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ORIGINAL ARTICLE

WP760, a melanoma selective drug

Mingzhong Zheng · Waldemar Priebe · Eugene T. Walch · Katherine G. Roth · Mikyung Han · Chi-Hui Tang · Sangkyou Lee · Nancy J. Poindexter · Izabela Fokt · Elizabeth A. Grimm

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Abstract
Purpose Our goal was to perform studies on the specificity and antimelanoma mechanism of a novel bisanthracycline, WP760. WP760 initially identified in the NCI 160 screen as anti-melanoma.
Methods The methyl thiazolyl tetrazolium reduction (MTT) assay was used to test tumor cell growth inhibition; confocal microscopy to view WP760 intracellular distribution; flow cytometry for cell-cycle arrest and apoptosis; and Western blotting was employed to identify and compare quantities and kinetics of cell growth related molecule levels.
Results WP760 induced G_2/M-phase cell-cycle arrest and apoptosis in melanoma cell lines and short-term melanoma explants established from clinical specimens in a time and concentration dependent manner at nM concentrations. In contrast, effects on fibroblasts and A549 lung cancer cells required higher concentrations, suggesting that WP760 possesses selectivity for melanoma. Molecular studies indicated that WP760 induced p53 stabilization, checkpoint kinase 2 and $p27^{Kip1}$ protein upregulation, and activation of caspase-3. Endogenous nitric oxide (NO) production has been implicated in the chemoresistance of melanoma; WP760 caused inhibition of the inducible nitric oxide synthase (iNOS) protein as well as inhibition of phosphorylation of ERK, known to drive the iNOS pathway. Based on WP760 localization into mitochondria, and caspase-3 inhibitor block the killing of WP760, the intrinsic pathway of apoptosis appears to have been activated.
Conclusions Our results indicate that WP760 affects a critical and unique set of growth regulatory effects in melanoma, and is a promising candidate for further preclinical studies.

Keywords Melanoma · Apoptosis · Cell cycle · iNOS · WP760

M. Zheng · W. Priebe · E. T. Walch · K. G. Roth · M. Han · C.-H. Tang · S. Lee · N. J. Poindexter · I. Fokt · E. A. Grimm (✉)
Department of Experimental Therapeutics, Unit 362, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
e-mail: egrimm@mdanderson.org

Introduction

Melanoma tumors are considered highly resistant to chemotherapy and radiotherapy [1]. Well analyzed drug-resistance mechanisms in melanoma include enhanced intracellular and extracellular drug transport, induction of redundant growth promoting pathways, and altered drug–target interactions [2]. Functional inhibition of apoptotic pathways in melanoma tumor cells is well noted; including inhibition driven by constitutively expressed low levels of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) [3]. To date most apoptosis-inducing agents stimulate melanoma cells to activate survival pathways, including the MAPK/ERK [4], phosphatidylinositol 3-kinase [5], and nuclear factor kappa B (NF-kB) [6]. Therefore, the development and evaluation of new agents with potential for growth regulation of melanoma remains a high priority.

As part of our drug discovery program, we have employed a "modular" approach to synthesizing unique libraries of growth inhibiting drugs [7]. One of

