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27 April 2024

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Abstract

The anti-proliferative activity of mitotane (o,p'DDD) in adrenocortical cancer is mediated by its metabolites o,p'DDE and o,p'DDA. We previously demonstrated a functional link between ribonucleotide reductase M1(RRM1) expression and o,p'DDD activity, but the mechanism is unknown. In this study we assessed the impact of RRM1 on the bioavailability and cytotoxic activity of o,p'DDD, o,p'DDE and o,p'DDA in SW13 and H295R cells. In H295R cells, mitotane and its metabolites showed a similar cytotoxicity and RRM1 expression was not influenced by any drug. In SW13 cells, o,p'DDA only showed a cytotoxic activity and did not modify RRM1 expression, whereas the lack of sensitivity to o,p'DDE was associated to RRM1 gene up-modulation, as already demonstrated for o,p'DDD. RRM1 silencing in SW13 cells increased the intracellular transformation of mitotane into o,p'DDE and o,p'DDA. These data demonstrate that RRM1 gene interferes with mitotane metabolism in adrenocortical cancer cells, as a possible mechanisms of drug resistance.

Keywords: RRMI, mitotane, metabolites, adrenocortical cancer, cell lines

1. Introduction

Adrenocortical carcinoma (ACC) is a rare malignant endocrine tumor with a variable but generally poor prognosis (Fassnacht *et al.* 2013). Mitotane (o,p'DDD), an isomer of the insecticide dichlorodiphenyltrichloroethane, is the reference drug for advanced ACC either alone or in combination with chemotherapy (Terzolo *et al.* 2007, Fassnacht *et al.* 2012). Metabolic activation is thought to be essential for the therapeutic effect of mitotane. In fact, mitotane is transformed into two metabolites, 1,1-(o,p-dichlorodiphenyl)-2,2 dichloroethene (o,p'DDE) and 1,1-(o,p-dichlorodiphenyl) acetic acid (o,p'DDA), by α- and β-hydroxylation, respectively (Lindhe *et al.* 2002).

In ACC patients, o,p'DDA plasma levels increase rapidly and roughly parallel mitotane, whereas o,p'DDE levels rise slowly (Andersen *et al.* 1999). A study suggested that the plasma concentrations of o,p'-DDE were more closely related to clinical improvement or remission than those of mitotane (Kasperlik-Zaluska *et al.* 2005). By contrast, a recent retrospective multicenter study on a large series of patients showed that only measurement of o,p'DDA may provide additional information in refining prediction of response (Hermsen *et al.* 2011). However, monitoring of mitotane metabolites is not widely available and has not entered clinical practice, yet.

The molecular mechanisms of mitotane action and the specific contribution of mitotane and its metabolites on cellular functions are poorly investigated and remain to be disentangled. Mitotane has been shown to significantly inhibit cell proliferation and cortisol secretion in a dose-dependent manner in H295R cell line (Lindhe *et al.* 2010). Mitotane has also been shown to sensitize H295R and SW13 cells to ionizing radiation by attenuating DNA repair and interfering with cell proliferation (Cerquetti *et al.* 2010). Recently, it has been found that non-cytotoxic concentrations of mitotane are able to impair gene transcription of a number of steroidogenic enzymes in H295R cells thus inhibiting cortisol secretion (Lin *et al.* 2012). Moreover, Poli et al. found that mitotane treatment at doses corresponding to the therapeutic window significantly reduced cell proliferation and viability through disrupting mitochondrial morphology and function (Poli *et al.* 2013). In another recent study, mitotane has been shown – although at high concentrations - to interfere with mitochondrial respiratory chain in H295R and SW13 cells, as a putative inhibitory mechanism of cell proliferation and steroidogenesis (Hescot *et al.* 2013).

Previous research from our group suggested that mitotane activity may be influenced by the expression of Ribonucleotide Reductase Large Subunit (RRM1) enzyme. A reduced expression of this gene was associated with prolonged recurrence-free survival in ACC patients treated adjuvantly, and specific silencing of RRM1 was able to restore mitotane sensitivity in poorly mitotane-sensitive SW13 ACC cells, *in vitro* (Volante *et al.* 2012). Moreover, we also demonstrated that in ACC cells RRM1 gene modulation is associated to specific profiles of sensitivity to mitotane in combination with gemcitabine, a chemotherapeutic agent used in advanced ACC which specifically targets RRM1 (Germano *et al.* 2014). However, the functional interplay between RRM1 and mitotane is unknown, although it might be argued that RRM1 enzyme interferes with mitotane metabolization due to its reductase activity.

