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Loss of C/EBP- β LIP drives cisplatin resistance in malignant pleural mesothelioma

This is the author's manuscript
Original Citation:
Availability:
This version is available http://hdl.handle.net/2318/1665635 since 2018-08-27T14:28:57Z
Published version:
DOI:10.1016/j.lungcan.2018.03.022
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(Article begins on next page)

Elsevier Editorial System(tm) for Lung

Cancer

Manuscript Draft

Manuscript Number: LUNGCANCER-D-17-01176R1

Title: Loss of C/EBP- β LIP drives cisplatin resistance in malignant pleural mesothelioma

Article Type: Research paper

Keywords: malignant pleural mesothelioma; CAAT/enhancer binding protein; cisplatin resistance; endoplasmic reticulum stress; immunogenic cell death

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Abstract: Objectives. Cisplatin-based chemotherapy is moderately active in malignant pleural mesothelioma (MPM) due to intrinsic drug resistance and to low immunogenicity of MPM cells. CAAT/enhancer binding protein $(C/EBP)-\beta$ LIP is a pro-apoptotic and chemosensitizing transcription factor activated in response to endoplasmic reticulum (ER) stress. Materials and methods. We investigated if LIP levels can predict the clinical response to cisplatin and survival of MPM patients receiving cisplatin-based chemotherapy. We studied the LIP-dependent mechanisms determining cisplatin-resistance and we identified pharmacological approaches targeting LIP, able to restore cisplatin sensitiveness, in patient-derived MPM cells and animal models. Results were analyzed by a one-way analysis of variance test.

Results. We found that LIP was degraded by constitutive ubiquitination in primary MPM cells derived from patients poorly responsive to cisplatin. LIP ubiquitination was directly correlated with cisplatin chemosensitivity and was associated with patients' survival after chemotherapy. Overexpression of LIP restored cisplatin's pro-apoptotic effect by activating CHOP/TRB3/caspase 3 axis and up-regulating calreticulin, that triggers MPM cell phagocytosis by dendritic cells and expanded autologous anti-tumor CD8+CD107+T-cytotoxic lymphocytes. Proteasome inhibitor carfilzomib and lysosome inhibitor chloroquine prevented LIP degradation. The triple combination of carfilzomib, chloroquine and cisplatin increased ER stress-triggered apoptosis and immunogenic cell death in patients' samples, and reduced tumor growth in cisplatin-resistant MPM preclinical models.

Conclusion. The loss of LIP mediates cisplatin resistance, rendering LIP a possible predictor of cisplatin response in MPM patients. The association of proteasome and lysosome inhibitors reverses cisplatin resistance by restoring LIP levels and may represent a new adjuvant strategy in MPM treatment.

Torino, 12 March 2018

To the kind attention of

Professor Ross A Soo

Associated Editor

Lung Cancer

Dear Professor Soo,

attached please find our revised manuscript # LUNGCANCER-D-17-01176.

On the basis of the Reviewers' criticisms, we extensively changed text an Figures. We added two new Supplementary Figures and one new Supplementary Table. We would like to thank the Reviewers for their thoughtful comments, which have improved the manuscript substantially.

We hope that the revised version of the manuscript will be considered worthy of publication in Lung Cancer.

All the authors have read and approved the submission of the manuscript.

The manuscript has not been published previously and is not being considered concurrently by another journal.

The authors also declare that there are no conflicts of interest.

Sincerely yours,

Chiara Riganti

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Reply to Reviewers

Reply to Reviewer 1

Reviewer #1: The authors examined the importance of the biomarker of "C/EBP-beta LIP" in predicting cisplatin resistance and patient prognosis with malignant pleural mesothelioma. A lot of experiments, using a variety of techniques have been carried out in this body of work and I commend the authors for that. The main findings were the loss of LIP mediates cisplatin resistance and preclinically, proteasome and lysosome inhibitors reverse cisplatin resistance by restoring LIP levels. If this conclusion is validated by other groups, it could be an important advance in malignant mesothelioma, given the limited treatment options and the generally grim prognosis.

The paper is generally well written and the discussion is clear. However, given the audience of Lung Cancer (the journal) is generally clinical, I think more simple explanations may be needed in the results part to make it easier to understand for the clinical audience.

1) My main concern is that perhaps the key findings do not support the conclusion of the paper and I think the authors will need to explain this a lot better. I refer to the results in Figure 1. This part of the experiments plays a key role and forms the rationale for the rest of the experiments and overall story.

- Figure 1 legend (line 465, page 20) states, Arrow, upper panel. However, there is no arrow in figure b, upper panel. So I assume it should be middle panel as there is an arrow stating UQ-C/EBP-beta LIP

- If my assumption is correct, the western blot shows that the highest ubiquitinated LIP appears to be in patient 7, 8, 9 which represents the sarcomatous patients. If this is true, those patients appear to have low survival (Supplementary table 2; stating survival of 6, 10, 5 months respectively)

- This is then in direct contradiction to figure 1d, which is meant to show LIP ubiquitination level. The Kaplan meier curve would suggest that LIP high (high LIP ubiquitination) have longer survival - contradicting to the data in figure 1b and supplementary table 2)

We apologize for the mistake in the legend of Figure 1. The arrow refers to the middle panel, as the Reviewer noted. We corrected the legend in the revised version. It is true that the highest ubiquitinated C/EBP- β LIP appears to be in patients 7, 8, 9, i.e. the patients with sarcomatous histotype and the lowest survival.

Ubiquitinated LIP is a typical sign of its degradation: when a high ubiquitination signal is detected (Figure 1b, middle panel), C/EBP- β LIP protein is low, owing to its continuous degradation via proteasome and lysosome [Li Y et al, J. Biol. Chem. 283 (2008) 22443-22456; Chiribau C et al, Mol. Cell. Biol. 30 (2010) 3722-3731; Riganti C et al, J. Natl. Cancer Inst. 107 (2015) piidjv046]. This event, that occurs in most ubiquitinated proteins [Dikic I, Annu. Rev. Biochem. 86 (2017) 193-224], explains the very low (patient 8) or absent (patient 7, 9) detection of C/EBP- β LIP (Figure 1b, upper panel). Patients with high ubiquitinated C/EBP- β LIP correspond to the category "LIP low" patients of the old Figure 1d. These patients (patients number 3, 4, 7, 8, 9) had low survival.

We apologize for not having clearly explained these correlations. We modified Results (page 10, line 226; page 11, line 230), Discussion (page 14, line 329), Figure 1f (the old Figure 1d) and its legend to make this point clearer. We added two new references.

- Line 470, page 20, should it be Figure 1b, rather than Figure 1a?

We corrected the figure legend.

Line 471, page 20, should it be Figure 1b again, rather than Figure 1a?We corrected the figure legend.

- Fiugre 1c itself, there is an arrow of HMC pointing to very low IC50. This is a little unusual, as my understanding is that generally normal mesothelial cells tend not to be sensitive to cisplatin at all. Please explain.

We agree that the IC₅₀ in immortalized commercially available cell lines such as Met-5A is significantly higher than the IC₅₀ found in the present work and comparable to the IC₅₀ observed in malignant pleural mesothelioma (MPM) cells. In the present work, however, we used primary human mesothelial cells (HMC) that undergo to a rate of spontaneous apoptosis of about 10% cells at each passage when put in culture. Although HMC were used before passage number 4 to avoid an excessive population of apoptotic cells in the cultures and we verified that the cell viability (measured by Trypan Blue staining) before each experiments was \geq 85%, we cannot exclude that the intrinsically-occurring apoptosis synergized with the pro-apoptotic effects induced by cisplatin, producing such low IC₅₀. The mean IC₅₀ to cisplatin for the three HMC samples was 3.47 \pm 1.96 µM. This data is in line with previously data on published on primary HMC [Salaroglio IC et al, Oncotarget 6 (2015) 1128-1142].

However, I may have mis-interpreted the results due to the ?mislabelling of figure 1 legend. Nevertheless, the authors need to correct the legend and explains this better. It would make more sense that ubiquitination LIP high would be predictive of low survival (as this would represent low LIP itself) and that the hypothesis of low LIP predicting for cisplatin resistance would make more sense. It would then also pave the way for the rest of the paper, which is trying to increase t he LIP level to reverse the cisplatin resistance.

We apologize again for the mislabeling of Figure 1 that led to data mis-interpretation. As stated before, we corrected the Figure and modify the text to clarify the key hypothesis of our work: "high LIP ubiquitination, i.e. low LIP level, means cisplatin resistance".

There are other more minor points that I think authors need to address to strengthen the paper:

1. Figure 2a: did the authors look at what happens to Ubiquitination of LIP? Did it reduce corresponding to an increase of LIP?

We thanks the Reviewer for the interesting observation. We measured the ubiquitination of LIP on the same extracts used in Figure 2a. The results are shown in the new Supplementary Figure 1. Although cisplatin slightly increased LIP amount, in consequence of the cisplatin-induced ER stress [Mandic A et al, J. Biol. Chem. 278 (2003) 278 9100-9106], it did not significantly change the basal rate of LIP ubiquitination compared to untreated cells (Supplementary Figure 1a, Supplementary Table 4b), meaning that induced LIP is ubiquitinated and destroyed by the cell. This explains the absent activation of pro-apoptotic LIP/CHOP/TRB3/caspase 3 axis and the consequent resistance to cisplatin (Figure 2). The ratio ubiquitinated LIP/total LIP, calculated by densitometric analysis, was unchanged compared to untreated cells (Supplementary Table 4b). However, the ratio between ubiquitinated LIP/total LIP was decreased in this case (Supplementary

Figure 1b). This trend suggests that the excess of exogenous LIP, as it occurs in LIPtransduced cells, likely saturates the maximal ubiquitination capacity of MPM cells. Therein, in this case a significant amount of LIP remains not-ubiquitinated and triggers the pro-apoptotic CHOP/TRB3/caspase 3 axis (Figure 2). This effect was particularly pronounced in LIP-transduced cells treated with cisplatin (Figure 2a): the sum of endogenous LIP induced by cisplatin and exogenous LIP produced by the transfection with a LIP-overexpressing vector results in huge increase of notubiquitinated LIP, allowing the induction of LIP-dependent apoptosis. We reported these data in the Results (page 11, line 252) and commented them in the Discussion (page 16, line 360). We modified Supplementary materials accordingly.

2. Figure 2d: I note that the cells with treated with cisplatin with a concentration of 25microM. For MPM1, I note that the IC50 was ~28microM as per Supplementary Table 3). Therefore, the Pt treated plate for MPM1 in figure 2d does not seem to represent an IC50 of 28 (if treated by Pt concentration of 25). The plates for MPM 7 in figure 2d makes sense with IC50 for MPM 7 being ~71)

IC₅₀ (Supplementary Table 3) was calculated by performing the Neutral Red staining, a quantitative assay that is more sensitive than crystal violet staining, used in Figure 2d. Crystal violet staining was used as a qualitative assay. The different sensitivity of the techniques - together with the poor ability of primary MPM cells to uptake crystal violet in an homogeneous way compared to other rapidly dividing cell lines - may explain the apparent differences between the IC₅₀ calculated in Supplementary Table 3 and the image of the scanned well in Figure 2d. To give a more quantitative measure of the staining, we dissolved crystal violet dye with 1% v/v acetic acid and we read the absorbance of each well at 570 nm (HT Synergy 96-well microplate

reader, Bio-Tek Instruments, Winoosky, VT). The mean of absorbance units of untreated cells was considered 100%; the absorbance units of the other experimental conditions were expressed as percentage towards untreated cells. The results of the quantification are shown in the right panel of Figure 2d, and demonstrated that 25 μ M cisplatin reduced cell viability in MPM1 to around 63.4%±. 7.12, that is not significantly different from the IC₅₀ calculated in Supplementary Table 3. We modified Materials and methods (page 7, line 150) and Figure 2 accordingly.

3. Graphic quality could be improved in Figure 4C and Figure 6d. The scale in the figures seems a little inconsistent?

We improved the quality of the Figure 4c and 6d. We apologized for the copy-andpaste mistake reporting the length of the scale bar in the figure legends. We corrected it.

4. Figure 4b: it seems that cisplatin alone also increase LIP somewhat. Please comment.

One of the mechanisms of cisplatin-induced toxicity is the induction of ER stressmediated apoptosis [Mandic A et al, J. Biol. Chem. 278 (2003) 278 9100-9106] that is mediated by LIP activation [Riganti C et al, J. Natl. Cancer Inst. 107 (2015) piidjv046]. In human MPM cells we indeed observed a small increase in LIP in cells treated with cisplatin (see for instance MPM1 cell extracts of Figure 2a), likely as a consequence of a low level of ER stress induced by the drug. In solid tumors, ER stress often occurs as a consequence of hypoxia or nutrient t shortage [Chevet E et al, Cancer Discov. 5 (2015) 586-597]. These conditions are sufficient to induce LIP activation [Meir O et al, PLoS One. 5 (2010) e9516]. The ER tress dependent on tumor environment and the ER stress elicited by cisplatin can produce the small induction of LIP observed in tumor extracts of Figure 4b. However, this induction of LIP was insufficient to trigger ER-dependent apoptosis, as demonstrated by the absent intratumor activation of CHOP and caspase 3 (Figure 4c).

We added a comment in the Discussion (page 16, line 377) on this point.

5. Figure 5d: Did the authors examine what happens with PT (cisplatin) alone? Also what happens with ubiquitination LIP? Did it reduce as expected with increased LIP? The effects of cisplatin treatment alone have been already reported in Figure 2a: to avoid redundancy, we did not report these data again in Figure 5d. We added a sentence in the legend of Figure 5 stating this.

We measured the ubiquitination of LIP on the same extracts of Figure 5d. The results are reported in the new Supplementary Figure 7. The treatment with carfilzomib and chloroquine, alone or in combination, increased the accumulation of ubiquitinated LIP in whole cell extracts compared to untreated cells (Supplementary Figure 7a, Supplementary Table 4c). The same increase in ubiquitinated LIP was detected in cells treated with the triple combination carfilzomib + chloroquine + cisplatin. Since carfilzomib and chloroquine inhibit proteasome and lysosome, respectively - that act downstream the ubiquitination system - they prevent the degradation of ubiquitinated proteins by proteasome and lysosome [Dikic I, Annu. Rev. Biochem. 86 (2017) 193-224]. However, the ratio between ubiquitinated LIP/total LIP is lowered in carfilzomib - and chloroquine-treated cells, in particular in the combination treatments carfilzomib + chloroquine or carfilzomib + chloroquine + cisplatin (Supplementary Figure 7b). This trend suggests that carfilzomib and chloroquine produce an "action mass"-like effect: by blocking the degradation of ubiquitinated LIP via proteasome

and lysosome, the drugs increase the amount of ubiquitinated LIP to a level saturating the ubiquitination capacity of MPM cells (see also the reply to minor point 1). This saturation ultimately results in the accumulation of not-ubiquitinated LIP that triggers the CHOP/TRIB3/caspase 3 pro-apoptotic pathway (Figure 5d).

We reported these data in the Results section (page 13, line 303) and commented them in the Discussion (page 17, line 403). We modified Supplementary materials accordingly.

6. Figure 6a: What about cisplatin treated alone?

The effects of cisplatin treatment alone have been reported in Figure 2c: to avoid redundancy, we did not report the data in Figure 6a. We added a sentence in the legend of Figure 6 stating this.

7. Did the MPM patients 1 to 9 receive any other treatments post cisplatin based therapy? If so, would progression free survival (PFS) or response rate (RR) be a more appropriate endpoint to look at rather than OS, as OS may be confounded by subsequent treatment effects?

The patients selected for this study received in most case second-line treatments after cisplatin + pemetrexed first-line chemotherapy. We reported the treatment scheme in the Supplementary Table 2, along with the progression free survival.

