JLK1486, a *N,N*-[(8-hydroxyquinoline)methyl]-substituted benzylamine analogue, inhibits melanoma proliferation and induces autophagy

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Abstract

Objectives: To investigate anti-proliferatory activity of a selected *N,N*-[(8-hydroxyquinoline)methyl]-substituted benzylamine (JLK1486) on melanoma cells and to characterize its mechanism of cell population growth inhibition.

Materials and methods: *In vitro* cultures of B16F10 (mouse melanoma) cells were used as a model to characterize anti-proliferatory activity of JLK1486 using MTT growth assay, trypan blue viability assessment, cell cycle analysis, melanin production, β -galactosidase and acridine orange staining.

Results: Proliferating B16F10 and also MeWo (human melanoma) cells were strongly growth inhibited by JLK1486, displaying IC₅₀ values of 196 nM and 110 nM respectively. Anti-proliferatory effects were independent of cell death and were characterized by a distinct accumulation of cells in G₀/G₁ phase. Tyrosinase activity and relative melanin content remained unchanged indicating that the anti-proliferatory activity was not due to phenotype differentiation. Although treated B16F10 cells stained strongly positive for senescence marker β -galactosidase, cells regained near normal proliferatory activity after removal of JLK1486. Increased acridine orange staining and presence of perinuclear vacuoles suggested induction of autophagy in B16F10 cells. Furthermore, JLK1486 pre-treatment completely abolished melphalan and antimycin A-induced apoptosis.

Conclusion: JLK1486 provides a promising chemical scaffold to develop new anti-melanoma drugs or

combination therapies, due to its potent inhibition of cell proliferation and induction of autophagy, at pharmacologically relevant concentrations.

Introduction

Malignant melanoma continues to increase in incidence throughout the world and represents one of the most aggressive types of skin cancer; it has high metastatic potential. Its extraordinary opposition to conventional chemotherapeutic agents creates urgency for development of novel therapeutic strategies with which to treat this fatal disease. Resistance of melanoma cells to induction of apoptosis by available anti-cancer drugs is believed to be the result of inappropriate activation of survival signalling pathways, specially those mediated by RAF/mitogen-activated protein kinase kinase (MEK)/extracellular-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt pathways (1).

Temozolomide (TMZ) is a second-generation alkylating agent considered to be reference therapy for metastatic melanoma, with ability to cross the blood–brain barrier. Limited efficacy of this drug to treat melanoma has prompted numerous studies to investigate its/any potential synergism with classical chemotherapeutic agents or other targeted therapies. Unfortunately outcome of some completed phase II clinical studies has indicated that overall survival was not significantly altered and that haematological toxicity may be worse with combined treatment (2).

Hydroxyquinoline is a privileged structural moiety found in many biologically active natural products and has been extensively used as a chemical scaffold to develop new therapeutic agents, for a wide range of pathologies. Using diversity-oriented synthesis, a series of 8-hydroxyquinoline substituted amines has previously been synthesized and reported to possess potent anti-tumour activity; however molecular mechanisms of their action was not

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fully elucidated. JLK1486, a *N,N*-(8-hydroxyquinoline methyl)-substituted benzylamine, has been shown to be a potent inhibitor of glioma cell proliferation (3,4) with capability of crossing the blood–brain barrier (5), and may thus be equally well suited to treatment of metastatic melanoma. Although anti-proliferative activity of these compounds correlates with generation of relatively stable and moderately reactive quinone methide intermediates (which react preferentially with thiols but not with DNA amino groups), the precise molecular mechanism through which JLK1486 inhibits cell growth, also, has not been clarified. In this study, we determined that JLK1486 halted progression of B16F10 melanoma cells into S-phase and induced features typical of autophagy.

