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Collateral Sensitivity of σ_2 Receptor ligands: Potentials in the Treatment of Multidrug Resistant Tumors

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Abstract

Tumors remain one of the main causes of human illnesses and death with MultiDrug Resistance (MDR) being the most severe limitation to the success of chemotherapy. MDR is mainly due to the overexpression of drug efflux transporters, such as P-glycoprotein (P-gp), but attempts to inhibit P-gp have not been clinically successful so far. Lately, some agents were found to be more effective against P-gp overexpressing cells, showing a property termed "collateral sensitivity" (CS). The molecular bases of CS are poorly understood and hypersensitivity to reactive oxygen species (ROS) is one of the hypotheses that accounts for it. We recently identified a few sigma-2 (σ_2) receptors ligands endowed with CS, likely because of their interaction with P-gp. In fact, a number of CS agents are P-gp substrates: they are actively effluxed by P-gp, and it is believed that they activate a futile ATP cycle, increase oxidative phosphorylation leading to higher ROS production and oxidative stress. Therefore, we verified ROS involvement to study the CS properties of σ_2 receptors ligands/P-gp substrates (**F408** and siramesine) whose activity was measured in three parental and Pg-p-overexpressing cell line pairs. We also demonstrated the major consumption of ATP induced by these compounds in P-gp overexpressing *vs* the parental cells. We analyzed the effects of siramesine and **F408** on mitochondrial respiratory chain (source of ROS and intracellular ATP). Siramesine and **F408** decreased both the electron flux rate and the ATP levels, with MDR cells undergoing a much more pronounced decrease than parent cells. Therefore, we demonstrated depletion of mitochondrial ATP supply by siramesine and **F408** as the mechanism by which these σ_2 ligands likely induce CS. In conclusion, CS and σ_2 ligands-mediated actions warrant further investigation as a way to face MDR.

Introduction

Despite all of the efforts directed towards cancer research, tumors remain one of the main causes of human illnesses and death. Chemotherapeutic agents that are the standard treatment for malignancies are very often associated with serious side effects mainly due to their lack of tumor specificity.[1] Physical targeting techniques (e.g. directed radiotherapy and intratumoral injections) and the newer molecular targeted therapies aim to treat cancer cells more selectively. However, both of these strategies present limitations: with physical targeting, metastases are often difficult to be reached, and targeted therapies may present tissue toxicity and resistance to chemotherapy. Multidrug resistance (MDR) is the major limitation to the full efficacy of chemotherapy, and it is often due to the overexpression of plasma membrane transporters such as P-glycoprotein (P-gp), breast cancer resistant protein (BCRP), and multidrug resistant associated proteins (MRPs), all of which belong to the ATP binding cassette (ABC) protein superfamily.[2-4] One of the main mechanisms of MDR is the overexpression of P-gp that has been associated with poor chemotherapy response and prognosis. P-gp actively extrudes classical chemotherapeutic drugs (e.g. vinblastine, paclitaxel, doxorubicin) as well as new tyrosine kinase inhibitors decreasing or abolishing their effects.[5] Therefore, inhibition of P-gp represents a strategy to circumvent cellular resistance to chemotherapeutic agents. Co-administration of a P-gp inhibitor with a cytotoxic drug would prevent the drug efflux restoring its pharmacological activity. Three generations of P-gp inhibitors have been already developed with this purpose. Nevertheless, suitable results from clinical trials have not been obtained. Co-administration of the chemotherapeutic agent with the P-gp inhibitor results in inconvenient and unpredictable pharmacokenetic problems so that clinical applications remain distant and alternative strategies are needed to counteract transporter-mediated resistance. Recent high-throughput screenings of pharmacological libraries identified specific compounds which were unexpectedly more effective in Pg-p-overexpressing cells than in Pg-p-negative cells.[6-8] This property was named Collateral Sensitivity (CS) from Szybalaski and Brysonwho found hypersensitivity to unrelated drugs in resistant Escherichia Coli cells.[9] CS

