“Toll like receptor agonists: assessment of their potential in melanoma therapy“

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CONTENT

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ABBREVIATIONS

[γ-^{32}P] dATP	phospholabelled deoxyadinine triphosphate
µg	Microgram
µl	Microliter
µm	Micromolar
A	Adenine
Aa	amino acid(s)
ATCC	“American type cell culture”
ATP	adenosine triphosphate
APS	amonium peroxiddisulfate
Bp	base pair
BSA	bovine serum albumin
C	Cytosin
°C	degree Celsius
cDNA	copy DNA
Conc.	Concentration
C-term	carboxy terminal
Cpm	counts per minute
Da	Dalton
dATP	Desoxyadenosintriphosphat
DMEM	Dulbeccos modifiziertes Eagles Medium
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
dNTP	Deoxy ribonucleotide triphosphate
DPBS	Dulbecco’s Phosphate buffered Saline
DTT	1,4-dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signaling-regulated kinase
FBS	fetal bovine serum
Fig	Figure
FITC	fluorescein-isothiocyanate

H&E	hematoxylin and eosin
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
H	hour
IgG	immunoglobulin G
kD	kilo Dalton
kbp	kilo base pair
L	litre
LPS	lipopolysaccharide
M	molar
m^2	square meter
MAPK	mitogen-activated kinase
mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
MW	molecular weight
mRNA	messenger RNA
NaCl	sodium chloride
NaF	sodium fluoride
ng	nanogramm
NHM	normal human melanocytes
nm	nanometer
nt	nucleotide
N-terminal	amino terminal
OD	optical density
PAA	polyacryle amide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
RGP	radial growth phase
RNA	ribonucleic acid
cRNA	complementary RNA
siRNA	small interference RNA

Rnase A	ribonuclease A
Rpm	rotation per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
TBE	tris borate EDTA buffer
TBS	tris buffered saline
UV	ultraviolet light
VGP	vertical growth phase

1. Introduction

Melanoma is the most aggressive form of skin cancer that originates from melanocytes, specialized pigment-producing cells found in the basal layer of the epidermis and in the eye (Hurst et al., 2003). However, the exposure to ultraviolet light is considered one of the most environmental risk factor that is greatly increased among people with fair skin (Bauer and Garbe, 2003; Bliss et al., 1995). Patients with advanced disease, such as lymph node involvement and distant metastases, have 5-year survival rates of 50% and 10–20%, respectively (Elder et al., 2005). This poor prognosis largely results from notoriously high resistance to conventional chemotherapy, namely cytotoxic drugs. The basis for drug resistance in melanoma is most likely dysregulation of apoptosis. Thus, defects at multiple levels and in both major apoptotic pathways have been described in melanoma (Soengas and Lowe, 2003; Grossman and Altieri, 2001).

1.1 Architectural organization of human skin

1.1.1 Normal skin

The skin is the largest organ of our body accounting for about 15% of the body weight with a surface area of 1.5 - 2.0 m^2. It is one of the 7 channels of elimination containing about 70% water, 25% protein and 2% lipids and helps the main organs of elimination (liver, kidneys, intestines) get rid of waste buildup. In addition, it has diverse functions: as a mechanical barrier to infections, as waterproof barrier (sebum producing), thermoregulation, excretion, absorption, as well as a protective role in screening out potentially harmful ultraviolet (UV) rays from the sun by manufacturing melanin pigments, besides its role in the production of vitamin D.

The uppermost layer of the skin is known as epidermis and contains melanocytes, which produce the pigment melanin, one of the main contributors to skin color. The main Function of melanocytes is the synthesis of the pigment melanin, which is an important protective factor against the damaging effects of B-range ultraviolet light (Rager et al., 2005). Normal human melanocytes (NHM) is located in the basal/suprabasal layer of the epidermis and has a strong propensity to metastasize. In addition, it contains Langerhans' cells, which are part of the skin's immune system.

The dermis is the deeper layer that contains collagen and elastin, in addition to sebaceous glands, sweat glands, lymph vessels, hair follicles and nerves. The dermis is divided into two layers: the papillary dermis, including a periadnexal

component, and the reticular dermis. Below the dermis lies a layer of fat (Fat layer) that helps insulate the body from heat and cold, provides protective padding, and serves as an energy storage area.