Materials and methods

Materials

JLK1486 was prepared according to procedures already described in an early report (3). TMZ, curcumin, melphalan, antimycin-A, rapamycin, wortmannin, L-DOPA and IBMX were obtained from Sigma (St. Louis, USA). Tissue culture medium and foetal calf serum were from Lonza (Verviers, Belgium). All other chemicals used in the study were of analytical grade, obtained from reputable manufacturers.

Cell culture

B16F10, MeWo, MCF7, HeLa and Chang liver cells were obtained from Highveld Biological (Johannesburg, South Africa). 3T3-L1 pre-adipocytes (CL-173) were purchased from ATCC (Manassas, USA). All cells except Chang liver were cultured in DMEM supplemented with 10% foetal calf serum. Chang liver cells were grown in EMEM containing 10% foetal calf serum and 1% non-essential amino acids.

Proliferation and cell viability assays

Cells were seeded into 96-well plates at 5000 cells/well and proliferation was deduced from MTT reduction at 72 h relative to that at time 0. Cell viability was determined using trypan blue assay. Spent tissue culture medium containing detached cells, and all wash solutions, were combined and added to cells obtained from trypsinization of remaining adherent monolayers. After centrifugation, cells were stained with trypan blue (0.4%) and immediately analysed by flow cytometry (10 000 events). Histogram data were collected on red filter (675 nm) which revealed two distinct peaks. The first represented viable cells, dimly fluorescent and not per-

meable to trypan blue, while the second peak represented dead cells, which permeable to the dye and hence more brightly fluorescent.

Cell cycle analysis with propidium iodide

Cells were washed by centrifugation at $500\times g$ for 5 min, then re-suspended in 1 ml PBS. This washing step was repeated twice and cells finally re-suspended in 500 μ l PBS. DNA Prep kit from Beckman Coulter was used for DNA cell cycle analysis and the assay was performed as follows: 50 μ l lysis buffer was added, tubes very gently vortexed and incubated for 5 min at room temperature. Thereafter, 500 μ l propidium iodide (50 μ g/ml) was added and allowed to incubate for 15 min at 37 °C in the dark. Samples were then analysed on a Beckman Coulter FC 500 (Brea, USA) flow cytometer and the data were analysed using MultiCycle version 4.0 software (Phoenix Flow Systems, San Diego, CA).

Cell differentiation

B16F10 cells were seeded into 24-well plates and treated with 1 μ M JLK1486 and/or 100 μ M IBMX (melanogenesis enhancer) for 5 days. Melanin content and tyrosinase activity were used as markers of differentiation and were measured after removing spent medium and washing cells in PBS. To quantify melanin content, cells were harvested by trypsinization and solubilized in 2 M NaOH at 100 °C for 20 min. Melanin content was then determined spectrophotometrically by measuring absorbance at 405 nm; data were expressed as function of cell density as determined with crystal violet (0.1%) staining in identically treated wells. Tyrosinase activity was measured after fixation in 4% formaldehyde for 20 min followed by three successive washes in PBS. Cells were incubated overnight in the presence of 10 mM L-DOPA and absorbance was measured at 405 nm.

Cell senescence

Cells were seeded in 6-well tissue culture dishes at 30 000 cells per well and treated with 1 μ M JLK1486 for 96 h. Senescence associated β -galactosidase was detected using Senescence β -galactosidase staining kit (Cell Signalling Technologies (Beverly, MA)) according to the manufacturer's instructions.

Colony forming capacity

B16F10 cells were seeded into 6-well plates at 500 cells per well and allowed to attach overnight. Cells were treated with JLK1486 or temozolomide for the indicated

times, after which the drugs were removed, cells washed once in complete medium and cultured for a further 5 days. Colonies were visualized by staining with crystal violet (1%).

AVO formation

Cells were seeded in 6-well tissue culture dishes at 30 000 cells per well and treated with JLK1486 in the presence and absence of 4 μM wortmannin. Seventy-two hours following initial treatment, cells were incubated in medium containing 1 $\mu\text{g}/\text{ml}$ acridine orange for 15 min, followed by three successive washes in PBS. Cells were trypsinized, collected by centrifugation and analysed by flow cytometry. A minimum of 10 000 cells within the gated region was analysed for increased red fluorescence.

