chromatography and flash column chromatography were performed with 60 Å pore size silica gel as the stationary phase (1:30 w/w, 63–200 μm particle size, from ICN, and 1:15 w/w, 15–40 μm particle size, from Merck, respectively). Melting points were determined in open capillaries on a Gallenkamp electrothermal apparatus. High-performance liquid chromatography (HPLC) on an Agilent Infinity 1260 system equipped with diode array with a multiwavelength UV/vis detector set at λ = 230 nm, 254 nm and 280 nm, through a Phenomenex Gemini RP-18 column (250 × 4.6 mm, 5 μm particle size) was performed on target compounds confirming ≥ 95% purity. 1H NMR spectra were recorded on a Mercury Varian 300 MHz or on a 500-vnmrs500 Agilent spectrometer (499.801 MHz). The following data were reported: chemical shift (δ) in parts per million (ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration, and coupling constant(s) in hertz. 13C NMR (125 MHz) were recorded on a 500-vnmrs500 Agilent spectrometer (499.801 MHz) on novel final compounds: chemical shifts in ppm were reported. Recording of mass spectra was done on an Agilent 1100 series LCMSD trap system VL mass spectrometer; only significant m/z peaks, with their percentage of relative intensity in parentheses, are reported. High resolution mass spectroscopy (HRMS) was performed on a Agilent 6530 Accurate-Mass Q-TOF LC/MS spectrometer. For final compounds 10 and 11, 13C NMR and HRMS spectra were recorded on their hydrochloride salts. Chemicals were from Aldrich, TCI and Alpha Aesar and were used without any further purification.

4.2. 2-(4-Chlorobutyl)-6,7-dimethoxy-3,4-dihydroisoquinolin-1(2H)-one (4)

To a suspension of NaH (0.123 g, 5.12 mmol) in dry DMF (2 mL) a solution of 3 (0.425 g, 2.05 mmol) in dry DMF (3 mL) was added in a dropwise manner. The mixture was stirred at 0 °C for 15 min. Then a solution of 1-bromo-4-chlorobutane (0.3 ml, 2.66 mmol) in dry DMF (2 mL) was
added and the resulting mixture was stirred for 4 h at room temperature. After cooling at 0 °C, the reaction mixture was quenched with water and the solvent concentrated under reduced pressure. The residue was dissolved in H₂O and extracted with CH₂Cl₂ (3 × 10 mL). The collected organic layers were dried over Na₂SO₄ and the solvent was evaporated under reduced pressure to afford the crude as a yellow oil. Purification through column chromatography with CH₂Cl₂ as eluent afforded the title compounds as colorless oil (0.426 g, 70% yield). GC-MS m/z: 297 (M+, 20), 220 (100).

4.3. 1-(4-(6,7-Dimethoxy-1-oxo-3,4-dihydroisoquinolin-2(1H)-yl)butyl)indoline-2,3-dione (6)

A solution of 4 (0.426 g, 1.43 mmol) in CH₃CN (10 mL) was added with K₂CO₃ (2.87 mmol, 0.396 g) and isatin 5 (0.192 g, 1.3 mmol). The resulting mixture was refluxed overnight under stirring. After the removal of the solvent under reduced pressure the residue was taken up with H₂O and extracted with AcOEt (3 × 10 mL). The collected organic layers were dried (Na₂SO₄) and evaporated under reduced pressure to afford a crude oil, which was purified by column chromatography (CH₂Cl₂/AcOEt 1:1) to give the title compound (0.28 g, 50% yield).

1H NMR (500 MHz, CDCl₃) δ 1.72-1.81 (m, 4H, CH₂CH₂CH₂N), 2.92 (t, 2H, J = 6.85 Hz, NCH₂CH₂Ar), 3.53 (t, 2H, J = 6.85 Hz, NCH₂CH₂Ar), 3.64 (t, 2H, J = 6.85 Hz, NCH₂CH₂CH₂CH₂N), 3.81 (t, 2H, J = 6.85 Hz, NCH₂CH₂CH₂CH₂N), 3.90 (s, 3H, OCH₃), 3.92 (s, 3H, OCH₃), 6.64 (s, 1H, aromatic), 7.00 (d, 1H, J = 7.83 Hz, aromatic), 7.10 (t, 1H, J = 7.34 Hz, aromatic), 7.54-7.61 (m, 3H, aromatic).

4.4. General procedure for the synthesis of final compounds 7-9

To a solution of isatins 5 or 6 (0.68 mmol) in Ethanol (10 mL) 4,4-dimethyl-3-thiosemicarbazide (0.68 mmol) was added and the resulting mixture was refluxed overnight. Upon cooling, precipitation of the final product was achieved.