depends on the hypersensitivity of MDR cells to unrelated agents as a result of the drug selection process that led to the development of MDR. Therefore, CS is a weakness of MDR cells that could conveniently be exploited: agents endowed with CS could avoid that repeated chemotherapeutic cycles select resistant cells whose proliferation would lead to intractable tumors. Molecular bases of CS are far from being understood.[10] Few hypotheses have been formulated to account for such property, and hypersensitivity to reactive oxygen species (ROS) is one of the most recent. A number of CS agents are P-gp substrates and therefore, once inside the cells they are actively effluxed by P-gp with ATP hydrolysis as a source of energy. To support the ATP futile cycle which is activated, oxidative phosphorylation increases leading to higher ROS production and oxidative stress. However, the process that has always been thought to support the energy needs of cancer is glycolysis and not oxidative phosphorylation, and only limited evidence shows the connection between Pg-p substrates and oxidative stress. Thiosemicarbazones and other metal chelators[11] have shed light on alternative mechanisms for ROS generation other than interaction with P-gp. In fact, these compounds endowed with CS have been shown to not interact with P-gp although they are provided with CS. Their biological activity involves interaction with metal ions with formation of metal chelates. Complexes of these compounds with metals cycling between two redox states (e.g. FeII/III) may lead to increase in ROS production. The encouraging results from in vivo tumor models prompted the development of different series of thiosemicarbazones.[11,12] From a series of isatin-β-thiosemicarbazones (IBTs) Hall and coworkers selected compound NSC73306 (Figure 1) that displayed antiproliferative effect 4.3-fold higher in Pg-p overexpressing cells *vs* the parent cells.[8] NSC73306 did not interact with P-gp but its CS was dependent of the density of P-gp: the higher the density, the higher the CS, as demonstrated from the MTT assay in a wide series of parental and drug-resistant cell line pairs. As suggested, the presence of MDR determines hypersensitivity to the increase in ROS, so that MDR cells are more prone to cell death in the presence of these compounds. The promising results obtained with NSC73306 encouraged the synthesis of analogous thiosemicarbazones in order to obtain compounds with more potent CS

properties and with higher solubility in water. Structural changes of the R group led to improvement in the CS properties with one compound ((*Z*)-N-(4-*tert*-butylphenyl)-2-(2-oxoindolin-3-ylidene)hydrazinecarbothioamide, **1**, Figure 1) displaying 15-fold higher activity in human adenocarcinoma resistant cells than in the corresponding parent cells (KB3-I and KB-V1 respectively).[8]

In our continuous effort to produce sigma-2 (σ_2) receptor ligands useful to target σ_2 receptors and study their involvement in tumors, we developed diverse series of compounds. [13-17] σ_2 Receptors are endocellular binding sites overexpressed in a number of cancers with a higher expression in proliferative *vs* quiescent cancer cells, so that they have been proposed as biomarkers for the proliferative status of tumors.[18] The activation of σ_2 receptors with specific ligands leads to tumor cell death through different pathways which appear to depend on the tumor type and on the σ_2 ligand type. σ_2 Receptors have not yet been cloned and diverse hypotheses[19] have been formulated on their identity: the most recent hypothesis identifies σ_2 receptors as the PGRMC1,[20] and studies are ongoing to confirm it. Despite the fact that the real identity of the σ_2 protein is still ambiguous, the potential in cancer diagnosis and treatment keeps scientific interest in σ_2 related research high.[21-25] With the aim of producing σ_2 agonists with potent antiproliferative activity, we gathered our inspiration from the most potent σ_2 agonists present in the literature and we produced three series of compounds in which different basic moieties were linked to a tetraline or indole or carbazole ring (General Structure, Figure 2).[16]

All compounds were evaluated for their antiproliferative activity in human MCF7 breast cancer cells and the resistant counterpart MCF7adr cells, so that indication on the activity of these σ_2 ligands in resistant cells could be obtained. Most of the compounds displayed comparable antiproliferative activity in both the cell lines (EC_{50} values ranging from 5.9 μM to 35 μM), with a few compounds displaying unexpected CS properties. We also investigated the antiproliferative effect of σ_2 agonist siramesine (Figure 3) in MCF7 and MCF7adr. Siramesine is one of the best