Results

JLK1486 was a potent inhibitor of melanoma cell proliferation

Proliferating B16F10 (mouse melanoma) and MeWo (human melanoma) cells were strongly inhibited by JLK1486, displaying IC_{50} values of 196 nM and 110 nM respectively. Inhibition was, however, not specific to melanoma cells as the apoptosis proficient and non-tumorigenic cell line, 3T3-L1 (mouse pre-adipocytes), was similarly growth inhibited by JLK1486. Trypan blue and DAPI staining of cells treated for 72 h at concentration greater than 5-fold the IC_{50} value, revealed no evidence of cytotoxicity or aberrant nuclear morphology (Fig. 1). In contrast, B16F10 cells treated with melphalan or curcumin had significantly reduced cell viability, as evident from trypan blue staining, as well as increased presence of cells with enlarged nuclei. TMZ treatment (200 μM) was also not significantly cytotoxic to the melanoma cells, a finding in accord with reported resistance of B16F10 and MeWo cells to DNA alkylating agents (6), however 3T3-L1 cells were similarly resistant. Flow cytometric analysis of relative quantity of cells with less than 2 N DNA, corroborated the absence of apoptosis of TMZ- and JLK1486-treated cells (Fig. 1). Taken together these results indicate that JLK1486 was cytostatic to both melanoma cell lines tested, and that the anti-proliferatory effects were not due to induction of apoptosis or necrosis.

JLK1486 inhibited melanoma cell proliferation by preventing progression into the S-phase

JLK1486 treatment resulted in distinct accumulation of cells in G_0/G_1 phase with reciprocal reduction in S-phase

cells, a profile characteristic of G_1 arrest (Fig. 2a). In comparison to untreated control cells, JLK1486 halted progression into S-phase, providing support for strong anti-proliferatory activity. Dose-response curves for effects of JLK1486 on progression into S-phase in B16F10 cells yielded an IC_{50} value of 720 nM for cell cycle arrest, which is approximately 3-fold higher than that obtained by MTT assay for the anti-proliferatory effect (Fig. 2b). In contrast to JLK1486, TMZ induced G_2/M arrest in these cells. Tentori *et al.* reported that TMZ inhibited proliferation of B16 cells (IC_{50} value $702 \pm 5.6 \mu\text{M}$) and that TMZ induced G_2/M arrest without activation of apoptotic cell death pathways (7). In agreement with these studies, we show that anti-proliferatory activity of TMZ (200 μM) on B16F10 cells resulted, at least in part, from G_2/M arrest, and that under these conditions apoptosis was not induced, as judged by absence of sub- G_1 DNA and trypan blue, as well as DAPI staining showing normal nuclei (Fig. 1).

In addition to the two melanoma cell lines tested, JLK1486 also induced G_0/G_1 arrest and severe reduction in percentage of proliferating HeLa and MCF-7 cells in S-phase (Fig. 2c), however in proliferating 3T3-L1 pre-adipocytes, there was no significant increase in percentage cells in G_0/G_1 phase after 72 h treatment. A similar effect was also evident in identically treated Chang liver cells where there appeared to be a slight increase in number of S-phase cells (Fig. 2c).

