Concurrent MEK and autophagy inhibition is required to restore cell death associated danger-signalling in Vemurafenib-resistant melanoma cells

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Abstract

Vemurafenib (PLX4032), an inhibitor of BRAF\(^{V600E}\), has demonstrated significant clinical effects. However, the majority of treated patients develop resistance, due to a variety of molecular mechanisms including MAPK reactivation through MEK. The induction of a cell death modality associated with danger-signalling resulting in surface mobilization of crucial damage-associated-molecular-patterns (DAMPs), e.g. calreticulin (CRT) and heat shock protein-90 (HSP90), from dying cells, is crucial for therapeutic success. Both cell death and danger-signalling are modulated by autophagy, a key adaptation mechanism stimulated during melanoma progression. However, whether cell death induced by MAPK inhibition is associated with danger-signalling, and the reliance of these mechanisms on autophagy, has not yet been scrutinized.

Using a panel of isogenic PLX4032-sensitive and resistant melanoma cell lines we show that PLX4032-induced apoptosis associated with caspase-dependent DAMPs exposure in the drug-sensitive melanoma cell lines, but failed to do so in drug-resistant partners, which displayed heightened MEK activation. Treatment with the MEK inhibitor, U0126, sensitised PLX4032-resistant cells to death, and re-established their danger-signalling capacity. Furthermore, only cells exposing death-induced danger-signals were phagocytosed and induced DC maturation. Although the PLX4032-resistant melanoma cells displayed higher basal and drug-induced autophagy, compromising autophagy, pharmacologically or by ATG5 knockdown, was not sufficient to re-establish their PLX4032 sensitivity. Interestingly, autophagy abrogation was particularly efficacious in boosting cell death and ecto-CRT/ecto-HSP90 in PLX4032-resistant cells upon blockage of MEK hyper-activation by U0126.

Thus combination of MEK inhibitors with autophagy blockers may represent a novel treatment regime to increase both cell death and danger-signalling in Vemurafenib-resistant metastatic melanoma.
1. Introduction

Approximately 40-60% of cutaneous melanomas carry mutations in v-raf murine sarcoma viral oncogene homolog B1 (BRAF), a key Ser/Thr kinase in the RAS-RAF-MEK-ERK axis of the mitogen-activated protein kinase (MAPK) pathway [1]. The most common mutation, that causes substitution of a glutamic acid with a valine at position 600 of the protein (V600E), accounts for approximately 90% of all BRAF mutations in melanoma and causes constitutive activation of the BRAF protein [2]. The subsequent constitutive activation of MAPK pathway leads to the uncontrolled induction of growth and survival signals, invasion and evasion of apoptosis [3-5].

Due to the high occurrence of BRAF mutations, a substantial amount of effort was devoted to the development of a drug that could specifically inhibit the BRAFV600E kinase. Vemurafenib, also known as PLX4032, RG7204, RO5185426 or Zelboraf® is approved for the treatment of patients with unresectable or metastatic melanoma with the BRAFV600E mutation (FDA website [online], (2011)) [6]. Vemurafenib treated patients show striking improvements within 1–2 weeks after treatment initiation representing clear evidence of early therapeutic benefit [1,7,8]. Nevertheless, after a time of fast partial or complete response, melanomas develop a resistance to the treatment and patients suffer from a more aggressive and usually incurable tumour re-growth [9]. As a result, there is substantial interest in identifying means to improve the durability of BRAFV600E inhibitor therapy. Till now, the main revealed resistance mechanisms include: (1) Enhanced MEK activity by increased ARAF/CRAF signalling [10] (2) silencing of BH3-only proteins (Bim-EL and Bmf) [11] and acquisition of various mutations eliciting a re-activation of the MAPK pathway, (3) up-regulation of epidermal growth factor receptor (EGFR) [12,13]; (4) mutational activation of NRAS and MEK [14,15]; and (5) activation via AKT signalling up-regulation [16]. Arising from these studies, new concepts of targeting BRAFV600E inhibitor-resistant cells have been proposed. One of them involves the concomitant inhibition of BRAFV600E (via dabrafenib) and MEK (via trametinib) [9,17,18].

In addition to alterations in MAPK signalling and cell death effectors, autophagy was recently implicated in the PLX4032 acquired-resistance [19,20]. Autophagy is an essential adaptive response that initiates the sequestration of damaged or ageing intracellular content leading to its degradation, thereby ensuring important quality control of the cytoplasmic materials along with the generation of building blocks to support growth and survival under condition of
nutrient deprivation [21]. This multistep catabolic process has been shown to play an especially important role in melanoma development [22] and acquisition of resistance to anti-cancer therapies [23]. Recently, PLX4032-induced autophagy was shown to be provoked and preceded by endoplasmic reticulum (ER)-stress [19,24]. Interestingly, reports from our lab as well as others, have linked the crosstalk between ER-stress and autophagy [25-27], with the regulation and modulation of danger-signalling – an important, therapeutic-success defining, molecular cascade originating from dying or stressed cancer cells [28,29]. Danger-signalling, incited by specific anticancer therapies, i.e. Hypericin-based photodynamic therapy (Hyp-PDT) or anthracyclines, results in the active mobilization and surface exposure of damage-associated-molecular-patterns (DAMPs) [30], which include among others surface exposed (ecto-) calreticulin (CRT) and ecto-heat shock protein 90 (HSP90) [25,31,32]. DAMPs, with extensive documentation for ecto-CRT, activate the host’s innate capacity to recognise, target and remove neoplastic cells [32-36]. To this end, considering the gap-in-knowledge about the PLX4032-DAMPs connection and the effects of PLX4032-induced autophagy on this link and melanoma cell death, we set out to determine the capacity of PLX4032 to potentiate danger-signalling as well as to investigate the role autophagy may play in PLX4032-sensitive and -resistant melanoma. We also aimed to investigate modalities that could incite cell death and danger-signalling in an established cellular model of PLX4032 acquired-resistance.
2. Materials and Methods

