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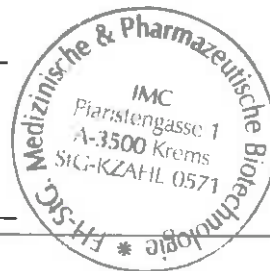Verfasserin Bachelorarbeit / *Author bachelor paper:* **Jana Kalina**

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Studiengangsleiter /*Programme director:* HARALD HUNDSBERGER

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Assessing the anti-tumor efficacy of bufalin in a panel of melanoma cell lines

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Jana KALINA

Internal Supervisor: Prof.(FH) Mag. Dr. Harald Hundsberger

External Supervisor: David de Semir, PhD

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Datum: 01.03.2016

Jana KALINA

Abstract

Bufalin is a traditional Chinese medicine extracted from the skin glands of the Asiatic toad. It is a cardioactive steroid that has been shown to induce apoptosis and inhibit proliferation in various human cancers. However, its effects on the proliferation of human malignant melanoma are mostly unknown. Nuclear receptor co-activator 3 (NCOA3) is a member of the p160 steroid receptor co-activator family and is commonly up-regulated in malignant melanoma and its metastasis. We investigated the effects of bufalin on the protein expression of NCOA3 in C8161.9, D04 and Mamel12 melanoma cell lines. Western blot analysis and immunofluorescence showed a significant decrease of NCOA3 after bufalin treatment. Furthermore, the RNA expression levels of the cyclins A1, B1 and B2 were down-regulated, while CHK2 was simultaneously up-regulated, suggesting an inhibition of proliferation as well as an activation of the DNA damage response following the down-regulation of NCOA3 upon treatment with bufalin. Interestingly, bufalin treatment prior to UVC exposure increased the sensitivity of the cells to UVC radiation.

Based on these results, we believe that bufalin inhibits cell proliferation of human malignant melanoma by down-regulation of NCOA3.

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List of Abbreviations

CAM	Complementary and alternative medicine
CCN	Cyclin
CDK	Cyclin dependent kinase
IR	ionizing radiation
NCOA3	Nuclear receptor co-activator 3
NR	Nuclear hormone receptor
o/n	overnight
qRT-PCR	quantitative real-time polymerase chain reaction
SRC	steroid receptor co-activator
TCM	Traditional Chinese Medicine
UV	ultraviolet

1   Introduction

1.1   Human malignant melanoma

As most aggressive, thus also most dangerous form of skin cancer, human malignant melanoma is responsible for more than 80% of all skin cancer deaths. The high death rate is only emphasized by the fact that malignant melanoma is actually a rather rare form of dermatologic cancer, diagnosed in only 4% of skin cancer patients. (Hsiao et al., 2012)

Especially in Caucasian populations, melanoma incidence has been increasing rapidly over the past decades. Most malignant melanoma develop due to unrepaired DNA damage to epidermal cells caused by UV radiation through intense, intermittent sun exposure. Unrepaired DNA then leads to mutations which, in turn, trigger uncontrolled cell growth and tumor formation. Although exposure to sunlight and frequent sunburns are an important factor in the development of melanoma, other (endogenous) risk factors like the presence of large nevi and genetic predispositions have to be considered (Bandarchi et al., 2010). Since malignant melanoma metastasize in the majority of cases, early diagnosis is crucial for patient survival. Unfortunately, the cancer is typically highly resistant to conventional chemotherapy and in general neither radiation nor surgery significantly increase the cure rate (Hsiao et al., 2012). This lack of efficient treatment leads to an average overall survival of patients with metastatic melanoma of only 8 to 18 months. (Finn et al., 2012)

Hence, the development of novel therapeutics with better efficacy and lower toxicity is a critical step to improve the survival rate of melanoma patients in the future.

1.2   Alternative and herbal medicines in cancer therapy

Although cancer is one of the leading causes of death in modern civilization, our prospects of successfully curing cancer patients are still very limited. Due to a lack of other therapeutic options, surgery, radiotherapy and chemotherapy are still the

major conventional cancer treatments. Their effectiveness, however, is often insufficient and also very restricted, especially considering the high recurrence rates as well as the significant side effects (e.g. nausea, vomiting, diarrhea, pain, hair loss and fatigue) that patients who undergo radio- or chemotherapy often suffer from. (F. Qi et al., 2010)

Traditional Chinese Medicine (TCM) has been used in China, Japan, Korea and other Asian countries for thousands of years to treat various health conditions such as cancer, inflammatory diseases or a weak immune system.

Chinese herbal medicines like Turmeric, Ginseng and garlic have preliminarily found their way into the Western world through their use as culinary spices or teas. With the increased demand for alternative and natural therapeutics, however, their use as complementary and alternative medicine (CAM) has become increasingly popular outside of Asia. In cancer treatment, TCM is nowadays mostly used as adjuvant therapy to enhance the efficacy and reduce the side effects of conventional cancer treatments such as chemo- and radiotherapy. (Z Meng et al., 2012; F. Qi et al., 2010)

Some of the most popular Chinese herbal medicines, which are frequently used in cancer therapy, are described in more detail below.

1.2.1   Turmeric

Turmeric (*Curcuma longa*) is a plant that belongs to the ginger family. Aside from its use as a culinary spice, it was traditionally used in Asia to treat inflammatory conditions. Curcumin, the primary active component in turmeric, has been determined to have a number of pharmacological properties including anti-inflammatory, antioxidant and anticancer effects. Curcumin has been shown to inhibit proliferation, angiogenesis and tumor cell invasion in various cancer cell lines.

In breast cancer, for instance, Curcumin has been demonstrated to inhibit angiogenesis by down-regulating the two major angiogenic factors, VEGF and b-FGF. Furthermore, it has also been found to enhance the efficacy of other

anticancer agents in the treatment of multidrug-resistant cancer. (Parekh et al., 2009; F. Qi et al., 2010)

1.2.2 Ginseng

Historically, *Panax ginseng* has been used as a restorative agent in China, Japan and South Korea for thousands of years, to improve circulation, increase blood supply, and rebuild strength after an illness. Recent studies have shown that Ginseng has potent immunomodulatory, anti-oxidative, anti-inflammatory and anticancer characteristics. Additionally, Ginseng seems to be capable of reducing the negative effects of radiation on normal human tissue and can markedly reduce the risk of cancer recurrence in patients (F. Qi et al., 2010). Epidemiological studies have shown a decreased risk of developing cancer with increased intake of Ginseng. (Yun et al., 2001)

1.2.3 Radix astragali

Astragalus propinquus is an herb that has been used as a TCM for thousands of years. Radix Astragali is the dried root of Astragalus. It was originally used to improve pulmonary function, and the functioning of adrenal glands and the gastrointestinal tract, as well as to increase metabolism, promote healing, and reduce fatigue. Recent studies have shown that Astragalus has potent immunomodulatory properties which include an increased production of interferons and tumor necrosis factor (TNF) and also activation of lymphocytes, natural killer cells and macrophages. Astragalus has proven to be efficient as an adjuvant cancer agent that can increase the resistance to the immunosuppressive effects of chemotherapeutic drugs. (F. Qi et al., 2010)

1.2.4 Allium sativum (Garlic)

Garlic has been used medicinally for thousands of years, traditionally to treat infections, diarrhea, rheumatism and snakebites. More recently, research has shown that garlic has cardiovascular, antineoplastic, and antimicrobial properties. Sulfur-containing compounds, including allicin, are abundant in garlic and appear to

be its primary, biologically active components. According to various preclinical studies, garlic has an anticancer effect on various cancers, especially colon cancer. (F. Qi et al., 2010; Tattelman, 2005)

1.3   Toad venom in TCM and cancer therapy

1.3.1   Chansu

Chansu, the dried secretion of the skin and parotid venom glands of the East Asian toad Bufo *gargarizans*, is an ancient TCM (Lee et al., 2014; J. Qi et al., 2014). It was originally used for its antimicrobial, anodyne, cardiotonic, anesthetic and anti-neoplastic effects, mostly in combination with other TCMs in popular recipes such as Liushenwan, Shexiangbaoxinwan and Niuhuangxiaoyanwan. Recent preclinical studies have identified Chansu as a potential anti-cancer and anti-inflammatory agent (F. Qi et al., 2010; J. Qi et al., 2014).

It has also been shown that the ethanol extract of Chansu inhibited the viability of cancer cells in T cell leukemia as well as breast and lung carcinoma. However, normal human lymphocytes were not significantly affected after treatment with bufalin. (Lee et al., 2014)

1.3.2   Huachansu

The sterilized hot water extract of Chansu is called Huachansu. It is the most extensively used, commercially available preparation of the dried toad skin secretion and is generally administered as an injection. (Z. Meng et al., 2009; J. Qi et al., 2014)

Research from the past three decades has gradually identified the two major active components in Huachansu and Chansu, namely indole alkaloids, including bufotenine, bufotenidine, cinobufotenine and serotonin, and steroidal cardiac glycosides. 28 cardiac glycosides have been identified in Huachansu, out of which the antitumor activity of the substance can be mainly ascribed to bufalin, cinobufagin and resibufogenin. (Z Meng et al., 2012)

Various studies have demonstrated the beneficial effects a treatment with Huachansu can have on cancer patients. A combined administration of Huachansu to patients with advanced gallbladder carcinoma, who were conventionally treated with gemcitabine-oxaliplatin, lead to an improvement of their QOL. (Qin et al., 2008)

Furthermore, a phase I clinical trial conducted by the Fudan University Cancer Hospital in Shangai in cooperation with the Unversity of Texas M.D. Anderson Cancer Center, showed that Huachansu was well tolerated when used as a single agent and could lead to disease stabilization in patients with hepatocellular carcinoma, non-small cell lung cancer or pancreatic cancer. (Z Meng et al., 2009)

Huachansu may also have potential as an adjuvant agent to improve the efficacy of radiotherapy. An *in vitro* study showed that treatment of human lung cancer cells with Huachansu leads to a decrease in cell viability and an increase in radio-sensitivity, which appears to be p53- dependent. (L. Wang et al., 2011)

1.4   Bufalin

1.4.1   General information

Bufalin (Figure 1) is the major immuno-reactive constituent of Chansu. Like other bufadienolides, it is a cardio-active C-24 steroid with cardiotonic, anesthetic, blood pressure stimulatory, respiratory and antineoplastic effects (Takai et al., 2008). Similar to other cardiac glycosides such as digoxin, found in the foxglove plant, and oleandrin, extracted from the Oleander bush, bufadienolides have been used for centuries to medicate patients with irregular heartbeats, edematous states or chronic heart failure. (Ying Wang et al., 2014)
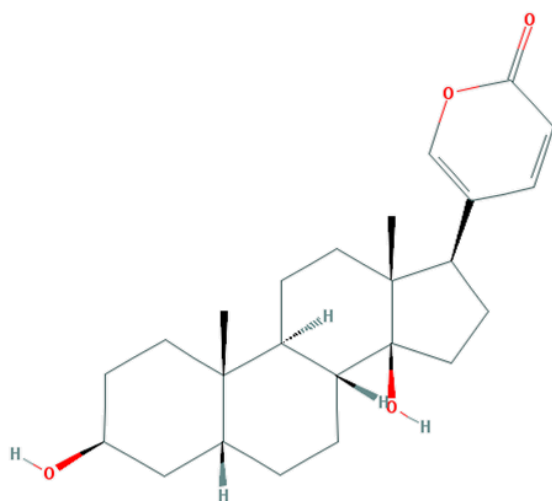
Figure 1 The chemical structure of bufalin[1]

Over the past decades, many studies have been carried out in order to reveal more about the effects bufalin has on various tumors. It has been shown that bufalin can inhibit the proliferation of tumor cells through cell cycle arrest and induction of apoptosis in a number of cancers like, for instance, leukemia, liver cancer, prostate cancer and lung cancer. (Takai et al., 2012)

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[1]Source: National Center for Biotechnology Information. PubChem Compound Database; CID=9547215, https://pubchem.ncbi.nlm.nih.gov/compound/9547215 (accessed Nov. 14, 2015).

The molecular mechanisms and pathways underlying said effects appear to be cancer type specific and are generally very diverse.

1.5 Bufalin as anti-cancer agent

1.5.1 Osteosarcoma

In human osteosarcoma, bufalin has been shown to inhibit cell proliferation and induce apoptosis by activation of the mitochondria-mediated, intrinsic apoptotic pathway. Mitochondria-mediated apoptosis is dependent on the fine balance between the pro- and anti-apoptotic proteins belonging to the bcl-2 family. A shift in the expression levels of those proteins either prevents or facilitates the release of apoptotic factors (e.g. cytochrome c) from the mitochondria.