known σ_2 agonists, whose antiproliferative σ_2-mediated activity was shown to be linked to ROS production. Siramesine, which served as lead compound for the development of the present series, demonstrated a 2-fold higher antiproliferative activity in MCF7adr (EC_{50} = 5.9 μM) than in MCF7 (EC_{50} = 12.3 μM), showing CS properties (Table 1).[16] In order to investigate this unexpected properties we evaluated compounds' interaction with P-gp. For all of the compounds modulation with P-gp was shown, with the most potent interaction displayed by indole derivative 6,7-Dimethoxy-2-[4-[1-(4-fluorophenyl)-1*H*-indol-3-yl]butyl]-1,2,3,4-tetrahydroisoquinoline (**F397**) (EC_{50} = 0.21 μM, Figure 3), and by carbazole derivative 9-[4-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)butyl]-9*H*-carbazole (**F408**) (EC_{50} = 0.42 μM, Figure 3). Also siramesine was shown to interact with P-gp with EC_{50} = 1.41 μM. Of the two novel compounds, only **F408** displayed a slight CS (Table 1).[16] Therefore, we evaluated ATP consumption induced by **F408**, **F397** and by reference compound siramesine in the cell line pairs. ATP content was reduced by **F408** and siramesine in MCF7adr (higher consumption) compared to MCF7, whereas ATP content was the same in both the cell lines treated with **F397** (Figure 4). These results demonstrated that **F408** and siramesine are P-gp substrates whereas **F397** is a P-gp inhibitor.

These pieces of evidence suggested how the CS properties of siramesine and **F408** might be linked to the activation of the futile ATP cycle and corresponding increased ROS production. In order to verify this hypothesis we first demonstrated the involvement of ROS in the antiproliferative action of all the novel compounds and siramesine by the pre-administration of the lipid anti-oxidant α-tocopherol. Pretreatment of MCF7 cells with 100 μM of α-tocopherol prior administration of all the σ_2 ligands of the series was able to completely rescue cells from death. This result demonstrated that ROS generation was at least in part responsible of the σ_2-mediated antiproliferative activity for these compounds as previously demonstrated for siramesine and other ligands of ours.[26-27] If ROS generation was responsible for CS properties of siramesine and **F408**, then ROS generated in MCF7adr should be higher than ROS generated in MCF7 upon treatment with these compounds.

Therefore, both cell lines were pretreated with increasing concentration of α-tocopherol (1 μM, 10 μM, 50 μM) before incubation with **F408** or siramesine (25 μM) (Figure 5). α-Tocopherol rescued cells viability in a dose dependent manner, with MCF7 cells surviving 20%-40% more than the corresponding resistant cells.[16] These results are in agreement with the hypothesis that the enhanced antiproliferative activity of siramesine and **F408** in resistant cells may be due to the increase in ROS production induced by the futile ATP cycle which is activated by P-gp substrates. In order to further support this hypothesis we investigated the effect of these two compounds in other two cell line pairs: human HT29 colorectal adenocarcinoma cells and the doxorubicin-resistant counterpart HT29-dx, human A549 lung carcinoma cells and the doxorubicin-resistant counterpart A549-dx. As in MCF7 and MCF7adr cells, **F408** and siramesine showed higher antiproliferative activity in resistant cell line than in parental cells, showing CS properties also in these cells (Table 1).

In HT29-dx cells, EC_{50} value for **F408** could not be determined because of its potent activity: 1μM of **F408** induced 60% of cells death. On the other hand, EC_{50} could be determined for this compound in HT29 cells (EC_{50} = 6.86 μM). In HT29-dx cell line, siramesine was 1.6-fold more potent than in the (EC_{50} = 8.35 μM in HT29-dx, EC_{50} =13.5 μM in HT29). In A549-dx cell line, **F408** was 2.3-fold more potent than in the corresponding parent cells (EC_{50} = 11.7 μM in A549-dx, EC_{50} = 26.4 μMin A549). In these same cell line pairs siramesine displayed a 3.3-fold higher activity in the MDR cells compared to the P-gp-negative control (EC_{50} = 4.63 μM in A549-dx, EC_{50} = 15.4 μM in A549).