Following the Reviewer's suggestions, we evaluated the progression free survival as end-point. The results are reported in the new Figure 1e and indicated a still significant difference between the "LIP low" and the "LIP high" patient groups. We modified the Results (page 11, line 230) and Discussion (page 15, line 338) sections, and the legend of Figure 1 accordingly. Overall, I think the potential for use of a FDA approved treatment in combination with cisplatin would represent an important advance in the treatment of MPM. The authors will need to discuss the above comments more clearly

We thanks the Reviewer for the useful comments, that substantially improved the manuscript. We hope to have exhaustively addressed all the comments throughout the manuscript.

Reply to Reviewer 2

Reviewer #2: This manuscript focused on the involvement of LIP, an isoform of the transcription factor c/EBP-beta, in the resistance to cisplatin of malignant pleural mesothelioma (MPM). This manuscript focused on the involvement of LIP, an isoform of the transcription factor c/EBP-beta, in the resistance to cisplatin of malignant pleural mesothelioma (MPM). The authors observed that MPM cell lines have lower expression of LIP than normal mesothelial cells linked to higher ubiquitination of this protein. They also found a correlation between LIP ubiquitination and cisplatin chemosensitivity of MPM cell lines and patients overall survival. Robust molecular functional studies demonstrate that LIP re-expression increase cisplatin sensitivity. Convincing in vitro and in vivo experiments show that LIP re-expression also led to cisplatin-induced apoptosis and immunogenic cell death. Proteasome and lysosome inhibitors restore LIP expression in MPM cells and also sensitizes MPM cells to cisplatin by increasing cisplatin-induced apoptosis and inducing immunogenic cell death.

1. The authors need to be more cautious in the abstract. They stated that "LIP levels

were directly correlated with cisplatin chemosensitivity and were associated with patients' survival after chemotherapy". They should precise that it is LIP ubiquitination, which is correlated. Furthermore, the statement about the "low immunogenicity of MPM cells" needs to be relativized based on published data. Overall, they should moderate some of their statement especially for the link between LIP ubiquitination and patient overall survival that should be validated in tumor samples from patients.

As suggested we modified the Abstract (page 2 line 44).

We better defined the "immune-evasive" attitude of MPM cells (Introduction; page 5, lines 82 and 90) and we added new references reporting the molecular mechanisms at the basis of the immune-suppressive environment and the low antigenicity of MPM cells.

We recognize that our preliminary data on the possible role of LIP ubiquitination as predictor of clinical response to cisplatin and survival must be validated in a larger cohort of tumor samples from patients. We modified the Discussion section (page 15, line 347; page 18, line 422) accordingly.

2. The authors found a correlation between cisplatin IC50 and LIP ubiquitination level, but not LIP protein expression. Based on data showed on Figure 2B, it seems there are no correlation between LIP expression and LIP ubiquitination level. The authors should at least address this point in the discussion. They also need to indicate, in a supplementary table, the LIP ubiquitination level, quantified from the western blot, in each cell line, for a better understanding.

Since the Figure 2b does not contained immunoblot analysis of total LIP or ubiquitinated LIP; we hypothesized that the Reviewer referred to Figure 1b. We apologize for not having clearly explained in the text that there was a correlation between ubiquitinated LIP and IC₅₀ of cisplatin. We clarified and addressed this point in the Discussion (page 15, line 335). Moreover, we included the quantification of the ratio between ubiquitinated LIP/total LIP reported in Figure 1b, calculated by densitometric analysis (figure 1 c in the revised version). We modified the Results section (page 10, line 226) and the legend of Figure 1 accordingly.

As suggested, we provided the quantification of the ubiquitinated LIP of Figure 1b in the new Supplementary Table 4s. Since the Reviewer 1 asked to investigate the ubiquitination level of LIP in the extracts of Figure 2a and 5d (reported in the Supplementary Figure 1 and 7), we included the quantification of ubiquitinated LIP also for these experimental sets in the Supplementary Table 4b-c. We commented the results of these quantification in the Discussion (page 15, line 335; page 16, line 360; page 17, line 403).

Highlights

- C/EBP-β LIP loss correlates with poor response to cisplatin in mesothelioma patients
- C/EBP-β LIP loss is due to its constitutive ubiquitination and degradation
- Lack of C/EBP-β LIP prevents cisplatin-induced apoptosis and immunogenic cell death
- Proteasome and lysosome inhibitors restoring C/EBP-β LIP rescue cisplatin efficacy

1	Loss of C/EBP-β LIP drives cisplatin resistance in malignant pleural mesothelioma
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32

33 Abstract

34 **Objectives.** Cisplatin-based chemotherapy is moderately active in malignant pleural mesothelioma

35 (MPM) due to intrinsic drug resistance and to low immunogenicity of MPM cells. CAAT/enhancer

binding protein (C/EBP)- β LIP is a pro-apoptotic and chemosensitizing transcription factor

activated in response to endoplasmic reticulum (ER) stress.

38 Materials and methods. We investigated if LIP levels can predict the clinical response to cisplatin

and survival of MPM patients receiving cisplatin-based chemotherapy. We studied the LIP-

40 dependent mechanisms determining cisplatin-resistance and we identified pharmacological

41 approaches targeting LIP, able to restore cisplatin sensitiveness, in patient-derived MPM cells and

42 animal models. Results were analyzed by a one-way analysis of variance test.

43 **Results.** We found that LIP was degraded by constitutive ubiquitination in primary MPM cells

44 derived from patients poorly responsive to cisplatin. LIP ubiquitination was directly correlated with

45 cisplatin chemosensitivity and was associated with patients' survival after chemotherapy.

46 Overexpression of LIP restored cisplatin's pro-apoptotic effect by activating CHOP/TRB3/caspase

47 3 axis and up-regulating calreticulin, that triggers MPM cell phagocytosis by dendritic cells and

48 expanded autologous anti-tumor CD8⁺CD107⁺T-cytotoxic lymphocytes.

49 Proteasome inhibitor carfilzomib and lysosome inhibitor chloroquine prevented LIP degradation.

50 The triple combination of carfilzomib, chloroquine and cisplatin increased ER stress-triggered

51	apoptosis and immunogenic cell death in patients' samples, and reduced tumor growth in cisplatin-
52	resistant MPM preclinical models.
53	Conclusion. The loss of LIP mediates cisplatin resistance, rendering LIP a possible predictor of
54	cisplatin response in MPM patients. The association of proteasome and lysosome inhibitors reverses
55	cisplatin resistance by restoring LIP levels and may represent a new adjuvant strategy in MPM
56	treatment.
57	
58	Highlights
59	- C/EBP- β LIP loss correlates with poor response to cisplatin in mesothelioma patients
60	- C/EBP- β LIP loss is due to its constitutive ubiquitination and degradation
61	- Lack of C/EBP- β LIP prevents cisplatin-induced apoptosis and immunogenic cell death
62	- Proteasome and lysosome inhibitors restoring C/EBP- β LIP rescue cisplatin efficacy
63	
64	Keywords: malignant pleural mesothelioma; CAAT/enhancer binding protein; cisplatin resistance;
65	endoplasmic reticulum stress; immunogenic cell death
66	
67	Abbreviations: ANOVA, analysis of variance; ATF6, activating transcription factor 6; C/EBP,
68	CAAT/enhancer binding protein; IRE1, inositol-requiring enzyme 1; CHOP/GADD153; C/EBP
69	homologous protein/growth arrest/DNA damage inducible 153; CEA, carcino-embrionic antigen;
70	ChIP, chromatin immunoprecipitation; DC, dendritic cells; ER, endoplasmic reticulum; EMA,
71	epithelial membrane antigen; EIF2AK3/PERK, eukaryotic translation initiation factor- 2α kinase 3;
72	FBS, fetal bovine serum; ICD, immunogenic cell death; HMC, human mesothelial cells; MPM,
73	malignant pleural mesothelioma; OS: overall survival; qRT-PCR, quantitative Real Time-PCR;
74	PFS: progression free survival; SPSS, Statistical Package for Social Science; TBS, Tris-buffered

- saline; TRB3, *tribbles*-related protein 3; UPN, Unknown Patient Number; WT1, Wilms tumor-1
- 76 antigen
- 77

78 **1. Introduction**

79 Malignant pleural mesothelioma (MPM), an asbestos-related cancer, is usually diagnosed at advanced stage, when chemotherapy - based on cisplatin associated with pemetrexed or raltitrexed -80 is the only available therapeutic option. [1,2]. The success of chemotherapy is limited by the 81 intrinsic chemoresistance [1] and immune-evasive nature of MPM. Such immune-evasive 82 83 microenvironment in MPM relies on the presence of immune-suppressive/immune-tolerant cells in 84 the MPM [3, 4], on the high levels of immune-suppressive immune-checkpoints on both MPM cells and surrounding T-lymphocytes [5, 6], on the low amount of tumor-associated antigens of MPM 85 cells [7], due to its low mutational burden [8]. 86 87 In sensitive cells, cisplatin induces DNA damage, hampers DNA repair [9] and elicits nuclearindependent effects, such as dispersal of Golgi apparatus [10] and apoptosis induced by 88 endoplasmic reticulum (ER) stress [11]. Upon ER stress, cancer cells expose on their surface the 89 90 "eat-me" signal calreticulin, leading to dendritic cells (DC)-mediated phagocytosis and activation of autologous anti-tumor cytotoxic CD8⁺T-lymphocytes [12]. This process is known as immunogenic 91 92 cell death (ICD) [12]. MPM cells however do not translocate calreticulin from ER to surface [13, 93 14], resulting ICD-refractory. This is an additional mechanism explaining the low immunogenicity of MPM cell. 94 95 Solid tumors respond to chemotherapy-induced ER stress by activating adaptation and survival pathways if the stress is limited, or pro-apoptotic pathways if the stress persists [15, 16]. The ER 96 stress-induced transcription factor CAAT/enhancer binding protein (C/EBP)-β is involved in both 97 98 responses. At the early ER stress phase, the pro-survival isoform C/EBP- β LAP is produced. Upon 99 prolonged ER stress, the isoform C/EBP-β LIP (LIP) is formed and activates C/EBP homologous protein/growth arrest/DNA damage inducible 153 (CHOP/GADD153) protein, which promotes 100 apoptosis by activating tribbles-related protein 3 (TRB3) and caspase 3 [17-20]. At the present there 101

are no data available about gene alterations (mutation, amplification or deletion) in the 87 MPM

103 evaluated by the Tissue Cancer Genome Atlas (<u>https://cancergenome.nih.gov</u>), nor about the

104 expression of C/EBP-β LAP/LIP isoforms in MPM, according to Protein Tissue Atlas

105 (<u>http://www.proteinatlas.org</u>).

We recently reported that chemoresistant tumors lack LIP, because of its constitutive ubiquitination.
LIP loss mediates chemoresistance by increasing the expression of the drug efflux transporter Pglycoprotein and by preventing the ER stress-dependent pro-apoptotic response [12].
Here, we investigated if LIP mediates the resistance to cisplatin in MPM. We identified clinically
feasible pharmacological strategies that restore the sensitivity of MPM to cisplatin by preventing
LIP degradation.

112 **2.** Materials and methods

113 **2.1.Chemicals.** Cell culture plastic ware were obtained from Falcon (Becton Dickinson, Franklin

114 Lakes, NJ). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA).

115 Carfilzomib was purchased from Biorbyt Ltd. (Cambridge, UK) and torin 1 was from Selleckchem

116 (Munich, Germany). The protease inhibitor cocktail set III was obtained from Millipore (Billerica,

MA). Unless specified otherwise, all reagents were purchased from Sigma Chemicals Co (St. Louis,MO).

119 2.1.Cells. Primary human mesothelial cells (HMC) were isolated from three patients with pleural fluid secondary to congestive heart failure, with no history of a malignant disease. Nine primary 120 human MPM samples (3 epithelioid MPM, 3 biphasic MPM, 3 sarcomatous MPM) were obtained 121 from diagnostic thoracoscopies. Histological and clinical features are shown in Supplementary 122 Tables 1-2. All patients, identified with Unknown Patient Numbers (UPN), received 5 cycles of 123 cisplatin 75 mg/m² every 21 days. Tissue was digested in medium containing 1 mg/ml collagenase 124 and 0.2 mg/ml hyaluronidase for 1 h at 37°C. Cells were seeded in culture and used within passage 125 6. The Ethical Committee of Biological Bank of Mesothelioma, S. Antonio e Biagio Hospital, 126 Alessandria, Italy, and San Luigi Gonzaga Hospital, Orbassano, Italy, approved the study 127 (#9/11/2011; #126/2016). Murine AB1 cells were purchased from Sigma Chemicals Co. Cells were 128

grown in Ham's F10 nutrient mixture medium (primary HMC/MPM cells) or DMEM (AB1 cells),
supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin-streptomycin.

131 2.3.Quantitative Real Time-PCR (qRT-PCR). RNA was extracted and reverse-transcribed using the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories). gRT-PCR was performed using IQTM 132 SYBR Green Supermix (Bio-Rad Laboratories). The same cDNA preparation was used for 133 measuring genes of interest and the housekeeping gene S14. Primer sequences were designed using 134 qPrimerDepot software (http://primerdepot.nci.nih.gov/). Relative gene expression levels were 135 136 calculated using Gene Expression Quantitation software (Bio-Rad Laboratories). 2.4.Immunoblotting. Protein or tumor extracts (20 µg) were subjected to SDS-PAGE and probed 137 with the following antibodies: C/EBP-β (directed against the common C-terminus of LIP and LAP, 138 139 Santa Cruz Biotechnology Inc., Santa Cruz, CA), CHOP/GADD153 (Abcam, Cambridge, UK), TRB3 (Proteintech, Chicago, IL), caspase-3 (GeneTex, Hsinhu City, Taiwan), β-tubulin (Santa 140 Cruz Biotechnology Inc.). To detect ubiquitinated C/EBP- β , 100 µg protein extracts were immuno-141 precipitated overnight with the anti-C/EBP-β antibody, using 25 μl of PureProteome Magnetic 142 Beads (Millipore). Immunoprecipitated samples were then probed with an anti-mono/polyubiquitin 143 antibody (Axxora). Blotting was followed by the peroxidase-conjugated secondary antibody. The 144 membranes were washed with Tris-buffered saline/Tween 0.01% v/v and proteins were detected by 145 enhanced chemiluminescence. Band density was calculated using ImageJ software 146 147 (http://www.rsb.info.nih.gov/ij/). **2.5.Cell viability and growth**. Cell viability with neutral red staining and crystal violet staining 148 were performed as reported [20]. IC_{50} was calculated with the CompuSyn software 149 150 (http://www.combosyn.com). Quantitation of crystal violet staining was performed by dissolving

151 crystal violet with 1% v/v acetic acid and reading the absorbance of each well at 570 nm (HT

152 Synergy 96-well microplate reader, Bio-Tek Instruments, Winoosky, VT). The mean absorbance of

untreated cells was considered 100%; the absorbance units of the other experimental conditionswere expressed as percentage towards untreated cells.

2.6.Cell cycle analysis. 1×10^4 cells were harvested, washed with PBS, treated with 0.25 mg/ml RNAse and stained for 15 min with 50 µg/ml propidium iodide. Cell cycle distribution was analyzed by Guava® easyCyte flow cytometer (Millipore, Billerica, MA), using the InCyte software (Millipore).

2.7.Over-expression of C/EBP-\beta LAP and LIP. The pcDNA4/TO expression vectors (Invitrogen Life Technologies, Milan, Italy) for LAP and LIP, produced as reported previously [17], were cotransduced with pcDNA6/TR vector (Invitrogen Life Technologies) in parental cells. Stable TetON clones were generated by selecting cells with 2 µg/ml blasticidin S (Invitrogen Life Technologies) and 100 µg/ml zeocin (InvivoGen, San Diego, CA). LAP and LIP induction was activated by adding 1 µg/ml doxycycline in the culture medium.