As shown by Western blot analysis, bufalin dose-dependently up-regulated the expression of the apoptosis promoting proteins bax, Apaf-1, cleaved PARP, and cleaved caspases 3, 7 and 9, whereas the expression of the anti-apoptotic protein bcl-2 was decreased. Another distinct indication for intrinsic apoptosis is the disruption of the mitochondrial transmembrane potential ($\Delta\psi_m$). With increasing concentrations of bufalin, $\Delta\psi_m$ was found to decrease in the osteosarcoma cells, thus further confirming the induction of the intrinsic apoptotic pathway via bufalin. (D. Wang & Bi, 2014)

In another recent study, the effects of bufalin on human osteosarcoma-derived cancer stem cells were researched. Although they represent only a very small percentage of cells within a tumor, cancer stem cells are thought to be one of the primary reasons for cancer recurrence and drug resistance, the major problem being that these cells are generally unaffected by conventional chemotherapeutic drugs. After treatment with bufalin, differentiation, proliferation and sphere formation of the cancer stem cells were significantly impaired. (Chang et al., 2014)

1.5.2   Leukemia

Microarray analysis showed that bufalin could inhibit cell growth of HL-60 leukemia cells by arresting the cell cycle in the G1/S-phase and induce apoptosis by activating the transcription factors AP-1 and NF-κB. (Chen et al., 2009)

It was also found that the multidrug resistance in K562/VCR vincristine-resistant leukemia cells could be, to some extent, reversed after treatment with bufalin. Apoptosis was activated via down-regulation of bcl-xL and up-regulation of bax. (Zhai et al., 2014)

1.5.3   Glioma

Treatment with bufalin significantly inhibited the proliferation of glioma cells. Furthermore, apoptosis was induced through the mitochondria-mediated pathway as well as through endoplasmic reticulum (ER) stress. Additionally, bufalin activated autophagy, which is a physiological catabolic mechanism with the function of maintaining homeostasis in a cell via degradation of dysfunctional proteins and cellular organelles. Although cancer cells might benefit from autophagy because the process enhances their fitness level and thereby facilitates survival, highly increased levels of autophagy might lead to autophagic cell death. However, inhibition of autophagy lead to enhanced bufalin-induced apoptosis and an increase of ER stress in the cells, suggesting that bufalin induced an interaction between apoptosis and autophagy through ER stress. (Shen et al., 2014)

1.5.4   Lung cancer

An *in vitro* study revealed that bufalin could inhibit proliferation and induce apoptosis of A 549 human lung adenocarcinoma cells in a time- and concentration-dependent manner via inhibition of the PI3K/Akt pathway. By inhibiting the phosphorylation (hence also the activation) of Akt, the expression of bax was up-regulated, bcl-2 was down-regulated and caspase-3 was activated. (Zhu et al., 2012)

1.5.5   Gastric cancer

Bufalin inhibited the proliferation of gastric cancer cells in a concentration-dependent and time-dependent manner. At a lower dosage, M-phase cell cycle arrest was induced. With increasing concentration, the bax/bcl-2 ratio was increased and caspase-3 was activated, thereby leading the cells into apoptosis. Upon bufalin treatment, PI3K/Akt signaling was first activated and later inhibited completely. The combination of bufalin with a PI3K-specific inhibitor was shown to increase apoptosis, suggesting that the PI3K/Akt pathway might play a role in bufalin-induced apoptosis. (D. Li et al., 2009)

1.5.6   Hepatocelluar carcinoma

It was recently demonstrated that bufalin can inhibit adhesion, migration, proliferation and invasion of hepatocellular carcinoma cells HCCLM3 and HepG2 *in vitro*. Furthermore, treatment with bufalin resulted in decreased protein expression levels of pAKT, pGSK3β, matrix metalloprotease 2 (MMP-2) and MMP-9, and a simultaneous increase in the expression of GSK3β, as well as in the suppression of the nuclear translocation of β-catenin. Consequently, the canonical Wnt signaling pathway was inhibited. Due to the reduced nuclear presence of β-catenin, the transcription and expression of the transmembrane protein E-cadherin was considerably increased, in turn causing, the down-regulation of MMP-2 and -9. MMPs are enzymes which catalyze the degradation of the extracellular matrix and are therefore highly associated with cancer cell migration and the formation of metastasis. An invasion assay accordingly showed a decrease in invasiveness after bufalin treatment. (Qiu et al., 2013)

In 2007, an *in vivo* study showed that bufalin had noticeable anti-tumor effects on *in situ* tumor models of human hepatocellular carcinoma implanted into the liver of nude mice. The administration of bufalin significantly reduced the tumor volumes. Apoptosis was induced by up-regulation of the pro-apoptotic gene bax, and consequently decreased the bcl-2/bax ratio. No apparent toxicity to brain, heart, lungs, liver and kidneys was observed. (Han et al., 2007)

1.5.7   Pancreatic cancer

In PANC-1 and CFPAC-1 pancreatic cancer cells, bufalin arrested the cell cycle in the G2/M phase and induced apoptosis. Western blot analysis showed decreased expression of p-Akt, activation of caspases 3 and 9, lower expression of bcl-2 and an enhanced expression of bax. When combined with the conventional chemotherapeutic gemcitabine, bufalin improved the drugs growth inhibiting effects. (M. Li et al., 2014)

1.5.8   Gallbladder carcinoma

Cell proliferation was significantly inhibited in human gallbladder carcinoma through bufalin-induced S-phase cell cycle arrest. Apoptosis was induced in a dose-dependent manner, most likely via the intrinsic pathway, indicated by the disruption of the mitochondrial membrane potential $\Delta\psi_m$ and the activation of caspase 3 as well as caspase 9 upon treatment with bufalin. (Jiang et al., 2014)

1.5.9   Gynecologic cancer

An *in vitro* study investigated the effects of bufalin on endometrial and ovarian cancer cells. It was found that the compound could induce arrest of the cell cycle in the G0/G1 phase and apoptosis by down-regulation of cyclin A, cyclin D3, bcl-2 and bcl-xL and simultaneous up-regulation of $p21^{WAF1}$ and cleaved caspase-9. Normal endometrial cells, however, remained viable after the same doses of bufalin and their proliferation was only weakly affected. (Takai et al., 2008)

1.5.10   Breast cancer

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been found to induce the extrinsic, death-receptor-dependent, apoptotic pathway by attaching to death receptor 4 (DR 4) or DR5 located in lipid rafts within the plasma membrane. This action consequently leads to the formation of the death-inducing signaling complex (DISC) which is followed by the activation of effector caspases downstream of caspase-8. TRAIL has therefore been selected as a promising agent for cancer

therapy. Unfortunately, most breast cancer cell lines are in general resistant to TRAIL.

Bufalin treatment has been shown to redistribute DR4 and DR5 in the lipid rafts, thereby enhancing TRAIL-induced apoptosis in breast cancer cells. (Yan et al., 2014)

1.5.11 Melanoma

At higher drug concentrations (up to 550 nM), the exposure of melanoma A 375.S2 cells to bufalin has been shown to significantly reduce cell viability and induced apoptotic cell death. $\Delta\psi_m$ and the expression level of bcl-xL were clearly reduced, whereas the expression of cleaved caspases 3, 8 and 9, cytochrome c, bax, Fas and FasL were elevated. These findings indicated that bufalin triggered apoptosis in A 375.S2 melanoma cells via both the intrinsic and also the extrinsic apoptotic pathway. (Hsiao et al., 2012)

However, the effects of lower bufalin doses on the proliferation of melanoma, as well as the underlying mechanisms have not been studied.

1.6 Nuclear Receptor Co-activator -3 (NCOA3)

Nuclear receptor co-activator-3 (NCOA3, also known as SRC3, p/CIP, AIB1, RAC3, ACTR and TRAM1) is one of three homologous members of the p160 steroid receptor co-activator (SRC) family. SRCs promote the transcriptional activation of steroid hormone receptors in a hormone-dependent manner. Recent studies have shown that SRC genes are frequently amplified and overexpressed in various human cancers. (Xu et al., 2009)

1.6.1 Physiological functions

As transcriptional co-activators, SRC proteins interact with nuclear receptors (NRs) and other specific transcription factors to mediate their transcriptional functions. By recruiting chromatin remodeling and other transcriptional enzymes, they alleviate the assembly of transcription factors for transcriptional activation.

As previously mentioned, SRCs do not exclusively serve as co-activators for NRs, they also co-activate other transcription factors such as NF-κB, E2F1, Smads, STATs (=signal transducers and activators of transcription) and p53. Gene transcription may further be stimulated by various interactions of SCRs with kinases, phosphatases, ubiquitin ligases, small ubiquitin-related modifier (SUMO) ligases, histone acetyl-transferases and histone methyltransferases. As SCRs regulate the gene expression of a broad range of transcription factors, they consequently regulate many diverse physiological functions including cell cycle and energy metabolism pathways. (Xu et al., 2009)

1.6.2   NCOA3 overexpression in cancer

NCOA3 has been found to be overexpressed and amplified in a number of human cancers, particularly in breast and prostate cancer. The underlying mechanisms, through which carcinogenesis is promoted, have been investigated in various studies. In breast cancer, NCOA3 overexpression is usually linked to the expressions of ERBB2, MMP2, MMP9 and polyoma enhancer activator 3 (PEA3) and is associated with larger tumor size, higher tumor grade and also poor disease-free survival. NCOA3 furthermore has a vital role in promoting the proliferation, migration and invasion of breast tumor cells through other mechanisms , such as up-regulating the function of E2F1 and estrogen receptor-α, and the activity of the insulin-like growth factor 1 (IGF1) signaling pathway (Xu et al., 2009). In addition, increased levels of NCOA3 were found to correlate with tamoxifen resistance of breast cancer cells (Osborne et al., 2003).

In prostate cancer, NCOA3 was found to activate the Akt-mTOR pathway and could stimulate cell growth by increasing the size of the cells. Knockdown of NCOA3 decreased cell proliferation and increased apoptosis. High NCOA3 levels were especially found in prostate tumor cells in more malignant stages.

Furthermore, amplification of NCOA3 has also been observed in other cancers, such as ovarian cancer, colorectal cancer, gastric cancer, endometrial carcinoma, hepatocellular carcinoma and pancreatic cancer, and often correlates positively with severity of the disease. (Anzick, 1997; Yi Wang et al., 2002; Xu et al., 2009)

1.6.2.1   NCOA3 in melanoma

The overexpression of NCOA3 in primary melanomas promotes tumor growth and is directly associated with an increased risk of relapse and the formation of metastasis, and consequently reduced patient survival. NCOA3 expression is remarkably higher in melanoma metastasis, especially in those of the lymph nodes. (Rangel et al., 2006)

1.6.3   Effects of bufalin on the expression of NCOA3

An extensive study recently revealed that bufalin could serve as a potential inhibitor of NCOA3 in breast cancer. Bufalin significantly reduced NCOA3 protein levels, decreased cell viability and blocked cancer cell growth at low nanomolar concentrations (Ying Wang et al., 2014).

As mentioned above, NCOA3 is frequently overexpressed in melanoma and its metastasis. Since high concentrations of bufalin have been shown to induce apoptosis in melanoma (see page 9, chapter 1.6.11), with this study we wanted to find out, whether low concentrations of the drug would similarly influence NCOA3 expression as has been observed in breast cancer.

2 Materials and Methods

2.1 Cell lines

Three melanoma cell lines were used for the experiments.