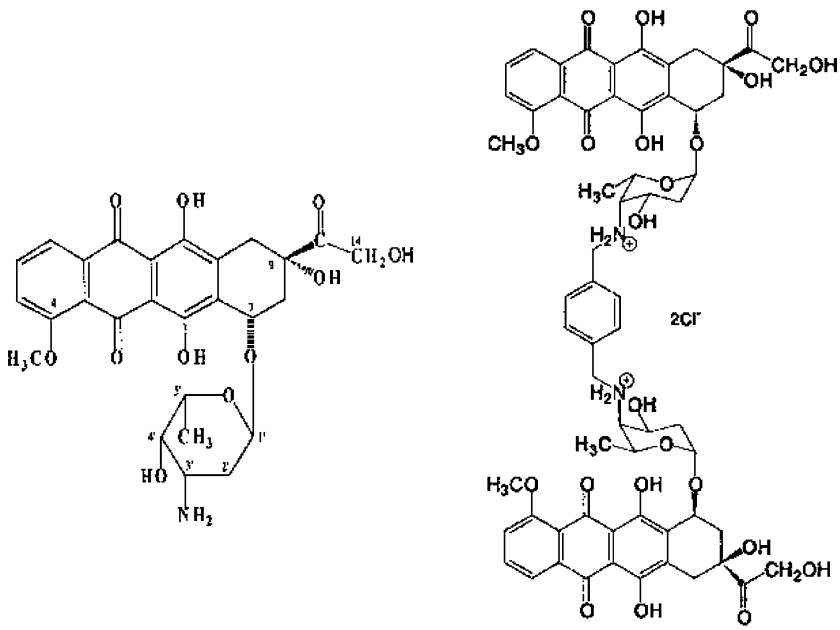

Fig. 1 Structures of WP760 and Dox

these new compounds, WP760 (Fig. 1), was prepared as part of a novel library of DNA binding agents which in its design exploited intercalating moieties derived from daunorubicin and doxorubicin, namely daunomycinone and adriamycinone, and two minor groove-binding sugars related to daunosamine and 4-amino-2,3,6-trideoxy-alpha-L-lyxo-hexopyranose and selected linkers [8]. This library was tested in preliminary NCI screening and the compound WP760 was identified as an agent selectively cytotoxic against melanoma. This report describes the initial in vitro functional activity of WP760, in comparison to other related and parent molecules, and provides evidence to support futher exploration of WP760 as a novel therapeutic as it overcomes the apoptosis resistance pathways and suppresses survival responses that are active in melanoma.

Materials and methods

WP760 and related drugs

WP760 (Fig. 1) was prepared by us [7] and its structure was confirmed using nuclear magnetic resonance (NMR) and purity assessed by TLC and ^{1}H-NMR [9]. Solutions containing 10 mM doxorubicin (Dox) (Sigma), WP652 or WP760 were prepared in dimethyl sulfoxide (DMSO), maintained at 4°C, and brought to the final concentrations with culture medium just before use.

Cell lines

The human metastatic melanoma cell line A375, with mutated B-raf and wt p53 [10] was obtained from the American Type Culture Collection (Manassas, VA, USA), and the metastatic human melanoma cell line MeWo, with homozygous wt B-raf and mutated p53, was provided by Dr. David Menter (The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA). Three low passage cell lines developed from explants of melanoma surgical biopsies (MEL-1, MEL-2, MEL-3) and fibroblasts from these same biopsies were prepared by culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml each of penicillin and streptomycin, and 2 mM L-glutamine in humidified 5% CO_2 at 37°C. DNA from MEL-1 was sequenced and found to contain the exon 15 V600E B-raf mutation, but MEL-2 and MEL-3 were both wt for B-Raf and N-ras.

In vitro growth inhibition. Cell viability and proliferation in response to WP760 was determined using the methyl thiazolyl tetrazolium (MTT) reduction assay performed as previously described [11]. Briefly, 1.5×10^4 cells were plated in triplicate in 96-well, flat-bottom tissue culture plates with treatments. After incubation for the indicated times (24, 48 or 72 h, 37°C, 5% CO_2), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide was added to each well to obtain a final concentration of 0.1 μg/ml (37°C, 5% CO_2) for 1 h. Medium was removed and 100 μl DMSO was added for 1 h at room temperature. Absorbance at

570 nm was read using an MRX microplate reader (DYNEX, Chantilly, VA, USA). All cells were greater than 90% viable at the onset of testing. All experiments were repeated at least three times.

Confocal microscopy assay

A375 Cells were grown on chamber slides and treated with Dox or WP760 (0–2 μM) for 2 h up to 24 h, followed by incubation for 15 min at 37°C in a medium containing 75 nM Mito Tracker Green FM (Molecular Probes, Eugene, OR, USA) to stain mitochondria. Confocal laser microscopy was performed with an Olympus Fluoview FV500 microscope system (Melville, NY, USA).

Western blotting

Melanoma cells were cultured with various concentrations of WP760, WP652, or Dox for 24 h or with 100 nM WP760 for 30 min to 48 h, and the cell lysates were examined for changes in selective growth and apoptotic markers. Whole-cell extracts were prepared using a cell lysis buffer containing 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1% Nonidet P-40, and a protease inhibitor cocktail. Whole-cell lysates (40 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis; transferred to nitrocellulose membranes; blocked with 5% milk in a buffer containing 10 mM tris base, 150 mM NaCl, and 0.1% tween 20 (pH 8.0); and probed with the appropriate antibodies. Apoptosis- and cell-cycle-related molecules, including p53, $p21^{WAF1}$, $p27^{Kip1}$, checkpoint kinase (CHK) 1/2, and cleaved caspase-3, and tumor cell proliferation-related molecules including phosphorylated ERK (p-ERK)1/2 and iNOS were examined. Rabbit antihuman cleaved caspase-3 immunoglobulin was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-β-actin monoclonal antibody was purchased from Oncogene Research Products (San Diego, CA, USA). All other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactivity was developed with either horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (Santa Cruz Biotechnology) using the enhanced chemiluminescence method (Amersham Biosciences, Sunnyvale, CA, USA).

