

ESMO Translational Research Fellowship

“Identification of novel molecular targets and biological agents for the chemotherapy of malignant pleural mesothelioma”

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Final Report

Host Institute:

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Rationale and Aim

Malignant pleural mesothelioma (MPM) is an aggressive tumour linked to chronic inhalation of asbestos fibers, with poor prognosis and increasing incidence in industrialized countries. Currently available chemotherapeutic regimens achieve a median overall survival of 12 months, and the major clinical problems of MPM management are the short duration of the response and the early relapse. The aim of this study was to identify novel biological agents to improve the cytotoxicity of the chemotherapeutic regimens currently used in MPM therapy, by forcing mesothelioma cells to undergo apoptosis. To this end, we engaged the TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) apoptotic pathway, to which many cancer cells exhibit an increased sensitivity compared to normal cells. In the context of MPM cells, the association between cisplatin and biologic agents against TRAIL receptors demonstrated a synergistic effect (Belyanskaya LL et al, Mol Cancer 2007). One of the mechanisms causing this sensitization seemed to be mediated by generation of oxidative stress.

An increase in ROS (reactive oxygen species) is associated with abnormal cancer cell growth and reflects a disruption of redox homeostasis due either to an elevation in ROS production or to a decline in ROS-scavenging capacity. Cancer cells, which exhibit a higher ROS set-point compared to normal cells, are thus likely to be more vulnerable to damage by further ROS insults. Therefore, we proposed to explore the efficacy of agents increasing ROS levels as a strategy to selectively kill cancer cells. We also proposed to search for molecular determinants deciding the cells' susceptibility to TRAIL-mediated cell death, for example the variation in expression levels of anti-apoptotic proteins.

AIMS: In the last two years we tested the possible beneficial effects of coupling TRAIL agonists with ROS-inducing treatments and with other TRAIL-sensitizing agents.

Experimental design

The study has been developed in three years: during the first two years we carried out in vitro assays using MPM cell lines and primary cultures to search for novel biological agents inducing death of MPM cells; we selected the most promising agents combinations and studied molecular markers involved in their

proapoptotic effect. During this third year we aim to validate the potential therapeutic efficacy of these agents in a mouse preclinical model; finally, the analysis of samples obtained from MPM patients before and after surgery is currently on going, to detect markers predicting treatment outcome and to explore the possibility of developing a phase I clinical trial based on the selected biologic agent(s) with or without standard chemotherapeutic regimens.

Results, Conclusions and Future Perspectives

1. Definition of novel biological agents to induce selective cell death of MPM: TRAIL dependent apoptosis.

In recent years, great emphasis was given to the use of Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) as an anticancer agent. TRAIL belongs to the TNF family of death ligands, and triggers the extrinsic apoptotic pathway. Treatment with TRAIL induces programmed cell death in a wide range of transformed cells, both in vitro and in vivo, without significant effects in normal cells. However, a significant proportion of human cancer cells are resistant to TRAIL induced apoptosis, and the mechanisms of resistance seem to differ among cell types (Zhao et al. 2012). We thus assessed the proapoptotic effect of TRAIL in MPM cell lines and primary cultures. First, we performed Annexin V assay and Fluorometric Homogenous Caspase Assay in epithelioid (ZL55) and sarcomatoid (ZL34) cell lines treated with different concentration of TRAIL for 24 hours (Figure 1).

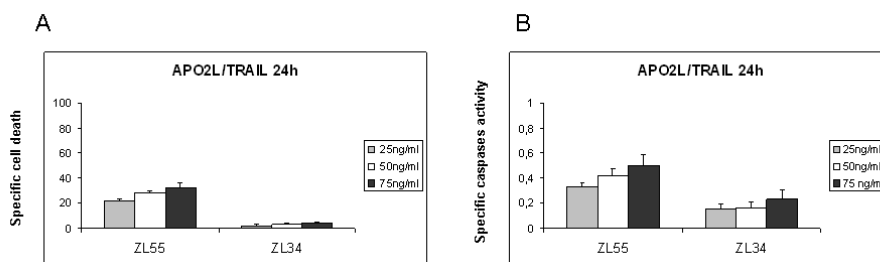


Figure 1. MPM cells were exposed to TRAIL for 24 hours at the indicated concentrations. **A)** Annexin V assay. Specific cell death was calculated by the following formula: (percentage of cell death in treated samples- percentage of cell death in untreated samples) / (100- the percentage of cell death in untreated samples) *100. **B)** Fluorometric Homogenous Caspase Assay . Specific caspases activity in treated cells was normalized compared to untreated control: (F treated – F untreated)/(F untreated).

The results showed that the sensitivity of sarcomatoid cell lines is lower than epithelioid.