3T3-L1 cells provided a further opportunity to investigate effects of JLK1486 on cell cycle kinetics as they possess the unique feature of being able to re-enter into an active phase of cell division after growth arrest, also known as the 'mitotic clonal expansion phase' (8,9). This allowed us to monitor kinetics of re-entry into the cell cycle in naturally synchronized cells without need for serum deprivation or other cell stress, to induce growth arrest. Confluent growth-arrested 3T3-L1 cells synchronously re-enter the cell cycle in response to hormonal stimuli such as addition of fresh serum, and complete an additional two rounds of cell division before initiating terminal differentiation (8). When our confluent 3T3-L1 cells were stimulated to re-enter cell division, there was a substantial increase in number of cells that were in the S-phase after 20 h, but by 48 h the majority of them had returned to G_0/G_1 , signifying completion of mitotic clonal expansion (Fig. 2d). In contrast, when 3T3-L1 cells were serum stimulated in the presence of 1 μM JLK1486, there was significant delay in rate of progression into S-phase which only peaked 48 h after induction. Considering similarity of the area under graphs for treated and untreated cells, it would appear that JLK1486 only reduced rate at which cells progressed to S-phase and not number of cells re-entering cell division. This cell-specific difference

Figure 1. JLK1486 inhibited proliferation independent of cell death. (a) Dose-dependent inhibition of cell proliferation in the presence of JLK1486. Proliferation rate calculated as ratio of MTT reduction at 72 h relative to that at time 0. Data represent mean $\pm$ SD for six replicate wells. IC₅₀ values were calculated using GraphPad Prism software (San Diego, USA). (b) Cell viability was determined by trypan blue exclusion staining and analysed by flow cytometry 72 h after initiation. Increased red fluorescence due to trypan blue uptake reflects reduced integrity of cell membranes and consequently cell viability. (c) Representative images of adherent cells after 72 h exposure to indicated compounds, and stained with DAPI to visualize nuclear morphology (magnification = 400 $\times$). (d) Sub-G₁ DNA content of treated B16F10 cells as analysed by flow cytometry after PI staining. (b–d) Concentrations tested were JLK1486 (1 μ M), melphalan (30 μ M), curcumin (30 μ M) and temozolomide (200 μ M).

between cell cycle parameters, together with the 3-fold difference in IC₅₀ value between MTT assay and cell cycle analysis, indicated that the primary anti-proliferatory mechanism of JLK1486 was not exclusively dependent on cell cycle arrest and suggested that G₀/G₁ arrest observed in the melanoma cells is a secondary effect in response to JLK1486 treatment.

Anti-proliferative activity of JLK1486 was independent of cell differentiation and classical senescence

Considering that induction of terminal differentiation is characterized by inhibition of cell cycle progression and with cell population growth arrest in G₀ phase, we investigated effects of JLK1486 on both production of

Figure 2. JLK1486 blocked progression to S-phase of B16F10 cells but not 3T3-L1 cells. (a) Cell cycle analysis of B16F10 cells 48 h after treatment with JLK1486 (1 μ M) and TMZ (200 μ M) relative to untreated proliferating cells. (b) Dose-dependent inhibition of S-phase of B16F10 cells treated with 1 μ M JLK1486 for 48 h. (c) Relative content of cells in the S-phase with and without treatment with 1 μ M JLK1486 (72 h) in indicated cell lines. (d) Re-entry of growth arrested 3T3-L1 cells after addition of fresh serum in presence and absence of 1 μ M JLK1486. Content of cells in the S-phase was assessed by propidium iodide staining and analysed by flow cytometry at the various time intervals indicated.

melanin and tyrosinase activity, classical differentiation markers for melanocytes. Treatment of proliferating B16F10 cells with IBMX, a melanogenesis enhancer, induced significant levels of melanin relative to untreated controls (Fig. 3a), while treatment with JLK1486 produced no visible signs of melanin accumulation nor increased tyrosinase activity. Quantification of relative melanin content and tyrosinase activity on a cell basis confirmed these observations (Fig. 3b,c), supporting the conclusion that anti-proliferation action of JLK1486 was independent of differentiation-induced growth arrest.

