The antidiabetic drug metformin affects H295R cells proliferation

R. Armignacco, G. Poli, G. Cantini, L. Canu, M. Mannelli, M. Luconi

Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

roberta.armignacco@unifi.it

Introduction

Adrenocortical carcinoma (ACC) is a rare, heterogeneous malignancy with a poor prognosis, particularly when metastatic at diagnosis. To date, radical surgery, possibly associated to mitotane adjuvant therapy, is the only available treatment [1]. However, the mean 5-year survival rate drops under 10% in metastatic ACC and chemo-resistance often develops [2]. Thus, more specific and effective drugs for ACC treatment are urgently required. The antidiabetic drug metformin, used in type II diabetes treatment, has been associated with decreased cancer incidence and mortality in several human cancers [3-8], suggesting us the possibility to test its potential efficacy on ACC too.

Methods

Cell culture. Subconfluent H295R cells, cultured in their specific complete medium, were treated with different stimuli added to 10% FBS medium.

Cell viability and proliferation. After treating cells for the indicated time points with increasing doses of metformin, alone or in combination with mitotane, MTS assay and direct cell count were performed to evaluate cell viability and cell proliferation, respectively. To confirm data regarding metformin effect on cell proliferation, $^{[3H]}$thymidine incorporation assay was also performed.

SDS page and Western blot analysis. Relative protein expression of protein extracts from treated cells was used to investigate the molecular pathways involved in mediating the drug effect.

Results

I. Metformin affects cell viability and proliferation

Metformin treatment results in a dose- and time-dependent decrease of H295R cells viability and proliferation, as evaluated by MTS assay (Fig.1A), direct cell count (Fig.1B) and thymidine incorporation assay (Fig.1C). For each type of assay, the half inhibitory dose (IC_{50}) was calculated at the different time points from the relative dose-response curves.

II. Metformin acts through AMPK and IGF-1R signaling pathways

Metformin treatment is able to induce a significant dose-related stimulation of the energy sensor kinase AMPK, which associates with a decrease of mTOR phosphorylation (Fig.2A), confirming that this pathway is active in H295R. Moreover, the IGF-1R downstream pathway seems to be affected by metformin too, since we observed a decrease of the receptor expression (Fig.2B, left panel) and of ERK1/2 phosphorylation (Fig.2B, right panel).

III. Metformin-mitotane combined effect of on H295R cells viability

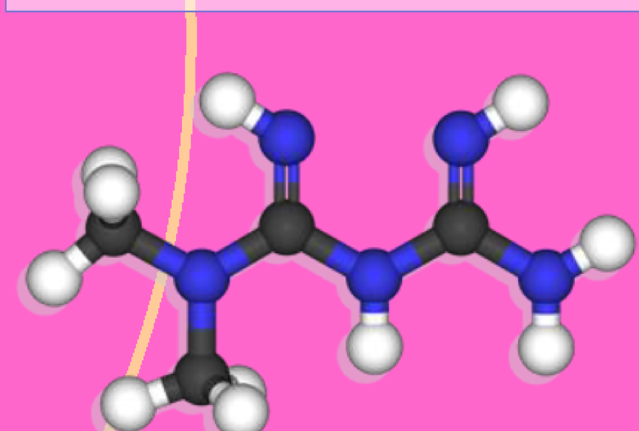
Since mitotane represents the current first line therapy for ACC, we wanted to assess whether the combination with metformin could result in an increased effect on cell viability. Adding increasing doses of metformin to a fix dose of mitotane (20 μM), at the indicated time points, results in a significant decrease of cell viability compared to the effect of mitotane alone (Fig.3A). Moreover, we observed that combining the two drugs results in a lower IC_{50} values at the indicated time points than metformin alone (Fig.3B).

Conclusions

Our data indicate that metformin is able to interfere with the *in vitro* cancer cell proliferation, showing an effect which depends on the drug concentration and treatment duration. Furthermore, the synergistic effect observed in the presence of mitotane suggests the possible use of metformin in combination with the current therapy for ACC. Further *in vivo* studies are necessary to prove metformin efficacy in adrenocortical carcinoma.

Objectives

- To evaluate the potential anti-cancer effects of metformin on H295R adrenocortical tumor cell line, looking for a possible alternative therapeutic approach to ACC treatment.
- To define the possible molecular pathways involved in metformin effect on cell viability and proliferation.



