Mitochondrial complex I inhibition triggers a mitophagy-dependent ROS increase leading to necroptosis and ferroptosis in melanoma cells

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Inhibition of complex I (CI) of the mitochondrial respiratory chain by BAY 87-2243 (‘BAY’) triggers death of BRAFV600E melanoma cell lines and inhibits in vivo tumor growth. Here we studied the mechanism by which this inhibition induces melanoma cell death. BAY treatment depolarized the mitochondrial membrane potential (Δψ), increased cellular ROS levels, stimulated lipid peroxidation and reduced glutathione levels. These effects were paralleled by increased opening of the mitochondrial permeability transition pore (mPTP) and stimulation of autophagosome formation and mitophagy. BAY-induced cell death was not due to glucose shortage and inhibited by the antioxidant α-tocopherol and the mPTP inhibitor cyclosporin A. Tumor necrosis factor receptor-associated protein 1 (TRAP1) overexpression in BAY-treated cells lowered ROS levels and inhibited mPTP opening and cell death, whereas the latter was potentiated by TRAP1 knockdown. Knockdown of autophagy-related 5 (ATG5) inhibited the BAY-stimulated autophagosome formation, cellular ROS increase and cell death. Knockdown of phosphatase and tensin homolog-induced putative kinase 1 (PINK1) inhibited the BAY-induced Δψ depolarization, mitophagy stimulation, ROS increase and cell death. Dynamin-related protein 1 (Drp1) knockdown induced mitochondrial filamentation and inhibited BAY-induced cell death. The latter was insensitive to the pancaspase inhibitor z-VAD-FMK, but reduced by necroptosis inhibitors (necrostatin-1, necrostatin-1s) and knockdown of key necroptosis proteins (receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and mixed lineage kinase domain-like (MLKL)). BAY-induced cell death was also reduced by the ferroptosis inhibitor ferrostatin-1 and overexpression of the ferroptosis-inhibiting protein glutathione peroxidase 4 (GPX4). This overexpression also inhibited the BAY-induced ROS increase and lipid peroxidation. Conversely, GPX4 knockdown potentiated BAY-induced cell death. We propose a chain of events in which: (i) CI inhibition induces mPTP opening and Δψ depolarization, that (ii) stimulate autophagosome formation, mitophagy and an associated ROS increase, leading to (iii) activation of combined necroptotic/ferroptotic cell death.

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To sustain their function and proliferation melanoma cells often shift their metabolism from mitochondrial towards glycolytic ATP production.1 However, various oncogenes and tumor suppressors (e.g. c-myc, Ras and Oct1), as well as hypoxia, stimulate mitochondrial metabolism.2–5 A key oncogenic event in melanoma is the occurrence of mutations in v-Raf murine sarcoma viral oncogene homolog B (BRAF). This protein kinase is involved in RAS–RAF–MEK–ERK mitogen-activated protein kinase signaling.1 Among the BRAF mutations, the V600E gain-of-function substitution is most commonly observed (i.e. in 40–60% of all melanomas). In addition, BRAF mutations were demonstrated in 40% of the thyroid cancers, 30% of the ovarian cancers and 20% of the colorectal cancers.6,7 Despite novel antitumour therapeutics, metastatic melanoma still has a poor prognosis due to the development of chemotherapy resistance.8 Importantly, acquired resistance to BRAF or MEK inhibitors was paralleled by increased mitochondrial biogenesis and activity in melanoma cells with BRAFV600E and NRAS mutations.9,10 This suggests that concomitant inhibition of mitochondrial function might constitute a potential therapeutic strategy.11,12 Proper mitochondrial functioning requires activity of the mitochondrial oxidative phosphorylation (OXPHOS) system.13–15 This system is embedded in the mitochondrial inner membrane (MIM) and consists of four electron transport chain (ETC) complexes (CI–CIV) and the F0F1-ATP-synthase (CV). OXPHOS generates ATP through chemiosmotic coupling by linking ETC-mediated proton efflux across the MIM to CV-mediated trans-MIM proton influx.16 The latter is driven by the inward-directed proton motive force across the MIM, which consists of an electrical (Δψ) and chemical (ΔpH) component, with Δψ contributing ~85% to the total PMF.17