In addition to differentiation, cells entering senescence may also undergo irreversible cell cycle arrest in G₁ phase of the cell cycle; thus, β -galactosidase activity was measured, as a marker of senescence. B16F10 cells exposed to JLK1486 revealed prominent increase in β -galactosidase activity. Unlike TMZ-treated cells, the vast majority of B16F10 cells were positive, melanin containing cells, only, appearing to lack β -galactosidase activity (Fig. 4a). Cells treated with TMZ displayed

enlarged and flattened morphology and β -galactosidase reactivity, however percentages of cells β -galactosidase positive was considerably less than those observed with JLK1486 treatment, indicating that only a portion of TMZ-treated cells developed typical senescent features.

As with differentiation, cell senescence is typically associated with permanent cessation of proliferation. To investigate degree of reversibility of anti-proliferation effects of JLK1486, we determined colony forming ability and cell cycle parameters of treated B16F10 cells after different recovery intervals. Proliferating cultures were treated with JLK1486 for 24 h then allowed to recover in the absence of JLK1486 for either 24 or 96 h. At these respective time points, cells were harvested, counted and sub-cultured for colony forming assay; remaining cells were subjected to cell cycle analysis. Cell density was maintained below 80% confluence to avoid growth arrest due to contact inhibition. Both cell cycle analysis and colony forming capacity indicated that treated cells were still severely growth restricted after 24 h recovery period, however, they

Figure 3. JLK1486 growth arrest was not due to differentiation. (a) Microscope images of B16F10 cells treated with melanogenesis inducer IBMX in presence and absence of JLK1486. (b) Melanin content of treated B16F10 cells treated with IBMX (100 μ M) and (c) tyrosinase activity in corresponding treatments.

regained near normal proliferative activity 96 h after removal of JLK1486. These results clearly indicated that JLK1486 did not induce classical senescence, but rather a type of reversible growth arrest with acquisition of a 'senescence-like' phenotype. This finding further established that JLK1486-induced inhibition of proliferation in B16F10 cells was fully reversible.

JLK1486 induced features typical of autophagy

Microscopic examination of cells treated with JLK1486 revealed occurrence of several perinuclear structures resembling vacuoles, thus raising the possibility of a lysosomal response other than senescence, such as autophagy (Fig. 5a). To identify development of acidic vacuoles (AVOs), a characteristic feature of autophagy, cells were stained with lysosomo-tropic dye, acridine orange (AO). To confirm upregulation of autophagy, we made use of wortmannin, well-established to be its

inhibitor (10). Treatment of B16F10 cells with 1 μ M JLK1486 or rapamycin led to significant increase in the red fluorescence, indicating increase in the lysosomal compartment (Fig. 5b). When treated cells were incubated with NH_4Cl for an hour before final analysis, there was prominent reduction in AVO staining content, confirming acidic vacuole specificity of AO staining. Simultaneous treatment with wortmannin also significantly reduced AVO content, indicating that both JLK1486- and rapamycin-treated cells induced autophagy in B16F10 cells, albeit to significantly different extents.

JLK1486 protected B16F10 cells from pro-apoptotic drugs

To further explore potential anti-apoptotic properties of JLK1486, we tested effects of pre-treatment with JLK1486 on efficacy of two known apoptosis inducers,

Figure 4. Induction of senescence phenotype and reversibility of JLK1486-induced growth arrest. (a) β -Galactosidase staining of B16F10 cells treated for 96 h with JLK1486 and TMZ. (b) Colony forming capacity 24 and 96 h after removal of JLK1486 and TMZ (representative wells from duplicate experiment). Proliferating B16F10 cells were treated with JLK1486 (1 μ M) or TMZ (200 μ M) for 24 h then allowed to recover in the absence of these compounds for either 24 or 96 h. At these respective time points, cells were harvested, counted and sub-cultured for colony forming assays, and the remaining cells subjected to cell cycle analysis. Representative images of individual colonies after 24-h recovery are depicted for each treatment. After 96-h recovery, individual colonies were essentially the same as untreated controls, and thus not shown. (c) Relative distribution of cells in G₀/G₁, S and G₂/M phases of the cell cycle at different times during recovery (post 24-h treatment in the absence of compounds). Control represents untreated proliferating B16F10 cells.