2.8.C/EBP- β LIP silencing. 2×10⁶ cells in 0.25 ml serum/antibiotic-free medium were transfected 165 either with non-targeting scrambled siRNA pools or siRNA pools specifically targeting LIP 166 sequence (customized ON-TARGETplus, Dharmacon RNAi Technologies; Dharmacon, Lafayette, 167 CO), employing DharmaFECT 1 reagent (Dharmacon), as per manufacturer's protocol. 168 169 **2.9.Chromatin immunoprecipitation (ChIP).** To determine the binding of LAP and LIP to calreticulin promoter we performed ChIP as described [21], using the anti-C/EBP-β antibody 170 directed against the common C-terminus of LAP and LIP. Putative binding sites of C/EBP-β were 171 172 identified using the Gene Promoter Miner software (http://gpminer.mbc.nctu.edu.tw/). PCR primers were designed using Primer3 software (http://primer3.ut.ee/). 173

2.10.Calreticulin expression, phagocytosi and T-lymphocyte activation. Surface calreticulin was
measured by flow cytometry as reported [22]. DC were generated from peripheral blood samples of
patients, collected before starting chemotherapy, as previously reported [13]. Phagocytosis assays
were performed as detailed in [22]. Active anti-tumor cytotoxic CD8⁺CD107⁺T-lymphocytes,

obtained from autologous T-lymphocytes (co-cultured 10 days with DC after phagocytosis) and isolated with the Pan T Cell Isolation Kit (Miltenyi Biotec., Bergisch Gladbach, Germany), were measured by flow cytometry [13]. The production of IFN- γ in the culture supernatant of CD8⁺Tcells co-cultured with DC or in the supernatant of tumor-draining lymph nodes - a second parameter of CD8⁺T-cells cytotoxic activity - was measured with the Human IFN- γ DuoSet Development Kit (R&D Systems, Minneapolis, MN).

2.11.*In vivo* tumor growth. 1×10^7 AB1 cells expressing LIP upon doxycycline administration in 184 drinking water, mixed with 100 µl Matrigel, were injected subcutaneously (s.c.) in 6-weeks-old 185 female immune-competent or immune-deficient (nude) balb/C mice (Charles River Laboratories 186 187 Italia, Calco), housed (5 per cage) under 12 h light/dark cycle, with food and drinking provided ad *libitum*. Tumor growth was measured daily by caliper, according to the equation $(LxW^2)/2$, where 188 L=tumor length and W=tumor width. When tumor reached the volume of 50 mm³, animals were 189 190 randomized and treated as reported in the Figure Legends. Tumor volumes were monitored by caliper and animals were euthanized at day 21 after randomization with zolazepam (0.2 ml/kg) and 191 192 xylazine (16 mg/kg). The hemocromocytometric analyses were performed with a UniCel DxH 800 Coulter Cellular Analysis System (Beckman Coulter, Miami, FL) on blood collected immediately 193 after sacrificing the mice. Hematochemical parameters were analyzed using the respective kits from 194 195 Beckman Coulter Inc. Animal care and experimental procedures, according to EU Directive 2010/63, were approved by the Bio-Ethical Committee of the Italian Ministry of Health 196 (#122/2015-PR). 197

2.12.Immunohistochemistry and intratumor immune infiltrate analysis. Tumors were resected
and fixed in 4% v/v paraformaldehyde, stained with hematoxylin/eosin or immunostained for
CHOP or cleaved(Asp175)-caspase 3 (Cell Signaling Technology Inc., Danvers, MA), followed by
a peroxidase-conjugated secondary antibody. Nuclei were counter-stained with hematoxylin.
Sections were examined with a Leica DC100 microscope. Excised tumors were digested with 1
mg/ml collagenase and 0.2 mg/ml hyaluronidase (1h at 37°C) and filtered using a 70 µm cell

strainer to obtain a single cell suspension. Infiltrating immune cells were collected by centrifugation
on Ficoll-Hypaque density gradient and subjected to immune phenotyping by flow cytometry, using
antibodies against CD11c for DC, CD3 and CD8 for T-lymphocytes (Miltenyi Biotec.). Draining
lymph nodes were collected, homogenized for 30 s at 15 Hz, using a TissueLyser II device (Qiagen,
Hilden, Germany) and centrifuged at 12000 x g for 5 minutes. The supernatant was used to measure
the amount of IFN-γ.

2.13.Proteasome, autophagy and lysosome activity. Proteasome activity was measured with the
Proteasome-GloTM Cell-Based Assays (Promega Corporation). Autophagy activity was measured
using the Autophagy Assay Kit (Sigma Chemicals Co.). The activity of cathepsin L, taken as an
index of lysosome activity, was measured as reported [23].

214 **2.14.Statistical analysis.** All data in the text and figures are provided as means±SD. The results 215 were analysed by a one-way analysis of variance (ANOVA), using Statistical Package for Social 216 Science (SPSS) software (IBM SPSS Statistics v.19). p<0.05 was considered significant. Overall 217 survival was defined as the time passed from the starting of cisplatin therapy to the date of death (all 218 patients were dead at the time of analysis). The Kaplan-Meier method was used to calculate overall 219 survival. Log rank test was used to compare the outcome of the two groups. The sample size was 220 calculated with the G*Power software (www.gpower.hhu.de), setting $\alpha \le 0.05$ and $1-\beta=0.80$.

221 **3. Results**

3.1.LIP is constitutively ubiquitinated in mesothelioma and correlates with cisplatin resistance 222 $C/EBP-\beta$ mRNA was equally expressed in primary non-transformed HMC and MPM cells (Figure 223 224 1a). By contrast, C/EBP-β LAP protein was detected in both HMC and MPM samples, LIP was detectable only in HMC. The absence or very low expression of LIP in MPM was due to its higher 225 ubiquitination: lower was the level of LIP in MPM (upper panel, Figure 1b), higher was LIP 226 ubiquitination (*middle panel*, Figure 1b; Figure 1c), suggesting that LIP ubiquitination can be 227 paralleled by its degradation, as it occurs for most ubiquitinated proteins [24] All MPM samples 228 were significantly more resistant to cisplatin in vitro compared to HMC (Supplementary Table 3). 229

Ubiquitinated LIP was directly correlated with the IC₅₀ of cisplatin (Figure 1d). LIP ubiquitination 230 was also significantly associated with patients' progression free survival (PFS; Figure 1e) and 231 overall survival (OS; Figure 1f) after cisplatin therapy: median PFS was 2.2 months, median OS 232 was 9 months (Supplementary Table 2) in the top 5 patients (UPN 3, 4, 7, 8, 9) with highest LIP 233 ubiquitination (LIP ubiquitination higher or equal to median value, Supplementary Table 4a). This 234 group was called "LIP low group" in Figure 1e-f, since patient-derived cell had lower levels of LIP 235 protein (Figure 1b, upper panel). Median PFS and OS were 5.3 and 16 months respectively 236 (Supplementary Table 2) in the top 4 patients (UPN 1, 2, 5, 6) with lowest LIP ubiquitination (LIP 237 ubiquitination lower or equal to median value, Supplementary Table 4a). This group was defined as 238 239 "LIP high group" in Figure 1e-f, since patient-derived cells had higher levels of LIP protein (Figure 1b, upper panel). 240

241 **3.2.Reconstitution of LIP restores cisplatin-induced cell death by activating ER stress**-

242 mediated apoptosis

To investigate whether there was a causal relationship between the loss of LIP and the resistance to
cisplatin, we induced overexpression of exogenous LIP in MPM cells (Figure 2a). LIP transduced
cells were more sensitive to cisplatin (Supplementary Table 5), activated the pro-apoptotic
CHOP/TRB3/caspase 3 axis (Figure 2a), increased the percentage of sub-G1 apoptotic cells,
reduced the percentage of cells entering the S-phase (Figure 2b), decreased cell proliferation (Figure 2a).

Cisplatin did not activate the LIP/CHOP/TRB3/caspase 3 axis, neither affected cell cycle, nor
proliferation, in non-transduced MPM cells, whereas LIP induction restored all these events. The
combination "LIP induction+cisplatin treatment" was superior to LIP induction only (Figure 2a-d).
Although cisplatin slightly increased LIP amount, it did not change the basal rate of LIP
ubiquitination, meaning that the induced LIP was ubiquitinated (Supplementary Figure 1a;
Supplementary Table 4b) and subsequently degraded by the cell. The ratio ubiquitinated LIP/total
LIP, calculated by densitometric analysis, was unchanged compared to untreated cells

(Supplementary Figure 1b). Also, in LIP-transduced cells, LIP protein underwent ubiquitination
(Supplementary Figure 1a; Supplementary Table 4b). However, the ratio ubiquitinated LIP/total
LIP was decreased in this case (Supplementary Figure 1b).

259 **3.3.LIP triggers immunogenic cell death in mesothelioma cells**

As observed above, MPM cells are refractory to ICD mediated by calreticulin [13, 14]. Two

261 predicted binding sites for C/EBP transcription factors are present in calreticulin promoter at

position 831-843 and 1302-1313 (Supplementary Figure 2a). The former one contains a CCAAT

box motif (Supplementary Figure 2b). To investigate whether C/EBP- β interacts with calreticulin

promoter, we used MPM clones constitutively overexpressing LAP or LIP (Figure 3a). Of note, LIP

bound the 831-843 site (Figure 3b). LIP-transduced cells increased calreticulin mRNA and surface

266 protein (Figure 3c-d), were easily phagocytized by DC (Figure 3e), expanded autologous activated

267 $CD8^+ CD107^+ T$ -lymphocytes producing IFN- γ (Figures 3f-g). By contrast, LAP overexpressing

cells did not differ from non-transduced MPM samples (Figure 3c-g).

As a proof of concept of the role of LIP in these process, we silenced the exogenously expressed

270 LIP in MPM cells (Supplementary Figure 3a). In LIP-silenced cells calreticulin expression,

271 phagocytosis and antitumor cytotoxic T-lymphocyte expansion were abrogated (Supplementary

Figures 3b-d), demonstrating the critical role of LIP as calreticulin inducer and ICD effector in

273 MPM.

274 **3.4.LIP** expression restores sensitivity to cisplatin and immune-mediated death *in vivo*

To validate the effects of LIP overexpression *in vivo*, we produced doxycycline-inducible LIP clones from AB1 cells (Supplementary Figure 4), a murine mesothelioma cell line syngeneic with balb/C mice. IC₅₀ of cisplatin was $91.08 \pm 17.18 \mu$ M in un-induced AB1 cells, in line with the most cisplatin-resistant human MPM cells (Supplementary Table 3). Accordingly, AB1-tumors were unresponsive to cisplatin when implanted in mice (Figure 4a). Upon induction of LIP (Figure 4b), the tumor growth was reduced in both immune-deficient and immune-competent mice (Figure 4a) and showed increased percentage of tumor cells positive for CHOP and cleaved caspase 3 (Figure

3c), in particular in LIP-induced/cisplatin-treated mice. The antitumor effects of LIP + cisplatin 282 283 were stronger in immune-competent mice, at least during the early development of MPM (Figure 4a), suggesting that immune system activation plays a significant role in delaying MPM growth. In 284 immune-competent mice, LIP induction increased intratumor infiltrating DC and CD8⁺ T-285 lymphocytes, and production of IFN-y in draining lymph nodes (Figure 4d-f). Cisplatin enhanced 286 all these events in LIP-expressing tumors (Figure 4d-f). 287

288 **3.5.LIP undergoes proteasomal and lysosomal degradation**

289

Since LIP was mono- and poly-ubiquitinated in MPM samples (Figure 1a), we searched which mechanisms are involved in LIP degradation. Compared to HMC, MPM cells displayed increased 290 291 proteasome (Figure 5a), autophagy (Figure 5b) and lysosome activity (Figure 5c). Treatment of MPM cells with the proteasome inhibitor carfilzomib and the lysosome inhibitor chloroquine 292 lowered the proteasome and lysosome activity of MPM to values comparable to HMC 293 294 (Supplementary Figure 5a-c). Either carfilzomib or chloroquine prevented LIP degradation and activated the downstream effectors CHOP, TRB3 and caspase 3 (Figure 5d). Chloroquine is an 295 296 inhibitor of autophagy and lysosomes [25, 26] (Supplementary Figure 5b-c). To better understand if 297 both mechanisms are equally involved in LIP degradation, we used the autophagy inhibitor 3methyladenine, which does not affect lysosome activity [25] (Supplementary Figure 5b-c). 3-298 299 methyladenine prevented LIP degradation and activated the CHOP/TRB3/caspase 3 axis (Supplementary Figure 6) at a lesser extent than chloroquine (Figure 5d), suggesting that autophagy 300 likely plays an ancillary role in the removal of LIP. 301

302 The combination of carfilzomib and chloroquine was even more effective than each agent alone in 303 activating the LIP/CHOP/TRB3/caspase 3 pathway (Figure 5d). The treatment with carfilzomib and chloroquine, alone or in combination, increased the accumulation of ubiquitinated LIP compared to 304

305 untreated cells (Supplementary Figure 7a; Supplementary Table 4c). The same increase in

ubiquitinated LIP was detected in cells treated with the triple combination carfilzomib + 306

chloroquine + cisplatin. The ratio between ubiquitinated LIP/total LIP was lowered in carfilzomib-307

and chloroquine-treated cells, in particular in the combination treatments carfilzomib + chloroquine
 or carfilzomib + chloroquine + cisplatin (Supplementary Figure 7b).

Carfilzomib and chloroquine combination was also the most effective in inducing ICD-related parameters (Supplementary Figure 8a-e). The immunologic effects of carfilzomib and chloroquine were likely due to the attenuated degradation of LIP, as demonstrated by the absence of calreticulin up-regulation in MPM cells lacking LIP (Supplementary Figure 8f). The triple combination of cisplatin with carfilzomib and chloroquine further activated LIP/CHOP/TRIB3/caspase 3 pathway and ICD (Figure 5d, Supplementary Figure 6a-e).

316 3.6.Combination of carfilzomib, chloroquine and cisplatin abrogates mesothelioma growth

317 Combined treatment of carfilzomib and chloroquine greatly reduced MPM cell proliferation (Figure

6a-b) and tumor growth *in vivo* (Figure 6c), in particular if associated with cisplatin. This triple

319 combination significantly increased the number of CHOP- and caspase 3-positive intratumor cells

320 (Figure 6d), and raised an anti-tumor immune response *in vivo*, as demonstrated by the increased

tumor-infiltrating DC and CD8⁺ T-lymphocytes (Figure 6e-f), and by the increased production of

322 IFN- γ in the draining lymph nodes (Figure 6g). Neither significant alterations in

323 hemocromocytometric parameters nor signs of liver, kidney, heart and muscle toxicity were

detectable in animals treated with this regimen (Supplementary Table 6).

325 **4. Discussion**

326 Aim of this study was to investigate the possible role of LIP as effector of resistance to cisplatin,

predictive factor of patient response to cisplatin, and possible target to improve the sensitivity tothis drug in MPM.