2.1. Tissue culture: A375 (ATCC), 451-LU and M1617 (a kind gift of Prof. M. Herlyn; both harbouring BRAF\textsuperscript{V600E} mutation [10,37,38]) metastatic melanoma cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium, Sigma-Aldrich, D6546) culture media containing 1% glutamine (or glutamax for M1617 cell lines) and penicillin/streptomycin (Sigma-Aldrich, G7513 and P0781 respectively) as well as 10% or 5% fetal bovine serum (FBS, HyClone) respectively. PLX4032 resistant 451-LU/RES and M1617/RES (BRAF\textsuperscript{V600E}) cells were cultured as in the same medium as their parental partners, further supplemented with 1 μM PLX4032 to maintain BRAF-acquired resistance [10]. The human thymocytes (THP-1) cells were cultured in RPMI media, supplemented with 50 μM beta mercapto-ethanol (Sigma), 10% FBS, 110 μg/ml sodium pyruvate (Sigma, S8636), 1% non-essential amino acids (Life Technologies, 11140035), L-glutamine and penicillin/streptomycin. Human immature dendritic cells (iDCs) were prepared as previously described [39]. All cells were maintained at 37°C, 5% CO\textsubscript{2} for a maximum of 10 passages.

2.2. Drug treatments: All experiments were carried out in required culture media with reduced (2%) FBS and the cells were treated for 72 hours (h) with PLX4032 (Selleckchem, S1267), U0126 (Promega, V1121) or PLX4720 (Abcam, ab141362), in comparison to vehicle (DMSO) treated controls. Caspase activation was inhibited by 2 h pre-treatment with 25 μM carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk, Bachem, N1560) prior to treatment with PLX4032 or U0126. Autophagic flux was observed by treatment with 10 nM Bafilomycin A1 (Baf A1; Sigma-Aldrich, B1793) for 24 h.

2.3. Western blotting: Following treatments, cells were scraped, pelleted, lysed in radio-immuno-precipitation assay buffer (RIPA) buffer (Enzo Life Sciences, 80-1284) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitors (Thermo Scientific, 88667). Protein concentration was determined with BCA kit (Thermo Scientific, 23228). Primary antibodies: eIF2α, p-eIF2α, cleaved caspase 3, total caspase 3, LC3B, Mek, p-Mek, p62 and PARP (Cell Signaling) or Actin (Sigma-Aldrich) were diluted 1:1000. Fluorescently labelled-secondary antibodies (anti-rabbit-DyLight 800 and anti-mouse-DyLight 680; Thermo Scientific, 35571 and 35519 respectively) were diluted 1:2000. The detection was done using the Odyssey infrared-imaging system (Li-Cor Biosciences). Densitometric analyses were generated using Image J.
2.4. **Cell Death:** Following treatment cells were trypsinized, washed twice with phosphate buffered saline (PBS) and fixed with 70% ice-cold ethanol. Percentage of propidium iodide-stained cells, positive for hypodiploid DNA (subG1 peak), were captured using an Attune flow cytometer (Life Technologies). Caspase-3 activity was measured in cells collected post-treatment and lysed in 100 mM Hepes pH 7.4 containing; 10% sucrose, 1% TritonX100, 2.5 mM EDTA, 5mM DTT, 1 mM PMSF, 2 µg/mL pepstatin, 2 µg/mL leupeptin. 25 µg (for A375 cells) or 50 µg (for 451-Lu and 451-Lu/RES) of total protein was incubated with fluorescent caspase-3 substrate acetylated-Asp-Glu-Val-Asp-(7-Amino-4-methylcoumarin) (Ac-DEVD-AMC; 50 µM; Bachem, I-1660) for 60 min at 37°C. After this, fluorescence was measured using a Flex Station (Molecular Devices) plate reader with an excitation of 360 nm and emission of 460 nm.

2.5. **Flow cytometry-based detection of DAMPs:** After treatment, cells were collected with TrypLE Express (Life Technologies, 12604-021), washed with PBS and with FC (Flow Cytometry) buffer (2% FBS, 1% BSA in PBS), incubated for 1 hr at 4°C with primary antibodies (1/100; CRT, Abcam, Ab 92516; HSP90, Enzo Life Sciences, ADI-SPA-830), washed and incubated for 1 hr at 4°C with secondary antibodies (1/500; anti-mouse-AlexaFluor 647, anti-rabbit-Alexa Fluor 488, both from Life Technologies). After final washes, cells were incubated in FC buffer containing 10 µg/mL 7-Aminoactinomycin D (7-AAD; Sigma-Aldrich, A9400) for 15 min and analysed on Attune Flow Cytometer (Life Technologies). The permeabilised cells were excluded from the analysis due to intracellular staining, and the fold changes in the MFIs for each DAMP were analysed. Cells were assessed for combined HSP90 and CRT positivity, relative to control.

2.6. **Cell viability:** Cells were seeded at 5000 cells per well of a 96-well plate. After treatment cells were washed with PBS and incubated with 0.01 mg/mL MUH (4-methylumbelliferyl heptanoate; Sigma, M2514; dissolved in PBS) for 30 min at 37°C. Fluorescence was measured with a Flex Station plate reader (Molecular Devices) with excitation 355nm, emission 460nm and cut off value of 455 nm.

2.7. **ATG5 knockdown:** ATG5 expression was reduced by siRNA-mediated knockdown as described previously [22]. Briefly, cells were transiently transfected either with Atg5-specific siRNA (Thermo Scientific, L-004374) or scrambled siRNA control (Qiagen, SI03650318) in FBS-free media using DharmaFECT (Dharmacon, T-2001) for 4 h prior to FBS addition and
incubation overnight. Cells were then used for experimentation. Following transfection, the reduction in protein expression was also verified by immunoblotting.

2.8. Fluorescent detection of autophagy: LC3 positive puncta were stained as previously described and imaged using a Leica confocal microscope [40]. Remaining cells were scraped and analysed using an Attune flow cytometer (Life Technologies).

