Cytotoxic activity of gemcitabine, alone or in combination with mitotane, in adrenocortical carcinoma cell lines.

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Cytotoxic activity of gemcitabine, alone or in combination with mitotane, in adrenocortical carcinoma cell lines

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Abstract

We aimed at investigating *in vitro* the cytotoxic activity (determined using WST-1, apoptosis and cell cycle assays) of gemcitabine, alone or in combination with mitotane, in mitotane-sensitive H295R and mitotane-insensitive SW-13 cells. Results of these experiments were compared with drug-induced modulation of RRM1 gene, the specific target of gemcitabine. In H295R cells, mitotane and gemcitabine combinations showed antagonistic effects and interfered with the gemcitabine-mediated inhibition of the S phase of the cell cycle. By contrast, in SW-13 cells, except when mitotane was sequentially administered prior to gemcitabine, the combination of the two drugs was synergistic. Such opposite effects were associated with opposite expression profiles of the target gene, with significant up-modulation in H295R but not in SW-13 under gemcitabine and mitotane combination treatment.

Keywords: adrenocortical cancer, gemcitabine, mitotane, cell lines

Highlights

- gemcitabine is an active cytotoxic agent in ACC cells *in vitro*
- mitotane/gemcitabine cytotoxicity differs in mitotane-sensitive and insensitive cells
- sequential use of mitotane→gemcitabine was antagonistic in both cell lines
- single/combinatory treatment response correlated to RRM1 gene modulation profiles

1. Introduction

Mitotane [1,1-dichcloro-2-(*o*-chloropheyl)-2-(*p*-chlorophenyl)-ethane] is an adreno-corticolytic drug and represents the standard treatment for adrenocortical carcinoma (ACC), a rare and clinically aggressive endocrine tumor generally associated with a poor prognosis (Wajchenberg et al., 2000; Dackiw et al., 2001; Fassnacht et al., 2011).

In clinical practice, the antitumor efficacy of mitotane has been demonstrated both in the adjuvant setting (Terzolo et al., 2007) and in the treatment of advanced/progressive disease. In the latter setting, mitotane is active either alone or in combination with different chemotherapeutic agents (Fassnacht et al., 2012). However, scarce data on antitumor efficacy of mitotane have been obtained *in vitro*. Mitotane is highly effective for blocking adrenocortical hormone secretion by inhibiting cholesterol chain cleavage and 11β-hydroxylation, but evidence of an anti-proliferative effect is incomplete and controversial. First, differential effects on tumor growth have been shown in the two most commonly investigated ACC cell line models, H295R and SW-13, the former being mitotane-sensitive and the latter lacking responsiveness even at very high doses (Volante et al., 2012). Second, the anti-neoplastic properties of mitotane seem to be adrenocortical-specific, since mitotane does not appear to be effective on other tumor cells lines, such as those of lung origin (Volante et al., 2012). Moreover, the mechanisms underlying its adrenocortical-specific anti-neoplastic properties are largely unknown and possibly related to different biological processes (including energetic metabolism, stress response and other cellular functions), as suggested by proteomic analysis of H295R cells (Stigliano et al., 2008). Finally, the possible interactions of mitotane with other antitumor agents, including the chemotherapeutic drugs commonly used for ACC treatment, are poorly explored. In this latter respect, mitotane has been shown to sensitize ACC cancer cells to ionizing radiation (Cerquetti et al., 2008) and to enhance cytotoxicity of chemotherapy by reversing P-glycoprotein-mediated multidrug resistance

with a specific effect on the cell cycle (Bates et al., 1991). However, very few studies determined *in vitro* the activity of mitotane in combination with other chemotherapeutic agents used in ACC patients (Villa et al., 1999).

From a clinical standpoint, the toxicity of mitotane is still a major limitation to its use in the treatment of ACC patients (Daffara et al., 2008) and predictive biomarkers correctly identifying patients who will profit from mitotane treatment are still missing. In a recent study from our group, high expression levels of Ribonucleotide Reductase Large Subunit 1 (RRM1) gene were shown to be negative predictors of response to mitotane administration as an adjuvant treatment (Volante et al., 2012). RRM1 is the specific molecular target of gemcitabine, a chemotherapeutic agent used in the treatment of different solid tumors. Gemcitabine requires intracellular phosphorylation to its active metabolites, 2'-2'-difluoro-dCDP and 2'-2'-difluoro-dCTP, which, specifically inhibits RRM1 and is incorporated into the DNA leading to chain termination (Gandhi et al., 1995). In ACC, a recent phase 2 study demonstrated that a combination of gemcitabine plus fluoropyrimidine derivatives (5-fluoruracilor capecitabine) and mitotane was active as second or third-line treatment (Sperone et al., 2010). However, no data are currently available on the cytotoxic efficacy of gemcitabine in ACC cells, *in vitro*.