Determination of cell-cycle arrest and apoptosis

A terminal deoxynucleotide transferase-mediated dUTP-FITC nick end labeling (TUNEL) assay was employed as described previously [3]. Briefly, cells were fixed and stained using an apoptosis detection kit according to the manufacturer's protocol (fluorescein FragEL, Oncogene Research Products, San Diego, CA, USA). To determine whether WP760 could induce cell-cycle arrest in human melanoma cells, A375 and MeWo cells were partially synchronized by growth in serum-free for 18 h followed by culture in complete medium with defined concentrations of WP760 (0–900 nm) for 24 h. The treated cells were centrifuged at 1,000×*g* for 5 min, fixed in 4% formaldehyde and PBS for 10 min, and stored in 80% ethanol at 4°C. The cells were rehydrated in tris-buffered saline [TBS, 10 mM tris base and 150 mM NaCl (pH 7.4)] for 10 min and rendered permeable by exposure to 20 μg/ml proteinase K for 5 min. After incubation in fluorescein FragEL equilibration buffer, cells were incubated in TUNEL reaction mixture for 90 min at 37°C in the dark. The cells were then washed in TBS and resuspended in propidium (PI) working solution (50 μg/ml RNAse-A and 10 μg/ml PI in PBS) for 15 min in the dark. TUNEL and PI staining was assessed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA).

Statistical analysis

All experiments were carried out in triplicate and repeated at least two times. Data are presented as mean ± SD. Data were analyzed by Student's *t* test and differences were considered significant at $P < 0.05$.

Results

Selective inhibition of melanoma cell growth

Initial NCI screenings of the NCI 60 cell lines for viability suggested that WP760 selectively inhibits melanoma cell growth (to be reported elsewhere). To assess the mechanisms of this inhibitory effect as well as confirm selectivity, we examined the growth inhibitory effects of WP760 on melanoma cell lines, A375 MeWo, as well as the lung cancer line A549, and normal human fibroblasts. We found that after treatment at varying concentrations up to 1,000 nM for 3 days, WP760 was much more effective at inhibiting growth in melanoma cells than Dox. The lung cancer cell line, A549, and fibroblasts were relatively resistant to WP760 (Fig. 2a). WP760 at 30 nM inhibited MeWo and A375 cell growth, 50 and 30% respectively, as measured by MTT assay. Notably at this concentration, WP760 did not have significant