2.9. Phagocytosis: Cancer cells were treated with 10 µM PLX4032 or U0126 for 72 h. Cancer cells and THP-1 thymocytes were detached with TrypLE™ Express (Life Technologies). Thymocytes were labeled with CellVue® NIR780 and cancer cells with CellVue® Jade (eBioscience™). Cancer cells and thymocytes were then incubated at a 1:1 ratio for 24h in thymocyte medium (see above). Cells were then harvested using TrypLE™ Express (Life Technologies) and analysed on flow cytometer (Attune, Life Technologies). The percentage of cancer cells that were phagocytosed was calculated by dividing the number of double-positive cell number by the number of the JADE positive cells.

2.10. Dendritic cell (DC) maturation: The protocol for co-incubation of cancer cells with immature human DCs (iDCs) has been previously described [25,28]. Briefly, iDCs were co-cultured with untreated or dying cancer cells (72h time point) at a 1:10 (iDCs : cancer cells) ratio for 24 h under standard culture conditions. For blocking experiments the cancer cells were pre-incubated for 1h at 37°C with blocking antibodies [1,25 µg/10⁶ cancer cells]: IgY (Promega, G116A), anti-HSP90 (Novus Bio, NB120-19104), anti-CRT (Thermo Scientific, PA1-902A); antibodies were present as well in the co-culture media. For detection of DC maturation, DCs were stained with: anti-CD1α antibody (BioLegend, 300120), anti-HLA-DR antibody (BD, MHLDR01) and anti-CD86 (BD, MHCD8605). 20 000 DCs (CD1α⁺cells) were acquired and analysed for HLA-DR and CD86 positivity using an Attune flow cytometer (Life Technologies).

2.11. Statistics: All data are the mean of 3 independent experiments ± standard deviation (SD). One-Way Analysis of variance (ANOVA) or Two-Way ANOVA with post hoc correction were used for comparison of tested time points and treatments. All analyses were performed using GraphPad Prism 5. */$ P<= 0.05, **/$$ P< = 0.01, $$$/$$$ P< = 0.001.
3. Results

3.1. MEK inhibitor, U0126, sensitises PLX4032-resistant melanoma cells to apoptosis

Mirroring the clinical paradigm of acquired Vemurafenib (from now on indicated as PLX4032) resistance [41,42], we used the well characterized BRAF-mutated A375 metastatic cell line, and two independent isogenic metastatic melanoma cell models of acquired resistance (PLX4032-sensitive: 451-LU or M1617 and PLX4032-resistant: 451-LU/RES or M1617/RES [10]) to investigate modalities for overcoming or bypassing drug resistance. The resistance mechanism of 451-LU/RES and M1617/RES has been previously described and found to largely rely on a switch to the CRAF or ARAF isoforms resulting in MEK1 hyperactivation after PLX4032 treatment [10].

Initial drug sensitivity was assessed by cell viability assays, following exposure to either PLX4032 or U0126 (Figure 1Ai and Aii, respectively). As reported in previous studies [10] BRAF-mutated melanoma cells displayed a significant loss in viability in response to doses of PLX4032 as low as 1 µM, while the isogenic resistant cell lines were clearly unaffected by this low PLX4032 dose or only slightly affected within the PLX4032 concentration range used (For either 451-LU vs. 451-LU/RES or M1617 vs. M1617/RES P ≤ 0.01). In line with the reported mechanism of acquired resistance [10], both parental and resistant melanoma cell lines were found to be sensitive to treatment with the MEK inhibitor U0126 (Figure 1Aii). Of note, similar dose-dependent effects were obtained with another selective BRAF inhibitor, PLX4720 [43], thus evidencing both similar potency and underlying mechanism of resistance (data not shown).

To gain further insights in the mechanisms of cell death, melanoma cells were treated with 0-25 µM PLX4032 for 72 h and apoptotic cell death sensitivity was determined by measuring an increase in the cell population harbouring a sub-G1 DNA content (Figure 1Bi). A dose-dependent increase in sub-G1 fraction of A375, 451-LU and M1617 cells (for all cell lines, from 1 µM P ≤ 0.01), with no significant increase for either 451-LU/RES or M1617/RES cells, was observed after PLX4032. Moreover, cell line comparison disclosed a significant reduction of cell death induction in both 451-LU/RES and M1617/RES cell lines (451-LU or M1617 vs. 451-LU/RES or M1617/RES P ≤ 0.001). Furthermore, when cell lines were exposed to U0126 (0-25 µM), to determine if cell death could be restored in the 451-LU/RES and M1617/RES cells (Figure 1Bii), a dose-dependent increases in cell death was observed (for 451-LU cells treated with 1 µM P < 0.05, for all cell lines from 10 µM U0126 P ≤ 0.001).
Thus, bypassing PLX4032-resistance by targeting MEK in the 451-LU/RES and M1617/RES cells resulted in a similar dose-dependent induction of apoptotic cell death.

To confirm that PLX4032 and U0126 target the MAPK pathway and that the induced cell death was caspase-dependent, following treatment, cells were assessed for MEK phosphorylation (pMEK), PARP cleavage (Figure 1C) and caspase-3 activity (Figure 2A), as well as sensitivity to the pan-caspase blocker zVAD-fmk (Figure 2B). Data indicated that following treatment with either MAPK inhibitor A375 cells and the parental 451-LU and M1617 cells showed a severe reduction in pMEK that coincided with increased PARP cleavage (Figure 1Ci-ii). Moreover, within the 451-LU resistant model, increased PARP cleavage was associated with increased caspase-3 activity (Figure 2A). Additionally, pre-treatment with 25 µM zVAD-fmk prior to PLX4032 (Figure 2B) resulted in a significant reduction in PLX4032-induced cell death (For A375 or 451-LU P < 0.01 and for 451-LU/RES P<0.05). Interestingly, in the resistant 451-LU/RES and M1617/RES cell lines, PLX4032 treatment increased pMEK status (Figure 1Ci), which could be inhibited by treatment with U0126 (Figure 1Cii). Importantly, U0126 (Figure 1Bii) re-established apoptosis in 451-LU/RES and M1617/RES cells, as shown by increased PARP cleavage and for 451-LU/RES caspase activation (Figure 1Cii and 2Aii respectively).

