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Photodynamic therapy with redaporfin targets the endoplasmic reticulum and Golgi apparatus

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14th November 2017

Thank you for the submission of your manuscript (EMBOJ-2017-98354) to The EMBO Journal, as well as giving additional input in your preliminary point-by-point response. As mentioned, your study has been sent to three referees, and we have received reports from all of them, which I copy below.

The referees acknowledge the potential interest and novelty of your work, although they also express major concerns. In particular, they raise reservations regarding overlap with previous literature on PDT generated ROS-ER stress and unclear physiological relevance of the findings (referees #1 and #3) as well as a lack of sufficient mechanistic insights into redaporfin-induced cell death and its molecular consequences at the Golgi as compared to other Golgi-disrupting compounds (referees #1 and #2). In additions, the referees point to a number of inconsistencies between data and missing controls, which would need to be resolved to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and can - based on your sensible preliminary point-by-point response - offer to invite you to revise your manuscript experimentally to address the referees' comments. Please note however, that we would need strong support from the referees on such a revised version of the manuscript to move towards publication.

REFEREE REPORTS

Referee #1:

In this manuscript, Gomes-da-Silva et al makes an attempt to unravel the mechanism behind which

PDT with redaporfin causes cancer cell death. The authors presented data indicating that redaporfin specifically accumulates in the endoplasmic reticulum (ER) in addition to the Golgi apparatus. Light-activated redaporfin causes selective damage to these subcellular compartments, elicits ER stress and irreversibly compromises Golgi-dependent secretion. The study is of high interest but there are some critical points to be addressed which are listed below.
Major points
It is well established fact that photodynamic therapy (PDT) often generates ROS-mediated ER stress eventually leading to cell death via immunogenic apoptosis. Garg et al 2012 have already shown that eIF2α phosphorylation and caspase-8 signalling are dispensable, suggesting that PDT perturbs ER dynamics eventually leading to cell death. Furthermore, since n-octanol:water partition coefficient of redaporfin is around 80, it is not surprising that it will localize in the ER and Golgi apparatus. Nevertheless, the attempt of this study to unravel the mechanistic details of the redaporfin induced cell death is encouraging. This study does not provide a detailed understanding of the mechanism behind redaporfin-induced cell death. This study reinforces previous findings (Garg et al 2012) having low novelty. It would have been better to evaluate the effect of redaporfin on all the arms of the UPR and subsequent downstream targets. Further, it does not show whether inhibition of secretory proteins is or is not the outcome of phenomenon of global protein attenuation. Moreover, the paper would gain significant impact if the findings were consolidated by in vivo experiments that remain the gold standard for cell death via immunogenic apoptosis.
Minor points
• Measurements of ROS by fluorescent dye methods are needed.

• Are there any effects of redaporfin on lipid peroxidation?

• Authors have not made any attempt to measure oxidative DNA damage.

• I would suggest to simplify their findings by diagrammatic representation.

• Please provide the value of redaporfin in mg/kg or µg/cells.

• Critical experiments could be repeated in Head and Neck cancer cell lines or other cell lines.

• What might be the possible reason for decrease in p-eIF2α/eIF2 ratio at 10 µM redaporfin in figure 3A and 3 B.

Referee #2:

The authors describe the effects of treatment of U2OS cells with redaporfin (RDP), a last generation photosensitizer presently being tested in clinical trials in advanced head and neck cancer patients. The authors report that RDP preferentially distributes to the ER and the Golgi,
and that PDT with RDP induces: 1. the disorganization of the Golgi complex and the reduction of several Golgi proteins; 2. an ER stress response; 3. an increase in cytosolic calcium; 4. the generation of reactive oxygen species; and 5. apoptosis.

By observing that BAPTA can prevent the increase in reactive oxygen species and reduce cell death, that an anti-oxidant can partially prevent Golgi damage and cell death and that caspase inhibitors prevent apoptosis but not the Golgi damage, the authors deduce that the cascade of events induced by RDP starts with oxidative stress that induces calcium release from the ER which in turn causes Golgi damage, ER stress and finally triggers apoptosis. The observation that two Golgi disrupting agents (BFA and GCA) partially prevent cell death caused by RDP leads the authors to propose that the Golgi complex involvement has a central role in the apoptotic cascade induced by RDP.

The manuscript reports a detailed description of the cell effects of the different and combined treatments but it raises rather than answers questions about the molecular mechanisms underlying the intriguing chain of events. Thus while the structural and functional damage to the Golgi complex induced by RDP are accurately described the mechanism leading to this damage, the molecular targets at the Golgi complex and the consequences of this disruptive effect on the Golgi on the

general cytotoxicity of RDP remain elusive.

Specific comments
1. localization of RDP at the Golgi complex: the IF images in Fig.1 do not allow to conclude that RDP is localized at the Golgi complex: higher resolution images are needed. The authors report the localization of RDP after 20h exposure: is this long time of exposure needed for the Golgi localization? What is the time course of accumulation of RDP in intracellular organelles? A diagnostic test such as fragmentation of the Golgi complex by nocodazole could help in defining the localization of RDP at the Golgi complex. Long nocodazole treatment induces the formation of peripheral Golgi ministacks: if any RDP is localized at the Golgi it should be found localized in the ministacks. As for the analysis of the subcellular fractions, the authors should show the extent of cross-contamination of the Golgi or ER enriched fractions by analyzing ER markers in the Golgi fractions and vice versa;
2. the effects of the two Golgi disrupting agents on RDP activity: the effects of these agents in reducing the extent of apoptosis and the number of dying cells are in fact too marginal to conclude that an "intact Golgi" is needed for the cytotoxic effect of RDP. However, what is surprising is that they do not aggravate but, even though marginally, alleviate the cell toxicity of RDP: this observation would lead to the conclusion that the dismantling of the Golgi complex has no causative role in the cell toxicity induced by RDP thus leaving open the question about the role of the disruption of the Golgi complex in the toxicity induced by RDP. Which are the mechanisms underlying this protective effect of Golgi disrupting agents? An interesting possibility that the Authors might want to consider is that the "protective role" of Golgi disrupting agents may be mediated by the recently described Golgi stress response involving ARF4. It is not clear whether the agents added 4 hours before irradiation are also present during the 6 hrs of irradiation.
3. Secretion of chemokines: the authors measure the extracellular levels of chemokines: a reduction in these levels may result from an impaired synthesis of chemokines. Thus the authors should measure also the intracellular levels of the chemokines and make a ratio of secreted vs intracellular.
4. Once again the question about the role of this impaired secretion in the cytotoxicity of RDP remains open, considering that BFA and GCA alleviate, albeit marginally, the cell toxicity. What is the underlying mechanism? Could BFA or GCA activate alternative pathways of secretion? The authors could test this possibility by measuring secretion of the same chemokines in cells exposed to BFA/GCA and RDP.