329 Using cells isolated from MPM patients treated with cisplatin but characterized by low response to

the drug (mean survival after the beginning of therapy: 13.11 months), we noticed that they had

lower amount of LIP compared to HMC cells and higher rate of LIP ubiquitination. Ubiquitination

of LIP is a typical sign of its degradation *via* proteasome and/or lysosome [18, 20, 27]. This is

333 consistent with the observation that in all MPM cells when ubiquitination of LIP was higher, the

protein was lower in comparison with HMC that had lower LIP ubiquitination and higher LIP 334 335 protein level. The rate of LIP ubiquitination strongly correlated with the in vitro resistance to cisplatin, measured as IC₅₀ of the drug: indeed, higher was LIP ubiquitination (i.e. lower LIP 336 protein was), higher was IC₅₀ of cisplatin. Hence, we concluded that LIP loss due to its 337 ubiquitination was related to resistance to cisplatin in vitro. We considered the PFS, defined as the 338 survival with stable disease from the beginning of cisplatin therapy, and the OS of each patient, 339 340 defined as the time passed from the starting of cisplatin therapy to the date of patient death. Patients with LIP ubiquitination higher or equal to median value were included in the so-called "LIP low" 341 group, since the patient-derived cell line had very low/undetectable LIP protein. Patients with LIP 342 343 ubiquitination lower or equal to median value were included in the so-called "LIP high" group, since the patient-derived cell line had higher LIP protein. "High LIP ubiquitination/LIP low" 344 patients had the shortest PFS and OS, patients with "low LIP ubiquitination/LIP high" patients 345 346 displayed the most prolonged PFS and OS after cisplatin treatment. Although the small number of patients and the absence of a control group receiving different 347 treatments makes difficult to separate the prognostic and the predictive role of LIP ubiquitination, 348 349 our work provides the rationale to measure LIP ubiquitination in larger series of MPM tumors, in order the evaluate if this parameter - beside being a good indicator of the sensitivity to cisplatin in 350 351 *vitro* - may be also predictive of the clinical response to cisplatin. In chemoresistant tumor cells, the loss of LIP upregulates the expression of the drug efflux 352 transporter P-glycoprotein [20], increasing the resistance of such cells to a broad spectrum of 353 354 chemotherapeutic drugs. Platinum-derived agents, however, are not substrates of P-glycoprotein [28]. Therefore, the restoration of drug sensitivity by LIP, after its genetic over-expression or 355 pharmacological inhibition of its degradation, must rely on a different mechanism. 356 357 Cisplatin triggers ER stress-dependent apoptosis [11], but MPM cells were refractory to these

events. Our findings in primary MPM samples and MPM preclinical models suggests that cisplatin

induces apoptosis via the ER-dependent CHOP/TRB3/caspase 3 axis only in the presence of LIP,

while LIP loss abrogated the cell death due to cisplatin-triggered ER stress. In cisplatin-treated 360 361 cells, the ratio between ubiquitinated LIP/total LIP was unchanged compared to untreated cells: the continuous ubiquitination of LIP likely explains the absence of any pro-apoptotic effect induced by 362 the drug in MPM cells. By contrast, the ubiquitinated LIP/total LIP ratio was decreased in LIP-363 transduced cells, suggesting that the excess of exogenous LIP may saturate the maximal 364 ubiquitination capacity of MPM cells. In these conditions, a significant amount of LIP remained 365 not-ubiquitinated and likely triggered the pro-apoptotic CHOP/TRB3/caspase 3 axis. This effect 366 was particularly pronounced in cells treated with cisplatin that slightly increased the amount of 367 endogenous LIP. The sum of endogenous LIP induced by cisplatin and exogenous LIP produced by 368 369 the transfection with a LIP-overexpressing vector produced a huge increase in not-ubiquitinated LIP, allowing the induction of LIP-triggered dependent apoptosis. 370 However, our finding that LIP induction attenuated tumor growth in mice even without cisplatin 371 372 indicates that LIP regulates additional mechanisms on top of ER stress. One such mechanism involves the anti-tumor immune response. Indeed, inducible intra-tumor 373 374 activation of LIP delayed tumor growth in immune-competent animals more than in immune-375 deficient mice, indicating that LIP attenuates MPM progression enhancing the anti-tumor immune response. Intriguingly, the combination cisplatin+LIP was even more effective than LIP induction. 376 377 In tumor extracts, cisplatin produced a small activation of LIP, without however reducing tumor growth. Since ER stress often occurs in tumor bulk as a consequence of hypoxia or nutrient 378 shortage [16] and these conditions are sufficient to induce LIP activation [17], it is likely that the 379 380 ER stress exerted by the tumor environment and the low ER stress elicited by cisplatin produced the small induction of LIP. Such induction, however, was insufficient to trigger ER-dependent 381 apoptosis, as demonstrated by the absent intratumor activation of CHOP and caspase 3. Previously, 382 cisplatin was not thought to trigger anti-tumor immune response [22, 29]. However, a recent report 383 revealed that in non-small cell lung cancer, cisplatin increased intratumor DC recruitment, tumor 384 cell phagocytosis and expansion of anti-tumor CD8⁺ T-lymphocytes [30]. Intratumor recruitment of 385

386 DC is mediated by cell surface expression of calreticulin [31]. ER stress (and thereby activation of 387 LIP) is necessary for triggering immune responses by cisplatin [32]. Our work demonstrates that 388 LIP induced calreticulin expression and the subsequent chain of immune responses, providing a 389 plausible mechanism by which LIP triggers an anti-tumor immune response and rescues the activity 390 of cisplatin via ER stress-dependent apoptosis and ICD.

Endogenous LIP is physiologically removed by proteasome and lysosomes [20]. Interestingly, a high activity of proteasome and lysosome correlates with bad patient prognosis in MPM [33, 34]. The inhibition of one of these two degradation pathways may be compensated by the increase in the other pathway, suggesting that only a simultaneous blockade of the two is required for restoring LIP-triggered apoptosis in MPM cells. To achieve this goal, we employed the FDA-approved proteasome inhibitor carfilzomib and the lysosome inhibitor chloroquine.

397 Bortezomib, another proteasome inhibitor, is known to induce apoptosis in MPM cells synergizing 398 with cisplatin and oxaliplatin if administered before platinum-based agents [33, 35]. Our work provides the rationale for this observation, suggesting that this synergy may be reached only after 399 400 the bortezomib-induced accumulation of LIP, which, in turn, enhanced cisplatin cytotoxicity. 401 Also chloroquine induces apoptosis of MPM cells if associated with nutritional stress [36] or PI3K/mTOR inhibition [20], two conditions that induce ER stress [15, 16, 37], suggesting that at 402 403 least part of the pro-apoptotic effect of chloroquine is triggered by ER dysfunctions. Since carfilzomib and chloroquine act downstream the ubiquitination system and prevent the degradation 404 of ubiquitinated proteins by proteasome and lysosomes [24], these drugs may produce an "action 405 mass"-like effect: by blocking the degradation of ubiquitinated LIP via proteasome and lysosomes, 406 407 carfilzomib and chloroquine increased the amount of ubiquitinated LIP to a level saturating the ubiquitination capacity of MPM cells. This saturation ultimately resulted in the accumulation of 408 409 not-ubiquitinated LIP that triggered the CHOP/TRIB3/caspase 3 pro-apoptotic pathway. In addition to ER stress-linked pro-apoptotic mechanisms, carfilzomib and chloroquine are known 410 ICD inducers [12, 38]. 411

Until now, synergistic anti-tumor effects exerted by combining proteasome and lysosome inhibitors 412 with platinum derivatives [33, 35, 39] were reported only in vitro. Our study is the first one 413 demonstrating that the combination treatment of carfilzomib, chloroquine and cisplatin is effective 414 in preclinical models of cisplatin-resistant MPM, by inducing ER stress-dependent apoptosis and 415 activating the host immune system against MPM. Such triple combination did not elicit detectable 416 signs of systemic toxicity in vivo. Moreover, each agent used in our preclinical model has well-417 known pharmacodynamic, pharmacokinetic and toxicological profiles for the extensive clinical use. 418 These considerations are encouraging in the perspective of translating the triple combination 419 cisplatin, carfilzomib and chloroquine to clinical settings. 420

421 Conclusions

Our work demonstrates that LIP levels strongly correlated with MPM response to cisplatin *in vitro*, providing the preliminary data to evaluate if the correlation between high LIP ubiquitination and low survival is valid in larger patients cohort. If the data reported in this study was confirmed, LIP ubiquitination may represent a factor predictive of clinical response to cisplatin. We suggest to include pharmacological inhibitors of LIP degradation, such as a combination of proteasome and lysosome inhibitors, in the number of strategies under evaluation to improve the response of MPM to the current treatment.

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430 Data availability

All data generated or analysed during this study are included in this published article and itssupplementary information files.

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435 None.

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437 Funding

- 438 This work was supported by Italian Association for Cancer Research (IG15232 to CR), Italian
- 439 Ministry of University and Research (EX60% Funding 2015 to SN and CR); Italian Ministry of
- 440 Health (to LR and GVS); De Benedetti-Cherasco Foundation (Torino-Weizmann Collaborative
- 441 Program: Scientific Cooperation and Exchange to MR and CR); Fondazione Cassa di Risparmio di
- 442 Torino (to CR). ICS is recipient of PhD scholarships from the Italian Institute for Social Security
- 443 (INPS). VM is a PhD fellow of Erasmus Mundus-ERAWEB Action 2 program.
- 444 The funding institutions had no role in the study design, data collection and analysis, or in writing445 the manuscript.
- 446

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554 Figure legends

555 Figure 1. Ubiquitination of LIP correlates with cisplatin resistance in mesothelioma

- **a.** Levels of $C/EBP\beta$ mRNA as determined by qRT-PCR in triplicates, in 3 primary human non-
- transformed mesothelial cells (HMC) and 9 primary human malignant pleural mesothelioma
- 558 (MPM) samples. Epi: epithelioid; Bip: biphasic; Sar: sarcomatous. b. Expression and ubiquitination
- of C/EBP-β LAP and LIP in HMC and MPM samples. Whole cell lysate was immunoprecipitated
- 560 (IP) with anti C/EBP- β antibody that recognize the common C-terminus C/EBP- β i.e. either LIP or
- 561 LAP and the blot was probed (IB) with the anti C/EBP- β antibody, to detect total levels of C/EBP-
- 562 β LAP and LIP isoforms, or with an anti-mono/poly-ubiquitin antibody, to detect the corresponding

ubiquitinated forms. Upper-middle panels, last lane: control immunoblot of MPM1 cell extract 563 564 immunoprecipitated in the absence of antibody. Arrow, *middle panel*: ubiquitinated LIP, according to the expected molecular weight. Before immunoprecipitation, an aliquot of cell lysate was probed 565 with an anti- β -tubulin antibody, to check that equal amounts of proteins from each extract were 566 loaded in immunoprecipitation. The figure is representative of 1 out of 3 experiments. c. The mean 567 band density of ubiquitinated LIP (indicated by the arrow, *middle panel*, Figure 1b) and the mean 568 569 band density of total LIP (upper panel, Figure 1b) was calculated with the ImageJ software and expressed as arbitrary optical density units, setting the mean ratio between ubiquitinated LIP/total 570 LIP in HMC to 1. Data are presented as means±SD (n=3). MPM vs HMC cells: *p<0.001. d. 571 Correlation between IC₅₀ of cisplatin (Pt; Supplementary Table 3) and LIP ubiquitination, 572 calculated with the ImageJ software . The mean band density of ubiquitinated LIP (indicated by the 573 arrow, Figure 1b) was expressed as arbitrary optical density units, setting the mean band density in 574 575 HMC samples to 1 (Supplementary Table 4a). e-f. LIP ubiquitination (indicated by the arrow, Figure 1b) was ranked according to mean band density of each patient (Supplementary Table 4a), 576 577 and median value was calculated. Patients were classified as "LIP low" if LIP ubiquitination was higher or equal to the median value (n=5, i.e. patient 3, 4, 7, 8, 9), "LIP high" if LIP ubiquitination 578 was lower or equal to the median value (n=4, i.e. patient 1, 2, 5, 6). Progression free survival (panel 579 580 e) and overall survival (panel f) probability was calculated using the Kaplan-Meier method. LIP high vs. LIP low group:*p<0.03 (panel e); *p<0.01 (panel f). UQ-LIP: ubiquitinated LIP 581 Figure 2. LIP reconstitution induces apoptosis and rescues cisplatin-cytotoxicity in 582 mesothelioma 583 a. Immunoblot of the indicated proteins in extracts of MPM1 (epithelioid MPM) and MPM7 584 (sarcomatous MPM) cells, stably transfected with the doxycycline-inducible LIP-expression vector, 585 cultured in the absence (-, Ctrl) or presence (+) of cisplatin (Pt, 25 μ M) and doxycycline for 24 h. 586 The figure is representative of 1 out of 3 experiments. **b**. Cell cycle analysis of cells treated as in **a**, 587 performed by flow cytometry in duplicates. Pooled data of MPM 1-9 as means±SD (n=3). LIP-588

treated vs. Ctrl cells:*p<0.01; LIP+Pt-treated cells vs. Pt-treated cells:°p<0.001. **c.** Cell proliferation

in cultures treated 8 h after seeding (time "0" in the graph) as in **a**. Pooled data of MPM 1-9 as

591 means±SD (n=4). LIP-treated vs. Ctrl cells (72-96 h):*p<0.01; LIP+Pt-treated cells vs. Pt-treated

cells (72-96 h):°p<0.001. **d**. Representative photographs of cells stained with crystal violet (96 h;

left panel) and quantitation of crystal violet-stained cells, expressed as percentage of viable cells

compared to untreated cells (*right panel*). Data of MPM1 and MPM7 cells are presented as

 $means \pm SD (n=4). Pt-treated vs. Ctrl cells: *p<0.05; LIP-treated vs. Ctrl cells: *p<0.001; LIP/LIP+Pt-treated vs. Ctrl cells: *p<0.001; LIP/LIP+treated v$

treated cells vs. Pt-treated cells: $^{\circ}p<0.001$.

597 Figure 3. LIP restoration primes mesothelioma cells for immunogenic cell death

a. Immunoblot of C/EBP β LAP and LIP in whole cell lysates of MPM1 and MPM7 cells,

transfected with the inducible expression vector for LAP (*left panel*) or LIP (*right pane*), cultured in

600 the absence (-, Ctrl) or presence (+) of doxycycline (Doxy). The figure is representative of 1 out of

601 3 experiments. **b**. ChIP showing the binding of LAP or LIP to the calreticulin (*CRT*) promoter (site

602 831-843). Bl: blank; DNA input: genomic DNA. The figure is a representative of 1 out of 3

603 experiments. c. Levels of calreticulin (*CRT*) mRNA as determined by qRT-PCR in triplicates.

Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing cells vs. un-induced cells:*p<0.001.

605 **d.** Surface calreticulin (CRT) as detected by flow cytometry in duplicates. The histograms represent

the results obtained on MPM1 and MPM7. e. Phagocytic index of MPM cells phagocytized by DC,

as determined by flow cytometry. Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing

608 cells vs. un-induced cells:*p<0.001. **f.** Percentage of CD8⁺CD107⁺T-lymphocytes as determined by

flow cytometry in duplicates. Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing cells

610 vs. un-induced cells:*p<0.001. **g.** IFN-γ levels in the supernatant of CD8⁺CD107⁺T-cells, measured

in duplicates. Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing cells vs. un-induced
cells:*p<0.001.