The aim of this study was to evaluated the cytotoxic effects of gemcitabine in H295R and SW-13 adrenocortical cancer cell lines, as a single agent or in combination with mitotane, and to compare the profiles of responsiveness with RRM1 drug-induced gene regulation.

2. Material and Methods

2.1 Cell culture and chemical reagents.

NCI-H295R and SW-13 ACC cell lines were supplied by the American Type Culture Collection (ATCC, Rockville, MD, USA). H295R cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's

Medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma, St. Louis, USA) supplemented with 1% L- glutamine (Sigma) and 2.5% of Nu-Serum (BD Biosciences, San Jose, CA) and enriched with 1% di ITS+Premix (BD Bioscience). SW-13 cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Biowest, France) and 1% L-glutamine (Sigma). Mitotane was purchased from Supelco and dissolved in 100% methanol (Sigma). Gemcitabine (Eli Lilly and Co, Indianapolis, IN) was dissolved in physiological buffer at 150 mM.

2.2 Treatment and cell viability assay.

Cell lines were seeded into 96-well plates in triplicates and treated with mitotane and gemcitabine (with a range between 100nM and 25µM for each drug), used alone or in simultaneous combinations for 48h and 72h in SW-13 and H295R cells, respectively. Time endpoints of 48h and 72h for SW-13 and H295R cells were selected based on the different proliferation profiles of these two cell lines, following previously published preliminary experiments (Volante et al., 2012). Sequential treatments were as follows: gemcitabine or mitotane for 24h, followed by the adjunct of mitotane or gemcitabine for 24h (total treatment: 48h) in SW-13 and for 48h (total treatment: 72h) in H295R cells. After incubation time, Cell Proliferation Reagent WST-1 (Roche Applied Science, Penzberg, Germany) was added to each well in order to measure cell proliferation, following the supplied protocol. The absorbance was determined using a microplate reader (iMARK microplate reader, Biorad Life Science Group, Hercules, CA USA) at a test wavelength of 450 nm and reference wavelength of 630 nm. Cell viability ratios were calculated using the sigmoid inhibition model (GraphPad PRISM 5, San Diego, CA, USA). Drug interaction between mitotane and gemcitabine was assessed using the combination index (CI), according to the following formula (Chou et al., 1984): $CI=C_{A,X}/IC_{X,A} + C_{B,X}/IC_{X,B}$; $C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A and drug B used in combination to achieve 50% drug effect. $IC_{x,A}$ and $IC_{x,B}$ are IC50

concentrations for single agents, that achieve the same effect. Values of CI < 1, equal to 1, and > 1 indicate synergistic, additive and antagonistic effects, respectively.

2.3 Apoptosis detection and cell cycle analysis.

Cytofluorimetric assays were performed on ACC cells treated in triplicates with mitotane and gemcitabine, alone or in simultaneous combination, at 48h and 72h for SW-13 and H295R cells, respectively. In apoptosis and cell cycle experiments, fixed drug concentrations of 5µM for both mitotane and gemcitabine - either used alone or in combination – were employed. Higher concentrations for both drugs were also tested but yielded insufficient rate of evaluable cells due to extensive cell necrosis (data not shown). To detect apoptotic events, cells were washed twice with PBS and the pellet was re-suspended in 1X Annexin binding buffer (Annexin V-FITC Apoptosis Detection Kit Immunostep, Roche Applied Science) at a concentration of 10^6 cells/100µl. Five µl of the Annexin V-FITC and 5 µl of Propidium iodide (PI) were added to each test, and samples were incubated for 15 minutes at room temperature in darkness. Additional 400 µl of 1X Annexin binding buffer was added to each tube and cells were analyzed using a BD FacsCanto flow cytometer (BD Biosciences). For cell cycle analysis, cells were fixed with EtOH 70% and re-suspended in PBS. PI/Rnase staining buffer (BD Biosciences) was added to each condition, following the manufacturer's instructions and samples were incubated for 15 minutes at room temperature in darkness. Flow cytometry setup was performed using DNA QC particles kit (BD Biosciences). For both cell cycle and apoptosis experiments, data were analyzed using the ModFit software (Verity Software, Topsham, ME,USA) and results were represented as fold changes in relative mean ratios between cells untreated as compared with the different treatment modalities.