As in MCF7 cell line pairs, we postulated that also in HT29/HT29-dx and A549/A549-dx, **F408** and siramesine determined CS effect, likely by the futile ATP-cycle and ROS production. Since one source of both ROS and intracellular ATP is the mitochondrial respiratory chain, we analyzed the effects of siramesine and **F408** on this parameter: interestingly, both HT29-dx and A549-dx cells had a basal higher level of electron flux through complex I and complex III of the mitochondrial

chain (Figure 6), coupled with a higher level of intramitochondrial ATP (Figure 7) than the parental cells. These data are consistent with our previous observations showing that these MDR cells have a more active aerobic metabolism than the drug sensitive counterpart.[28] Interestingly, siramesine and in particular **F408** decreased both the electron flux rate and the ATP levels: MDR cells underwent a much more pronounced decrease than drug sensitive cells, likely because the basal aerobic mitochondrial activity was higher in the former. In conclusion ATP depleting agents are inducers of CS[10] and we demonstrated depletion of mitochondrial ATP supply by siramesine and **F408**, suggesting that this is the mechanism by which these σ_2 ligands induce CS. Although more efforts are needed to fully understand the mechanisms for σ_2 mediated antiproliferative action and CS, compounds exerting these activities warrant further investigation as a strategy to face the challenge of resistant tumors treatments.

Materials and Methods

Cell cultures. Human MCF7 breast adenocarcinoma was purchased from ICLC (Genoa, Italy), human MCF7adr breast adenocarcinoma (resistant to doxorubicin), were kindly provided by Prof. G. Zupi (IRE, Rome, Italy). Human colon cancer doxorubicin sensitive HT29 cells (ATCC) and the doxorubicin resistant counterpart HT29-dx cells, human doxorubicin sensitive lung cancer A549 (ATCC) cells and the doxorubicin chemoresistant A549-dx cells were obtained and cultured as already reported.[29]. HT29 and A549 have low/undetectable levels of Pg-p, which is instead expressed in HT29-dx and A549-dx cells.[29] MCF7 cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere. MCF7adr and HT29 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, in a humidified incubator at 37 °C with a 5 % CO_2 atmosphere. A549 cells were grown in HAM'S F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, in a humidified incubator at 37 °C

with a 5 % CO_2 atmosphere. HT29-dx and A549-dx were grown in the same medium of parental cells supplemented with doxorubicin (150 nM for HT29-dx and 10nM for A549-dx, respectively).

Cell Viability. Determination of cell growth was performed using the MTT assay at 48 h.[30,31] On day 1, 25,000 cells/well were seeded into 96-well plates in a volume of 100 μL. On day 2, the various drugs concentration (0.1–100 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, 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 nm and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

Effect of α-tocopherol on cell viability. The interference of reactive oxygen species in cell viability was indirectly performed using MTT assay at 24 h. On day 1, 25,000 cells/well were seeded into 96-well plates in presence or absence of different concentrations of α-toco (1-100μM). On day 2, the drugs (25 μM) were added alone and in combination with different concentrations of α-toco (1-100μM). After incubation (24 h) with drugs, 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 nm and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

Bioluminescent ATP assay. This experiment was performed as reported in technical sheet of ATPlite 1 step Kit for luminescence ATP detection (PerkinElmer Life Sciences).[32] The MCF7 and MCF7adr cells were seeded into black CulturePlate 96/wells plate in 100 μL of complete medium at a density 2×10^4 cells/well. The plate was incubated O/N in a humidified atmosphere 5% CO_2 at 37 °C. The medium was removed and 100 μL of complete medium in the presence or absence of different concentrations of test compounds was added. The plate was incubated for 2h in a humidified atmosphere 5% CO2 at 37 °C. Then, 50 μL of mammalian cell lysis solution was added

to all wells and the plate stirred for 5 mitutes in an orbital shaker. In all wells, 50 μL of substrate solution was added and the plate stirred for 5 min in an orbital shaker. The plate was dark adapted for ten min and the luminescence was measured on the microplate reader Victor 3 from PerkinElmer Life Sciences.