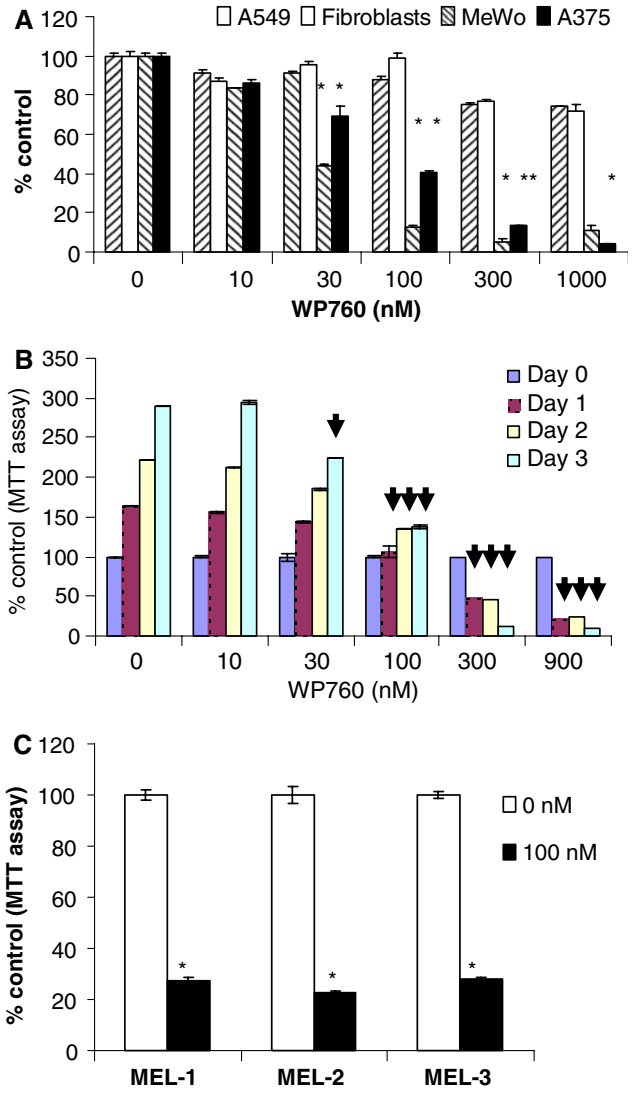

Fig. 2 **a** Effects of WP760 and Dox on cell proliferation. A375, MeWo, A549 cancer cells, and fibroblasts were treated with increasing concentrations of WP760 or Dox for 72 h. Cell proliferation was measured using the MTT assay. Data are presented as the mean of three experiments ± SD. *$P < 0.01$ compared to 0 nM control. **b** Inhibition of melanoma cell proliferation. A375 cells were treated with various concentrations of WP760 for up to 3 days. Cell proliferation was measured using the *MTT assay*. Data are presented as the mean of three experiments ±SD. *Arrow* indicated $P < 0.01$ compared to 0 nM control. **c** Three early passage melanoma cell lines developed from patient tumor tissue (*MEL*-1, *MEL*-2, and *MEL*-3) were treated with 100 nM WP760 or not treated. Cell proliferation was measured using the *MTT assay*. Data are presented as the mean of three experiments ± SD. *$P < 0.01$ compared to 0 nM control

growth inhibitory effects on A549 or fibroblasts. WP760 at100 nM inhibited about 60% of A375 and 80% of MeWo cell growth but still did not show any growth inhibitory effects on A549 lung cancer cells or fibroblasts.

Inhibition of melanoma cell line survival in a time- and concentration-dependent manner

To determine the growth-inhibitory effects of WP760 on melanoma cells, we treated A375 and MeWo cells with various concentrations of WP760, up to 900 nM and measured cell growth by MTT assay at different times points from 0 to 72 h. WP760 had similar effects on A375 and MeWo cells, therefore only the A375 data are presented. The in vitro growth inhibition studies (Fig. 2b) showed that melanoma cells were sensitive to WP760, which inhibited proliferation in a time- and concentration-dependent manner. The lowest tested concentration at which WP760 significantly ($P < 0.01$) inhibited A375 cell proliferation was 30 nM after 3 days, whereas 100 nM of WP760 significantly ($P < 0.01$) inhibited proliferation within 24 h. At a concentration of 300 nM for 24 h, WP760 inhibited about 90% proliferation in A375 cells compared to untreated control cells.

Inhibition of primary cultures of melanoma cell growth

As melanoma cell lines are not necessarily reflective of the melanoma cells from patient tumors, we wished to address the growth inhibitor effect on early passage melanoma derived from surgical specimens. Treatment of the three early passage lines from our clinical melanoma samples with 100 nM WP760 for 24 h resulted in a 70% decrease in cell proliferation (Fig. 2c).

Induction of G_2/M-phase cell cycle arrest and apoptosis

To address mechanisms of WP760 involved in inhibition of cell growth, we measured the percentage of cells in each cycle phase and the extent of DNA fragmentation. A375 cells were cultured in serum-free media for 18 h to partially synchronize the serum-dependent cells. The cells were washed and cultured for an additional 24 h in complete medium containing various concentrations of WP760 followed by PI staining for cell cycle analysis. The percentage of cells in the G_2/M phase was significantly ($P < 0.05$) higher for cells treated with WP760 at 100, 300, and 900 nM than for the control cells (Fig. 3a), suggesting that the drug induced cell-cycle arrest. To test whether the G_2/M-phase cell-cycle arrest may also be due to DNA damage; we performed TUNEL staining, which confirmed that WP760 induced DNA fragmentation in A375 cells in a concentration-dependent manner (Fig. 3b). Additional studies at later times demonstrated increased TUNEL staining at lower