In order to test whether the efficacy of TRAIL treatment was dependent from the histotype, we treated 7 MPM cell lines (3 epithelioid: ZL55, H226, M14K; 1 sarcomatoid: ZL34; 3 biphasic: MSTO-211, SPC111, ZL5) and one primary culture of sarcomatoid MPM (MPM1801) with TRAIL 50 ng/ml for 24 hours and analysed caspases activity by Fluorometric Homogenous Caspase Assay (Figure 2). The results showed that sensitivity to TRAIL treatment is very heterogeneous, independently from the histotype.

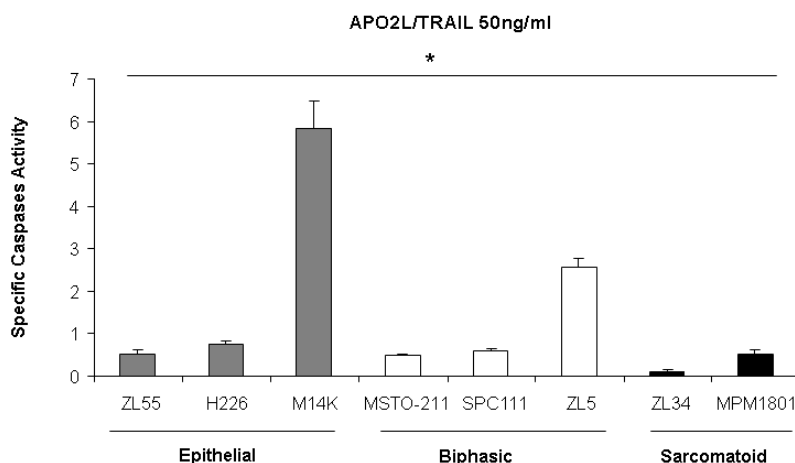


Figure 2. MPM cells were exposed to TRAIL (50 ng/ml) for 24 hours and caspases activity was evaluated by Fluorometric Homogenous Caspase Assay. Specific caspases activity in treated cells was normalized compared to untreated control: $(F_{\text{treated}} - F_{\text{untreated}}) / (F_{\text{untreated}})$. The results were represented as means \pm SE of 3 different experiments running in triplicate. *statistically significant difference of caspases activation between MPM cells treated with TRAIL compared with untreated cells ($p < 0.05$) by Mann-Whitney test.

2. Strategies to improve TRAIL dependent apoptosis

2a. ROS level modulation and K^+ channels.

Previous evidences demonstrated that ROS induction enhances TRAIL-induced apoptosis through the down-regulation of anti-apoptotic proteins (Kong et al. 2012; Kim et al. 2012).

Glibenclamide is a blocker of ATP-sensitive K^+ (K^+ATP) mitochondrial channels, which exerts antitumor activity through reactive oxygen species induction (Qian X. et al. 2008).

We thus tested the synergic effect of engaging the TRAIL apoptotic pathway associated with Glibenclamide (Gly) in MPM cell lines.

To this end MPM cells were treated with Gly 50 μ M with or without TRAIL 50 ng/ml for 24 hours and caspases activation assay was performed (Figure 3).

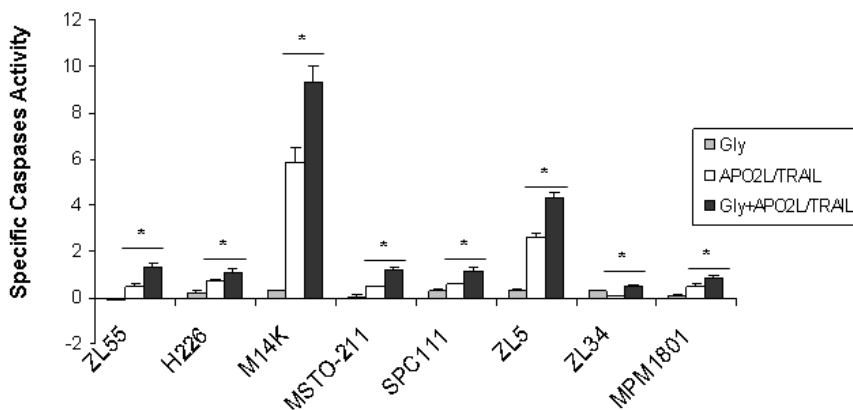


Figure 3. The cells were treated with Gly and/or TRAIL for 24 hours. Specific caspases activity in treated cells was normalized compared to untreated control: $(F_{\text{treated}} - F_{\text{untreated}}) / (F_{\text{untreated}})$. The results were represented as means \pm SE of 3 different experiments running in triplicate. *statistically significant difference of caspases activation between MPM cells treated with Gly + TRAIL compared with cells treated with TRAIL, ($p < 0.05$) by Mann-Whitney test.

The results showed a statistically significant increase of caspases activity in all cell lines treated with the combination of the two agents compared to untreated controls and to TRAIL and Glibenclamide used as single agents.