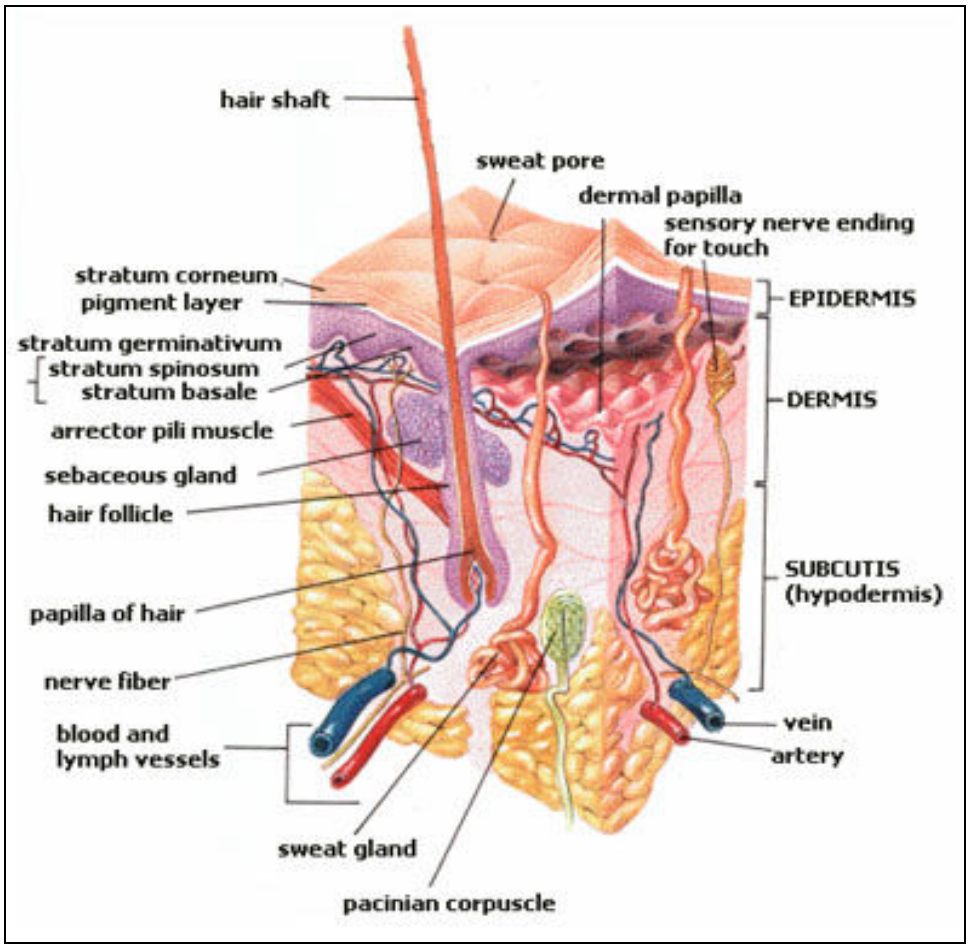


Fig.1: Architectural organization of human skin (courtesy: www.merck.com)

1.1.2 Melanoma

Melanoma predominantly affects adults, with a peak incidence in the fourth decade, and has no sex prevalence. A patient's risk of developing a second primary melanoma after diagnosis of the first one is 3-5% (Rager et al., 2005). Early diagnosis and identification of penetrating melanoma cells below the basal membrane zone is of utmost importance to prevent the dissemination of these cells in other organs and tissue. However, the neoplastic transformation of melanocytes gives rise to malignant melanoma.

Melanomas are histologically classified according to their location and stage of progression. Five distinct stages have been proposed in the evolution of melanoma

on the basis of such histological criteria: common acquired and congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; radial-growth phase (RGP) melanoma; vertical-growth phase (VGP) melanoma; and metastatic melanoma (Weyers et al., 2000).