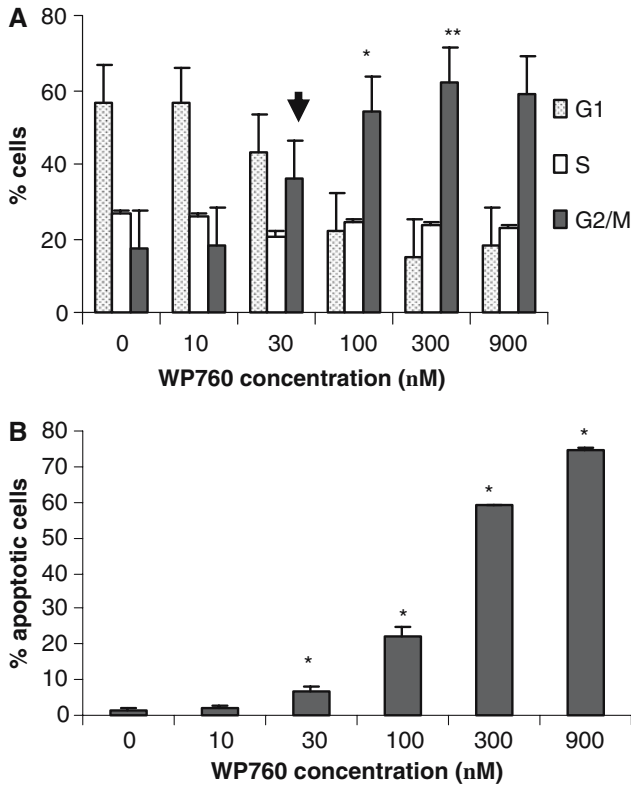

Fig. 3 **a** Induction of G_2/M-phase cell cycle arrest. A375 cells (1×10^6 cells/ml) were synchronized by incubation overnight in the absence of serum and treated with WP760 at the indicated concentrations for 24 h. The cells were then washed, fixed, stained with PI, and cell cycle analysis done by flow cytometry. The percentages of cells in each phase are presented as the mean of three experiments ±SD. *Arrow* indicated $P < 0.05$ and $^*P < 0.01$ compared to 0 nM control. **b** Induction of DNA fragmentation in A375 cells. A375 cells (1×10^6 cells/ml) were synchronized by incubation overnight in the absence of serum and treated with WP760 at the indicated concentrations for 24 h. The cells were then washed, fixed, stained with TUNEL reagent, and analyzed for DNA fragmentation by flow cytometry. Data are presented as the mean of three experiments ± SD. $^*P < 0.01$ compared to 0 nM control

concentrations of WP760 as the apoptotic cells accumulated with time (data not shown).

Matching mitochondria in intracellular distribution

Dox is well known as a DNA binding drug, and can be observed to localize in the nucleus of cells due to its red autofluoresence. WP760 was also expected to bind DNA and autofluoresence, therefore we compared the intracellular localization of these two drugs. Confocal microscopy images of A375 cells showed that WP760 mainly exists in the cytoplasm, while Dox was apparent in the nucleus as early as 2 h (Fig. 4a). Treatment for 6, 12 and 24 h did not change the cellular distribution of either WP760 or Dox. At no time did WP760 appear to localize to the nucleus. Staining of mitochondria with mitotracker was performed to test whether WP760 co-localized to mitochondria or remained in the cytosol, and surprisingly the mitotracker and WP760 merged completely indicating that under identical conditions, Dox localized, as expected, to the melanoma cell nucleus but WP760 localized to the mitochondria.

Induction of p53, active caspase-3, CHK2, $p27^{Kip1}$, and $p21^{WAF1}$

Because caspase-3 activation (cleavage) and p53 stabilization are molecule markers of apoptosis we examined WP760 treated cells for expression of p53 and cleaved caspase-3 by Western analysis WP760 induced both cleavage of caspase-3 and p53 stabilization (Fig. 4b), thus confirming that WP760-induced apoptosis may be p53 related. Also, WP760 induced caspase-9 and caspase-8 expression in A375 cells. Caspase-9 is a mitochondria-related apoptosis pathway indicator, and caspase-8 is non-mitochondria-related [12]. Therefore, these data suggest that multiple pathways of apoptosis are likely to be elicited by WP760.

CHK1, CHK2, $p27^{Kip1}$, and $p21^{WAF1}$ are important cell-cycle regulatory markers [12, 13]. Accumulation of these proteins in the cytosol inhibits proliferation by inducing cell-cycle arrest. Immunoblotting demonstrated that WP760 induced the expression of CHK2, $p27^{Kip1}$ and $p21^{WAF1}$ (Fig. 4b), whereas CHK1 was not detected in A375 or MeWo cells (data not shown).

Identification of caspase-3 activation and involvement

Because the caspase-3 is involved in the apoptosis of WP760 induced melanoma cells, we co-treat MeWo cells with or without 2 μg/ml caspase-3 inhibitor II and WP760. The growth inhibition by WP760 was blocked by caspase-3 inhibitor II (Fig. 4c).

Inhibition of MAPK/ERK cascade

Because the MAPK/ERK pathway plays a supporting role in melanoma proliferation and survival, and is often activated as a result of upstream mutated B-raf or N-ras [10] we performed immunoblotting to examine ERK phosphorylation changes. The phosphorylation was decreased in response to WP760 in a concentration-dependent manner (Fig. 4c) while total ERK did not change. Other components of MAPK/ERK family including p-p38, p-JNK1/2 are also down-regulated (data not shown).