613 Figure 4. LIP overcomes cisplatin-resistance and immune-resistance in vivo

a. 1×10^7 AB1 inducibly expressing LIP (clone 1, Supplementary Figure 3) were injected s.c. in 6-614 615 weeks-old female immune-competent or immune-deficient (nude) balb/C mice. When tumor reached the volume of 50 mm³, animals (n=10/group) were randomized and treated on days 1,7 and 616 14 as follows: 1) control (Ctrl) group, treated with 0.1 ml saline solution intravenously (i.v.); 2) LIP 617 group, treated with 1 mg/ml doxycycline in the drinking water to induce the LIP intratumorally; 3) 618 cisplatin (Pt) group, treated with 5 mg/kg cisplatin i.v.; 4) LIP+cisplatin (LIP+Pt) group, treated 619 with cisplatin and doxycycline to induce LIP. Data are means±SD. Pt/LIP+Pt groups vs. Ctrl group 620 (days 15-21:immune-competent mice, day 21:immune-deficient/nude mice):*p<0.001; LIP+Pt 621 group vs. Pt group (days 9-21:immune-competent and immune-deficient/nude mice):°p<0.001; 622 LIP+Pt group vs. LIP group (days 9-21: immune-competent and immune-deficient/nude mice):[#]p< 623 0.005; LIP+Pt group in immunocompetent vs. immune-deficient/nude mice: p<0.005 (not shown). 624 **b**. Immunoblotting of C/EBP_β LAP and LIP from tumor extracts, to check LIP induction in mice 625 626 treated with doxycycline. The figure is representative of extracts from two immune-deficient mice and two immune-competent mice. β-tubulin was used as control of equal protein loading. c. 627 628 Sections of tumors from each group of immune-competent animals, stained with hematoxylin and 629 eosin (HE; 20X objective; bar=100 µm), or immunostained with the indicated antibodies (63X objective; bar=10 µm). The photographs are representative of sections from 5 tumors/group. Data 630 are means±SD. Pt/LIP+Pt groups vs. Ctrl group:*p<0.005; LIP+Pt group vs. Pt group:°p<0.001; 631 LIP+Pt group vs. LIP group:[#]p<0.05. **d.** Percent of DC cells in tumors grown in immune-competent 632 mice, treated by Pt, doxycycline (LIP) or their combination. The percent of DC was determined in 633 duplicates by flow cytometry of single cell suspensions. e. Percent of CD3⁺CD8⁺T-lymphocytes 634 635 measured as in **d**. Data of panels **d** and **e** are means±SD. Pt/LIP+Pt groups vs. Ctrl group:*p<0.001; LIP+Pt group vs. Pt group:^op<0.001; LIP+Pt group vs. LIP group:[#]p<0.02. **f.** IFN-γ levels as 636 637 measured in duplicates in the supernatant of tumor-draining lymph nodes of immune-competent mice. Data of are means±SD. Pt/LIP+Pt groups vs. Ctrl group:*p<0.001; LIP+Pt group vs. Pt 638 group:°p<0.001; LIP+Pt group vs. LIP group:[#]p<0.05. 639

640 Figure 5. LIP undergoes proteasomal and lysosomal degradation in mesothelioma cells

a. Proteasome activity as analyzed in 3 primary HMC and 9 primary human MPM samples,

642 determined in duplicates by a chemiluminescence-based assay. Data are means±SD (n=3). MPM vs.

643 HMC:*p<0.001. **b.** Autophagy activity as measured spectrofluorimetrically in duplicates. Data are

644 means±SD (n=3). MPM vs HMC: *p<0.001. c. Lysosome activity as measured in duplicates by a

spectrophotometric assay. Data are means±SD (n=3). MPM vs HMC:*p<0.001. **d.** Immunoblot of

the indicated proteins in extracts of MPM1 and MPM7 cells, grown for 24 h in the absence (-), or

647 presence of the proteasome inhibitor carfilzomib (20 nM, Ca), the lysosome inhibitor chloroquine

 $(10 \ \mu M, Cq)$, or their combination, with or without, cisplatin (Pt, 25 μM), added for additional 24 h.

649 The effects induced by cisplatin alone are reported in Figure 2a. The figure is representative of 1 out

of 3 experiments.

Figure 6. Carfilzomib and chloroquine rescue cisplatin anti-tumor effects *in vitro* and *in vivo*

a. Growth curves of MPM cells, treated 8 h (time "0" in the graph) after seeding with fresh medium

653 (Ctrl), or media containing carfilzomib (20 nM, Ca), chloroquine (10 μM, Cq), or their

combination, without or with cisplatin (Pt, 25μ M). The effects induced by cisplatin alone are

reported in Figure 2c. Pooled data of MPM 1-9 as means±SD (n=4). All drug-treated cells vs. Ctrl

656 cells (72-96 h):*p<0.001; Ca+Cq+Pt cells vs. Ca+Cq cells (72-96 h):°p<0.001. **b**. Representative

657 photographs of cells stained with crystal violet (96 h). c. AB1 cells were injected s.c. in 6-weeks-old

growth in immune-competent balb/C mice (n=10/group). When tumor reached the volume of 50

 mm^3 , animals (n=10/group) were randomized and treated on days 1, 7 and 14 as follows: 1) control

660 (Ctrl) group, treated with 0.1 ml saline solution intravenously (i.v.); 2) carfilzomib (Ca) group,

treated with 5 mg/kg carfilzomib i.v.; 3) chloroquine (Cq), treated with 10 mg/kg chloroquine *per*

662 *os*; 4) carfilzomib+chloroquine (Ca+Cq) group, treated with both drugs; 5)

carfilzomib+chloroquine+cisplatin (Ca+Cq+Pt) group, treated with carfilzomib, chloroquine and 5

664 mg/kg cisplatin i.v. (24 h after carfilzomib+chloroquine). Data are means±SD. All drug-treated cells

vs. Ctrl group:*p<0.001; Ca+Cq+Pt group vs. Ca+Cq group:°p<0.001. **d.** Sections of tumors from

- each group, stained with hematoxylin and eosin (HE; 20X objective; bar=100 μ m), or
- immunostained with the indicated antibodies (63X objective; $bar=10 \mu m$). The percentage of
- 668 CHOP- and cleaved caspase 3-positive cells as determined by analyzing sections from 5 mice of
- each group (110-93 cells/field), using the Photoshop program. "Ctrl" group intensity was taken as
- 670 100%. Data are means±SD. Treated groups vs. Ctrl group:*p<0.002; Ca+Cq+Pt group vs. Ca+Cq
- group:°p<0.01. e. Percentage of infiltrating DC in tumors grown in mice that were treated as in d. f.
- Percentage of infiltrating $CD3^+CD8^+T$ -lymphocytes in these tumors. Data in panels **e** and **f**,
- determined in duplicates, are means±SD. All drug-treated groups vs. Ctrl group:*p< 0.001;
- 674 Ca+Cq+Pt group vs. Ca+Cq group: $^{\circ}p < 0.02$. **g.** IFN- γ levels in the supernatants of tumor-draining
- lymph nodes. Data determined in duplicates are means±SD. All drug-treated groups vs. Ctrl group:
- p < 0.001; Ca+Cq+Pt group vs. Ca+Cq group: p < 0.05.

1	Loss of C/EBP- β LIP drives cisplatin resistance in malignant pleural mesothelioma
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32

33 Abstract

34 **Objectives.** Cisplatin-based chemotherapy is moderately active in malignant pleural mesothelioma

35 (MPM) due to intrinsic drug resistance and to low immunogenicity of MPM cells. CAAT/enhancer

binding protein (C/EBP)- β LIP is a pro-apoptotic and chemosensitizing transcription factor

activated in response to endoplasmic reticulum (ER) stress.

38 Materials and methods. We investigated if LIP levels can predict the clinical response to cisplatin

39 and survival of MPM patients receiving cisplatin-based chemotherapy. We studied the LIP-

40 dependent mechanisms determining cisplatin-resistance and we identified pharmacological

41 approaches targeting LIP, able to restore cisplatin sensitiveness, in patient-derived MPM cells and

42 animal models. Results were analyzed by a one-way analysis of variance test.

43 **Results.** We found that LIP was degraded by constitutive ubiquitination in primary MPM cells

44 derived from patients poorly responsive to cisplatin. LIP ubiquitination was directly correlated with

45 cisplatin chemosensitivity and was associated with patients' survival after chemotherapy.

46 Overexpression of LIP restored cisplatin's pro-apoptotic effect by activating CHOP/TRB3/caspase

47 3 axis and up-regulating calreticulin, that triggers MPM cell phagocytosis by dendritic cells and

48 expanded autologous anti-tumor CD8⁺CD107⁺T-cytotoxic lymphocytes.

49 Proteasome inhibitor carfilzomib and lysosome inhibitor chloroquine prevented LIP degradation.

50 The triple combination of carfilzomib, chloroquine and cisplatin increased ER stress-triggered

51	apoptosis and immunogenic cell death in patients' samples, and reduced tumor growth in cisplatin-
52	resistant MPM preclinical models.
53	Conclusion. The loss of LIP mediates cisplatin resistance, rendering LIP a possible predictor of
54	cisplatin response in MPM patients. The association of proteasome and lysosome inhibitors reverses
55	cisplatin resistance by restoring LIP levels and may represent a new adjuvant strategy in MPM
56	treatment.
57	
58	Highlights
59	- C/EBP- β LIP loss correlates with poor response to cisplatin in mesothelioma patients
60	- C/EBP- β LIP loss is due to its constitutive ubiquitination and degradation
61	- Lack of C/EBP- β LIP prevents cisplatin-induced apoptosis and immunogenic cell death
62	- Proteasome and lysosome inhibitors restoring C/EBP- β LIP rescue cisplatin efficacy
63	
64	Keywords: malignant pleural mesothelioma; CAAT/enhancer binding protein; cisplatin resistance;
65	endoplasmic reticulum stress; immunogenic cell death
66	
67	Abbreviations: ANOVA, analysis of variance; ATF6, activating transcription factor 6; C/EBP,
68	CAAT/enhancer binding protein; IRE1, inositol-requiring enzyme 1; CHOP/GADD153; C/EBP
69	homologous protein/growth arrest/DNA damage inducible 153; CEA, carcino-embrionic antigen;
70	ChIP, chromatin immunoprecipitation; DC, dendritic cells; ER, endoplasmic reticulum; EMA,
71	epithelial membrane antigen; EIF2AK3/PERK, eukaryotic translation initiation factor- 2α kinase 3;
72	FBS, fetal bovine serum; ICD, immunogenic cell death; HMC, human mesothelial cells; MPM,
73	malignant pleural mesothelioma; OS: overall survival; qRT-PCR, quantitative Real Time-PCR;
74	PFS: progression free survival; SPSS, Statistical Package for Social Science; TBS, Tris-buffered

- saline; TRB3, *tribbles*-related protein 3; UPN, Unknown Patient Number; WT1, Wilms tumor-1
- 76 antigen
- 77

78 **1. Introduction**

79 Malignant pleural mesothelioma (MPM), an asbestos-related cancer, is usually diagnosed at advanced stage, when chemotherapy - based on cisplatin associated with pemetrexed or raltitrexed -80 is the only available therapeutic option. [1,2]. The success of chemotherapy is limited by the 81 intrinsic chemoresistance [1] and immune-evasive nature of MPM. Such immune-evasive 82 83 microenvironment in MPM relies on the presence of immune-suppressive/immune-tolerant cells in 84 the MPM [3, 4], on the high levels of immune-suppressive immune-checkpoints on both MPM cells and surrounding T-lymphocytes [5, 6], on the low amount of tumor-associated antigens of MPM 85 cells [7], due to its low mutational burden [8]. 86 87 In sensitive cells, cisplatin induces DNA damage, hampers DNA repair [9] and elicits nuclearindependent effects, such as dispersal of Golgi apparatus [10] and apoptosis induced by 88 endoplasmic reticulum (ER) stress [11]. Upon ER stress, cancer cells expose on their surface the 89 90 "eat-me" signal calreticulin, leading to dendritic cells (DC)-mediated phagocytosis and activation of autologous anti-tumor cytotoxic CD8⁺T-lymphocytes [12]. This process is known as immunogenic 91 92 cell death (ICD) [12]. MPM cells however do not translocate calreticulin from ER to surface [13, 93 14], resulting ICD-refractory. This is an additional mechanism explaining the low immunogenicity of MPM cell. 94 95 Solid tumors respond to chemotherapy-induced ER stress by activating adaptation and survival

pathways if the stress is limited, or pro-apoptotic pathways if the stress persists [15, 16]. The ER 96 stress-induced transcription factor CAAT/enhancer binding protein (C/EBP)-β is involved in both 97 98 responses. At the early ER stress phase, the pro-survival isoform C/EBP- β LAP is produced. Upon prolonged ER stress, the isoform C/EBP-β LIP (LIP) is formed and activates C/EBP homologous 99 protein/growth arrest/DNA damage inducible 153 (CHOP/GADD153) protein, which promotes 100 apoptosis by activating tribbles-related protein 3 (TRB3) and caspase 3 [17-20]. At the present there 101 are no data available about gene alterations (mutation, amplification or deletion) in the 87 MPM 102 evaluated by the Tissue Cancer Genome Atlas (https://cancergenome.nih.gov), nor about the 103

104 expression of C/EBP-β LAP/LIP isoforms in MPM, according to Protein Tissue Atlas

105 (<u>http://www.proteinatlas.org</u>).

We recently reported that chemoresistant tumors lack LIP, because of its constitutive ubiquitination.
LIP loss mediates chemoresistance by increasing the expression of the drug efflux transporter Pglycoprotein and by preventing the ER stress-dependent pro-apoptotic response [12].

Here, we investigated if LIP mediates the resistance to cisplatin in MPM. We identified clinically
feasible pharmacological strategies that restore the sensitivity of MPM to cisplatin by preventing

111 LIP degradation.

112 **2. Materials and methods**

2.1.Chemicals. Cell culture plastic ware were obtained from Falcon (Becton Dickinson, Franklin

114 Lakes, NJ). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA).

115 Carfilzomib was purchased from Biorbyt Ltd. (Cambridge, UK) and torin 1 was from Selleckchem

116 (Munich, Germany). The protease inhibitor cocktail set III was obtained from Millipore (Billerica,

MA). Unless specified otherwise, all reagents were purchased from Sigma Chemicals Co (St. Louis,MO).

119 2.1.Cells. Primary human mesothelial cells (HMC) were isolated from three patients with pleural fluid secondary to congestive heart failure, with no history of a malignant disease. Nine primary 120 human MPM samples (3 epithelioid MPM, 3 biphasic MPM, 3 sarcomatous MPM) were obtained 121 from diagnostic thoracoscopies. Histological and clinical features are shown in Supplementary 122 Tables 1-2. All patients, identified with Unknown Patient Numbers (UPN), received 5 cycles of 123 cisplatin 75 mg/m² every 21 days. Tissue was digested in medium containing 1 mg/ml collagenase 124 and 0.2 mg/ml hyaluronidase for 1 h at 37°C. Cells were seeded in culture and used within passage 125 6. The Ethical Committee of Biological Bank of Mesothelioma, S. Antonio e Biagio Hospital, 126 Alessandria, Italy, and San Luigi Gonzaga Hospital, Orbassano, Italy, approved the study 127 (#9/11/2011; #126/2016). Murine AB1 cells were purchased from Sigma Chemicals Co. Cells were 128

grown in Ham's F10 nutrient mixture medium (primary HMC/MPM cells) or DMEM (AB1 cells),
supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin-streptomycin.

131 2.3.Quantitative Real Time-PCR (qRT-PCR). RNA was extracted and reverse-transcribed using the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories). gRT-PCR was performed using IQTM 132 SYBR Green Supermix (Bio-Rad Laboratories). The same cDNA preparation was used for 133 measuring genes of interest and the housekeeping gene S14. Primer sequences were designed using 134 qPrimerDepot software (http://primerdepot.nci.nih.gov/). Relative gene expression levels were 135 136 calculated using Gene Expression Quantitation software (Bio-Rad Laboratories). 2.4.Immunoblotting. Protein or tumor extracts (20 µg) were subjected to SDS-PAGE and probed 137 with the following antibodies: C/EBP-β (directed against the common C-terminus of LIP and LAP, 138 139 Santa Cruz Biotechnology Inc., Santa Cruz, CA), CHOP/GADD153 (Abcam, Cambridge, UK), TRB3 (Proteintech, Chicago, IL), caspase-3 (GeneTex, Hsinhu City, Taiwan), β-tubulin (Santa 140 Cruz Biotechnology Inc.). To detect ubiquitinated C/EBP-β, 100 μg protein extracts were immuno-141 precipitated overnight with the anti-C/EBP-β antibody, using 25 μl of PureProteome Magnetic 142 Beads (Millipore). Immunoprecipitated samples were then probed with an anti-mono/polyubiquitin 143 antibody (Axxora). Blotting was followed by the peroxidase-conjugated secondary antibody. The 144 membranes were washed with Tris-buffered saline/Tween 0.01% v/v and proteins were detected by 145 enhanced chemiluminescence. Band density was calculated using ImageJ software 146 (http://www.rsb.info.nih.gov/ij/). 147 **2.5.Cell viability and growth**. Cell viability with neutral red staining and crystal violet staining 148 were performed as reported [20]. IC_{50} was calculated with the CompuSyn software 149 150 (http://www.combosyn.com). Quantitation of crystal violet staining was performed by dissolving

crystal violet with 1% v/v acetic acid and reading the absorbance of each well at 570 nm (HT

152 Synergy 96-well microplate reader, Bio-Tek Instruments, Winoosky, VT). The mean absorbance of

untreated cells was considered 100%; the absorbance units of the other experimental conditionswere expressed as percentage towards untreated cells.