Therefore, the aim of the present study was to evaluate whether modulation of RRM1 gene expression may alter metabolization and activity of mitotane, o,p'DDE and o,p'DDA in ACC cell lines.

2. Materials and Methods

2.1. Cell cultures and chemical reagents. H295R and SW13 ACC cell lines were supplied from ATCC (LGC Standards s.r.l., Sesto San Giovanni, Milan, Italy). 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-Aldrich, St. Louis, USA) supplemented with 2 mmol/L L-glutamine, penicillin (25 units/mL), and streptomycin (25 mg/mL, all from Sigma-Aldrich) and 2.5% of Nu-Serum (BD Biosciences, San Jose, USA) and enriched with 1% di ITS+Premix (BD Bioscience). SW13 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biowest, Nuaillè, France) and 2 mmol/L L-glutamine, penicillin (25 units/mL), and streptomycin (25 mg/mL, all from Sigma-Aldrich). Mitotane, o,p'DDE and p,p'DDA (Sigma-Aldrich) were dissolved in 100% methanol (Sigma-Aldrich).

2.2. RRM1 silencing. For RRM1 silencing experiments in SW13 cells, on-Target plus Smart Pool siRNAs targeting RRM1 and on-Target plus Smart Pool, non-targeting, siRNAs were used (GE Dharmacon, Lafayette, USA). INTERFERin siRNA transfection reagent was purchased from Polyplus transfection (Illrkich, USA). Briefly, using a reverse transfection, siRNAs (at a final concentration of 50

nmol/L) and INTERFERin were diluted in medium without serum and incubated for 10 minutes at room temperature. Lipoplexes were then transferred to multi-well tissue culture plates and overlaid with 25×10^4 cells/ well. After 24 hours, the medium was changed, and silenced-SW13 cells were treated as detailed below. Efficiency of RRM1 knockdown was assessed by means of real-time PCR (see below for methods; data not shown). To evaluated RRM1 silencing effects on global metabolism, ATP and pyruvate were measured by means of colorimetric/Fluorimentric assay kits, following the procedure as for a colorimetric assay provided by the manufacturer (BioVision, USA).

2.3. Treatments and cell viability assay. H295R and SW13 cell lines were seeded into 96-well plates in triplicates and treated for 72h and 48h, respectively, with o,p'DDE (from 0.1 to 20 µM; from <0.01 mg/l to 6.55 mg/l) and p,p'DDA (from 50 to 400 µM; from 14 mg/l to 119 mg/l). Drug concentrations were in a range comparable with therapeutic levels *in vivo*. 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, USA) at a test wavelength of 450 nm and reference wavelength of 630 nm. Cell viability was also measured in RRM1-silenced and non-targeting siRNA SW13 cells treated with o,p'DDE (from 0.1 to 20 µM) for 48h.

2.4. Analyte extraction, HPLC-UV analysis and chromatographic conditions. Concentrations of mitotane, o,p'DDE and o,p'DDA were analyzed in duplicates on equal amounts cell lysates and surnatants of H295R and SW13 cells, as well as in H295R RRM1-transduced and SW13 RRM1 silenced cells (and corresponding negative controls). Analyte concentrations were evaluated after 15 µM mitotane treatment for 48 and 72 hours, using an HPLC-UV system (High Pressure Liquid Cromatography, VWR-Hitachi system, LaChrom Elite, RADnor, USA). Extraction of mitotane, o,p'-DDE and p,p'-DDT, this latter used as internal standard (IS), was performed by vortex mixing of 500 µl of samples with 100 µl of IS (100mg/ml) and 750 µl of acetone. Samples were then centrifuged at 12000 rpm for 5 min and 500 µl of organic layer were transferred to an HPLC-UV vial mixed with 500 µl of recovering phase (HPLC grade water-methanol-acetonitrile, 40:10:50, v/v/v) for injection. Separation was then achieved with a C18 reverse-phase column

(LiChroCART® 250-4 HPLC Cartridge, LiChrospher® 100, RP-18, 5 mm, VWR) preceded by a specific guard column. Chromatographic analysis was carried out at 30°C by a gradient of HPLC-grade water, methanol, acetonitrile (0-6.5 min 10:10:80, v/v/v; 6.6-9.7 min 5:5:90, v/v/v; 9.7-15 min 10:10:80, v/v/v) at the constant flow rate of 1.0 ml/min. The eluate was monitored at 218 nm (De Francia S *et al.* 2006).