Figure 5. JLK1486 induced features typical of autophagy. (a) Phase contrast images of B16F10 cells treated for 72 h with 1 μ M JLK1486 or 200 μ M temozolomide (b) AVO quantification of JLK1486-treated B16F10 cells by flow cytometry. Cells were treated with 1 μ M JLK1486 or 1 μ M rapamycin in the presence or absence of 4 μ M wortmannin. One hour before acridine orange staining and flow cytometric analysis, samples from each treatment group were incubated with 5 mM NH₄Cl to confirm specificity of fluorescence response for staining acidic vacuoles.

melphalan and antimycin-A. Pre-treatment of B16F10 cells with 1 μM JLK1486 24 h prior to induction of apoptosis, completely abolished melphalan- and antimycin-A-associated occurrence of cells with sub-G1 DNA (Fig. 6), suggesting that JLK1486 may be anti-apoptotic or at least prevent DNA fragmentation.

NAC did not attenuate JLK1486-induced anti-proliferative effect

Previous studies have established that 8-hydroxyquinoline derivatives can undergo formation of quinone methide intermediates (Figs 4,7a) raising the possibility that JLK1486-induced inhibition of cell proliferation may be a direct consequence of GSH depletion and generalized redox imbalance with subsequent activation of the

unfolded protein response. As a strategy to counteract GSH depletion, NAC was used to restore cell GSH levels. Addition of NAC to JLK1486 treatment, did not however change anti-proliferative properties in any of the cell lines tested (Fig. 7b), indicating that as an alkylating agent GSH is not the molecular target of JLK1486 and implies that alkylating properties of this compound more likely target specific protein(s) with vulnerable thiol groups.

Discussion

Previous studies have aimed at development of hydroxyquinoline-based compounds as novel anticancer agents (3,4), and highlighted JLK1486 as a promising anti-glioblastoma compound. Although these investigations revealed activation of various transcription factors which

Figure 6. JLK1486 pre-treatment attenuated apoptosis. B16F10 cells were pre-treated for 24 h in absence or presence of 1 μM JLK1486 followed by 48 h treatment with various pro-apoptotic drugs. (a) representative DNA histograms of B16F10 cells treated with apoptosis inducers in the presence and absence of JLK1486 pre-treatment. (b) Quantitative analysis % cells containing fragmented DNA. Data represent the mean from three independent experiments.

Figure 7. Anti-proliferatory activity of JLK1486 was not due to GSH depletion. (a) Structure and proposed formation of QM intermediate capable of alkylating thiol groups. (b) Effect of JLK1486 treatment (48 h) on proliferation of various cells in presence and absence of 2.5 mM NAC. 2-deoxy-ribose was used to deplete cell GSH levels and to demonstrate efficacy of NAC to restore proliferative capacity.

in turn may regulate cell proliferation, the precise molecular mechanism was not established. In this study, we investigated potential anti-melanoma activity of JLK1486 in the highly metastatic melanoma cell line B16F10, and attempted to characterize its anti-proliferative mechanism. Consistent with earlier studies, JLK1486 also strongly inhibited cell proliferation of the two melanoma cell lines tested; however, there was no evidence of apoptotic cell death. Even at concentrations 5-fold greater than its IC_{50} value, it had no significant effect on viability of B16F10 cells when treated for 72 h (data not shown). This strong cytostatic and non-cytotoxic effect is similar to that reported for inhibition of glioblastoma proliferation and is in accord with its neuroprotective properties against stressed HT-22 cells (11); however, it differs from pro-apoptotic effects observed in a number of carcinoma cell lines including M4 melanoma cells (4). In contrast to cytostatic effects observed in various glioblastoma cell lines, JLK1486 induced extensive apoptotic cell death in primary glioblastoma (12). These contrasting effects indicate that the anti-proliferative mechanism of JLK1486 is highly cell type dependent.