4.4.1. (Z)-N,N-Dimethyl-2-(2-oxoindolin-3-ylidene)hydrazinecarbothioamide (7) Crystallization from H₂O/EtOH afforded the title compound as orange crystals (0.084g, 50% yield); mp = 250-250 °C; 1H NMR (500 MHz, DMSO-d₆) δ 3.35 (s, 6H, CH₃), 6.92-6.94 (m, 1H, aromatic), 7.06-7.09
18

(m, 1H, aromatic), 7.32-7.35 (m, 1H, aromatic), 7.51-7.53 (m, 1H, aromatic), 11.28 (s, 1H, isatin NH), 13.41 (s, 1H, NHCS); 13C NMR (500 MHz, DMSO- d6) 43.75; 43.83; 117.96; 119.45; 124.34; 129.54; 131.31; 134.22; 141.23; 169.51; 177.83. LC-MS (ESI+) m/z: 271 [M+Na]+; LC-MS-MS 271: 226; LC-MS (ESI) m/z 247 [M-H]; LC-MS-MS 247: 204, 174; QTOF (m/z) Calcd for C11H12N4OS [M+Na]+: 271.0630, found: 271.0621. Compound was > 98% pure by HPLC analysis performed with MeOH/H2O, 80 : 20 v/v, at a flow rate 0.8 mL min-1.

4.4.2. (Z)-2-(2-Oxindolin-3-ylidene)hydrazinecarbothioamide (8) Crystallization from H2O/EtOH afforded the title compound as orange crystals (0.07g, 50% yield); mp = 253 – 255 °C; 1H NMR (500 MHz, DMSO- d6) δ 6.90-6.92 (m, 1H, aromatic), 7.06-7.09 (m, 1H, aromatic), 7.32-7.36 (m, 1H, aromatic), 7.63-7.65 (m, 1H, aromatic), 8.67 (s, 1H, NH), 9.03 (s, 1H, NH), 11.19 (s, 1H, isatin NH), 12.46 (s, 1H, NHCS); 13C NMR (500 MHz, DMSO- d6) 117.66; 119.51; 124.54; 129.44; 131.25; 134.65; 141.32; 168.72; 180.82. LC-MS (ESI+) m/z: 243 [M+Na]+; LC-MS-MS 243: 226, 185; LC-MS (ESI) m/z 219 [M-H]; LC-MS-MS 219: 160; QTOF (m/z) Calcd for C9H8N4OS [M+Na]+: 243.0317, found: 243.0307. Compound was > 98% pure by HPLC analysis performed with MeOH/H2O, 80 : 20 v/v, at a flow rate 0.8 mL min-1.

4.4.3. (Z)-2-(1-(4-(6,7-Dimethoxy-1-oxo-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2-oxoindolin-3-ylidene)-N,N-dimethylhydrazinecarbothioamide (9) Crystallization from H2O/EtOH afforded the title compound as yellow crystals (0.173 g, 50% yield); mp = 201-202 °C; 1H NMR (500 MHz, CDCl3) δ 1.67-1.83 (m, 4H, CH2CH2CH2N), 2.88 (t, 2H, J = 6.85 Hz, NCH2CH2Ar), 3.48-3.54 (m, 8H, CH2NCH2CH2Ar and N(CH3)2), 3.58-3.64 (m, 2H, CH2NCH2CH2Ar), 3.80-3.87 (m, 2H, NCH2CH2CH2CH2N), 3.92 (s, 3H, OCH3), 3.93 (s, 3H, OCH3), 6.63 (s, 1H, aromatic), 6.96 (d, 1H, J = 7.34 Hz, aromatic), 7.12 (t, 1H, J = 7.34 Hz, aromatic), 7.33 (d, 1H, J = 7.34 Hz, aromatic), 7.60 (s, 1H, aromatic), 7.83 (t, 1H, J = 7.34 Hz, aromatic), 13.62 (s, 1H, NH); 13C NMR (500 MHz, CDCl3) 24.74; 25.01; 27.72; 39.44; 46.17; 46.35; 56.04; 56.08; 109.23; 109.33; 110.44; 119.96; 121.66; 121.85; 123.27; 130.76; 131.54; 134.77; 142.04; 147.98; 151.88; 161.78; 164.62; 180.13.
QTOF ($m/z$) Calcd for C$_{26}$H$_{31}$N$_3$SO$_4$ [M+Na]$^+$: 532.1994, found: 532.1988. Compound was $> 98\%$ pure by HPLC analysis performed with MeOH/H$_2$O, 80 : 20 v/v, at a flow rate 0.8 mL min$^{-1}$.

4.5. General procedure for the synthesis of 10-11

A solution of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline or 1-cyclohexylpiperazine (2.90 mmol) in CH$_3$CN was added with K$_2$CO$_3$ (0.49 g, 3.50 mmol) and 1-bromobutane (0.38 ml, 3.50 mmol). The resulting mixture was refluxed overnight under stirring. After the removal of the solvent under reduced pressure the residue was taken up with H$_2$O and extracted with CH$_2$Cl$_2$ (3 × 10 mL). The collected organic layers were dried (Na$_2$SO$_4$) and evaporated under reduced pressure to afford a crude oil which were purified by column chromatography (CH$_2$Cl$_2$/MeOH 95:5) to give the title compounds which were transformed into the corresponding hydrochloride salts, recrystallized from MeOH/Et$_2$O.