Using a panel of BRAFV600E melanoma cell lines, we recently demonstrated that BAY 87-2243 (BAY; Ellinghaus et al.18), a potent inhibitor of the first OXPHOS complex (CI or NADH:ubiquinone oxidoreductase; EC 1.6.5.3), dose-dependently decreases cell viability.19 The effect of BAY was paralleled by increased levels of cellular ROS and a
BAY treatment induces cell death in BRAFV600E melanoma cells. In this study, we used two BRAFV600E melanoma cell lines (G361 and SK-MEL-28) to investigate the mechanism of BAY-induced cell death. We previously demonstrated that BAY treatment for 72 h reduced the viability of these cells in a dose-dependent manner with IC50 values in the nanomolar range (Figure 1a). Within this timeframe, BAY did not affect the viability of human epidermal melanocytes (Hema-LP) and primary human skin fibroblasts (CT5120; Supplementary Figure S1A). Experiments were performed at an ambient glucose concentration of 5 mM. Importantly, regular refreshment of the culture medium did not prevent the BAY-induced reduction in cell viability, arguing against glucose depletion being responsible for this reduction (Supplementary Figure S1B). In agreement with our previous study, it was found that BAY displayed a half-maximal inhibition of cell viability (T1/2) at 20 and 66 h for G361 and SK-MEL-28 cells, respectively (Figure 1b). This means that BAY treatment (2 min) increased the number of mPTP openings are photoinduced by controlled illumination. To assess whether TRAP1 and mPTP opening play a role in BAY-induced cell death, we used a previously described method to measure mPTP opening. In brief, cells are stained with the fluorescent cation tetrakis(dimethylamino)ethylene methyl ester (TMRM), which accumulates in the mitochondrial matrix in a Δψ-dependent manner. Next, reversible mPTP openings are photoinduced by controlled illumination of TMRM (Supplementary Figure S2 and Supplementary Movie). For quantification, individual mPTP openings were manually counted from ‘difference images’ calculated by subtracting the (n−1)th image from the nth image in the recorded TMRM image sequence (Figure 2a; e.g. img420−img421). In these difference images individual mPTP openings are highlighted in black (Figure 2a; arrowhead). Acute BAY treatment (2 min) increased the number of mPTP openings to a similar extent in both cell lines (Figure 2b). Of note, given the short incubation time we here used a 40 nM BAY concentration. This was the lowest BAY concentration that maximally inhibited oxygen consumption in G361 and SK-MEL-28 cells during acute treatment. The acute effect of BAY on mPTP opening (Figure 2b) and its chronic effect on cell death (Figure 2d) were inhibited by pre-treatment (2 h) with the mPTP inhibitor cyclosporin A (CsA). TRAP1 overexpression (Supplementary Figure S3A) mimicked both effects of CsA (Figures 2b–e). These results suggest that TRAP1 inhibits mPTP opening to prevent cell death in BRAFV600E melanoma cells and that TRAP1 overexpression is required to inhibit BAY-induced mPTP opening and cell death. In agreement with this idea, TRAP1 knockdown (Supplementary Figure S3A) increased the sensitivity to BAY-induced cell death as compared with siCTRL (Figure 2e). TRAP1 overexpression significantly reduced the increase in cellular ROS levels after 24 h of BAY treatment (Figure 2c). Taken together, these results suggest that stimulation of mPTP opening is a key component of BAY-induced cell death and that endogenous TRAP1 levels and/or TRAP1 activity are too low to protect against this death. The fact that TRAP1 overexpression lowers ROS levels in BAY-treated cells suggests that TRAP1 directly reduces these levels or that mPTP opening induces ROS.