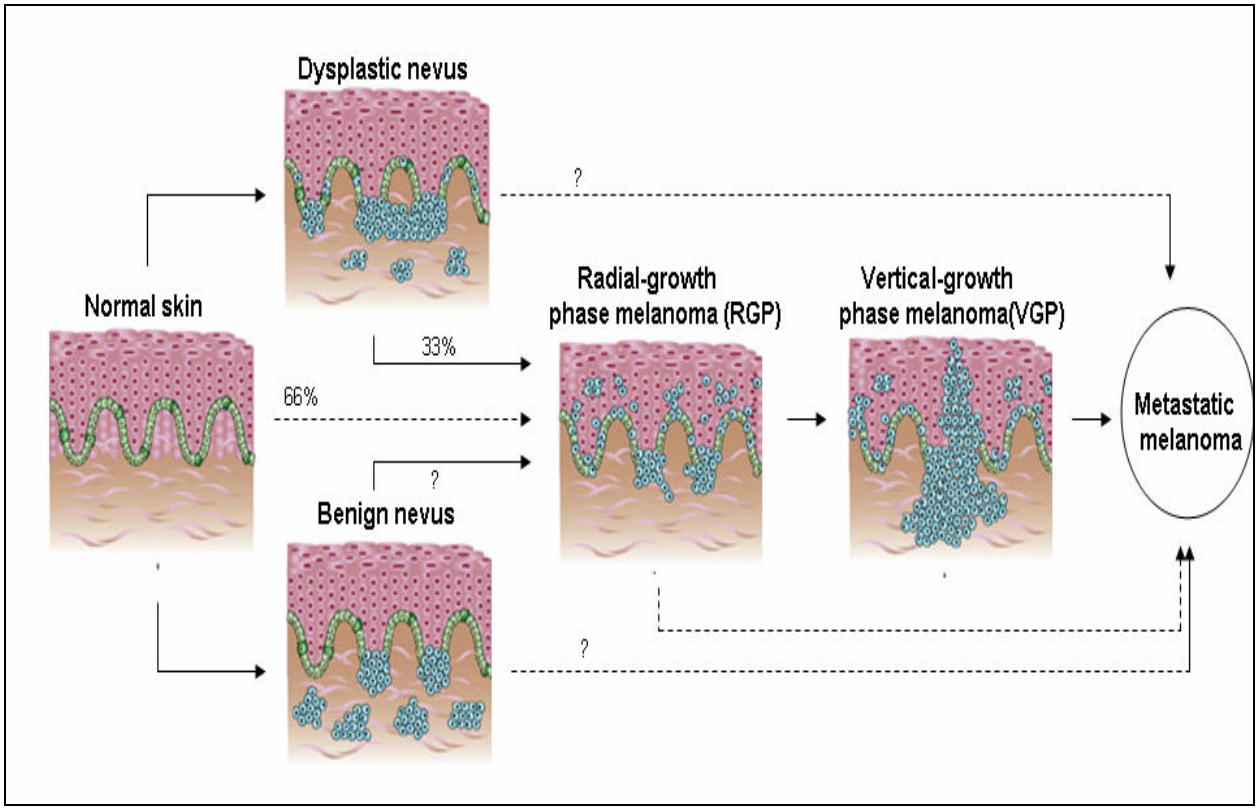


Fig 2: Distinct stages in the evolution of melanoma (modified after Chin L et al., 2003)

The common acquired nevi, or moles, are composed of nevus cells (melanocytes) grouped in collections within the first and/or second layers of the skin. They are characterized by a wide range of presentation and can occur on any surface of the skin or mucous membranes, including the nail beds. They usually first appear between the ages of 6 to 12 months. There is a higher prevalence of nevi in people with light skin compared to those with dark skin. They become more numerous in childhood and the teenage years, and tend to regress later in life (Grichnik., 2008).

The congenital nevi are seen on over 1 percent of newborns. They can be small, or they can cover most of the skin surface. Large nevi are at highest risk for malignant changes (sowa et al., 2008)

The dysplastic nevi is one of the relatively common moles that seens to be broader and larger than an ordinary mole and tends to be irregular in color and shape. People

with dysplastic moles have a greater than average chance of developing melanoma.(Rabkin., 2008).

The radial-growth phase melanoma is an invasive melanoma, but have a cure rate that approaches 100% with surgery alone. The superficial spreading type is the most common form in white populations. This type is associated with excessive, intermittent sun exposure (especially in childhood and youth). RGP melanoma can be treated efficiently by surgical dissection, with low risk of relapse or metastasis. However, if left untreated, the melanoma can progress to the vertical growth phase (Abramova et al ., 2002).

Vertical-growth phase melanoma are the late pattern of growth of cutaneous malignant melanoma in which tumor cells spread from the epidermis into the dermis . It is believed that the transition from radial to vertical-growth phases is the crucial step in the evolution of melanoma that presages the acquisition of metastatic potential and poor clinical outcome (Chin et al., 2003).

Metastatic melanoma are the melanoma that has spread to other parts of the body by way of the bloodstream or the lymphatic system (Pawelek and Chakraborty., 2008).

1.1.2.1 Melanoma resistance to conventional therapy

Melanoma cells have low levels of spontaneous apoptosis *in vivo* compared with other tumour cell types, and are relatively resistant to drug-induced apoptosis *in vitro* (Soengas and Lowe, 2003). Most chemotherapeutic drugs function by inducing apoptosis in malignant cells, so resistance to apoptosis is likely to underlie drug resistance in melanoma (Soengas and Lowe, 2003), and this extraordinary resistance to chemotherapy, radiotherapy and immunotherapy is a major barrier to successful treatment of melanoma.

There are several approved postoperative adjuvant therapies for malignant melanoma (Tarhini and Agarwala, 2006). Interferon (IFN) is the most commonly used adjuvant immunotherapy for advanced melanoma, although the effectiveness is still widely debated. High-dose interleukin-2 (IL-2) has also been approved, but response rates are low accompanied with high toxicity. Dacarbazine (DTIC) is the reference approved chemotherapeutic agent for the treatment of advanced melanoma, and drugs such as carmustine (BiCNU), paclitaxel (taxol), temozolomide and cisplatin have shown single-agent activity in metastatic disease (Balch and Cascinelli, 2001; Tarhini and Agarwala, 2006). In addition, many different immunotherapies have been

tested, but so far none of these approaches has reached regulatory approval (Kirkwood et al., 2006). However, recent trials of genetically engineered lymphocyte transfer have shown promise, with 2 (out of 17) patients achieving full remission for 18 months (Morgan et al., 2006). Unfortunately, these therapies all contribute little to overall patient survival, so the identification of the signalling pathways that are central to melanoma initiation and progression is opening an exciting new area for melanoma treatment. It provides the opportunity to develop targeted therapies.

1.2 Apoptosis

Apoptosis or programmed cell death (PCD) is a naturally occurring process defined as a mechanism of cellular suicide, which occurs after sufficient cellular damage. It is a normal component of the development and health of multicellular organisms. It is involved in tissue homeostasis and tumorigenesis and characterized morphologically by chromatin condensation and formation of apoptotic bodies and biochemically by DNA fragmentation into oligonucleosome-sized fragments (Wyllie et al., 1997). The dying cell separates from its neighbors and undergoes a period of membrane blebbing, condensation of cytoplasm and increase in cell density. Simultaneously the nuclear chromatin becomes compact, segregates and forms sharply delineated masses along the nuclear envelope. The nucleus splits into discrete fragments and finally, the cell splits into a cluster of membrane bound apoptotic bodies, each containing a variety of organelles (Catchpoole and Stewart., 1995). Apoptotic bodies are ingested by nearby cells and macrophages before they cause an inflammatory reaction.