4.5.1. N-Butyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (10) was obtained as white crystals (0.5 g, 90% yield); mp = 191-193 °C. $^1$H NMR (500 MHz, MeOH-$d_4$) $\delta$ 1.03 (t, 3H, J = 7.3 Hz, NCH$_2$CH$_2$CH$_2$CH$_3$), 1.44-1.49 (m, 2H, NCH$_2$CH$_2$CH$_2$CH$_3$), 1.78-1.84 (m, 2H, NCH$_2$CH$_2$CH$_2$CH$_3$), 3.10-3.12 (m, 2H, NCH$_2$CH$_2$CH$_2$CH$_3$), 3.23-3.26 (m, 2 H, ArCH$_2$CH$_2$NCH$_2$), 3.29-3.31 (m, 2H, ArCH$_2$CH$_2$NCH$_2$), 3.80 (s, 6H, 2 OCH$_3$), 4.86 (s, 2H, ArCH$_2$NCH$_2$CH$_2$CH$_2$CH$_3$), 6.77 (s, 1H, aromatic), 6.82 (s, 1 H, aromatic); $^{13}$C NMR (500 MHz, MeOH-$d_4$) 13.83; 18.75; 23.73; 25.74; 51.43; 53.67; 56.10; 59.56; 111.41; 111.63; 116.76; 130.45; 146.71; 146.92; GC-MS m/z: 248 (M+, 0.5), 206 (100); QTOF ($m/z$) Calcd for C$_{15}$H$_{23}$NO$_2$ [M+H]$^+$: 250.1807, found: 250.1800. Compound was $> 98\%$ pure by HPLC analysis performed with CH$_3$CN/HCOONH$_4$ (20 mM, pH = 5) $75 : 25$ v/v, at a flow rate 1.0 mL min$^{-1}$.

4.5.2. 1-Butyl-4-cyclohexyl-piperazine (11) was obtained as white crystals (0.5 g, 77% yield); mp = 250-252 °C. $^1$H NMR (500 MHz, MeOH-$d_4$) $\delta$ 1.01 (t, 3H, J = 7.34 Hz, NCH$_2$CH$_2$CH$_2$CH$_3$), 1.19-2.20 (m, 14H, cyclohexyl CH$_2$ and NCH$_2$CH$_2$CH$_2$CH$_3$), 3.20-3.66 (m, 11H, piperazine CH and CH$_2$ and NCH$_2$CH$_2$CH$_2$CH$_3$); $^{13}$C NMR (500 MHz, MeOH-$d_4$) 13.82; 18.90; 24.32; 25.74; 25.65;
28.86; 50.10; 52.43; 53.82; 59.46; GC-MS m/z: 224 (M+, 30), 181 (100); QTOF (m/z) Calcd for C_{14}H_{29}N_{2} [M+H]^+: 225.2331, found: 225.2324. Compound was > 98% pure by HPLC analysis performed with CH_{3}CN/HCOONH_{4} (20 mM, pH = 5) 75 : 25 v/v, at a flow rate 1.0 mL min^{-1}.

4.6. Biology

4.6.1. Materials

[^3]H-DTG (50 Ci/mmol) and CulturePlate 96/wells plates were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). DTG was purchased from Tocris Cookson Ltd, UK. (+)-Pentazocine and calcein-AM were obtained from Sigma-Aldrich-RBI s.r.l. (Milan, Italy). Male Dunkin guinea-pigs (200-250 g) and Wistar Hannover rats (250-300 g) were from Harlan, Italy. Cell culture reagents were purchased from EuroClone (Milan, Italy). Protease inhibitor cocktail, was obtained from Sigma-Aldrich (Milan, Italy). Anti-P-Glycoprotein antibody produced in mouse (C219) was purchased from Calbiochem (Merck-Millipore, Germany). Anti-β-actin, secondary peroxidase antibodies and all reagents for western blotting were purchased from Life Technologies Italia (Monza, Italy).

4.6.2. Compounds

σ_{2} Receptor ligands 1 [(Z)-2-(1-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2-oxoindolin-3-ylidene)-N,N-dimethylhydrazinecarbothioamide hydrochloride], 2 [(Z)-2-[1-[4-(4-Cyclohexylpiperazin-1-yl)butyl]-2-oxoindolin-3-ylidene]-N,N-dimethylhydrazinecarbothioamide di-hydrochloride], intermediate 3 [6,7-dimethoxy-3,4-dihydroisoquinolin-1(2H)-one], and σ_{1} fluorescent ligand 5-(dimethylamino)-2-(6-((5-(4-(4-methylpiperidin-1-yl)butyl)-5,6,7,8-tetrahydronaphthalen-2-yl)oxy)hexyl)isoindoline-1,3-dione were synthesized in our laboratories according to published methods [20,33,42]. Caspase-3 inhibitor Z-DEVD-FMK was purchased from Tocris Bioscience, α-tocopherol and N-Acetyl-L-cysteine from Sigma-Aldrich (St. Louis, MO). Compounds were dissolved in DMSO with final concentrations less than 0.3%.