Figure 1  Dose- and time-dependent effect of BAY and medium refreshment on cell viability. (a) Dose-dependent effect of BAY (N = 2, n = 4) on the viability of G361 and SK-MEL-28 cells (at 72 h). A Boltzmann equation was used to determine the IC50 (x0) value: \[ y = \frac{A_2 - A_1}{1 + \exp\left(-\frac{x - x_0}{d}\right)} + A_1 \] \[ x = \frac{(A_2 - A_1)\left(1 + \exp\left(-\frac{x - x_0}{d}\right)\right)}{A_2 - A_1} + A_1 \] Statistics: In panel b, significant differences with respect to the SK-MEL-28 cell line are indicated by \( P < 0.05 \) and \( **P < 0.0001 \).
of the number of green GFP puncta per cell, representing autophagosomes, revealed a significant BAY-induced increase (Supplementary Figure S4A and B and Figure 3a). Co-incubation with bafilomycin A1 (BafA1), which prevents fusion of autophagosomes with lysosomes,25 further increased the number of green GFP puncta. This suggests that BAY does not inhibit autophagosome fusion but stimulates autophagosome formation. We previously demonstrated that the antioxidant α-tocopherol (TOC) inhibits the BAY-induced increase in cellular ROS levels and cell death.19 Here we observed that 24 h cotreatment with TOC fully prevents BAY-induced stimulation of autophagosome formation (Figure 3a). To further investigate the role of autophagosome formation in BAY-induced cell death, we performed knockdown of autophagy-related 5 (ATG5; Supplementary Figure S3B). This protein is required for autophagosome formation26 and its knockdown significantly reduced the BAY-induced increase in green puncta (Figure 3a). Remarkably, ATG5 knockdown also prevented the ROS increase after 24 h BAY treatment (Figure 3b). Analysis of cellular mitochondrial content using Mitotracker Green FM (MG) revealed that BAY treatment reduced MG staining and that this reduction was prevented by ATG5 knockdown (Figure 3c). This suggests that BAY-induced stimulation of ATG5-dependent autophagosome formation mediates mitochondria-specific autophagy (i.e. mitophagy). ATG5 knockdown inhibited BAY-induced loss of cell viability (Figure 3d). Taken together, these data suggest that TOC-sensitive ‘triggering ROS’ is required for mPTP opening and subsequent ATG5-mediated autophagosome formation. Moreover, our results suggest that ATG5-mediated autophagosome formation is required for sustained elevated ROS and increased mitophagy and eventually BAY-induced cell death.

**PINK1 knockdown inhibits BAY-stimulated mitophagy, Δψ depolarization, ROS increase and cell death.** To demonstrate the potential involvement of mitophagy in BAY-induced cell death, cells were transfected with GFP-LC3 (marking autophagosomes) and stained with MitoTracker Red (MR) to highlight mitochondria. Then, the number of green GFP puncta colocalizing with MR was determined to quantify the amount of mitophagy (Supplementary Figure S4C;
arrowheads). BAY treatment (24 h) stimulated mitophagy (Figure 4a) and induced Δψ depolarization (Figure 4b). Phosphatase and tensin homolog-induced putative kinase 1 (PINK1) is a key regulator of mitophagy that recruits autophagy receptors to mitochondria upon Δψ depolarization.27 PINK1 knockdown (Supplementary Figure S3C) inhibited the BAY-induced changes (Figures 4a–d) as well as the BAY-induced reduction in cell viability (Figure 4e). The extent of mitophagy induction was positively correlated with the degree of Δψ depolarization (Supplementary Figure S1C), compatible with the key role of Δψ depolarization in mitophagy induction.28 These results suggest that BAY induces Δψ depolarization, thereby stimulating PINK1-dependent mitophagy and an ensuing increase in cellular ROS levels.