2.4 RRM1 gene expression evaluation by means of Real Time PCR.

H295R and SW-13 cell lines were cultured in triplicate into six-well plates and treated with mitotane and gemcitabine either alone or in simultaneous/sequential combinations, at a fixed concentration of 5µM for mitotane and gemcitabine, in both cell lines. Duration of treatment was set as detailed above at 48h for SW-13 and 72h for H295R cells**.** Total RNA was extracted using Qiazol Reagent (Qiagen, Japan). Complementary DNA was generated using M-MLVT RT (200U/µl) (Invitrogen, California) and oligodT primers (500µl/ml) (Invitrogen) from 1µg of total RNA. Relative cDNA quantification of RRM1 and a housekeeping gene (beta-actin) were examined by quantitative real-time PCR using primers and PCR conditions previously reported (Ceppi et al., 2006). Target gene expression was analyzed at different treatment combinations normalizing RRM1 mRNA levels to beta-actin, then calculating ΔΔCt and expressing corresponding values as $2^{-\Delta\Delta Ct}$. A change in RRM1 gene expression levels above 2 folds was considered significant.

3. Results

3.1 Gemcitabine is an active cytotoxic agent in adrenocortical cancer cells.

Gemcitabine induced a dose-dependent decrease of cell viability in the two adrenal cancer cell lines (**Figure 1**), with IC50 values of 28.87 µM (standard error ±0.04) (at 48h of treatment) and 45.99 µM (standard error ±2.61) (at 72h of treatment) in SW-13 and H295R cells, respectively. The effect of gemcitabine on apoptosis (**Figure 2**) was mild in H295R cells, with a fold increase of 2.37 (standard error ±0.43; Student's t test: p=0.13) and 3.12 (standard error ±0.37; Student's t test: p=0.02) in the early and late apoptotic phases, respectively. In SW-13 cells, a 2.16 (standard error ±0.07; Student's t test: p=0.025) fold increase was observed in the early apoptotic phase whereas a consistent increase in the population of late apoptotic/necrotic cells was detected, with a fold increase of 54.25 (standard error ±5.25; Student's t test: p=0.009). In cell cycle experiments

(**Figure 3**), as expected by the specific role of gemcitabine to block the S phase, a striking reduction of the rate of cells in the G2 phase was observed in both H295R (Student's t test: p=0.0005) and SW-13 cells (Student's t test: p=0.0002).

3.2 Opposing effect of gemcitabine/mitotane combination in mitotane-sensitive and insensitive adrenocortical cancer cells.

As expected by the known profiles of drug-responsiveness of the two cell lines, mitotane determined a cytotoxic effect, as measured by cell viability assay, in H295R cells, with a IC50 value of 30.62 µM (standard error ±2.02) (at 72 h of treatment), but not in SW-13 cells, with a IC50 value of $6{,}20x10^2$ µM (standard error $\pm 0.17x10^2$) (at 48 h of treatment) (**Figure 1**). Early and late apoptotic cell ratios were not modified by mitotane treatment neither in H295R nor in SW-13 cells (Student's t test: all p values >0.1) (**Figure 2**); moreover, mitotane treatment induced mild changes in the different phases of the cell cycle in H295R cells, only (Student's t test corresponding p values for fold changes in G1, S and G2: 0.018, 0.019, 0.016, respectively) (**Figure 3**).

The combination of mitotane and gemcitabine showed opposing effects in mitotane-sensitive and insensitive cells. In H295R cells, that responded in terms of cell viability to both mitotane and gemcitabine used as single agents, the simultaneous combination of these two drugs resulted in an antagonistic effect on cell growth (CI= 5.44±0.87). Such drug antagonism was evident also in sequential treatments and significantly increased when mitotane was used as the first drug (gemcitabine→mitotane: CI= 2.12±0.21; mitotane→gemcitabine: CI =82.11±1.03).

In mitotane-insensitive SW-13 cells, the combination of gemcitabine + mitotane showed a slightly significant synergistic anti-proliferative effect (CI=0.80±0.05) that potentiated the cytotoxic effect observed by using gemcitabine alone (**Figure 1**). Sequential gemcitabine→mitotane treatment

showed effects similar to the simultaneous treatment (CI= 0.90±0.07), whereas sequential mitotane→gemcitabine treatment was antagonistic (CI= 2.48±0.78).