4.6.3. Cell Culture
Human pancreas cancer cell lines BxPC3, AsPC1, MiaPaCa-2, and Panc1 were obtained from American Type Culture Collection (ATCC, Bethesda, MD). Murine pancreas adenocarcinoma Panc02 was a gift from Bryan Clary (Duke University). The mouse KCKO cell line isolated from a spontaneously developing pancreatic cancer overexpressing human MUC1 [41] was kindly provided by Dr. Pinku Mukherjee (University of North Carolina, Charlotte, NC). The mouse KP02 line was derived from pancreatic cancer tumor tissue obtained from p48-CRE/LSL-KrasG12D/p53flox/+ mice (backcrossed C57BL/6, n = 6). The MCF7σ1 was produced in our laboratory [43]. AsPC-1, BxPC-3 and Panc02 cells were cultured in RPMI-1940 medium with 10% fetal bovine serum (FBS). MIAPaCa-2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS and 2.5% horse serum. PANC-1 and MCF7σ1 cells were cultured in DMEM with 10% FBS. KCKO cells were cultured in RPMI-1940 medium with 10% FBS, 1% sodium pyruvate, 1% HEPES buffer, and 1% L-glutamine. KP02 cells were cultured in 1:1 mixture of DMEM and Ham’s F-12 Nutrient Mixture with 10% FBS. Penicillin (100 mg/mL) and streptomycin (100 mg/mL) were added to all media; cells were maintained in a humidified incubator at 37° C with 5% CO2.

4.6.4. Competition σ2 Binding Assays

All the procedures for the binding assays were previously described. σ2 Receptor binding were carried out according to Berardi et al [44]. The specific radioligand and tissue sources was: [3H]-DTG in the presence of 1 μM (+)-pentazocine to mask σ1 receptors, rat liver membranes. The following compounds were used to define the specific binding reported in parentheses: DTG (85-96%). Concentrations required to inhibit 50% of radioligand specific binding (IC50) were determined by using six to nine different concentrations of the drug studied in at least three experiments with samples in duplicate. Scatchard parameters (Kd and Bmax) and apparent inhibition constants (Ki) values were determined by nonlinear curve fitting, using the Prism, version 3.0, GraphPad software [45].
4.6.5. $\sigma_1$ Binding by Flow Cytometry studies

The procedure for $\sigma_1$ binding by flow cytometry studies were carried out according to Abate et al 2016 [42]. MCF7$\sigma_1$ cells were incubated with increasing concentrations (0.1, 1, 10, and 100 nmol/L and 1 and 10 µM) of (+)-pentazocine or PB212 [46-49] or thiosemicarbazones 1, 2 and 7, followed by 100 nmol/L of either $\sigma_1$ fluorescent compound ($\sigma_1$FC, 5-(dimethylamino)-2-(6-((5-(4-(4-methylpiperidin-1-yl)butyl)-5,6,7,8-tetrahydronaphthalen-2-yl)oxy)hexyl)isoindoline-1,3-dione) for 75 min at 37 °C. To mask $\sigma_2$ receptors, 2-(3-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)propyl)-5-methoxy-3,4-dihydroisoquinolin-1(2H)-one F390 [28,42] (10 µM) was co-incubated. At the end of the incubation periods, cells were washed twice with PBS, detached with 200 mL of Cell Dissociation Solution (Sigma Chemical Co.) for 10 min at 37 °C, centrifuged at 13,000 g for 5 min and resuspended in 500 µL of PBS. The fluorescence was recorded using a Bio-Guava® easyCyte™ 5 Flow Cytometry System (Millipore, Billerica, MA), with a 530 nm band pass filter. For each analysis, 50,000 events were collected and analyzed with the InCyte software (Millipore).

4.6.6. Calcein-AM experiment

These experiments were carried out as already described [50]. MDCK-MDR1 cell line (50,000 cells per well) was seeded into black CulturePlate 96/wells plate with 100 µL medium and allowed to become confluent overnight. 100 µL of different concentrations of test compounds (0.1-100 µM) were solubilized in culture medium and added to each well. The 96/wells plate was incubated at 37 °C for 30 min. 100 µL of Calcein-AM, solved in Phosphate Buffered Saline (PBS), was added to each well to yield a final concentration of 2.5 µM, and the plate was incubated for 30 min. The plate was washed 3 times with 100 mL ice cold PBS. Saline buffer (100 µL) was added to each well and the plate was read by a PerkinElmer Victor3 spectrofluorimeter at excitation and emission wavelengths of 485 nm and 535 nm, respectively. In these experimental conditions, Calcein cell accumulation in the absence and in the presence of tested compounds was evaluated and
fluorescence basal level was estimated by untreated cells. In treated wells the increase of fluorescence with respect to basal level was measured. EC50 values were determined by fitting the fluorescence increase percentage versus log[dose].