Drp1 knockdown induces mitochondrial filamentation and inhibits BAY-stimulated cell death. In a starvation model,29 autophagosomal degradation of mitochondria was prevented by mitochondrial elongation (i.e. filamentation) mediated by downregulation of dynamin-related protein 1 (Drp1). This GTPase is one of the key mediators of mitochondrial fission.30 Two Drp1 isoforms, the brain (‘b’) and ubiquitous (‘u’) form, were detected in G361 and SK-MEL-28 cells. Knockdown of both isoforms (Supplementary Figure S3D) induced mitochondrial filamentation, both in the absence and presence of BAY (Figures 5a and b). Drp1 knockdown also inhibited the BAY-induced reduction in cell viability (Figure 5c). Compatible with these results (Supplementary Figure S1D), BAY-induced cell death was inhibited by a chemical inhibitor of Drp1 activity (Mdivi1; Cassidy-Stone et al.31). Mdivi1 also inhibited the BAY-induced stimulation of mitophagy (Supplementary Figure S1E). These results suggest that BAY-induced mitophagy stimulation and cell killing are inhibited by mitochondrial filamentation.
BAY-induced cell death involves necroptosis and ferroptosis rather than apoptosis. Stimulation of autophagy was linked to induction of both apoptotic and necroptotic cell death.\textsuperscript{32–36} The broad-spectrum caspase inhibitor z-VAD-FMK was unable to prevent BAY-induced cell death (Figure 6a), arguing against involvement of apoptosis. On the other hand, Nec-1, which blocks necroptosis by inhibiting the activity of receptor-interacting serine/threonine-protein kinase 1 (RIPK1),\textsuperscript{37,38} inhibited BAY-induced cell death (Figures 6a and b). However, Nec-1 not only inhibits RIPK1 but also indoleamine-2,3-dioxygenase, which catalyzes the conversion of tryptophan into kynurenine.\textsuperscript{39,40} Therefore, we next investigated the effect of Nec-1s (or 7-Cl-O-Nec-1), a RIPK1 inhibitor displaying increased stability and specificity.\textsuperscript{39,41} Nec-1s also inhibited BAY-induced cell death, albeit to a lesser extent than Nec-1 (Figure 6b), suggesting that this death is partially mediated by necroptosis. Compatible with this conclusion, knockdown of RIPK1 inhibited the BAY-induced loss in cell viability (Supplementary Figure S3E and Figure 6c).

However, RIPK1 kinase activity is not only required for necroptosis but also for apoptosis.\textsuperscript{42} For this reason, we performed knockdown of the mixed lineage kinase domain-like protein (MLKL), the presence of which is essential for necroptosis induction.\textsuperscript{41,43} Similar to RIPK1, MLKL knockdown inhibited the cell viability loss in BAY-treated cells (Supplementary Figure S3F and Figure 6d). Nec-1s only partially blocked BAY-induced cell death (Figure 6b), suggesting that necroptosis is not the only death mechanism involved. In this sense, autophagy can also promote ferroptosis,\textsuperscript{44} a mode of cell death that is negatively regulated by glutathione peroxidase 4 (GPX4) and characterized by increased iron-dependent ROS production, glutathione (GSH) depletion and lipid peroxidation.\textsuperscript{41} Supporting the involvement of ferroptosis, BAY treatment increased cytosolic ROS levels (see above), reduced cellular GSH levels (Figure 7a) and stimulated TOC-sensitive lipid peroxidation (Figure 7c). Moreover, we previously demonstrated that cotreatment with the GSH precursor N-acetyl cysteine inhibited the BAY-induced increase in cellular.

**Figure 4** Effect of PINK1 knockdown on the BAY-induced stimulation of mitophagy, Δψ depolarization, reactive oxygen species (ROS) levels and reduction in cell viability. (a) Effect of BAY treatment, siPINK1-no. 1 and siPINK1-no. 2 on the number of green puncta colocalizing with mitochondria in G361 and SK-MEL-28 cells (at 24 h; N = 3, n ≥ 14). (b) Similar to panel a, but now for the effect on mitochondrial membrane potential (i.e. the JC-1 red/green ratio signal; N = 3, n = 6). (c) Similar to panel a, but now for the effect on cellular ROS levels (N = 3, n = 6). (d) Similar to panel a, but now for the effect on cellular MG fluorescence intensity (G361: at 24 h; SK-MEL-28: at 24 h; N = 3, n = 6). (e) Effect of BAY on the viability of cells (G361: at 48 h; SK-MEL-28: at 72 h; N = 3, n = 6) transfected with siCTRL, siPINK1-no. 1 and siPINK1-no. 2. Statistics: Significant differences relative to the indicated conditions are marked by *P < 0.05, **P < 0.01 and ***P < 0.001. In panel c statistical analysis was performed using a one-sample t-test against a value of 100. In panel d, NS indicates nonsignificant and significant differences with the (siCTRL+10 nM BAY condition) are marked by *&. In panel (e) significant differences (P < 0.05) between conditions are marked by symbols ($, &).
The ferroptosis inhibitor ferrostatin-1 (Fer-1; Dixon et al.) partially prevented BAY-induced cell death (Figures 6a and b). Overexpression of the ferroptosis-inhibiting enzyme GPX4 (Supplementary Figure S3G) inhibited the BAY-stimulated increase in cellular ROS levels (Figure 7b) and lipid peroxidation (Figure 7c). In agreement with this result, GPX4 overexpression and knockdown (Supplementary Figure S3G) inhibited and potentiated the BAY-induced reduction in cell viability, respectively (Figure 7d). Overall, these results suggest that BAY treatment does not induce apoptotic cell death but triggers combined necroptosis and ferroptosis.