Referee #3:

The MS from Guido Kroemer and colleagues, links redaporfin PDT mediated inhibition of anterograde transport to induction of mitochondrial cell death and inhibition of the release of certain cytokines. The link between PDT and loss of secretory potential, which is the strongest suggestion of the paper, is made fairly convincing by the authors.
This makes moreover sense of the finding that various previous studies dealing with the effects of the light activation of ER/Golgi localizing dies have conclusively shown that these photoactivatable compounds require BAX/BAK effectors to trigger mitochondrial apoptosis that is executed largely through caspase activation and that, not surprisingly, antioxidants prevent this type of ROS-induced cell death. Also some of these compounds have been previously shown to reduce the overall secretory capacity of the cells.
In general, at the biochemical level the study is well performed and organized. However, it remains rather descriptive in nature and the observation that ER-Golgi disrupting agents offer partial protection from PDT induced mitochondrial cell death, thus implicating the integrity of ER/Golgi secretory compartment in cell death induction after PDT, is rather weak. Moreover, a number of questions remain unsolved; what distinguishes redaporfin PDT from classical Golgi-disrupting compounds? Does PDT trigger an unspecific downregulation of GA markers as a result of ROS production or are there specific, early transport steps of the secretory machinery that are compromised?
Furthermore, it is important that the authors show that these effects are biologically relevant at lower concentration of redaporfin. Would redaporfin in therapeutic settings reach intratumorally the high micro molar concentrations required to observe the disrupting effects on the secretory efficiency and cell death?

Some specific comments are listed below:
Compromising secretory pathway function may lead to the accumulation of proteins in the ER leading to ER stress and ultimately cell death. The authors show that redaporfin after light activation induces some features of the UPR. However, whether there is a hierarchical link between (apical?) GA damage and ER stress followed by mitochondrial cell death has not been experimentally tested. Fig. 2: Loss of Golgi markers, GBF1, GOLGA2, GALT1 and certain ER markers, like EIF2AK3 and PDIA3, occurs already when low(er) doses of redaporfin PDT are used, 0.3-0.6 uM. Likewise, activation of the UPR is readily detectable in response to similar doses, which neither induce substantial cell death or loss of clonogenic growth (Fig. 5). Also, the partial protective effect of BFA is only observed at higher, micromolar concentrations of redaporfin and the strongest inhibitory effects on cytokine secretion as well. This argues against the primary role of ER-to Golgi trafficking disturbance in driving cytotoxic effects of redaporfin, which requires doses at which -as the authors themselves mention- relevant and collateral damage to cytoplasmic targets has been caused. Under these conditions is then not surprising that the accumulating cytoplasmic damage converges into (several) pathways and activates different mechanisms that eventually favour BAX/BAK dependent mitochondrial apoptosis.

It is not clear which is the eIF2a-P kinase involved as the expression of the EIF2AK3 = PERK is downregulated after PDT.

It is often not clear which controls are shown in various immunofluorescence analysis (see e.g. Fig.3 etc), light but no compound? Compound without light? And at which concentration?

Basically all or most of the assays are performed at 6 h post treatment why? Kinetics analysis should be performed as well. Please note that in several Figures (e.g. imaging analysis) the concentration of redaporfin used is not mentioned making it very hard to gauge whether the PDT effect is specific and a direct consequence of the ER/Golgi targeting.

Fig. 4: There is no clear concentration dependency in ROS production after PDT, which is strange. Overall ROS production seems rather low, based on this measurement and it should be important to compare it to a positive control such as hydrogen peroxide as well as to show how it evolves during time. Likewise, in Fig. 6: the effects of PDT on cytosolic calcium elevations are also rather minor. This seems to be a secondary effect of the many alterations induced by PDT when high concentrations of redaporfin are used. Also, here the authors should add a positive control for store depletion in these cells (e.g. thapsigargin or IP3 generating agents like ATP) to be compared with and show the kinetics of this event, which is claimed to be immediate.Fig. 6 why is BAPTA alone inducing BAX aggregation?

One of the most important conclusion that the authors draw is that the release of a set of cytokines is reduced by PDT. However although the authors mention that redaporfin PDT mediates a form of cell death with important immunomodulatory consequences, the impact of this observation is not explored while the authors are top-notch experts in this domain.

1st Revision - authors' response 16th March 2018

Point-by-point reply to Referee #1:

General critique by Reviewer No. 1: In this manuscript, Gomes-da-Silva et al makes an attempt to unravel the mechanism behind which PDT with redaporfin causes cancer cell death. The authors presented data indicating that redaporfin specifically accumulates in the endoplasmic reticulum (ER) in addition to the Golgi apparatus. Light-activated redaporfin causes selective damage to these subcellular compartments, elicits ER stress and irreversibly compromises Golgi-dependent secretion. The study is of high interest but there are some critical points to be addressed which are listed below.