In order to confirm the results obtained with measurement of caspases activity, we performed the Annexin V assay in ZL55 and ZL34; we observed an increase of apoptotic cell death only in ZL55 cell lines, while no difference was observed in sarcomatoid cell lines (Figure 4).

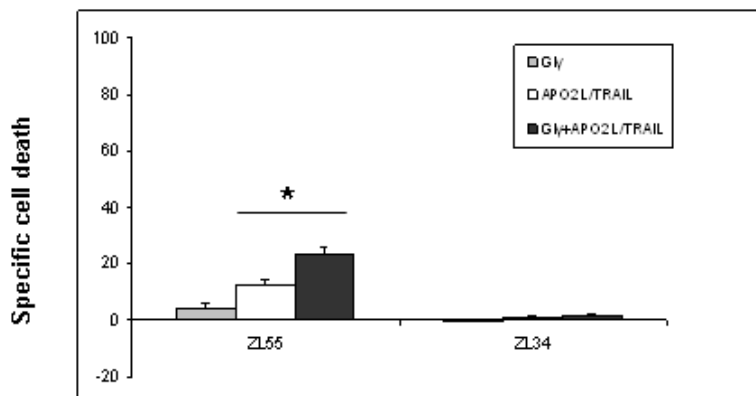


Figure 4. The cells were treated with Glibenclamide and/or TRAIL for 24 hours. Specific cell death was calculated by the following formula: (percentage of cell death in untreated samples- percentage of cell death in treated samples) / (100- the percentage of cell death in untreated samples) *100. The results were represented as means \pm SE of 3 different experiments running in triplicate.*statistically significant difference of specific cell death between MPM cells treated with Gly +TRAIL compared with cells treated with TRAIL, ($p < 0.05$) by Student t test.

We next performed a time-lapse analysis of ROS production upon treatment with Gly and/or TRAIL in ZL55 and ZL34 cells. We observed ROS induction in epithelioid cell lines treated with Glibenclamide with or without TRAIL compared to no treatment (nt), while no higher ROS levels were assessed in sarcomatoid cell lines treated with Gly with or without TRAIL compared to untreated (Figure 5).

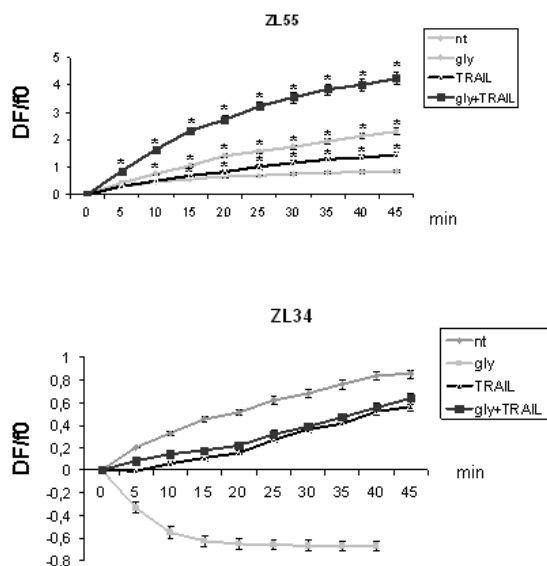


Figure 5. f_0 is the fluorescent signal measured at the beginning of the experiment, and DF is the difference in fluorescent signal measured at each individual time point subtracted of the f_0 value. Mean and Standard Error of DF/ f_0 recorded in at least 30 cells per time point per sample in 3 independent experiments were calculated. *statistically significant ROS induction in MPM cells treated with Gly with or without TRAIL compared to untreated, ($p < 0.05$) by Student t test.

In order to verify whether increase of caspases activity in presence of Gly + TRAIL in ZL55 cell lines was due to ROS production, ZL55 and ZL34 were previously treated with a ROS scavenger, N-Acetyl-Cysteine (NAC) 1mM and then with Gly and/or TRAIL.

As expected, we observed a statistically significant reduction of caspases activation (Gly+TRAIL) only in ZL55 cell lines in presence of NAC, while no difference was observed in sarcomatoid cell lines (Figure 6).

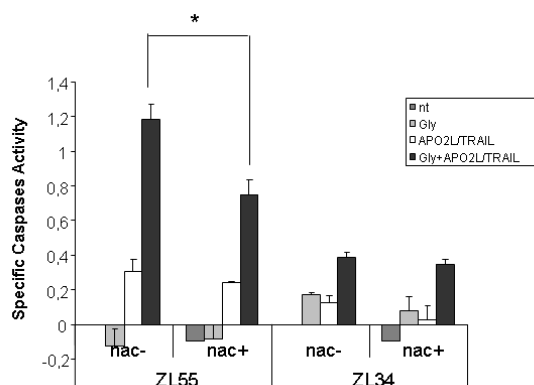


Figure 6. The cells were pre-treated with ROS scavenger NAC and then with Gly and/or TRAIL for 24 hours. Specific caspases activity in treated cells was normalized compared to untreated control: $(F \text{ treated} - F \text{ untreated}) / (F \text{ untreated NAC-})$. The results were represented as means \pm SE of 3 different experiments running in triplicate. *statistically significant difference ($p < 0.05$) of caspases activation between ZL55 treated with Gly+TRAIL without and with NAC, by Mann-Whitney test.