2.1.1 C8161.9
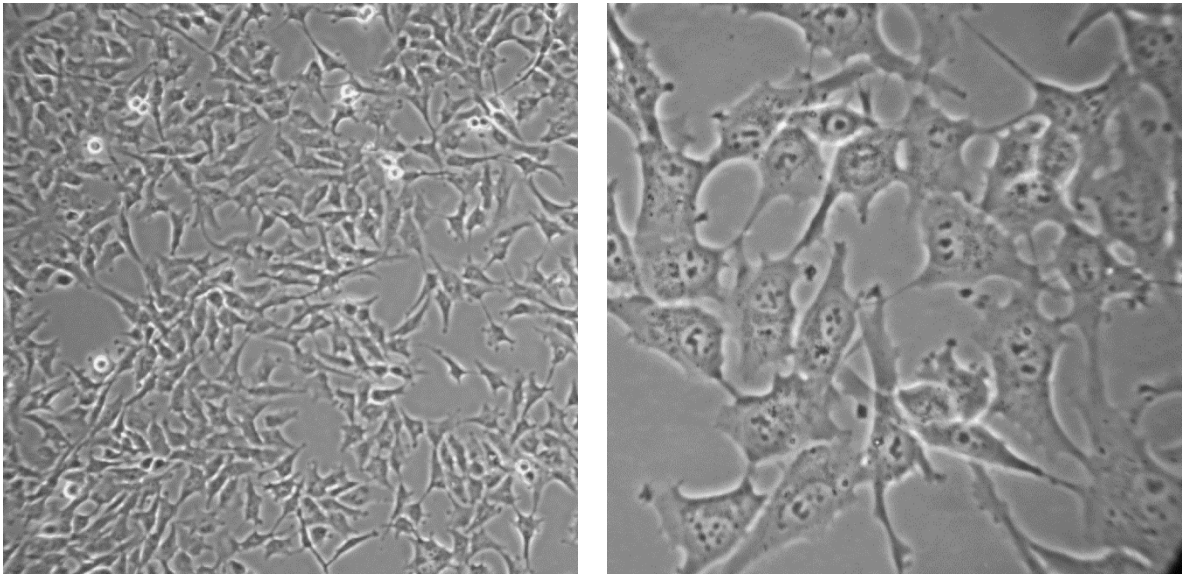
Figure 2 Morphology of C8161.9 cells in 100x magnification (left) and 400x magnification (right)

C8161.9 cells were obtained from Dr. Danny Welch, University of Alabama, Birmingham, AL).

These melanoma cells have an icosahedral shape and grow rapidly.

2.1.2 D04
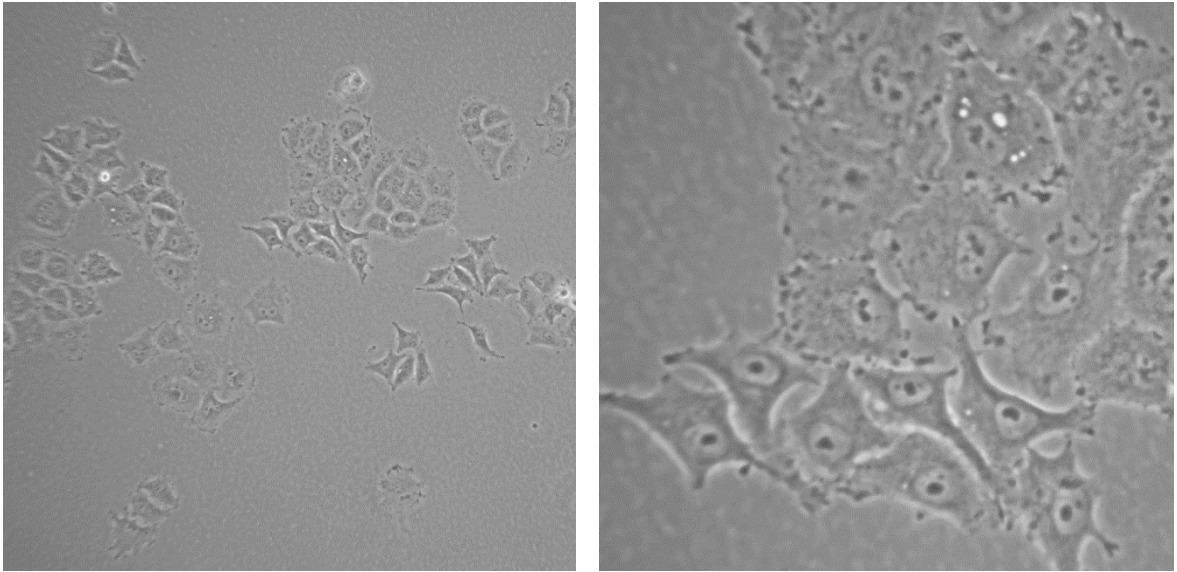
Figure 3 Morphology of D04 cells at 100x magnification (left) and 400x magnification (right)

D04 cells have a similar morphology as C8161.9 cells. Their shape, however, is less icosahedral and they tend to grow in clumps.

The cells were a kind gift from Dr. Boris Bastian (UCSF Cancer Center).

2.1.3 Mamel12
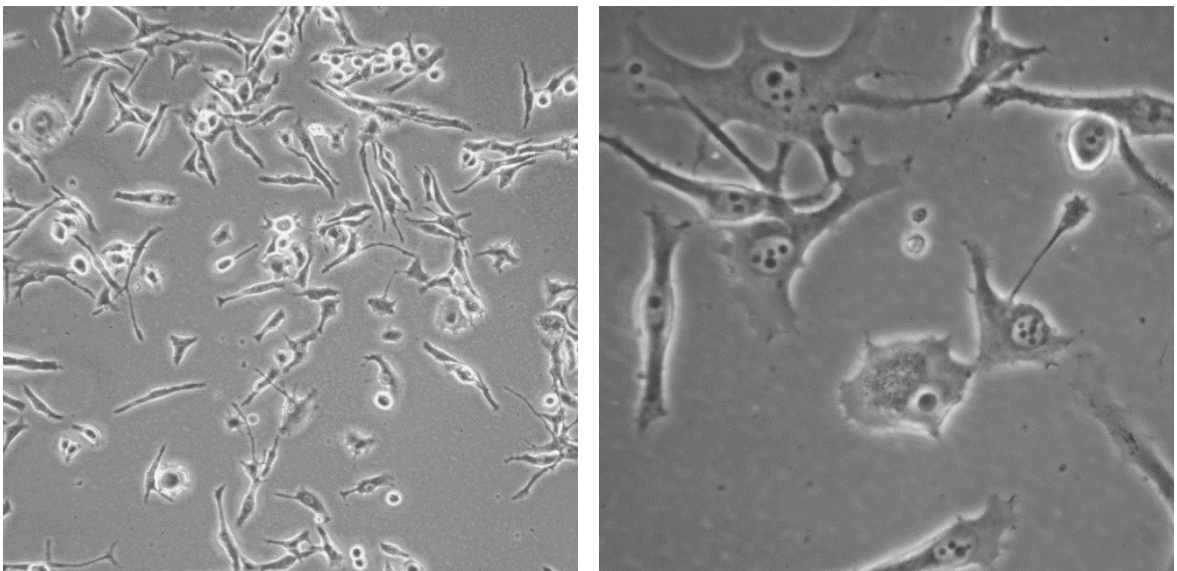
Figure 4 Morphology of Mamel12 cells at 100x magnification (left) and 400x magnification (right)

Mamel12 cells were obtained from Dr. Dirk Schadendorf (Department of Dermatology, University Hospital Tübingen, Germany).

These melanoma cells are small in size and show a lot of variability in their morphology.

2.2   Cell culture

C8161.9 cells were grown in Dublecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12; Corning Cat# 10-092-CM) containing 5% FBS (JRScientific; Cat# 43640-500) and 1x penicillin/streptomycin (P/S, GE Healthcare Life Sciences, Cat# SV30010)

DO4 cells were cultured in RPMI-1640, 1x (Corning, Cat# 10-040-CM) containing 5% FBS and 1x P/S

Mamel12 cells were maintained in RPMI-1640, 1x supplemented with 10% FBS and 1x P/S.

Table 1 Overview on the media compositions for the used melanoma cell lines

Cell line	Medium	FBS (%)	P/S (%)
C8161.9	DMEM/F-12	5	1
D04	RPMI	5	1
Mamel12	RPMI	10	1

All cells were cultured with humidity at 37°C under 5% CO_2 atmosphere.

2.3   Chemicals

Bufalin (Sigma Aldrich, Cat# B0261) dissolved in DMSO

DMSO (Corning, Cat# MT-25950CQC)

2.4   Cell viability assay

Cell viability assays are used to determine the effectiveness of cytotoxic agents. Two different cell survival assays were performed in this study.

In order to determine a bufalin concentration suitable for further experiments, 5000 cells per well were seeded in 96-well-plates and treated with increasing concentrations of bufalin (0, 5, 10, 20 and 40 nM) for 2 days. The relative number of viable cells was measured with the DOJINDO Cell counting Kit-8 (500 tests, # CK04). IC values were determined with a spectrophotometric multiwell plate reader (Spectramax 190) using SoftMax Pro software.

A variation of the assay was used to test the effect of bufalin on the UVC sensitivity was of melanoma cells. 5000 cells per well were seeded in 96-well-plates and treated with the bufalin concentration respectively determined for each cell line (C8161.9 and D04: 2.5nM; Mamel12: 5nM) for 6 hours. The bufalin-containing medium was then removed from the cells and they were exposed to increasing doses of UVC light (0,10, 20 and 40 J/m^2). Medium was replaced without bufalin immediately after UVC treatment. The relative number of viable cells was measured after 48 hours as described above.

2.5   Western blotting assay

2.5.1   Protein extraction

A cell pellet was collected for protein extraction. The pellet was resuspended in 75µl of RIPA lysis buffer (Santa Cruz Biotechnology Cat# sc-24948) mixed with protease and phosphatase inhibitor (100x, Thermo Scientific, Cat# 1861281). RIPA contains sodium dodecyl sulfate (SDS), which gives proteins an overall negative charge. To facilitate cell lysis, the suspension was mixed up and down with an insulin syringe, transferred into an Eppendorf tube and then incubated for 30 minutes on ice. The cell lysate was then centrifuged for 10 minutes at 4°C and maximum speed. The supernatant containing the proteins was carefully transferred into a fresh Eppendorf tube.

2.5.2   Protein quantification

To get comparable protein bands, it is crucial that the protein load is the same for every sample. It is therefore necessary to determine the concentration of proteins present in every sample.

Protein concentration was measured via Bradford assay. The samples were measured in triplicates. 160 µl HBS and 40 µl of Bradford reagent (Bio Rad, Cat# 500-0006) were added per used well of a 96-well-plate. 1µl of the protein lysate was added per well (dilution factor 1:200). The standard curve was prepared in duplicates containing 40µg, 20µg, 10µg, 5µg, 2.5µg and 0µg of BSA. The sample concentrations were determined via spectrophotometric multiwell plate reader using SoftMax Pro software.

2.5.3   SDS-Page

Polyacrylamide gel electrophoresis is a method used to separate proteins of different sizes according to their electrophoretic mobility.

50µg of protein sample were mixed 1:4 with loading dye (NuPage LDS Sample Buffer 4x, Novex; Cat# NP00007), heated in a heating block at 95°C for 5 minutes and spun down briefly. The samples and protein marker (Page Ruler Plus, Thermo Scientific; Cat # 26619) were loaded into the wells of a 7.5% SDS-page gel placed in 1x running buffer (TGS,10x, Bio-Rad, Cat# 161-0772).

Table 2 Composition of separating gel and stacking gel

	Separating gel (7.5%)	Stacking gel (4%)
H_2O	5.1 ml	3.1 ml
40 % Acrylamide	1.7 ml	0.5 ml
Tris-HCl	2 ml (1.5 M)	1.25 ml (0.5 M)
10 % SDS	80 µl	55 µl
APS (10%)	75 µl	50 µl
Temed	8 µl	5 µl

An electric field was applied (100V), leading the negatively charged proteins to migrate to the anode small molecules travelling further than larger molecules. Voltage was applied, until the bands of interest (shown by the marker) were well separated.

2.5.4 Protein Transfer

After separation by size, the proteins were transferred to a nitrocellulose membrane by wet transfer, which is used for proteins with high molecular weight (like NCOA3, the protein of interest with a mass of approximately 160 kDa) Therefore, the gel and the membrane were placed between two filter papers and fixated in a cassette, which was then placed into transfer cell filled with transfer buffer (1x TGS containing 30% MetOH). An ice tray was added to avoid overheating of the buffer during transfer, which was set for 60 minutes at 100V in the cold room at 4°C.

2.5.5 Antibodies and development

After the transfer, the membrane was blocked by placing it into 1xTBST (TBS,10x, Corning; Cat# 46-012CM), (Tween-20, Santa Cruz Cat# sc-29113) containing 5% milk powder for 30 minutes. Following three washing steps with 1x TBST for 5 minutes, primary antibody was added to the membrane in TBST containing 5% milk powder(NCOA3 – dilution 1:1000; Santa Cruz, Cat# sc-9119) (GAPDH - dilution 1:2000; EMD Millipore, Cat# MAB374) (ß-Aktin – dilution 1:4000; Sigma Aldrich, Cat# A5441) and incubated overnight at 4°C.