This observation was paralleled by data resulting from apoptosis (**Figure 2**) and cell cycle (**Figure 3**) investigations. In fact, the combination of gemcitabine + mitotane in H295R cells had a minor influence on the apoptotic rate in late phase, only (Student's t test: p=0.019). Moreover, the proportion of cells in the G2 phase, although lower than untreated cells, was significantly increased as compared to gemcitabine alone (Student's t test: p=0.001), thus showing a significant interference in the inhibitory effect of gemcitabine to the S phase. By contrast, in SW-13 cells the combination of gemcitabine + mitotane potentiated the effect of gemcitabine to induce late apoptotic/necrotic (Student's t test: p=0.038) events.

3.3 Modulation of RRM1 gene expression by gemcitabine and mitotane.

Mitotane and gemcitabine alone showed no effect in the modulation of RRM1 gene in H295R cells, whereas mitotane induced an up-modulation of RRM1 gene expression levels in SW-13 cells (**Figure 4**). Interestingly, the opposing anti-proliferative effects of the combination gemcitabine plus mitotane were paralleled by opposite effects on RRM1 gene modulation. In fact, the antagonism of the two drugs in H295R cells, either as simultaneous or sequential treatments, was associated with up-modulation of RRM1 transcription. In SW-13 cells, mitotane-induced RMM1 gene up-modulation was absent in synergistic combinations of simultaneous mitotane + gemcitabine and sequential gemcitabine→mitotane treatments, whereas was observed in antagonist mitotane→gemcitabine combination.

4. Discussion

The present study demonstrates that gemcitabine is effective as an anti-neoplastic agent in ACC *in vitro*, and shows that the drug interacts differently with mitotane provoking variable effects on cell viability and modulation of its target gene RRM1.

The concept of this study stems from different viewpoints. Primarily, no preclinical data on gemcitabine activity on ACC cell lines have been published, so far, although this drug represents an antitumoral compound used in advanced ACC patients (Sperone et al., 2010). Very few studies in the literature analyzed the effects of different chemotherapeutic agents on the growth and survival of ACC cells *in vitro*. However, gemcitabine was not included in these studies, which were also based on a single cell line (SW-13) (Montoya et al., 2008; Villa et al., 1999). Our data show that gemcitabine is highly effective in the two most commonly studied ACC cell lines, specifically blocking the transition from the S to the G2 phase and inducing late apoptotic events, this latter being more evident in the SW-13 cell line model. As compared to gemcitabine, and in line with previous data from the literature including those from our group (Volante et al., 2012), mitotane was effective at inducing a reduction of cell viability in H295R but not in SW-13 cells. However, this effect on cell proliferation was not paralleled by a significant influence on apoptosis and cell cycle in mitotane-sensitive H295R cells. This observation, already presented in previous reports detailing the effects of mitotane on cell cycle used as a single agent (Cerquetti et al., 2008), supports a cytostatic rather than cytotoxic effect of mitotane in ACC cells.

A secondary endpoint was the investigation of the interactions between gemcitabine and mitotane used as combination treatment in ACC cells. In this respect, very few data are available in the literature on the activity of mitotane in association with any of the chemotherapeutic agents commonly used in ACC patients' treatment (Villa et al., 1999), generally with an additive effect. This aspect is of particular interest since mitotane is the mainstay of treatment in ACC patients and is present in every chemotherapeutic regimen currently proposed in advanced or progressive

disease (Fassnacht et al., 2012). However, it is already established in other tumor models that the combination of active drugs, if used alone as antitumor agent, is not necessarily favorable in terms of global anti-neoplastic efficacy. Our data are rather provocative, since differential effects of the mitotane plus gemcitabine combination were demonstrated in mitotane-sensitive and mitotane-insensitive ACC cells. In fact, in H295R cells, sensitive to mitotane used as a single agent, the combination with gemcitabine was antagonistic and repressed the antineoplastic properties of both agents. By contrast, in SW-13 cells mitotane was not effective alone but potentiated the antitumor effects of gemcitabine when used as a combination modality. It is worth noticing that in both cell lines sequential treatments having mitotane as the first drug showed an antagonistic effect. Our results suggest that a combination modality may not be always favorable for ACC patients. It has been already established in other tumor models that the combination of active drugs is not necessarily associated with greater anti-neoplastic efficacy. In lung cancer models, gemcitabine has been demonstrated to be either synergistic or antagonistic with topotecan depending on the type and timing of combination (Giovannetti et al., 2005). However, caution should be used to translate our results into clinically meaningful data. In fact, our findings were obtained *in vitro,* only, using the two commercially available ACC cell lines, that do not necessarily represent the best model for assessing chemotherapeutic agent sensitivity profiles in ACC. Moreover, *in vivo* studies are needed to better understand the interactions at the pharmacodynamic and metabolic levels between mitotane and gemcitabine (or other chemotherapeutic agents used in ACC patients), and to validate our findings into a clinical perspective.