Together these observations indicate that targeting the BRAF-MEK-ERK pathway, by treatment with the MEK inhibitor U0126, induces apoptosis in PLX4032-resistant metastatic melanoma cells.

3.2. PLX4032 and U0126 induce cell death-related surface exposure of CRT and HSP90

Due to the emerging importance of danger-signalling in melanoma cell death [44-46], and its regulation by ER-stress and autophagy cross-talk [25,33,44], we investigated the effect of acquired PLX4032-resistance on the exposure of the ‘eat-me’ signal ecto-CRT (which favours uptake of dying cells by innate immune cells) and ecto-HSP90 (which facilitates recognition and phagocytosis of dying cells and antigen presentation) following BRAF inhibition in PLX4032-sensitive and -resistant cells. PLX4032 significantly increased both ecto-CRT and ecto-HSP90, in A375 and 451-LU and M1617 cell lines (Figure 3Ai-ii, for ecto-CRT or ecto-HSP90 in either cell line from 10 µM P ≤ 0.05). Instead, 451-LU/RES and M1617/RES cells demonstrated a complete absence of PLX4032-induced danger-signalling (Figure 3Ai-ii, for
451-LU vs. 451-LU/RES or M1617 vs M1617/RES for either ecto-CRT or ecto-HSP90 P < 0.001).

To confirm that the inability of PLX4032 to induce danger-signalling in cells with acquired-resistance to PLX4032 is not due to gross defects in intracellular pathways trafficking CRT and HSP90 to the surface in response to anticancer treatments, these melanoma cells were treated for 24 h with increasing concentrations of bortezomib, a known inducer of ecto-CRT and ecto-HSP90-based danger-signalling [30-32]. Treatment with the proteasome blocker bortezomib induced cell death and exposure of both ecto-CRT and ecto-HSP90, with no significant differences between cell lines, indicating that failure to stimulate apoptotic cell death in PLX4032-resistant melanoma also compromises their ability to mobilize CRT and HSP90 (Figure 3B-C, 0-150 nM). In line with this, U0126 treatment in PLX4032-resistant melanoma cells restored cell killing (Figure 1) and ecto-CRT and ecto-HSP90 exposure, with no significant difference between cell lines (Figure 3D, from 10 µM for all P < 0.001). Interestingly, treatment of A375 and 451-LU cells with 25 µM PLX4032 or U0126 was associated with increased eIF2α phosphorylation (peIF2α), a marker of the ER-stress module involved in the caspase-dependent trafficking mechanism of CRT to the cell surface (Figure 3E)[25,26]. Whereas peIF2α was not stimulated by PLX4032 in 451-LU/RES cells, this ER-stress marker was clearly evoked following U0126 (Figure 3E), correlating well with the re-establishment of cell death and danger-signalling in these resistant cells (Figure 3E, P < 0.001). Furthermore, caspase inhibition by zVAD-fmk significantly reduced both ecto-CRT and ecto-HSP90 on PLX4032-treated A375 and 451-LU cells (Figure 3F, for ecto-CRT P ≤ 0.05 and ecto-HSP90 P ≤ 0.001). Of note, caspase-dependent cell death triggered by cisplatin did not elicit ecto-CRT exposure and failed to stimulate eIF2α phosphorylation in these melanoma cells (data not shown) as previously reported in other cancer cell models [27].

All together these results highlight the close relationship between caspase-dependent cell death and the danger-signalling capacity of melanoma cells in response to the targeted inhibition of the MAPK pathway.

3.3. PLX4032 and U0126 incite phagocytosis and DC maturation in a death-induced danger-signalling dependent manner
Due to the lack of relevant *in vivo* models, to extrapolate the importance of PLX4032 or U0126-induced danger-signalling, 451-LU and 451-LU/RES cells following 72 h exposure to either agent were co-incubated with THP-1 thymocytes for 24 h prior to phagocytosis analysis (Figure 4A). Data demonstrated that when MAPK-inhibitory agents were proficient in the incitement of cell death and danger-signalling, a significant increase in the phagocyted melanoma cells was observed (for 451-LU treated with PLX4032 P < 0.05 or either 451-LU and 451-LU/RES with U0126, P < 0.01). Notably, treatment with PLX4032 which incites neither cell death nor danger signals in the 451-LU/RES cells, also failed to induce an increase in phagocytosis compared to untreated cells (Figure 4A).

Furthermore, to assess the biological importance of the surface exposed DAMPs after MAPK-inhibition, following treatment with PLX4032 or U0126, melanoma cells were incubated with immature human dendritic cells (iDCs) (i.e. the major antigen presenting cells of the innate immune system), and their maturation status was assessed by measuring the expression of the maturation markers CD86 and HLR-DR by flow cytometry. A significant increase in the matured DC population was found following co-incubation of iDCs with 451-LU cells treated with PLX4032 (P < 0.001) or either 451-LU or 451-LU/RES following U0126 (for both P < 0.001) (Figure 4B and C). Once more, as observed for phagocytosis, 451-LU/RES cells treated with PLX4032 were not capable of stimulating iDCs maturation and, remarkably, even repressed the basal activation induced by exposing DCs to control/untreated melanoma cells (Figure 4B). To determine the physiological importance of ecto-CRT and ecto–HSP90, 451-LU cells following treatment with PLX4032 were pre-incubated with a blocking antibody mix targeting CRT and HSP90 or isotype specific controls, prior to exposure to iDCs (Figure 4C). Interestingly, under the presence of blocking antibodies, the maturation capacity of PLX4032-treated cells was significantly reduced (P < 0.001).

Together these data highlight the importance of the cell death-associated and danger-signalling-mediated capacity of melanoma cells for the clearance and incitement of an immune response, following successful inhibition of the MAPK signalling pathway.