Cells that undergo apoptosis break apart and are recycled by a type of white blood cell called a macrophage. Apoptosis protects the body by removing genetically damaged cells that could lead to cancer, and it plays an important role in the development of the embryo and the maintenance of adult tissues. Cancer results from a disruption of the normal regulation of the cell cycle. When the cycle proceeds without control, cells can divide without order and accumulate genetic defects that can lead to a cancerous tumor (Bataille., 2003). In recent years, Apoptosis has received a lot of press as a possible key to new therapies for a host of ailments-from cancer to Parkinson's disease (Kostrzewa., 2000). During the past 10 years, the interest of basic scientists and clinicians in the influence of programmed cell death

(apoptosis) on the sensitivity of tumours to anticancer treatment has risen and continues to rise dramatically.

Apoptosis takes place in a variety of biologically significant situations including embryogenesis, organogenesis and the maintenance of homeostasis as well as normal function of the immune system (Cummings et al., 1997). Abnormal regulation of apoptosis has been implicated in the onset and progression of diseases both in the form of inhibited and excessive apoptosis (Antonsson et al., 2001). Two major apoptosis pathways have been identified, namely the death receptor pathway (also called extrinsic pathway) and the mitochondrial (intrinsic) pathway. The death receptor pathway involves at least five transmembrane receptors belonging to the TNF (tumor necrosis factor)/NGF (nerve growth factor) -receptor superfamily (Timmer et al., 2002). Fas is a type I cell surface protein belonging to the TNF/NGF receptor family (Itoh et al., 1991). FasL is a type II membrane protein that belongs to the TNF family and is expressed predominantly in activated T lymphocytes and in tissues including small intestines, kidney, testis, and lung (Suda et al., 1993). The mitochondrial pathway is mediated by mitochondrial membrane permeabilization and the release of cytochrome c (Antonsson, 2001). In both of these pathways, the final result is the activation of the caspase cascades, which lead to proteolysis of structural and regulatory proteins and cell death, bcl-2 family proteins and p53 regulating apoptosis (Antonsson, 2001; Timmer et al., 2002).

1.2.1. Regulation of apoptosis

Apoptosis is markedly influenced both positively and negatively by a variety of genes, many of which are mutated, and/or dysfunctionally regulated, in human cancers (Kinoshita et al., 2006). Among the most important of these are the tumour suppressor gene p53 and members of the bcl-2 gene family (Radhakrishnan et al., 2006; Berardo et al., 1998). The fact that apoptosis is a genetically defined pathway has led to two principal expectations: (a) that the tumour genotype will be predictive of the outcome of current anticancer therapy; and (b) that new therapies based on apoptosis will be superior to present-day anticancer treatments. The requirement for wild-type p53 for apoptosis after genotoxic damage caused by anticancer agents including irradiation has been well demonstrated, particularly in oncogenically transformed rodent cells and in tissues of lymphoid origin (Sanchez-Prieto et al., 1995). However, the influence of p53 and other genes on apoptosis in malignant tissues of nonhematological origin is clear. There have also been reports indicating

that apoptosis does not correlate with the total cell death measured by other means following anticancer therapies (Chada et al., 2006).

Many genes, which affect the extent to which certain cell types undergo apoptosis have been identified during normal development and after pathological stress (Mooi et al., 2006; Zimmermann et al., 2001). Together with the assumption that apoptosis plays a major role in cell death by DNA-damaging agents, these genetic studies have led to the present hypothesis that tumours with mutations in p53, high levels of bcl-2, or high ratios of bcl-2 should be resistant to cancer treatment as p53 is the transcription factor for the bax gene that can be blocked by bcl-2 (Hockenbery et al.,1993; Korsmeyer et al., 1993). Because there is a lot of data from clinical studies in which the clinical outcome has been correlated with the status of these and other genes affecting apoptosis, this hypothesis would seem an easy one to test. However, a major problem with such analysis is that it is often impossible to separate treatment sensitivity from patient prognosis. For example, tumours with mutated p53 can be more anaplastic, can have a higher proportion of proliferating cells, can be more metastatic, and in general can have a more aggressive phenotype than tumours with wild-type p53 (Ridgeway et al., 2006). This can lead to a bad prognosis for patients whose tumours have mutated p53 independent of treatment sensitivity (Murakami et al., 2000).

The IAPs are a family of anti-apoptotic proteins that are conserved across several species. They were first discovered in baculoviruses, a group of viruses specific to insects, and their human counterparts include the neuronal apoptosis inhibitory protein (NAIP), cIAP1, cIAP2, XIAP, livin, and survivin. All IAPs are characterized by the presence of at least one tandem baculovirus IAP repeat (BIR) motif. cIAP1, cIAP2, XIAP, and survivin can bind to and inhibit the active forms of the terminal caspases 3 and 7. cIAP1, cIAP2, and XIAP also bind to the zymogen form of caspase 9, thereby preventing its proteolytic processing as well as the processing of downstream proteases, such as caspases 3, 6, and 7 (Kasof et al., 2001). Some members of the IAP family proteins are expressed in certain human cancers, and therefore the removal of their inhibitory effects seems to be potentially useful in sensitizing the cancer cells to anticancer drugs (Deveraux et al., 1999).