Major point 1 raised by Reviewer No. 1: It is well established fact that photodynamic therapy

(PDT) often generates ROS-mediated ER stress eventually leading to cell death via immunogenic apoptosis. Garg et al 2012 have already shown that eIF2α phosphorylation and caspase-8 signalling are dispensable, suggesting that PDT perturbs ER dynamics eventually leading to cell death. Furthermore, since n-octanol:water partition coefficient of redaporfin is around 80, it is not surprising that it will localize in the ER and Golgi apparatus. Nevertheless, the attempt of this study to unravel the mechanistic details of the redaporfin induced cell death is encouraging. This study does not provide a detailed understanding of the mechanism behind redaporfin-induced cell death. This study reinforces previous findings (Garg et al 2012) having low novelty. It would have been better to evaluate the effect of redaporfin on all the arms of the UPR and subsequent downstream targets. Further, it does not show whether inhibition of secretory proteins is or is not the outcome of phenomenon of global protein attenuation. Moreover, the paper would gain significant impact if the findings were consolidated by in vivo experiments that remain the gold standard for cell death via immunogenic apoptosis.

Our response: We concur with the reviewer that the monumental paper by Garg et al. (published in EMBO Journal 2012) is the absolute reference in the literature on PDT-induced ER stress, and we will express this idea in a much more outspoken fashion in the Introduction of our paper. Our present paper deals with another PDT compound, redaporfin (instead of hypericin) and tried to indicate that this compound not only affect the ER but that it also has profound effects on the Golgi apparatus. The reviewer provides some indications how we can improve the paper:

"It would have been better to evaluate the effect of redaporfin on all the arms of the UPR and subsequent downstream targets." **We recently have generated knockout cells for each of the eIF2alpha kinases including PERK (official name: EIF2AK3) to test whether any of them is required for redaporfin-induced eIF2alpha phosphorylation and cell killing. Only one of these kinases, namely EIF2AK1 (but not EIF2AK2, EIF2AK3 or EIF2AK4) contributed to eIF2alpha phosphorylation in response to redaporfin-mediated PDT. Only the knockout of one of these kinases, again EIF2AK1 (but not EIF2AK2, EIF2AK3 or EIF2AK4) sensitized to cell death induction by redaporfin-mediated PDT. Moreover, we knocked down elements of the other two arms of the ER stress response pathway (ATF6 and IRE1alpha) and found that this manipulation failed to sensitize to cell killing by redaporfin-mediated PDT. These results have been added to the paper (Figure 3C-F and Figure S4L,M).**

"[The paper] does not show whether inhibition of secretory proteins is or is not the outcome of phenomenon of global protein attenuation." **In response to this critique, we addressed the question whether PDT mediated by redaporfin causes a general shutdown of protein synthesis. For this, we used a new technology based on the non-radioactive aminoacid analogue L-azidohomoalanine that can be used for the click chemistry-mediated fluorescent detection of new translation products (Nat Protoc. 2017 Dec;12(2):279-288). We found that redaporfin-mediated PDT led to a partial but not complete inhibition of protein synthesis. This result has been included in the paper (Figure S5).**

"[…]the paper would gain significant impact if the findings were consolidated by in vivo experiments that remain the gold standard for cell death via immunogenic apoptosis." **We have performed a series of vaccination experiments to show that PDT mediated by redaporfin is indeed immunogenic. We added these results to paper (Figure 3I, J and Figure S7).**

Minor point 1 raised by Reviewer No. 1: Measurements of ROS by fluorescent dye methods are needed.

Our response: We used two fluorescent probes (namely dihydroethidine and CellRox Green) to measure ROS induced by redaporfin-mediated PDT. These results have been included in the paper (Figure 4 and Figure 8A-H).

Minor point 2 raised by Reviewer No. 1: Are there any effects of redaporfin on lipid peroxidation?

Our response: We have been unable to detect lipid peroxidation when using antibodies specific for 4-hydoxynonenal, a product of lipid peroxidation.

Minor point 3 raised by Reviewer No. 1: Authors have not made any attempt to measure oxidative DNA damage.

Our response: We used an antibody specific for phosphorylated histone H2X (g-H2AX), using immunofluorescence to detect so-called DNA damage foci in the nuclei of PDT-treated cells as a proxy for oxidative DNA damage. We have detected the induction of g-H2AX foci in the context of redaporfin-mediated PDT, and this induction was blunted by the addition of tocopherol, an antioxidant, supporting the idea that g-H2AX foci are indeed induced by oxidative stress. These results have been added to the paper (Figure 4A,B and Figure S8G,H).

Minor point 4 raised by Reviewer No. 1: I would suggest to simplify their findings by diagrammatic representation.

Our response: We provided a sort of graphical abstract.

Minor point 5 raised by Reviewer No. 1: Please provide the value of redaporfin in mg/kg or μg/cells.

Our response: We have included this information in the Materials and Methods when we describe the standard procedure of redaporfin-mediated PDT.

Minor point 6 raised by Reviewer No. 1: Critical experiments could be repeated in Head and Neck cancer cell lines or other cell lines.

Our response: The most critical experiment (namely brefeldin A or golgicide-reduce cell killing) were repeated in three additional human cancer cell lines to illustrate the general validity of our findings. These results have been added to the supplemental material (Figure S9A-F).

Minor point 7 raised by Reviewer No. 1: What might be the possible reason for decrease in p-eIF2α/eIF2 ratio at 10 μM redaporfin in figure 3A and 3 B.

Our response: All redaporfin doses (2.5, 5, 10 μM) combined with light induced eIF2alpha phosphorylation as compared to untreated controls (no redaporfin) or the application of redaporfin (10 μM) without light. The reviewer points out that redaporfin+light-induced eIF2alpha phosphorylation is less strong at 10 μM than at lower concentrations. It is possible that cell stress pathways such as ER stress leading to eIF2alpha phosphorylation are overwhelmed by an excess of local stress.