Our results point also to the fact that a better genetic and pathological profiling of ACC is needed to identify tissue biomarkers that may predict response to therapy. In this latter respect, a third aim of the study was to analyze in parallel with cytotoxic properties of the two compounds, the

modulation profiles of RRM1, the specific target of gemcitabine that in a recent study from our group was also found to be associated with mitotane responsiveness, being high gene expression levels correlated to a lower sensitivity (Volante et al., 2012). In line with the drug-sensitivity profiles, synergistic mitotane plus gemcitabine combination treatment in SW-13 cells, simultaneous or sequential gemcitabine→mitotane, did not up-modulate RRM1 gene expression rendering the cells more sensitive to both drugs, whereas the antagonist sequential mitotane→gemcitabine combination induced RRM1 gene up-modulation, similar to mitotane alone. By contrast, the antagonism showed in H295R cells by the mitotane and gemcitabine combination, either simultaneous or in the two different sequential modalities, was associated with a striking up-modulation of RRM1 gene, a finding not evident using the two compounds individually, possibly conferring resistance of this cell model to both agents. Far from being a molecular explanation of the differential effects of mitotane and gemcitabine combination treatment in ACC cells, these findings strongly support our previous data on a major role of the RRM1 enzyme in determining profiles of responsiveness to mitotane in ACC tumor cells.

5. Conclusions

In summary, this study shows that i) gemcitabine is an active cytotoxic agent in ACC cells *in vitro*, thus substantiating the use of this chemotherapeutic agent in ACC patients; ii) its efficacy in combination with mitotane is heterogeneous, antagonistic in mitotane-sensitive and synergistic in mitotane-insensitive cells depending on the sequential schedule, and iii) the drug effects are strongly associated with a different modulation of its target gene - RRM1.

Further studies are therefore needed to better understand *in vivo* the profiles of responsiveness to the combination of gemcitabine and mitotane, as well as to clarify the influence of RRM1 gene in the pharmacokinetics of the two compounds, either used alone or in combination.

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Figure legends

Figure 1. Cell viability after single or combined mitotane and gemcitabine treatment. Cytotoxic response to mitotane and gemcitabine and their combination in H295R and in SW-13 cell lines. Data result from three different experiments (±SD, n=3) having three replicates for each experiment, and are expressed as ratios of proliferating cells as compared to basal conditions. Logarithm of doses correspond to the following drug concentrations: -7=0.1µM; -6=1µM; -5.6=5µM; -5=10µM; -4.6=25µM.

Figure 2. Apoptosis analysis by means of flow cytometry after single or combined mitotane and gemcitabine treatment. Percentages of different type of apoptotic cells are reported in the upper Table as the result of three different experiments, having three replicates for each experiment. A representative flow cytometry analysis is shown in the lower panels. Early apoptotic cells correspond to FITC Annexin V positive and PE Propidium Iodide negative (lower right quadrants in the lower panels), whereas late apoptotic/necrotic cells correspond to cells positive for both FITC Annexin V and PE Propidium Iodide (upper right quadrants in the lower panels). The lower left quadrant of each panel shows the viable cells (negative for both FITC Annexin V and PE Propidium Iodide). MIT: mitotane; GEM: gemcitabine.

Figure 3. Cell cycle analysis by means of flow cytometry after single or combined mitotane and gemcitabine treatment. Percentages of cells in the different phases of the cell cycle are reported in the upper Table as the result of three different experiments having three replicates for each experiment, and illustrated as graphs in the lower panels. MIT: mitotane; GEM: gemcitabine.

Figure 4. RRM1 gene expression after single or combined mitotane and gemcitabine treatment.