4.6.7. **Cell Viability and ROS interference**

Determination of cell growth was performed using the MTT assay at 48 h [51,21]. On day 1, 25,000 cells/well were seeded into 96-well plates in a volume of 100 μL. On day 2, the various drug concentrations (1 μM-100 μM) were added. In all the experiments, the various drug-solvents (EtOH, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs (48 h), MTT (0.5 mg/mL) was added to each well, and after 3-4 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized using 100 μl of DMSO/EtOH (1:1) and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences. The interference of ROS in cell viability was indirectly determined by MTT assay reported above at 24 h. On day 1, 25000 cells per well were seeded into 96-well plates in the presence or absence of α-tocopherol (100 μM). On day 2, the drugs (1 μM-100 μM) were added alone and in combination with α-tocopherol (100 μM). After incubation (24 h) with drugs, MTT assay was performed as above. The interference of ROS in cell viability was indirectly determined by MTT assay reported above at 48 h. On day 1, cells were plated at a density of 2 x 10^4 cells/well in opaque 96-well, clear-bottom plates 24 hours prior to treatment. On day 2, cells were treated with the drugs in the presence or absence of α-tocopherol (100 μM) or N-Acetyl-L-cysteine (100 μM). After incubation (48 h) with drugs, MTT assay was performed as above.

4.6.8. **Western blotting**

The experiment was carried out according to Niso et al. with minor modification [52]. All cells were washed twice with 10 ml phosphate-buffered saline (PBS), scraped in 1 ml PBS and centrifuged for 1 min at 11,000 g. Proteins were extracted from cells by homogenization in cold
RIPA buffer (Life Technologies) containing 1X protease inhibitor cocktail and centrifuged at 14,000 g for 15 min at 4°C. The supernatant was recovered and the protein concentration was measured using the microLowry kit. 30 μg of protein extract was separated on 10% polyacrylamide gel (Life Technologies) and then transferred onto a polyvinylidene difluoride membrane (PVDF) by iBlot® Gel Transfer Device (Life Technologies). Membrane was blocked for 30 min at room temperature with blocking buffer (1% BSA, 0.05% Tween 20 in Tris-buffered saline, TBS). The membrane was then incubated overnight at 4°C with anti-P-Glycoprotein (1:500 mouse monoclonal) or for 1h at room temperature anti-β-actin (1:1000 mouse monoclonal) antibodies, diluted in blocking buffer. After incubation time, membrane was washed with washing buffer (0.05% Tween 20 in Tris-buffered saline, TBS) for three times and incubated with a secondary peroxidase antibody (1:2000 anti-mouse for P-glycoprotein and β-actin) for 1h at room temperature. After washing, the membrane was treated with the enhanced chemiluminescence (ECL, Life Technologies) according to the manufacturer's instructions and the blot was visualized by UVITEC Cambridge (Life Technologies). The expression level was evaluated by densitometric analysis using UVITEC Cambridge software (Life Technologies) and β-actin expression level was used to normalize the sample values.

4.6.9. Detection of Caspase-3 activity in vitro

Caspase-3 activity was measured in KP02 cell lines with a Caspase-Glo® Assay Systems (Promega) according to protocol in which the reagent contain luminogenic caspase substrates that cleaved by activated caspase. Cells were seeded at a density of 1 × 10^4 in black 96-well, clear bottom plates for 24 hours before treatment with 25 μM of compounds in presence or absence of α-tocopherol (100 μM) or Z-DEVD-FMK (1μM) for 5 hours after treatment. The contents were then mixed using plate shaker for 30 seconds, and incubated at room temperature for 90 minutes. Luminescence signal was measured using multi-mode microplate reader (BioTek). Assay was
performed in triplicates, and caspase activity was plotted compared cells treated with DMSO as a control.

4.6.10. Detection of Mitochondrial Superoxide by flow cytometry and ROS interference

MitoSOX™ Red reagent is a novel fluorogenic dye specifically targeted to mitochondria in live cells. Oxidation of MitoSOX™ Red reagent by superoxide produces red fluorescence. Mitochondrial superoxide is generated as a byproduct of oxidative phosphorylation. In an otherwise tightly coupled electron transport chain, approximately 1–3% of mitochondrial oxygen consumed is incompletely reduced; those “leaky” electrons can quickly interact with molecular oxygen to form superoxide anion, the predominant reactive oxygen species (ROS) in mitochondria [53-56]. MitoSOX™ Red reagent is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species (RNS)–generating systems. KP02 cells were seeded into 12-well plates 24 hours before treatment with iron chelators (50 μM) for 2 hours at 37°C in presence or absence of α-tocopherol (100 μM) followed by staining with MitoSOX™ Red (5 μM). Two hours after red dye addition, the cells were washed twice with PBS and harvested with trypsin/EDTA buffer. The cells were washed twice with PBS before analysis with FACSCalibur (BD Bioscience, San Jose, CA). The oxidation product of MitoSOX™ Red by mitochondrial superoxide fluoresces with an emission maximus of 580 nm and was detected in the FL3 channel. Experiment was performed in triplicates.