Point-by-point reply to Referee #2:

General critique by Reviewer No. 2: The authors describe the effects of treatment of U2OS cells with redaporfin (RDP), a last generation photosensitizer presently being tested in clinical trials in advanced head and neck cancer patients.
The authors report that RDP preferentially distributes to the ER and the Golgi, and that PDT with RDP induces: 1. the disorganization of the Golgi complex and the reduction of several Golgi proteins; 2. an ER stress response; 3. an increase in cytosolic calcium; 4. the generation of reactive oxygen species; and 5. apoptosis.

By observing that BAPTA can prevent the increase in reactive oxygen species and reduce cell death, that an anti-oxidant can partially prevent Golgi damage and cell death and that caspase inhibitors prevent apoptosis but not the Golgi damage, the authors deduce that the cascade of events induced by RDP starts with oxidative stress that induces calcium release from the ER which in turn causes Golgi damage, ER stress and finally triggers apoptosis. The observation that two Golgi disrupting agents (BFA and GCA) partially prevent cell death caused by RDP leads the authors to propose that the Golgi complex involvement has a central role in the apoptotic cascade induced by RDP.

The manuscript reports a detailed description of the cell effects of the different and combined treatments but it raises rather than answers questions about the molecular mechanisms underlying the intriguing chain of events. Thus, while the structural and functional damage to the Golgi complex induced by RDP are accurately described the mechanism leading to this damage, the molecular targets at the Golgi complex and the consequences of this disruptive effect on the Golgi on the general cytotoxicity of RDP remain elusive.

Our response: The reviewer accurately summarizes our paper and then points out that "it raises rather than answers questions". We have spent a lot of time thinking about an appropriate solution to solve the central problem of our hypothesis. In the initial version of this paper, we limited ourselves to show that redaporfin plus light (photodynamic therapy, PDT) can cause oxidative damage to cellular structures including the Golgi apparatus and that dispersal of the Golgi by specific drugs (brefeldin A and golgicide) can attenuate the cytotoxic effect of redaporfin-mediated PDT. We have devised a strategy to induce oxidative damage to the Golgi in a specific way, namely by engineering cells in which the Golgi apparatus can be subjected to oxidative damage in a specific way. This system is based on the expression of a peroxidase (horse radish peroxidase or HRP) in the lumen of the Golgi apparatus by fusion with a Golgi targeting sequence. The addition of low concentrations of diaminobenzidine (DAB) and H_2O_2 induces the formation of a brown insoluble precipitate (a DAB oxidation product) that specifically inactivates Golgi dynamics and function (Jollivet et al., 2007, Mol Biol Cell. 18:4637-47). We have used this system to show that HRP targeted to the Golgi or to the ER sensitizes cells to killing by DAB and H_2O_2. In contrast, targeting HRP to the cytosol did not sensitize the cells to DAB/H_2O_2 contrasting with the fact that the cells did produce the brown precipitate that results from DAB oxidation. We interpret these results to mean that oxidative damage to the ER and the Golgi is sufficient to kill cells (Figure 6 and Figure S11).

General comment No. 1 by Reviewer No. 2: Localization of RDP at the Golgi complex: the IF images in Fig.1 do not allow to conclude that RDP is localized at the Golgi complex: higher resolution images are needed. The authors report the localization of RDP after 20h exposure: is this long time of exposure needed for the Golgi localization? What is the time course of accumulation of RDP in intracellular organelles? A diagnostic test such as fragmentation of the Golgi complex by nocodazole could help in defining the localization of RDP at the Golgi complex. Long nocodazole treatment induces the formation of peripheral Golgi ministacks: if any RDP is localized at the Golgi it should be found localized in the ministacks. As for the analysis of the subcellular fractions, the authors should show the extent of cross-contamination of the Golgi or ER enriched fractions by analyzing ER markers in the Golgi fractions and vice versa.

Our response: We provided confocal fluorescence microscopy images to document the localization of redaporfin (RDP) at the Golgi apparatus in the supplemental material.

Moreover, we quantified images to provide an accurate kinetic characterization of the redistribution of RDP towards the Golgi. We also took up the suggestion by the reviewer to treat cells with nocodazole for a protracted period (24 hours) and to investigate the redistribution of RDP toward Golgi ministacks. This manipulation caused RDP to relocalize from one single perinuclear site (corresponding to the Golgi) to multiple small cytoplasmic dots. Finally, we investigate the cross-contamination of Golgi and ER-enriched fractions, as suggested by the reviewer (Figure S1D-J).

General comment No. 2 by Reviewer No. 2: the effects of the two Golgi disrupting agents on RDP activity: the effects of these agents in reducing the extent of apoptosis and the number of dying cells are in fact too marginal to conclude that an "intact Golgi" is needed for the cytotoxic effect of RDP. However, what is surprising is that they do not aggravate but, even though marginally, alleviate the cell toxicity of RDP: this observation would lead to the conclusion that the dismantling of the Golgi complex has no causative role in the cell toxicity induced by RDP thus leaving open the question about the role of the disruption of the Golgi complex in the toxicity induced by RDP. Which are the mechanisms underlying this protective effect of Golgi disrupting agents? An interesting possibility that the Authors might want to consider is that the "protective role" of Golgi disrupting agents may be mediated by the recently described Golgi stress response involving ARF4. It is not clear whether the agents added 4 hours before irradiation are also present during the 6 hrs of irradiation.

Our response: We investigated whether knockdown of ARF4 with suitable siRNAs would attenuate the partial rescue effects of brefeldin A and golgicide against redaporfin (RDP)-mediated PDT. However, we did not find such an effect. As pointed out in the response to the general critique by the reviewer, we examined whether oxidative stress-dependent damage to the Golgi is sufficient to cause cell death via the pathway described here. Finally, we pointed out in the Figure legends that the Golgi-disrupting agents were present throughout the duration of the experiment.

Figures for Referee not shown.