3.4. PLX4032-resistant cells possess higher autophagic flux under basal and MAPK-inhibition
The acquired resistance of melanoma cells to PLX4032 induces intracellular adaptation mechanisms that abolished apoptosis-associated danger-signalling. Interestingly, along with ER-stress, PLX4032 has been shown recently to incite autophagy in melanoma cells [19].

To evaluate if acquired PLX4032-resistance affects both basal and stress-induced autophagy, autphagic flux and key autophagy markers [21] were monitored in untreated and PLX4032 or U0126 treated melanoma cells (Figure 5). Autophagic flux was assessed by western blot detection of p62 and autophagosome-associated LC3 II, two pro-autophagic proteins that are degraded during autophagy, in the absence or presence of Bafilomycin A1 (Baf A1), an inhibitor of the vacuolar H+-ATP pump. By alkalinizing the lysosomes, Baf A1 prevents the late stage of autophagy, i.e. fusion of the autophagosomes with lysosomes and degradation of their cargo. Western blot analysis of LC3 II and p62 revealed their increased accumulation in the presence of Baf A1 (Figure 5A), indicating an active autophagosome turnover in these cells (i.e. autophagic flux). Noticeably, the autophagic flux was significantly enhanced in 451-LU/RES and M1617/RES cells as compared to their isogenic, PLX4032-sensitive 451-LU or M1617 cells, as well as to A375 cells (Figure 5Aii, for 451-LU/RES vs. 451-LU or M1617/RES vs. M1617 P ≤ 0.05) [22,47]. Furthermore, in A375 cells and both parental and resistant cell lines autophagy was stimulated in response to the cellular stress caused by the targeted inhibition of MAPK pathway, but notably 451-LU/RES as well as M1617/RES cell lines displayed the greatest PLX4032 and U0126-stimulated autophagic response (Figure 5B, for 451-LU/RES vs. 451-LU or M1617/RES vs. M1617 P ≤ 0.01).

To confirm differences in basal autophagic flux across the melanoma cell lines observed by western blot [48], confocal microscopy experiments of methanol-fixed LC3 stained cells, were performed and the presence of stereotypical LC3-positive puncta were scored (Figure 5Di) [22]. Additionally, quantitative analysis of LC3-based fluorescence was carried out by flow cytometry (Figure 5Dii). Both imaging and quantitative LC3 fluorescence analysis, confirmed that PLX4032-resistance was associated with a significant increase in LC3 puncta both under basal and after MAPK-inhibition conditions (Figures 5C).

In aggregate, these results indicate that PLX4032-resistant cells display heightened autophagy, both in basal and after MAPK-targeted treatments, irrespective of their ultimately cell fate; i.e. cell survival after PLX4032 or cell death after U0126 treatment.
3.5. Inhibition of autophagy does not sensitise PLX4032-resistant cells to apoptosis induction

Based on the observations indicating an increased autophagic flux in PLX4032-resistant melanoma cells and because of the known cytoprotective role of autophagy in melanoma [19,49], we then tested the effect of pharmacological-based autophagy inhibition on PLX4032- or U0126-induced cell death (Figure 6). Pre-treatment of the melanoma cells with Baf A1 potentiated cell death induced by either PLX4032 or U0126 in A375, 451-LU and M1617 cells (Figure 6, P ≤ 0.05 for A375, 451-LU and M1617 cells treated with PLX4032 or P ≤ 0.01 for U0126), suggesting that autophagy enhances melanoma cell’s resistance against cell death in response to MAPK-targeted inhibition. Intriguingly, autophagy blockage by Baf A1 was unable to restore PLX4032 cell death sensitivity in either the 451-LU/RES or M1617/RES cells whereas this combined treatment significantly enhanced their response following U0126 (Figure 6, P ≤ 0.01 for either cell line).

All together these data demonstrate that in spite of their heightened basal and drug-induced autophagy, PLX4032-resistant melanoma cells exhibit a predominant MEK-dependent mechanism(s) enabling them to resist PLX4032-induced cell death.

3.6. Concomitant targeting of MEK and autophagy restores apoptosis-associated danger-signalling in the PLX4032-resistant cells

Understanding that autophagy inhibition could potentiate cell death in response to PLX4032 and U0126, it was important to correlate this with their danger-signalling modulation capacity [28].

To this end, we assessed ecto-CRT (Figure 7A) or ecto-HSP90 (Figure 7B) exposure in these parental and resistant melanoma cell lines after treatment with PLX4032 or U0126, with or without Baf A1 co-treatment. As for cell death, blocking the late stages of autophagy in melanoma cells by the addition of Baf A1 significantly enhanced danger signals exposure following PLX4032 in all sensitive cells (For ecto-CRT or ecto-HSP90; P ≤ 0.05 for A375, 451-LU and M1617), whereas no ecto-CRT or ecto-HSP90 increase was observed in either of the resistant cell lines after treatment (Figure 7). Interestingly, treatment with U0126, in combination with Baf A1, potentiated danger-signalling in all cell lines (For ecto-CRT or ecto-HSP90 P≤0.05 for all cell lines). Of note, following concurrent Baf A1 and U0126
treatment danger-signalling response was significantly higher in the drug resistant 451-LU/RES and M1617/RES cells when compared to that observed for either of their isogenic partners (Figure 7, 451-LU/RES vs. 451-LU or M1617/RES vs. M1617 P < 0.01).