4.6.11. In vivo assessment of tumor growth

Animal studies were performed according to the animal studies protocol (20130073) approved by the Washington University Institutional Animal Care Facility. In this pre-clinical model, we utilized the KP02 cell line. In vivo studies with mice were performed to compare the effect of σ2 ligands with iron chelator 7. Mice treated with vehicle alone (25% Cremophor in H2O) served as the control cohort. Female C57BL/6 mice (8 weeks old, National Cancer Institute Laboratories) were injected in the right flank with 200 μL of a single-cell suspension of KP02 cells in non-supplemented RPMI medium (2.5 x 10^5 cells per mouse). Treatment began when the mean tumor diameter was ~ 5-6
Mice received daily intraperitoneal (i.p.) injections of the σ₂ ligands 1 and 2 (750nmol), or 7 (750nmol) in 100 µL vehicle or vehicle alone (control) for 2 weeks. Tumors were measured three times weekly in two dimensions with a digital caliper, and tumor volumes were calculated by the standard formula of Tumor Volume = Length x Width² x 0.5. All mice were euthanized when tumors reached a diameter of 15 mm or had ulcerated. The experiment was repeated using a double concentration of 1 and 2 following the same procedure described above. Several mice from each treatment cohort were assessed for pathologic evaluation (Digestive Diseases Research Core Center at Washington University School of Medicine, St. Louis, MO). Blood was collected for complete blood count (CBC) and biochemical analysis (AST, ALT, BUN, total protein, glucose and Cr). Organs were examined grossly and histologically.

4.6.12. Statistical analysis

Statistical analyses and data plotting were performed using GraphPad Prism software version 6.03 (San Diego, CA). Results were expressed as mean ± standard error of the mean of at least 3 biological replicates. EC₅₀ values were calculated by curve fitting normalized viability versus drug concentration. Differences in viability, caspase-3 activity, and tumor volume were analyzed using two-way ANOVA to identify differences and confirmed with paired two tailed t-tests. Mann-Whitney test was used to compare the difference in CBC and biochemistry analyses. Kaplan-Meier survival analyses were used to assess differences between treatment groups and were compared using a log-rank test. A p-value < 0.05 was considered significant for all analyses.
References


[38] B.J. Vilner, C.S. John, W.D. Bowen, Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines, Cancer Res. 55 (1995) 408-413.


[44] F. Berardi, C. Abate, S. Ferorelli, V. Uricchio, N.A. Colabufo, M. Niso, R. Perrone, Exploring the importance of piperazine N-atoms for sigma(2) receptor affinity and activity in a series of


[50] M. Niso, M.L. Pati, F. Berardi, C. Abate, Rigid versus flexible anilines or anilides confirm the bicyclic ring as the hydrophobic portion for optimal σ2 receptor binding and provide novel tools for the development of future σ2 receptor PET radiotracer, RSC Adv. 6 (2016) 88508-88518.


Figure 1.

![Chemical structures](image)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>( R )</th>
<th>Calcein-AM</th>
<th>MCF7</th>
<th>MCF7dx</th>
<th>A549</th>
<th>A549dx</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^b)</td>
<td>A</td>
<td>3.04 ± 0.18</td>
<td>24.6 ± 3.6</td>
<td>15.7 ± 1.6</td>
<td>10.4 ± 1.6</td>
<td>3.89 ± 0.5</td>
</tr>
<tr>
<td>2(^b)</td>
<td>B</td>
<td>2.83 ± 0.35</td>
<td>1.81 ± 0.4</td>
<td>2.95 ± 0.4</td>
<td>2.20 ± 0.3</td>
<td>2.83 ± 0.4</td>
</tr>
</tbody>
</table>

\(^a\) Values represent the mean of \( n \geq 3 \) separate experiments in duplicate ± SEM; \(^b\) From ref 20.
Figure 2.
Figure 3.

A  C57BL/6 mice w/ subcutaneous KP02 tumor: tumor volume

B  C57BL/6 mice w/ subcutaneous KP02 tumor: tumor volume
Figure 4.

C57BL/6 mice w/ subcutaneous KP02 tumor: body weight

Body Weight (gm)

Days of Treatment

Vehicle
1 (1500nmol)
2 (1500nmol)
7 (750nmol)
Legends of Figures

**Figure 1.** Novel $\sigma_2$ thiosemicarbazone ligands and their antiproliferative activity

**Figure 2.** Caspase-3 activation, involvement of Reactive Oxygen Species (ROS) and Mitochondrial Superoxide detection in KP02 cell lines.