General comment No. 3 by Reviewer No. 2: Secretion of chemokines: the authors measure the extracellular levels of chemokines: a reduction in these levels may result from an impaired synthesis of chemokines. Thus the authors should measure also the intracellular levels of the chemokines and make a ratio of secreted vs intracellular. Once again the question about the role of this impaired secretion in the cytotoxicity of RDP remains open, considering that BFA and GCA alleviate, albeit marginally, the cell toxicity. What is the underlying mechanism? Could BFA or GCA activate alternative pathways of secretion? The authors could test this possibility by measuring secretion of the same chemokines in cells exposed to BFA/GCA and RDP.

Our response: We measured extracellular and intracellular cytokines in the presence of BFA or GCA to solve these questions. We found that BFA and GCA increased intracellular cytokines and decreased the concentration of extracellular cytokines. There was not a single case in which BFA or GCA would increase the concentration of cytokines in the supernatant of the cells. We concluded that the cytokines that we measured here are secreted through conventional (rather than alternative) pathways of secretion.

Figures for Referee not shown.

Point-by-point reply to Referee #3:

General critique by Reviewer No. 3: The MS from Guido Kroemer and colleagues, links redaporfin PDT mediated inhibition of anterograde transport to induction of mitochondrial cell death and inhibition of the release of certain cytokines. The link between PDT and loss of secretory potential, which is the strongest suggestion of the paper, is made fairly convincing by the authors.

This makes moreover sense of the finding that various previous studies dealing with the effects of the light activation of ER/Golgi localizing dies have conclusively shown that these photoactivatable compounds require BAX/BAK effectors to trigger mitochondrial apoptosis that is executed largely through caspase activation and that, not surprisingly, antioxidants prevent this type of ROS-induced cell death. Also some of these compounds have been previously shown to reduce the overall secretory capacity of the cells.
In general, at the biochemical level the study is well performed and organized. However, it remains rather descriptive in nature and the observation that ER-Golgi disrupting agents offer partial protection from PDT induced mitochondrial cell death, thus implicating the integrity of ER/Golgi secretory compartment in cell death induction after PDT, is rather weak. Moreover, a number of questions remain unsolved; what distinguishes redaporfin PDT from classical Golgi-disrupting compounds? Does PDT trigger an unspecific downregulation of GA markers as a result of ROS production or are there specific, early transport steps of the secretory machinery that are compromised?

Furthermore, it is important that the authors show that these effects are biologically relevant at lower concentration of redaporfin. Would redaporfin in therapeutic settings reach intratumorally the high micro molar concentrations required to observe the disrupting effects on the secretory efficiency and cell death?

Our response: The reviewer summarizes our study and acknowledges that some aspects of our paper are "fairly convincing" and that our work is "well performed and organized", while asking a series of several questions that prompted us to perform additional experiments.

"[…] the observation that ER-Golgi disrupting agents offer partial protection from PDT induced mitochondrial cell death, thus implicating the integrity of ER/Golgi secretory compartment in cell death induction after PDT, is rather weak." **In response to this remark, we have to acknowledge that the rescuing effects of brefeldin A and golgicide, two agents that cause dismantling of the Golgi apparatus, on the phototoxic death of cells treated by a combination of redaporfin and light is partial yet not complete. We therefore decided to inverse the question: instead of asking whether dispersion of the Golgi would reduce killing by brefeldin A and golgicide, we addressed the problem whether local damage of the Golgi induced by targeting a transgene-encoded peroxidase to this organelle would be able to kill cells through the pathway that we delineate here (see also our response to the general critique by reviewer No. 2). We presented these new data in the revised version of our paper. In essence, we targeted a ROS-producing enzyme (horseradish peroxidase) to the Golgi apparatus and showed that local ROS production (in response to H_2O_2 challenge combined with DAB) was sufficient to kill cells (Figure 6 and Figure S11).**

"What distinguishes redaporfin PDT from classical Golgi-disrupting compounds?" **We believe that there are multiple differences between redaporfin PDT and classical Golgi-disrupting compounds. Apart from the fact that redaporfin PDT causes cell death while classical Golgi-disrupting agents are barely cytotoxic, a fact that is already treated in this paper, redaporfin PDT induces local damage and strong oxidation, which is not the case for classical Golgi-disrupting agents. This has been shown by measuring ROS production with fluorescent biosensors as well by determining the depletion of Golgi proteins that could be inhibited by the antioxidant tocopherol (Figure 4 and Figure S8).**

"Does PDT trigger an unspecific downregulation of GA markers as a result of ROS production or are there specific, early transport steps of the secretory machinery that are compromised?" **We have used an antioxidant to address this question. Cells were exposed to redaporfin PDT in the absence or presence of the lipophilic antioxidant tocopherol and the abundance of GA and ER**

markers were assessed by immunofluorescence or immunoblot. Tocopheral largely inhibited the destruction of Golgi proteins (Figure 4C-G).

"Would redaporfin in therapeutic settings reach intratumorally the high micro molar concentrations required to observe the disrupting effects on the secretory efficiency and cell death?" **Redaporfin concentration has been measured in the plasma and tumors from mice (based on the fluorescence of this compound), leading to the conclusion that redaporfin can reach the concentrations that mediate the effects described here within the tumor (Saavedra et al, 2014).**

Saavedra R, Rocha LB, Dabrowski JM, Arnaut LG (2014) Modulation of biodistribution, pharmacokinetics, and photosensitivity with the delivery vehicle of a bacteriochlorin photosensitizer for photodynamic therapy. *ChemMedChem* **9:** 390-398

Specific comment No. 1 by Reviewer No. 3: Compromising secretory pathway function may lead to the accumulation of proteins in the ER leading to ER stress and ultimately cell death. The authors show that redaporfirin after light activation induces some features of the UPR. However, whether there is a hierarchical link between (apical?) GA damage and ER stress followed by mitochondrial cell death has not been experimentally tested.