1.2.2 Apoptosis and tumour sensitivity to anticancer agents

Mutations in p53 or other genes may affect tumour aggressiveness and patient prognosis, and it is difficult to obtain an answer from clinical data to the question of the role of p53 or of apoptosis in treatment sensitivity. However, experimental models cannot only be free of such biases, they can also use modern gene knockout, transgene, and other molecular techniques to answer the important question of: "Does the level of apoptosis and/or genes controlling apoptosis affect the sensitivity of cancer cells for killing by genotoxic agents?"

It has become widely accepted that cell death after DNA damage by anticancer agents is primarily the result of apoptosis, and that cells that can evade apoptosis will be resistant to cell killing. Often cited for this view, and in particular the role of mutated p53 in radiation and anticancer drug resistance, are pioneering studies with dominant oncogene-transformed normal fibroblasts from embryos of p53 wild-type (p53+/+) and p53 knockout mice (p53-/-) (Kiaris et al., 2005), as well as highly significant associations of mutated p53 with drug resistance (Weinstein et al., 1997).

However, despite the seemingly strong case that cells die from cancer treatment due to apoptosis largely controlled by wild-type p53, several investigators have reported results that contradict this hypothesis, particularly when they have measured both apoptosis and overall cell killing by colony-forming assays. For example, it has been reported that large changes in apoptosis do not lead to any changes in eventual cell killing (Zhang et al., 2006), or that the status of p53 does not affect sensitivity to DNA-damaging agents (Christophorou et al., 2006). There are also several examples in the literature where apoptosis clearly contributes to the overall sensitivity of cells to treatment with radiation or chemotherapeutic agents as assessed either by *in vivo* treatments (Inoue et al., 2002) or *in vitro* (Selimovic et al., 2008, Pettit et al., 1999; Wu et al., 1998). However, there is a major difference between these studies and the ones that have failed to find a link between apoptosis and overall sensitivity to cytotoxic agents. It is, therefore, likely that the majority of tumours have evolved past the point where they may have been apoptotically hypersensitive to genotoxic and nongenotoxic stress. In human tumours, this phenotypic evolution can occur with or without alteration of genes such as p53 or those of the bcl-2 family, but is associated with a loss of the rapid induction of apoptosis after genotoxic damage (Urist et al., 2004). This selection does not necessarily eliminate the ability of the cell to carry out apoptosis altogether; in some cases, the majority of tumour cells may still die by this

process, although usually in a more delayed manner occurring several days after treatment and usually after cell division (Ricci et al., 2006; Chin et al., 2006; Gizatullin et al., 2006). Selection against the apoptotically sensitive phenotype is the explanation for widely reported findings demonstrating that apoptosis in human tumour cells has minimal impact on the overall cellular sensitivity to present anticancer therapy (Wijnholds et al., 1997.). Several of these studies showed that even in cases where the level of apoptosis can be modified in human tumour cell lines by p53 (Brown et al., 1999), p21 (Komarova et al., 2000), or bcl-2 (Brown et al., 1999), the overall sensitivity to various genotoxic agents was unrelated to the level of apoptosis. This finding indicates that in these cases, apoptosis after genotoxic treatment occurs only in cells that have already lost their capacity to continue growth, likely as a result of unrepaired or misrepaired DNA lesions.

1.2.3 Mitogen-activated protein (MAP) kinase signalling pathways and their role in the regulation of apoptosis

Mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction cascades that play a regulatory role in cell growth, differentiation and apoptosis (Hassan et al., 2009b; 2008, selimovic et al., 2008, Romashko et al., 2003; Mansouri et al., 2003; Deng et al., 2003). Although the MAP kinase signalling pathways JNK, p38 and ERK share structural similarities, the outcome of their activation is quite different. The activation of JNK and p38 is mediated mainly by apoptosis signalling regulated kinase 1(ASK1) (Sarker et al., 2003.), whereas the activation of ERK is mediated mainly by ras/raf pathway (Hsu et al. 2007). MAP kinases are activated in response to their phosphorylation within a TXY motif, by dual specificity MAP kinase kinases (MAPKKs). The MAPKKs form a highly conserved group that is activated through phosphorylation of conserved serine and threonine residues by a somewhat more diverse group of MAP kinase kinase kinases (MAPKKKs). A number of MAPKKKs have been identified as upstream activators of the JNK and p38 pathways, including ASK1 (Yamagishi et al., 2003) that is mainly implicated in the regulation of both JNK and p38 pathways. Activation of the JNK and p38 signalling pathways lead to phosphorylation of a number of targets including the transcription factors ATF-2 and AP-1 (Ahmed et al., 2003, Hassan et al., 2009b; 2008) resulting in an increase in their transcriptional activity. Ras regulates