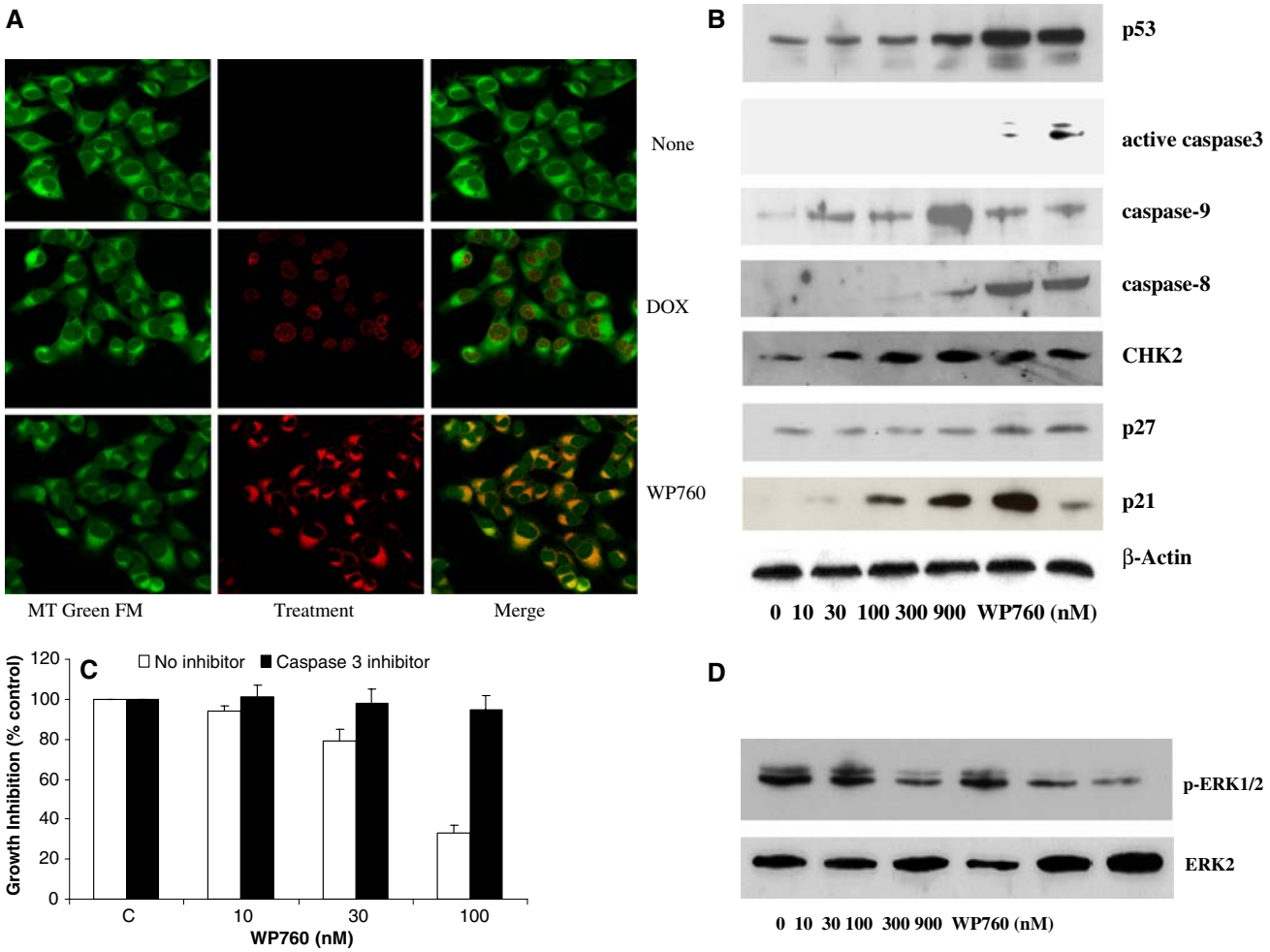

Fig. 4 **a** Comparison of the intracellular distribution of WP760 and Dox. A375 cells were treated with 0.5 μM Dox or WP760 for 2 h and incubated for 15 min with 75 nM Mito tracker green (MF Green) FM (localizes to the mitochondria and produces green fluorophore). The images were obtained by confocal microscopy. The images in the *left panel* were *Mito Tracker Green FM* produced green fluorophore. The images in the *middle panel* were WP760 or Dox produced red fluorophore. The images in the right panel were the merged images from both the *left* and *middle panels*. **b** Stimulation of cell cycle and apoptotic regulators. After treatment with WP760 at the indicated concentrations for 24 h, whole-cell A375 and MeWo lysates were prepared from 2×10^6 cells. Equal quantities of protein were loaded. Expression of p53, cleaved caspase-3, caspase-8, caspase-9, CHK2, $p27^{Kip1}$ and $p21^{WAF1}$ proteins were then determined by immunoblotting and data shown for MeWo (similar results for both cell lines). **c** Caspase-3 inhibitor blocks the growth Inhibition of WP760 in MeWo cells. 1×10^6 MeWo cells/well were incubated for overnight in six well plates. The media was then replaced with fresh media containing WP760 and/or caspase-3 inhibitor II at the indicated concentrations for an additional 72 h. Cell proliferation was measured using the MTT assay. Data are presented as the mean of three experiments ± SEM. **d** Inhibition of ERK phosphorylation in A375 cells. A total of 1×10^6 A375 cells/well were incubated for 18–24 h in 6 well plates. The media was then replaced with fresh media containing WP760 at the indicated concentrations for an additional 24 h. Whole-cell A375 lysates were extracted from 2×10^6 cells. Equal quantities of protein were loaded. p-ERK1/2 and ERK2 expression were determined by immunoblotting.