Our response: We have addressed this question by inhibiting each of the arms of the UPR (by targeting different UPR-relevant genes/proteins by CRISPR/Cas9 technology or RNA interference). In particularly, we knocked out all 4 eIF2a kinases (EIF2K1 to 4) and knocked down ATF6 and IRE1. We found that knockout of EIF2K1 was the sole manipulation that sensitized cells to cell killing by redaporfin and light, while knockout of EIF2K2, EIF2K3 or EIF2K2, as well as knockdown of ATF6 or IRE1 failed to change cell killing by PDT in this setting. These results have been added to the revised paper (Figure 3C-F and Figure S4L,M).

Specific comment No. 2 by Reviewer No. 3: Fig. 2: Loss of Golgi markers, GBF1, GOLGA2, GALT1 and certain ER markers, like EIF2AK3 and PDIA3, occurs already when low(er) doses of redaporfin PDT are used, 0.3-0.6 uM. Likewise, activation of the UPR is readily detectable in response to similar doses, which neither induce substantial cell death or loss of clonogenic growth (Fig. 5). Also, the partial protective effect of BFA is only observed at higher, micromolar concentrations of redaporfin and the strongest inhibitory effects on cytokine secretion as well. This argues against the primary role of ER-to Golgi trafficking disturbance in driving cytotoxic effects of redaporfin, which requires doses at which -as the authors themselves mention- relevant and collateral damage to cytoplasmic targets has been caused. Under these conditions is then not surprising that the accumulating cytoplasmic damage converges into (several) pathways and activates different mechanisms that eventually favour BAX/BAK dependent mitochondrial apoptosis.

Our response: This point raised by the reviewer is well taken. The damage to the Golgi and ER occurs at an early stage and at low concentrations of redaporfin PDT. We have performed additional kinetic and dose-response analyses to relate these effects to delayed cell killing. Moreover, as explained in the response to the general critique, we have addressed the question whether oxidative damage to the Golgi in a specific way can lead to cell death by investigating another system of local oxidative damage. This system is based on the expression of a peroxidase (horse radish peroxidase or HRP) in the lumen of the Golgi apparatus by fusion with a Golgi targeting sequence. The addition of low concentrations of diaminobenzidine (DAB) and H_2O_2 the formation of an insoluble precipitate that specifically inactivates Golgi dynamics and function (Jollivet et al., 2007, Mol Biol Cell. 18:4637-47). This system has been used to show that ROS-mediated damage initiated at the level of the Golgi or the ER can kill cells (Figure 6 and Figure S11).

Specific comment No. 3 by Reviewer No. 3: It is not clear which is the eIF2a-P kinase involved as the expression of the EIF2AK3 = PERK is down regulated after PDT.

Our response: We have generated cell lines in which each of the four EIF2alpha kinase (EIF2K1, EIF2K2, EIF2K3 and EIF2K4) has been knocked out, and we have shown that the sole EIF2alpha kinase responsible for EIF2alpha phosphorylation induced by redaporfin PDT is EIF2K1 (Figure 3C-G).

Specific comment No. 4 by Reviewer No. 3: It is often not clear which controls are shown in various immunofluorescence analysis (see e.g. Fig.3 etc), light but no compound? Compound without light? And at which concentration?

Our response: The requested information has been added to the manuscript.

Specific comment No. 5 by Reviewer No. 3: Basically all or most of the assays are performed at 6 h post treatment why? Kinetics analysis should be performed as well. Please note that in several Figures (e.g. imaging analysis) the concentration of redaporfin used is not mentioned making it very hard to gauge whether the PDT effect is specific and a direct consequence of the ER/Golgi targeting.

Our response: We have performed additional kinetic and dose-response analyses to relate the redaporfin effects to cell killing (Figure S1A,B). Moreover, we indicated the concentrations of redaporfin used in each figure in the legends.

Specific comment No. 6 by Reviewer No. 3: Fig. 4: There is no clear concentration dependency in ROS production after PDT, which is strange. Overall ROS production seems rather low, based on this measurement and it should be important to compare it to a positive control such as hydrogen peroxide as well as to show how it evolves during time. Likewise, in Fig. 6: the effects of PDT on cytosolic calcium elevations are also rather minor. This seems to be a secondary effect of the many alterations induced by PDT when high concentrations of redaporfin are used. Also, here the authors should add a positive control for store depletion in these cells (e.g. thapsigargin or IP3 generating agents like ATP) to be compared with and show the kinetics of this event, which is claimed to be immediate.

Our response: We performed additional ROS measurements by means of fluorescent biosensors such as dihyroethidien (Figure S8) using menadione as positive control. Moreover, we studies the magnitude of the calcium elevation in comparison to appropriate positive controls, showing that PDT can cause an increase in intracellular calcium levels that is equivalent to the one induced by thapsigargin.

Specific comment No. 7 by Reviewer No. 3: One of the most important conclusions that the authors draw is that the release of a set of cytokines is reduced by PDT. However although the authors mention that redaporfin PDT mediates a form of cell death with important immunomodulatory consequences, the impact of this observation is not explored while the authors are top-notch experts in this domain.

Our response: We added an experiment showing that redaporfin PDT can induce immunogenic cell death (ICD) to demonstrate the capacity of this agent to stimulate anticancer immune responses (Figure 3I,J and Figure S7).

2nd Editorial Decision 18th April 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. It has now been seen by the three original referees, whose comments are enclosed below. As you will see, all referees find that their concerns have been sufficiently addressed and are now broadly in favour of publication, pending minor issues are convincingly addressed.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending satisfactory revision of the remaining issues related to integration of published work and interpretation of the current data.

REFEREE REPORTS

Referee #1:

The authors have generally addressed all of my major comments from the original submission with new experiments and additional revisions to the text. The manuscript has been improved from these revisions and now seems suitable to for publication of these results.

Referee #2:

The authors have partially addressed the concerns raised in my previous review, but some issues still remain unclear.