2b. ROS level modulation and DNA damage.

It was known that platinum compounds could exert their action through ROS induction, therefore we assessed whether carboplatin plus pemetrexed (CP) could induce ROS generation and whether ROS induction could sensitize MPM cell lines to TRAIL-mediated apoptosis.

We first analyzed apoptosis induction in MPM cell lines ZL55 and ZL34 after treatment with carboplatin *plus* pemetrexed.

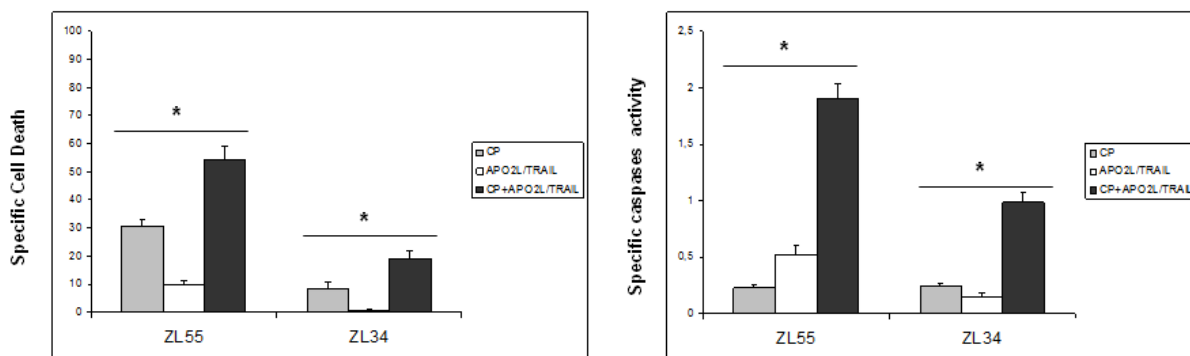


Figure 7. MPM cells were pre-treated with CP (carboplatin 27 μ M; pemetrexed 58 μ M) for 24 hours followed by incubation with TRAIL (50 ng/ml) for 24 hours. **A)** Annexin V assay. Specific cell death was calculated by the following formula: $(\text{percentage of cell death in treated samples} - \text{percentage of cell death in untreated samples}) / (100 - \text{percentage of cell death in untreated samples}) * 100$. **B)** Fluorometric Homogenous Caspase Assay. Specific caspases activity was calculated as the ratio of specific fluorescence signal of the treated cells $(F \text{ treated} - F \text{ untreated}) / F \text{ untreated cells}$. The results were represented as means \pm SE of 3 different experiments running in triplicate. *statistically significant difference of specific cell death between MPM cells treated with CP + TRAIL compared with cells treated with TRAIL, ($p < 0.001$) by Mann-Whitney test.

We observed an increase of specific cell death (SCD) and specific caspases activity in both cell lines treated with the combination of the two agents compared to untreated controls and to TRAIL and CP used as single agents (Figure 7).

In order to assess whether the increase of cell death could be due to ROS induction by CP, we performed a time-lapse analysis of ROS production upon CP treatment in ZL55 and ZL34 cell lines. Results showed no increase of ROS production in both cell lines (Figure 8).

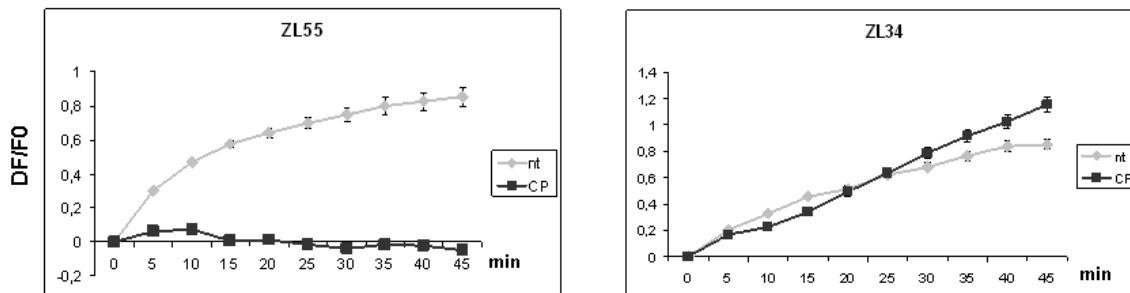


Figure 8. The cells were pre-incubated with 25 nM H2-MTR for 30 min and then treated with CP. Fluorescence was analysed every 5 minutes for at least 45 minutes. f_0 is the fluorescent signal measured at the beginning of the experiment, and DF is the difference in fluorescent signal measured at each individual time point subtracted of the f_0 value. Mean and Standard Error of DF/f_0 recorded in at least 30 cells per time point per sample in 3 independent experiments were calculated.