After three more 5-minute-washes with TBST on the next day, secondary antibody (Goat Anti-Rabbit IgG (H+L)-Conjugate; Bio-Rad Cat# 172-1019) (Goat-Anti-Mouse IgG (H+L)-Conjugate; Bio-Rad Cat# 170-6516) was added to the membrane in TBST and incubated for 60 minutes. The membrane was again washed three times for 5 minutes with TBST. Before the membranes could be developed on an x-ray film (Thermo Scientific, Cat# 34090), luminol reagent (Santa Cruz, Cat# sc-2048) was added to the membranes for protein detection.

2.6   Quantitative Realtime-PCR

2.6.1   RNA extraction

RNA extraction was done by using RNeasy Mini Kit (Qiagen, Cat# 74106) according to the user's guide provided by the manufacturer.

Cell pellets were collected for RNA extraction. To eliminate any possibly present RNAses from the samples, 10µl of ß-mercaptoethanol (Sigma Aldrich, Cat# M3148-25ML) were added per 1ml of RLT lysis buffer. The pellet was resuspended in 350µl of the mixture and the resulting lysate was added to a QIAshredder spin column (Qiagen, Cat# 79656) and centrifuged for two minutes at maximum speed, to ensure disruption of the cells and homogenization of the lysate. The flow-through was mixed with one volume (350µl) of 70% ethanol.

The total volume of 700µl was transferred to an RNeasy Mini spin column in a 2ml collection tube and centrifuged for 15 seconds at 8000g. The flow-through was discarded, and 700µl of RW1 buffer were added to the spin column. After another centrifugation step (15 seconds at 8000g), the flow-through was again discarded and 500µl of RPE buffer were added. Followed by another centrifugation at 8000g for 15 seconds, the flow through was discarded. Again, 500µl of RPE buffer were added and the spin column was centrifuged at 8000g, this time for two minutes. The flow-through was discarded. To ensure the membrane was dry, the column was placed into a fresh 2ml collection tube and centrifuged for one minute at full speed.

The spin column was then placed into a 1.5ml collection tube. Depending on the pellet size, 30 to 50µl of RNase-free water was added to the column, left to soak the filter in the spin column for a minute, and then centrifuged for one minute at 8000g to eluate the RNA. For further use the RNA was kept on ice to avoid degradation or could be stored at -20°C.

RNA concentration and purity were measured with a NanoDrop 2000 UV-Vis Spectrophotometer.

2.6.2   cDNA synthesis

Harvested RNA has to be converted into cDNA by reverse transcription, before it can be quantified by rt-PCR. Since the purified mRNA is spliced, which means that the introns (uncoding sequences) have already been excluded, the cDNA synthesized from it contains only exons (coding sequences).

For the conversion of the RNA samples to cDNA, the iScript cDNA Synthesis Kit from Bio-Rad (Cat# 170-8691) was used. For a final volume of 10µl per PCR tube, 1 µg of RNA was mixed with 2.5 µl of a Mix containing primers, DNA polymerase and the enzyme reverse transcriptase and also nuclease free water.

Table 3 cDNA synthesis reaction volumes

RNA	x µl (containing 1µg RNA)
Nuclease free water	x µl (dependent on RNA volume)
5x iScript reaction mix	2 µl
iScript reverse transcriptase	0.5 µl
Total volume	10 ml

The temperature steps of the PCR cycle program used for cDNA synthesis were defined as follows:

25°C for 5 minutes
42°C for 30 minutes
85°C for 5 minutes
4°C until further use (storage at -20°C)

2.6.3   RT-PCR with TaqMan probes

Per cDNA sample, at least two different probes were used in one PCR run, one coding for the control gene and one coding for the gene of interest. Since for each used probe the sample was added in triplicates to the wells of a 96-well PCR plate, 6 sample replicas (and consequently 6 wells of the plate) were needed for the use of 2 probes.

The procedure and the reagent volumes are further described for the use of two or three probes. If more than two genes of interest were tested, the numbers and amounts were scaled up to the desired amount of replicas (for example 12 replicas per were needed for simultaneous use of 4 probes on one cDNA sample).

Table 4 RT-PCR reaction volumes used for 6 (left column) or 9 (right column) sample replicas

Master Mix	35.6 µl	53.4 µl
Nuclease free water	21.4 µl	32.1 µl
cDNA (previously diluted 1:5)	11.5 µl	16.5 µl
Total Volume	68 µl	102 µl

Nuclease free water, TaqMan Fast Advanced Master Mix (Thermofisher Cat# 444557) and cDNA (previously diluted 1.5 with nuclease free water) were added to an Eppendorf tube in the volumes displayed in Table 4 and mixed thoroughly. The final volume was then split into Eppendorf tubes according to the number of used probes, one tube per probe, each tube containing 34µl of the mixture. As final step, 1.7µl of probe were added per tube. From each tube, respectively 11µl were pipetted into three wells of the PCR plate.

Finally, the plate was covered with sealing film and the PCR run was performed with 7500 Fast RT-PCR system.

2.7   Immunofluorescence

Immunofluorescence microscopy is a method that is used to detect and visualize proteins or other structures in a cell by antibody binding.

Melanoma cells were seeded in 6-well plates on microscope cover glass slips (Fisher Scientific, Cat# 12-541-13). After cell attachment, the cover slips were washed carefully three times with PBS and then fixed with 4% Formaldehyde (Fisher Scientific, Cat# F79-500) in PBS for 10 minutes. After three more washes with PBS, the cells were permeabilized with 0.2 % Triton X-100 (MP Biomedicals Cat# 807426) in PBS for another 10 minutes. In the next step, the cover slips were washed three

more times with PBS and then blocked with 3% BSA (Albumin, from bovine serum; Sigma-Aldrich, Cat# A7906-100G) in PBS for 10 minutes. After one more wash with PBS, NCOA3 primary antibody (Santa Cruz, Cat# sc-9119), previously diluted 1:1000 in 250µl Dako Antibody diluent (Cat# 53022) was added per coverslip and incubated overnight at 4°C.

Subsequently, the cover slips were washed again three times with PBS. The secondary antibody (Alexa Fluor goat anti rabbit IgG, life technologies, Cat# A11072) was diluted in DAKO diluent (1:1000), 500 µl were added per cover slip and incubated for 1 hour at room temperature in the dark.

After incubation of the secondary antibody, the cover slips were washed once with 1x TBST and twice with PBS, followed by dehydration with an ethanol series, namely 70%, 90% and finally 100% EtOH). Thereafter, the slides were mounted on microscope glass slides (Fisher Scientific, Cat# 22-038-103) with VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Cat# H-1200), finally ready for examination under the fluorescent microscope.

2.8   Colony formation assay

Similar to cell viability assays, colony formation assays are used to test for the efficacy of a substance on the proliferation and the survival of cells. The method is based on the capability of single cells to form colonies.

The assay was used to test the effect of bufalin on the UVC sensitivity of melanoma cells. For each cell line, 5000 cells per well were seeded in 6-well-plates with 3 ml of the corresponding cell culture medium. Following the attachment of the cells to the bottom of the wells, the cells in three wells respectively were treated with bufalin for 6 hours. DMSO served as control in the other three wells. After the incubation time, the drug-containing medium was removed. Since UVC light cannot penetrate liquids, the cells without medium were exposed to 20 J/m^2 or 40 J/m^2 UVC light. Immediately afterwards, the medium was replaced without DMSO or bufalin. The cells were incubated at 37°C for 6 to 10 days, until the formation of colonies could be observed.

Upon colony formation, the medium was removed and the bottom of the wells was covered with crystal violet solution (Sigma Aldrich, Cat# HT90132-1L). The dye was incubated for 10 minutes to stain the colonies and then removed, the wells were washed thoroughly with plenty of water and the colonies were counted.

3 Results

3.1 Effects of bufalin on melanoma cell viability

To confirm that treatment with bufalin has an effect on the viability of the C8161.9, D04 and Mamel12 melanoma cells, a cell survival assay was done as an initial experiment. As depicted in Figure 5, cell viability was significantly decreased with increasing dosage of bufalin in all three melanoma cell lines 48 hours after treatment, indicating a dose-dependent toxicity of bufalin to all three melanoma cell lines.
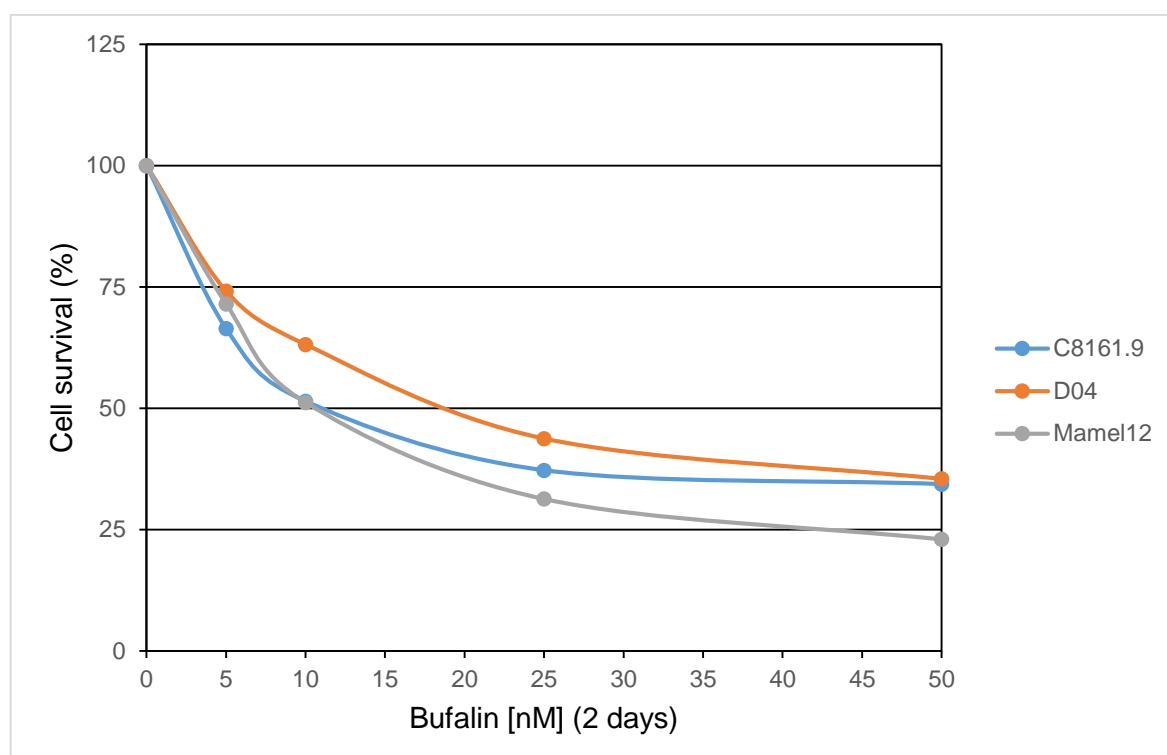
Figure 5 Effects of bufalin on the cell viability of C8161.9, D04 and Mamel12 cells at the indicated concentrations after 48h as determined by cell survival assay. IC_{50}: for C8161.9 and Mamel12 approximately 10nM, for D04 approximately 20nM

For further experiments in order to explore the influence of bufalin on the NCOA3 expression in melanoma, we needed viable cells and thus a drug concentration that was low enough to present only minor toxicity to the cells. Although the cell survival assay showed similar viability for the three cell lines at low bufalin dosage (around 5nM), the optimization processes for the following experiments showed that the

appropriate bufalin concentration for the C8161.9 and D04 cells was 2.5nM whereas 5nM turned out to be a more suitable concentration for Mamel12.

3.2 Changes in the protein expression of NCOA3

As member of the p160 steroid receptor co-activator family, NCOA3 plays a physiologically important role in promoting the ligand-dependent transcriptional activity of nuclear receptors, which regulate genes involved in crucial aspects of cell proliferation, differentiation and homeostasis, and other specific transcription factors (Torchia et al., 1997). NCOA3 has been shown to be regularly overexpressed in human malignant melanoma and its metastasis (Rangel et al., 2006).
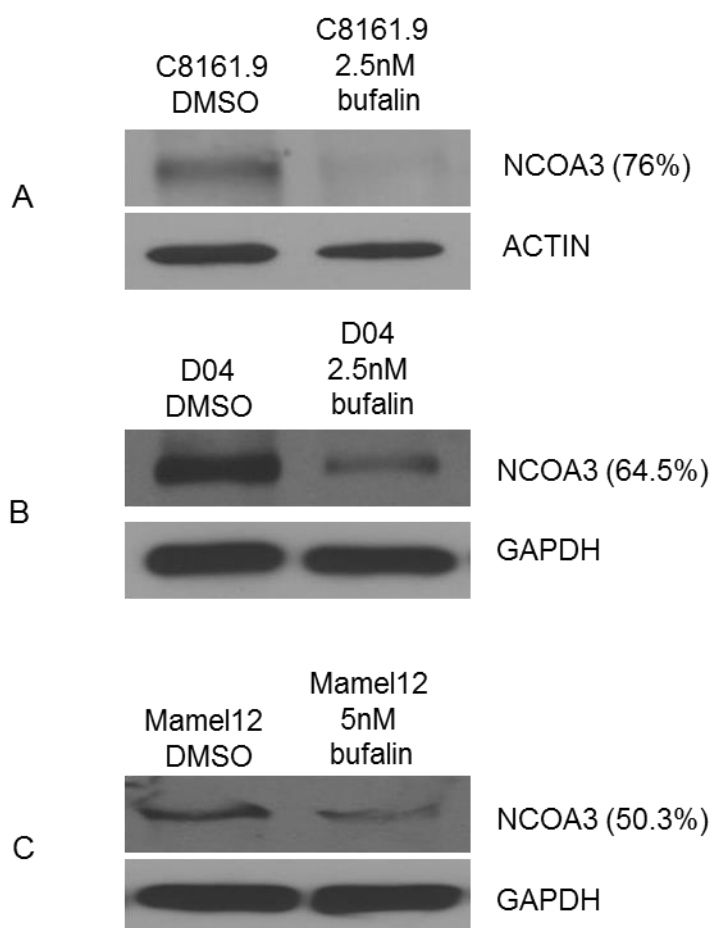
Figure 6 Protein levels of NCOA3 after 6h treatment with bufalin vs. DMSO at indicated concentrations, normalized to Actin/GAPDH. A: decrease of NCOA3 in C8161.9 by 76%,B: in D04 by 64.5%, C: in Mamel12 by 50.3%

A recent study demonstrated that bufalin can inhibit the protein levels of NCOA3 in breast cancer (Ying Wang et al., 2014). Hence, we hypothesized that treatment with bufalin might have a similar effect on melanoma cells.

Western blotting assay showed a significant decrease of NCOA3 protein expression only 6 hours after the melanoma cells had been treated with bufalin. Figure 6A shows that in the C8161.9 cells, bufalin promoted down-regulation of NCOA3 by 76 percent. In the D04 cells, the NCOA3 protein level was decreased by 64.5% (Figure 6B) and in the Mamel12 cell line by 50.3% (Figure 6C).

For two cell lines, C8161.9 and Mamel12, similar results could be achieved 24 hours after bufalin treatment via immunofluorescence staining.
Figure 7 depicts C8161.9 cells. The two upper pictures show the control cells, treated with only DMSO. The lower pictures show the bufalin-treated cells. DAPI was incorporated in the nuclei, shown in blue in the pictures on the right. NCOA3 expression is represented in red in the pictures on the left-hand side. The intensity of the red color is visibly lower in the bottom left picture showing the bufalin-treated cells, thereby indicating a decreased NCOA3 expression in comparison to the control in the upper left picture showing a much brighter red color.
Quantification analysis of the fluorescent microscope pictures is shown in Figure 8 and confirms a decrease in the expression of NCOA3 in the cells treated with bufalin.

Figure 9 shows Mamel12 cells 24h after bufalin was added. The pictures are assorted in the same way as described above for Figure 7. The difference in mean pixel intensity for bufalin versus DMSO treated cells is even more prominent than for the C8161.9 cells, revealing that bufalin induced an even stronger decrease of the NCOA3 protein levels in the Mamel12 cells. Determination of the mean pixel intensity again verified this observation, as can be seen in Figure 10, since the distance between the two curves is larger than in Figure 8.

Figure 7 effect of treatment with 2.5 nM bufalin for 24h, versus DMSO as control, on C8161.9 cells shown by immunofluorescence staining. Pictures taken with fluorescent microscope at 200x magnification. Blue stain: nuclei stained with DAPI; Red stain: NCOA3 expression.

Figure 8 Analysis of immunofluorescence microscopy pictures of C8161.9 cells quantifying differences in NCOA3 expression after 2.5 nM bufalin treatment versus DMSO. Higher pixel intensity depicts higher protein level of NCOA3. Mean Pixel intensity is stronger in the control (blue line) than in the bufalin-treated cells (red line), indicating decreased expression of NCOA3 after bufalin treatment.
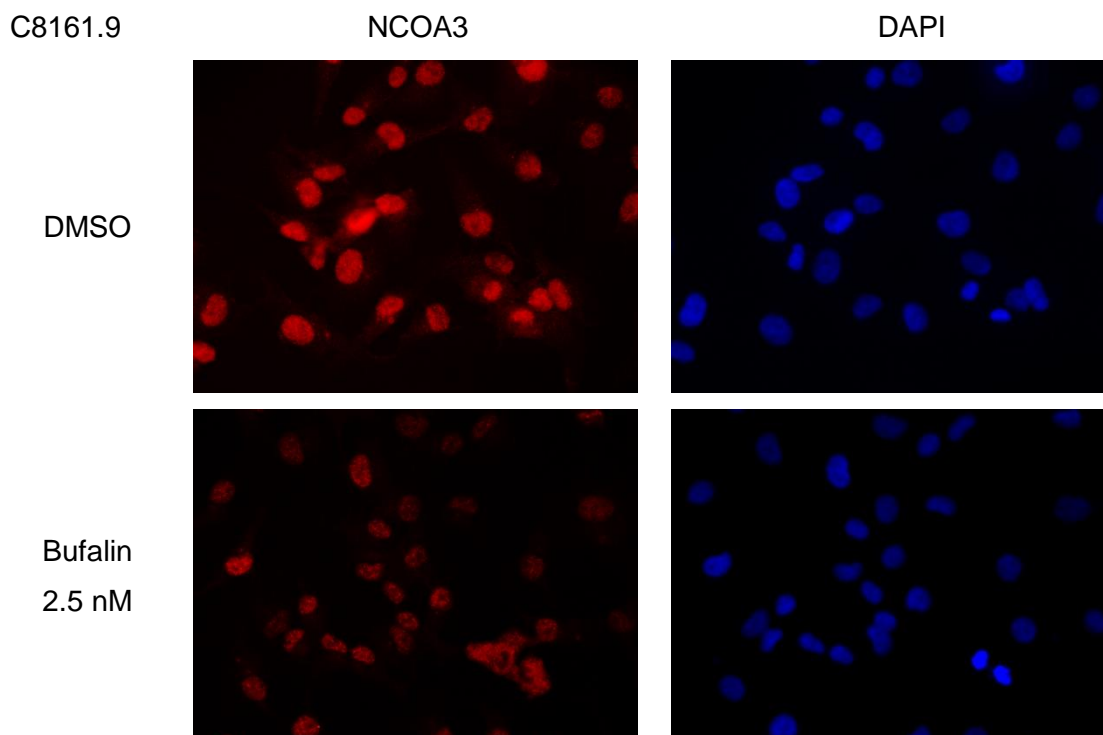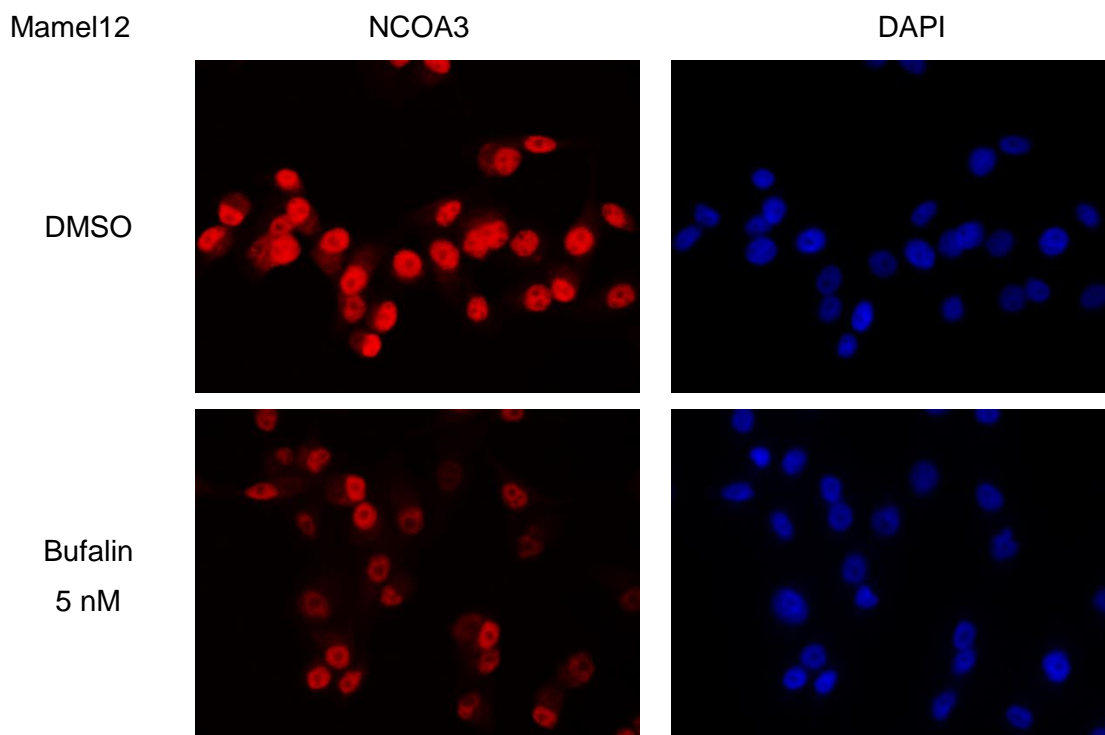
Figure 9 effect of treatment with 5 nM bufalin for 24h, versus DMSO as control, on Mamel12 cells shown by immunofluorescence staining. Pictures taken with fluorescent microscope at 200x magnification. Blue stain: nuclei stained with DAPI; Red stain: NCOA3 expression

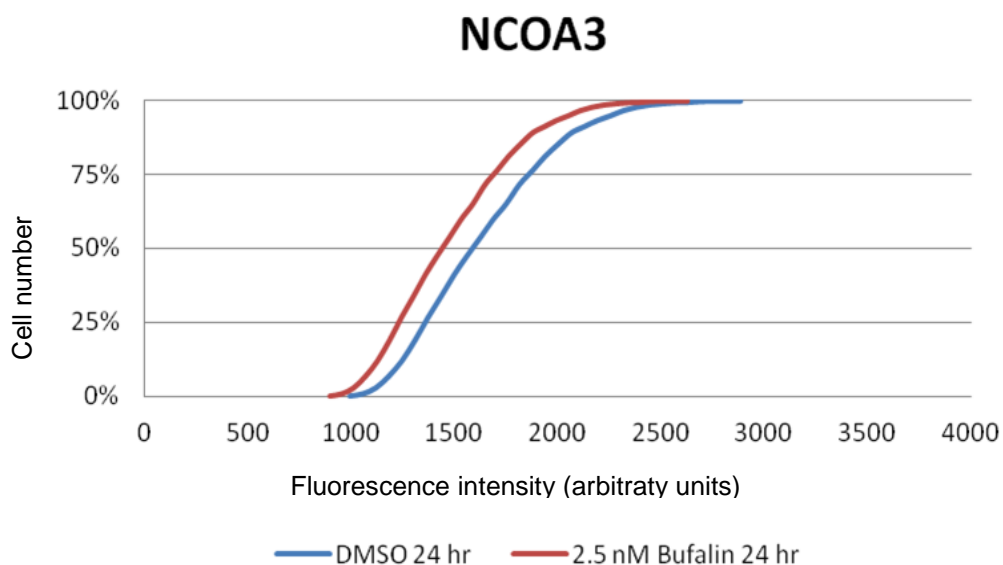*Figure 10 Analysis of immunofluorescence microscopy pictures of Mamel12 cells quantifying differences in NCOA3 expression after 5 nM bufalin treatment versus DMSO. Higher pixel intensity depicts higher protein level of NCOA3. Mean Pixel intensity is stronger in the control (blue line) than in the bufalin-treated cells (red line), indicating decreased expression of NCOA3 after bufalin treatment.*
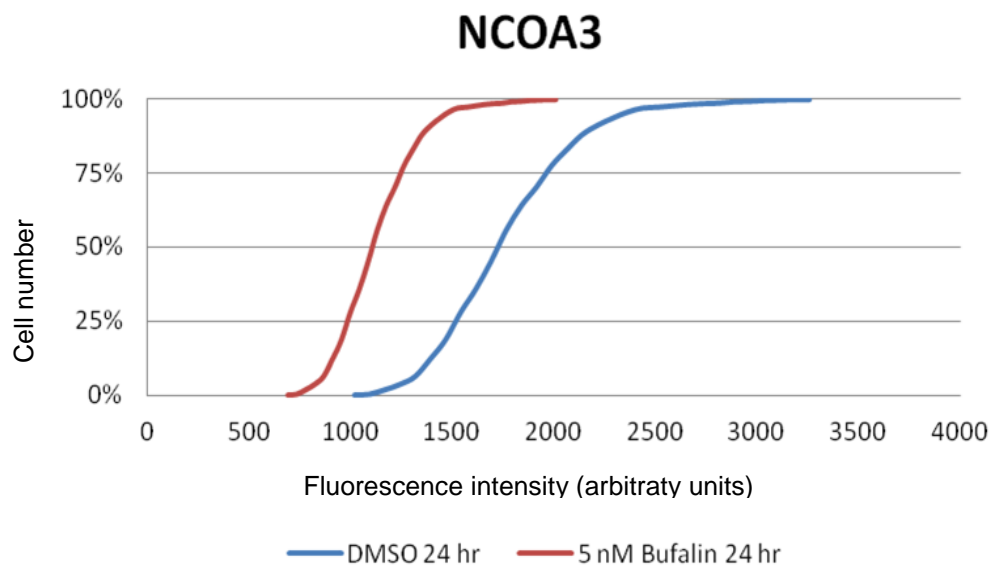
3.3   Bufalin affects the cell cycle of melanoma cells

To get an insight on whether bufalin, and consequently NCOA3, has an effect on the cell cycle of melanoma cells, we tested for RNA expression levels of the Cyclins A1, B1 and B2 in the C8161.9 cell line, 24 hours after the cells had been treated with bufalin. The presence of cyclin A1 is crucial in the S-phase of the cell cycle where it binds to cyclin- dependent kinase 2 (CDK2) and also in the transition to the G2-phase, where it is bound to CDK1 (also known as CDC2), B-type cyclins are highly expressed during M-phase and bind to CDK1.

Quantitative RT-PCR showed that the RNA expression of the tested cyclins was considerably decreased after bufalin treatment. As depicted in Figure 11, the RNA levels of cyclin A1 were down-regulated by 59.2 % and those of cyclin B1 were decreased by 67.2%. Down-regulation of cyclin B2 was not as prominent, but still amounted to 24.9% in comparison to DMSO- treated cells.

Although further analysis is required, it can be assumed that bufalin might influence proliferation and cell cycle progression in melanoma cells.
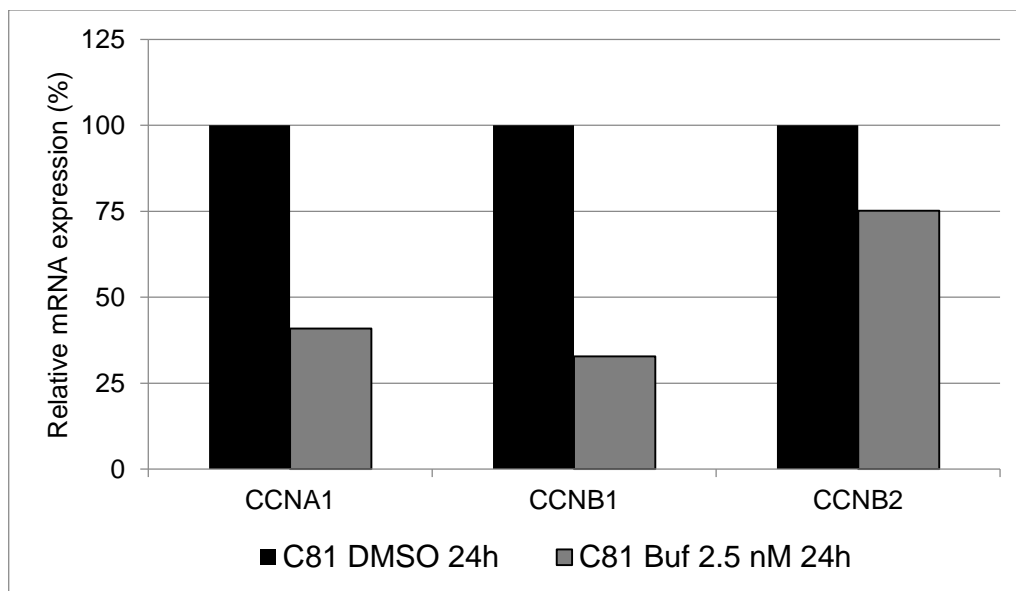
Figure 11 Results of quantitative RT-PCR showing the effects of 24h bufalin treatment (2.5 nM) versus DMSO on RNA expression (in %) of Cyclins A1, B1 and B2 in C8161.9 melanoma cells. CCNA1 down-regulated by 59.2%, CCNB1 by 67.2%, CCNB2 by 24.9%

3.4   Bufalin affects DNA damage response in melanoma

The development of melanoma is strongly associated with the excessive exposure of the skin to ultraviolet (UV) light, which causes DNA damage to the cells of the epidermis.

Figure 12 The DNA damage repair pathway[2]

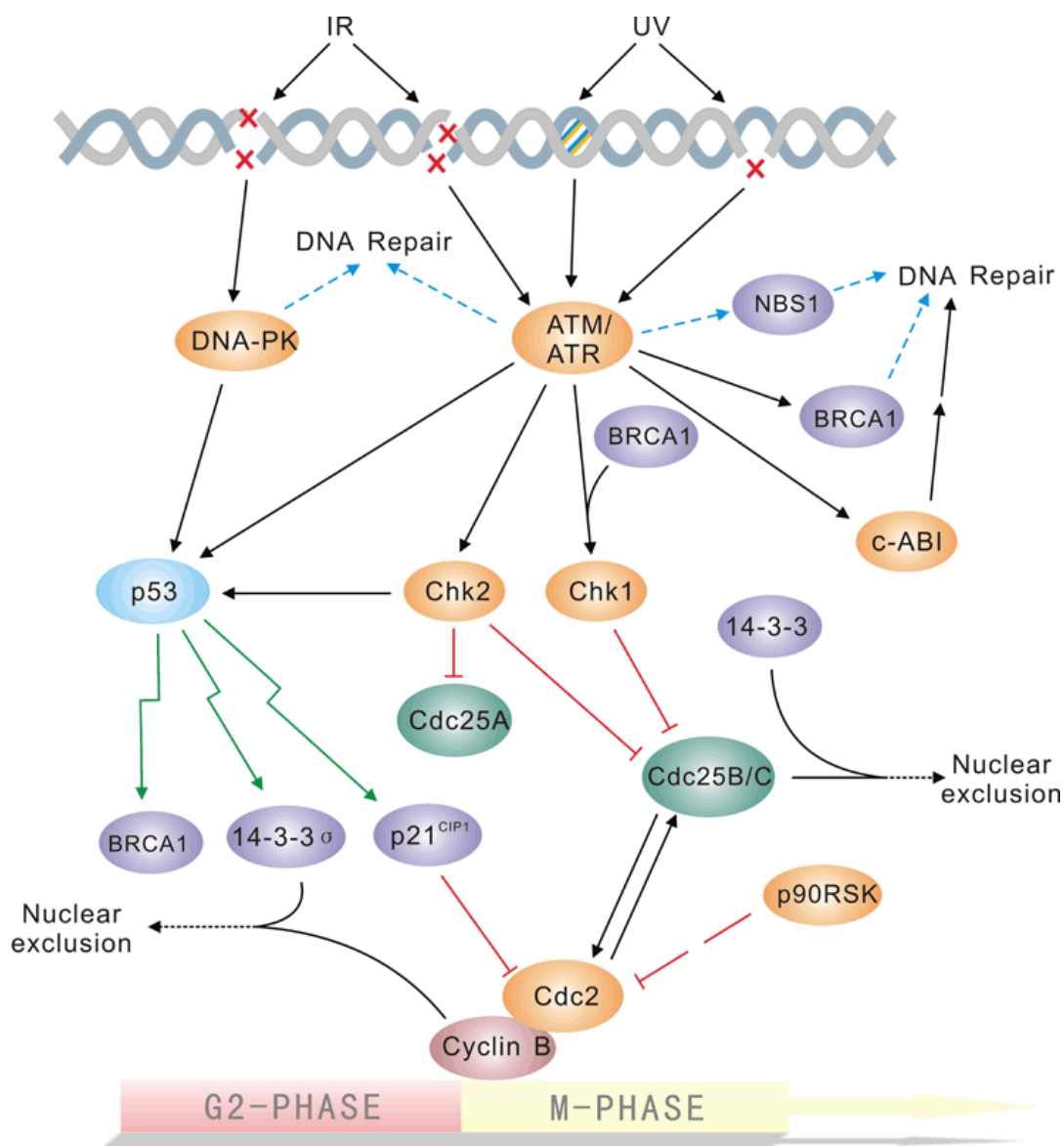Physiologically, DNA damage has a great influence on the progression of the cell through the cell cycle. If damage is induced by exposure to certain chemicals,

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[2] Source: Signalway Antibody - http://www.sabbiotech.com/a-110-DNA-Damage-Repair-Pathway.html (accessed Jan 26, 2015)

ionizing radiation (IR) or ultraviolet (UV) rays, it is detected by the cell cycle control system and the cell cycle is arrested at one of the two DNA damage check points. Upon detection of DNA damage, the DNA damage repair pathway (see Figure 12) is initiated. As first event, ATM and ATR kinases get activated, which in turn phosphorylate and thereby activate two other protein kinases, namely CHK1 and CHK2. CHK2 then activates the downstream tumor suppressor protein p53 and initiates degradation of the phosphatase CDC25A, which acts as a CDK activator. Up-regulation of p53 stimulates the transcription of p21, a CDK inhibitor protein, which supports cell cycle arrest. Depending on the severity of damage to the DNA, it can either be repaired, or the cell undergoes apoptosis.

If the DNA damage response is not functioning properly, DNA damage remains unrepaired and mutations can accumulate in the cell, which in the long run can lead to the development of cancer. (Alberts et al., 2008)

In order to get an insight on the effects of bufalin on the DNA damage response of melanoma cells, we tested for the RNA expression of CHK2 in C8161.9 cells upon treating them with bufalin for 24 hours. We found that bufalin could increase the RNA expression of CHK2 by 1.43 fold (Figure 13). Hence we assume that bufalin can positively influence the DNA damage response in melanoma.
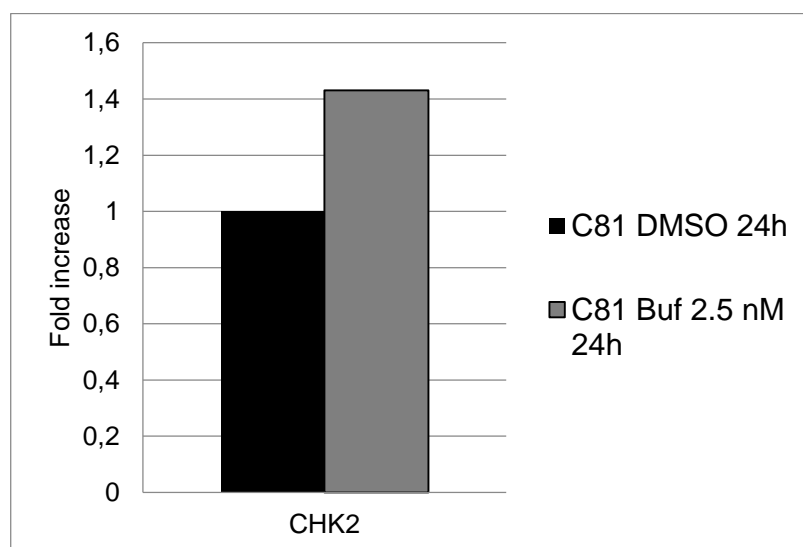
Figure 13 Effects of 24h bufalin treatment (2.5 nM) versus DMSO on RNA expression of CHK2 in C8161.9 melanoma cells. Expression of CHK2 increased by 1.43 fold Bufalin influences UVC sensitivity of melanoma cells

3.5   Bufalin influences UVC sensitivity of melanoma cells

As previously stated, the development of melanoma is linked to the exaggerated exposure of the skin to UV light, which in turn leads to DNA damage. Bufalin treatment up-regulated the RNA levels of CHK2, which is relevant in the DNA damage repair pathway. Consequently, we wanted to find out if bufalin treatment 6 hours prior to UV exposure would influence the sensitivity of melanoma cells to UV light.

Short-wavelength ultraviolet light (UVC) was used in these experiments. UVC light is very weak at the earth's surface, since it is mostly absorbed by the ozone layer of the atmosphere. However, it is a very aggressive form of UV light. If the skin is exposed to it, it leads to sunburn very quickly. Using UVC light was therefore a suitable option to test whether bufalin can generally alter the reaction of melanoma cells to ultraviolet light.

The graph in Figure 14 shows a dose-dependent decrease in cell viability of the C8161.9 cells treated with only DMSO, after exposure to increasing doses of UVC light. Cell viability was decreased to 50% by a UVC dose of approximately 40 J/m^2
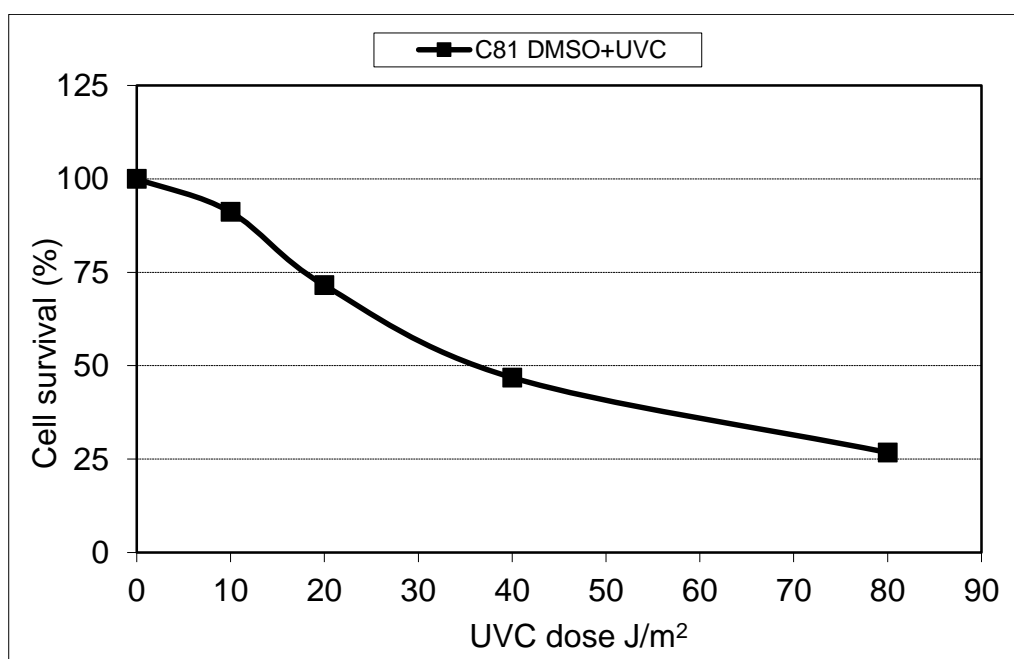
Figure 14 Effects of increasing doses of UVC light on the viability of C8161.9 cells treated with DMSO, 24 hours after exposure. Cell viability decreases with increasing doses of UVC. Cell viability decreased to 50% after exposure to roughly 40 J/m^2 UVC

Bufalin treatment 6 hours prior to UVC exposure significantly added to the negative effects of UVC exposure on the viability of C8161.9 cells.

As depicted in Figure 15, the bufalin pretreated C8161.9 cells contained a much lower percentage of surviving cells after low UVC doses (10 J/m^2 and 20 J/m^2) in comparison to the control cells, which were treated with DMSO only. A 50% decrease of viable cells was reached with 20J/m^2 UVC among the cells which had been pretreated with bufalin.
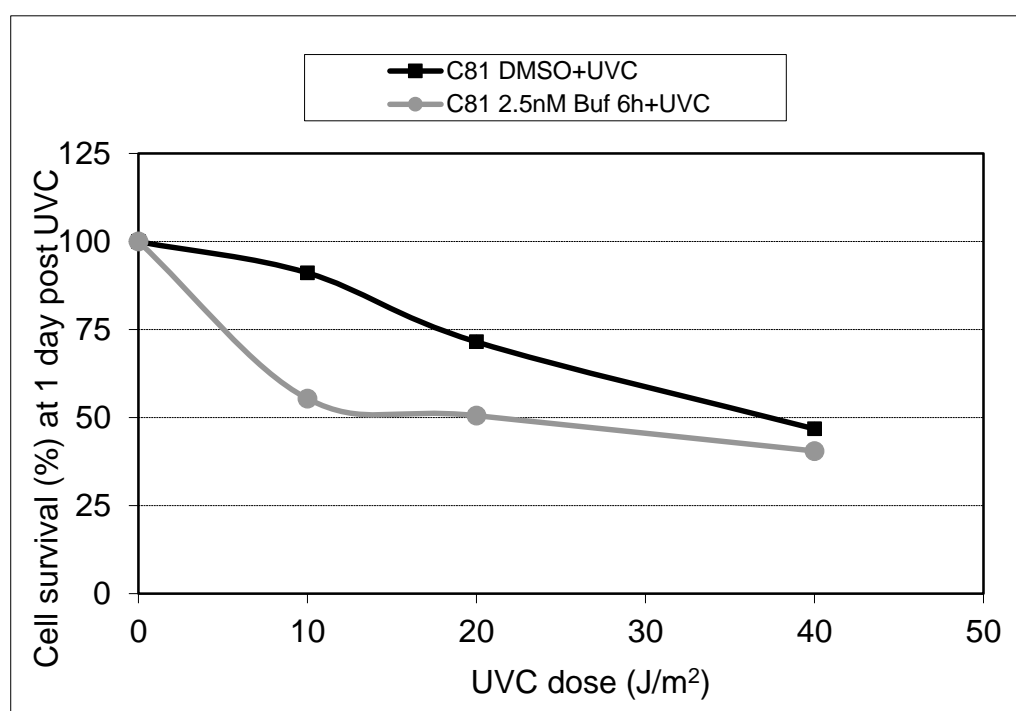
Figure 15 C8161.9 cells were seeded in a 96-well plate, treated with 2.5 nM bufalin versus DMSO for 6h and afterwards exposed to respectively increasing doses of UVC light. Medium was replaced without bufalin. Cell viability was tested 24h after UVC exposure.

Comparable results, although not as distinct as for the C8161.9 cells, could be achieved with the D04 and Mamel12 cell lines 6 hours post bufalin treatment. After a UVC dose that was as low as 20 J/m^2, the viability of both bufalin pretreated D04 (Figure 16) and Mamel12 cells (Figure 17) was decreased, whereas the cells of the control, treated with DMSO only, showed a slight increase in the number of viable cells in comparison to the number of viable cells in the DMSO treated cells that were not exposed to UVC light.

Figure 16 DO4 cells were treated with 2.5 nM bufalin versus DMSO for 6h, followed by exposure to 20 J/m^2 of UVC versus no UVC exposure as control. Medium was replaced without bufalin after UVC treatment. Cell viability was tested 24h after UVC exposure
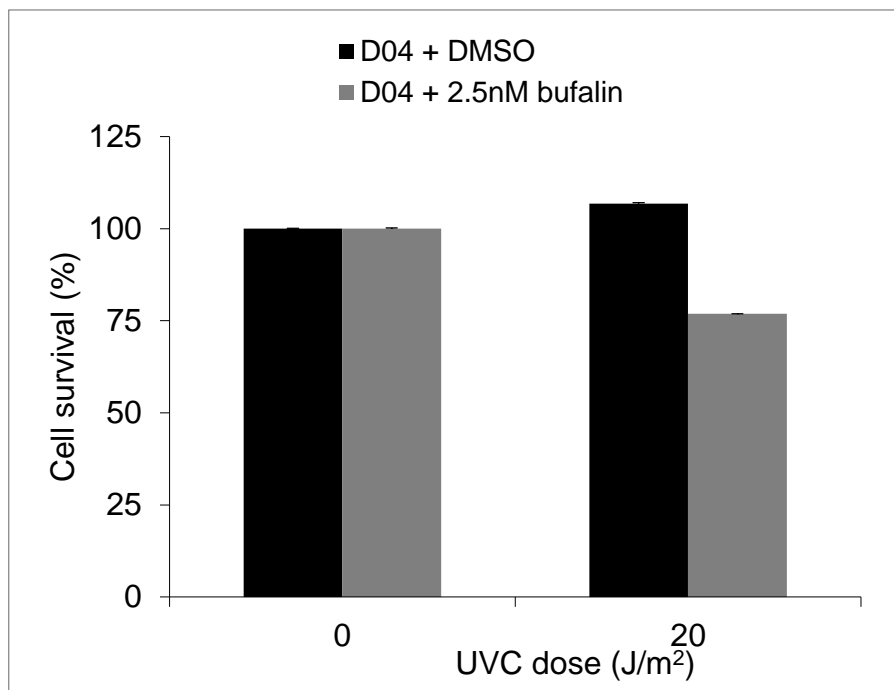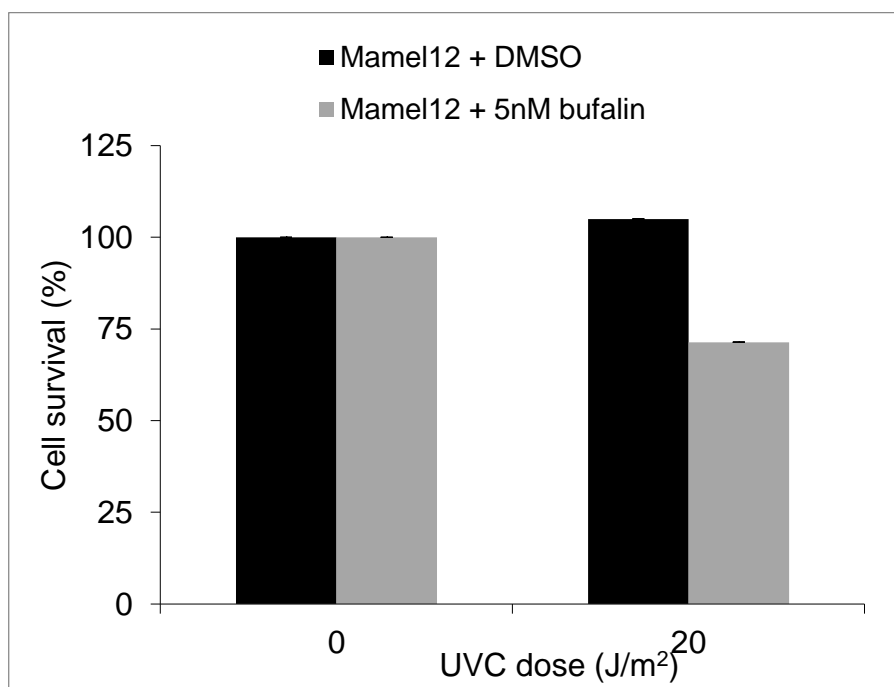

Figure 17 Mamel12 cells were treated with 5nM bufalin versus DMSO for 6h, followed by exposure to 20 J/m^2 of UVC versus no UVC exposure as control. Medium was replaced without bufalin after UVC treatment. Cell viability was tested 24h after UVC exposure

Following the results obtained from the cell viability assays, we investigated whether the treatment of melanoma with bufalin before exposure to low doses of UVC light would also influence the effect of UVC on the ability of single melanoma cells to form colonies.