Mitochondrial respiration and ATP synthesis. Mitochondria were extracted as described earlier.[33] A 50 μL aliquot was sonicated and used for the measurement of protein content with BCA kit (Sigma Chemical Co.) or Western blotting; the remaining part was stored at −80 °C until use. To confirm the presence of mitochondrial proteins in the extracts, 10 μg of each sonicated sample was subjected to SDS-PAGE and probed with an anti-VDAC/porin antibody (Abcam, Cambridge, UK; not shown). The activity of of Complex I-III was measured on 10 μg of non sonicated mitochondria, re-suspended in 0.2 ml of buffer A (5 mM KH_2PO_4, 5 mM $MgCl_2$, 5% w/v bovine serum albumin) plus 0.1 ml of buffer B (25% w/v saponin, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 5% w/v bovine serum albumin, 0.12 mM oxidized cytochrome c, 0.2 mM NaN_3). The reaction was started with 0.15 mM NADH and was followed for 5 min, using a Packard EL340 Microplate Reader (Bio-Tek Instruments). The results were expressed as nmol reduced cytochrome c/min/mg mitochondrial proteins. In the same extracts, the amount of ATP was measured with the ATP Bioluminescent Assay Kit (FL-AA, Sigma Aldrich Co.), using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek). ATP was quantified as arbitrary light units; data were converted into nmol ATP/mg mitochondrial proteins.

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Figure Legends

Figure 1. Thiosemicarbazones

Figure 2. General Structure of novel σ_2 receptor ligands: Each of the hydrophobic moieties (**R**) was connected through a propyl or butyl linker to each of the basic moieties (**Y**).

Figure 3. σ_2 Receptor agonists

Figure 4. ATP consumption in MCF7 and MCF7adr by siramesine, F397 and F408. ATP content in MCF7 (grey bars) and MCF7adr (black bars) treated with **siramesine,** or **F397** or **F408 (**25 μM). Control = untreated cells.

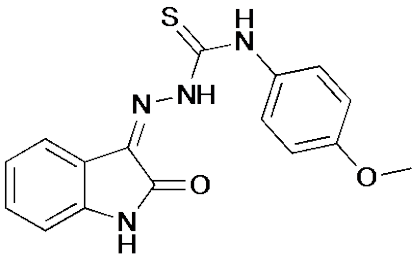
Figure 5. α-Tocopherol effect on cells viability. Antiproliferative effect of (A) 25 μM siramesine or (B) 25 μM **F408** in MCF7 and MCF7adr cells. Cells were pre-treated with increasing concentrations of α-tocopherol.

Figure 6. Mitochondrial respiratory activity. The electron flux between Complex I and Complex III was measured spectrophotometrically in isolated mitochondria from HT29 and HT29-dx cells (A), A549 and A549-dx cells (B) incubated with 5 μM siramesine or 15 μM **F408**

Figure 7. Mitochondrial ATP level. The amount of intramitochondrial ATP was measured by chemiluminescence in isolated mitochondria from HT29 and HT29-dx cells (A), A549 and A549-dx cells (B) incubated with 5 μM siramesine or 15 μM **F408**

Table 1. Antiproliferative Action (IC_{50} μM) of siramesine, **F408** and **F397** in parental and drug-resistant cell line pairs.

Figure 1



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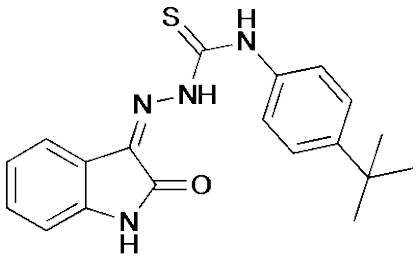
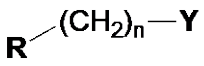


1

Figure 2



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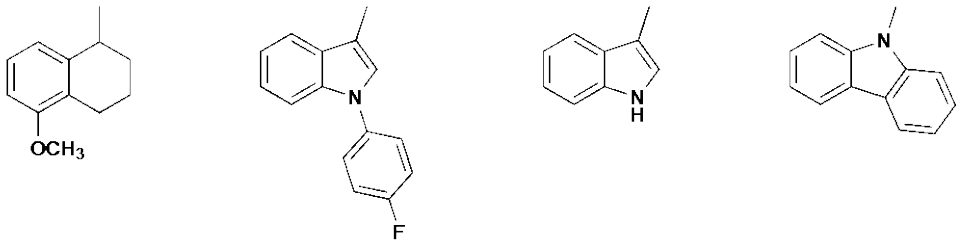