(A) Indirect measurement of ROS involvement following 48 hours treatment with 4 $\mu$M 1, 2, 7 or 9 in the presence of 100 $\mu$M lipophilic antioxidant $\alpha$-tocopherol or hydrophilic antioxidant N-acetyl-L-cysteine (NAC) in KP02 cells $p < 0.001$; (B) Caspase-3 activation was measured by Caspase-GloR Assay in KP02 cells treated with 25 $\mu$M of different compounds for 5 hours and expressed relative to vehicle. Cells treated with 2 had significant increase in caspase-3 $p < 0.0001$. (C) Mitochondrial Superoxide detection in KP02 after 2 hours treatment with 50 $\mu$M of compounds alone or in combination with the lipid antioxidant $\alpha$-tocopherol (1 mM), 1 generated high mitochondrial superoxide production $p < 0.0001$. Values are the means of $n \geq 3$ independent experiments in triplicates with SEM.

**Figure 3.** Thiosemicarbazones efficacy in C57BL/6 bearing KP02 tumors.

$2.5 \times 10^5$ KP02 cells were inoculated subcutaneously into female, 8 weeks old C57BL/6 mice and when tumors had reached a mean diameter of 5-6 mm, daily thiosemicarbazones treatment (750nmol/100$\mu$L 1, 2 and 7 (A); 1500 nmol/100$\mu$L 1 and 2 (B)) began by i.p. injection. 7 treatment (A) decreased tumor volume $p < 0.001$, while at the same dosage 1 and 2 treatment (A) was not effective $p > 0.05$. Treatment with a higher concentration of 1 and 2 (B) decreased significantly tumor volume $p = 0.004$ and $p = 0.012$ respectively. Data represent Means ± SEM, $n = 7$-10 per group.
Figure 4. C57BL/6 mice tolerated the drugs well without signs of weight loss. Data represent Means ± SEM, n = 7-10 per group, p > 0.05.
Scheme 1. Synthesis of Thiosemicarbazones 7-9.\textsuperscript{a}

\textsuperscript{a}Reagents and Conditions: (a) 1-Bromo-4-chlorobutane, NaH, DMF; (b) K$_2$CO$_3$, CH$_3$CN; (c) appropriate thiosemicarbazide, EtOH.
Scheme 2. Synthesis of 11-14.a

\[ \text{Reagents and Conditions: } (a) \text{ 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline or 1-cyclohexylpiperazine, } K_2CO_3, \text{ CH}_3\text{CN}; \text{ (b) 1-Bromo-4-chlorobutane, KOH, TBAB, DMF.} \]
Table 1. $\sigma_2$ Receptor affinity and P-gp activity.

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R</th>
<th>$R_1$</th>
<th>$\sigma_2$</th>
<th>EC$_{50}$ ((\mu)M)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>CH$_3$</td>
<td>34.1$^b$</td>
<td>3.04$^b$</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>CH$_3$</td>
<td>35.4$^b$</td>
<td>2.83$^b$</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td>CH$_3$</td>
<td>&gt;10000</td>
<td>n.a.$^c$</td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>H</td>
<td>&gt;10000</td>
<td>n.a.$^c$</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>CH$_3$</td>
<td>&gt;10000</td>
<td>11.0±1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>X</th>
<th>BM</th>
<th>$\sigma_2$</th>
<th>EC$_{50}$ ((\mu)M)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>H</td>
<td>A</td>
<td>15.0±0.9</td>
<td>76.4±3.4</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>B</td>
<td>65.2±1.4</td>
<td>n.a.$^c$</td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td>A</td>
<td>3.66$^d$</td>
<td>6.09±0.8</td>
</tr>
<tr>
<td>14</td>
<td>C</td>
<td>B</td>
<td>1.90$^d$</td>
<td>0.46±0.06</td>
</tr>
</tbody>
</table>

$^a$Values represent the mean of \(n \geq 3\) separate experiments in duplicate ± SEM; $^b$From ref 20; $^c$n.a. = not active; $^d$From ref 29.
Table 2. Viability screening on pancreatic cancer cell lines following 48 hr treatment.