Inhibition of iNOS protein expression

Endogenous NO plays an important role in supporting melanoma cell proliferation [14] as well as apoptosis resistance [3]. To assess whether iNOS-driven NO generation is controlled as part of the WP760 mechanism of action, we examined iNOS protein levels by immunoblotting. The expression of iNOS proteins in A375 cells was downregulated in a concentration- (Fig. 5a) and time-dependent (Fig. 5b) manner. WP760 at 100 nM (or higher) significantly inhibited iNOS protein in A375 cells as early as 2 h and decreased to its lowest level after 6 h. Levels of iNOS then appeared to partially recover although never returning to the level of expression prior to treatment with WP760. In addition, the three early passage melanoma cells lines expressed endogenous iNOS. Treatment of these cells for 24 h with 100 nM WP760 caused downregulation of iNOS expression in two of three lines tested (Fig. 5c).

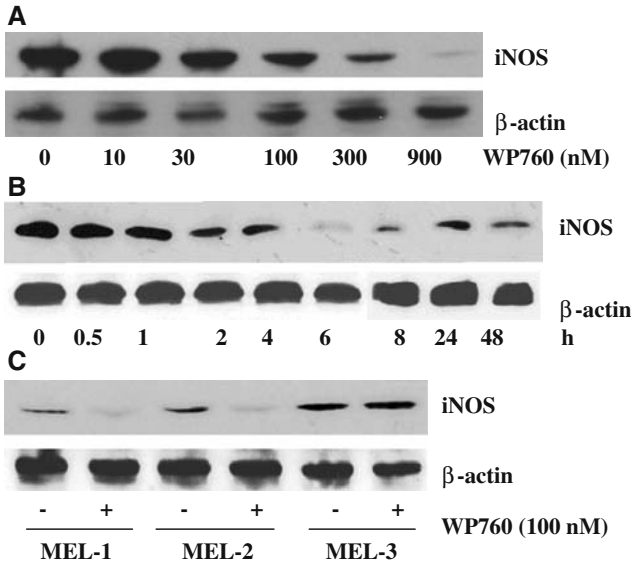

Fig. 5 Inhibition of constitutive iNOS expression in A375 cells in a concentration-dependent (**a**) and time-dependent (**b**) manner. After treatment with WP760 at the indicated concentrations for 24 h (**a**) or with 100 nM WP760 for the indicated times (**b**), whole-cell lysates were extracted from 2×10^6 cells/ml. Equal quantities of protein were loaded. The iNOS protein expression was then examined by Western blotting. **c** Inhibition of constitutive iNOS expression in early passage melanoma cell lines developed from patient tumor tissues. After treatment with 100 nM WP760 for 24 h, whole-cell lysates were extracted from 2×10^6 cells. iNOS protein expression in each melanoma cell line was then determined by immunoblotting

Discussion

WP760 appears to be a unique melanoma selective drug, capable of interrupting growth and apoptosis resistance pathways, and inducing apoptosis via the mitochondrial depolarization. WP760 was effective in controlling melanoma growth of both long term cultured cell lines and early passage tumor cells developed from surgical specimens. Normal human fibroblasts or lung cancer lines were relatively resistant to WP760, requiring 10 to 100-fold more WP760 for growth inhibitory effects. This finding indicates that malignant melanoma cells are differentially sensitive to WP760 as compared to culture human fibroblasts and a lung cancer cell line. While the molecular mechanisms unique to melanoma growth appear to be multifactorial, and are the target of much current research, it is clear from our studies that WP760 induces G2/M-phase cell-cycle arrest and apoptosis. It is expected that WP760 is a DNA-damaging agent, however the lack of nuclear DNA localization and apparent mitochondrial targeting raises the possibility of additional functional interactions, or a direct interaction with mitochondrial DNA. These possibilities are currently under consideration in further studies.

Our data further demonstrate that the G_2/M-phase cell-cycle arrest and apoptosis induced by WP760 involves multiple pathways related to p53, $p27^{Kip1}$, and CHK2. Because p53 is critical in the maintenance of genome integrity [15] by regulating the cell cycle ($p21^{WAF1}$ and cyclin G), DNA damage (GADD45), and apoptosis (Bax), restoring apoptosis via regulation of p53 is an important strategy in cancer gene therapy [16]. P53 either causes cell-cycle arrest or induces apoptosis [17]. Our data show that WP760 induces p53 in A375 cells which is known to contain wt p53, suggesting that p53 is critical to the induction of cell-cycle arrest and apoptosis mediated by WP760. P53-dependent apoptosis is well known to be activated in response to DNA-damaging agents, such as carcinogens, ultraviolet light, and chemotherapeutic drugs [18]. The induction of G_2/M-phase cell-cycle arrest and apoptosis by WP760 may be explained by its Dox-like structure [19]. The antitumor activity of Dox is the result of an apoptotic mechanism, and the modification in its chemical structure in the development of WP760 abolished multidrug resistance, thus improving the ability of the chemical to induce apoptosis. Both $p27^{Kip1}$ and CHK2 are important cell-cycle regulating factors, and $p27^{Kip1}$ proteins play an important role in human cancer cell apoptosis and cell cycle arrest [20]. CHK2 acts as a key integrator of DNA-damage signals regulating cell-cycle progression, DNA repair, and cell death by phosphorylating a variety of substrates, including the p53 tumor-suppressor protein [21, 22]. The results of immunoblotting showed that WP760 induced cleaved caspase-3 and caspase-8 in a dose-dependent fashion, suggesting that WP760 may have also induced apoptosis via the traditional nuclear DNA damage pathways. However, the WP760-induced caspase-9 and cytochrome C release indicates that an earlier mitochondria-related apoptosis was activated by WP760. Our data showed that unlike its parent molecule, Dox, WP760 is mainly distributed into the mitochondria within 2 h of exposure and therefore is likely to be the initiating event. At this time, we propose that the mitochondrial depolarization is a key event, but that sequaelae occur consistent with both pathways of apoptosis.

These results also confirm our previous work as well as the work of other labs [10] that the MAPK/ERK pathway is constitutively activated in melanoma. We show here that WP760 inhibits this activation in a concentration-dependent manner. The MAPK/ERK pathway plays an important role in the survival mechanisms

of melanoma and other cancer cells [23, 24]. B-raf mutation is found that in approximately 60% of melanoma tissue and cell lines, including the A375 line used in these studies. This mutation mimics RAS activation and automatically induces MAPK/ERK cascades. Our data showed that WP760 caused inhibition of the phosphorylation of ERK1/2 as well as a decrease in the expression of the downstream product, iNOS. Therefore, WP760 should also be considered as an MAPK/ERK inhibitor, and this inhibition could pay a critical role in the mechanisms of selective anti-proliferation in melanoma.

Endogenous NO produced via activation of iNOS in human tumors has been shown to stimulate tumor progression and metastasis [25, 26]. In this study WP760 was found to inhibit iNOS expression in a time- and concentration-dependent manner. Constitutively expressed iNOS is a known melanoma cell survival marker [27]. In previous studies, Dox was found to inhibit the in vivo production of NO and iNOS in rats [28] and in vitro in human colorectal cells [29]. WP760 maintains the iNOS-inhibitory characteristics of Dox, but with increased selectivity for melanoma. NO has been shown to activate the MAPK/ERK pathway to promote tumor cell migration [30], scavenge mitochondrial superoxide anions, and inactivate caspase-3 (S-nitrosylation) to suppress apoptosis [31, 32]. Thus, WP760-mediated inhibition of the expression of iNOS protein could be a means of inhibiting proliferation of tumor cells and inducing apoptosis. iNOS is considered a downstream component in NF-κB activation, which is constitutively activated in human melanoma cells probably in part from the MAPK [33]. Determining whether NF-κB and its other downstream molecules, such as Bcl-2 and survivin, are involved in WP760-induced apoptosis will require further study.

In conclusion we demonstrated that WP760 effectively inhibits melanoma cell proliferation by inducing G_2/M-phase cell-cycle arrest and apoptosis. WP760-induced apoptosis is p53 related. Our data confirm that both mitochondria-related and unrelated pathways could be involved in WP760's induction of apoptosis. We showed that $p27^{Kip1}$ and CHK2 accumulations occur in response to WP760, and these accumulations are important in inhibiting cell cycle progression. Moreover, our results show that WP760 downregulates iNOS levels and suppresses the MAPK/ERK cascade, both of which are critical for melanoma cells proliferation and survival. While many unresolved questions of the apparently unique mechanism of action of WP760 have now been raised, our study demonstrates that WP760 is a potential therapy for chemoresistant tumors such as melanoma and should be considered as high priority for further investigation.

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