To confirm whether pharmacological inhibition of autophagy could be phenocopied by its genetic inhibition, the expression of ATG5 (a key pro-autophagic protein involved in the initiation of the autophagic process [21]) was significantly reduced in A375, 451-LU and 451-LU/RES cell lines by siRNA-mediated knock-down (Figure 8A, for all P < 0.001), in comparison to scrambled controls. Consistent with the Baf A1 observations, inhibition of autophagy by ATG5 knock-down (siATG5) was sufficient to enhance cell death induced by PLX4032 in the sensitive melanoma cell lines or by U0126 in all cell lines (Figure 8B, for 451-LU with PLX4032 P < 0.05 or U0126 in all cell lines P ≤ 0.05). This is in line with the general cytoprotective role of autophagy observed against various anti-melanoma therapies [19,49,50]. Moreover, genetic blockage of autophagy by siATG5 did not alter the cell death response in PLX4032 treated 451-LU/RES cells (Figure 8B), as observed previously with co-treatment with Baf A1 (Figure 6). Interestingly, U0126 combined with siATG5, as observed for Baf A1 combinations, demonstrated the greatest incitement of cell death in the 451-LU/RES (For 451-LU/RES vs. 451-LU or A375 for U0126 combined with Baf A1 P ≤ 0.05 or siATG5 P ≤ 0.01) (Figure 6 and 8B).

In line with the effects of Baf A1 and with the cell death induction, when autophagy was inhibited by siATG5 (Figure 8C), a similar enrichment in cell surface exposed danger signals was observed following PLX4032 (For ecto-CRT in 451-LU and ecto–HSP90 in A375 or 451-LU P ≤ 0.05) treatment, in the sensitive/parental cell lines (Figure 8C). Treatment with U0126 combined with siATG5, potentiated danger-signalling of all cell lines (For ecto-CRT or ecto-HSP90 P≤0.05 for all cell lines). Of note, independent of the inhibitory mechanism (Baf A1 vs. siATG5) following U0126 treatment danger-signalling response was significantly higher in the drug resistant cells (Figure 4, 451-LURES vs. A375 or 451-LU P < 0.01).

Taken together these results indicate that although autophagy plays a contributing role in melanoma survival [22,51], its inhibition is not sufficient to overcome PLX4032 resistance in 451-LU/RES or M1617/RES cell lines. However when autophagy is inhibited concurrently with the blockage of MEK, a significant enrichment in melanoma cell death and danger signals is achieved.
4. Discussion

Targeting mutant BRAF was the most promising breakthrough for melanoma therapy in decades [41]. However, BRAF\textsuperscript{V600E} targeted therapy with Vemurafenib induces enrichment in a pre-existing subset of resistant cells resulting in the recurrence of therapy-resistant disease [8,52,53]. Due to this phenomenon, research has shifted not only toward understanding the complex mechanisms behind the acquired-resistance, but also to ways to bypass and accentuate the therapeutic outcome. The results shown in this study demonstrate that targeting MEK downstream to BRAF\textsuperscript{V600E} is a valid mechanism to promote cell death accompanied with danger-signalling potential, of PLX4032-resistant melanoma cells. Moreover, this potential can be further enhanced by the combination with an autophagy inhibitor.

The acquisition of resistance to PLX4032 in the M1617 and 451-LU cell lines was established by persistent treatment with low dose PLX4032 [10] where the cell evolved by regulatory adaptations to rely more heavily on signalling transmitted through ARAF and CRAF, thus bypassing the inhibition of proliferative MEK signalling. Within this study we show that upon PLX4032 treatment, melanoma cells that have acquired a drug-resistant phenotype displayed indeed an increased MEK phosphorylation, a reported consequence of intrinsic resistance that is often only observable following therapy [14]. This incitement of the MAPK pathway might be a consequence of numerous molecular adaptations [19]. However, what is prominent is the outcome, a cancer type with a notoriously aggressive and proliferative phenotype [19] that can be suppressed by targeting MEK [10]. In line with this, the re-sensitisation of PLX4032-resistant melanoma cells towards cell death by the MEK inhibitor U0126 observed in this study, further highlights the clinical relevance of MEK-based targeted therapy [17,18].

Importantly, we show here that the apoptotic cell death induced by MAPK-inhibition (both PLX4032-mediated in sensitive melanoma cells or triggered by U0126 in the resistant isogenic melanoma cell line) was coupled with the exposure of ecto-CRT and ecto-HSP90, key mediators of danger-signalling. This implies that the apoptosis induced by BRAF/MEK-inhibition might have a promising danger-signalling-based immune-stimulatory capacity. Such a capacity is important considering that danger-signalling has been found to be crucial for the success of anti-cancer therapies [36]. In line with this, anticancer therapies capable of inducing crucial DAMPs like ecto-CRT and ecto-HSP90 (e.g. anthracyclines, bortezomib, oxaliplatin) have been found to perform better in the clinic than those that cannot (e.g. cisplatin) [54,55]. Moreover, the inability to incite danger-signalling has been associated with
negative prognosis or therapy non-responsiveness in cancer patients [36,56,57]. All this highlights that BRAF/MEK-inhibitor induced danger-signalling can have important clinical implications for anti-cancer immunity in patients – a premise that needs to be investigated and confirmed using available clinical samples.

Interestingly, treatment with PLX4032 reduced the basal p-eIF2α expression in 451-LU/RES cells. ER-stress, in particular the PERK arm of the unfolded protein response, characterised by eIF2α activity, has been implicated in danger-signalling activation [25,26]. Here we show a lack of danger-signalling for the resistant 451-LU/RES and M1617/RES cell lines that can be regained following U0126 treatment, likely by reinstating the induction of the ER-stress and p-eIF2α-dependent module, as well as caspase-dependent cell death. This study hence corroborates previous observations reporting that caspase activity is instrumental for ecto-CRT exposure following certain conventional chemotherapeutics (like mitoxantrone and doxorubicin) [26]. However, it should be mentioned that for other ER stress-inducing agents, such as Hyp-PDT, caspase signalling is dispensable for ecto-CRT trafficking [25,28], thus underscoring that the association between ER stress and caspase-reliant danger-signalling, is dependent on the therapeutic modality inciting cancer cell death.