**Discussion**

Here we provide mechanistic insight into how BAY-induced inhibition of mitochondrial CI induces death in BRAF*V600E* melanoma cells. A chain of events is proposed (Figure 8), in which CI inhibition stimulates mPTP opening, Δψ depolarization, autophagosome formation and mitophagy induction. The latter increases cellular ROS levels that stimulate lipid peroxidation and GSH depletion, leading to combined necroptotic and ferroptotic cell death.

**Specificity of BAY for melanoma cells.** Within the used timeframe, BAY-induced CI inhibition effectively killed G361 and SK-MEL-28 melanoma cells, without affecting the viability of non-cancer cells. It was proposed that cancer cells displaying a higher basal ROS level than normal cells can be therapeutically targeted by ROS-inducing anticancer agents. This suggests that BAY treatment increases ROS levels beyond a death-inducing threshold in cancer cells but not in non-cancer cells. Compatible with this idea, we observed that G361 cells displayed a fourfold higher basal ROS level than SK-ML-28 cells (Supplementary Figure S1F). This might explain why BAY-induced killing occurred at a much earlier time point in G361 cells than in SK-MEL-28 cells.

**Role of external glucose.** We previously demonstrated in C2C12 myoblasts that acute (30 min) inhibition of CI or CIII stimulates glycolytic ATP production to prevent ATP shortage. Similarly, chronic (5 weeks) CI inhibition induced a fully glycolytic phenotype in primary skin fibroblasts, associated with an extreme sensitivity to glucose withdrawal. These findings led us to propose that BAY-induced CI inhibition might, in addition to increasing cellular ROS levels, induce a shortage of glucose contributing to cell death. Compatible with this idea and our current results,
evidence was provided that chronic glucose depletion induces autophagic cell death in B16F1 melanoma cells.\textsuperscript{50} Moreover, we previously observed that increasing the external glucose concentration from 5 to 25 mM inhibited BAY-induced cell death.\textsuperscript{19} Here we demonstrate that regular medium refreshment does not markedly inhibit the BAY-induced reduction in cell viability, arguing against glucose shortage having a role in this process. In this sense, our observation that 25 mM glucose also reduces the BAY-induced ROS increase\textsuperscript{19} suggests that the inhibitory effect of high external glucose on BAY-induced cell death is ROS-mediated.

**Role of mPTP opening.** Our results highlight an important role for mPTP opening in the death-inducing mechanism of BAY. TRAP1 overexpression also reduced the BAY-induced increase in cellular ROS levels. Supported by evidence in the literature,\textsuperscript{22,51} this suggests that (part of the generated) ROS acts as an upstream activator of mPTP opening (Figure 8: ‘triggering ROS’). Inhibition of BAY-induced cell death by TOC might be due to this antioxidant preventing mPTP activation (by scavenging the ‘triggering ROS’). Compatible with this explanation, preliminary evidence suggests that the TOC derivative Trolox inhibits mPTP opening in primary human skin fibroblasts (Werner J.H. Koopman, personal observation). Alternatively, TOC might prevent cell death induction by lowering the levels of mitophagy-induced ‘killing ROS’ (Figure 8). TRAP1 knockdown potentiated the BAY-induced reduction in cell viability, likely caused by increased ROS levels.\textsuperscript{52} This potentiation, in combination with the protective effect of TRAP1 overexpression, suggests that endogenous TRAP1 has a role in protecting the cells against BAY-induced cell death but that these levels and/or TRAP1 activity are insufficiently high. PINK1 can phosphorylate TRAP1 to prevent apoptosis induced by oxidative stress.\textsuperscript{53} This would mean that PINK1 knockdown should reduce TRAP1 activity, thereby potentiating BAY-induced cell death. In contrast, we observed that PINK1 knockdown antagonized the effects of BAY, arguing against PINK1 acting via TRAP1.