RRM1 gene expression analysis in H295R and SW-13 cell lines under different treatments. Data result from three different experiments having two replicates for each experiment, and are expressed as fold changes ($2^{-\Delta\Delta Ct}$). A fold change >2 was considered significant. MIT: mitotane; GEM: gemcitabine

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Highlights

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Figure

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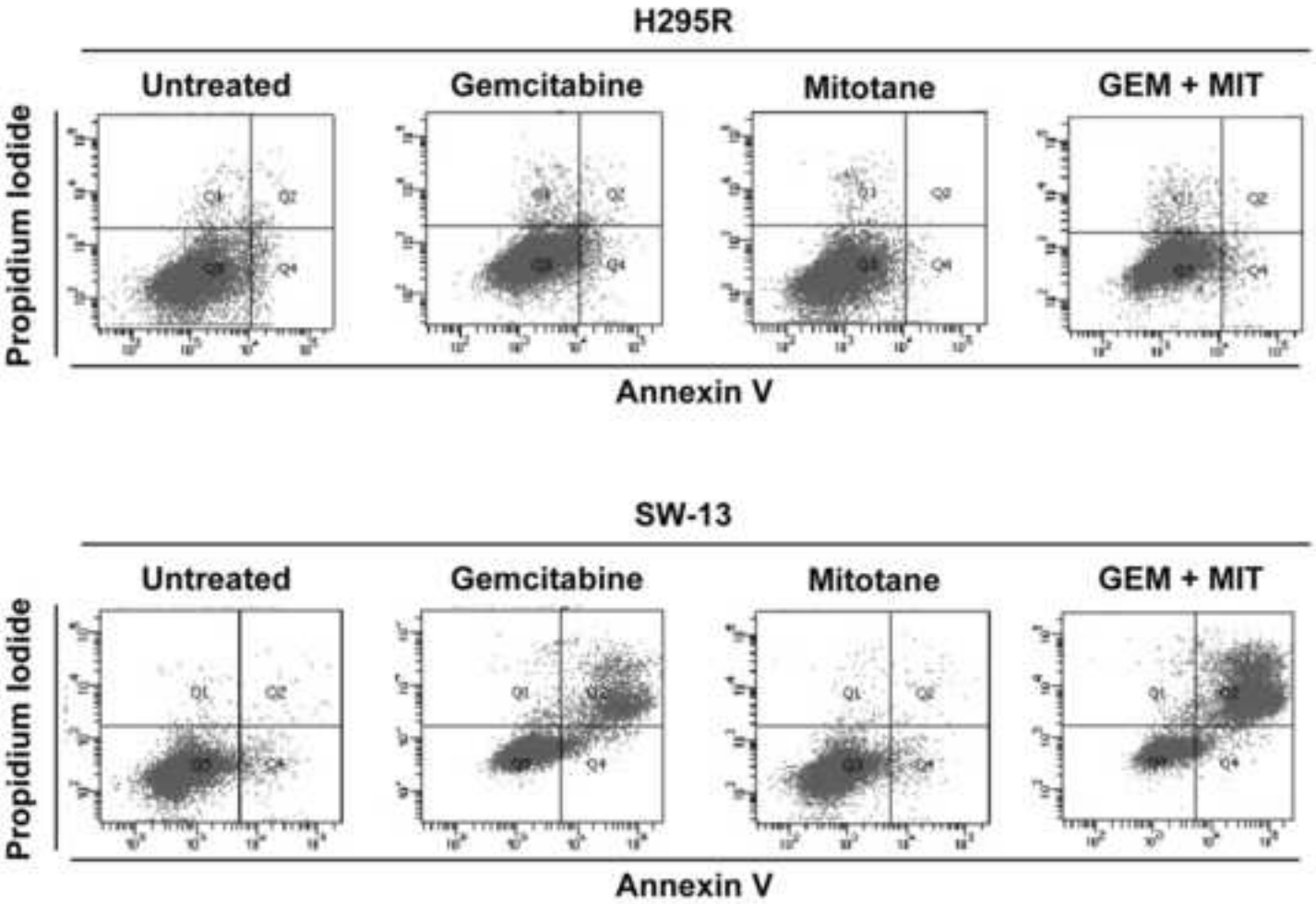
Cell line	Treatment	% ANNEXIN positive/PI negative	FOLD INCREASE			% ANNEXIN positive/PI positive	FOLD INCREASE		
H295R	UNTREATED	4.7±0.1	2.37±0.43	1.64±0.33	0.86±0.11	0.3±0.1	3.12±0.37	2±1	2.37±1.12
	GEMCITABINE	11.2±2.30				0.9±0.2			
	MITOTANE	7.75±1.75				0.55±0.15			
	GEM/MIT	4.05±0.45				0.65±0.05			
SW-13	UNTREATED	1.9±0.19	2.16±0.07	0.68±0.03	1.53±0.1	0.2±0	54.25±5.25	1.25±0.25	143.75±17.75
	GEMCITABINE	4.10±0.30				10.85±1.05			
	MITOTANE	1.3±0.2				0.25±0.05			
	GEM/MIT	2.9±0.10				28.75±3.45			