2.6.Cell cycle analysis. 1×10^4 cells were harvested, washed with PBS, treated with 0.25 mg/ml RNAse and stained for 15 min with 50 µg/ml propidium iodide. Cell cycle distribution was analyzed by Guava® easyCyte flow cytometer (Millipore, Billerica, MA), using the InCyte software (Millipore).

2.7.Over-expression of C/EBP-\beta LAP and LIP. The pcDNA4/TO expression vectors (Invitrogen Life Technologies, Milan, Italy) for LAP and LIP, produced as reported previously [17], were cotransduced with pcDNA6/TR vector (Invitrogen Life Technologies) in parental cells. Stable TetON clones were generated by selecting cells with 2 µg/ml blasticidin S (Invitrogen Life Technologies) and 100 µg/ml zeocin (InvivoGen, San Diego, CA). LAP and LIP induction was activated by adding 1 µg/ml doxycycline in the culture medium.

2.8.C/EBP- β LIP silencing. 2×10⁶ cells in 0.25 ml serum/antibiotic-free medium were transfected either with non-targeting scrambled siRNA pools or siRNA pools specifically targeting LIP

167 sequence (customized ON-TARGETplus, Dharmacon RNAi Technologies; Dharmacon, Lafayette,

168 CO), employing DharmaFECT 1 reagent (Dharmacon), as per manufacturer's protocol.

2.9.Chromatin immunoprecipitation (ChIP). To determine the binding of LAP and LIP to

170 calreticulin promoter we performed ChIP as described [21], using the anti-C/EBP- β antibody

171 directed against the common C-terminus of LAP and LIP. Putative binding sites of C/EBP- β were

identified using the Gene Promoter Miner software (<u>http://gpminer.mbc.nctu.edu.tw/</u>). PCR primers

173 were designed using Primer3 software (<u>http://primer3.ut.ee/</u>).

2.10.Calreticulin expression, phagocytosi and T-lymphocyte activation. Surface calreticulin was
 measured by flow cytometry as reported [22]. DC were generated from peripheral blood samples of
 patients, collected before starting chemotherapy, as previously reported [13]. Phagocytosis assays

were performed as detailed in [22]. Active anti-tumor cytotoxic CD8⁺CD107⁺T-lymphocytes,

obtained from autologous T-lymphocytes (co-cultured 10 days with DC after phagocytosis) and isolated with the Pan T Cell Isolation Kit (Miltenyi Biotec., Bergisch Gladbach, Germany), were measured by flow cytometry [13]. The production of IFN- γ in the culture supernatant of CD8⁺Tcells co-cultured with DC or in the supernatant of tumor-draining lymph nodes - a second parameter of CD8⁺T-cells cytotoxic activity - was measured with the Human IFN- γ DuoSet Development Kit (R&D Systems, Minneapolis, MN).

2.11.*In vivo* tumor growth. 1×10^7 AB1 cells expressing LIP upon doxycycline administration in 184 drinking water, mixed with 100 µl Matrigel, were injected subcutaneously (s.c.) in 6-weeks-old 185 female immune-competent or immune-deficient (nude) balb/C mice (Charles River Laboratories 186 187 Italia, Calco), housed (5 per cage) under 12 h light/dark cycle, with food and drinking provided ad *libitum*. Tumor growth was measured daily by caliper, according to the equation $(LxW^2)/2$, where 188 L=tumor length and W=tumor width. When tumor reached the volume of 50 mm³, animals were 189 190 randomized and treated as reported in the Figure Legends. Tumor volumes were monitored by caliper and animals were euthanized at day 21 after randomization with zolazepam (0.2 ml/kg) and 191 192 xylazine (16 mg/kg). The hemocromocytometric analyses were performed with a UniCel DxH 800 Coulter Cellular Analysis System (Beckman Coulter, Miami, FL) on blood collected immediately 193 after sacrificing the mice. Hematochemical parameters were analyzed using the respective kits from 194 195 Beckman Coulter Inc. Animal care and experimental procedures, according to EU Directive 2010/63, were approved by the Bio-Ethical Committee of the Italian Ministry of Health 196 (#122/2015-PR). 197

2.12.Immunohistochemistry and intratumor immune infiltrate analysis. Tumors were resected
and fixed in 4% v/v paraformaldehyde, stained with hematoxylin/eosin or immunostained for
CHOP or cleaved(Asp175)-caspase 3 (Cell Signaling Technology Inc., Danvers, MA), followed by
a peroxidase-conjugated secondary antibody. Nuclei were counter-stained with hematoxylin.
Sections were examined with a Leica DC100 microscope. Excised tumors were digested with 1
mg/ml collagenase and 0.2 mg/ml hyaluronidase (1h at 37°C) and filtered using a 70 µm cell

strainer to obtain a single cell suspension. Infiltrating immune cells were collected by centrifugation
on Ficoll-Hypaque density gradient and subjected to immune phenotyping by flow cytometry, using
antibodies against CD11c for DC, CD3 and CD8 for T-lymphocytes (Miltenyi Biotec.). Draining
lymph nodes were collected, homogenized for 30 s at 15 Hz, using a TissueLyser II device (Qiagen,
Hilden, Germany) and centrifuged at 12000 x g for 5 minutes. The supernatant was used to measure
the amount of IFN-γ.

2.13.Proteasome, autophagy and lysosome activity. Proteasome activity was measured with the
Proteasome-GloTM Cell-Based Assays (Promega Corporation). Autophagy activity was measured
using the Autophagy Assay Kit (Sigma Chemicals Co.). The activity of cathepsin L, taken as an
index of lysosome activity, was measured as reported [23].

214 **2.14.Statistical analysis.** All data in the text and figures are provided as means±SD. The results 215 were analysed by a one-way analysis of variance (ANOVA), using Statistical Package for Social 216 Science (SPSS) software (IBM SPSS Statistics v.19). p<0.05 was considered significant. Overall 217 survival was defined as the time passed from the starting of cisplatin therapy to the date of death (all 218 patients were dead at the time of analysis). The Kaplan-Meier method was used to calculate overall 219 survival. Log rank test was used to compare the outcome of the two groups. The sample size was 220 calculated with the G*Power software (www.gpower.hhu.de), setting $\alpha \leq 0.05$ and $1-\beta=0.80$.

221 **3. Results**

3.1.LIP is constitutively ubiquitinated in mesothelioma and correlates with cisplatin resistance

223 $C/EBP-\beta$ mRNA was equally expressed in primary non-transformed HMC and MPM cells (Figure

1a). By contrast, C/EBP- β LAP protein was detected in both HMC and MPM samples, LIP was

detectable only in HMC. The absence or very low expression of LIP in MPM was due to its higher

226 ubiquitination: lower was the level of LIP in MPM (upper panel, Figure 1b), higher was LIP

227 ubiquitination (*middle panel*, Figure 1b; Figure 1c), suggesting that LIP ubiquitination can be

228 paralleled by its degradation, as it occurs for most ubiquitinated proteins [24] All MPM samples

were significantly more resistant to cisplatin *in vitro* compared to HMC (Supplementary Table 3).

Ubiquitinated LIP was directly correlated with the IC_{50} of cisplatin (Figure 1d). LIP ubiquitination 230 was also significantly associated with patients' progression free survival (PFS; Figure 1e) and 231 overall survival (OS; Figure 1f) after cisplatin therapy: median PFS was 2.2 months, median OS 232 was 9 months (Supplementary Table 2) in the top 5 patients (UPN 3, 4, 7, 8, 9) with highest LIP 233 ubiquitination (LIP ubiquitination higher or equal to median value, Supplementary Table 4a). This 234 group was called "LIP low group" in Figure 1e-f, since patient-derived cell had lower levels of LIP 235 protein (Figure 1b, *upper panel*). Median PFS and OS were 5.3 and 16 months respectively 236 (Supplementary Table 2) in the top 4 patients (UPN 1, 2, 5, 6) with lowest LIP ubiquitination (LIP 237 ubiquitination lower or equal to median value, Supplementary Table 4a). This group was defined as 238 239 "LIP high group" in Figure 1e-f, since patient-derived cells had higher levels of LIP protein (Figure 1b, *upper panel*). 240 **3.2.Reconstitution of LIP restores cisplatin-induced cell death by activating ER stress-**241 242 mediated apoptosis To investigate whether there was a causal relationship between the loss of LIP and the resistance to 243 cisplatin, we induced overexpression of exogenous LIP in MPM cells (Figure 2a). LIP transduced 244 cells were more sensitive to cisplatin (Supplementary Table 5), activated the pro-apoptotic 245 CHOP/TRB3/caspase 3 axis (Figure 2a), increased the percentage of sub-G1 apoptotic cells, 246 247 reduced the percentage of cells entering the S-phase (Figure 2b), decreased cell proliferation (Figure 2c-d). 248 Cisplatin did not activate the LIP/CHOP/TRB3/caspase 3 axis, neither affected cell cycle, nor 249 proliferation, in non-transduced MPM cells, whereas LIP induction restored all these events. The 250 combination "LIP induction+cisplatin treatment" was superior to LIP induction only (Figure 2a-d). 251 Although cisplatin slightly increased LIP amount, it did not change the basal rate of LIP 252 ubiquitination, meaning that the induced LIP was ubiquitinated (Supplementary Figure 1a; 253 Supplementary Table 4b) and subsequently degraded by the cell. The ratio ubiquitinated LIP/total 254 LIP, calculated by densitometric analysis, was unchanged compared to untreated cells 255 11

- 256 (Supplementary Figure 1b). Also, in LIP-transduced cells, LIP protein underwent ubiquitination
- 257 (Supplementary Figure 1a; Supplementary Table 4b). However, the ratio ubiquitinated LIP/total
- 258 LIP was decreased in this case (Supplementary Figure 1b).

259 **3.3.LIP triggers immunogenic cell death in mesothelioma cells**

- As observed above, MPM cells are refractory to ICD mediated by calreticulin [13, 14]. Two
- 261 predicted binding sites for C/EBP transcription factors are present in calreticulin promoter at
- position 831-843 and 1302-1313 (Supplementary Figure 2a). The former one contains a CCAAT
- box motif (Supplementary Figure 2b). To investigate whether C/EBP- β interacts with calreticulin
- promoter, we used MPM clones constitutively overexpressing LAP or LIP (Figure 3a). Of note, LIP
- bound the 831-843 site (Figure 3b). LIP-transduced cells increased calreticulin mRNA and surface
- 266 protein (Figure 3c-d), were easily phagocytized by DC (Figure 3e), expanded autologous activated
- 267 $CD8^+ CD107^+ T$ -lymphocytes producing IFN- γ (Figures 3f-g). By contrast, LAP overexpressing
- cells did not differ from non-transduced MPM samples (Figure 3c-g).
- As a proof of concept of the role of LIP in these process, we silenced the exogenously expressed
- 270 LIP in MPM cells (Supplementary Figure 3a). In LIP-silenced cells calreticulin expression,
- 271 phagocytosis and antitumor cytotoxic T-lymphocyte expansion were abrogated (Supplementary
- Figures 3b-d), demonstrating the critical role of LIP as calreticulin inducer and ICD effector in
- 273 MPM.

274 3.4.LIP expression restores sensitivity to cisplatin and immune-mediated death in vivo

To validate the effects of LIP overexpression *in vivo*, we produced doxycycline-inducible LIP clones from AB1 cells (Supplementary Figure 4), a murine mesothelioma cell line syngeneic with balb/C mice. IC₅₀ of cisplatin was $91.08 \pm 17.18 \mu$ M in un-induced AB1 cells, in line with the most cisplatin-resistant human MPM cells (Supplementary Table 3). Accordingly, AB1-tumors were unresponsive to cisplatin when implanted in mice (Figure 4a). Upon induction of LIP (Figure 4b), the tumor growth was reduced in both immune-deficient and immune-competent mice (Figure 4a) and showed increased percentage of tumor cells positive for CHOP and cleaved caspase 3 (Figure 3c), in particular in LIP-induced/cisplatin-treated mice. The antitumor effects of LIP + cisplatin were stronger in immune-competent mice, at least during the early development of MPM (Figure 4a), suggesting that immune system activation plays a significant role in delaying MPM growth. In immune-competent mice, LIP induction increased intratumor infiltrating DC and CD8⁺ Tlymphocytes, and production of IFN- γ in draining lymph nodes (Figure 4d-f). Cisplatin enhanced

all these events in LIP-expressing tumors (Figure 4d-f).

288 **3.5.LIP undergoes proteasomal and lysosomal degradation**

289 Since LIP was mono- and poly-ubiquitinated in MPM samples (Figure 1a), we searched which

290 mechanisms are involved in LIP degradation. Compared to HMC, MPM cells displayed increased

proteasome (Figure 5a), autophagy (Figure 5b) and lysosome activity (Figure 5c). Treatment of

292 MPM cells with the proteasome inhibitor carfilzomib and the lysosome inhibitor chloroquine

lowered the proteasome and lysosome activity of MPM to values comparable to HMC

294 (Supplementary Figure 5a-c). Either carfilzomib or chloroquine prevented LIP degradation and

activated the downstream effectors CHOP, TRB3 and caspase 3 (Figure 5d). Chloroquine is an

inhibitor of autophagy and lysosomes [25, 26] (Supplementary Figure 5b-c). To better understand if

both mechanisms are equally involved in LIP degradation, we used the autophagy inhibitor 3-

298 methyladenine, which does not affect lysosome activity [25] (Supplementary Figure 5b-c). 3-

299 methyladenine prevented LIP degradation and activated the CHOP/TRB3/caspase 3 axis

300 (Supplementary Figure 6) at a lesser extent than chloroquine (Figure 5d), suggesting that autophagy

301 likely plays an ancillary role in the removal of LIP.

302 The combination of carfilzomib and chloroquine was even more effective than each agent alone in

activating the LIP/CHOP/TRB3/caspase 3 pathway (Figure 5d). The treatment with carfilzomib and

304 chloroquine, alone or in combination, increased the accumulation of ubiquitinated LIP compared to

305 untreated cells (Supplementary Figure 7a; Supplementary Table 4c). The same increase in

306 ubiquitinated LIP was detected in cells treated with the triple combination carfilzomib +

307 chloroquine + cisplatin. The ratio between ubiquitinated LIP/total LIP was lowered in carfilzomib-

and chloroquine-treated cells, in particular in the combination treatments carfilzomib + chloroquine
 or carfilzomib + chloroquine + cisplatin (Supplementary Figure 7b).

Carfilzomib and chloroquine combination was also the most effective in inducing ICD-related parameters (Supplementary Figure 8a-e). The immunologic effects of carfilzomib and chloroquine were likely due to the attenuated degradation of LIP, as demonstrated by the absence of calreticulin up-regulation in MPM cells lacking LIP (Supplementary Figure 8f). The triple combination of cisplatin with carfilzomib and chloroquine further activated LIP/CHOP/TRIB3/caspase 3 pathway

and ICD (Figure 5d, Supplementary Figure 6a-e).

316 3.6.Combination of carfilzomib, chloroquine and cisplatin abrogates mesothelioma growth

317 Combined treatment of carfilzomib and chloroquine greatly reduced MPM cell proliferation (Figure

6a-b) and tumor growth *in vivo* (Figure 6c), in particular if associated with cisplatin. This triple

319 combination significantly increased the number of CHOP- and caspase 3-positive intratumor cells

320 (Figure 6d), and raised an anti-tumor immune response *in vivo*, as demonstrated by the increased

tumor-infiltrating DC and CD8⁺ T-lymphocytes (Figure 6e-f), and by the increased production of

322 IFN- γ in the draining lymph nodes (Figure 6g). Neither significant alterations in

323 hemocromocytometric parameters nor signs of liver, kidney, heart and muscle toxicity were

detectable in animals treated with this regimen (Supplementary Table 6).