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R</th>
<th>R₁</th>
<th>Panc02</th>
<th>KP02</th>
<th>KCKO</th>
<th>MIAPaCa-2</th>
<th>BxPC3</th>
<th>AsPC1</th>
<th>Panc-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>CH₃</td>
<td>1.33±0.2</td>
<td>6.92±0.9</td>
<td>6.49±1.0</td>
<td>14.6±2.2</td>
<td>2.34±0.8</td>
<td>2.01±0.6</td>
<td>14.6±2.1</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>CH₃</td>
<td>1.17±0.4</td>
<td>7.32±0.8</td>
<td>6.18±0.3</td>
<td>10.8±1.3</td>
<td>6.15±0.6</td>
<td>3.86±0.2</td>
<td>8.73±0.8</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td>CH₃</td>
<td>1.21±0.1</td>
<td>2.83±0.6</td>
<td>3.14±0.4</td>
<td>18.3±2.1</td>
<td>2.52±0.5</td>
<td>2.17±0.3</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td>H</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>CH₃</td>
<td>2.18±0.3</td>
<td>5.62±1.1</td>
<td>4.51±0.8</td>
<td>10.3±1.9</td>
<td>5.93±0.7</td>
<td>5.74±0.5</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Activity, EC₅₀ (µM)ᵃ

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>X</th>
<th>BM</th>
<th>Panc02</th>
<th>KP02</th>
<th>KCKO</th>
<th>MIAPaCa-2</th>
<th>BxPC3</th>
<th>AsPC1</th>
<th>Panc-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>H</td>
<td>A</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>B</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>n.d.</td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td>A</td>
<td>&gt;100</td>
<td>49±2.5</td>
<td>77±5.2</td>
<td>40±3.5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td>C</td>
<td>B</td>
<td>&gt;100</td>
<td>70±4.6</td>
<td>67±4.5</td>
<td>79±6.1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

ᵃValues represent the mean of n ≥ 3 separate experiments in duplicate ± SEM; ᵇn.d. = not determined.
Table 3. Thiosemicarbazones do not induce changes in blood cytology (CBC) following treatment of KP02 tumor-bearing C57BL/6 mice.

A.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>7</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^3/µL)</td>
<td>7.8 ± 0.55</td>
<td>7.93 ± 1.35</td>
<td>5.75 ± 0.45</td>
<td>8.87 ± 0.63</td>
</tr>
<tr>
<td>RBC (10^6/µL)</td>
<td>9.35 ± 0.11</td>
<td>8.63 ± 0.71</td>
<td>9.19 ± 0.15</td>
<td>9.94 ± 0.92</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>13.8 ± 0.1</td>
<td>12.9 ± 1.1</td>
<td>13.45 ± 0.05</td>
<td>14.9 ± 0.9</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>47.5 ± 3</td>
<td>42.65 ± 2.85</td>
<td>45.45 ± 4.15</td>
<td>51 ± 3.8</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>50.7 ± 0.76</td>
<td>49.45 ± 0.75</td>
<td>49.5 ± 0.1</td>
<td>51.4 ± 0.9</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.8 ± 0.1</td>
<td>14.95 ± 0.05</td>
<td>14.65 ± 0.15</td>
<td>15 ± 0.5</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>29.13 ± 0.83</td>
<td>30.2 ± 0.6</td>
<td>29.6 ± 0.3</td>
<td>29.25 ± 0.45</td>
</tr>
<tr>
<td>Platelets (10^3/µL)</td>
<td>951.6 ± 86.3</td>
<td>865 ± 25</td>
<td>976.5 ± 81.5</td>
<td>854 ± 47</td>
</tr>
</tbody>
</table>

B.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>7</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dL)</td>
<td>27.3 ± 2.66</td>
<td>29.5 ± 2.5</td>
<td>22 ± 2</td>
<td>21.5 ± 1.5</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.33 ± 0.02</td>
<td>0.35 ± 0.12</td>
<td>0.31 ± 0.02</td>
<td>0.34 ± 0.1</td>
</tr>
<tr>
<td>ALT (µ/L)</td>
<td>54.3 ± 6.33</td>
<td>257 ± 27</td>
<td>95 ± 41</td>
<td>147.5 ± 86.5</td>
</tr>
<tr>
<td>AST (µ/L)</td>
<td>98.6 ± 0.05</td>
<td>573.5 ± 37.4</td>
<td>172 ± 11.2</td>
<td>416 ± 21.2</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>5.55 ± 0.15</td>
<td>5.5 ± 0.3</td>
<td>5.1 ± 0.1</td>
<td>5.75 ± 0.35</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>168 ± 3.3</td>
<td>309 ± 4</td>
<td>285 ± 24.5</td>
<td>288.5 ± 26.5</td>
</tr>
</tbody>
</table>

(A) Blood cytology analysis of C57BL/6 mice (n = 3 mice/group) treated with thiosemicarbazone 7 (750nmol/100µL), σ₂ ligands 1 and 2 (1500nmol/100 µL) and vehicle (control) for 2 weeks. The differences in complete blood count laboratory values between the two groups are not statistically
significant, $p > 0.05$. (B) Biochemical analysis of C57BL/6 mice ($n = 3$ mice/group) treated with thiosemicarbazone 7 (750nmol/100μL), $\sigma_2$ ligands 1 and 2 (1500nmol/100 μL) and vehicle (control) for 2 weeks. The differences in serum chemistries between the groups are not statistically significant, $p > 0.05$. 
Less toxicity of $\sigma_2$ target compounds 1 and 2 versus metal chelator 7