Figure

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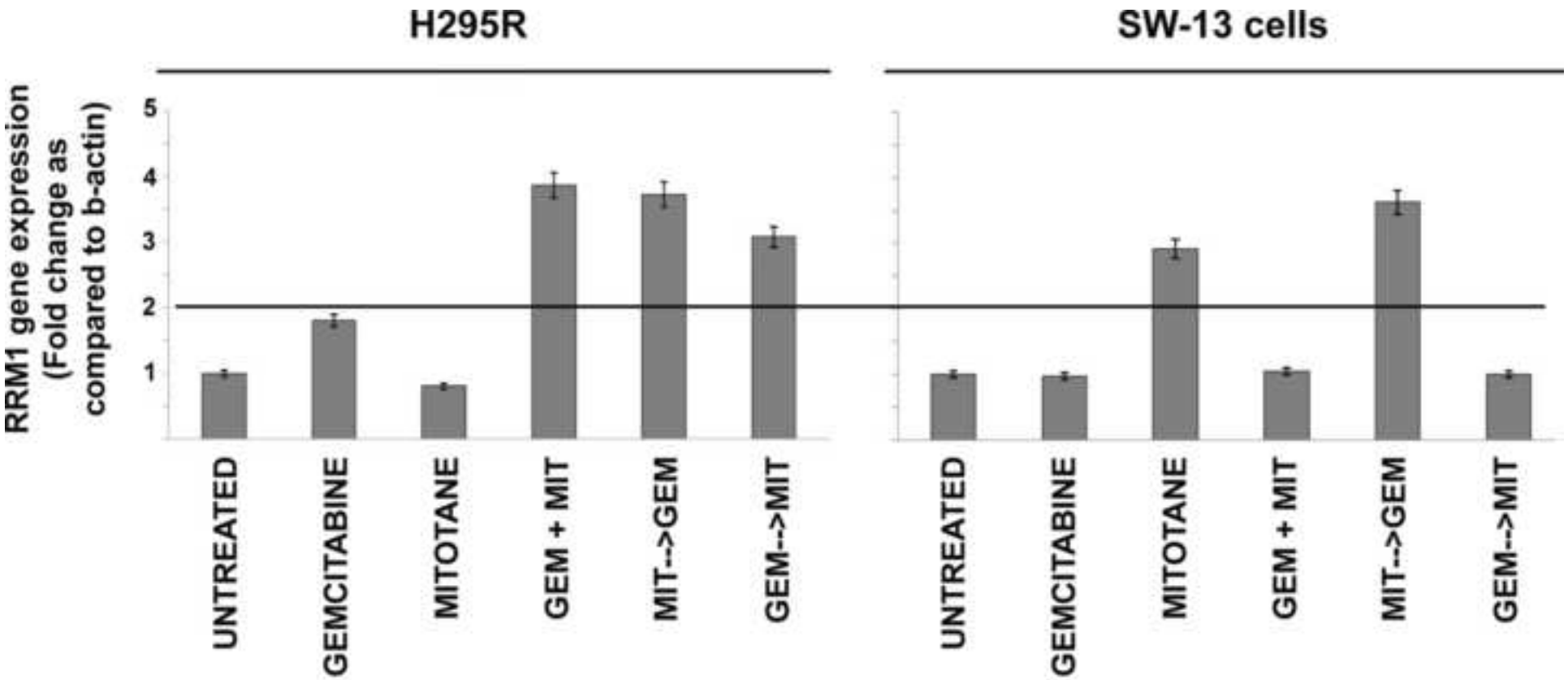