multiple signaling pathways, of which the best understood is the Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway. Ras and raf are protooncogenes, and expression of these genes activates signaling pathways, which in turn control cellular growth. Therefore, the Ras/Raf signaling pathway has been recognized as an important process in cancer biology (Sridhar et al., 2005; Dhillon and Kolch, 2002). Raf was the first identified and is the best-characterized downstream effector kinase of Ras. Despite several findings and new insights on this signaling pathway, the role of Raf in cancer cells remains controversial, yet interesting. In this pathway, extracellular signals mediated by growth factors transmitted through cellular receptors lead to activation of Ras by the confirmation change due to the conversion of GDP to GTP. Ras signalling often leads to activation of Raf, a cytosolic serine/threonine kinase. Raf is the general mediator of Ras and MEK1/2 is the general downstream effector substrate for Raf. Activated Raf then phosphorylates two serine residues of MEK1/2 in the activation loop. Interestingly, MEK acts as a dual specific kinase which can phosphorylate both threonine and tyrosine residues (Dhanasekaran and Premkumar Reddy, 1998). Then activated MEK phosphorylates downstream ERK1/2. The presence of two ERKs (ERK1 and 2) and MEKs (MEK1 and 2) is interesting, and perhaps surprising, since their functions are similar. However, ERK acts on several substrates ranging from cytoskeletal proteins, phosphatases, kinases to transcription factors (Lewis et al., 1998). These phosphorylation events lead to activation of transcription factors that play a critical role in tumorigenesis (Hassan et al., 2009a; 2005a, Kolch, 2000). Activated ERK 1 and 2 induce cell growth, differentiation and angiogenesis (Hassan et al., 2009a). These kinases could be a suitable target, not only to prevent resistance towards chemotherapy, but also to inhibit angiogenetic effects that are important for the growth of tumour cells *in vivo* (Sobke et al, 2006). Therefore, the examination of the role of the three MAP kinase signalling pathways JNK, p38 and ERK in the regulation of anticancer agents mediated cell growth inhibition and/or cell death in melanoma cell lines is considered.

1.3 Toll like receptors

Toll-like receptors (TLRs) are a class of single membrane-spanning non-catalytic receptors that recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated

molecular patterns (PAMPs). To date, 11 human TLRs and 13 mouse TLRs have been identified, and each TLR appears to recognize distinct PAMPs derived from various microorganisms, including bacteria, viruses, protozoa and fungi (Akira et al., 2006). TLRs are a conserved set of receptors that trigger innate immune activation in the presence of invading microbes (Büchau et al., 2007). Activation of the TLRs leads not only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity. Although there are a limited number of studies on the association between TLR expression and human malignancy, several recent reports on the expression of TLRs and cancers have been published. The expression or up-regulation of TLRs has been detected in many tumor cell lines or tumors, especially epithelial derived cancers (YU and Chen, 2008). TLR4 and TLR5 were detected to express in gastric epithelium with intestinal metaplasia and dysplasia, and strongly express in tumor cells of gastric carcinoma patients, but not in non-inflamed gastric mucosa (Schmausser et al., 2005). TLR2, TLR3, TLR6, and TLR9 were consistently expressed in hepatocellular carcinoma cell HepG2 (Nishimura et al., 2005). TLR9 protein is also expressed in human breast cancer cells and clinical breast cancer samples (Merrell et al., 2006). High expression of TLR9 was detected in clinical samples and cell lines of lung cancer as well (Droemann et al., 2005). Although the TLR profile varies on different tumor cells, the current evidences indicate that the expression of TLRs is functionally associated with tumor progression (YU and Chen, 2008). TLR expression may promote malignant transformation of epithelial cells. Engagement of TLRs increases tumor growth and tumor immune escape, and induces apoptosis resistance and chemoresistance in some tumor cells. These findings demonstrate that TLR is a promising target for the development of anticancer drugs and make TLR agonists or antagonists the potential agents for tumor therapy.

1.3.1 Toll like receptor agonists

The imidazoquinolineamine derivative 1-(2-methyl propyl)-1H-imidazole [4,5-c]quinoline-4-amine imiquimod is low-molecular-weight immune response modifiers that can induce the synthesis of interferon-alpha (IFN-α) and other cytokines in a variety of cell types. It belongs to a family of synthetic small nucleotide-like molecules with potent immuno-modulatory activity mediated through Toll-like receptor (TLR)-7 (and 8) signaling. When applied tropically, these compounds display immune-mediated anti-tumoral activity without damaging normal tissues (Stockfleth et al.,

2003; Ray et al., 2005). Imiquimod has been shown to induce immunologic activity by stimulating significant increases in numerous cytokines, including interferon-α, tumor necrosis factor (TNF) and interleukin-12 (IL-12) (Tyring et al., 2002). The innate immune response is stimulated *via* the induction, accompanied with synthesis and release of specific cytokines. The induction of interferon (IFN)-α, interleukin (IL)-6 and tumor necrosis factor (TNF)-α by imiquimod has been observed *in vitro* and in both human and animal studies (Reiter et al., 1994; Gibson et al., 1995)

Imiquimod-induced apoptosis in tumor cells could not to be antagonized by functional blockade of various membrane-bound death receptors, including Fas/APO-1 (CD95), TRAIL, and TNF receptors, which suggests that imiquimod-induced apoptosis is independent from apoptosis mediated by membrane-bound death receptors (Schön et al., 2003).

Imiquimod has demonstrated a high efficacy in the treatment of basal cell carcinomas or actinic keratoses (Reiter et al., 1994; Megyeri et al., 1995). At higher concentrations imiquimod exerts direct proapoptotic activity against various tumor cell population *in vitro* and *in vivo* (Schön et al., 2003). In addition, it stimulates binding of several induction-specific nuclear complexes: the NF-kappa B-specific complexes binding to the kappa B enhancer present in the promoters of all cytokine genes but not in IFNA genes (Pitha et al., 2007). Synthetic imidazoquinoline-like molecules, imiquimod (R-837) and resiquimod (R-848) have been shown to activate NF-κB through TLR7, whereas resiquimod also activates NF-κB through TLR8 (Hemmi et al., 2002). Both imiquimod and resiquimod have been identified as TLR7 agonists based on their inability to induce DC maturation or TNF- , IL-12, or IFN- production in TLR7-deficient mice. Additionally, imiquimod induces NF-κB activation in human embryonic Kidney cell line HEK293 transfected with human or mouse TLR7 (Gibson et al., 2002). The potential importance of these TLR agonists in the treatment of Melanoma cell lines is suggested by their ability to sensitize tumor cells to cytotoxic agents, and their future probably lies in combination with radiotherapies, chemotherapies, monoclonal antibodies and cancer vaccines.

1.4 The aim of the work

Melanoma is characterized by disrupted intracellular signaling which includes blocking of pro-apoptotic pathways and activation of survival signals. This reflects the

tumor's extraordinary resistance to current chemotherapy. Imiquimod is a tropical immune response modifier (imidazoquinoline) with both antiviral and antitumor properties. The aim of this study was to assess the killing efficiency of TLRs agonists (imiquimod and its chemical derivatives) in melanoma cells and to investigate the molecular mechanism(s), which contribute to the modulation of Toll like receptors agonists (imiquimod and its derivatives)-induced apoptosis of melanoma cells. Therefore, this work is focused in the following subjects:

- Assessment of the inhibitory or killing efficiency of Toll like receptor agonists in melanoma cell using cell viability assay and apoptosis specific assays.
- Determination of the molecular mechanisms, which are involved in the modulation of Toll like receptor agonists-induced apoptosis of melanoma cells using cell viability assay, flow cytometry analysis, Western blot analysis, *in vitro* kinase assay and electrophoretic mobility shift assay.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, enzymes, and antibodies

If not stated otherwise chemicals, enzymes and antibodies were of highest purity grade and purchased from Pharmacia Biotech (Uppsala, Sweden), Biometra (Göttingen, Germany), Peqlab (Erlangen, Germany), Amersham Buchler (Braunschweig, Germany), Perkin Elmer (New Jersey, USA), Sigma-Aldrich (Deisenhofen, Germany), Roche, Molecular Biochemicals (Mannheim, Germany), Upstate Biotechnology (Eching, Germany), GeneCraft (Münster, Germany), Roche (Heidelberg, Germany), Clontech (Palo Alto, California, USA), Qiagen (Hilden, Germany), Chemicon (Hofheim, Germany), Promega (Mannheim, Germany), Santa Cruz (Santa Cruz, USA), Calbiochem (Bad soden, Germany), Gibco BRL (Eggenstein, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Bio-Rad (München, Germany), Invitrogen (NV Leek, Neitherlands), and Biolabs (Schwalbach, Germany).

2.1.1.1 Chemicals

Adenosine Triphosphate (ATP): Sigma (St.Louis, USA)
Agar: Difco laboratories, Detroit, USA
Ampicillin: Gibco BRL (Eggenstein, Germany)
AMV Reverse Transcriptase: Boehringer Mannheim (Mannheim, Germany)
Biotherm-Taq DNA-polymerase: Genecraft (Münster, Germany)
Cell proliferation Kit I (XTT): Boehringer Mannheim (Mannheim, Germany)
Didesoxynucleotide (dNTP): Sigma (St.Louis, USA)
Glutathione-Sepharose: Pharmacia (Freiburg, Germany)
NaCL: Difco laboratories (Detroit, USA)
Poly T-oligonucleotide: Birsner/Grob (Denzlingen, Germany) Boehringer, Mannheim
Protease-inhibitor (Tablette): Boehringer Mannheim (Mannheim, Germany)
RNAse A: Roche (Mannheim, Germany)
RNAse-inhibitor: Roche (Mannheim, Germany)
Trypton: Difco laboratories (Detroit, USA)
HCL: Karl Roth GmbH (Karlsruhe, Germany)
U0126: Biomol (Lörrach, Germany)

SP600125: (Biomol, Germany)

SB203580: (Biomol, Germany)

-P^{32}.dATP. (Hartmann analytic, Germany)

2.1.1.2 Enzymes for RT-PCR and DNA digestion

RT-Enzymes (Genecraft,Germany)

Taq-polymerase (Genecraft,Germany)

dNTP nucleotides (Genecraft,Germany)

Primers (Genecraft,Germany)

2.1.1.3 Antibodies

I. Primary antibodies

Anti-PARP (Cell Signaling Technology, Inc., USA)

Anti-p38 (Santa Cruz Biotechnology, Inc., USA)

Anti-JNK (Santa Cruz Biotechnology, Inc., USA)

Anti-ERK (Santa Cruz Biotechnology, Inc., USA)

Anti- Actin (SC-1615, Santa Cruz Biotechnology, Inc., USA)

Anti-Cytochrom c (Abcam, USA)

Anti-caspase 3 (Cellular signalling, Germany)

Anti-Bap-31 (Santa Cruz Biotechnology, Inc., USA)

Anti-NFkB (Santa Cruz Biotechnology, Inc., USA)