Moreover, Annexin V assay performed in both cell lines pre-treated with NAC 1mM revealed that there was no different levels of cell death between samples pre-treated with NAC compared to samples treated with CP+TRAIL only (Figure 9). On the basis of these results we concluded that ROS induction by chemotherapy could not sensitize MPM cells to TRAIL-mediated apoptosis.

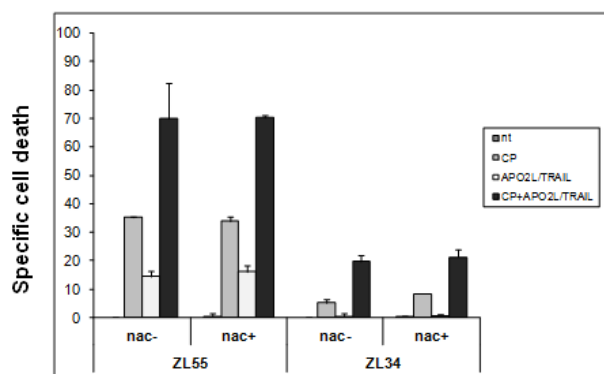


Figure 9. The cells were pre-treated with ROS scavenger NAC (1mM) and then with CP and/or TRAIL. Specific cell death in treated cells was normalized compared to untreated control: $(\text{percentage of cell death in treated samples} - \text{percentage of cell death in untreated samples} - \text{NAC}) / (100 - \text{percentage of cell death in untreated samples} - \text{NAC}) * 100$. The results were represented as means \pm SE of 3 different experiments running in triplicate.

2c. p53 activation.

Considering that DNA-damaging agents could activate p53 in p53 wild-type cells, and that mesothelioma cells showed wild type p53 in most of the cases, we assessed the effect of chemotherapy with CP and nutlin 3, an inhibitor of MDM2-p53 interaction that lead to p53 activation, on p53 activation in ZL55 and ZL34 cell lines.

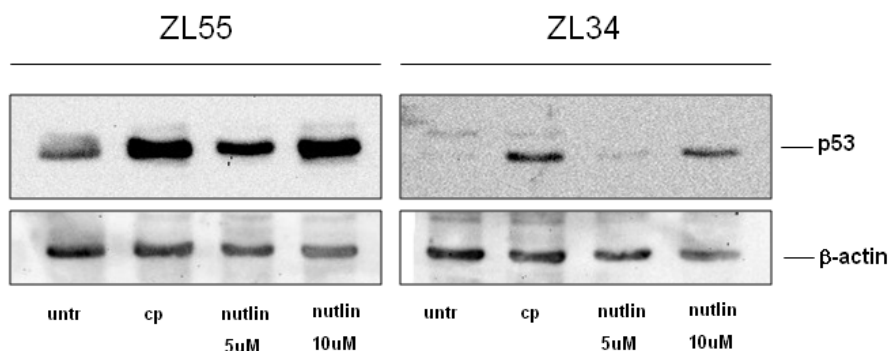


Figure 10. MPM cells were exposed to CP or nutlin 3 for 24 hours and lysates were analysed by western blotting using p53 and β -actin (as loading control) antibodies.

Western blot analysis revealed that CP treatment was able to activate p53 in MPM cell lines. Moreover, treatment with nutlin 3 was also able to activate p53 in a dose dependent manner.

In order to assess whether p53 activation could increase TRAIL-mediated cell death, we performed apoptosis assays of ZL55 and ZL34 cell lines treated with nutlin 3 in presence or in absence of TRAIL. Interestingly, we observed that nutlin 3 improved TRAIL induced apoptosis in both cell lines with a greater effect in sarcomatoid cells rather than in epithelioid cells (Figure 11); in fact, the percentage of apoptotic ZL55 cells after treatment with nutlin plus TRAIL was lower than after treatment with CP plus TRAIL, while the administration of nutlin was more effective than the administration of CP in increasing TRAIL induced apoptosis of sarcomatoid cell lines (Figures 7 and 11). These results confirmed that p53 could sensitize to TRAIL-dependent apoptosis; probably, p53 is not the only apoptotic signal transduction pathway activated in response to CP administration in epithelioid cell lines, whereas nutlin 3 could activate p53 bypassing the activation of resistance mechanisms activated in response to CP treatment in sarcomatoid cell lines.