In the revised manuscript the authors have introduced a new set of experiments based on the approach developed by one of the co-authors involving cells expressing Golgi-targeted HRP. A similar approach has also been reported by Guizzunti et al. (PMID: 27791030) who also showed that, once treated with DAB/H2O2, these cells undergo a mitotic block (due to the inability of the Golgi complex to undergo mitotic fragmentation) and consequent cell death. The authors should consider these published data when they hypothesize that the only mechanism sensitizing these cells to killing by DAB/H2O2 is due to oxidative stress at the Golgi complex.

As suggested, the authors have applied nocodazole treatment as a diagnostic test of the association of RDP with the Golgi complex. As expected, they got the dispersal of the Golgi complex into small punctae (i.e. ministacks), and RDP also appears localized in multiple cytoplasmic dots that, however, do not co-localize with the Golgi marker, at least in the images shown in Fig. S1I,J. This mismatching might possibly be due to the extremely prolonged nocodazole treatment (24hr) that the authors applied, over-interpreting my suggestion of a prolonged treatment, which in fact implied a typical 3hr treatment.

As for the possibility that BFA could rescue the RDP toxicity via a Golgi stress response involving ARF4, the authors mention in their reply that they tested whether the ARF4-KD would attenuate the BFA-induced rescue of RDP toxicity, but they found this not to be the case. However, in their reply they do not show these data, but data referring to ARF4 and ARF4-KD in cells treated with RDP and not with RDP+BFA.

As for the question concerning cytokine secretion, the authors have assessed that the majority of cytokines are secreted via conventional (i.e. BFA-sensitive) secretion. These data should induce the authors to reconsider their interpretation/hypothesis that the impaired cytokine secretion induced by RDP might contribute to the RDP toxicity since BFA, which in fact blocks cytokine secretion, rescues in part the RDP toxicity. I also noticed in the cytokine array, provided by the authors in their reply, that the intracellular content of many cytokines (and not only their secreted fraction) is also reduced by RDP, suggesting that RDP treatment is likely to induce a complex transcriptional response that may take part to its toxicity.

Referee #3:

The authors have improved the MS and put serious effort to experimentally support the main conclusions of their work.

However, one unclear aspect that has emerged here from the inclusion of the in vitro/in vivo data is why, 5 micromolar redaporfin-PDT -while eliciting even better than the 10 micromolar redaporfin-PDT dose the hallmarks of ICD, namely CALR, ATP and HMGB1, in vitro (S7), yet fails completely to increase the anticancer vaccination potential in vivo (Fig. 3K), for which the highest dose is required?
Since all the mechanistic data about ER/GA cellular damage, eIF2a-P, ROS and cell death, cytokines release, are induced (better or equally well) by the 5 uM redaporfin+light, something else beyond cell death and the pathways analyzed here seems to be required for the tumor-rejecting ability of redaporfin-PDT-based anticancer vaccines.

While it is clear that the MS focuses mainly on the mechanistic details of the ER/GA damage and cell death pathway induced in response to redaporfin-PDT, given the addition of the in vivo data, it would be important to provide an explanation about this PDT-dose discrepancy in the current MS. The authors should consider to expand the discussion of their own additional mechanistic (e.g. on the cytoprotection exerted by the EIF2AK1 kinase?) as well as in vivo data (e.g. on immunogenicity of redaporfin-PDT?) as well.

Minor:
Please add the (commercial?) source and purity of hypericin.

2nd Revision - authors' response 23rd April 2018

Point-by-point reply to the Editors:

Point-by-point reply to Referee #2:

General critique by Reviewer No. 2: The authors have partially addressed the concerns raised in my previous review, but some issues still remain unclear.

Our response: We have addressed each of the remaining issues in our point-by-point reply, as stated below.

General comment No. 1 by Reviewer No. 2: In the revised manuscript, the authors have introduced a new set of experiments based on the approach developed by one of the co-authors involving cells expressing Golgi-targeted HRP. A similar approach has also been reported by Guizzunti et al. (PMID: 27791030) who also showed that, once treated with DAB/H2O2, these cells undergo a mitotic block (due to the inability of the Golgi complex to undergo mitotic fragmentation) and consequent cell death. The authors should consider these published data when they hypothesize that the only mechanism sensitizing these cells to killing by DAB/H2O2 is due to oxidative stress at the Golgi complex.

Our response: We have cited the paper by Guizzanti et al. as we mention the system in which cells expressing Golgi-targeted horse-radish peroxidase are killed by local oxidative stress.

General comment No. 2 by Reviewer No. 2: As suggested, the authors have applied nocodazole treatment as a diagnostic test of the association of RDP with the Golgi complex. As expected, they got the dispersal of the Golgi complex into small punctae (i.e. ministacks), and RDP also appears localized in multiple cytoplasmic dots that, however, do not co-localize with the Golgi marker, at least in the images shown in Fig. S1I,J.
This mismatching might possibly be due to the extremely prolonged nocodazole treatment (24hr)

that the authors applied, over-interpreting my suggestion of a prolonged treatment, which in fact implied a typical 3hr treatment.

Our response: We used different incubation periods (4 and 24 h) of treatment with nocodazol to disperse the Golgi, yet observed the best effect for a prolonged incubation period. Importantly, redaporfin colocalizes with the GALT1-GFP marker when other GA-disrupting agents (GCA and BFA) were used, which is in accordance with redaporfin´s GA tropism.

General comment No. 3 by Reviewer No. 2: As for the possibility that BFA could rescue the RDP toxicity via a Golgi stress response involving ARF4, the authors mention in their reply that they tested whether the ARF4-KD would attenuate the BFA-induced rescue of RDP toxicity, but they found this not to be the case. However, in their reply they do not show these data, but data referring to ARF4 and ARF4-KD in cells treated with RDP and not with RDP+BFA.