**Role of autophagosome formation and mitophagy.** BAY treatment stimulated autophagosome formation, which was inhibited by the antioxidant TOC. In parallel, TOC also inhibited the BAY-induced increase in cellular ROS levels and lipid peroxidation. This suggests that increased ROS
and/or lipid peroxidation are required for stimulation of autophagosome formation, supported by previous findings.\(^{28,54-56}\) Upon \(\Delta \psi\) depolarization, mitophagy is triggered by PINK1 accumulation on the surface of mitochondria ultimately leading to their autophagosomal uptake and lysosomal degradation.\(^{57}\) Knockdown of ATG5 and PINK1 inhibited autophagosome formation and mitophagy, respectively, suggesting that BAY-induced mPTP opening stimulates autophagosome formation,\(^{24,26}\) allowing subsequent mitochondrial removal by mitophagy.\(^{27}\) Supporting this mechanism is the fact that mitochondrial content was reduced by up to 50% in BAY-treated cells and that this reduction was inhibited by knockdown of ATG5 or PINK1. Importantly, both ATG5 and PINK1 knockdown prevented the BAY-induced increase in cellular ROS levels and cell viability reduction. Stimulation and inhibition of autophagy in an angiogenesis model also increased and decreased ROS levels, respectively.\(^{58}\) This strongly suggests that increased cellular ROS levels and triggering of cell death are downstream effectors of autophagy/mitophagy (Figure 8). Although it might be possible that mitochondria produce increased amounts of ROS at some time during their mitophagic degradation, the exact mechanism by which autophagy/mitophagy increases ROS levels requires further investigation. PINK1 knockdown/knockout has been associated with mitochondrial fragmentation, increased ROS levels, \(\Delta \psi\) depolarization and stimulation of mPTP opening.\(^{59-61}\) In contrast, here we observed that PINK1 knockdown by itself induced apparent \(\Delta \psi\) hyperpolarization and, consistently, inhibited the BAY-induced stimulation of mitophagy, \(\Delta \psi\) depolarization and ROS increase. These effects might be a melanoma- or context-specific phenomenon that, to the best of our knowledge, was not described previously. Whether mitophagy requires prior Drp1-mediated mitochondrial fragmentation is still controversial.\(^{62}\) For instance, mitochondrial division during mitophagy can occur in a Drp1-independent manner.\(^{63}\) On the other hand, Drp1-dependent mitochondrial fragmentation might facilitate mitophagy by creating small-size fragments facilitating autophagosomal uptake.\(^{62}\)

### Role of mitochondrial morphology

We demonstrated that mitochondria display a non-filamentous morphology in G361 and SK-MEL-28 cells and that BAY-induced cell death is not
associated with detectable changes in this morphological phenotype. Drp1 knockdown induced a filamentous mitochondrial morphology that was not affected by BAY treatment and also inhibited the BAY-induced reduction in cell viability. BAY-induced mitophagy and cell death were reduced by Mdiv1, a chemical inhibitor of Drp1 activity. These results suggest that mitochondrial filamentation prevents BAY-induced stimulation of mitophagy and thereby cell death. In parallel, mitochondrial filamentation might also mitigate mitochondrial dysfunction by damage and/or antioxidant sharing, thereby inhibiting the ROS trigger for mPTP-induced mitophagy induction (Figure 8).

In addition to its role in mitophagy PINK1 is a known regulator of the mitochondrial fission and fusion machinery. Using primary neurons and COS-7 cells, it was demonstrated that PINK1 knockdown induces mitochondrial filamentation. This might suggest that in our study part of the inhibitory effect of PINK1 knockdown on BAY-induced cell death is mediated by stimulation of mitochondrial filamentation. Interestingly, melanoma cells that were made resistant against the BRAFV600E-specific inhibitor and chemotherapy drug Vemurafenib possessed filamentous mitochondria. Conversely, knockdown of the fusion-promoting protein mitofusin 2 in melanoma cells induced a more fragmented mitochondrial phenotype and increased cell death upon Vemurafenib treatment. Therefore, our results might suggest that prevention or reversal of mitochondrial filamentation could be a strategy to overcome Vemurafenib resistance in BRAFV600E melanoma cells.