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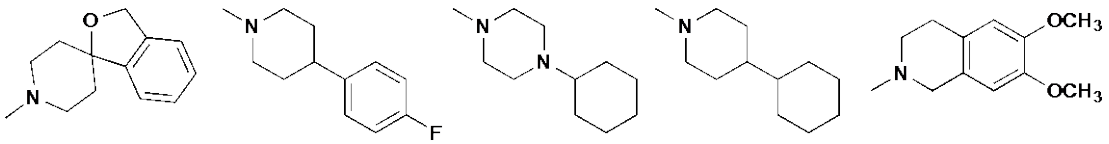
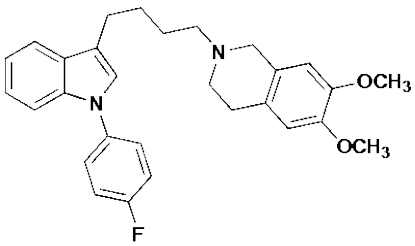
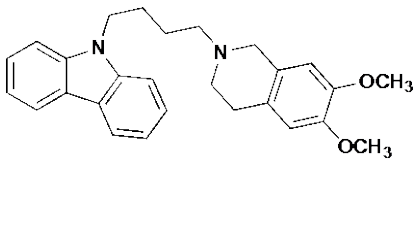


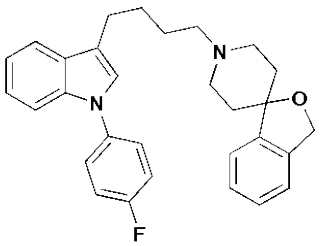
Figure 3



F397



F408



Siramesine

Figure 4

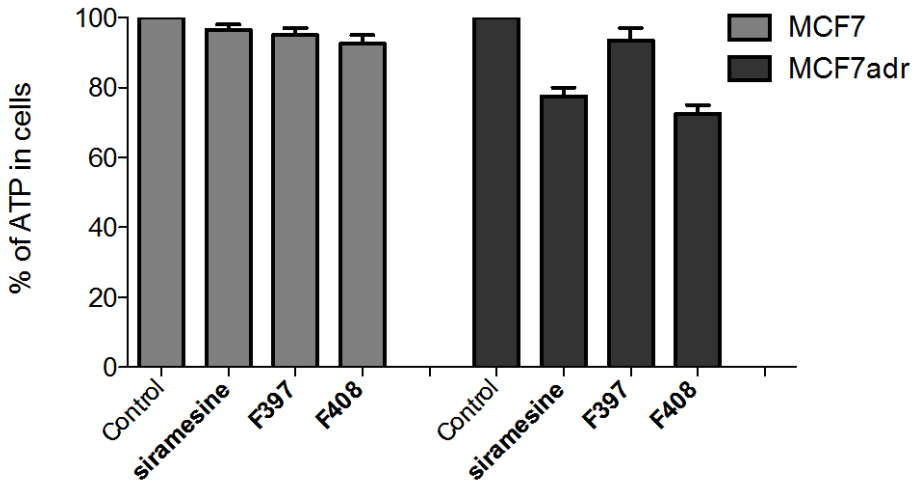
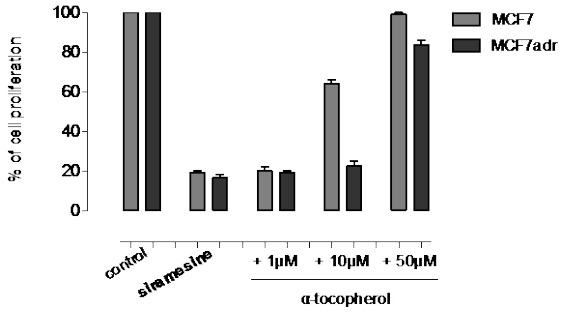