JLK1486 induced prominent growth arrest in G_0/G_1 phase of the cell cycle in B16F10 cells but not in 3T3-L1 cells. Given similar cytotoxicity profiles, the

seemingly modest effect of JLK1486 on cell cycle parameters of 3T3-L1 cells after 72 h treatment relative to that on B16F10 cells, appears contradictory to anti-proliferatory capacity of JLK1486 in these two cell lines. Our results indicate that 3T3-L1 cells seemed to be slightly more sensitive to anti-proliferative effects of JLK1486 (IC_{50} values: 3T3-L1 = 112 nM and B16F10 = 196 nM). This cell-specific difference together with approximately 3-fold difference in IC_{50} between MTT proliferation assay and cell cycle analysis indicates that the anti-proliferatory mechanism of JLK1486 is not exclusively dependent on cell cycle arrest and suggests that G_0/G_1 arrest observed in the melanoma cells is a secondary effect, in response to JLK1486 treatment, possibly ER stress as has been reported for U87 glioblastoma cells (4). Bruyère *et al.* also reported absence of any significant changes in cell cycle profile when glioblastoma cells were treated with 100 nM JLK1486 despite significant growth inhibition at this concentration (IC_{50} values for anti-proliferative activity ranged between ~80 and 290 nM for glioblastoma cells investigated) (13).

A more perplexing question is how can it be that a non-cytotoxic compound with potent anti-proliferatory activity reduces cell population expansion without any major change to the cell cycle, as seen in 3T3-L1 and

Chang liver cells? One possible explanation may be that in these cells, JLK1486 delays the cell cycle, but with uniform effect on rate of progression throughout each phase of the cell cycle. This results in lower rate of cell division without altering relative percentage of cells present in any given phase. Our results on effects of JLK1486 on mitotic clonal expansion in 3T3-L1 cells (in which there was clear delay in number of cells that progressed to S-phase) as well as on synchronized proliferating 3T3-L1 cells (in which majority of treated cells were still in S phase 18 h after reinitiating the cell cycle), as opposed to untreated control cells [which had progressed beyond S phase during the same time interval (data not shown)] supports such a hypothesis. However, at this stage we can not rule out the possibility that 3T3-L1 and Chang liver cells are intrinsically more resistant to phase-specific G₀/G₁ arrest induced by JLK1486, relative to B16F10 cells, and that delay in overall cell cycling in 3T3-L1 and Chang liver cells represents the same initial discrepancy between G₀/G₁ arrest and anti-proliferative potency, described above.

Considering the prominent G₀/G₁ arrest on JLK1486 treatment, and absence of any indication of terminal differentiation, we investigated two additional cytostatic mechanisms, namely senescence and autophagy. When B16F10 cells were treated with 1 μ M JLK1486, there was a dramatic increase in expression of senescence marker β -galactosidase, however within 96 h of removal of JLK1486, cells regained full proliferative capacity, indicating that although the senescent phenotype was extensive it was transient and thus not considered indicative of classical senescence which is irreversible. Similar elevation in senescence marker β -galactosidase, without permanent growth arrest, has been reported in U87 glioma cells (12), although treatment induced extensive cell death of primary glioblastoma cells through activation of both caspase and cathepsin activities. In this study, there were no convincing morphological changes in JLK1486-treated cells regarding other characteristics typically associated with senescence, such as changes in side scatter properties or presence of heterochromatin foci in DAPI-stained nuclei. Although β -galactosidase activity has long been considered a specific marker for senescence, its functional relevance to this anti-proliferative mechanism has not been established (14). Given that this enzyme is associated with the lysosomal compartment, it is tempting to speculate that its dramatic increase in JLK1486-treated cells may equally represent induction of a further lysosomal pathway, namely autophagy, which is also characterized by formation of autolysosomes. Thus, appearance of β -galactosidase without permanent growth arrest does not reliably assign JLK1486-treated cells as being truly

senescent, and may simply represent overlap between pathways of senescence and autophagy which are both characterized by expansion of the lysosomal compartment.