325 4. Discussion

326 Aim of this study was to investigate the possible role of LIP as effector of resistance to cisplatin,

predictive factor of patient response to cisplatin, and possible target to improve the sensitivity tothis drug in MPM.

329 Using cells isolated from MPM patients treated with cisplatin but characterized by low response to

the drug (mean survival after the beginning of therapy: 13.11 months), we noticed that they had

lower amount of LIP compared to HMC cells and higher rate of LIP ubiquitination. Ubiquitination

of LIP is a typical sign of its degradation *via* proteasome and/or lysosome [18, 20, 27]. This is

consistent with the observation that in all MPM cells when ubiquitination of LIP was higher, the

protein was lower in comparison with HMC that had lower LIP ubiquitination and higher LIP 334 335 protein level. The rate of LIP ubiquitination strongly correlated with the *in vitro* resistance to cisplatin, measured as IC₅₀ of the drug: indeed, higher was LIP ubiquitination (i.e. lower LIP 336 protein was), higher was IC_{50} of cisplatin. Hence, we concluded that LIP loss due to its 337 ubiquitination was related to resistance to cisplatin in vitro. We considered the PFS, defined as the 338 survival with stable disease from the beginning of cisplatin therapy, and the OS of each patient, 339 340 defined as the time passed from the starting of cisplatin therapy to the date of patient death. Patients with LIP ubiquitination higher or equal to median value were included in the so-called "LIP low" 341 group, since the patient-derived cell line had very low/undetectable LIP protein. Patients with LIP 342 343 ubiquitination lower or equal to median value were included in the so-called "LIP high" group, since the patient-derived cell line had higher LIP protein. "High LIP ubiquitination/LIP low" 344 patients had the shortest PFS and OS, patients with "low LIP ubiquitination/LIP high" patients 345 346 displayed the most prolonged PFS and OS after cisplatin treatment. Although the small number of patients and the absence of a control group receiving different 347 348 treatments makes difficult to separate the prognostic and the predictive role of LIP ubiquitination, 349 our work provides the rationale to measure LIP ubiquitination in larger series of MPM tumors, in order the evaluate if this parameter - beside being a good indicator of the sensitivity to cisplatin in 350 351 *vitro* - may be also predictive of the clinical response to cisplatin. In chemoresistant tumor cells, the loss of LIP upregulates the expression of the drug efflux 352 transporter P-glycoprotein [20], increasing the resistance of such cells to a broad spectrum of 353 354 chemotherapeutic drugs. Platinum-derived agents, however, are not substrates of P-glycoprotein [28]. Therefore, the restoration of drug sensitivity by LIP, after its genetic over-expression or 355 pharmacological inhibition of its degradation, must rely on a different mechanism. 356 357 Cisplatin triggers ER stress-dependent apoptosis [11], but MPM cells were refractory to these events. Our findings in primary MPM samples and MPM preclinical models suggests that cisplatin 358

induces apoptosis via the ER-dependent CHOP/TRB3/caspase 3 axis only in the presence of LIP,

while LIP loss abrogated the cell death due to cisplatin-triggered ER stress. In cisplatin-treated 360 361 cells, the ratio between ubiquitinated LIP/total LIP was unchanged compared to untreated cells: the continuous ubiquitination of LIP likely explains the absence of any pro-apoptotic effect induced by 362 the drug in MPM cells. By contrast, the ubiquitinated LIP/total LIP ratio was decreased in LIP-363 transduced cells, suggesting that the excess of exogenous LIP may saturate the maximal 364 ubiquitination capacity of MPM cells. In these conditions, a significant amount of LIP remained 365 not-ubiquitinated and likely triggered the pro-apoptotic CHOP/TRB3/caspase 3 axis. This effect 366 was particularly pronounced in cells treated with cisplatin that slightly increased the amount of 367 endogenous LIP. The sum of endogenous LIP induced by cisplatin and exogenous LIP produced by 368 369 the transfection with a LIP-overexpressing vector produced a huge increase in not-ubiquitinated LIP, allowing the induction of LIP-triggered dependent apoptosis. 370 However, our finding that LIP induction attenuated tumor growth in mice even without cisplatin 371 372 indicates that LIP regulates additional mechanisms on top of ER stress. One such mechanism involves the anti-tumor immune response. Indeed, inducible intra-tumor 373 374 activation of LIP delayed tumor growth in immune-competent animals more than in immune-375 deficient mice, indicating that LIP attenuates MPM progression enhancing the anti-tumor immune response. Intriguingly, the combination cisplatin+LIP was even more effective than LIP induction. 376 377 In tumor extracts, cisplatin produced a small activation of LIP, without however reducing tumor growth. Since ER stress often occurs in tumor bulk as a consequence of hypoxia or nutrient 378 shortage [16] and these conditions are sufficient to induce LIP activation [17], it is likely that the 379 380 ER stress exerted by the tumor environment and the low ER stress elicited by cisplatin produced the small induction of LIP. Such induction, however, was insufficient to trigger ER-dependent 381 apoptosis, as demonstrated by the absent intratumor activation of CHOP and caspase 3. Previously, 382 cisplatin was not thought to trigger anti-tumor immune response [22, 29]. However, a recent report 383 revealed that in non-small cell lung cancer, cisplatin increased intratumor DC recruitment, tumor 384 cell phagocytosis and expansion of anti-tumor CD8⁺ T-lymphocytes [30]. Intratumor recruitment of 385

386 DC is mediated by cell surface expression of calreticulin [31]. ER stress (and thereby activation of 387 LIP) is necessary for triggering immune responses by cisplatin [32]. Our work demonstrates that 388 LIP induced calreticulin expression and the subsequent chain of immune responses, providing a 389 plausible mechanism by which LIP triggers an anti-tumor immune response and rescues the activity 390 of cisplatin via ER stress-dependent apoptosis and ICD.

Endogenous LIP is physiologically removed by proteasome and lysosomes [20]. Interestingly, a

high activity of proteasome and lysosome correlates with bad patient prognosis in MPM [33, 34].

393 The inhibition of one of these two degradation pathways may be compensated by the increase in the

other pathway, suggesting that only a simultaneous blockade of the two is required for restoring

395 LIP-triggered apoptosis in MPM cells. To achieve this goal, we employed the FDA-approved

396 proteasome inhibitor carfilzomib and the lysosome inhibitor chloroquine.

397 Bortezomib, another proteasome inhibitor, is known to induce apoptosis in MPM cells synergizing 398 with cisplatin and oxaliplatin if administered before platinum-based agents [33, 35]. Our work provides the rationale for this observation, suggesting that this synergy may be reached only after 399 400 the bortezomib-induced accumulation of LIP, which, in turn, enhanced cisplatin cytotoxicity. 401 Also chloroquine induces apoptosis of MPM cells if associated with nutritional stress [36] or PI3K/mTOR inhibition [20], two conditions that induce ER stress [15, 16, 37], suggesting that at 402 403 least part of the pro-apoptotic effect of chloroquine is triggered by ER dysfunctions. Since carfilzomib and chloroquine act downstream the ubiquitination system and prevent the degradation 404 of ubiquitinated proteins by proteasome and lysosomes [24], these drugs may produce an "action 405 mass"-like effect: by blocking the degradation of ubiquitinated LIP via proteasome and lysosomes, 406 407 carfilzomib and chloroquine increased the amount of ubiquitinated LIP to a level saturating the ubiquitination capacity of MPM cells. This saturation ultimately resulted in the accumulation of 408 409 not-ubiquitinated LIP that triggered the CHOP/TRIB3/caspase 3 pro-apoptotic pathway. In addition to ER stress-linked pro-apoptotic mechanisms, carfilzomib and chloroquine are known 410

411 ICD inducers [12, 38].

412	Until now, synergistic anti-tumor effects exerted by combining proteasome and lysosome inhibitors
413	with platinum derivatives [33, 35, 39] were reported only in vitro. Our study is the first one
414	demonstrating that the combination treatment of carfilzomib, chloroquine and cisplatin is effective
415	in preclinical models of cisplatin-resistant MPM, by inducing ER stress-dependent apoptosis and
416	activating the host immune system against MPM. Such triple combination did not elicit detectable
417	signs of systemic toxicity in vivo. Moreover, each agent used in our preclinical model has well-
418	known pharmacodynamic, pharmacokinetic and toxicological profiles for the extensive clinical use.
419	These considerations are encouraging in the perspective of translating the triple combination
420	cisplatin, carfilzomib and chloroquine to clinical settings.
421	Conclusions
422	Our work demonstrates that LIP levels strongly correlated with MPM response to cisplatin <i>in vitro</i> ,
423	providing the preliminary data to evaluate if the correlation between high LIP ubiquitination and
424	low survival is valid in larger patients cohort. If the data reported in this study was confirmed, LIP
425	ubiquitination may represent a factor predictive of clinical response to cisplatin. We suggest to
426	include pharmacological inhibitors of LIP degradation, such as a combination of proteasome and
427	lysosome inhibitors, in the number of strategies under evaluation to improve the response of MPM
428	to the current treatment.
429	
430	Data availability
431	All data generated or analysed during this study are included in this published article and its
432	supplementary information files.
433	
434	Conflict of interests
435	None.
436	
437	Funding

- 438 This work was supported by Italian Association for Cancer Research (IG15232 to CR), Italian
- 439 Ministry of University and Research (EX60% Funding 2015 to SN and CR); Italian Ministry of
- 440 Health (to LR and GVS); De Benedetti-Cherasco Foundation (Torino-Weizmann Collaborative
- 441 Program: Scientific Cooperation and Exchange to MR and CR); Fondazione Cassa di Risparmio di
- 442 Torino (to CR). ICS is recipient of PhD scholarships from the Italian Institute for Social Security
- 443 (INPS). VM is a PhD fellow of Erasmus Mundus-ERAWEB Action 2 program.
- 444 The funding institutions had no role in the study design, data collection and analysis, or in writing445 the manuscript.
- 446

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554 Figure legends

555 Figure 1. Ubiquitination of LIP correlates with cisplatin resistance in mesothelioma

- **a.** Levels of $C/EBP\beta$ mRNA as determined by qRT-PCR in triplicates, in 3 primary human non-
- transformed mesothelial cells (HMC) and 9 primary human malignant pleural mesothelioma
- 558 (MPM) samples. Epi: epithelioid; Bip: biphasic; Sar: sarcomatous. b. Expression and ubiquitination
- of C/EBP-β LAP and LIP in HMC and MPM samples. Whole cell lysate was immunoprecipitated
- 560 (IP) with anti C/EBP- β antibody that recognize the common C-terminus C/EBP- β i.e. either LIP or
- 561 LAP and the blot was probed (IB) with the anti C/EBP- β antibody, to detect total levels of C/EBP-
- 562 β LAP and LIP isoforms, or with an anti-mono/poly-ubiquitin antibody, to detect the corresponding

563	ubiquitinated forms. Upper-middle panels, last lane: control immunoblot of MPM1 cell extract
564	immunoprecipitated in the absence of antibody. Arrow, <i>middle panel</i> : ubiquitinated LIP, according
565	to the expected molecular weight. Before immunoprecipitation, an aliquot of cell lysate was probed
566	with an anti- β -tubulin antibody, to check that equal amounts of proteins from each extract were
567	loaded in immunoprecipitation. The figure is representative of 1 out of 3 experiments. c. The mean
568	band density of ubiquitinated LIP (indicated by the arrow, <i>middle panel</i> , Figure 1b) and the mean
569	band density of total LIP (upper panel, Figure 1b) was calculated with the ImageJ software and
570	expressed as arbitrary optical density units, setting the mean ratio between ubiquitinated LIP/total
571	LIP in HMC to 1. Data are presented as means±SD (n=3). MPM vs HMC cells: *p<0.001. d.
572	Correlation between IC_{50} of cisplatin (Pt; Supplementary Table 3) and LIP ubiquitination,
573	calculated with the ImageJ software . The mean band density of ubiquitinated LIP (indicated by the
574	arrow, Figure 1b) was expressed as arbitrary optical density units, setting the mean band density in
575	HMC samples to 1 (Supplementary Table 4a). e-f. LIP ubiquitination (indicated by the arrow,
576	Figure 1b) was ranked according to mean band density of each patient (Supplementary Table 4a),
577	and median value was calculated. Patients were classified as "LIP low" if LIP ubiquitination was
578	higher or equal to the median value (n=5, i.e. patient 3, 4, 7,8, 9), "LIP high" if LIP ubiquitination
579	was lower or equal to the median value (n=4, i.e. patient 1, 2, 5, 6). Progression free survival (panel
580	e) and overall survival (panel f) probability was calculated using the Kaplan-Meier method. LIP
581	high vs. LIP low group:*p<0.03 (panel e); *p<0.01 (panel f). UQ-LIP: ubiquitinated LIP
582	Figure 2. LIP reconstitution induces apoptosis and rescues cisplatin-cytotoxicity in
583	mesothelioma
584	a. Immunoblot of the indicated proteins in extracts of MPM1 (epithelioid MPM) and MPM7
585	(sarcomatous MPM) cells, stably transfected with the doxycycline-inducible LIP-expression vector,
586	cultured in the absence (-, Ctrl) or presence (+) of cisplatin (Pt, 25 $\mu M)$ and doxycycline for 24 h.
587	The figure is representative of 1 out of 3 experiments. b . Cell cycle analysis of cells treated as in a ,
588	performed by flow cytometry in duplicates. Pooled data of MPM 1-9 as means±SD (n=3). LIP-

treated vs. Ctrl cells:*p<0.01; LIP+Pt-treated cells vs. Pt-treated cells:°p<0.001. **c.** Cell proliferation

- in cultures treated 8 h after seeding (time "0" in the graph) as in **a**. Pooled data of MPM 1-9 as
- 591 means±SD (n=4). LIP-treated vs. Ctrl cells (72-96 h):*p<0.01; LIP+Pt-treated cells vs. Pt-treated
- cells (72-96 h):°p<0.001. **d**. Representative photographs of cells stained with crystal violet (96 h;
- *left panel*) and quantitation of crystal violet-stained cells, expressed as percentage of viable cells
- compared to untreated cells (*right panel*). Data of MPM1 and MPM7 cells are presented as
- 595 means±SD (n=4). Pt-treated vs. Ctrl cells:[#]p<0.05; LIP-treated vs. Ctrl cells:*p<0.001; LIP/LIP+Pt-
- treated cells vs. Pt-treated cells: $^{\circ}p<0.001$.