Figure

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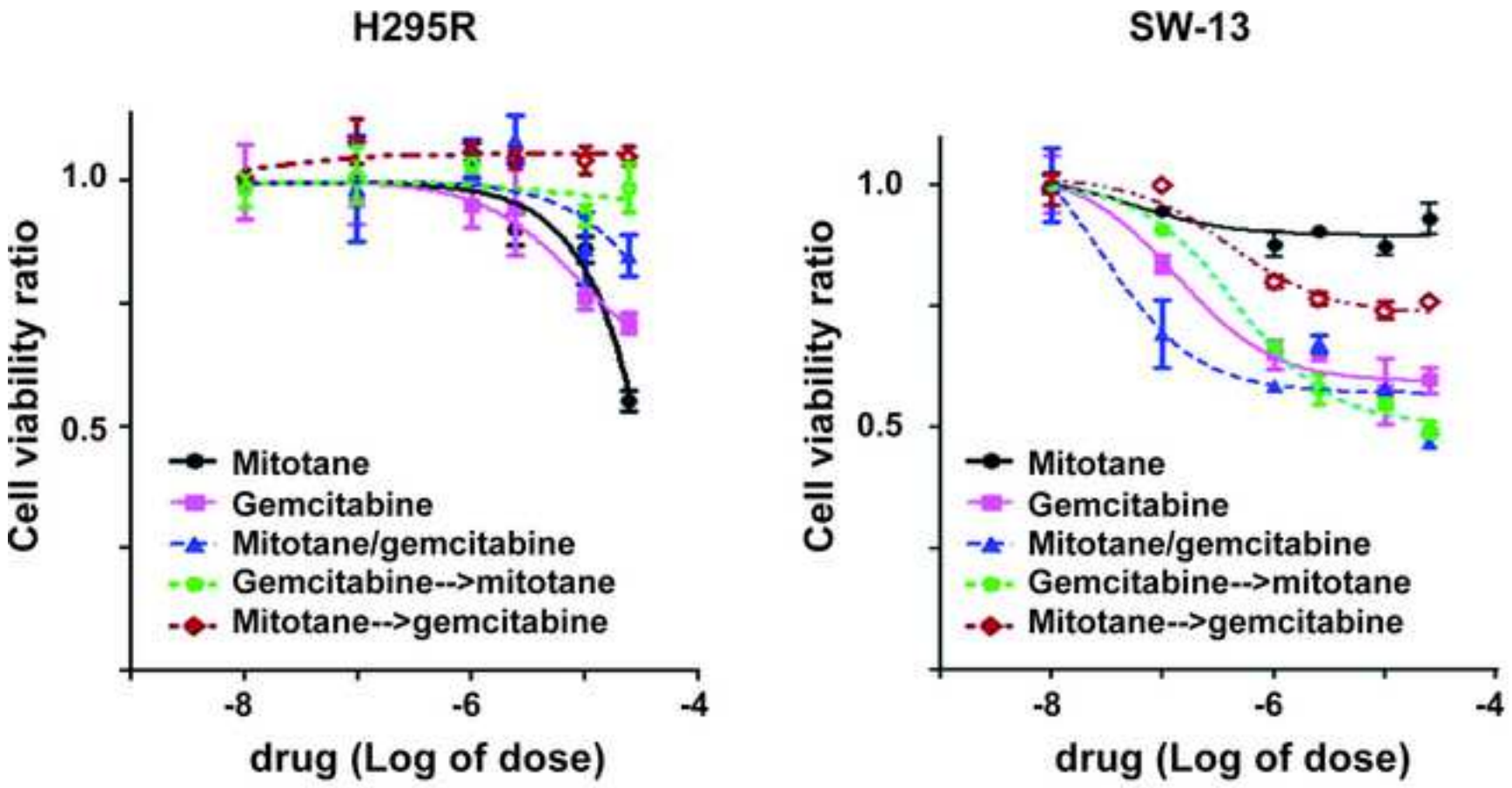

COMBINATION INDEX (CI)	SW-13	H295R
Mitotane/Gemcitabine	0.80±0.05	5.44±0.87
Gemcitabine→Mitotane	0.90±0.07	2.12±0.21
Mitotane→Gemcitbine	2.49±0.78	82.11±1.03

Figure

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Cell line	Treatment	%G1	FOLD INCREASE	%S	FOLD DECREASE	%G2	FOLD DECREASE
H295R	UNTREATED	69.7±0.68	0.88±0.03 / 1.07±0.01 / 0.96±0.03	21.94±0.9	0.57±0.06 / 1.21±0.01 / 0.67±0.01	8.35±0.19	84.48±1.9 / 1.23±0.04 / 3.09±0.05
	GEM	61.61±3.12		38.39±3.12		01.1±0	
	MIT	75.12±0.29		18.12±0.2		6.75±0.09	
	GEM/MIT	63±1.8		32.6±0.21		2.7±0.11	
SW-13	UNTREATED	59.08±0.11	1.28±0.48 / 0.94±0.06 / 1.23±0.01	32.32±0.10	1.37±0.17 / 0.87±0.03 / 1.28±0.01	8.41±0.14	84.1±1.4 / 1.13±0.1 / 8±1.4
	GEM	76.16±2.56		23.83±2.56		0.1±0	
	MIT	55.58±3.17		36.86±1.24		7.5±0.6	
	GEM/MIT	73.09±0.36		26.91±0.36		0.1±0