JLK1486 induced AVO formation, which further supports involvement of a lysosomal response such as autophagy and/or ER stress. Rapamycin, an inhibitor of mTOR and well-known inducer of autophagy, was considerably less effective than JLK1486 in elevating formation of AVOs. Relative insensitivity of B16F10 cells to effects of rapamycin may be related to presence of mutated B-RAF, and that oncogenic B-RAF is reported to confer resistance to mTOR-dependent induction of autophagy (15). Thus, autophagy activation is considered to have a pro-survival function in B-RAF wild-type cells, which would be more sensitive to autophagy inhibition. On the other hand, ER stress-induced autophagy has been shown to be significantly less in B-RAF mutated compared to wild-type melanoma cells (15), and consequently inhibition of autophagy in this setting would be relatively ineffective in enhancing cell death. Interestingly, addition of the autophagy inhibitor, chloroquine did not promote cell death in JLK1486-treated B16F10 cells (data not shown) which is consistent with ER stress response in B-RAF mutated cells.

Pro-survival characteristics of JLK1486 are further illustrated by complete ablation of apoptosis of melphalan and antimycin-A-treated cells. It is known that melphalan induces apoptosis by affecting DNA alkylation and may induce both S and G₂/M arrest, however cell death is restricted to S phase only (16). Under our experimental conditions, we found that melphalan-treated B16F10 cells significantly accumulated in both S and G₂/M phases, with concomitant increase in apoptosis as judged from the sub-G₁ peak. Thus, it is not surprising that pre-treatment with JLK1486 (which almost completely prevents progression to S-phase), would attenuate cytotoxic efficacy of melphalan, and thus appear anti-apoptotic. Consequently apparent anti-apoptotic characteristics cannot be offered as proof for induction of autophagy, due to dependency of this drug on actively proliferating cells. In contrast, antimycin-A directly targets mitochondrial membrane potential and thus leads to activation of the intrinsic apoptotic pathway, presumably independent of the cell cycle. Pre-treatment with JLK1486 completely blocked appearance of sub-G₁ DNA which is in line with protective effects of autophagy. Interestingly, pre-treatment with Nelfinavir (a protease inhibitor that has been shown to induce ER stress and subsequent autophagy in melanoma cells) prior to anti-A treatment, also prevented apoptosis, but unlike JLK1486, Nelfinavir did not induce G₁ arrest in B16F10 cells (data not shown). These findings

suggest that JLK1486-induced G₀/G₁ arrest may be independent of induction of the survival response and is in accord with absence of G₀/G₁ arrest in 3T3-L1 and Chang liver cells, despite potent anti-proliferatory effects.

Cell-specific differences observed in this study, as well as previously reported in the literature, may be due to differential sensitivity of cells to successfully mount a counter response when challenged with JLK1486. On the one hand, induction of autophagy can engage a strong survival response, as we have determined in B16F10 cells [and possibly also in glioblastoma and neuronal cells (11)], however in certain lines response to JLK1486-induced cell stress may not be sufficiently counteracted by autophagy, or autophagy in itself may trigger cell death. Considering that the majority of aggressive malignant melanomas are highly resistant to therapeutic strategies targeting a single signalling pathway, JLK1486 provides an additional chemical scaffold with potent anti-proliferatory activity, with which to exploit existing chemotherapeutic approaches to potentially enhance efficacy. As a potent inducer of ER stress and concomitant autophagy, JLK1486 represents a valuable addition to the existing and somewhat limited repertoire of available drugs which target this recognized anti-cancer mechanism. On the other hand, our results also indicate that cell cycle arrest induced by JLK1486 can protect malignant melanoma cells from chemotherapeutic drugs that target proliferating cells; this needs to be taken into consideration when designing possible synergistic drug treatment regimes.

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