A different method was adopted for o,p'-DDA to eliminate interferences at the analyte retention time. Extraction of o,p'-DDA and nilotinib, used as IS, was performed by protein precipitation: 50 µl of IS working solution (200mg/ml) were added to 500 µl of samples. After 750 µl of protein precipitation solution (HPLC-grade water and methanol, 50:50, v/v) were added to each sample, tubes were vortexed for 30 sec and then centrifuged at 12000 rpm for 10 min. Finally 800 µl of organic layer were transferred to an HPLC vial for injection. Separation was then achieved with a C18 reverse-phase column (LiChroCART® 250-4 HPLC Cartridge, LiChrospher® 100, RP-18, 5 mm, VWR) preceded by a specific guard column. Chromatographic analysis was carried out under isocratic elution at 35°C by a mobile phase consisting of 40% solvent A (72.5% water, 25% methanol, 2.5% triethylamine), 20% methanol, and 40% acetonitrile at the costant flow rate of 0.9 ml/min. The eluate was monitored at 267 nm (De Francia S *et al.* 2006). System management and data acquisition were performed with the EzChrom Elite software. Analytes quantification was performed by IS calibration; a linear regression was used to obtain the best fit for all calibration points. Regression coefficient (r^2) of all calibration curves was higher than 0.99 for all analytes.

2.5. RNA extraction and quantitative PCR analysis of RRM1 gene expression. Total RNA was extracted from H295R and SW13 cell lines at basal conditions or following treatment with o,p'DDE (1µM) and p,p'DDA (50µM), as well as from RRM1-silenced SW13 cells at basal conditions, using Qiazol Reagent (Qiagen, Hilden, Germany). Complementary DNA was generated using M-MLVT RT (200U/µl) (Invitrogen, USA) and oligodT primers (500µl/ml) (Invitrogen, Carlsbad, USA) from 1µg of total RNA. Relative cDNA quantification for RRM1 and a housekeeping gene (β-actin) were examined by quantitative real-time PCR using primers and PCR conditions previously reported (Ceppi P *et al.* 2006). To analyze target gene expression, levels of RRM1 mRNA were normalized to housekeeping gene β-actin, then ΔΔCt calculation was performed and corresponding values were expressed as $2^{-\Delta\Delta Ct}$.

2.6. Statistical analysis. Student's t-test was used to compare extra- and intracellular concentrations of mitotane and its metabolites under different conditions, and the efficacy of drugs on cell viability of SW13 cells RRM1-silenced vs non-targeting siRNA. Cell viability ratios were calculated using the sigmoid inhibition model (GraphPad PRISM 5, San Diego, USA). Statistical significance was set at $p<0.05$.

3. Results

3.1. Cytotoxic effects of o,p'DDE and o,p'DDA and their correlation with RRM1 gene expression in ACC cells. H295R cells were sensitive in terms of cell viability to mitotane (IC50 = 30.6 μM), o,p'DDE (IC50 <1 μM) and p,p'DDA (IC50 = 292 μM) (**Figure 1a**). SW13 cells were poorly sensitive to mitotane (IC50= 6.2 x 10^2 μM) and o,p'DDE (IC50 = 122 μM) but significantly responsive to p,p'DDA at concentrations similar to H295R cells (IC50 = 282 μM) (**Figure 1a**). Treatment with o,p'DDE or p,p'DDA did not modulate RRM1 gene expression in H295R cells. By contrast, incubation with o,p'DDE in SW13 cells was associated with up-regulation of RRM1 gene expression (up to 3 times over baseline at 1 μM concentration). However, p,p'DDA incubation at 50 μM concentration did not modify RRM1 gene expression (**Figure 1b**). RRM1 silencing conferred sensitivity to o,p'DDE in SW13 cells since IC50 was significantly lower in RRM1-silenced cells that in non-targeting siRNA cells (38 μM vs.122 μM, p=0.0018) (**Figure 2**).

3.2. Uptake and distribution of mitotane in ACC cells. Levels of mitotane (as mean values of two different experiments) were significantly lower in surnatants of H295R as compared to SW13 cells after mitotane treatment (p=0.005). In the same experiments, the extracellular and intracellular levels of o,p'DDE were higher in SW13 cells than H295R cells (p<0.0001 and p=0.048, respectively) while the extracellular and intracellular levels of o,p'DDA were lower in SW13 cells than H295R cells (p=0.004 and p=0.004) (**Figure 3**).

3.3. Effects of RRM1 knockdown on mitotane bioavailability in SW13 cells. After 48h, silencing of RRM1 gene in SW13 induced a significant increase in the intracellular concentrations of o,p'DDE and o,p'DDA (p=0.04 and p=0.003) and a decrease in the intracellular concentrations of mitotane (p<0.0001) (**Figure 4**). Silencing of RRM1 gene in SW13 induced also a slight increase in the intracellular concentrations of pyruvate (not reaching statistical significance) and a significant decrease in the intracellular concentrations ATP (**supplementary Figure 1**)

4. Discussion

Mitotane is considered an active treatment for ACC, either in an adjuvant setting or in advanced disease. Mitotane induces adrenolytic effects and inhibits a number of steroidogenic enzymes; however, the mechanisms of action remain largely unknown (Terzolo *et al.* 2014). No robust predictor of response is currently available to select patients to treatment. Although it is generally thought that the activity of mitotane depends on metabolization into active molecules, o,p'DDE and o,p'DDA, few studies addressed specifically the effect of such metabolites (Johansson *et al.* 2002, Asp *et al.* 2010, Lindhe *et al.* 2010).

The first aim of the present study was to test *in vitro* the anti-proliferative effects of o,p'DDE and p,p'DDA in two ACC cell lines showing different sensitivity to mitotane (Volante *et al.* 2012). We herein confirmed and extended previous observations that o,p'DDE is able to reduce significantly cell viability of H295R cells (Johansson *et al.* 2002, Asp *et al.* 2010, Lindhe *et al.* 2010), while it does not affect cell viability of SW13 cells, which are also unresponsive to mitotane. By contrast, o,p'DDA reduced cell viability in both cell lines, with IC50 concentrations and dose-response curves comparable between the two cellular models.

Our second aim was to test the bioavailability profiles of mitotane in the two cell models that demonstrated a different profile, characterized by higher o,p'DDE concentrations in SW13 cells and higher o,p'DDA concentrations in H295R cells. These observations suggest that SW13 may have a distinct metabolic pathway of mitotane leading to defective generation of o,p'DDA. However, we may disclose the limit that differences between intra- and extra-cellular compartments may be also influenced by active transportation. The finding that in SW13 cells mitotane is inactive while o,p'DDA is able to reduce cell growth is consistent with the hypothesis that transformation of mitotane into o,p'DDA is a necessary step of mitotane action.

Transposing this observation into clinical practice, it is interesting to note that o,p'DDA plasma levels were found to be correlated with tumor response to mitotane treatment independently from mitotane levels (Hermsen *et al.* 2011). Moreover, our experimental results support the view that the ability to metabolize mitotane may differ among ACC cases, because of intracellular alterations in the metabolic process which may lead to different responses to mitotane treatment (Schteingart *et al.* 2007). Thus, a better understanding of intra-tumoral mitotane metabolization may help to select those tumors with the highest probability of response.

Our third aim was to evaluate an association between metabolic profiles of mitotane and expression of the RRM1 gene. The interest on RRM1 stemmed from previous data from our group on the role of RRM1 gene expression as a predictor of response to mitotane either *in vitro* or *in vivo* (Volante *et al.* 2012). We analyzed RRM1 gene expression in H295R and SW13 cells treated with mitotane metabolites. In the H295R cell line, o,p'DDE and o,p'DDA were both effective on reducing cell viability and did not modify RRM1 gene expression, as we have demonstrated for mitotane (Volante *et al.* 2012). In SW13 cells, o,p'DDE was not cytotoxic and up-modulated RRM1 gene expression, as we have observed with mitotane in the same cell type (Volante *et al.* 2012). By contrast, o,p'DDA was effective in reducing SW13 cell proliferation and did not modify RRM1 gene expression. The finding that RRM1 silencing induced an anti-proliferative effect of o,p'DDE in SW13 cells and restored a metabolic pathway of mitotane metabolism alike that of H295R cells strengthens the inverse relationship between RRM1 and mitotane activity. The present data demonstrate that RRM1 impairs mitotane metabolization and this may represent a major mechanism to influence mitotane sensitivity in ACC cells. These data are new and unexpected since RRM1 is a well known molecule involved in DNA synthesis and damage repair. Due to this mechanism, preclinical studies have shown that RRM1 is involved in resistance to gemcitabine in NSCLC (Bergman *et al.* 2005) and lower RRM1 expression was associated with a high response rate to platinum agents and gemcitabine in different cancer types (Jordheim *et al.* 2011), although in other reports RRM1 was either not or inversely associated with the survival of NSCLC patients receiving gemcitabine-containing regimens (Gong *et al.* 2012). However, in the model of adrenocortical cancer - at least *in vitro* - RRM1 plays a different "predictive" role since it is not the target of a specific agent but, as supported by our data in SW13 cells, interferes with the anti-proliferative activity of mitotane reducing its transformation into active metabolites (**Figure 5**). This effect, which is mediated *in*

vitro by mitotane-induced RRM1 up-modulation rather than expression at baseline, is apparently adrenocortical-specific since not observed in other cell models, such as lung cancer, as previously demonstrated by our group (Volante *et al.* 2012). However, it remains to be ascertained if the effect of RRM1 on mitotane metabolism is direct and specific or - at least in part - associated to a global alteration in metabolism. In fact, we observed that RRM1 knock down modified ATP and pyruvate concentrations in SW13 cells, as a possible negative interference of RRM1 down-modulation with the Krebs cycle.

A limitation of the present study is represented by the limited number of ACC cell lines commercially available. In fact, H295R and SW13 cells differ not only in terms of mitotane sensitivity but also in terms of morphology, proliferation rate and hormone secretion. Therefore, experimental results obtained in these two cell lines, that do not necessarily represent the best model for assessing metabolic and therapeutic responsiveness profiles in ACC, should be translated into clinically meaningful data with caution. However, the finding that RRM1 can interfere on intracellular mitotane metabolism suggest a further mechanism by which this enzyme can modulate the sensitivity/resistance of cancer cells to antineoplastic drugs. Moreover, these data provide further evidence that RRM1 expression should be tested in clinical trials as a predictive marker of response to mitotane in ACC patients, and new molecules blocking ribonucleotide reductase holoenzyme can potentially overcome the resistance of ACC to mitotane (Zhou *et al.* 2013)

5. Conclusions

In summary, the present study demonstrates that: i) metabolites of mitotane have anti-proliferative effects in ACC cells which are specific of the cell type; ii) similar to what already demonstrated for mitotane, responsiveness to p,p'DDA and o,p'DDE in ACC cells is associated to modulation of RRM1 gene expression; iii) although proven in a single knock down cell model, our data suggest that RRM1 interference with mitotane intracellular metabolization may represent the potential mechanism of resistance to antineoplastic effects of mitotane in ACC cells.

Acknowledgments.

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Figure legends

Figure 1. **Cell viability assays of mitotane metabolites responsiveness in H295R and SW13 cells** (panel a) **and RRM1 gene expression levels after drugs treatments** (1 μM o,p'DDE and 50 μM p,p' DDA) (panel b). Three replicate wells were used to determine each data point of cell viability measurements; untreated cells were used as the reference.

Figure 2. **Cell viability assays of o,p'DDE responsiveness in wild type (nontargeting siRNA) and RRM1siRNA SW13 cells.** Three replicate wells were used to determine each data point of cell viability measurements.

Figure 3. Intracellular and extracellular concentration of mitotane metabolites in H295R and SW13 cells, measured by means of HPLC-UV. Four replicates were used to determinate each data point of drugs levels.

Figure 4. Effects of RRM1 silencing in the metabolization of mitotane in SW13 cells: intracellular concentration of mitotane, o,p'DDE and o,p'DDA were measured in nontargeting siRNA and RRM1 siRNA cells. Two replicates were used to determinate each data point of drugs levels. Student's t test was used to compared drug levels.

Figure 5. Schematical illustration of the potential link between RRM1 and loss of anti-neoplastic activity of mitotane in SW13 cells. Ribonucleotide reductase is a multimeric enzyme made of 2 subunits (RRM1 and RRM2) which catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates. Moreover, RRM1 subunit is involved in the suppression of cell proliferation, cell migration and metastases (see Jordheim *et al.* 2011 for reference). In SW13 cells, RRM1 is specifically up-regulated by mitotane and interferes with the transformation of mitotane into its active metabolites, thus reducing mitotane anti-proliferative activity.

Supplementary Figure 1. Effects of RRM1 silencing in the intracellular concentrations of pyruvate and ATP in SW13 cells. Intracellular concentration of pyruvate and ATP were measured in nontargeting siRNA and RRM1 siRNA cells by a colorimetric assay. Three replicates were used to determinate each data point. Student's t test was used to compared drug levels.

Highlights

i) mitotane metabolites have cell type-specific anti-proliferative effects;

ii) responsiveness to mitotane metabolites is associated to modulation of RRM1 expression;

iii) RRM1 directly interferes with mitotane intracellular metabolization
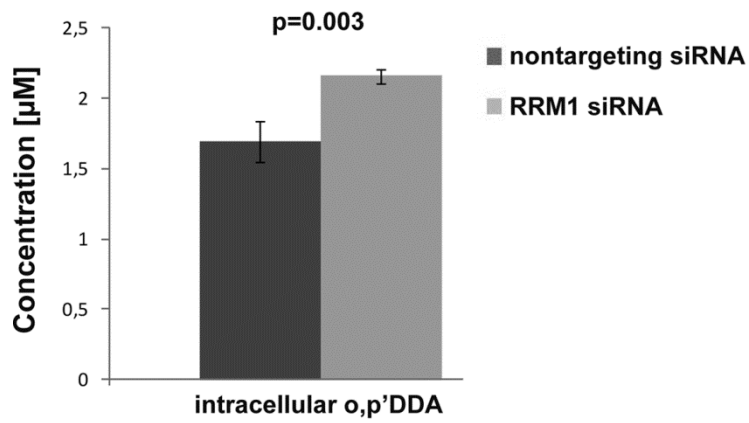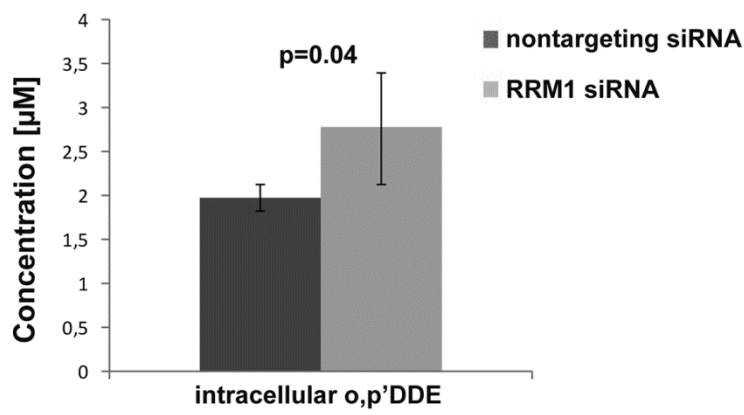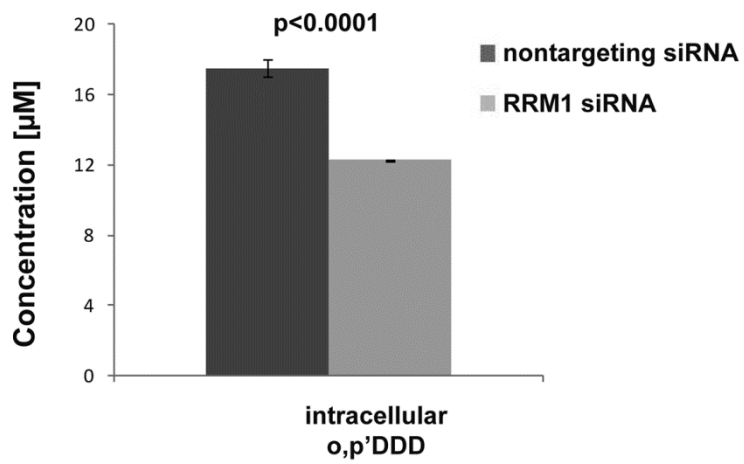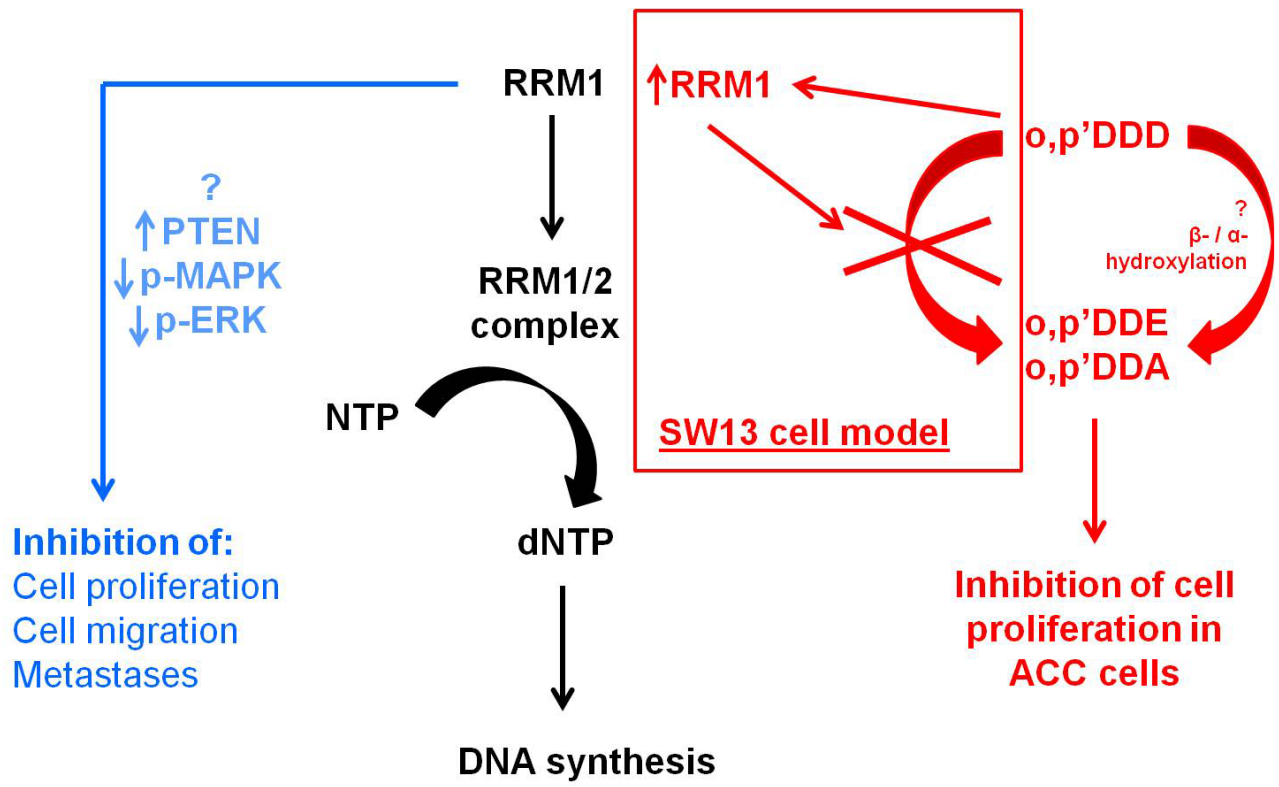
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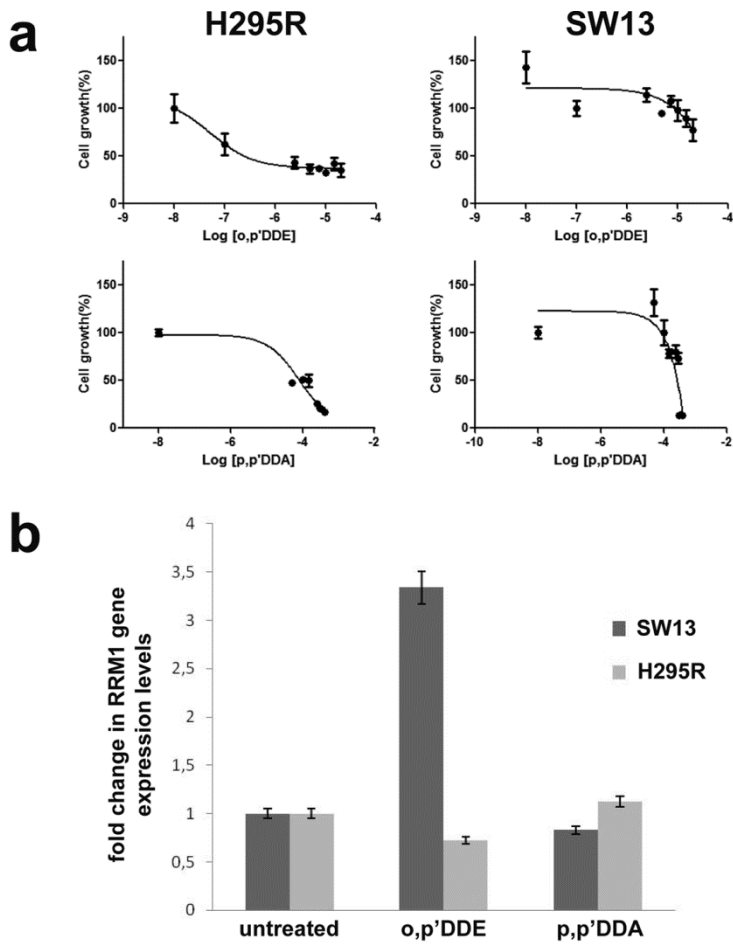