Our response: Increased ARF4 was observed at short time points after redaporfin-PDT and after prolonged incubation time (24 h) with BFA. However, no additional differences were found in the levels of ARF4 when PDT was combined with BFA (6 h), which suggests that the ARF4 pathway is not involved in the BFA-mediated cytoprotective effect. Moreover, Jan H. Reiling *et al* (PMID: 24185178) showed that ARF4 silencing only preserves viability, GA integrity and secretion when chronic treatment with low concentrations (20 ng/μL) of BFA were used. At high concentration (> 1 μg/mL) of BFA no cytoprotection was achieved by silencing ARF4. Thus, the BFA concentration used in our work (5 μg/mL) is not expected to be sensitive to the ARF4 pathway. Brefeldin A treatment reduced the lethal action of redaporfin-PDT on cells. This has been made clear in the revised version of the paper.

General comment No. 4 by Reviewer No. 2: As for the question concerning cytokine secretion, the authors have assessed that the majority of cytokines are secreted via conventional (i.e. BFA-sensitive) secretion. These data should induce the authors to reconsider their interpretation/hypothesis that the impaired cytokine secretion induced by RDP might contribute to the RDP toxicity since BFA, which in fact blocks cytokine secretion, rescues in part the RDP toxicity. I also noticed in the cytokine array, provided by the authors in their reply, that the intracellular content of many cytokines (and not only their secreted fraction) is also reduced by RDP, suggesting that RDP treatment is likely to induce a complex transcriptional response that may take part to its toxicity.

Our response: The reviewer is right, RDP does reduce general transcription, and this had been shown in the revised version of the paper.

Point-by-point reply to Referee #3:

General critique by Reviewer No. 3: The authors have improved the MS and put serious effort to experimentally support the main conclusions of their work.

Our response: We thank the reviewer for appreciating our effort. Responses to the remaining specific points of critique are listed below.

Specific comment No. 1 by Reviewer No. 3: However, one unclear aspect that has emerged here from the inclusion of the in vitro/in vivo data is why, 5 micromolar redaporfin-PDT -while eliciting even better than the 10 micromolar redaporfin-PDT dose the hallmarks of ICD, namely CALR, ATP and HMGB1, in vitro (S7), yet fails completely to increase the anticancer vaccination potential in vivo (Fig. 3K), for which the highest dose is required?
Since all the mechanistic data about ER/GA cellular damage, eIF2a-P, ROS and cell death, cytokines release, are induced (better or equally well) by the 5 uM redaporfin+light, something else beyond cell death and the pathways analyzed here seems to be required for the tumor-rejecting

ability of redaporfin-PDT-based anticancer vaccines. While it is clear that the MS focuses mainly on the mechanistic details of the ER/GA damage and cell death pathway induced in response to redaporfin-PDT, given the addition of the in vivo data, it would be important to provide an explanation about this PDT-dose discrepancy in the current MS.

Our response: It is important to point out that both concentrations (5 μM and 10 μM of redaporfin-PDT) significantly confer anti-tumor protection and indeed, vaccines generated with 5 μM of redaporfin-PDT strongly impaired tumor growth (Fig 3J).
The hallmarks of ICD induced by redaporfin-PDT were tested on human U2OS osteosarcoma (eIF2α) and mouse non small cell lung cancer TC-1 (CALR, ATP and HMGβ1) in vitro and then on mouse TC-1 in vivo (because only mouse cancer cell can be introduced into immunocompetent mice) was performed. Vaccines were prepared at 1.5 h after cells irradiation whereas the ICD hallmarks were assessed in vitro at 2.5 h for eIF2α and 5 h for CALR, ATP and HMGB1. Hence subtle species or cell type-and time points-related differences may account for the criticized minor discrepancy in dose response curves.

Specific comment No. 2 by Reviewer No. 3: The authors should consider to expand the discussion of their own additional mechanistic (e.g. on the cytoprotection exerted by the EIF2AK1 kinase?) as well as in vivo data (e.g. on immunogenicity of redaporfin-PDT?) as well.

Our response: We briefly touched on the two new aspects of the paper, namely the role of EIF2AK1 in cell death killing/immunogenicity in the Discussion of the paper.

Minor comment by Reviewer No. 3: Please add the (commercial?) source and purity of hypericin.

Our response: This has been done.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Guido Kroemer
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2017-98354

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Experiements have been conducted in triplicates and have been repeated several times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Ten animals per group have been used for in vivo studies to evaluate tumor growth and vaccination efficacy.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals have been excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	We randomly distributed animals to different treatment groups.
For animal studies, include a statement about randomization even if no randomization was used.	We randomly distributed animals to different treatment groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	We randomly distributed animals to different treatment groups.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	One or two-way ANOVA or t-test have been used where appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	A gaussian distribution was assumed.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Primary antibodies against CALR (ab ab2907), TOMM20 (ab78547), GBF1 (ab 86071), GM130 (ab 52649), ERp57 (ab 10287) and actin-HRP (ab 49900) were purchased from Abcam (Cambridge, UK). Antibodies for PERK (3192S), P-eIF2α (Ser51) (ab32157), eIF2α (9722S) and P-histone H2AX (9720) came from Cell Signaling Technology (Danvers, MA, USA) and the antibody against B4GALT1 (Abnova, PAB20512) from Abnova (Taipei, Taiwan)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Mycoplasma-free human osteosarcoma U2OS, U2OS biosensor cells (GALT1-GFP, CALR-GFP, GFP-ATF4, GFP-ATF6, XBP1-DBD-Venus (XBP1-GFP), BAX-GFP, SMAC-GFP), TC1, Hela, A549 and HCT116 were routinely checked for contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mus musculus; C57BL/6; female; 6-8 weeks of age; WT. All mice were kept in a pathogen–free, environmental-controlled animal facility with 12 h light/dark cycles and had food and water ad libitum. Six- to eight-week-old female wild-type C57BL/6 mice were obtained from ENVIGO France (Gannat, France).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed in compliance with the EU Directive 63/2010 and specific ethic protocol (Protocol 2354-2015102013453410 v2 that was approved by the Ethical Committee of the Gustave Roussy Campus Cancer, CEEA IRCIV/IGR no. 26, registered at the French Ministry of Research)
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA