RNA interference

Lentiviral vectors used to downregulate Bcl-2, Bcl-x_L, Mcl-1, and Noxa have been previously reported (56, 119). In summary, the sequences used to generate short hairpin interfering RNAs were as follows. Bcl-2 (1): nt 197-215; Bcl-2 (2): nt 500-518 Bcl-x_L: nt 714-732; Mcl-1: nt 2343-2362; and Noxa: nt 1177-1195. Non-specific oligonucleotides were also designed to generate control shRNA. Viruses were generated from 293FT cells, and titers providing > 80% infection efficiency were used. The efficacy and specificity of each construct was determined by protein immunoblotting (see text). When indicated, treatment with bortezomib was initiated 3 days after infection with the corresponding shRNA-expressing viruses.

Analysis of drug response in animal models (mouse xenografts)

Female athymic nude mice (Taconic Farms, Hudson, NY) were kept in pathogen-free conditions and used at 8 to 12 weeks of age. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals of the University of Michigan. To analyze localized growth of melanoma cells *in vivo*, 0.5×10^6 GFP-tagged melanoma cells were injected subcutaneously (s.c.) in the rear flanks of athymic nude mice (n=10 tumors per experimental condition). As surrogate for metastatic dissemination of melanoma cells, 1×10^6 GFP-expressing melanoma cells were injected intravenously (i.v.) into the mouse tail vein (n=10). Treatment was initiated 48 hours or 72 hours after the s.c. or i.v. tumor implantations, respectively. Animals

were weighed every two days to ensure maintenance of total weight within 80% of control populations. Bortezomib was administered systemically by intraperitoneal (i.p.) injection (1.0-1.3 mg/kg) in a qOD (every other day) schedule (56). Similar results were obtained for treatments performed via i.v. administration (not shown). (-)-Gossypol was administered orally at a daily dose of 15-20 mg/kg as described (111). Imaging of tumor cells *in vivo* was performed with an Illumatool TLS LT-9500 fluorescence light system (Lightools Research, Encinitas, CA) and the emitted fluorescence from tumor cells was captured with a Hamamatsu Orca 100 CCD camera. Volumes of the s.c. xenografts were estimated as $V=L*W^2/2$, where L and W stand for tumor length and width, respectively. Pathological analyses of internal organs were guided by fluorescence imaging. Lungs and lymph nodes were harvested and weighed, and external metastases were counted manually and scored by number and size. Metastatic involvement was monitored independently by analysis of hematoxilin-eosin staining of paraffin sections. Mice were euthanized when control populations showed signs of discomfort or respiratory defects.

Statistical analyses. Statistical evaluation of tumor growth and metastasis *in vivo* was performed using the Statistical Package of the Social Sciences (SPSS) Version 11.5 for Windows. Non-parametric Kruskal-Wallis *H* test was used for X-group comparisons and Mann-Whitney *U* test for two-group comparisons. Two-tailed *p*-values smaller than 0.05 were considered statistically significant.

CHAPTER III

AUTOPHAGY IN THE REGULATION OF MELANOMA DRUG RESPONSE

Abstract

Melanoma progression is invariably associated with the acquisition of multiple defects in cell death pathways. Overerexpression of anti-apoptotic Bcl-2 family members, dysregulation of caspase inducers, and deficiencies in p53-dependent death programs are all common features of aggressive melanomas. Anti-melanoma therapeutic strategies aim to overcome these negative apoptotic signals, yet the execution of death programs remains slow and inefficient, even when using pleiotropic agents. These results suggest the existence of yet unidentified mechanisms influencing melanoma cell survival. In the course of investigating drug resistance in melanoma, chemotherapy-treated melanoma cells were examined by electron microscopy. Multilamellar structures within the cytosol, consistent with autophagosomes, were noted in response to a range of different treatments. These electron dense structures were particularly evident and persistent upon treatment with the drug cyclopamine, an inhibitor of Sonic Hedgehog (SHH) signaling. Cyclopamine, while not an efficient killer of melanoma cells, was cytostatic to a broad range of melanoma cell lines, representative of the diversity of metastatic melanomas. In investigating this effect, we noted a marked number of multilamellar cytoplasmic structures in cyclopamine-treated melanoma cells, consistent with autophagosomes. Studies utilizing the autophagy protein LC3 as a marker validated the increase in

autophagosomes in response to cyclopamine. These results were unexpected; although the SHH pathway is known to regulate the proliferative capacity of a variety of tumor cell types, it has not previously been demonstrated to regulate autophagy, nor had it been linked to melanoma. Autophagic response to cyclopamine is tumor cell-selective, occurring in melanoma cells, but not in treated melanocytes; furthermore, the autophagic response to cyclopamine is not specific to melanoma, but indeed is seen in a broad range of cancer cell lines derived from different tumor types. The cytostatic induction of autophagy by cyclopamine seen *in vitro* is maintained *in vivo*, as demonstrated using a xenograft mouse model of melanoma. These data indicate a role for autophagy in the control of melanoma cell survival. Understanding this pathway, previously unexplored in melanoma, may reveal new targets for drug design.

Introduction

Melanoma represents a prototype of an aggressive solid cancer, with increasing incidence and extremely poor prognosis at advanced stages (129). The only agents approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma are the alkylating agent dacarbazine (DTIC) and the immunomodulator IL-2 (1). However, durable and complete responses in metastatic melanoma rarely benefit more than 5% of patients, and secondary toxicities can be severe (130, 131). Consequently, the current average survival of patients with metastatic melanoma is 6 to 10 months, and therefore, the development of novel therapies is a priority in the treatment of this disease (129).

High throughput histo-genetic analyses and systematic functional studies have significantly advanced our understanding of melanoma initiation and progression (95, 132). Consistent defects and alterations in BRAF/MAPK; PI3K/AKT, NF-κB or NOTCH signaling cascades have been identified, providing an exciting platform for rational drug design (133). Death programs controlled by mitochondria and/or by the endoplasmic reticulum are also under evaluation (111, 134, 135). However, targeted therapy has not yet been proven effective in melanoma trials (63). Importantly, compensatory mechanisms can be activated during treatment, resulting in for cell populations with an even higher chemoresistance (136-138).

One area of rapidly burgeoning interest amongst cancer biologists and oncologists is the regulation of autophagy, and its role in carcinogenesis and drug resistance. Besides its role in degradation of proteins and organelles,

autophagy can play a decisive role in cellular survival during starvation, by providing an energy source from recycled cellular components. However, when stressors become excessive, autophagy can act as a cell death pathway via the digestion of essential cellular proteins and structures (139, 140). Autophagy can therefore act as either a survival mechanism during starvation, or paradoxically as a cell death pathway in response to overwhelming stress, particularly when other cell death mechanisms, such as apoptosis, are deficient (67, 141). Consequently, it is unclear whether autophagy acts to augment melanoma treatment response, or exacerbates melanoma chemoresistance. Furthermore, none of the more than 20 autophagy genes described up to date in mammalian cells (142) have been investigated in any detail in melanoma. Therefore, whether autophagy is regulated in a differential manner in melanoma and normal cells is unknown, but might provide a window for therapeutic intervention.

In this chapter, I will present molecular evidence for a role of autophagy in melanoma drug response. Electron microscopy analyses of chemotherapy-treated melanoma cells revealed the presence of multilamellar structures within the cytosol. These structures have morphologies consistent with those of autophagosomes. The structures were particularly evident and persistent upon treatment with cyclopamine, an inhibitor of the Sonic Hedgehog pathway protein Smoothened. Expanding the scope of our studies to include cell lines from other cancer types, we found that the accumulation of autophagosomes in response to cyclopamine treatment was widespread. Finally, we used cyclopamine to treat melanomas *in vivo*, using our nude mouse xenograft model of melanoma;

importantly, we found that the induction of autophagy seen in tissue culture settings is maintained in this more clinically relevant system.

Overall, these results support two novel findings: 1) a role of autophagy in melanoma drug response, and 2) a connection between Sonic Hedgehog signaling and autophagy regulation. The latter finding was unexpected, as no prior work has linked autophagy and SHH, although both processes are critical to cancer cell cycle regulation and tumorigenesis. Given our current poor knowledge of mechanisms of melanoma drug resistance, these results have potential clinical implications, both in the optimization of current therapies and in the development of new treatment agents.

Results

Chemotherapy-induced autophagy in melanoma cells: A new mechanism of action for cyclopamine?

Our laboratory is broadly interested in understanding the aggressive behavior of melanomas, and specifically why melanoma cells are so resistant to many standard chemotherapeutics. Transmission electron microscopy (TEM) is a highly informative technique to uncover ultrastructural details of drug response, particularly with respect to the integrity of membrane-bound organelles. Specifically, TEM is one of the most accepted approaches to assess autophagosome generation. Therefore, melanoma cells were treated with a panel of standard chemotherapeutic agents, known to be poorly effective in the clinic, to address whether autophagy might be responsible for drug resistance. As shown in Figure 3.1a we noted that a range of compounds induced the formation of vacuoles surrounded by double membranes and sequestering cellular structures. These autophagosome-like structures were particularly obvious after treatment with doxorubicin, with the BH3 mimetic gossypol, and with cisplatin (Fig. 3.1). These results are interesting as they support a widespread role of autophagy in drug response.

One chemotherapeutic agent, however, resulted in a remarkable number of electron-dense structures, increased in size, number, and complexity over the other agents (Fig. 3.1, Fig. 3.2a). This compound was the drug cyclopamine, which is a natural compound that is classically used as an inhibitor of the protein Smoothened (SMO) a key effector of the so-called Sonic-Hedgehog pathway

(Fig.3.2b). The Sonic Hedgehog pathway has been previously reported to be hyperactivated in melanoma cells (143). However, there has been no connection between SMO and autophagy neither in melanoma cells nor in other systems. Therefore, we investigated the mechanistic effects of cyclopamine in melanoma cells, as with the goal of gaining new insight into mechanisms of tumor cell survival.

Figure 3.1

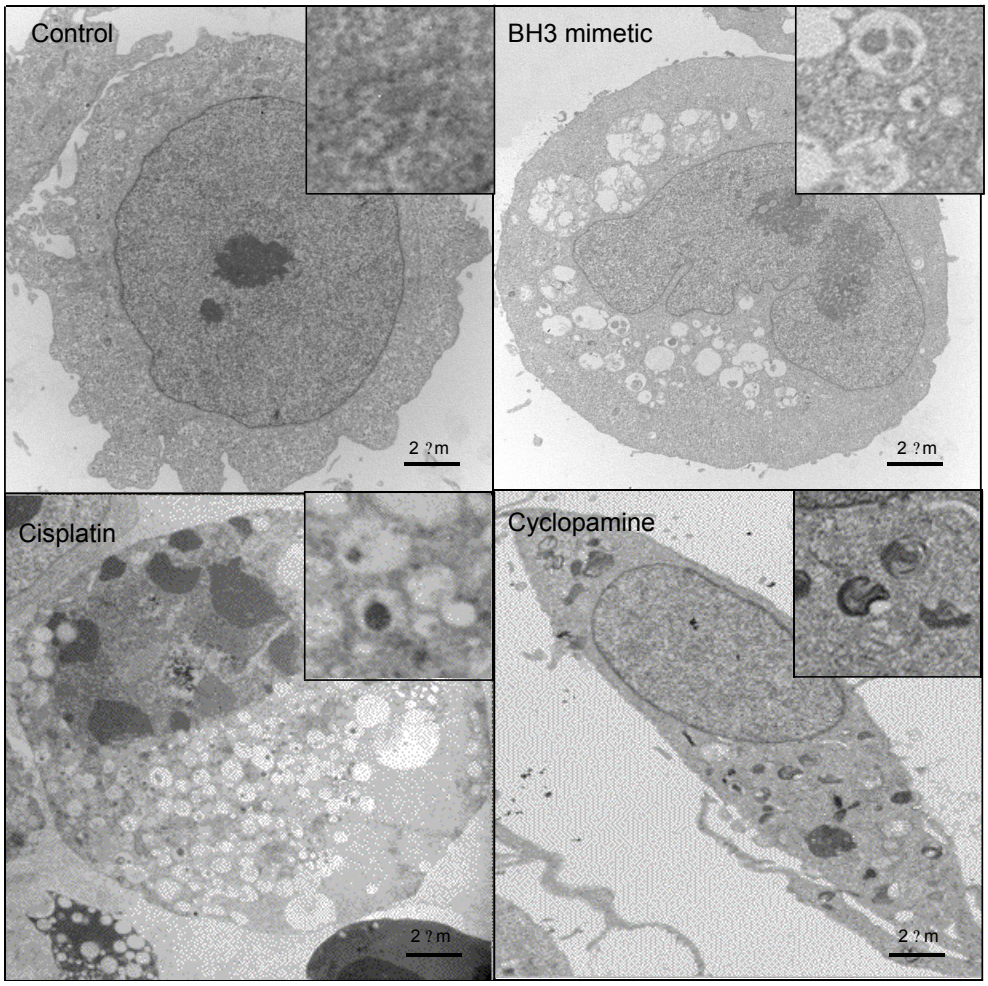


Figure 3.1 Electron microscopy reveals autophagy-like features in melanoma cells treated with various chemotherapeutic agents. Shown are electron micrographs of the melanoma cell line SK-Mel-103 treated for 24 h with control media, 5 μM (-) gossypol, 50 nM cisplatin or 10 μM cyclopamine. These concentrations were chosen to have selective effects towards tumor cells (i.e. without secondary toxicities to normal melanocytes). Note that all the indicated treatments induce the formation of membrane-bound vacuoles with cytosolic debris. With (-) gossypol and cisplatin treatments, there is clear evidence of progressive, degradative processes within the autophagic vacuoles; however, these remain quite dense with cyclopamine treatment.

Figure 3.2

a **b**

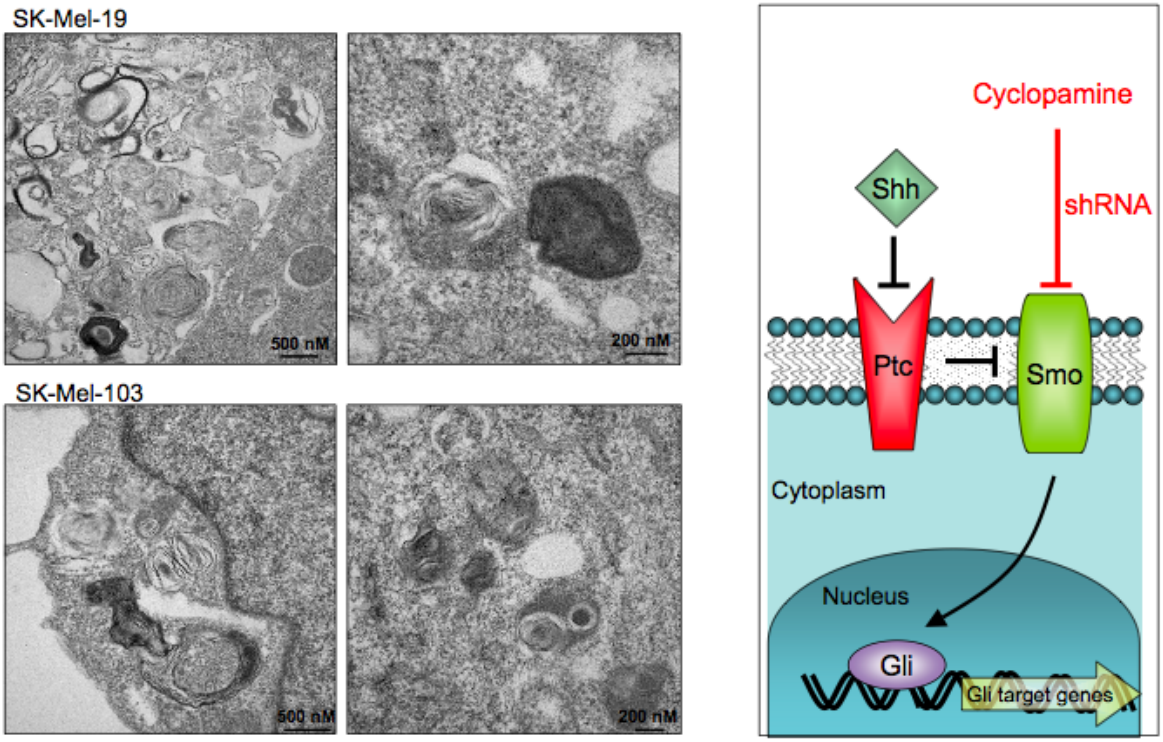


Figure 3.2 Multilamellar structures induced in melanoma cells by cyclopamine, an inhibitor of the SHH pathway. (a) High magnification electron micrographs showing clear multi-membrane organelles sequestering electron dense structures after treatment of the indicated melanoma cell lines with cyclopamine (10 μM, 24h). **(b)** Schematic of the Sonic Hedgehog (SHH) pathway involving the positive effector Smoothened (SMO) and the negative regulator Patched (PTCH), whose net balance determines whether or not the Gli transcription factors become activated. SMO is the main known target of cyclopamine. Adapted from Ruiz I Altaba et al, 2002.

Progressive and sustained accumulation of autophagosomes by cyclopamine

Traditionally, autophagy is defined as a stepwise process in which autophagosomes fuse with lysosomes for subsequent degradation of the sequestered cytosolic components. The timeframe required for the initiation, fusion and completion of this self-cannibalism is largely dependent on the cell type, stimuli and microenvironmental conditions (68, 71). The acute depletion of serum, for example, can engage autophagy within hours (144, 145). A similarly efficient autophagy has been described in response to rapamycin, an inhibitor of mTOR. The formation and resolution of autophagosomes can be conveniently monitored by fluorescence imaging, following changes in the distribution of a fluorescently-tagged version of the LC3 protein (namely GFP-LC3). Thus, while GFP-LC3 is diffusely expressed in the cytosol of resting cells, it accumulates as foci after processing, lipidation and insertion in the membrane of autophagosomes. Interestingly, LC3 is degraded or de-lipidated once lysosomal hydrolases become activated in the autolysosome. Therefore, GFP-LC3 is also a useful tool to assess both the initiation and the resolution of autophagosome formation (76, 145).

For real-time imaging of short- and long-term autophagy-associated events, I generated a lentiviral expression vector to transduce GFP-LC3 in a stable manner in normal and tumor cells. Cells were then treated with rapamycin (as a control for bona fide autophagy programs) or with cyclopamine, and images

were captured at different times post-treatment. A summary of results obtained is shown below in Fig. 3.3.

Figure 3.3

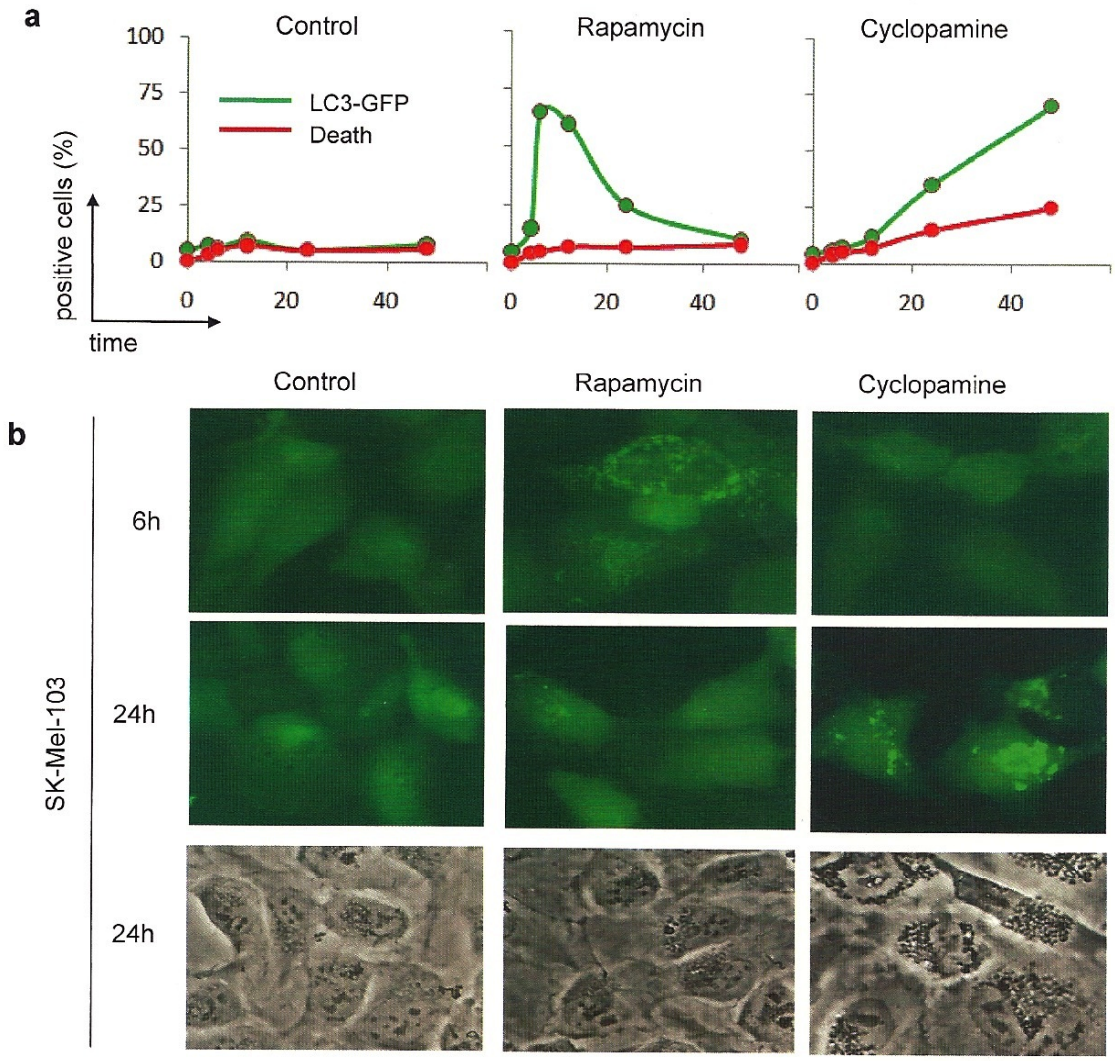


Figure 3.3 Analysis of autophagosome formation by fluorescence-based imaging of GFP-LC3. (a) Differential effect of rapamycin and cyclopamine in melanoma cells determined by quantifying the percent of cells with focal GFP-LC3 staining (indicative of autophagosome formation) at the indicated times post-treatment. **(b)** Representative fluorescence and bright field micrographs of control, rapamycin and cyclopamine-treated cells. Note the transient accumulation of LC3-GFP foci by rapamycin and the stable induction by cyclopamine.

As indicated in Fig. 3.3, a basal level of GFP-LC3 foci was present in untreated SK-Mel-103 cells, although in these conditions most of the fluorescence was homogeneously distributed throughout the cells. Rapamycin engaged an acute focal re-localization of GFP-LC3 (autophagosomes), which interestingly was resolved 20 h after treatment. Cyclopamine was a relatively slower inducer of GFP-LC3 puncta, but these were stable and progressively accumulated with time (Fig. 3.3a). Parallel visualization of cell morphology by optical microscopy revealed also a sustained accumulation of intracytosolic granules (Fig. 3.3.b). Intriguingly, despite the obvious formation of these granules, cells remained viable up to 50 h after treatment (Fig 3.3a, b). Independent analyses of ultrastructural changes by electron microscopy, confirmed the accumulation of increasingly larger multi-membrane cytosolic aggregates at late time points after cyclopamine treatment (see examples for SK-Mel-103 and MM-608 at 60 and 54h, respectively; Fig. 3.4). Altogether, these data indicate that cyclopamine is an effective inducer of large intracellular aggregates with features of autophagosomes (i.e. membrane-bound organelles decorated with LC3, and containing cellular components). However, our results also have uncovered significant differences between cyclopamine and standard autophagy inducers such as rapamycin, suggesting a novel mechanism of stress response to anticancer agents. Moreover, our data emphasizes the ability of melanoma cells to mount protective responses to maintain their viability even under conditions of obvious distress of cytosolic compartments.

Figure 3.4

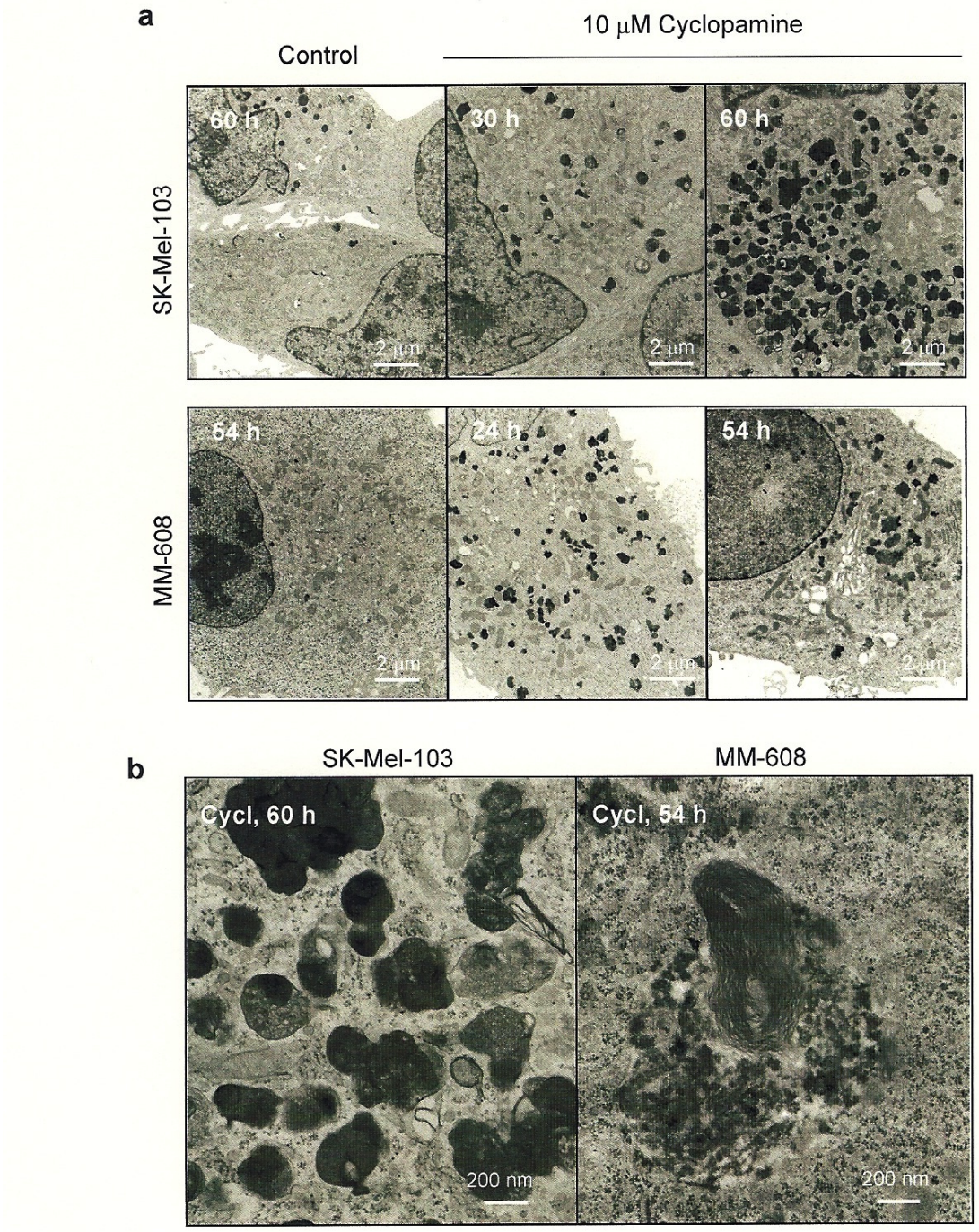


Figure 3.4 Sustained induction of autophagosomes by cyclopamine, visualized by electron microscopy in the indicated melanoma cells **(a)**. Note the massive accumulation of large cytosolic granules in both cell lines at late time points of treatment with cyclopamine (10 μM). High magnification pictures **(b)** reveal membranous structures encircling cellular debris.

Knockdown of *Smoothened* Expression via shRNA

To validate that the autophagy effects observed following cyclopamine treatment were indeed the result of the targeting of the Sonic Hedgehog pathway (and not simply an off target effect), we used a genetic approach to replicate the effects of the drug. As mentioned above, the target of cyclopamine is Smoothened (SMO), which when inhibited results in a blockade of Shh signaling (Fig. 3.2b). Therefore, we designed and manufactured two different lentiviral shRNA vectors to specifically target and knock-down the expression of SMO in infected cells (shown in Figure 3.5a, please also see materials and methods.) These vectors were then used to infect SK-Mel-103 melanoma cells.

As there are no commercially-available antibodies which can reliably and selectively detect hSMO protein, we utilized RT-PCR to evaluate the effectiveness of our shRNA-lentiviral infections. As shown in Figure 3.5a (bottom panels), the level of hSMO message was significantly reduced in SK-Mel-103 infected with either shRNA construct, but was not affected in cells infected with the control construct. We observed that the morphology of the SMO depleted melanoma cells resembled closely that of the cyclopamine-treated cells: cell proliferation decreased as determined by BrdU incorporation and total cell counts (Fig. 3.5b,c). Importantly, cells accumulated significant numbers of cytoplasmic granules, as shown in Figure 3.5d. Additional experiments to examine the status of LC3 in the SMO depleted melanoma cells are currently ongoing.

Figure 3.5

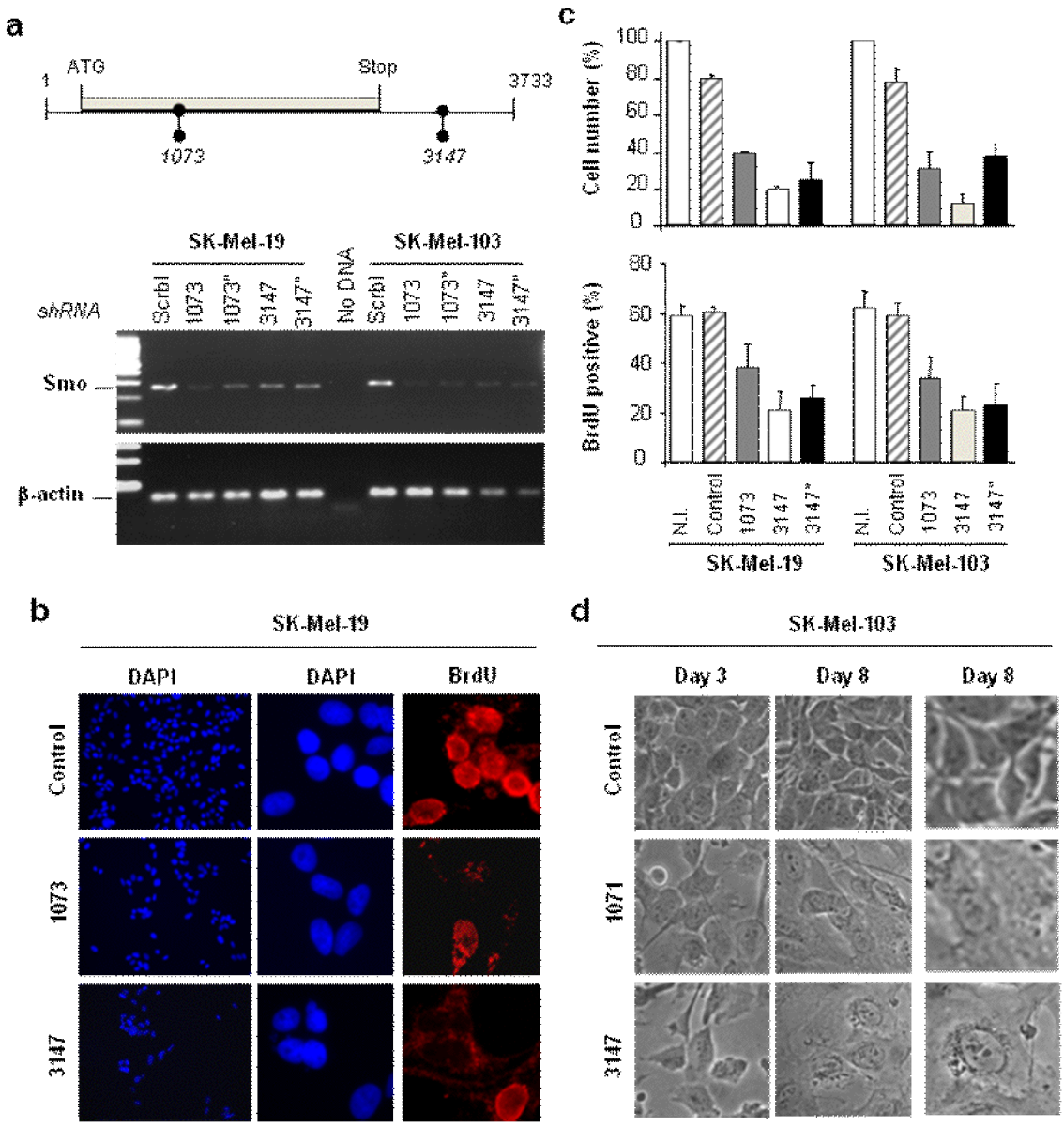


Figure 3.5 Cytostatic effects of SMO downregulation. (a) Schematic representation of the shRNAs used to knock down the endogenous expression of SMO. The efficacy of the shRNAs used (in duplicates and in independent experiments) was tested by RT-PCR. BrdU staining **(b)** and total cell counts (**c)** were performed to compare the relative effect of control and SMO shRNAs on cell proliferation. **(d)** Morphological features of melanoma cells 3 and 6 days after infection with lentiviruses coding for the indicated control or SMO shRNA.

Tumor cell selective cytostatic effects of cyclopamine

Melanoma cells acquire a large number of genetic and epigenetic alterations in multiple signaling cascades (5, 146, 147). To determine whether the cytostatic effects of cyclopamine identified in SK-Mel-103 and MM-608 (Fig. 3.4) are a general feature of melanomas, drug response was analyzed in a panel of melanoma cell lines, chosen to represent various known defects in survival and apoptotic pathways found in this tumor type (see Table 3.1).

Table 3.1

Cell type/ line	Code	p53	p53 induct	p14 (mRNA)	p16 (mRNA)	B-RAF (V599)	N-Ras (exon 3)	Apaf-1 (prot)	Casp8 (prot)	Bcl-2 (prot)	Bcl-xL (prot)	Mcl1 (prot)	Adr
NHEM	**Mel**	ND	ND	ND	ND			++	++	++	+	-/+	+
SK-Mel- 2	**1**	G245S	-*	ND	ND	wt	Q61R	+	++	ND	ND	ND	++
SK-Mel-5	**2**	wt^R	+	ND	ND	wt/mutant	wt	-		++	++	++	+
SK-Mel-19	**3**	wt	+	+	+'**	mutant	wt	++	++	+++	++	ND	++
SK-Mel-28	**4**	$R273H^R$	-*	ND	+'**	mutant	wt	-/+	++	++	++	++	-
SK-Mel-29	**5**	wt	+	+	+'**	mutant	ND	++	++	+++	++	++	++
SK-Mel-85	**6**	wt	+	-	-	mutant	wt	++	-/+				++
SK-Mel-94	**7**	wt	+	+	+	mutant	wt	-	-/+	+++	++	ND	-
SK-Mel-100	**8**	wt^R	+*	ND	-	wt/mutant	wt	-/+	-/+	ND	ND	ND	-
SK-Mel-103	**9**	wt^R	+	+	+	wt	Q61R	-	+	++	+++	+++	-
SK-Mel-147	**10**	wt^R	+	-	+'**	wt	Q61R	-	+	++	+++	+++	-
SK-Mel-173	**11**	wt^R	+	ND	-	wt	wt	++	-/+	+++	++	+++	-
SK-Mel-187	**12**	R273H	+*	+	+	wt/mutant	wt	-	ND	ND	ND	ND	-
SK-Mel-197	**13**	wt^R	+	+	-	wt/mutant ?	Q61R	-	ND	ND	ND	ND	-
G-361	**14**	wt^R	+	+	-	wt/mutant	wt	-	-	+++	++		+/++
Malme-3M	**15**	wt^R	+	-	-	wt/mutant	wt	+	ND	++	++	ND	-
M14-Mel	**16**	wt^R	-*	+	+	wt/mutant	wt	+	++	ND	ND	ND	-/+
UACC-62	**17**	wt	+	-	-	mutant	wt	+	++	++	+	ND	++
UACC-257	**18**	wt^R	+	+	+	wt	wt	-	++	+++	++	ND	-
LOX-LIVM	**19**	wt	+	-	-	wt/mutant	wt	++	++	ND	ND	ND	-/+
WM-1366	**20**	ND	ND	ND	ND	wt	Q61R	++	+++	++	+++	++	-

Table 3.1 Genetic background of the human metastatic melanoma lines used in this study. p53 mutational status was determined by direct sequencing of exons 2-10 by RT-PCR. Samples with polymorphism P72R are indicated as ^{R}. The inducibility of p53 was determined by immunoblotting of extracts treated with doxorubicin (0.5 mg/ml, 12h). Lines with high endogenous levels of p53 are indicated with an asterisk. Apaf-1, Casp-8, Bcl-2, Bcl-xL and Mcl-1 levels were determined by immunoblotting and normalized to control melanocytes. BRAF and NRAS mutational status was determined by direct sequencing of PCR-amplified genomic fragments of exons 15 and 3 respectively. Responses to doxorubicin (Adr; 0.5 g/ml, 30h) are categorized into ++, +, -/+, -, for percentages of cell death of 100-70, 70-50, 50-30 and <30%, respectively.

Cells were exposed to various levels of cyclopamine and cell death was assessed by the MTT assay, which quantifies cellular metabolic activity as a surrogate for viability (148). Primary human melanocytes were included as a normal cell control. Antitumoral effects of cyclopamine in a variety of cancer types have been previously described for doses of 10μM to 25 μM (143, 149-151). Therefore we used three cyclopamine concentrations within that range (i.e. 10μM, 15μM, and 20μM) to test our melanoma panel. As summarized in Figure 3.6a, some of the melanoma cell lines showed reductions in viability in response to cyclopamine within the experimental time-course (lines SK-19, SK-29, G361, MM-426, and Malme-3M). This is consistent with a previous report (143). However, for the majority of the melanoma cell lines tested, cell viability was only affected at 20μM cyclopamine; at lower doses, the detected metabolic activity was at or above 70% of untreated controls. Interestingly, all cell lines (although to various extents) showed a markedly granular cytoplasm. This phenotype was sustained, and in most cases did not lead to significant cell death (Fig. 3.6b). Comparing these results with those for similar MTT assays using other chemotherapeutics such as doxorubicin (not shown), cyclopamine is a cytostatic agent and not a potent killer of melanoma cells. Still, stress responses induced by cyclopamine are tumor cell selective. Thus, normal melanocytes did not show a granular cytosol by optical microscopy (Fig. 3.6b), nor accumulate focal GFP-LC3 upon treatment (Fig. 3.7).

Figure 3.6

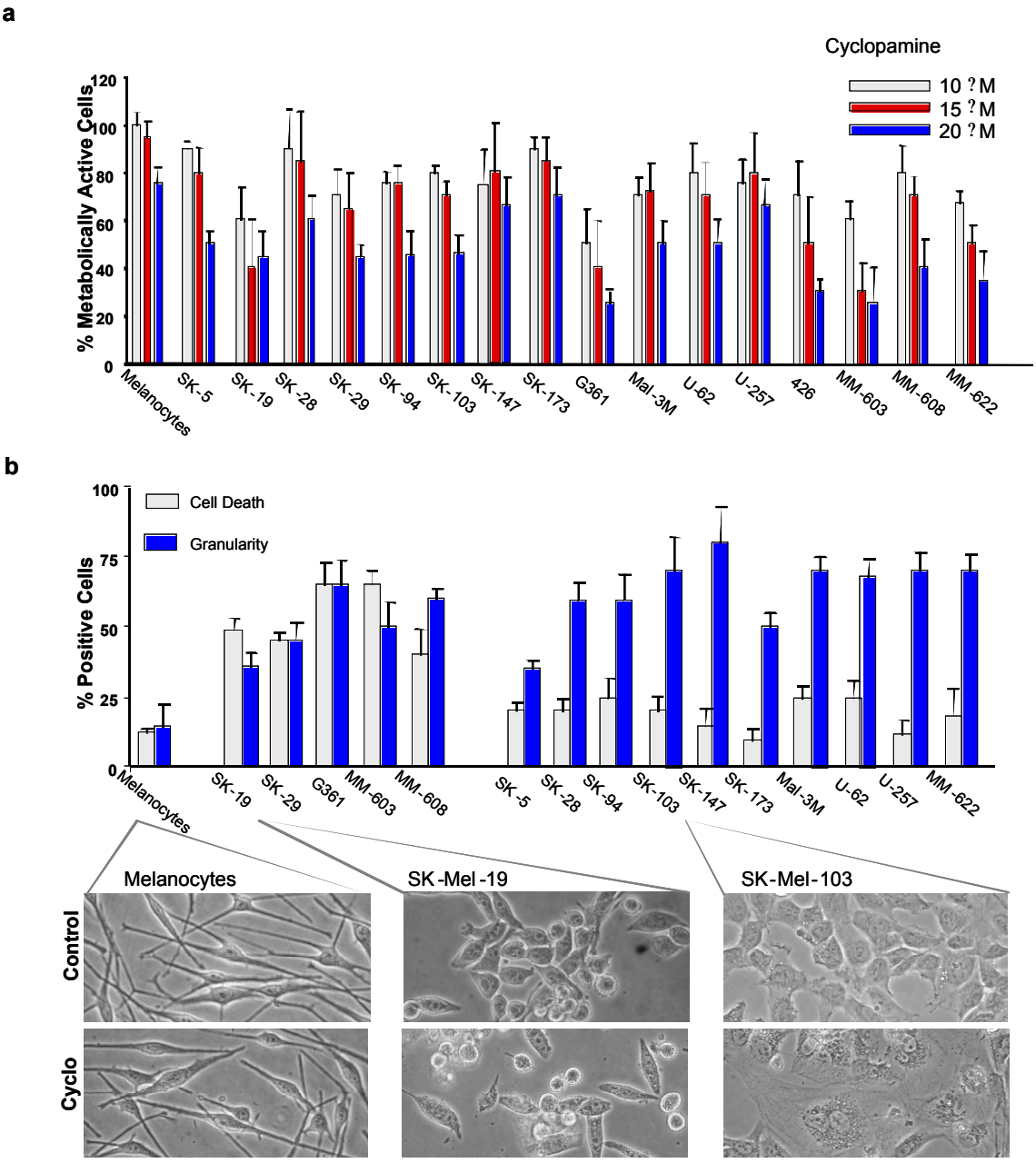


Figure 3.6 Cytostatic activity of cyclopamine in melanoma cells. (a) Percent of metabolically active cells determined by MTT assays 36 h after treatment with the indicated doses of cyclopamine. **(b)** Extent of cell death and intracytosolic granularity in the indicated melanoma cell lines treated for 36 h with 10 μM cyclopamine. Shown are also micrographs to show the impact of this drug on the morphology and viability of melanocytes and melanoma cells. Under conditions that induce a marked granularity in melanoma cells, normal melanocytes remained viable and without obvious signs of cellular stress.

Figure 3.7

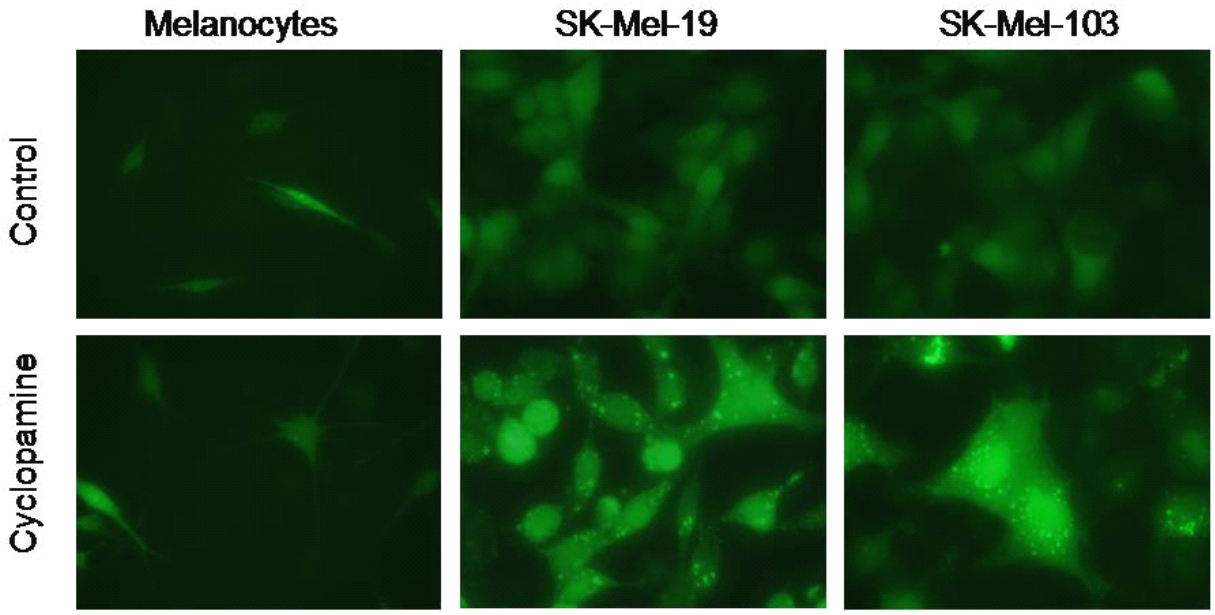


Figure 3.7 Selectivity of cyclopamine-driven cytostatic effect. Fluorescence images of normal melanocytes and the indicated melanoma cells stably transduced with GFP-LC3 and treated with solvent control or 10 mM cyclopamine. Note the homogeneous distribution GFP-LC3 in treated melanocytes under conditions where melanoma cells show a clear focal localization of this protein.

Effects of cyclopamine on proteins regulating apoptosis and autophagy: Differences from other chemotherapeutic agents.

The results shown above support the notion that cyclopamine is a cytostatic agent, but a relatively poor cytotoxic agent in melanoma cells. To further define protective responses that may be unique to cyclopamine, we decided to compare it with respect to various chemotherapeutic agents in their ability to alter the endogenous levels of proteins involved in autophagy (LC3) and apoptosis (caspases and Bcl-2 family members). We chose to compare the effect of cyclopamine with two agents: doxorubicin and bortezomib, able to activate the apoptotic machinery in melanoma cells. Doxorubicin was chosen as a classical DNA damaging agent, whose pro-apoptotic activities depend, at least in part on its ability to activate p53. Bortezomib, in turn, was selected as a prototype of novel proteasome inhibitors, which our lab and others have shown to be a potent inducer of the pro-apoptotic protein NOXA (56) (57). Interestingly, in contrast to the sustained lipidation of LC3 induced by cyclopamine, the LC3 II form was only transiently induced by doxorubicin, or not detectable in the case of bortezomib (Fig. 3.8). Thus, these compounds do not activate autophagy for cell protection. In fact, caspase processing was clearly detectable in doxorubicin- or bortezomib-treated cells, but absent in the case of cyclopamine (Fig. 3.8). Neither NOXA, Bcl-2, Bcl-x_L nor Mcl-1 changed their levels after treatment with cyclopamine (Fig. 3.8 and results not shown). Therefore, it is unlikely that potentiation of anti-apoptotic Bcl-2 family members contribute to the resistance to cyclopamine. This is in contrast to other agents such as bortezomib (Fig. 3.8), where Mcl-1 is

readily upregulated (see chapter 2). Additional analyses of protein expression revealed a striking difference at the level of p53, in the response to cyclopamine and doxorubicin or bortezomib. Of these three compounds, only cyclopamine maintained low p53 levels. These results are surprising, because p53 is a central sensor of intra and extracellular stress stimuli, including those that can activate autophagy (refs). Thus, the massive accumulation of cytosolic granules with autophagosome-like features induced by cyclopamine (Figs. 3.2, 3.4), does not lead to a dysregulation of the cell physiology that could be sensed as stress by p53. In the next chapter we will exploit the functional implication of these results (i.e. by combining cyclopamine with p53 inducers to shift the outcome of autophagy for cell survival to cell death).

Figure 3.8

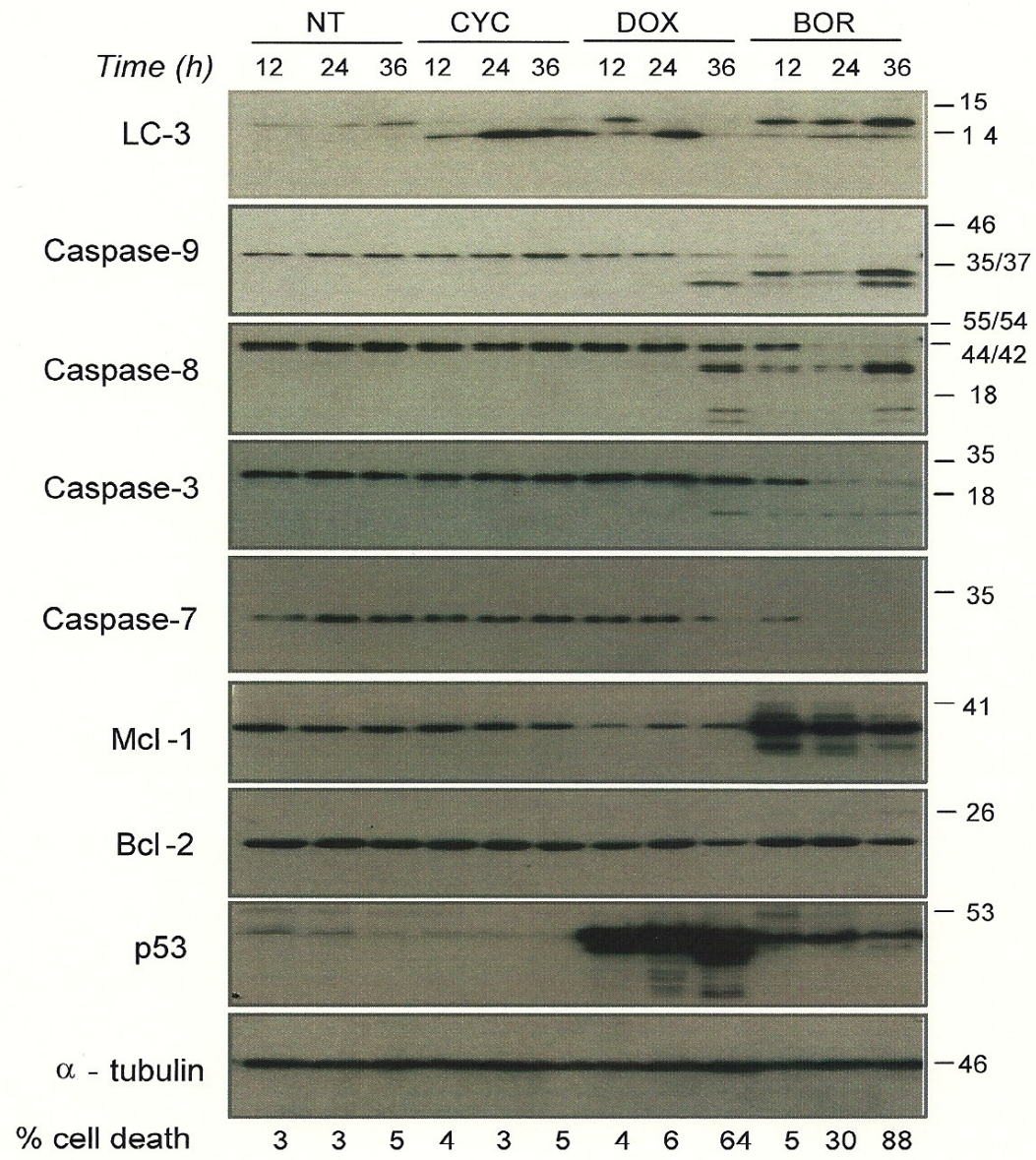


Figure 3.8 Differential activation of autophagy and apoptosis proteins by cyclopamine (CYC), doxorubicin (DOX) and Bortezomib (Bor). Shown are immunoblots of SK-Mel-103 collected at the indicated times post-treatment and probed for the expression of LC3, apoptotic caspases, Bcl-3 family members and p53. Note the sustained lipidation of LC3 by cyclopamine (but not for doxorubicin or bortezomib), in the absence of caspase activation and p53 induction.

Similar Response to Cyclopamine in Diverse Cancer Types

Our finding that cyclopamine treatment of melanoma cells caused autophagosome accumulation led us to question whether other cancer cell types would behave similarly; i.e. is the autophagic effect of cyclopamine generalized, or is it specific to melanoma? To this end, cyclopamine was added to a panel of cell lines representing a variety of human cancer types. Broadly speaking, we noted similar findings: inhibition of proliferation and granular appearance under light microscopy, without a generalized induction of cell death (see examples in Fig. 3.9b). Next, we examined the status of LC3 in the treated cancer cells. As shown in Figure 3.9a, there was a robust conversion of LC3 from the LC3-I (inactive) to the LC3-II (active) form in most of the cancer cell lines examined in response to cyclopamine treatment. In fact, the increase in LC3-II levels in some of these lines, such as the pancreatic cancer line CFPAC-1 and the breast cancer line MDA-MB-231, was more robust than any we had seen in the melanoma cell lines.

In addition to examining the morphology of these cells via light microscopy, we also utilized the GFP-LC3 lentiviral construct to visualize autophagosome formation in different tumor cell lines. As seen in Fig. 3.9b, upon treatment with 10-15μM, GFP-LC3 redistributed from a diffuse to a punctate pattern, consistent with its retention in autophagosomes.

Figure 3.9

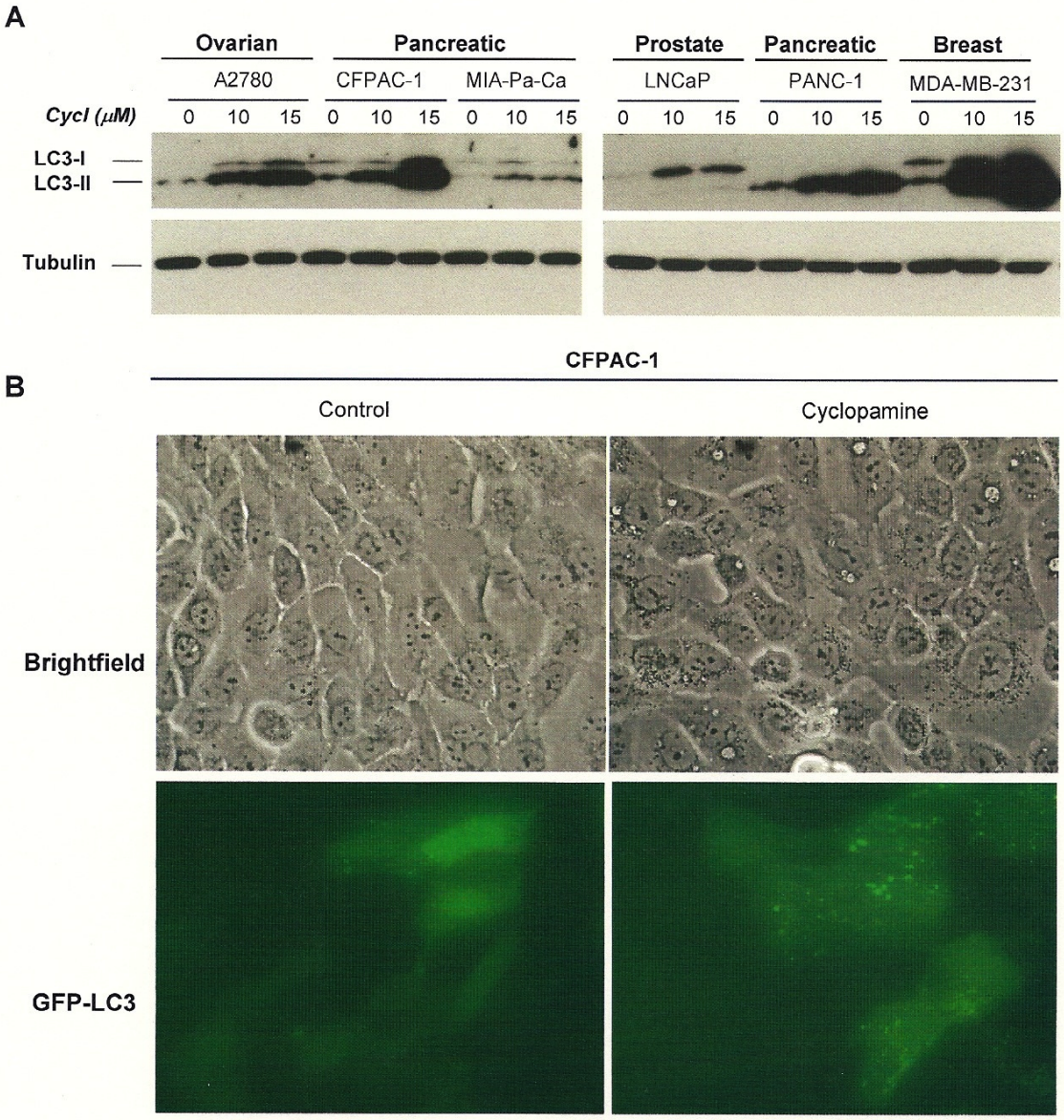


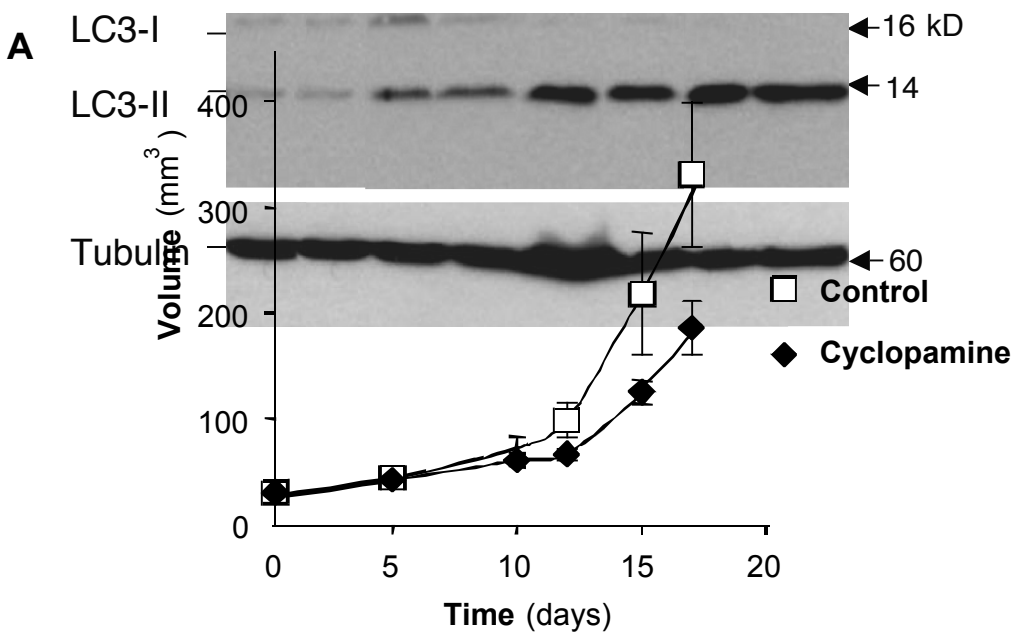
Figure 3.9 Broad activation of autophagy hallmarks by cyclopamine in various tumor cell types. (A) Protein immunoblots showing LC3 lipidation (by means of the increase in the LC3 II form) after treating the indicated cell lines with 0, 10 or 15 μM cyclopamine for 24 h. **(B)** Brightfield and fluorescent micrograpahs of the pancreatic cell line CFPAC-1 showing the formation of cytosolic granular structures that recruit LC3.

Autophagy Induction by Cyclopamine in Melanomas *In Vivo*

Finally, we wanted to extend our findings into surrogate models of melanoma *in vivo*, to determine whether cyclopamine was cytostatic and activates autophagy in the more relevant biological milieu of a living organism. To that end, we again utilized the xenograft mouse model of melanoma, as discussed in Chapter 2. GFP- labeled cells of the SK-Mel-103 human melanoma line were implanted in the flanks of nude mice, and the animals were treated using cyclopamine injected twice daily at 2mg/kg, starting at 72 hours post-implantation. As shown in Figure 3.10a, the anti-tumor effect of cyclopamine treatment was modest, leading to only a slowing of tumor growth versus vehicle control. This is consistent with the poor killing activity of cyclopamine in cultured cells.

When these tumors were removed and their proteins extracted, the presence of increased active (lipidated) LC3 in the cyclopamine-treated tumor samples supports the concept of autophagy induction; that is, the ratio of LC3 (II) to LC3 (I) was significantly increased in tumors from cyclopamine-treated animals versus vehicle-control treated ones. The effect was reproducible in multiple tumor samples, as seen in Figure 3.10b. This finding is crucial, as it validates the potential for regulation of autophagy via cyclopamine (or related drugs) in the dynamic setting of a growing tumor in a living organism.

Figure 3.10



B

Figure 3.10 Impact of cyclopamine *in vivo*. (A) Volume of subcutaneous xenografts generated by implanting SK-Mel-103 in nude mice and treating with vehicle or cyclopamine (2 mg/ml) as indicated in the text. **(B)** Analysis of LC3 status in tumors from control and cyclopamine-treated groups generated in (A). 4 specimens of each condition were processed for total protein isolation, and were separated by PAGE to be probed with LC-3 antibody.

Discussion

In this chapter, I have discussed a novel finding: a connection between cyclopamine and autophagy in melanoma cells. These results were surprising, as cyclopamine has been previously characterized as a specific inhibitor of the Hedgehog (Hh) signaling pathway, via targeting of the transmembrane protein Smoothened (SMO), but has not been linked to the self-degradation of intra-cytosolic components. Furthermore, while a recent publication represented the first evidence for a role of hedgehog signaling in melanoma (143), there is no established connection between hedgehog signaling, autophagy and cell survival.

First isolated from the Western corn lily (*Veratrum californicum*) following an 11 year search for the culprit behind an outbreak of sheep birth defects (152), cyclopamine earned its name from its potent teratogenic effect: ewes in Idaho, after grazing on corn lilies, gave birth to lambs displaying a range of severe congenital abnormalities, including cyclopia and holoprosencephaly (153). The isolation of cyclopamine and the related alkaloid jervine from the corn lily plants provided the causative agent, but it would be 30 years before the molecular mechanism behind cyclopamine's teratogenic effect was elucidated. It was noted that the phenotype of mice lacking *Sonic hedgehog (SHH)* closely resembled that of the cyclopamine-exposed lambs, displaying a severe holoprosencephaly. Subsequent investigations determined that cyclopamine indeed inhibited SHH signaling (154), specifically by preventing activation of Smoothened (SMO) (155), a protein downstream in the SHH signaling cascade.

Hedgehog signaling is of great importance in organism development, but also in tumor biology. In vertebrates, the soluble signaling factor SHH functions by binding to and inactivating the cell surface receptor Patched-1. Active Patched-1 inhibits the activation of SMO. Once not held in check by Patched-1, SMO is able to signal to the nucleus, leading to the activation of Gli transcription factors, including GLI1, GLI2, and GLI3 (156). The Gli factors then regulate expression of the hedgehog responsive genes; they derive their name from the gliomas in which the were first isolated (157). In addition to gliomas, a number of human cancers display inappropriate elevations in SHH signaling, including basal cell carcinomas, pancreatic carcinomas, medulloblastomas, and rhabdomyosarcoma (158-161). Intriguingly, SHH signaling is critical to neural crest development, the source of the melanocytes from which melanomas arise (162). Prior to initiating the investigations discussed herein, there was no published evidence of a role for SHH signaling in melanoma, but a recent report agrees with our data that SHH signaling does occur in melanoma and is important to tumor growth (143). We do find some cell lines that eventually die in response to cyclopamine (e.g. cell line SK-Mel-19 or MM-608 (Fig. 3.6). These lines are usually sensitive to standard anticancer agents, and have a more intact apoptotic program than the typically chemoresistant melanoma lines (15, 56, 111). In the most aggressive lines, however, we found no evidence of caspase activation in melanoma cells in response to cyclopamine treatment.

Our data suggest a novel mode of action for cyclopamine, and consequently, a different role for Hh signaling in tumor control. Cyclopamine

treatment leads to a progressive accumulation of membrane bound organelles within melanoma cells; in some cases these structures appear to contain cellular remnants. Furthermore, the structures are large enough to be observed via light microscopy. Our studies looking at LC3 activation, as demonstrated by native LC3 protein electrophoretic mobility shift, and by the focalization of exogenous GFP-LC3 to the granular structures, support our hypothesis that cyclopamine increases autophagosome levels in melanoma cells, as well as in other tumor types.

An obvious question to address is whether the impact of cyclopamine on autophagy is due to its validated mechanism of inhibiting hedgehog signaling by disrupting SMO function, or if the autophagy effect is due to a separate, unknown, “off-target” mechanism of cyclopamine. While additional investigations will be needed to confirm it, the results from the SMO knockdown experiments support a connection between hedgehog signaling and autophagy. However, while SMO downregulation led to the formation of cytosolic granules, the effect was less dramatic than treatment with cyclopamine. Thus, it is possible that indirect or secondary effects of cyclopamine are influencing autophagy in our experiments, distinct from SMO. It is interesting to speculate that cyclopamine impacts membrane processing in Hh signaling. SMO and other components of the Hh pathway are modified to regulate their localization (i.e. they transition from membrane bound to soluble states). Cyclopamine may interfere with this trafficking, and thus lead to aberrant membrane-bound structure accumulation. We plan to do additional experiments to explore this, including looking at the

importance of downstream regulators of Hh signaling, such as the Gli transcription factors. We will also utilize a constitutively active mutant form of SMO (161), which we have cloned into a lentiviral vector, to examine if the reverse is true: does increasing hedgehog signaling impair or diminish autophagy in melanomas?

Another intriguing question is why some melanoma lines died in response to cyclopamine, while others survived, albeit with growth arrest. Does this represent a differential dependence on, or sensitivity to, autophagy in the different lines? A related question is how universal the autophagy/cyclopamine connection is in cancer. Our results from a panel of lines from different cancers suggest that it is widespread indeed; in fact, it may be even more robust in other tumor types than it is in melanoma. In most lines, and in particular, in those with defects in the apoptotic machinery, the induction of autophagy by cyclopamine does not efficiently lead to cell death. The fact that the most resistant cells to cyclopamine are also resistant to standard chemotherapeutic agents that activate apoptotic programs suggests that bypassing apoptotic defects may shift the outcome of autophagy from survival to cell killing. In Chapter 4, I will present our data supporting this hypothesis. I will also examine whether cyclopamine is enhancing the on-rate of autophagy (i.e. autophagosome formation) and/or blocking its off-rate (i.e. interfering with lysosomal-dependent degradation).

Materials and Methods

Cells

The melanoma cell lines SK-Mel-5, SK-Mel-14, SK-Mel-19, SK-Mel-29, SK-Mel-94, SK-Mel-103, and SK-Mel-147 were obtained from the Memorial Sloan-Kettering Cancer Center, New York; melanoma cell lines G361, MM-603, MM-608, MM-622; U-62, U-257, and Mal-3M are from various other laboratories. Melanoma cells were cultured in Dulbecco's modified Eagles's medium (DMEM) (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Nova-Tech, Inc., Grand Island, NY). Primary human melanocytes were isolated from human neonatal foreskins as described (56) and maintained in Medium 254 supplemented with melanocyte growth factors (HMG-1) containing 10 ng/ml phorbol 12-myristate 13-acetate (Cascade Biologics, Portland, OR). The other cancer cell lines, including Panc-1, CFPAC, LnCaP, A2780, MIA-Pa-Ca, and MDA-MB-231 were kind gifts of other researchers at the University of Michigan Cancer Center.

Cell death assays / Cell viability assays

The MTT assay (Roche Diagnostics, Mannheim, Germany) was utilized to measure cell growth inhibition in response to drug treatment. Briefly, melanocytes and melanoma cell lines in culture were trypsinized, counted, and plated at 5,000 to 20,000 cells per well in 96-well plates, and were incubated overnight. The following day, indicated drugs were added to each well in 300 µL of appropriate media, and cells were incubated for incubated for 48 hours. 3-

(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were then performed according to the manufacturer's protocol. The MTT assay measures cell metabolic activity, based on mitochondrial conversion of MTT from a soluble tetrazolium salt into an insoluble colored formazan precipitate. This precipitate is dissolved in dimethyl sulfoxide, and levels are then quantified by spectrophotometry (148). Assays were performed in duplicate wells in each experiment, and the results averaged; the experiments were repeated in triplicate. Percentage of activity is expressed, relative to matched untreated controls for a given cell type / cell line, and represents an established assay of overall cell viability.

The percentage of cell death at the indicated times and drug concentrations was estimated by standard trypan blue exclusion assays. Briefly, floating and adherent cells were pooled, stained with a 0.4% trypan blue solution (Gibco Laboratories, Grand Island, NY) and scored under a light microscope (a minimum of 500 cells per treatment were counted). The determine percentage of cells displaying an "autophagic" phenotype, adherent cells were examined and subjectively scored as positive or negative for granularity.

Reagents

Cyclopamine was purchased from Toronto Research Chemicals, (North York, ON, Canada) and resuspended in 95% ethanol; stocks were maintained at –80°C and kept no longer than 2 weeks. Doxorubicin hydrochloride (Adriamycin) was

from Fisher Scientific (Fair Lawn, NJ). Bortezomib (Velcade™; formerly PS-341) was obtained from Millennium Pharmaceuticals (Cambridge, MA). The MEK inhibitor 4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) was purchased from Calbiochem (La Jolla, CA). The cell permeable pan-caspase inhibitor zVAD-FMK [Z-Val-Asp(OMe)-FMK] was from MP Biomedicals (Aurora, OH). The pLV vector was a gift of Dr. Mikhail Nikiforov; the GFP-LC3 construct was obtained from Dr. Gabriel Nunez.

Protein Immunoblotting

For immunoblotting, total cell lysates were subjected to electrophoresis in 12%, 15% or 4-15% gradient SDS gels under reducing conditions, and subsequently transferred to Immobilon-P membranes (Millipore, Bedford, MA). Protein bands were detected by the ECL system (GE Healthcare, Buckinghamshire, UK). Primary antibodies included those against: caspase-9 and caspase-3 from Novus Biologicals (Littleton, CO); caspase-8 (Ab-3) from Oncogene Research Products (San Diego, CA); caspase-7 from Cell Signaling Technology (Beverly, MA); Bcl-x_L from BD Transduction Laboratories (Franklin Lakes, NJ); Bcl-2 from Dako Diagnostics (Glostrup, Denmark); Mcl-1 from Santa Cruz Biotechnology (Santa Cruz, CA), p53 from Novocastra (Newcastle upon Tyne, UK), LAMP-1 from Abgent (San Diego, CA); LC3/ATG8 from Abgent (San Diego, CA); Noxa from Calbiochem (San Diego, CA); and tubulin (clone AC-74) from Sigma Chemical (St Louis, MO). Secondary antibodies were either anti-mouse or anti-

rabbit from GE HealthcareCaspase processing was analyzed by immunoblotting with specific antibodies.

Transmission Electron Microscopy

For electron microscopy, cells were seeded onto gelatin-coated eight-well Lab-Tek chamber slides. After treatment, cells were fixed in wells with half-strength Karnovsky's fixative in 0.2 M sodium cacodylate buffer at pH 7.2 for 30 min at room temperature. The cells were washed three times in buffer and in some cases incubated in a 0.1% solution of dihydroxyphenylalanine (L-DOPA) for 2 h twice at 37°C for the histochemical identification of tyrosinase. Cells were then postfixed with 1% osmium tetroxide containing 1.5% potassium ferrocyanide for 30 min. The cells were washed, stained *en bloc* with 0.5% uranyl acetate for 30 min, dehydrated, and embedded in Eponate 12. Cells were sectioned on an RMC, Inc. (Tucson, AZ) MT 6000-XL ultramicrotome, stained with aqueous solutions of uranyl acetate (2%) and lead citrate (0.3%) for 15 min each, and then viewed and photographed in a JEM-100CX transmission electron microscope (JEOL, Tokyo, Japan). All tissue processing supplies were purchased from Ted Pella, Inc. (Tustin, CA).

RNA interference

The sequences used to generate short hairpin interfering RNAs against hSMO were as follows: 5' –GTTATTCTCTTCTACGTCA-3', corresponding to nucleotides 1073 to 1091 in the hSMO mRNA; and 5'-

GTATTCCTCTCCCAGGTGT-3', corresponding to nucleotides 3147 to 3165 in the hSMO mRNA. Oligonucleotides corresponding to this sequence in the forward and reverse orientations, separated by a 9 nucleotide linker sequence, were cloned into a lentiviral vector under control by the H1 promoter. A non-specific shRNA vector was used as control, (as described previously). Viruses were generated from 293FT packaging cells and titers were used to provide > 80% infection efficiency. The efficacy and specificity of each construct was determined by RT-PCR.

CHAPTER IV

THERAPEUTIC IMPLICATIONS OF CYCLOPAMINE-MEDIATED INHIBITION OF AUTOPHAGY IN MELANOMA

Abstract

Autophagy has emerged as a critical pathway in the determination of cancer cell response to stressors. We have recently discovered the ability of cyclopamine, an inhibitor of hedgehog signaling, to cause growth arrest in melanoma cells, while simultaneously leading to the accumulation of multilamellar cytoplasmic bodies with characteristics consistent with autophagosomes. In this chapter, I will discuss additional investigations of this phenomenon, which focus on two primary questions: 1) the mechanistic basis of the effect of cyclopamine on melanoma, and 2) the clinical implications of these findings. In contrast to classical genotoxic-induced stress programs, the response to cyclopamine was determined to be independent of p53. When compared to known autophagy inducers, cyclopamine treatment results in a slower onset of autophagosome formation, but greater persistence, with a marked, progressive accumulation of the LC3-GFP puncta in cyclopamine-treated melanoma cells, and differntial patterns of protein expression on immunoblots. Lowering the level of the autophagy protein ATG7 via shRNA indicates that this protein is required for LC3-GFP focal staining seen in response to cyclopamine. Utilizing various inhibitors of autophagy, which work at different steps in the autophagic process, we conclude that the cyclopamine activity is

counteracted by early-stage autophagy inhibitors, but not greatly altered by late-stage autophagy inhibitors. Co-localization experiments with markers of lysomsomes confirm that the autophagosomes formed in response to cyclopmamine fail to fuse with lysosomes. This lead us to hypothesize the effect of cyclopamine is due largely, and perhaps primarily, to the blockade of autophagy completion, causing cells to accumulate autophagosomes that do not fuse with lysosomes. Therefore, although cyclopamine is an inefficient killer as a single agent, it is not neutral to cells, as it does interfere with the autophagy machinery. To test this possibility, we performed a series of experiments combining autophagy induction by classical stressors, such as hypoxia or growth factor withdrawal, with cyclopamine treatment. Interestingly, the combination of these autophagic stimuli with cyclopamine resulted in a significant killing of melanoma cells. Consequently, cyclopamine may compromise the autophagy-dependent survival mechanisms which are activated in response to chemotherapeutic agents. Indeed, similar synergistic interactions were found between cyclopamine and DNA damaging agents, opening the door to possible novel treatment strategies to bypass the traditional chemoresistance of malignant melanoma.

Introduction

In the previous chapter, I presented evidence that the treatment of melanoma cells with the drug cyclopamine induces a program of autophagosome accumulation. Specifically, we found that cyclopamine caused a marked accumulation of membrane-bound structures within melanoma cells, with an appearance consistent with autophagosomes. We also showed that cyclopamine induced the biochemical modification of the endogenous pool of the autophagosome protein LC3, an accepted marker of autophagy activation. Finally, we reported that cyclopamine affected the redistribution of an exogenous, fluorescently-tagged LC3 protein into clusters akin to those seen in classical autophagy systems. While some melanoma cell lines treated died in response to cyclopamine, most lines, including the more aggressive ones, survived for extended periods of time. Therefore, we hypothesized that cyclopamine was not toxic to melanoma cells, in part because the activation of protective stress-response programs that involve autophagy mediators. Furthermore, these cyclopamine effects appear to be non-specific to melanoma; similar findings were made when a variety of different cancer cells types were treated with the drug.

Next, we sought to define the mode of action of cyclopamine, and place it in a clinically relevant context. First, we investigated classical autophagy-dependent programs. In this context, the tumor suppressor p53 was an interesting candidate, since wild-type (functional) p53 is present in the majority of melanomas (163), and autophagy has been recently described among the multiple roles of p53 as a drug-response factor (88, 164). The mTOR pathway

was also assessed, as it represents a primary autophagy modulator (165-168). In addition, we studied the intermediate and late stages of autophagy induction (i.e. involving autophagosome-lysosome fusion and resolution). The ultimate goal of these studies was to define to which extent cyclopamine works as a standard (macro)autophagy inducer or it has unique effects of the autophagy machinery. Finally, we tested the impact of cyclopamine in both “naturally-occurring” pro-autophagy tumor contexts (i.e. low oxygen / growth factor deprivation) and in pro-autophagy states induced by chemotherapies. I will show data that suggest the targeted deregulation of autophagy may serve as a powerful adjunct to current melanoma treatments.

Results

Cyclopamine-Mediated Effects on Melanoma Cells are p53 Independent

The tumor suppressor protein p53 is well known for its pleiotropic roles in cell viability and cell death. The activation of apoptotic programs by transcriptionally dependent and independent functions of p53 has been actively pursued in the clinic. More recently, positive and negative effects of p53 on autophagy programs have also been described (88, 169). Since cyclopamine was an efficient inducer of autophagosome-like structures, but a poor killer of melanoma cells, we hypothesized that cyclopamine would activate p53 in an "autophagy-mode", instead on an "apoptotic mode". The drugs bortezomib, doxorubicin, and etoposide were utilized as p53-inducing chemotherapeutic agents; each is known to induce, stabilize, and/or activate p53 to promote cell death (59, 170-172). As shown in Figure 4.1a, melanocytes and the melanoma cell lines SK-Mel-103 and G-361 both demonstrate increased levels of p53 following treatment with each of these three standard chemotherapies. Surprisingly, there was little or no increase in p53 levels in response to cyclopamine treatment; in fact, the p53 levels appear to decrease slightly in the melanoma cells, relative to untreated controls.

We also investigated whether p53 was required for the cyclopamine response. Our lab had previously generated a very effective lentiviral shRNA construct for the knockdown of p53 (56), (see Fig. 4.1b, left panel). Using this tool, we found that p53 downregulation had no effect on cyclopamine-driven LC3 lipidation (Fig. 4.1b, right panel). In contrast, knockdown of p53 very markedly

reduced the cytotoxicty of the DNA-damaging agent doxorubicin to melanoma cells (not shown).

Figure 4.1

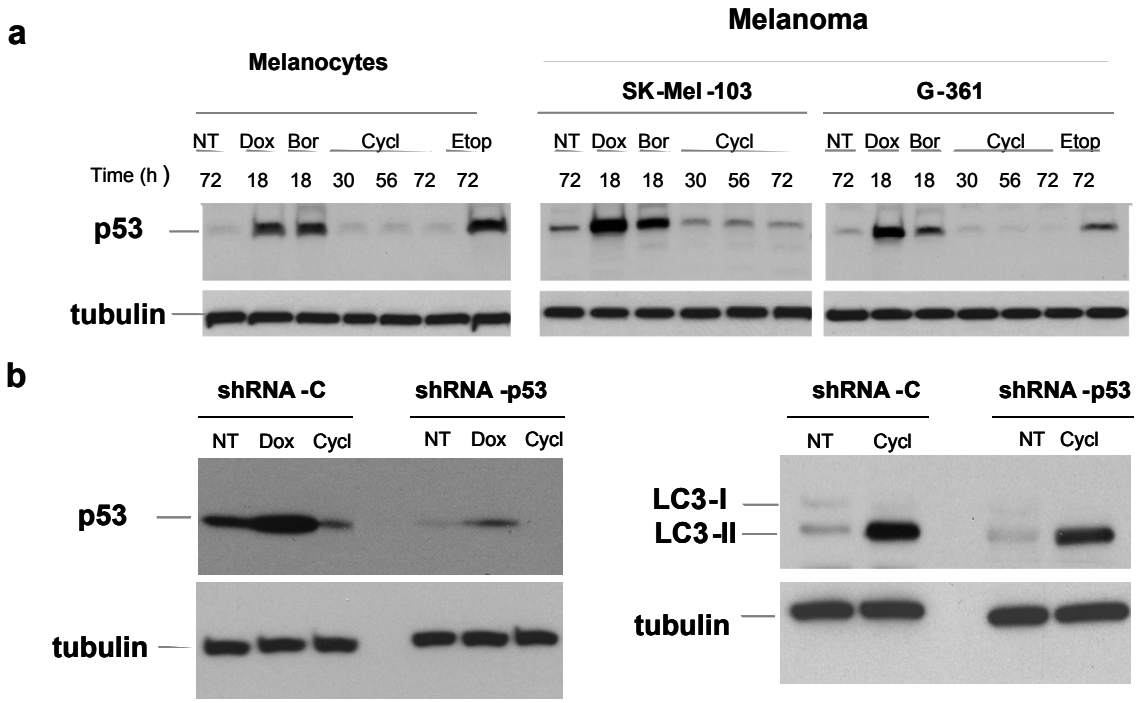


Figure 4.1 p53-independent induction of autophagy by cyclopamine. (a) Protein immunoblots showing p53 accumulation in response to doxorubicin (Dox), Bortezomib (Bor) and Etoposide (Etop), but not by cyclopamine (Cycl) in melanocytes or the indicated melanoma cells. **(b)** Lentiviral-mediated transduction of shRNA against p53 inhibit p53 protein expression (left panel), but not cyclopamine-driven LC3 lipidation (shown in right panel as the accumulation of LC3-II protein).

No downregulation of mTOR pathway by Cyclopamine

As mentioned before, the inactivation of the mTOR pathway (for example with rapamycin) is a well-described inducer of autophagy. In chapter 3, we showed that cyclopamine was unlikely to be equivalent to rapamycin, as the two drugs had very different kinetics. Rapamycin is a rapid, yet transient, inducer of GFP-LC3 foci (autophagosomes). However, over time the GFP puncta resolve, and the GFP returns to a diffuse distribution. In contrast, melanoma cells treated with cyclopamine demonstrate a continuous, progressive accumulation of the GFP puncta (Fig. 3.3). One possible interpretation of this result in that the pharmacokinetics of the drugs in tissue culture are different (e.g. differences in solubility). However, given the low solubility and inherent lability of the cyclopamine (173), it seems unlikely that it gets incorporated into cells more efficiently than rapamycin or that it persists in the culture media for a longer period of time.

To explore differences between cyclopamine and rapamycin responses in melanoma, we performed additional immunoblotting to examine effects of the drugs on two classical targets of the mTOR pathway, the p70S6K and the S6K proteins (165, 174). U0126 (a MEK inhibitor) was used as an agent that can induce cell cycle arrest but not autophagy in melanoma cells (M. Verhaegen, unpublished results). As shown in Fig. 4.2 mTOR abrogated the phosphorylation of p70S6K and the S6 proteins. The level of phosphorylated S6 gradually decreased following cyclopamine treatment. However, this is likely a reflection of cell cycle inhibitory effects of cyclopamine rather than any induction of

autophagy. The downregulation of S6 which occurred after LC3-II formation was a late effect, occurring only after LC3 lipidation, and was also found in response to U0126, which arrests melanoma cells in G1/S (111), but does not induce LC3 lipidation (Fig. 4.2). We also noted (Figure 4.3) a lack of an effect of cyclopamine on AKT activation. Altogether, these results exclude mTOR, MEK and AKT downregulation as putative drivers of cyclopamine-mediated autophagosome formation.

Figure 4.2

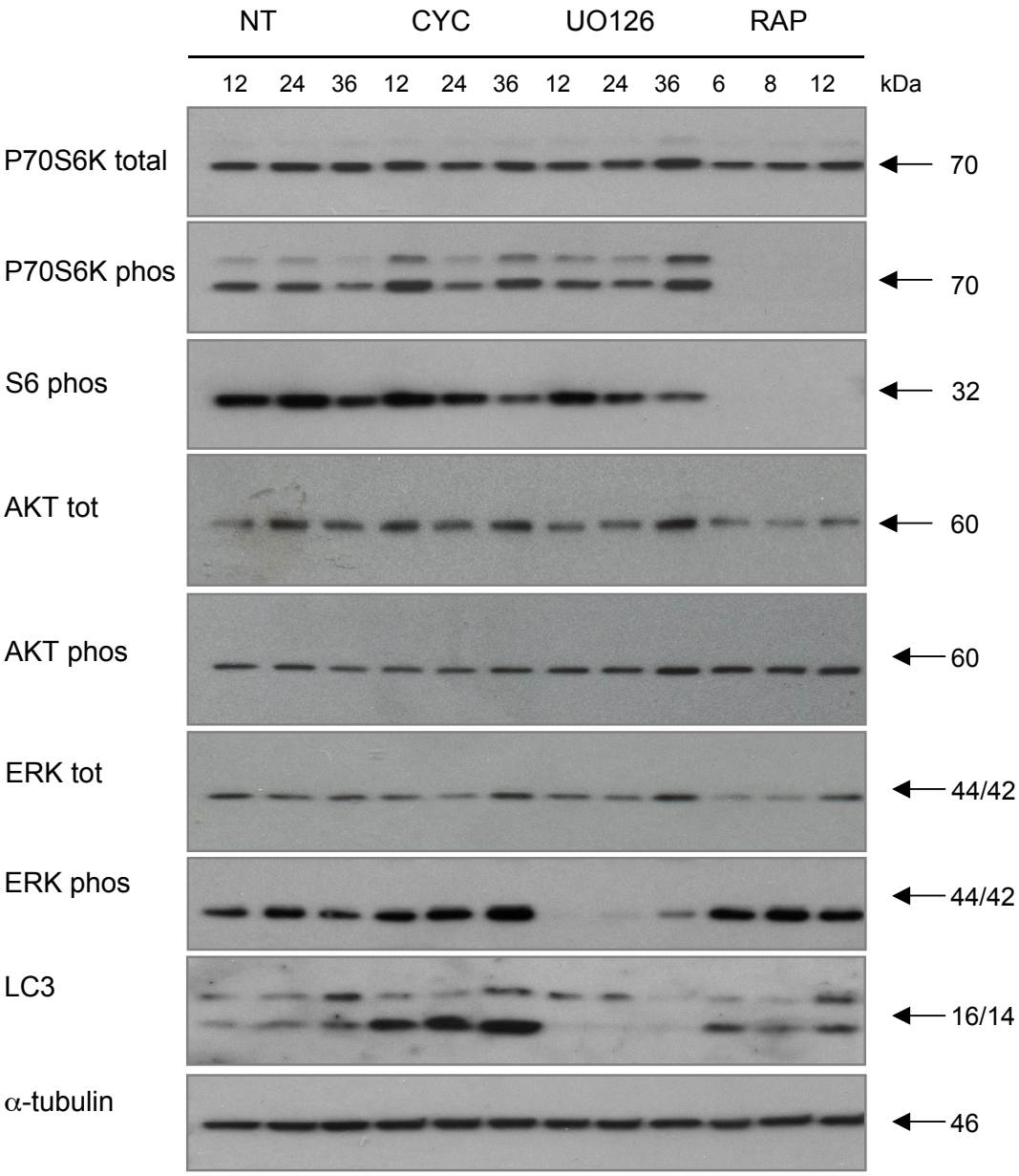


Figure 4.2 mTOR and AKT-independent induction of autophagy by cyclopamine. Protein immunoblots showing mechanistic differences among rapamycin and cyclopamine. Rapamycin (but not cyclopamine) interfered with the mTOR pathway (shown by means of abrogation of the phosphorylation of p70S6 and the S6 proteins). Note that active (phosphorylated) AKT and ERK are not significantly altered by cyclopamine. U0126 is shown as an example of MEK inhibitor that blocks ERK phosphorylation.

Knockdown of ATG7 Partially Inhibits Cyclopamine Response

Next, we assessed genetic determinants of the effect of cyclopamine upon melanoma cells. We chose to inactivate ATG7, an E1-like protein critical to both the ATG12-ATG5 and the ATG8 / LC3 conjugation pathways in autophagy (175-177). The knockdown of ATG7 using siRNA has previously been shown to inhibit autophagy in transformed mammalian cells (178).

As seen in Figure 4.3a, the knockdown of ATG7 using pooled siRNA transfection inhibited the accumulation of GFP-LC3 puncta in response to cyclopamine. This is consistent with the previously described role for ATG7 in LC3 lipidation, an event required prior to its accumulation on the autophagosomal membrane (see Figure 1.6). However, the knockdown of ATG7 via siRNA did not prevent the formation of multiple inclusions within the cytosol of cyclopamine-treated melanoma cells (Fig. 4.3b). In fact, cytoplasmic inclusions driven by cyclopamine were even more evident in ATG-7 shRNA than in control shRNA-cells. These results suggest autophagy may be activated to remove intercellular aggregates induced by cyclopamine treatment.

Figure 4.3

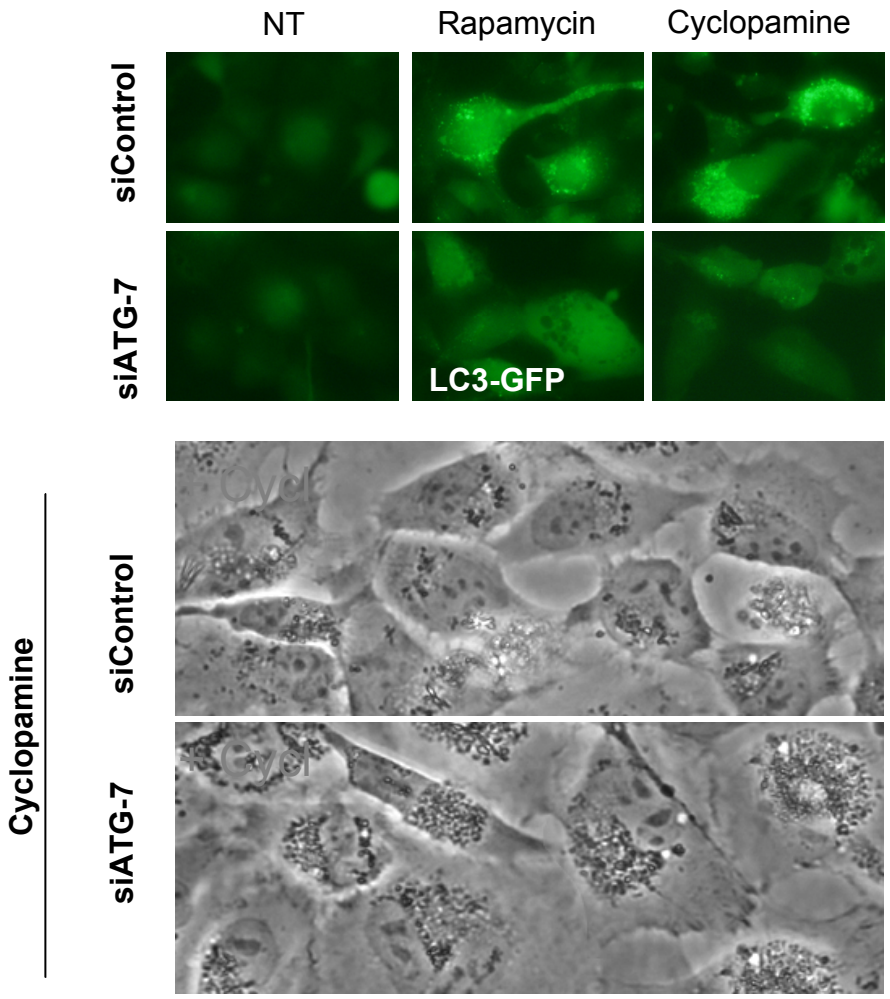


Figure 4.3 ATG7 downregulation blocks LC3 relocalization, but enhances the accumulation of intracellular inclusions driven by cyclopamine. (a) Immunophotographs of SK-Mel-103 expressing GFP-LC3 and transduced with control or ATG7 siRNAs. Cells were imaged 12h after treatment with vehicle control, rapamycin (25 nM) or cyclopamine (10 μM). **(b)** Brightfield pictures of the indicated populations of SK-Mel-103 treated with 10 μM cyclopamine for 24 h. Note the increased granular cytosol in cells transduced with ATG7 siRNA.

Pharmacological Analyses of Cyclopamine-Driven Autophagy

The results discussed above indicate that cyclopamine may impact autophagy by 1) enhancing the induction of autophagy, 2) decreasing the resolution of autophagosomes, or 3) some combination of both events. In order to further elucidate the mechanisms underlying the cyclopamine response, we utilized drugs that are known to inhibit either the early or the late stages of autophagy (3-methyladenine and chloroquine, respectively, see Fig. 4.4). 3-methyl adenine (3MA) is a nucleotide analogue that, at concentrations in the 5-10mM range, compromises autophagic activity (179). 3MA is an inhibitor of class III phosphatidylinositol 3-kinases; thus, 3MA blocks Vps34, the PI3-kinase required in the vesicle nucleation step of phagophore formation (180). Conversely, chloroquine is a lysomotropic quinine that inhibits the final step of autophagy. This drug becomes protonated and "trapped" in the low pH environment of lysosomes. Chloroquine acts to raise lysosomal pH, and thereby interferes with autolysosome resolution (181). Chloroquine is also is believed to inhibit lysosomal fusion with maturing autophagosomes (182) (Fig. 4.4).

Figure 4.4

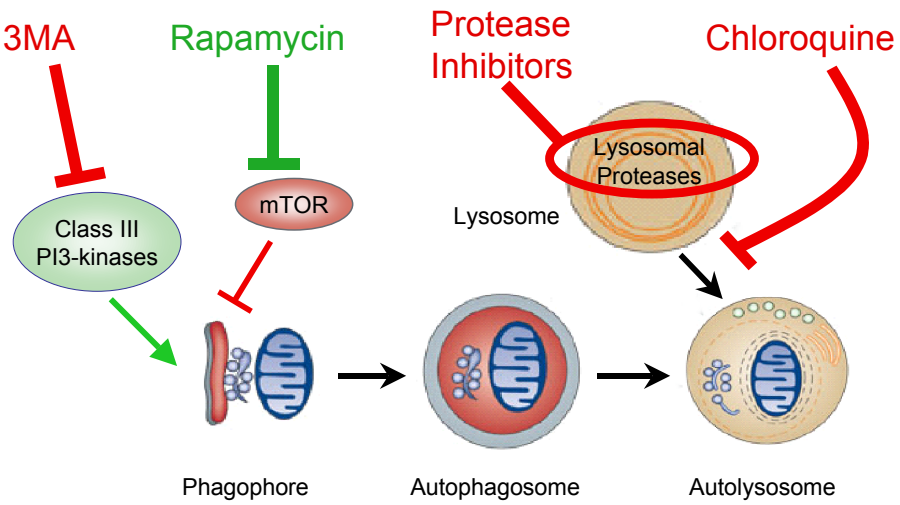


Figure 4.4 Inhibitors used in this study to block early and late stages of autophagy induction. Various compounds impact autophagy at different stages. 3-methyladenine, or 3MA, acts on class III PI3 kinases to prevent phagophore formation. Thus it is an early, or upstream, inhibitor of autophagy. Chloroquine acts downstream, by concentrating in acidic lysosomes and raising their pH, an effect that disrupts the function of lysosomal enzymes. Chloroquine also may block the fusion of lysosomes with autophagosomes. The protease inhibitors pepstatin A and E64d also act downstream, by blocking the activity of lysosomal proteases, and thus interfering with autolysosomal digestion.

As shown in Fig. 4.5, incubating cells with 3MA blocked the formation of GFP-LC3 puncta in response to both cyclopamine and the classical autophagy inducer rapamycin. These results reinforce the concept that the induction of autophagy-like phenotypes by cyclopamine is dependent on gene expression. Interestingly, neither chloroquine nor a combination of pepstatin A and E64d had a significant effect on GFP-LC3 puncta formation by cyclopamine, although these compounds enhanced the effect of rapamycin (Fig. 4.5). This is consistent with the blockade of autophagy resulting from cyclopamine occurring at an intermediate point, falling between the more upstream phagophore step and the downstream autolysosome stage.

Figure 4.5

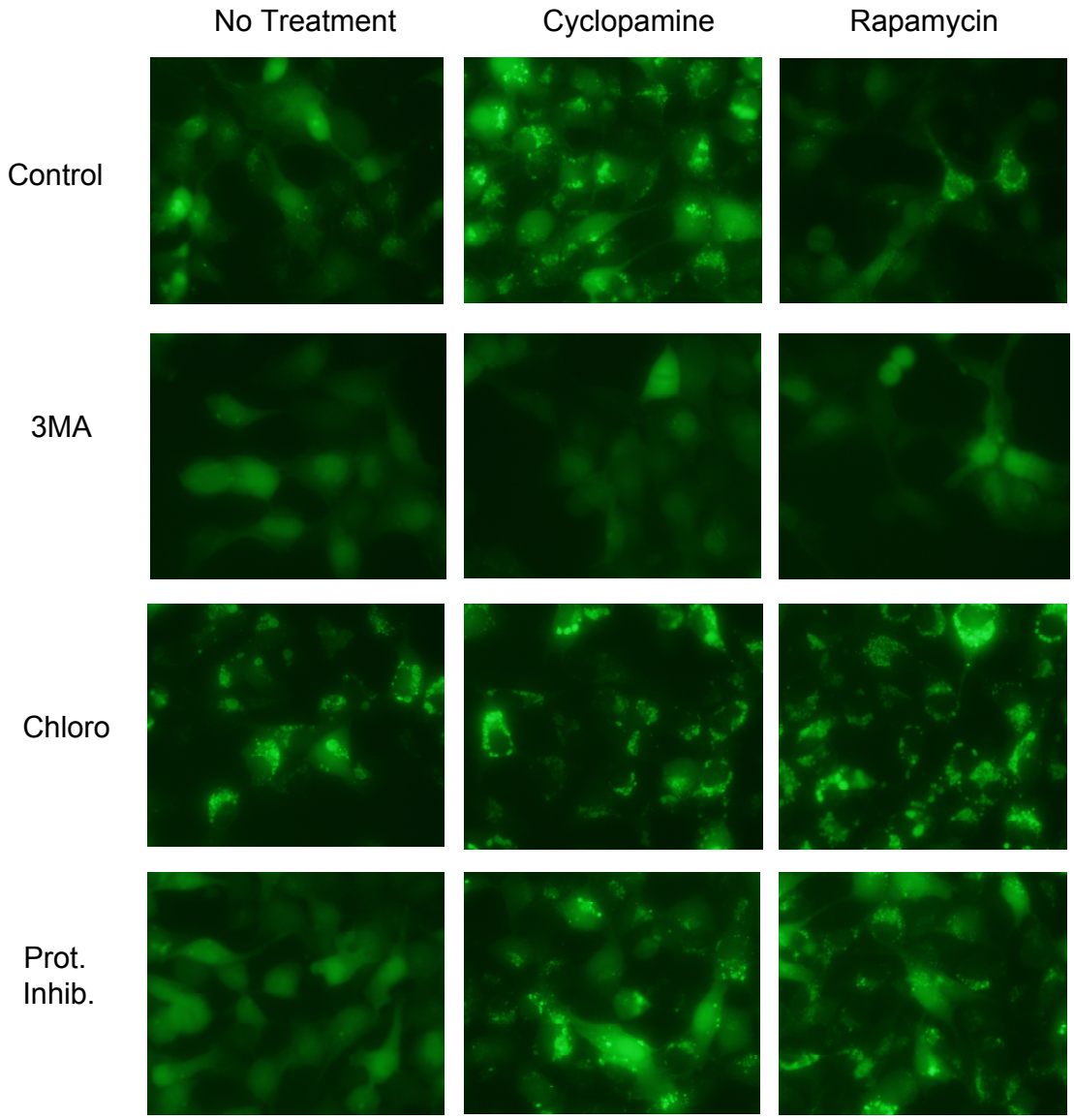


Figure 4.5 Regulation of late stages of autophagy by cyclopamine and rapamycin. Fluorescence imaging of SK-Mel-103 treated transduced with GFP-LC3 and treated with vehicle, 10 μM cyclopamine and 10 nM rapamycin in the absence or presence of either 3-MA, chloroquine (chloro) or a combination of pepstatin A (20μg/mL) and E64d (10μg/mL) (Prot Inhib). While 3MA blocks both cyclopamine and rapamycin driven LC3-relocalization, chloroquine and the lysosomal hydrolase inhibitors affect primarily rapamycin.

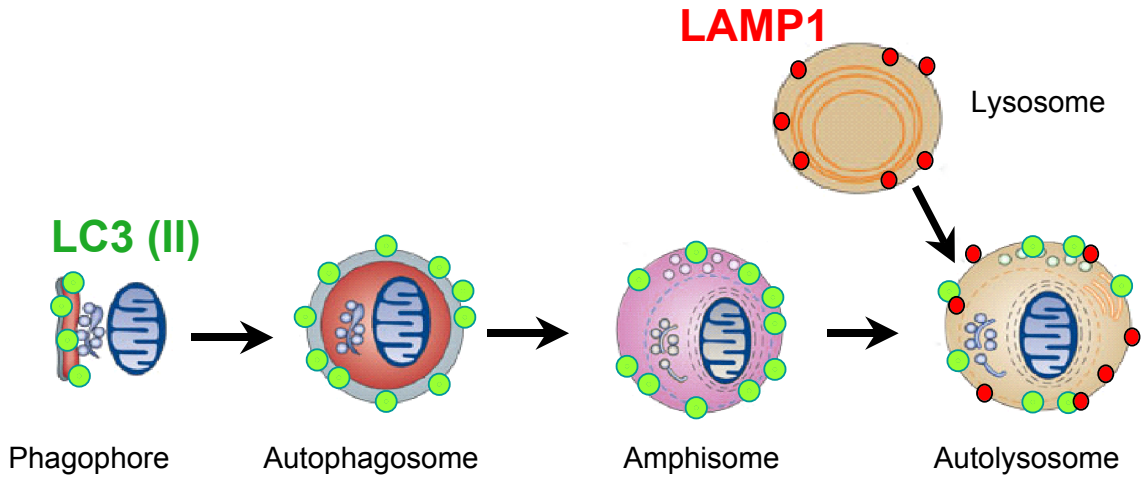
Lack of cyclopamine-induced autophagosome fusion with lysosomes

Our next step in defining the mechanism of cyclopamine action was to examine the location of lysosomes within the cyclopamine treated cells. Using the lysosomal marker LAMP-1, we sought to establish whether lysosomal fusion occurs with the GFP-LC3 labeled puncta (see Figure 4.6a). LAMP-1 (lysosome-associated membrane protein 1) is a transmembrane glycoprotein primarily targeted to lysosomes, with some present in late endosomes (183). Melanoma cells expressing the GFP-LC3 construct were treated with cyclopamine. After puncta formation, the cells were fixed and prepared for immunohistochemistry. As shown in Figure 4.7b, the localization of LAMP-1 does not overlap significantly with the GFP-LC3 puncta, indicating that the formation of autophagosomes in response to cyclopamine does not proceed efficiently to lysosomal fusion / autolysosome formation. Similar results were observed with Lysotracker™, a fluorescent dye marker of lysosomal compartments (not shown).

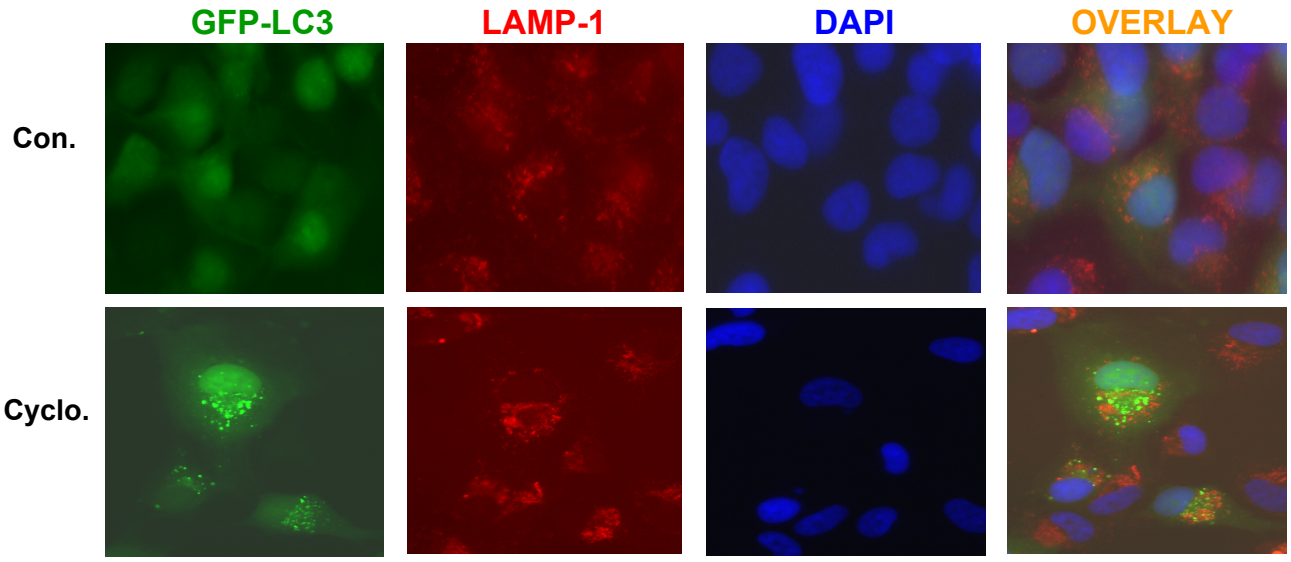
Figure 4.6 Assessing autophagosome-lysosome fusion. (a) Schema showing the points of integration of markers LC3 and LAMP-1 into the autophagy pathway. **(b)** SK-Mel 103 melanoma cells transduced with GFP-LC3 were treated with carrier control (Con) or with cyclopamine (Cyclo) for 24h and fixed to assess GFP-LC3 (green) and LAMP1 (red). Note the absence of significant co-localization of both proteins in the cyclopamine treated cells.

Figure 4.6

a


LAMP1
Lysosome
LC3 (II)
Phagophore
Autophagosome
Amphisome
Autolysosome

b


GFP-LC3
LAMP-1
DAPI
OVERLAY
Con.
Cyclo.

Cyclopamine plus conditions favoring autophagy results in cell death

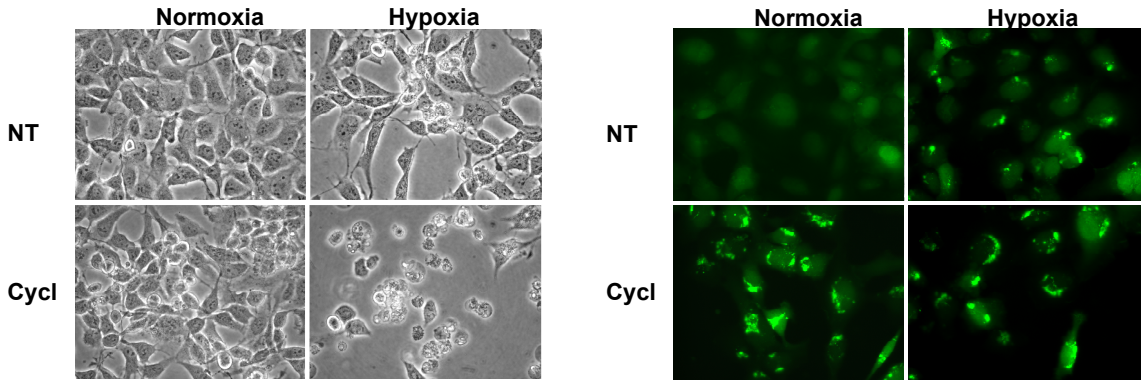
Given our finding that cyclopamine prevents autophagy from proceeding to completion, we hypothesized that further stimulating the influx of autophagy, might result in melanoma cell death. Hypoxia and growth factor withdrawal are two of the classical cues known to induce cellular autophagy (72, 184). Therefore, we sought to test our hypothesis using experimental models of these situations.

Cell culture in hypoxic conditions (created via a low oxygen tissue culture chamber) led to autophagosome formation in melanoma cells (visualized by focal GFP-LC3 staining; Figure 4.7a). Under conditions where cyclopamine had a minimal impact on melanoma cell death under normoxia, incubation in hypoxia accelerated GFP-LC3 formation by about 12 hours, ultimately leading to cell detachment and cell death (Fig. 4.7a).

We also tested the affect of serum starvation since it is a also a well-described autophagy inducer (185-187). Interestingly, low serum content (2%) also favored the cytotoxic effect of cyclopamine in a variety of melanoma cell lines (see Figure 4.7b). Interestingly, this increased killing by cyclopamine was found to preferentially affect tumor cells, as normal melanocytes remained viable (not shown).

Figure 4.7

a



b

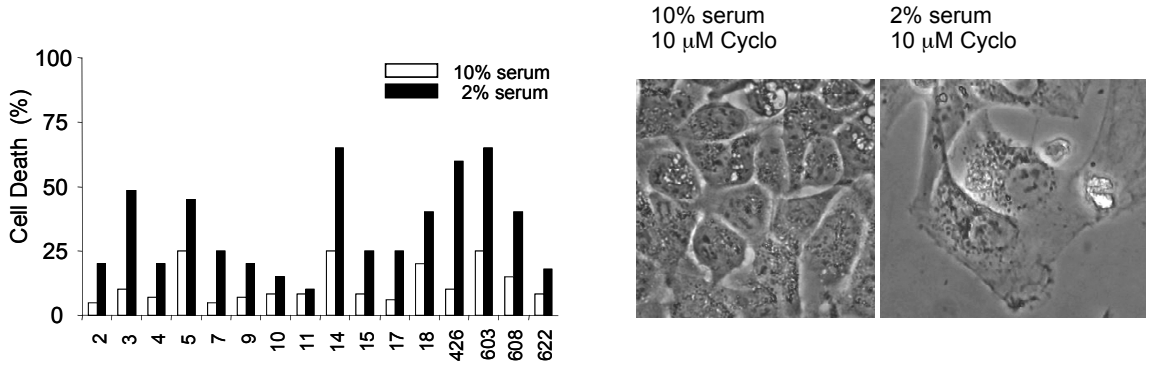


Figure 4.7 Synergistic interaction of cyclopamine with hypoxia (a) or low serum (b), defined by brightfield microscopy, plus visualization of LC3/ autophagosome formation in SK-Mel 103 cells **(a)** or quantification of the extent of cell death (trypan blue exclusion) across a panel of melanoma cell lines **(b)**. For full names of cell lines used in **(b)** see Table 3.1.

Cyclopamine enhances melanoma cell death from chemotherapies

Perhaps of greatest potential relevance to the clinical treatment of melanoma, the combination of autophagy inhibition with standard chemotherapeutics might result in favorable synergies. As discussed previously, autophagy is posited by oncologists to represent a mechanism by which cancer cells can escape from the cell death, a death that would otherwise occur in response to chemotherapeutic injury. We reasoned that since cyclopamine does not induce p53 in melanoma cells, then the combination of cyclopamine, which impairs autophagic maturation, with DNA damage-inducing agents that do induce cellular stress programs and activate p53 might result in additive or synergistic killing. Alternatively, the combination of proteosome inhibition, which can enhance the accumulation of misfolded proteins due to defective protein clearance, with cyclopamine-driven oversaturation of lysosomal functions would be expected to overwhelm the treated melanoma cells and thus lead to their death.

We tested a panel of chemotherapeutics against different melanoma cell lines, both in the presence and the absence of cyclopamine. As seen in Figure 4.8, the combination of cyclopamine with either the DNA damaging agent doxorubicin or the proteosome inhibitor bortezomib lead to increased killing of melanoma cells of different lineages, in settings where either single agent was ineffective. While these results are preliminary, they support our hypothesis that the inhibition of autophagy, perhaps via cyclopamine or its derivatives, may serve

as a means of enhancing the ability of a range of chemotherapies to kill melanomas.

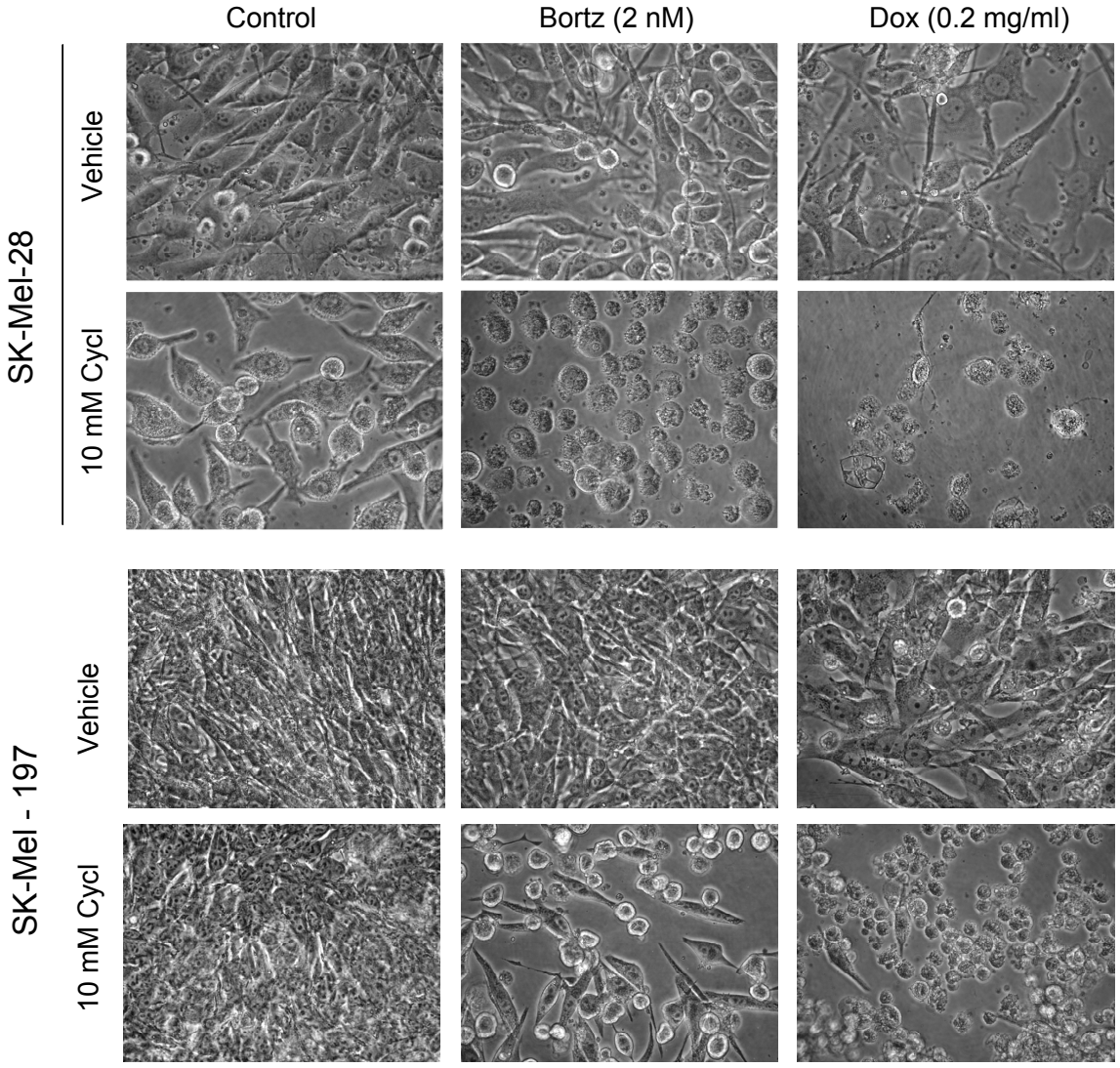


Figure 4.8

Figure 4.8 Synergistic interaction of cyclopamine with doxorubicin (Dox) and bortezomib (Bortz). Pictures correspond to brightfield images of the indicated melanoma cell lines treated with vehicle control of cyclopamine.

Discussion

Autophagy is emerging as a key stress response program in cancer ells However, the final cellular outcome of autophagy induction and execution, and its contribution to cancer progression and drug response, is a matter of active investigation (188-193). There is evidence indicating that cells with misbalanced autophagy programs are more tumorigenic, and that autophagy, like apoptosis, represents a means of auto-elimination of hyperproliferative cells.(194) Conversely, other data suggest that autophagy is a stress-response pathway that can enable cells to remove damaged structures and organelles, that autophagy inversely regulates apoptosis (and *vice versa*), and that cancer cells rely on autophagy to escape cell death from various stresses such as hypoxia, starvation, and chemotherapeutic toxicity (65, 195). Likely, both concepts are valid, and the effect of autophagy on cell fate is dependant on the context. The findings I have presented in this chapter strongly support the notion that deregulating autophagy, either by exacerbating its influx or by blocking a normal resolution mediated by lysosomal-driven degradation of autophagosome components, could be of great potential therapeutic benefit to melanoma patients.

In this chapter, I have first presented evidence cyclopamine differs from other chemotherapeutic agents. We first sought to investigate the effect of p53

on the cyclopamine response, both via measuring p53 levels after treatment, and also assessing the impact of p53 knockdown on drug response. We were surprised to find that the cyclopamine response was essentially p53 independent: the levels of p53 did not change, and the depletion of the protein had no observed effect on cyclopamine response. This was unexpected, as p53 is so broadly involved in cancer cell drug response and cell fate decisions. Functioning as both a transcriptional regulator and as a direct activator of apoptosis (196, 197), p53 is often referred to as the 'guardian of the genome.' The >50% rate of mutation or inactivation of p53 in a diverse range of cancer types (198) substantiates its critical role in preventing tumor growth, and p53 has furthermore been validated as an effecter of drug response to a range of cytotoxic or DNA-damaging chemotherapies, including ionizing radiation, etoposide, doxorubicin, 5-fluorouracil, and cisplatin (199-201). The role of p53 in the cyclopamine response is thus noteworthy for its absence.

Equally intriguing is the fact that, while many different chemotherapeutics seemed to incite an acute autophagic response in melanoma cells, cyclopamine lead to a slower, but progressive and robust, accumulation of autophagosomal structures. Furthermore, the cyclopamine-induced autophagosomes behaved differently from those in the classical rapamycin-induced autophagy paradigm, in that the autophagosomes did not resolve after treatment with cyclopamine ended; the pathway of autophagy did not proceed to completion, and the late step of cyclopamine-induced autophagosomal fusion with lysosomes was found

inefficient. In effect, “exiting” from the autophagy program was defective, resulting in accumulation of autophagosomal structures in the treated cells.

An additional important difference between cyclopamine and autophagy inducers such as rapamycin relates to the mTOR pathway. Rapamycin inhibits mTOR-dependent targets such as p70S6K and S6K, whereas cyclopamine does not. Still, cyclopamine-driven generation of cytosolic aggregates and the accumulation of autophagosomes are not inconsequential. By lowering the level of the critical autophagy protein ATG7 using siRNA, we found an exacerbation of aggregate formation in cells treated with cyclopamine, and furthermore a sensitization to other stress inducers.

While these findings are novel in and of themselves (as autophagy is poorly characterized in melanoma) the promise of a possible clinical application of provides practical relevance to our studies. Autophagy *in vitro* can be induced by growth factor starvation and by hypoxia (202). Significantly, both these conditions are likely to exist in tumors *in vivo*, as cancers often outgrow their blood supply and thus exist in a nutrient-poor, oxygen-poor environment (66). In addition, some degree of hypoxia is found in nearly all solid tumors (203, 204), and tumor hypoxia is predictive of worse outcome, in part because of increased DNA damage and genetic instability from the hypoxia (205). Hypoxia can also activate autophagy mechanisms, although the specific contribution of this effect is still unclear.

If tumor microenvironments select for cells able to use autophagy to overcome stressors, than the disruption of autophagy might represent a

vulnerability, and therefore a treatment target. We believe that the rational combination of an autophagy blocker such as cyclopamine with treatments that either damage cancer cells or induce autophagy in them may thus hold promise for improving melanoma therapy. While the data presented at the end of this chapter are more of starting point than a conclusive proof of this hypothesis, we are excited about the possibilities.

Materials and Methods

Cells / cell culture

All cell types used were described in the Materials and Methods Section of Chapter III. Cell culture modifications utilized in the specific experiments described in this chapter include: 1) low serum growth media, which is Dulbecco's modified Eagles's medium (DMEM) (Life Technologies, Rockville, MD) supplemented with 2% (instead of the usual 10%) fetal bovine serum; and 2) hypoxia, which was created in a specialized cell culture incubator equipped with an oxygen meter and a nitrogen injection system, which was capable of maintaining an atmospheric environment of 0.5%-1.0% molar content oxygen per gas volume.

Reagents

Reagents described previously were as before (see Materials and Methods Section, Chapter III). Additional reagents used in this chapter are: Rapamycin was purchased from LC Laboratories (Woburn, MA). Chloroquine, 3-methyladenine (3MA) leupeptin, pepstatin A, and E-64d were obtained from Sigma-Aldrich (St. Louis, MO). The lentiviral vector used to downregulate p53 has been previously reported (56, 119).

siRNA for RNA Interference

To knockdown the autophagy regulatory gene ATG7, "Stealth" small interference RNA (siRNA) were purchased from Invitrogen (Carlsbad, CA), provided as three

pairs of matched RNA molecules, specific for the targeted (ATG7) gene plus a scrambled (control) pair. The ATG7 sequences were as follows: 5'-UUCUCUUGCUUGCAGCAAUGACGGC-3' and complement; 5'-AAACCUUUGAUCCAAACCCACUGGC-3' and complement; and 5'-AUUUCUGGAAGCUUCACUUCGAAGA-3' and complement. Cells were transfected with either a pool of the 3 sets of siRNA against ATG7 or the nonspecific RNA at 100 pmol/ml using DharmaFECT1 Reagent (Dharmicon/Thermo Fischer Scientific, Lafayette, Colo,). Two sequential transfections of 4-6 hours were performed for each experiment; fresh media was then added, followed by 36 hours of maintenance in cell culture to allow for ATG7 protein depletion.

Protein Immunoblotting

Technique for immunoblotting of cell lysates was as described before see Materials and Methods Section, Chapter III). Other primary antibodies used in this chapter include: anti-AKT, anti-phos AKT, anti-ERK, anti-phos ERK, anti-P70S6K, anti- P70S6K phos, and anti-S6 phos.

Immunofluorescence Labeling of Fixed Cells

The localization of LAMP-1 was performed on cells grown in 6-well culture plates. Cells were washed with PBS, then fixed with 4% formaldehyde treatment for 15 minutes. 0.2% Triton-X in PBS was used to permeabilize the cells. Anti-human LAMP-1 monoclonal mouse antibody [LAMP-1 (E-5):sc-17768 from Santa Cruz

Biotechnology (Santa Cruz, CA)] was diluted 1:100 in 5% goat serum in PBS, and placed on the cells overnight at 4°C in a humidified chamber. After washing, the cells were stained with Alexa Fluor 594 goat anti-mouse IgG [Molecular Probes, Eugene, OR], diluted 1:2000 in 5% goat serum in PBS, for 45 minutes in a humidified chamber at room temperature. After washing, cells were mounted with a cover slip for preservation.

CHAPTER V

CONCLUSIONS

The Problem of Malignant Melanoma: A Modern Black Plague

Malignant melanoma represents a daunting, yet growing, clinical problem. In the United States, the current lifetime risk of acquiring the disease is estimated to be 1 in 75 amongst Caucasians (206); worldwide, the incidence of this disease has doubled in the past 20 years (133). Once the disease becomes metastatic, the median survival time is 6 months (207), and 5 year survival rates remain less than 5% (1). Melanoma is responsible for more than 50% of global skin cancer deaths; the percentage being even higher in the industrialized world (133). In comparison to other cancer types, melanoma strikes younger patients, and has a propensity for rapid, early spread. Obviously, novel treatment strategies and options are desperately needed.

While the comparatively high malignant potential of melanoma (versus other cancer types) is complex, a central reason underlying the difficulty in treating melanoma is its particularly strong resistance to chemotherapy. Different cancer chemotherapeutics act by targeting a variety of cellular processes, yet most ultimately function to trigger some form of programmed tumor cell death. Unfortunately, a deficiency in apoptosis is a hallmark of melanoma (208), and accounts, at least in part, for the poor response rate of melanoma to chemotherapy and biotherapy. The current standard of care for treatment of metastatic melanoma includes the alkylating agent dacarbazine,

which has a response rate of only 5-15%, and delays disease progression for several months but does not improve overall survival (209). Interferon alpha, which is used in patients with positive lymph nodes (Stage III melanoma), yields a 3% benefit in survival at 5 years, but at considerable cost, in both patient morbidity and financial terms (210, 211). Thus, novel approaches, which could either restore melanoma cell death induction or supersede its blockade, are needed if treatment outcomes are to improve.

A Functional Hierarchy of Apoptosis Regulation in Melanoma

Disruptions in apoptosis induction can and do occur at multiple points in melanoma progression (212, 213). In Chapter 2, we explored the therapeutic implications in melanoma of the recently discovered differences between the various Bcl-2 family members. In the past, it was generally assumed that the various anti-apoptotic multidomain Bcl-2 proteins in the mitochondrial cell death pathway non-specifically opposed the numerous different BH3-only death effector proteins. However, the pro-death BH3-only protein Noxa is now known to display a preferential binding affinity for the pro-survival family member Mcl-1 (30) (see Figure 1.3). This may prove to be a crucial finding in the effort to understand why melanoma is so resistant to chemotherapy: Mcl-1 expression in particular is linked to melanoma progression and resistance to chemotherapy (44).

Past efforts to target the Bcl-2 family in melanoma raised hopes but ultimately disappointed, at least as single agents (214). However, it is likely that,

armed with more recent discoveries, cancer biologists can develop new approaches that more specifically target the particular defects present in melanoma, and hopefully will yield greater treatment success (215). Optimal melanoma treatment will most likely be achieved with a combination of drugs targeting different pathways within the malignant cells. Our group (56, 216) and others (57, 97) have shown that proteasome inhibition in melanoma leads to increased levels of both pro-death Noxa and anti-death Mcl-1. In the work described in Chapter 2, we expanded upon these findings to map a functional hierarchy of the anti-death Bcl-2 proteins in melanoma after proteasome inhibition. We established by genetic means the critical role of Mcl-1 in continued melanoma cell survival, and the more dispensable function of Bcl-x_L and Bcl-2. We found that, of these anti-apoptotic proteins, Mcl-1 is an efficient mediator of the resistance of melanoma cells to various chemotherapeutic agents, including bortezomib. In addition, we validated a novel targeted drug therapy (TW-37), a BH3 mimetic, as acting in part by disrupting the protective effect of Mcl-1 in melanoma cell survival. These results suggest the means to rationally improve melanoma drug response. Thus, we combined bortezomib with TW-37 and showed a significant killing of the melanoma cells in both *in vivo* and *in vitro.* To our knowledge, this is the only pharmacological approach in melanoma that is directly aimed to exploit differential requirements of normal and tumor cells on proteasome targets and anti-apoptotic mediators. We are cautiously optimistic that these drug combination results can be quickly translated to the clinic, as the therapeutics involved have already been used safely as single agents with

acceptable toxicities.

The Role of Autophagy in Melanoma Drug Response

While our knowledge of apoptosis has become much more sophisticated in the past decade, non-apoptotic mechanisms of cell death exist, and are emerging as important determinants of cancer cell fate. Autophagy is a particularly complex pathway in this regard, as it can serve to either ensure cell survival or to induce cell death, dependant upon the context and the degree of autophagic activity (65). In chapter 3, we described our initial findings with regard to autophagy and melanoma. This is largely an uncharted area: while autophagy in cancer has generated recent intense interest, we know of no published studies looking specifically at autophagy in melanoma.

We began by assessing the response of melanoma cells in culture to chemotherapies, and found evidence via electron microscopy that a variety of agents lead to notable autophagosome increases within the cell. Surprisingly, the most robust induction of autophagosomes was following treatment with cyclopamine, a drug previously characterized as an inhibitor of Sonic hedgehog signaling. Subsequently, we showed that the effect of the drug on autophagosome formation was partially recapitulated by knockdown of the target molecule Smoothened. Cyclopamine is not a potent killer of melanoma, but had a cytostatic effect, leading to the accumulation of large numbers of autophagosomes in melanoma cells. Importantly, this effect was selective for cancer cells, as it occurred in melanoma cells but not in melanocytes. More

globally, we found similar results in other cancer types, and also in melanoma xenografts treated *in vivo.*

One implication of our findings with cyclopamine is that Sonic hedgehog signaling may have an important role to play in the regulation of autophagy, particularly in cancer. During the course of these investigations, the first work linking Shh signaling to melanoma was reported (143). Broadly speaking, the authors report that cyclopamine is cytostatic and/or cytotoxic to melanoma cell lines in culture, but requires long treatment periods and high drug doses. Similar to our findings, they could reproduce much of the effect of cyclopamine on melanoma cells by knockdown of Gli, via siRNA. Much like the work I described in chapter 3, this report includes an *in vivo* correlation, although they use the impractical and potentially confounding technique of injecting cyclopamine directly into tumors to achieve the optimal effect. Interestingly, the authors noted only modest increases in apoptosis in melanoma cells in response to cyclopamine. The basis for the resistance of melanomas to cyclopamine was unknown, but strongly suggests that other mechanisms an involved in the effect seen in melanoma after cyclopamine treatment In Chapter 3, I provided several lines of evidence indicating that cyclopamine dysregulates autophagy, and subsequently showed that this dysregulation can be exploited therapeutically (Chapter 4). Thus, this work has revealed new insights into the mode of action of a drug that is widely used to define mechanistic aspects of the Sonic Hedgehog pathway. Importantly, we showed that autophagosome formation induced by cyclopamine occurred not just in melanoma cells, but was in fact detected in a

variety of cell lines from different tumor types. We believe that this finding could have a broad impact in cancer biology.

The involvement of autophagy in cancer is not well understood, as positive and negative effects on tumor development have been described. but the observation of a connection between autophagy and cancer treatment is not novel. Chemotherapies (217, 218) and radiation (219) have been noted to induce autophagosome formation in cancer cells. What remains unclear is whether this represents an adaptive response and thus a survival mechanism, or a precursor to self-destruction. It seems plausible that it can be either, and maybe even both, depending on context: tumor type, treatment modality, microenvironmental factors, etc. Inhibiting autophagy can push apoptosis-resistant cancers towards death in response to chemotherapy (89). Conversely, however, the induction of autophagy via rapamycin can augment the death of cancer cells from gamma irradiation (220) and chemotherapy (221). Our data indicates that in fact, autophagy can be induced and proceed via different pathways. Thus, we showed kinetic and phenotypic differences between the induction of classical autophagy programs with rapamycin and with cyclopamine. In contrast to rapamycin and many other classical autophagy inhibitors, cyclopamine can induce autophagosome formation without interfering with the mTOR pathway, suggesting the existence of alternative mechanisms leading to autophagosome initiation. Furthermore, while rapamycin-driven autophagy is initiated and resolved efficiently within a few hours, cyclopamine leads to a sustained accumulation of autophagosomes. Consequently, our studies have

uncovered new mechanisms involved in melanoma cell maintenance. The use of autophagy modulators in clinical oncology, while nascent, is on the rise. As our understanding of the mechanisms underlying the physiology of autophagy in cancer increases, autophagy-based treatments are sure to continue to increase, both in width of tumor applicability and in depth of targeting specificity.

Therapeutic Implications of the Autophagy / Cell-death Axis

In chapter 4, we more fully characterized the response of melanoma cells to cyclopamine. A striking finding was the lack of activation of p53 upon cyclopamine treatment. Generally, p53 is induced by a variety of chemotherapeutic drugs and it is a central sensor of genotoxic and metabolic dysfunction. However, we found that the cyclopamine response is not dependant upon p53, and does not impact p53 levels. Given the massive accumulation of cytosolic granular structures caused by cyclopamine, it was expected that these cells would have activated p53 as a stress response mechanism. While surprising, this finding also presented an opportunity. Since cyclopamine response includes an element of autophagy blockade, we reasoned that combinations of cyclopamine with agents that further increase autophagy influx would be deleterious, because the stalled autophagy secondary to cyclopamine together p53 with induction from classical chemotherapies could lead to effective tumor cell killing. We then confirmed this experimentally, showing an additive lethal effect of cyclopamine and doxorubicin (e.g. at doses that none of these compounds would induce cell death at single agents). We provided further

support for this hypothesis by showing synergistic effects of cyclopamine with hypoxia or low serum, both of are known to promote autophagy.

While future studies will be needed to define the precise interplay between cyclopamine and standard chemotherapeutic agents and autophagy enhancers, our data presented in Chapter 4 suggest that autophagy programs can be harnessed in melanoma cells to promote their self-destruction.

Apoptosis, Autophagy and Melanoma: Looking to the Future

The findings presented in this thesis represent only the first steps in unraveling the intricacies determining how melanoma cells respond to stressors, and why and how they utilize autophagy to avoid cell death. Further analyses will be needed to define the specific requirement of the various autophagy genes (e.g. Beclin, STG5, ATG7 and others) to autophagy induction by cyclopamine. We also will need to determine why autophagosomes do not efficiently fuse to lysosomes in this setting, and ultimately clarify the contribution of Smoothened and other components of the SHH pathway to the regulation of autophagy in melanoma cells. Nevertheless, we are excited by the tantalizing prospect of a possible treatment strategy provided by our results: disabling autophagy while enhancing cellular stress to effect melanoma cell death. Combination therapies are currently the focus of the majority of the clinical trials on melanoma treatments. To date, however, the elimination of autophagic activity has not been included as a target in melanoma treatment. We hope that in the near future, the combination of autophagic inhibition or regulation with more traditional

chemotherapeutic apoptosis induction will be explored, and hopefully result in improvements in the treatment of this very devastating disease.

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