METFORMIN (MET)

H295R CELL LINE

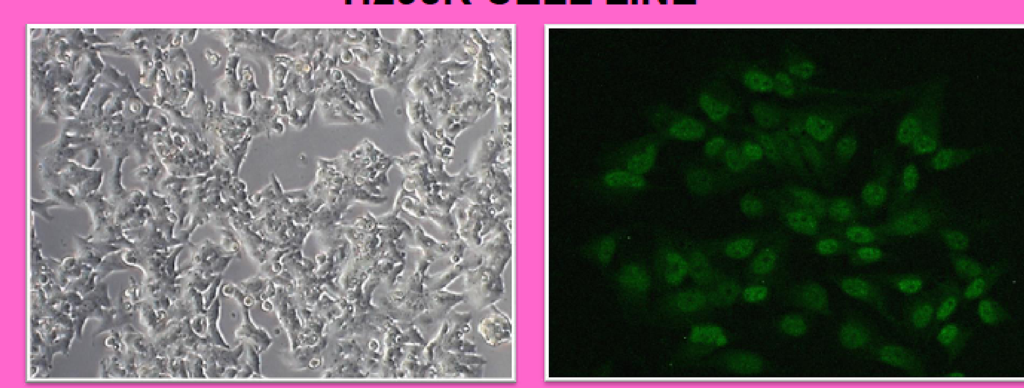