Therefore the cells were seeded in low numbers, bufalin was added for 6 hours and afterwards the cells were treated with $20J/m^2$ or $40J/m^2$ UVC. Formation of colonies was examined 6 to 10 days later.
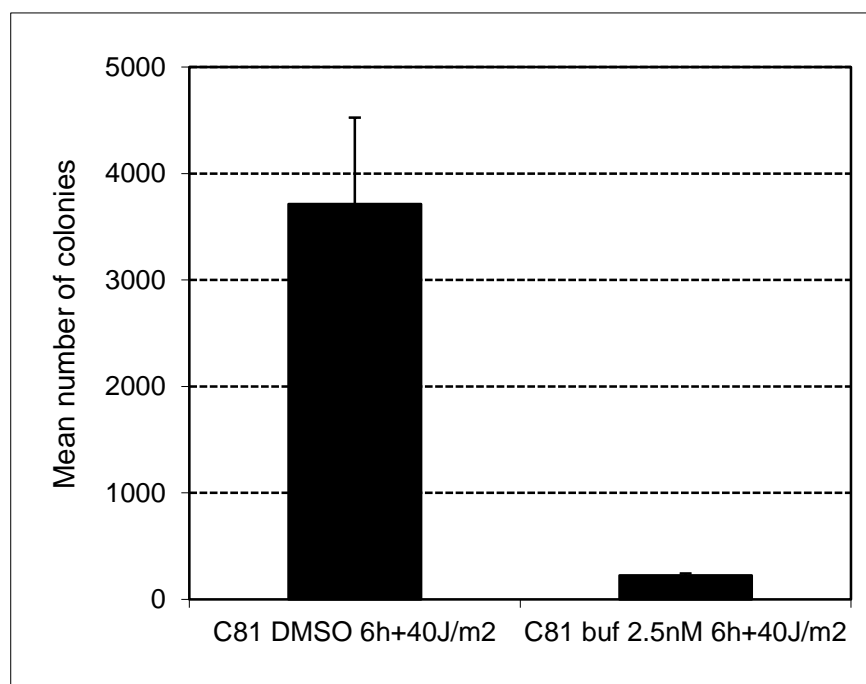
Figure 18 C8161.9 cells were seeded in 6-well plates (5000 cells/well), starved o/n at 0.1% FBS and then treated with bufalin 2.5nM versus DMSO as control for 6h, followed by exposure to 40 J/m² UVC light. Medium was replaced without bufalin after UVC treatment. Colonies were examined after 6 days of growth.

In Figure 18, the effect of bufalin administration previous to UVC exposure on the colony formation ability of C8161.9 cells is shown. The left column shows the mean number of colonies formed in the control wells. These cells were treated with only DMSO before exposure to UVC. The right column shows the mean number of colonies formed by C8161.9 cells pretreated with bufalin before UVC. Seeing as the number of colonies in the bufalin treated well is much lower, bufalin remarkably enhanced the effect of UVC on the colony formation ability of C8161.9 cells.

Comparable results could be obtained for the D04 (Figure 19) and Mamel12 (Figure 20) cell lines. Bufalin prior to UVC treatment strongly inhibited colony formation in both cell lines.

Taking all results from cell viability and colony formation assays into account, bufalin treatment prior to UVC exposure significantly decreased cell proliferation in C8161.9, D04 and Mamel12 melanoma cell lines, compared to UVC alone. Consequently, it can be assumed that bufalin increases the sensibility of melanoma cells to UVC light.

Figure 19 D04 cells were seeded in 6-well plates (5000 cells/well), starved o/n and then treated with bufalin 2.5nM versus DMSO as control for 6h, followed by exposure to 40 J/m^2 UVC light. Medium was replaced without bufalin after UVC treatment. Colonies were examined after 10 days of growth.
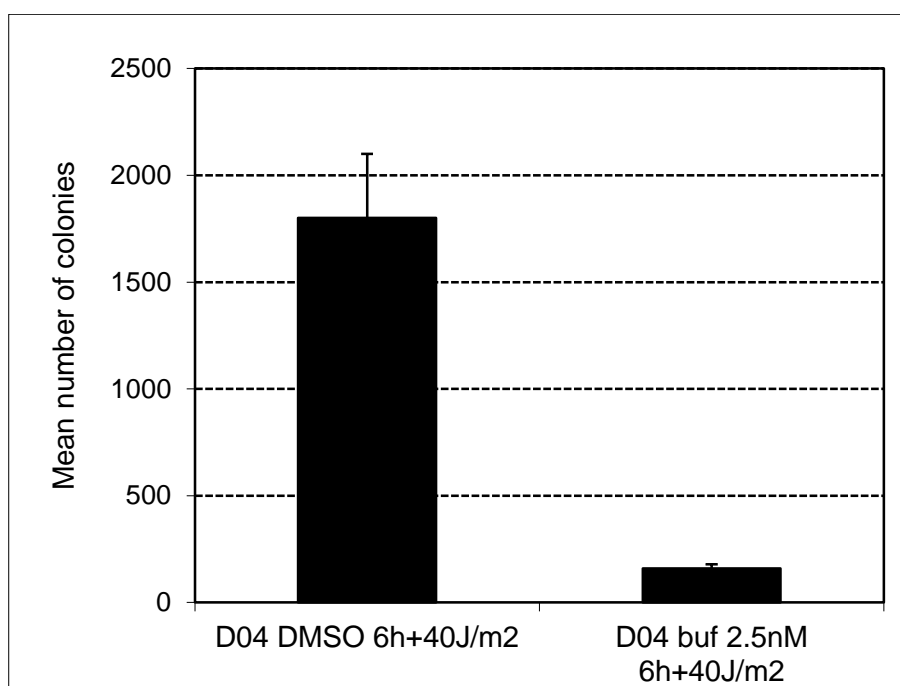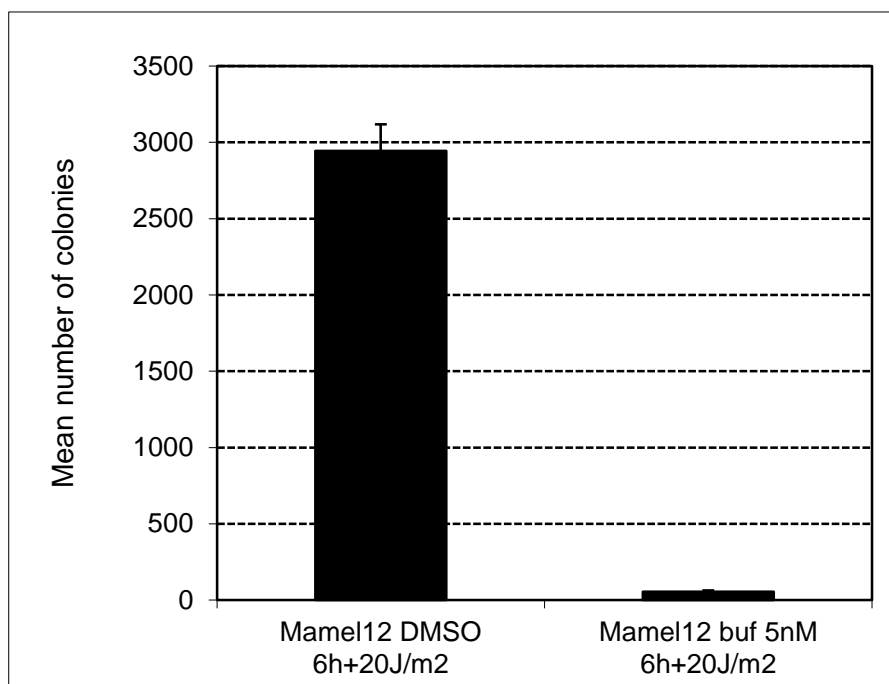
Figure 20 Mamel12 cells were seeded in 6-well plates (5000 cells/well), starved o/n at 1% FBS and then treated with bufalin 5nM versus DMSO as control for 6h, followed by exposure to 20 J/m² UVC light. Medium was replaced without bufalin after UVC treatment. Colonies were examined after 10 days of growth.

4 Discussion

Human malignant melanoma is a highly aggressive, therapy-resistant form of skin cancer. Of all types of dermatologic malignancies, melanoma is responsible for the largest number of patient deaths, its incidence increasing steadily across the globe over the past decades (Hsiao et al., 2012; Markovic et al., 2007). Hence, melanoma presents a significant and continuing public health problem. (Rigel & Carucci, 2000).

The development of melanoma is dependent on the interactions between genetic risk factors, such as fair skin, the presence of large nevi, and a family history of melanoma, and environmental risk factors. The most significant environmental risk factor is the exposure of the skin to UV radiation (Bandarchi et al., 2010; Markovic et al., 2007). Many genes are involved in the development, proliferation and progression of melanoma. The nuclear receptor co-activator 3 (NCOA3) has been identified as a potent prognostic marker, since it is often overexpressed in melanoma and especially its metastasis, leading to the assumption that high levels of NCOA3 might promote tumor growth (Rangel et al., 2006).

Malignant melanoma metastasize at a high rate, which makes early detection and treatment crucial for patient survival. However, this cancer is notoriously difficult to treat. Neither surgical resection, nor chemo- or radiotherapy are particularly effective, and response rates greater than 25% are rarely achieved. (Hocker, Singh, & Tsao, 2008). The development of new therapeutics is therefore critical to improve patient survival.

Due to low efficacy and significant side effects of conventional cancer treatments, the use of alternative and complementary medicine, such as Traditional Chinese Medicine, has become more and more popular worldwide over the past years (F. Qi et al., 2010). The TCM bufalin has been the research focus of many groups. Bufalin is the major immuno-reactive component of the venom extracted from the skin and parotid venom glands of the Asiatic toad. The substance has been found to inhibit tumor growth in a number of cancers (Takai et al., 2012). Its effects on the proliferation of melanoma cells, however, are only scarcely known.

Our results showed that bufalin significantly inhibits viability and proliferation of the melanoma cell lines C8161.9, D04 and Mamel12.

According to a recent study, bufalin can inhibit the protein expression levels of NCOA3 in breast cancer, and thereby decrease viability and growth of the cells (Ying Wang et al., 2014). Since NCOA3 is frequently overexpressed in melanoma, we investigated whether the bufalin had a similar effect on the NCOA3 expression of the cells. Western blot analysis and immunofluorescence microscopy showed that after treatment with a low dose of bufalin (2.5 nM or 5 nM), the protein levels of NCOA3 were significantly decreased in the three tested melanoma cell lines.

Furthermore, we found that bufalin treatment lowered the RNA levels of Cyclins A1, B1 and B2 and simultaneously up-regulated the RNA expression of CHK2 in C8161.9 cells. Further analysis, for example by Propidium-Iodide staining and FACS analysis, would be required to assess whether bufalin arrests the cell cycle in melanoma cells. However, an effect of the drug on proliferation and cell cycle progression of the cells is indicated by the decreased cyclin levels. The increase of CHK2 expression, which plays an important role in DNA damage repair as it up-regulates the downstream p53 and inhibits the CDK1 activator CDC25A, is further associated with the inhibition of cell proliferation.

Since the down-regulation of NCOA3 lead to down-regulation of Cyclins A1, B1 and B2 and at the same time to the up-regulation of CHK2, we consequently assume that an elevated NCOA3 expression promotes melanoma progression by inhibiting the DNA damage response.

The main environmental cause for the development of melanoma is accumulated and unrepaired DNA damage in the melanocytes of the epidermis due to excessive UV exposure (Bandarchi et al., 2010). Based on the findings explained above, we conducted experiments to find out if the down-regulation of NCOA3 and simultaneous activation of the DNA damage repair pathway would have an effect on the sensitivity of the cells to ultraviolet light. UVA is ultraviolet radiation between 400-320nm wavelength, UVB is between 320-290nm wavelength and UVC is between 290-100nm. The shorter the wavelength, the higher the energy of radiation so as a proof of principle we used UVC as being the strongest UV.

Cell viability and colony formation assays both showed that bufalin treatment prior to UVC exposure of C8161,9, D04 and Mamel12 melanoma cells markedly increased the damaging effects of ultraviolet light on the viability of the cells, showing that bufalin and consequently the down-regulation of NCOA3 increases the sensitivity of melanoma cells to UVC light.

However, UVC radiation is very low at the earth's surface, since it is almost completely absorbed by the ozone layer of the atmosphere, and is therefore not the type of UV radiation that causes melanoma in patients. UVA rays account for 90 to 95% of UV radiation that reaches the earth. While UVB makes up only 5-10% of solar radiation, its high energy damages surface skin epidermal layers and causes sunburn. UVA penetrates deeper layers of skin and causes tanning. Being UVA and/or UVB more clinically relevant, further experiments using these two UV lights are therefore needed to conclude whether similar results can be obtained.

5 Conclusion

Taken together, our results showed that bufalin reduced the proliferation of C8161.9, D04 and Mamel12 melanoma cells by down-regulating NCOA3 protein expression as well as specific cell cycle cyclins and by activating the DNA damage response pathway.

Although more experiments need to be carried out in order to get a better understanding of the mechanisms of cell toxicity mediated by bufalin in melanoma cells, the present findings suggest that bufalin might be a potential therapeutic agent for the effective treatment of human malignant melanoma in the future.

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