Mode of cell death. We observed that z-VAD-FMK neither displayed cytotoxic effects by itself nor prevented BAY-induced cell death, arguing against involvement of apoptosis. The inhibitory effects of Nec-1s and Fer-1 suggest that combined necroptosis and ferroptosis are responsible for the BAY-induced reduction in cell viability. Involvement of necroptosis is further supported by the inhibitory effects of RIPK1 and MLKL knockdown on the BAY-induced reduction in cell viability. Compatible with the proposed mechanism (Figure 8), increased ROS levels have been demonstrated to promote stabilization of the RIPK1/RIPK3 necrosome. The latter study also provided evidence that RIPK1, RIPK3 and MLKL stimulate ROS production, which further promotes necroptosome stabilization. Involvement of ferroptosis is supported by the observation that BAY treatment increases ROS levels, stimulates lipid peroxidation and induces GSH depletion, all of which are hallmarks of ferroptotic cell death. It was recently demonstrated that oxidation of specific phosphatidylethanolamines (PEs) in endoplasmic reticulum-associated cell compartments depends on acyl-CoA synthase 4 and is required for ferroptosis induction. Compatible with our TOC results, tocopherols suppressed ferroptosis by inhibiting lipooxygenase, which generates doubly and triply oxygenated (15-hydroperoxy)-diacyl PE species that act as death signals. Knockdown and overexpression of GPX4, an essential regulator of ferroptotic cell death, potentiated and antagonized the BAY-induced reduction in cell viability, respectively. This again suggests involvement of ferroptosis in BAY-induced cell death. It is currently unclear how increased cellular ROS levels induce specific activation of necroptosis or ferroptosis. Nec-1s and Fer-1 inhibited the BAY-induced reduction in cell viability to a similar extent, suggesting that both death mechanisms are equally activated. Erastin-induced ferroptosis was promoted by autophagic degradation of ferritin and ATG5 knockdown inhibited this ferroptosis. Therefore, BAY-induced stimulation of autophagy might directly activate ferroptosis. Knockdown of ATG5 or PINK1 prevented the BAY-induced reduction in cell viability to a similar extent, suggesting that PINK1-dependent mitophagy, occurring downstream of ATG5-dependent autophagosome formation, is primarily responsible for cell death induction.

Conclusion

Using BAY as a tool, we here provide evidence that CI inhibition induces the death of BRAFV600E melanoma cells by stimulating mPTP opening and inducing Δψ depolarization. These events increase cellular ROS levels in a mitophagy-dependent manner, leading to induction of combined necroptotic/ferroptotic cell death.

Materials and Methods

Cell culture. BRAFV600E human melanoma cell lines G361 (no. ATCC-CRL-1424) and SK-MEL-28 (no. ATCC-HTB-72) were derived from a skin melanoma site and obtained from American Type Culture Collection (ATCC; LGC Standards GmbH, Wesel, Germany). Melanoma cells were routinely cultured at

Figure 8 Proposed mechanistic model and experimental evidence. Treatment with BAY inhibits mitochondrial CI. This induces a (local) increase in reactive oxygen species (ROS) levels (‘triggering ROS’), which stimulates mPTP opening and autophagosome formation. Simultaneously, CI inhibition induces depolarization of the mitochondrial membrane potential (Δψ) leading to mitophagy induction. The latter induces ROS levels (‘killing ROS’) leading to parallel stimulation of necrosome formation (RIPK1/MLKL), lipid peroxidation and GSH depletion. Increased necrosome formation further stimulates ROS levels and leads to induction of necroptosis, whereas lipid peroxidation and GSH depletion stimulate ferroptosis. Experimental evidence presented in this study is marked in red (inhibitory effect) and green (stimulatory effect)
37 °C and 5% CO₂ in ATCC-recommended media supplemented with 10% (v/v) fetal calf serum (FCS; Gibco-Thermo-Fisher, Waltham, MA, USA). Before experiments, the above cells were cultured for 12 h in a hypoxia-free medium (no. A1443001; Gibco Thermo Fisher Scientific Inc., Waltham, MA, USA) to which was added: 5 mM o-glucose (Sigma-Aldrich, St. Louis, MO, USA), 2 mM Glutamax (no. 35050038; Gibco) and 5% (v/v) dialyzed FCS (no. 26400044; 10 000 MW cutoff; Gibco). Cells were routinely cultured in a humidified atmosphere (95% air, 5% CO₂, 37 °C).