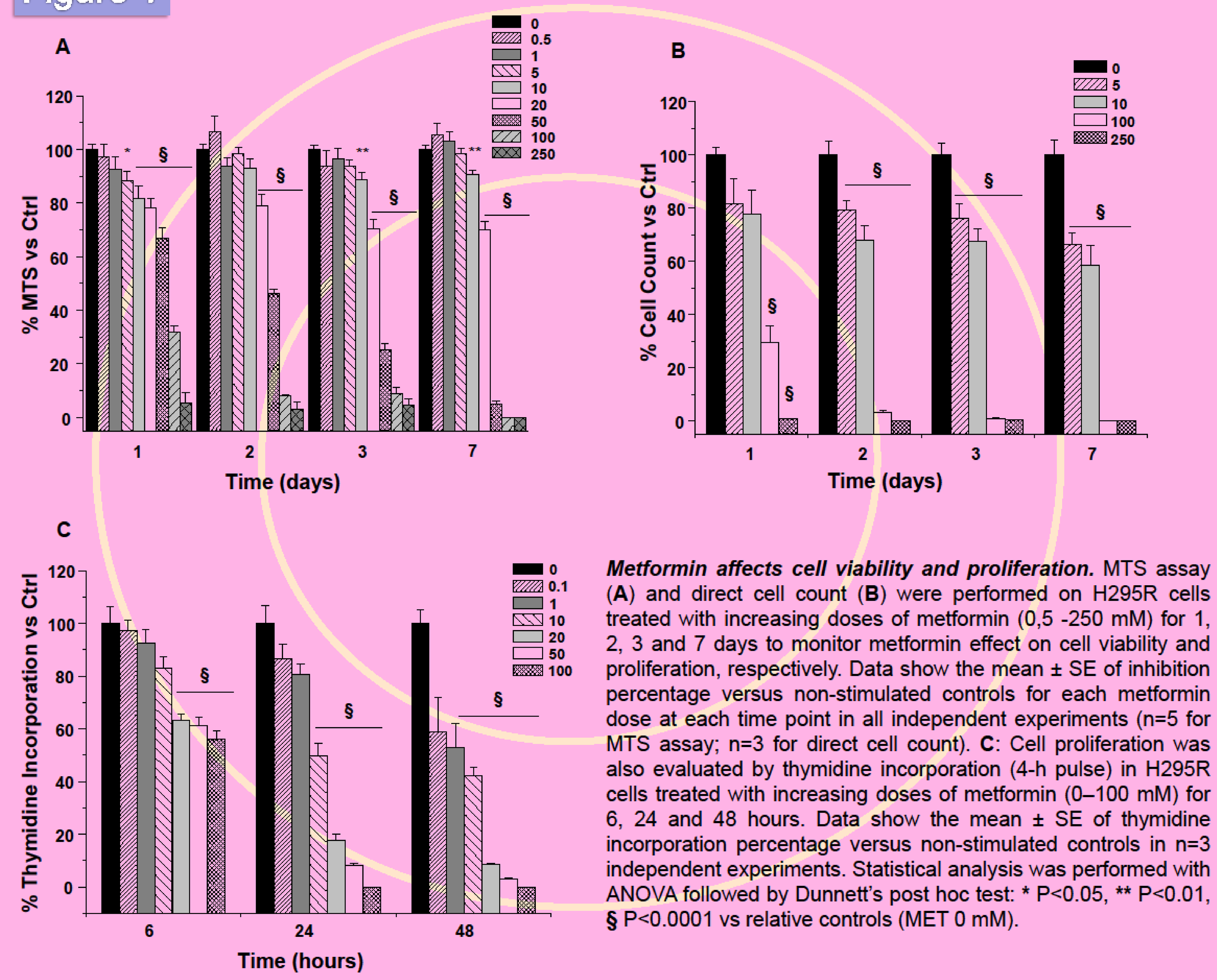
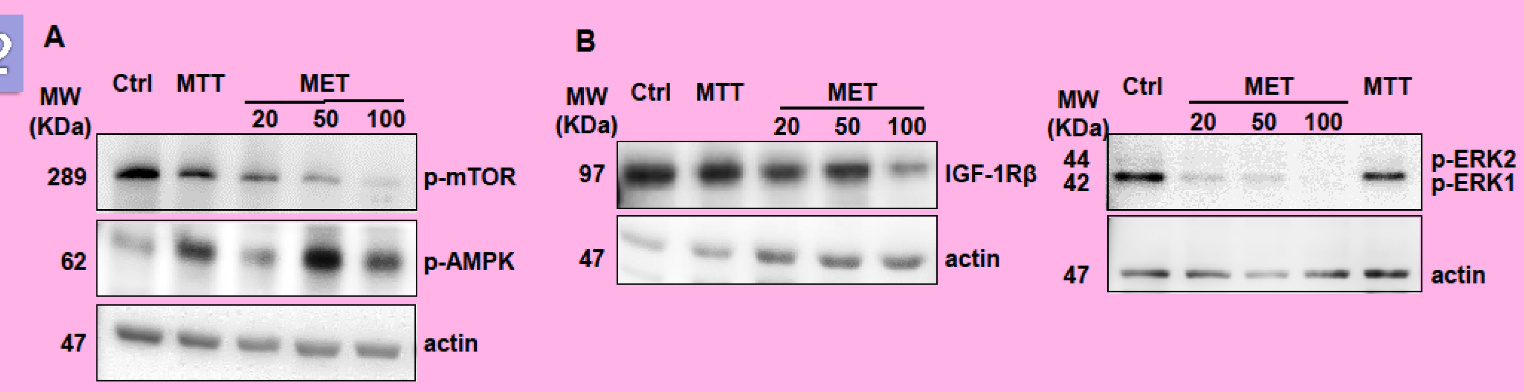