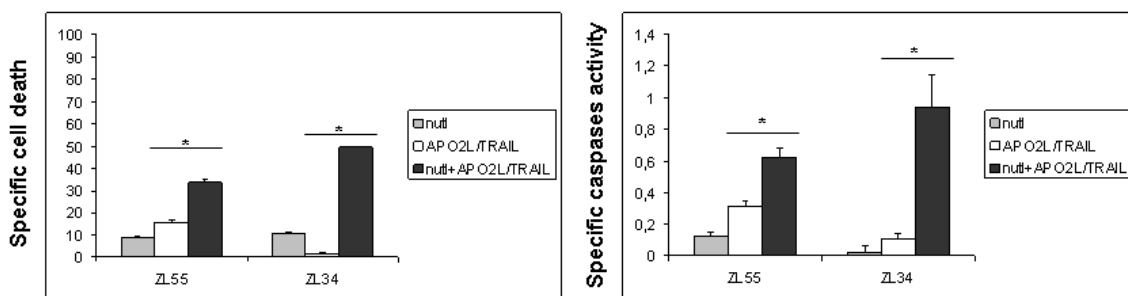


Figure 11. MPM cells were treated with nutlin 3a (10uM) with or without TRAIL for 24 hours. **A)** Annexin V assay. Specific cell death was calculated by the following formula: (percentage of cell death in treated samples- percentage of cell death in untreated samples) / (100- the percentage of cell death in untreated samples) *100. **B)** Fluorometric Homogenous Caspase Assay . Specific caspases activity was calculated as the ratio of specific fluorescence signal of the treated cells (F treated – F untreated)/ F untreated cells. The results were represented as means \pm SE of 3 different experiments running in triplicate.*statistically significant difference of specific cell death between MPM cells treated with nutl + TRAIL compared with cells treated with TRAIL, ($p < 0.001$) by Student t test.

p53 is a transcription factor directly implied in the regulation of both extrinsic and intrinsic apoptosis pathways. The sensitization to TRAIL-induced apoptosis might be due to the transcriptional up-regulation of TRAIL Receptors DR4/DR5, down-regulation of non functional decoy receptors (DcR1 and DcR2) which are able to sequester TRAIL causing suppression of apoptosis (Zhang et al., Cancer Gene Ther 2005), as well as to the ability to induce BCL-2 pro-apoptotic family members such as BAX, Bid, Noxa and Puma. Moreover some anti-apoptotic proteins such as Survivin, BCL-2 and BCL-XL can be repressed by p53 (Zhao et al., Cancer Lett 2012).

In order to explore TRAIL-sensitizing effects of p53, we first investigated whether p53-inducing treatments were able to modulate mRNA expression levels of TRAIL-Rs. To this end, we treated ZL55 and ZL34 cell lines with CP or nutlin 3 and then we evaluated mRNA levels of DR4/DR5, DCR1/DCR2 by Real-Time PCR.

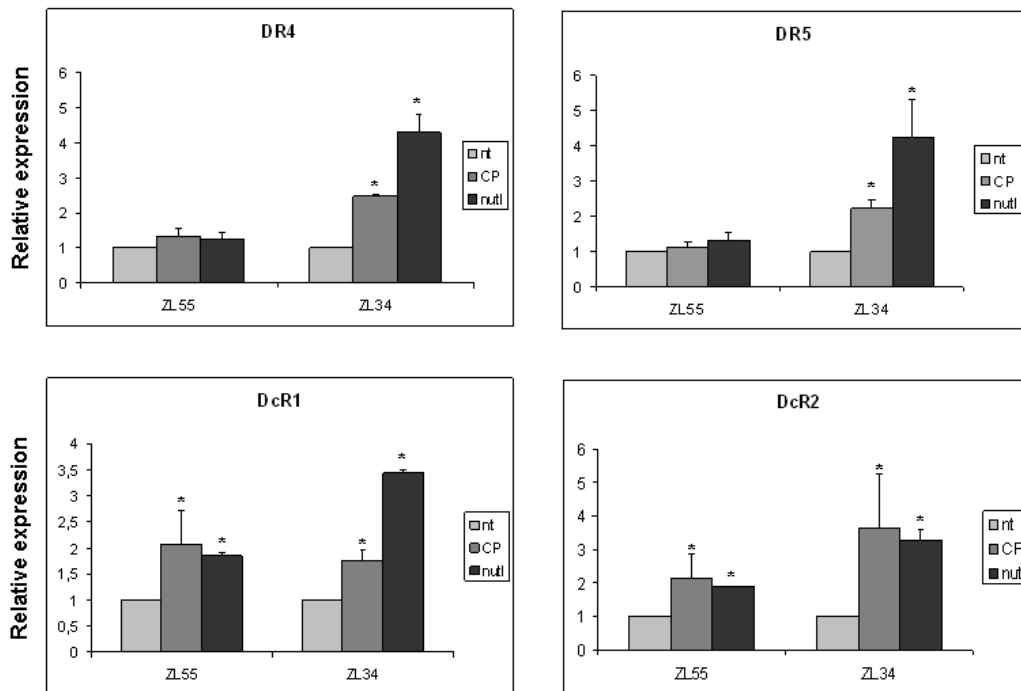


Figure 12. MPM cells were treated with CP or nutlin 3a for 24 hours and mRNA expression levels were evaluated by Real-Time PCR. The results were represented as means \pm SE of 3 different experiments running in triplicate. *statistically significant difference in mRNA expression levels of cells treated with CP or nutlin 3 compared with untreated cells ($p < 0.05$) by Mann-Whitney test.