597 Figure 3. LIP restoration primes mesothelioma cells for immunogenic cell death

a. Immunoblot of C/EBP β LAP and LIP in whole cell lysates of MPM1 and MPM7 cells,

transfected with the inducible expression vector for LAP (*left panel*) or LIP (*right pane*), cultured in

- 600 the absence (-, Ctrl) or presence (+) of doxycycline (Doxy). The figure is representative of 1 out of
- 601 3 experiments. **b**. ChIP showing the binding of LAP or LIP to the calreticulin (*CRT*) promoter (site
- 602 831-843). Bl: blank; DNA input: genomic DNA. The figure is a representative of 1 out of 3
- 603 experiments. c. Levels of calreticulin (*CRT*) mRNA as determined by qRT-PCR in triplicates.
- Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing cells vs. un-induced cells:*p<0.001.
- 605 **d.** Surface calreticulin (CRT) as detected by flow cytometry in duplicates. The histograms represent
- the results obtained on MPM1 and MPM7. e. Phagocytic index of MPM cells phagocytized by DC,
- as determined by flow cytometry. Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing
- 608 cells vs. un-induced cells:*p<0.001. **f.** Percentage of CD8⁺CD107⁺T-lymphocytes as determined by
- flow cytometry in duplicates. Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing cells
- 610 vs. un-induced cells:*p<0.001. g. IFN- γ levels in the supernatant of CD8⁺CD107⁺T-cells, measured
- in duplicates. Pooled data of MPM 1-9 as means±SD (n=3). LIP-expressing cells vs. un-induced
 cells:*p<0.001.
- 613 Figure 4. LIP overcomes cisplatin-resistance and immune-resistance in vivo

a. 1×10^7 AB1 inducibly expressing LIP (clone 1, Supplementary Figure 3) were injected s.c. in 6-614 615 weeks-old female immune-competent or immune-deficient (nude) balb/C mice. When tumor reached the volume of 50 mm³, animals (n=10/group) were randomized and treated on days 1,7 and 616 14 as follows: 1) control (Ctrl) group, treated with 0.1 ml saline solution intravenously (i.v.); 2) LIP 617 group, treated with 1 mg/ml doxycycline in the drinking water to induce the LIP intratumorally; 3) 618 cisplatin (Pt) group, treated with 5 mg/kg cisplatin i.v.; 4) LIP+cisplatin (LIP+Pt) group, treated 619 with cisplatin and doxycycline to induce LIP. Data are means±SD. Pt/LIP+Pt groups vs. Ctrl group 620 (days 15-21:immune-competent mice, day 21:immune-deficient/nude mice):*p<0.001; LIP+Pt 621 group vs. Pt group (days 9-21:immune-competent and immune-deficient/nude mice):°p<0.001; 622 LIP+Pt group vs. LIP group (days 9-21: immune-competent and immune-deficient/nude mice):[#]p< 623 0.005; LIP+Pt group in immunocompetent vs. immune-deficient/nude mice: p<0.005 (not shown). 624 **b**. Immunoblotting of C/EBP_β LAP and LIP from tumor extracts, to check LIP induction in mice 625 626 treated with doxycycline. The figure is representative of extracts from two immune-deficient mice and two immune-competent mice. β -tubulin was used as control of equal protein loading. c. 627 628 Sections of tumors from each group of immune-competent animals, stained with hematoxylin and 629 eosin (HE; 20X objective; bar=100 µm), or immunostained with the indicated antibodies (63X objective; bar=10 µm). The photographs are representative of sections from 5 tumors/group. Data 630 are means±SD. Pt/LIP+Pt groups vs. Ctrl group:*p<0.005; LIP+Pt group vs. Pt group:°p<0.001; 631 LIP+Pt group vs. LIP group:[#]p<0.05. **d.** Percent of DC cells in tumors grown in immune-competent 632 mice, treated by Pt, doxycycline (LIP) or their combination. The percent of DC was determined in 633 duplicates by flow cytometry of single cell suspensions. e. Percent of CD3⁺CD8⁺T-lymphocytes 634 635 measured as in **d**. Data of panels **d** and **e** are means±SD. Pt/LIP+Pt groups vs. Ctrl group:*p<0.001; LIP+Pt group vs. Pt group:^op<0.001; LIP+Pt group vs. LIP group:[#]p<0.02. **f.** IFN-γ levels as 636 637 measured in duplicates in the supernatant of tumor-draining lymph nodes of immune-competent mice. Data of are means±SD. Pt/LIP+Pt groups vs. Ctrl group:*p<0.001; LIP+Pt group vs. Pt 638 group:°p<0.001; LIP+Pt group vs. LIP group:[#]p<0.05. 639

640	Figure 5. LIP undergoes proteasomal and lysosomal degradation in mesothelioma cells
641	a. Proteasome activity as analyzed in 3 primary HMC and 9 primary human MPM samples,
642	determined in duplicates by a chemiluminescence-based assay. Data are means \pm SD (n=3). MPM vs.
643	HMC:*p<0.001. b. Autophagy activity as measured spectrofluorimetrically in duplicates. Data are
644	means±SD (n=3). MPM vs HMC: *p<0.001. c. Lysosome activity as measured in duplicates by a
645	spectrophotometric assay. Data are means±SD (n=3). MPM vs HMC:*p<0.001. d. Immunoblot of
646	the indicated proteins in extracts of MPM1 and MPM7 cells, grown for 24 h in the absence (-), or
647	presence of the proteasome inhibitor carfilzomib (20 nM, Ca), the lysosome inhibitor chloroquine
648	(10 μ M, Cq), or their combination, with or without, cisplatin (Pt, 25 μ M), added for additional 24 h.
649	The effects induced by cisplatin alone are reported in Figure 2a. The figure is representative of 1 out
650	of 3 experiments.
651	Figure 6. Carfilzomib and chloroquine rescue cisplatin anti-tumor effects in vitro and in vivo
652	a. Growth curves of MPM cells, treated 8 h (time "0" in the graph) after seeding with fresh medium
653	(Ctrl), or media containing carfilzomib (20 nM, Ca), chloroquine (10 μ M, Cq), or their
654	combination, without or with cisplatin (Pt, 25 μ M). The effects induced by cisplatin alone are
655	reported in Figure 2c. Pooled data of MPM 1-9 as means±SD (n=4). All drug-treated cells vs. Ctrl
656	cells (72-96 h):*p<0.001; Ca+Cq+Pt cells vs. Ca+Cq cells (72-96 h):°p<0.001. b . Representative
657	photographs of cells stained with crystal violet (96 h). c. AB1 cells were injected s.c. in 6-weeks-old
658	growth in immune-competent balb/C mice (n=10/group). When tumor reached the volume of 50
659	mm ³ , animals (n=10/group) were randomized and treated on days 1, 7 and 14 as follows: 1) control
660	(Ctrl) group, treated with 0.1 ml saline solution intravenously (i.v.); 2) carfilzomib (Ca) group,
661	treated with 5 mg/kg carfilzomib i.v.; 3) chloroquine (Cq), treated with 10 mg/kg chloroquine per
662	os; 4) carfilzomib+chloroquine (Ca+Cq) group, treated with both drugs; 5)
663	carfilzomib+chloroquine+cisplatin (Ca+Cq+Pt) group, treated with carfilzomib, chloroquine and 5
664	mg/kg cisplatin i.v. (24 h after carfilzomib+chloroquine). Data are means±SD. All drug-treated cells
665	vs. Ctrl group:*p<0.001; Ca+Cq+Pt group vs. Ca+Cq group:°p<0.001. d. Sections of tumors from
- each group, stained with hematoxylin and eosin (HE; 20X objective; $bar=100 \mu m$), or
- immunostained with the indicated antibodies (63X objective; $bar=10 \mu m$). The percentage of
- 668 CHOP- and cleaved caspase 3-positive cells as determined by analyzing sections from 5 mice of
- each group (110-93 cells/field), using the Photoshop program. "Ctrl" group intensity was taken as
- 670 100%. Data are means±SD. Treated groups vs. Ctrl group:*p<0.002; Ca+Cq+Pt group vs. Ca+Cq
- group:°p<0.01. e. Percentage of infiltrating DC in tumors grown in mice that were treated as in d. f.
- Percentage of infiltrating $CD3^+CD8^+T$ -lymphocytes in these tumors. Data in panels **e** and **f**,
- determined in duplicates, are means±SD. All drug-treated groups vs. Ctrl group:*p< 0.001;
- 674 Ca+Cq+Pt group vs. Ca+Cq group: $^{\circ}p < 0.02$. **g.** IFN- γ levels in the supernatants of tumor-draining
- lymph nodes. Data determined in duplicates are means±SD. All drug-treated groups vs. Ctrl group:
- p < 0.001; Ca+Cq+Pt group vs. Ca+Cq group: p < 0.05.

Conflict of interests

None.



Figure 2



Figure 3





Figure 5



Figure 6



Supplementary Figure (for online use only) Click here to download Supplementary Figure (for online use only): Supplementary Figures_LC_R1.docx

Supplementary Tables

MPM							
(UPN)	CALR	PANCK	POD	EMA	CEA	WT1	CK5
1	POS	POS	NEG	NEG	NEG	POS	NEG
2	POS	POS	NEG	NEG	NEG	POS	NEG
3	POS	POS	NEG	POS	NEG	POS	NEG
4	POS	POS	NEG	NEG	NEG	POS	NEG
5	POS	POS	NEG	NEG	NEG	NEG	NEG
6	POS	POS	NEG	NEG	NEG	FOC	NEG
7	NEG	NEG	NEG	NEG	NEG	POS	NEG
8	FOC	FOC	NEG	NEG	NEG	FOC	NEG
9	NEG	POS	NEG	NEG	NEG	NEG	NEG

Supplementary Table 1. Histological characterization of mesothelioma samples

Results of the immunohistochemical staining of MPM samples for calretinin (CALR), pancytokeratin (PANCK), podoplanin (POD), epithelial membrane antigen (EMA), carcino-embryonic antigen (CEA), Wilms tumor-1 antigen (WT1), cytokeratin 5 (CK5). POS: positive; NEG: negative; FOC: focal positivity. UPN: unknown patient number.

MPM	Histotype	Sex	Age	Asbestos	Surgery	Radiotherapy	First-line	Second-line	Progression	Overall
(UPN)			(years)	exposure			chemotherapy	treatments	free survival	survival
							with cisplatin		(months)	(months)
1	epithelioid	М	51	Р	No	No	Yes	Gemcitabine	4.1	23
								Vinorelbine		
2	epithelioid	М	77	Р	No	Yes	Yes	Gemcitabine	10.3	16
3	epithelioid	F	47	Е	No	Yes	Yes	Trabectedin	5	12
4	biphasic	М	66	Е	Yes	No	Yes	None	2	9
5	biphasic	М	64	Р	No	No	Yes	Cisplatin	5.5	16
								+pemetrexed		
6	biphasic	М	72	Р	No	No	Yes	Trabectedin	5.1	21
7	sarcomatous	F	87	ND	No	No	Yes	None	1.8	6
8	sarcomatous	М	69	Е	No	No	Yes	Trabectedin	2.6	10
9	sarcomatous	М	79	Р	No	No	Yes	Trabectedin	2.2	5

Supplementary Table 2. Clinical features of mesothelioma patients

Histological classification of MPM samples, anagraphic and clinical data of patients. M: male; F: female; P: professional; E: environmental; U: unlikely; ND: not-determined. UPN: unknown patient number. First-line chemotherapy: 5 cycles of cisplatin 75 mg/m² every 21 days. Progression free survival: survival with stable disease from the beginning of cisplatin therapy. Overall survival: survival from the beginning of cisplatin therapy until patients exitus.

Supplementary Table 3. IC₅₀ of cisplatin

Samples	IC ₅₀ (µM)
HMC 1	1.43±0.37
HMC 2	5.28±0.46
HMC 3	3.72±0.11
MPM 1	28.14±5.77 *
MPM 2	55.81±9.28 *
MPM 3	49.45±8.72 *
MPM 4	78.26±10.75 *
MPM 5	42.32±5.18 *
MPM 6	48.11±11.33 *
MPM 7	71.34±16.32 *
MPM 8	74.05±12.15 *
MPM 9	93.52±16.93 *

 1×10^4 cells were seeded in quadruplicate in 96-well plates, treated for 96 h with cisplatin at scalar concentrations (from 10^{-10} to 10^{-3} M), then stained with Neutral red solution. IC₅₀ was calculated with the CompuSyn software. Data are means±SD (n=4). MPM cells vs. each HMC: *p<0.001.

a	Figure 1b			
condition	UQ LIP	total LIP		
HMC	1	2.669884		
MPM1	1.692308	0.374548		
MPM2	2.153846	0.37125		
MPM3	2.333333	0.424765		
MPM4	2.5	0.920423		
MPM5	1.846154	0.951585		
MPM6	1.903846	1.055493		
MPM7	2.50641	1.056983		
MPM8	2.666667	1.054902		
MPM9	2.942308	1.046657		

Supplementary Table 4. Quantification of ubiquitinated LIP

b	Figure 2a			
condition	UQ LIP	total LIP		
MPM1 Ctrl	1	1		
MPM1 LIP	0.538232	0.866023		
MPM1 Pt	0.688645	0.851552		
MPM1 LIP+Pt	0.317735	0.851552		
MPM7 Ctrl	1	1		
MPM7 LIP	0.834996	0.834996		
MPM7 Pt	0.604407	0.698161		
MPM7 LIP+Pt	0.249762	0.687504		

c	Figure 5d			
condition	UQ LIP	total LIP		
MPM1 Ctrl	1	1		
MPM1 Ca	0.75358	1.319789		
MPM1 Cq	0.742566	1.14256		
MPM1 Ca+Cq	0.613394	1.731273		
MPM1 Ca+Cq+Pt	0.598165	2.538498		
MPM7 Ctrl	1	1		
MPM7 Ca	0.688755	1.256245		
MPM7 Cq	0.690439	1.269075		
MPM7 Ca+Cq	0.689884	1.748149		
MPM7 Ca+Cq+Pt	0.595311	2.464859		

Band density of ubiquititinated (UQ) LIP and total LIP of the blots reported in Figure 1b (Table **a**), Figure 2a and Supplementary Figure 1a (Table **b**), Figure 5d and Supplementary Figure 7a (Table **c**), was calculated using ImageJ software (<u>http://www.rsb.info.nih.gov/ij/</u>). The ratio between ubiquitinated LIP/total LIP was reported in Figure 1c, Supplementary Figure 1b and Supplementary Figure 7b.

MPM	IC ₅₀ (µM)	Sf
(UPN)		
1	8.05 ± 0.23	3.49
2	11.23 ± 0.51	4.96
3	7.24 ± 0.91	6.83
4	8.36 ± 0.15	9.36
5	14.23 ± 4.12	3.18
6	7.11 ± 0.81	6.76
7	10.23 ± 0.85	6.97
8	15.63 ± 1.91	4.73
9	24.58 ± 3.14	3.80

Supplementary Table 5. Cisplatin sensitization in mesothelioma cells overexpressing LIP

 1×10^4 cells were seeded in quadruplicate in 96-well plates, treated for 96 h with cisplatin at scalar concentrations (from 10^{-10} to 10^{-3} M), then stained with Neutral red solution. IC₅₀ was calculated with the CompuSyn software. Data are presented as means±SD (n=4). IC₅₀ in LIP-overexpressing MPM cells vs. wild-type MPM cells (reported in Supplementary Table 3): *p<0.001 (not shown in this Table). Sensitization factor (Sf) was obtained by dividing IC₅₀ in wild-type MPM cells (Supplementary Table 3) and IC₅₀ in LIP-overexpressing cells (Supplementary Table 4).

	Ctrl	Ca	Cq	Ca+Cq	Ca+Cq+Pt
RBC (x10 ⁶ /µl)	13.09±1.88	11.72±2.35	12.45±3.89	11.09±3.78	11.38±2.11
Hb (g/dl)	13.78±2.91	12.52±2.18	13.21±1.98	12.81±2.71	12.09±1.93
WBC (x10 ³ /µl)	13.23±2.81	12.21±3.11	12.84±2.29	13.78±2.11	11.83±2.48
PLT (x10 ³ /μl)	1145±239	901±175	1093±269	1193±207	946±152
LDH (U/l)	5879±982	5729±498	6230±502	6183±529	5936±610
AST (U/l)	187±42	229±41	173±45	179±55	209±47
ALT (U/l)	52±11	51±15	62±9	52±13	45±17
AP (U/l)	126±38	101±25	136±42	145±33	121±34
Creatinine (mg/l)	0.031±0.006	0.027±0.005	0.034±0.007	0.030±0.007	0.035±0.006
CPK (U/l)	345±67	411±109	398±163	372±152	361±129

Supplementary Table 6. Hematochemical parameters of the animals

Immunocompetent balb/C mice (n=10/group) were treated as described in Figure 6. Blood was collected immediately after euthanasia and analyzed for red blood cells (RBC) counts, hemoglobin (Hb), white blood cells (WBC), platelets (PLT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), creatinine, creatine phosphokinase (CPK). Data are means±SD.