Recent studies have demonstrated that blockage of the MAPK pathway of BRAFV600E melanoma cells can reverse suppression of DC function [58], and increase abundance of tumour infiltrating lymphocytes [59] as well as CD8+T/FoxP3+CD4+ T cell ratio and NK cells [60]. However the cancer cell-associated molecular mediators eliciting these favourable immune responses were not investigated in these studies. Unfortunately, due to the inability to monitor human cancer cell models in an immune-competent model, coupled with the lack of mouse isogenic models of PLX4032-acquired resistance, as well as the differences in immune responses between species [32,61], to date there are no accepted/available in vivo models to investigate the effects of BRAF-inhibitor acquired resistance on cancer cell modulated immune responses. However in the present study we found that treatment with either PLX4032 in parental cells or U0126 in the PLX4032-sensitive or -resistant melanoma models, induces cell death-associated danger-signalling resulting in increased phagocytosis of dying cancer cells and stimulating DC maturation. Importantly, we found that a caspase-dependent danger-signalling that mediates the exposure of ecto-CRT and ecto-HSP90 on the surface of the dying melanoma cells, is required to incite DC maturation following MAPK-inhibition. Our findings thus reveal the immunological relevance of this PLX4032/U0126 mediated
apoptotic cell death pathway, and define a modality linking cancer cell death to the previously described immunological responses observed in response to BRAF and MEK inhibition.

Exploring the mechanisms underlying the resistant phenotype, interestingly we found that the PLX4032-resistant melanoma cells used in our study not only showed enhanced basal autophagic flux as compared to their isogenic parental counterparts, but also displayed a significantly higher increase in autophagy after treatments with the BRAF or MEK inhibitors. Although enhanced MEK activity could ultimately modulate autophagy through various ERK-dependent mechanisms [62-64], it is unlikely that the higher levels of drug-induced autophagy in the PLX4032-resistant melanoma cells are mechanistically linked to the MEK status, as they were observed after MEK activation (PLX4032) as well as inhibition (U0126).

A recent work of Ma and co-workers [18] showed that targeting autophagy sensitized both BRAF inhibitor sensitive and resistant melanoma cells to PLX4032. Notably, both the latter and our study highlight the induction of ER stress in response to PLX4032 treatment, in our case however, this was the case only in PLX4032-sensitive cell lines whereas autophagy was induced in both isogenic cell lines after MPAK inhibition, though prevalently in the drug-resistant cells. Moreover, both studies underscore that drug-induced autophagy is a cytoprotective mechanism, as MAPK-inhibitor induced cell death was significantly enhanced following autophagy inhibition. The adaptive and pro-survival role of autophagy in melanoma is further supported by the recent findings that chemical inhibitors of autophagy in combination with PLX4032 further sensitized melanoma cells to apoptosis, compared to single agent [65].

However, in the study of Ma et al, blockage of autophagy was shown to subvert survival and growth also in the PLX4032-resistant melanoma cells [18]. Instead, our findings show that autophagy inhibition was not sufficient to reignite cell death of the PLX4032-resistant cells, a process achieved only in response to MEK inhibition, thus disclosing the predominant reliance of these melanoma cells on MEK-dependent pro-survival mechanisms. This discrepancy could be a direct consequence of the experimental models used, since Ma et al. used melanoma cell lines displaying different sensitivity to PLX4032 to show their reliance on autophagy as a protective mechanism [18]. Instead, within the present study, we used isogenic models of bona fide acquired resistance to PLX4032, where melanoma cells evolve mechanisms to bypass PLX4032-induced BRAF inhibition, often depicted by hyperactivation of MEK, to ensure that the MEK-ERK pro-survival signalling can precede unabated [13] and
to a degree that can still support their drug resistance, even under conditions of autophagy mitigation [14][14][14][14][13].

Importantly however, this study highlights that in cells with BRAF-acquired resistance, when heightened autophagy was inhibited concurrently with U0126, an agent that was able to stimulate eIF2α phosphorylation, cell death and danger-signalling were both synergistically potentiated and to a higher degree than that observed in PLX4032-sensitive melanomas. This suggests that heightened autophagy in metastatic melanoma and even more in drug-resistant cells, impedes danger-signalling downstream to ER stress [18]. This observation is also in line with our recent reports showing that cytoprotective autophagy, induced downstream to the potent ER stressor Hyp-PDT, helps in the suppression of ecto-CRT based danger-signalling in human melanoma and bladder carcinoma [28]. Intriguingly, in other contexts where therapy-induced autophagy was not cytoprotective (i.e. for mitoxantrone and oxaliplatin), it also failed to suppress ecto-CRT [66]. Moreover, this current study extends this danger-signalling suppressive role of autophagy to ecto-HSP90. Thus, based on our past and current studies as well as others, it can be proposed that cytoprotective autophagy represents a mechanism of danger-signalling suppression in dying cancer cells [67]. Due to melanomas’ addiction to autophagy [22], especially in late stage metastatic disease [22,68], autophagy inhibition appears to be a viable strategy to increase MEK-inhibition-induced cell death associated with danger-signalling, thus advocating the need for autophagy inhibitor-based trials in a BRAFV600E related context. Moreover, the recent clinical finding that single agent targeting of MEK was insufficient to illicit a response may further implicate the need for combinational therapy, with an autophagy inhibitor or highlight a mechanism to potentiate the effects of ipilimumab, the CTL A-4 blockade therapy that has demonstrated promising anti-melanoma effects clinically by prolonging T-cell activation and restoring T-cell proliferation, thus amplifying T-cell-mediated immunity and the patient's capacity to mount an effective anti-tumour immune response [69]. The combination with MEK inhibitor induced danger-signalling may allow for reduced ipilimumab dosage, yielding cancer cell targeting without the significant non-specific auto-immune reactions induced by ipilimumab [70], as CTLA-4 plays a pivotal role in regulating tolerance to self-antigens [70].
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Conflicts of interest

No conflict of interest
References


Figure Legends

Figure 1: Targeting MEK, using U0126, restores cell death induction in PLX4032-resistant melanoma cells. (A) Viability of all cell lines was assessed by MUH-based cytotoxicity assays following 72 h treatment with PLX4032 (Ai) or U0126 (Aii). (B) Furthermore, (C) cell death of A375, 451-LU, 451-LU/RES, M1617 and M1617/RES cell lines was determined following 72 h PLX4032 (Bi) or U0126 (Bii, for both agents 0-25 μM) by propidium iodide stained flow cytometry. Western blot analysis of melanoma cells treated with PLX4032 (Ci) or U0126 (Cii) prior to probing for phosphorylated vs. total MEK (pMEK vs. MEK) to assess inhibition of the MAPK pathway and PARP cleavage as an indicator of apoptosis. Data are the average of 3 independent experiments ± SD. Western blot images are a representative image of reproducible experiments.