As shown in figure 12, neither CP nor nutlin were able to increase mRNA level of DR4 and DR5 in ZL55 cell lines, while a mild increase of the decoy receptors was seen.

Conversely, both CP and nutlin 3 were able to increase mRNA levels of all TRAIL-Rs in ZL34 cells, but while CP has a similar effect on the mRNA expression levels of four receptors, nutlin 3 increased more mRNA of DR4 and DR5 compared to DcR1 and DcR2.

The analysis of the surface TRAIL-Rs protein expression levels by flow cytometry is currently on going. We next analysed mRNA expression levels of some pro and anti apoptotic proteins that could be regulated by p53 induction. To this end we treated ZL55 and ZL34 cell lines with CP or nutlin 3 with or without TRAIL and real-time PCR was performed (Figure 13A, and B).

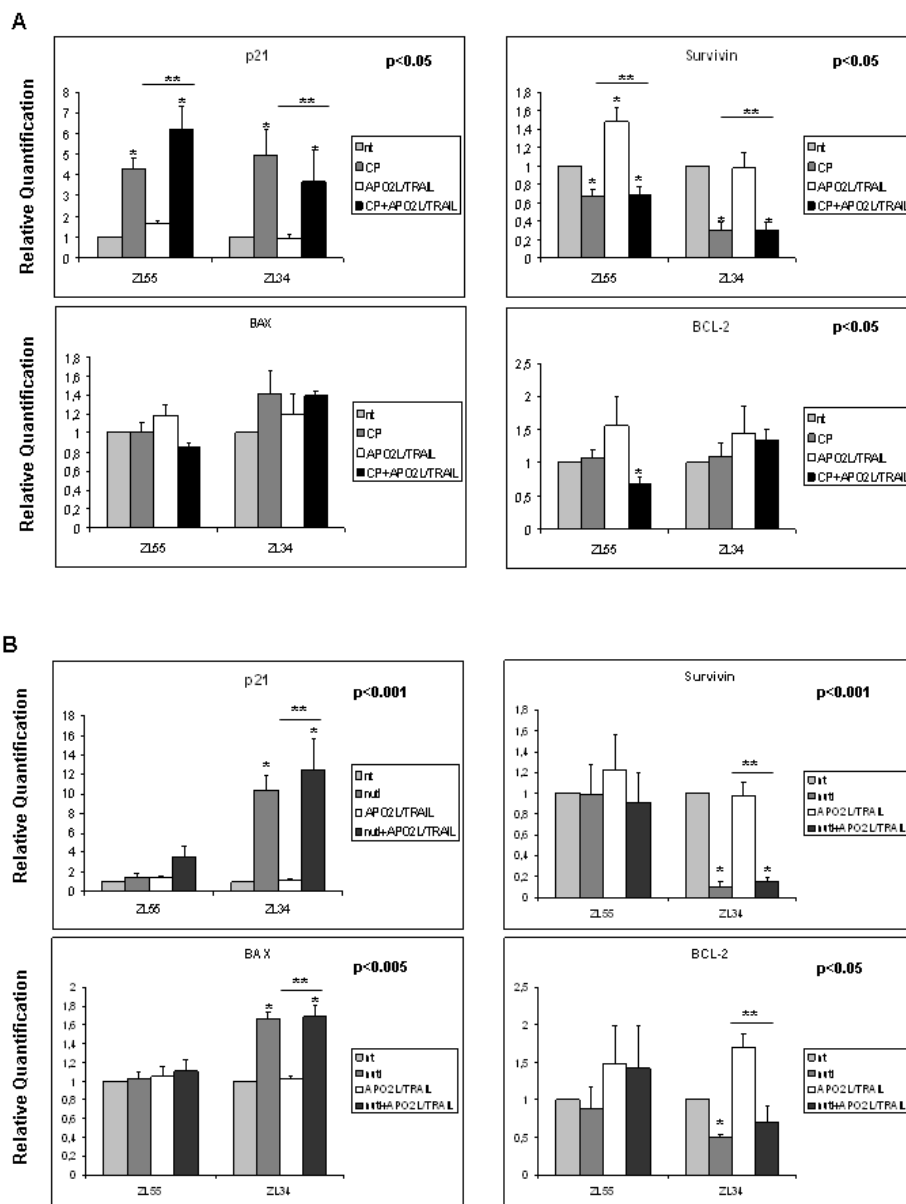


Figure 13. MPM cells were treated with CP or nutlin 3a with or without TRAIL and mRNA expression levels were evaluated by Real-Time PCR. The results were represented as means \pm SE of 3 different experiments running in triplicate. *statistically significant difference in mRNA expression levels of cells treated with CP or nutlin 3 or TRAIL compared with untreated cells ($p < 0.05$) by Mann-Whitney test. ** statistically significant difference in mRNA expression levels of cells treated with CP or nutlin 3 plus TRAIL compared with cells treated with TRAIL only ($p < 0.05$) by Mann-Whitney test

We observed that CP treatment increases p21 mRNA expression and decreased survivin mRNA expression in both cell lines, with a stronger effect in ZL34 than in ZL55 cell lines. No significant differences were observed in BAX and BCL-2 mRNA expression levels.

On the contrary, nutlin 3 had no effect on p21, survivin, BAX and BCL2 expression levels in ZL55 cell lines, but had a stronger activity in sarcomatoid cells.

These results suggested that:

a) in ZL55 cell lines the increase of cell death with nutlin + TRAIL treatment could be p53 independent (probably due to activation of other target of MDM2 or to an off-target effect of nutlin 3). In order to clarify the involvement of p53 in response to CP or nutlin treatment we are testing the effect of p53 siRNA in ZL55

and ZL34 cell lines treated with CP or nutlin 3 with or without TRAIL.

b) In sarcomatoid cell lines the activation of p53 by nutlin 3 was more effective than the activation by CP, probably due to a failure in activation of pro-survival signalling pathway triggered by CP.

SUMMARY OF RESULTS AND CONCLUSIONS

1. Glibenclamide sensitizes MPM cell lines to TRAIL-mediated apoptosis. When we analyzed the effects of Glibenclamide in the two MPM histotypes, different mechanisms of action were shown in epithelioid and sarcomatoid cell lines. In fact, ROS induction was observed in epithelioid but not in sarcomatoid cell lines treated with Glibenclamide.
2. TRAIL-dependent apoptosis sensitization by chemotherapy (carboplatin *plus* pemetrexed), observed both in epithelioid and in sarcomatoid cell lines, is probably not due to ROS induction but to p53 activation; p53 is not the only apoptotic signal transduction pathway activated in response to CP administration in epithelioid cell lines.
3. Nutlin 3 sensitizes both epithelioid and sarcomatoid cell lines to TRAIL-induced cell death, with a greater effect in sarcomatoid cells rather than in epithelioid cells; it is possible that nutlin 3 could activate p53 bypassing the activation of resistance mechanisms activated in response to CP treatment in sarcomatoid cell lines.

FUTURE PERSPECTIVES

In vivo experiments are currently on going which will demonstrate the antitumor effect of new drugs with or without chemotherapy in MPM.

Further experiments are warranted in order to clarify the molecular pathways involved in apoptosis sensitization with such new drugs, and to show any difference between the two histotypes.

Finally, the collection and analysis of samples obtained from MPM patients before and after surgery is currently ongoing, to detect markers predicting treatment outcome.

List of Publications Resulting from the Grant

Giulia Pasello, Loredana Urso, Micol Silic-Benussi, Marco Schiavon, Vincenzo Ciminale, Federico Rea, Adolfo Favaretto: Glibenclamide sensitizes malignant pleural mesothelioma cells and primary cultures to TRAIL-mediated apoptosis. Abstract and Poster, XI international conference of the International Mesothelioma Interest Group, Boston 11-14 September 2012

Selection of Courses & Workshops Attended During the Fellowship

- THE 10TH INTERNATIONAL MESOTHELIOMA INTEREST GROUP (IMIG) CONFERENCE; 31 AUGUST-02 SEPTEMBER 2010
- INTERNATIONAL ASSOCIATION FOR THE STUDY OF LUNG CANCER, AMSTERDAM, JULY 3-7 2011
- ADVANCES IN MEDICINE; 23 MARCH 2012, UNIVERSITY OF PADUA
- 2012 AMERICAN SOCIETY OF CLINICAL ONCOLOGY (ASCO) MEETING 1-5 JUNE 2012, CHICAGO (IL)
- ENDOTHELIAL PROGENITORS CELLS FROM DISCOVERY TO THERAPY 21JUNE 2012, PADOVA
- THE 11TH INTERNATIONAL MESOTHELIOMA INTEREST GROUP (IMIG) CONFERENCE; 11-14 SEPTEMBER 2012, BOSTON (MA)
- MALIGNANT PLEURAL MESOTHELIOMA: CLINICAL PATHOLOGICAL AND LEGAL FEATURES; 8 NOVEMBER 2012, PADOVA

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