Figure 5

A



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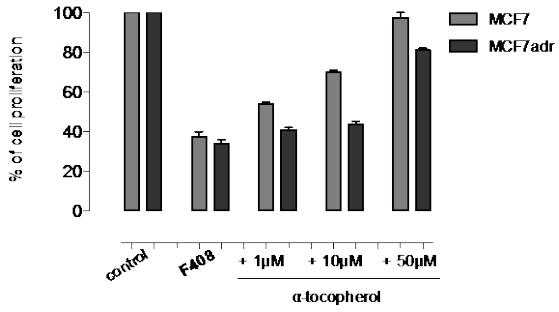
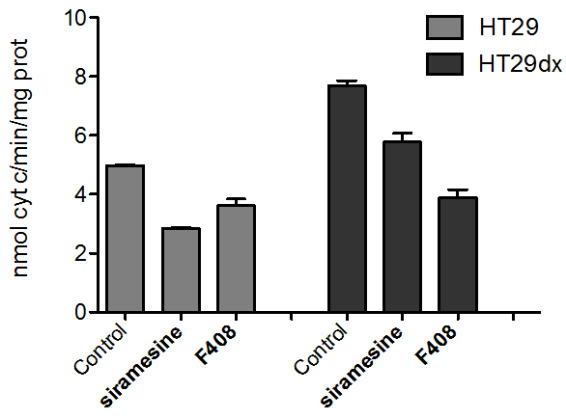


Figure 6

A



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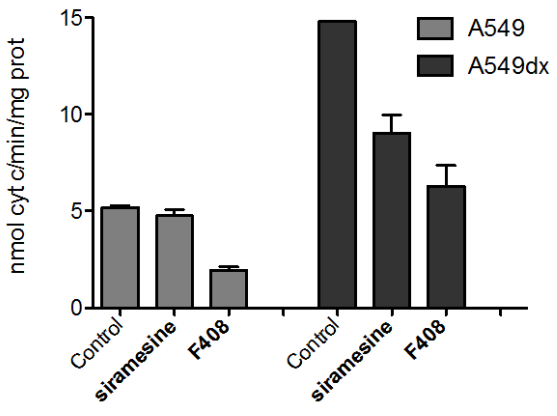
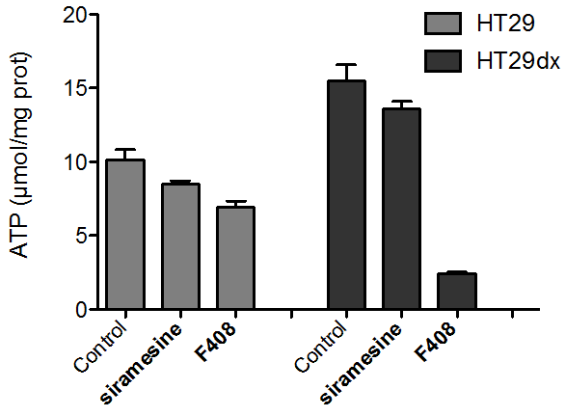


Figure 7

A



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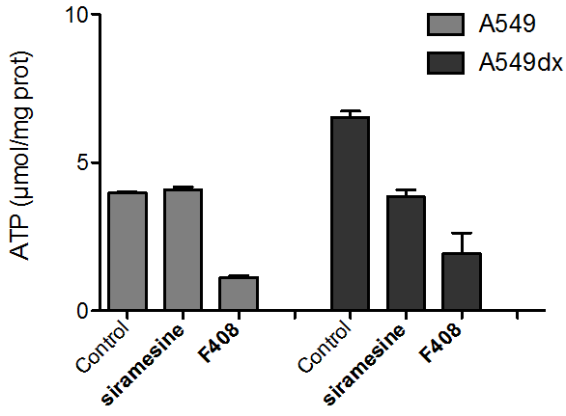

Table 1.

compd	Cell Lines	IC50 (μM)[a]	CS
siramesine	MCF7	12.3±0.6	2.1
	MCF7adr	5.90±1.2	
	A549	15.4±2.2	3.3
	A549dx	4.63±0.5	
	HT29	13.5±1.4	1.6
	HT29dx	8.35±0.9	
F408	MCF7	28.2±5.0	1.6
	MCF7adr	17.1±1.2	
	A549	26.4±2.6	2.3
	A549dx	11.7±1.1	
	HT29	6.86±0.7	ND
	HT29dx	60%[b]	
F397	MCF7	17.8±0.4	
	MCF7adr	21.8±1.5	

[a] Values are the means of $n \geq 2$ separate experiments, in duplicate. [b] IC50 could not be determined since 1 μM compound determined the death of 60% cells.