Figure 2: Targeting MAPK induced caspase-dependent cell death. (A) Induction of apoptosis was confirmed by the assessment of caspase-3 activity following 72 h exposure to PLX4032 (Ai) or U0126 (Aii). (B) Moreover, apoptosis was inhibited, in all cell lines, by a 2 h pre-incubation with zVAD-fmk prior to 25 μM PLX4032 treatment for 72h and cell death assessed by propidium iodide stained flow cytometry. Data are the average of 3 independent experiments ± SD.

Figure 3: Silencing of danger-signalling in PLX4032-resistant melanomas can be overcome by MEK inhibition. (A) A375, 451-LU, 451-LU/RES, M1617 and M1617/RES melanoma cell lines were assessed for ecto-CRT (Ai) or ecto-HSP90 (Aii) by flow cytometry following 72 h treatment to a dose range (0-25 μM) of PLX4032. (B-C) A375, 451-LU and 451-LU/RES melanoma cell lines after treatment with bortezomib (0-150 nM) for 24 h were assessed for cell death induction (propidium iodide positive cells) (B) and ecto-CRT (Ci) or ecto-HSP90 (Cii) by flow cytometry. (D) A375, 451-LU, 451-LU/RES, M1617 and M1617/RES melanoma cell lines were assessed for ecto-CRT (Di) or ecto-HSP90 (Dii) by flow cytometry following 72 h treatment to a dose range (0-25 μM) of U0126. (E) A375, 451-LU and 451-LU/RES melanoma cell lines treated with vehicle, PLX4032 or U0126, as indicated, were immunoblotted for phosphorylated and total eIF2α. (F) Assessment of ecto-CRT or ecto-HSP90 exposure by flow cytometry in cell lines exposed to PLX4032 (25 μM for 72 h) following a 2 h pre-incubation with zVAD-fmk (25 μM). All data sets are the mean of 3 independent experiments ± SD.
Figure 4: Cell death-induced danger signals induced by MAPK inhibition incite physiological responses. (A) The phagocytosis of 451-LU and 451-LU/RES melanoma cells by THP-1 cells was measured after 24 h co-incubation. The cancer cells were untreated (Control) or treated with PLX4032 (10 µM) or U0126 (10 µM) for 72 h. Percentage of phagocytosis was determined by the percentage of JADE+ cells within NIR+ cancer cell gate. (B) The phenotypic maturation of human iDCs (defined as percentage of CD86+/HLA-DR+ cells within CD1α+ gate) was investigated after 24 h co-incubation with control or treated 451-LU and 451-LU/RES cells (72 h time point). (C) Untreated or PLX4032-treated 451-LU cells (72 h time point) were co-incubated with human iDCs in the presence, or not, of control antibody (IgY) or ecto-CRT and ecto-HSP90 blocking antibodies (αCRT/αHSP90) and the phenotypic maturation of DCs was assessed. All data sets are the mean of replicate experiments ± SD.

Figure 5: PLX4032 acquired resistance enhances basal and stress-induced autophagy. (A) Autophagic flux of A375, 451-LU and 451-LU/RES, as well as M1617 and M1617/RES cells were assessed by western blotting for p62 and LC3 following 24 h exposure to Baf A1. (B) Induction of autophagy of all cell lines was assessed following 72 h treatment with 10 µM PLX4032 or U0126 by western blotting for LC3 (Bi), summarized by densitometry analysis (Bii). (C) Basal autophagy was compared by (Ci) western blotting-based densitometry analysis or (Cii) flow cytometry comparison of mean fluorescent intensities following LC3 staining of control samples of all cell lines. (Di) Autophagy was confirmed by LC3-based immunofluorescent- and (Dii) flow cytometric-based assessment of methanol-fixed melanoma cells following 10 µM PLX4032 or U0126 time?. Western blot images are representative blots of 3 independent samples and expressions normalized to β-Actin loading control. All data sets are the mean of 3 independent experiments ± SD. Scale bars = 20 µm. Image inserts are digital magnifications to highlight puncta formation.

Figure 6: Autophagy contributes to melanoma survival independent of PLX4032-resistance. (A) Cell death induction of 10 µM PLX4032 or U0126 following perturbation of autophagy by Baf A1 treatment was assessed by propidium iodide stained flow cytometry. All data sets are the mean of 3 independent experiments ± SD.

Figure 7: Pharmacological-based inhibition of autophagy potentiates MEK-induced danger-signalling in dying melanoma cells. Melanoma cell lines were assessed for ecto-CRT (Ai) or ecto-HSP90 (Aii) by flow cytometry following 72h exposure to 10 µM PLX4032
or U0126 with or without autophagy suppression by Baf A1. All data sets are the mean of 3 independent experiments ± SD.

Figure 8: Genetic-based inhibition of autophagy sensitizes melanoma cells to MEK-induced cell death and potentiates danger-signalling. (A) Transient siRNA-based knockdown of ATG5 was monitored by western blotting for ATG5; achieving greater than a 70% reduction in protein expression in a cell lines (Aii). (B) Cell death induction of 10 µM PLX4032 or U0126 following perturbation of autophagy by siRNA-mediated knockdown of ATG5, in comparison to scrambled control, was assessed by propidium iodide based flow cytometry. (C) Danger-signalling incited by PLX4032 or U0126 combined with siRNA-mediated targeting of ATG5 was assessed by flow cytometry for ecto-CRT (Ci) and –HSP90 (Cii). All data sets are the mean of 3 independent experiments ± SD. Western blot images are representative blots of 3 independent experiment and expressions normalized to β-Actin loading control.