**Knockdown/overexpression studies and western blot analysis.** Knockdown/overexpression was performed as described in the Supplementary Information. For western blotting, cell pellets were lysed in modified RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 1× protease inhibitor cocktail (no. 11697488001; Roche, Mannheim, Germany). Protein concentration in the lysates was determined using a NanoDrop UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), and 100 μg of protein per well was separated by SDS-PAGE and transferred to a PVDF membrane using an IBlot gel transfer stack (Invitrogen, Carlsbad, CA, USA). Antibodies and their detection are described in the Supplementary Information.

**Cell viability, death assay and mPTP opening.** Cell viability was determined using crystal violet staining. Cell death was assessed by flowcytometric analysis of propidium iodide (PI)-stained cells using a FACSCalibur flow cytometer (BD Biosciences, Breda, The Netherlands). Analysis of mPTP opening was performed using a previously described approach. Additional information is provided in the Supplementary Information.

**Quantification of autophagy, mitophagy and mitochondrial morphology.** For autophagy analysis, cells were seeded on Nunc Lab-Tek glass-bottomed coverslips (no. 595470, Thermo Scientific), transfected with a construct encoding tandem mCherry-GFP-tagged LC3 (no. 21074; Addgene, Cambridge, MA, USA) and cultured for 24 h. Autophagy was analyzed by transfecting the cells with a GFP-LC3 construct (no. P36235, Invitrogen), followed by subsequent costaining with MR (Invitrogen). Mitochondrial morphology was analyzed by staining the cells with MGI (Invitrogen). Details are provided in the Supplementary Information.

**Analyses of cellular ROS levels, GSH levels, lipid peroxidation, mitochondrial membrane potential and mitochondrial content.** To measure ROS and GSH levels, cells were stained with CM-H2DCFDA or 5,5′,6,6′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) staining. Mitochondrial content was determined using MG staining. Details are provided in the Supplementary Information.

**Chemicals.** N-Benzoyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (no. ALX260202; Z-VAD-FMK) was obtained from Enzo Life Sciences (Raleigh, NC, USA), and Nec-1 (no. SC20142) from Santa Cruz Biotech (Dallas, TX, USA). (-)-Tocris (no. T3251), BafA1 (no. B1793), Crystal Violet solution (no. HT90132), CsA (no. 30024), Fer-1 (no. SML683), Mdiv1 (no. M0199) and PI (no. P4170; PI) were obtained from from Sigma-Aldrich. Nec-1s (no. HY-14622A) was purchased from Santa Cruz Biotech (Dallas, TX, USA) and Nec-1 (no. SC200142) from Santa Cruz Biotech (Dallas, TX, USA) and Nec-1 (no. SC200142) from Santa Cruz Biotech (Dallas, TX, USA) and Nec-1 (no. SC200142) from Santa Cruz Biotech (Dallas, TX, USA).

**Data and image analysis.** The number of independent experiments (days and replicates (assays, individual cells) are marked by N and n, respectively. Unless stated otherwise, statistical significance was assessed using an independent two-population Student’s t-test (*P<0.05; **P<0.01; ***P<0.001; relative to the indicated condition), and results from multiple experiments are represented by their average value ± S.E.M. Curve fitting and statistical analysis was performed using Origin Pro 6.1 (OriginLab, Northampton, MA, USA). Image visualization, processing and quantification was carried out using Image Pro Plus 6.1 (Media Cybernetics, Rockville, MD, USA) and FIJI software (http://fiji.sc).

**Conflict of Interest**
FB, LMPEVO, HMB, SEvEdV, JCWH, SG, PHGMW and WJHK declare no competing interests. LS, CK and MH are full-time employees of Bayer Pharma AG, which developed BAY 87-2243.

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**Author contributions**
FB: designed experiments, performed experiments, analyzed data, prepared figures, wrote the manuscript; LMPEVO: performed experiments, analyzed data; LS: designed experiments, discussed and proofread the manuscript; HMB: performed experiments, analyzed data; SEvEdV: performed experiments; JCWH: performed experiments, analyzed data, GC: discussed and proofread the manuscript; MH: discussed and proofread the manuscript; PHGMW: designed experiments, wrote the manuscript; WJHK: designed experiments, analyzed data, prepared figures, wrote the manuscript, supervised the research.


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