Figure 1



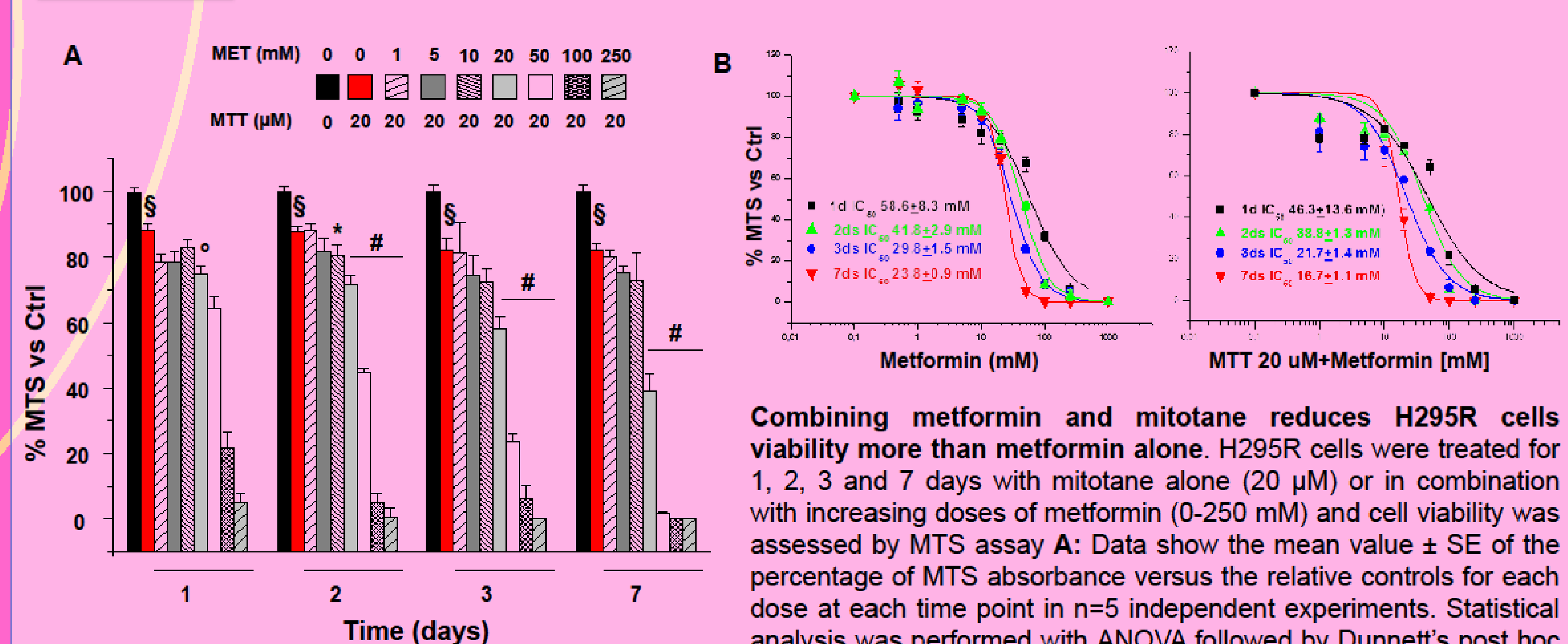
Metformin affects cell viability and proliferation. MTS assay (**A**) and direct cell count (**B**) were performed on H295R cells treated with increasing doses of metformin (0,5 -250 mM) for 1, 2, 3 and 7 days to monitor metformin effect on cell viability and proliferation, respectively. Data show the mean ± SE of inhibition percentage versus non-stimulated controls for each metformin dose at each time point in all independent experiments (n=5 for MTS assay; n=3 for direct cell count). **C**: Cell proliferation was also evaluated by thymidine incorporation (4-h pulse) in H295R cells treated with increasing doses of metformin (0–100 mM) for 6, 24 and 48 hours. Data show the mean ± SE of thymidine incorporation percentage versus non-stimulated controls in n=3 independent experiments. Statistical analysis was performed with ANOVA followed by Dunnett's post hoc test: * P<0.05, ** P<0.01, § P<0.0001 vs relative controls (MET 0 mM).

Figure 2



Metformin affects AMPK and IGF-1R signaling pathways in a dose-dependent manner. Western Blot analysis was performed on protein extracts derived from H295R cells treated with mitotane (MTT 20 μM) or different doses of metformin (MET 20, 50, 100 mM). **A:** After 6 hour treatment with metformin, a significant increase of AMPK activation, associated with a decreased m-TOR phosphorylation, was observed. **B:** Treating cells for 24 hours with metformin leads to a dose-dependent decrease of IGF-1R (right panel) and of ERK1/2 phosphorylation (left panel). Actin was used as internal loading control.

Figure 3



Combining metformin and mitotane reduces H295R cells viability more than metformin alone. H295R cells were treated for 1, 2, 3 and 7 days with mitotane alone (20 μM) or in combination with increasing doses of metformin (0-250 mM) and cell viability was assessed by MTS assay **A:** Data show the mean value ± SE of the percentage of MTS absorbance versus the relative controls for each dose at each time point in n=5 independent experiments. Statistical analysis was performed with ANOVA followed by Dunnett's post hoc test: § P<0,001 MTT vs control; * P<0.05, ° P<0.001, # P<0.0001 MTT vs MTT+MET. **B:** Dose-response curves of cell viability related to the indicated time points were used to calculate the respective IC_{50} of metformin, indicated in the inset.

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Adrenal

Roberta Armignacco

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