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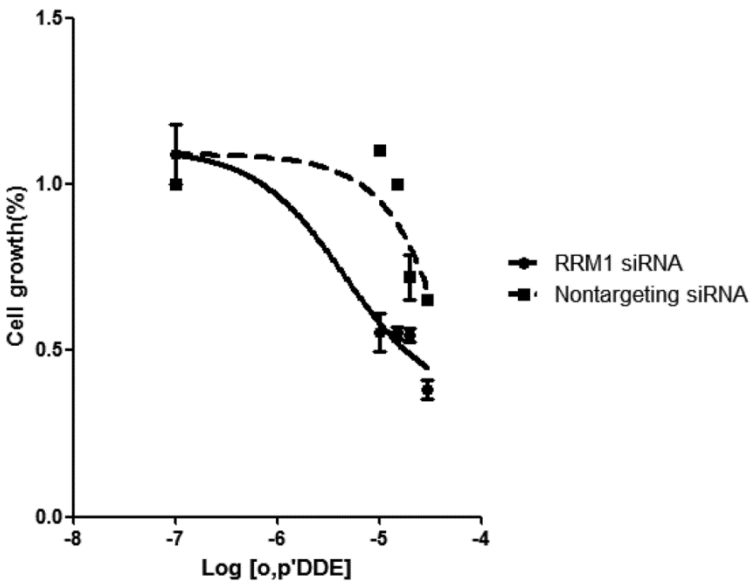
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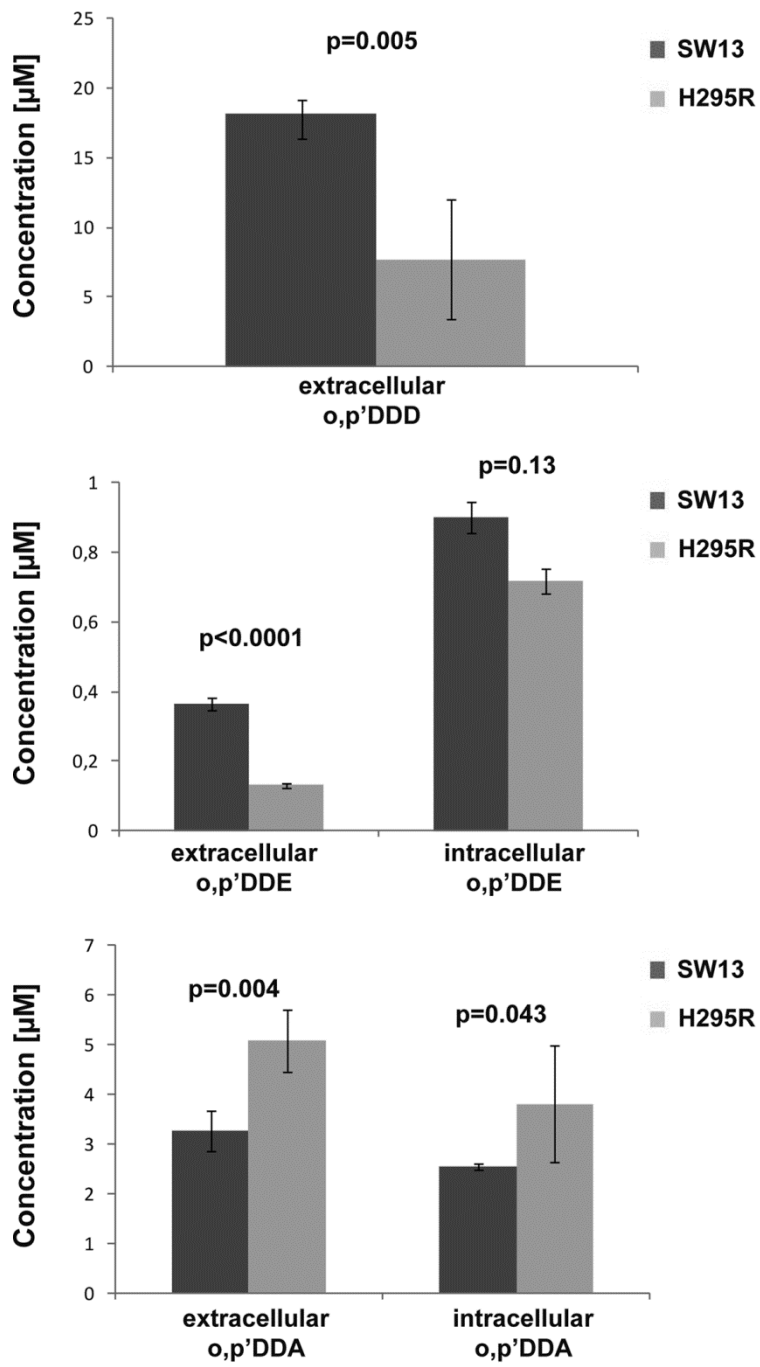
Figure 4.

Figure 5.

Supplemental Figure 1.