Anti-Tom 20 (Santa Cruz Biotechnology, Inc., USA)

Anti-IKKα (Santa Cruz Biotechnology, Inc., USA)

Anti-IkBα (Santa Cruz Biotechnology, Inc., USA)

Anti-p65 (Santa Cruz Biotechnology, Inc., USA)

Anti-XIAP (BD Biosciences, USA)

II. Secondary antibodies

Alkaline phosphatase anti-rabbit: Santa Cruz (Santa Cruz Biotechnology, Inc., USA)

Alkaline phosphatase anti-mouse: Santa Cruz (Santa Cruz Biotechnology, Inc., USA)

Alkaline phosphatase anti-goat: Santa Cruz (Santa Cruz Biotechnology, Inc., USA)

2.1.2 Kits for molecular biology

Qiagen plasmid isolation kits.(Qiagen, Germany)
QIAquick PCR purification/ gel extraction/ nucl. removal Kit (Qiagen, Germany)
Rneasy for RNA extraction (Qiagen, Germany)
Bio-Rad protein Assay kit (Bio-Rad, Germany)

2.1.2.1 Substances for *in vitro* kinase assay

IKBα -substrat (Santa Cruz, USA)
Sepharose protein A (Sigma Aldrich, USA)

2.1.2.2 Oligonucleotides for electrophoretic mobility shift assay (EMSA)

NF-κB binding site : 5`-AGTTGAGGGGACTTTCCCAGGC-3` (Promega, USA)

2.1.3 Human cell lines

Human melanoma cell lines A375 and BLM (ATCC: American type culture collection). The cell line was maintained in Dulbecco's Modified Eagle medium (DMEM-F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 1% Antibiotic solution at 37°C in a humidified atmosphere of 5% CO_2.

2.1.4 Media for cell culture

DMEM/F12 (1:1)-medium: Serva (Heidelberg, Germany)
Dulbecco's Phosphate buffered Saline (DPBS): Sigma (Deisenhofen, Germany)
Doxycycline: Clontech (Palo Alto, USA)
Dulbecco's Modified Egale Medium (DMEM): Gibco BRL (Eggenstein, Germany)
Fetal Bovine Serum (FBS): Gibco BRL(Eggenstein, Germany)
Trypsine/EDTA solution: Seromed/Biochem (Berlin, Germany)
Penicillin/streptomycine: Gibco BRL(Eggenstein, Germany)

2.1.5 X-ray films

Hyperfilm ECL : Amersham Biosciences (Buckinghamshire, UK)

2.1.6 Buffers and solutions for DNA electrophoresis

0.5xTBE buffer (Tris-borate-EDTA)

0.045 M Tris-borate

0.001 M EDTA

1xTAE buffer (Tris-acetate-EDTA)

0.04 M Tris-acetate

0.001M EDTA

6x loading buffer

0.25% Bromophenol blue

0.25% Xylenecyanol FF

30% Glycerol in water, mix well and store at 4°C.

2.1.7 Buffers and solutions for the preparation of nuclear and whole cell extracts

RIPA buffer

50 mM Tris (pH 8.0)

150 mM NaCl

1.0% NP-40

0.5% DOC

0.1% SDS

Modified RIPA buffer

50 mM Tris (pH 7.4)

150 mM NaCl

1 mM EDTA

1 mM $Na3VO_4$

1 mM NaF

1.0% NP-40

0.25% DOC

NP-40 Lysis buffer

50 mM Tris (pH 8.0)

150 mM NaCl

1.0% NP-40

High salt lysis buffer

50 mM Tris (pH 8.0)
500 mM NaCl
1.0% NP-40

Low salt lysis buffer

50 mM Tris (pH 8.0)
1.0% NP-4

Buffer A

20 mM HEPES; pH 7.9.
10 mM NaCl
0.2 mM EDTA
2 mM DTT

Store at 4°C until use. Before use add the protease inhibitor to buffer A (1 volume protease stock solution: 24 volumes buffer A).

Buffer C

20 mM HEPES; pH 7.9
0.75 mM spermidin
0.15 mM spermin
420 mM NaCl
0.2 mM EDTA
2 mM DTT
25% Glycerol

Store at 4°C until use. Before use add the protease inhibitor to buffer C (1 volume protease stock solution: 24 volumes buffer C).

Protease inhibitors

Prepare as 25x stock solution; store at -20°C; and add fresh to the lysis buffer

2.1.8 Buffers and solutions for protein electrophoresis and western blot

30% acrylamide stock solution

30g acrylamide: bisacrylamide (19:1)

Adjust the volume to 100 ml with deionized H_2O. Store at 4°C.

4x Resolving buffer

181.7 g Tris base

40 ml 10% SDS

Adjust the pH to 8.8 and add deionized H_2O to final volume of 1l.

Store at room temperature.

4x Stacking buffer

60.6 g Tris-base

40 ml 10% SDS

Adjust the pH to 6.8 and add deionized H_2O to final volume of 1l.

Store at room temperature.

5x running buffer stock

15g Tris-base

72g glycine

Adjust the volume to 1l with deionized H_2O. Store at room temperature.

1x running buffer

200 ml 5x running buffer stock

0.1% SDS

Adjust the volume to 1l with deionized H_2O.

Staining solution

25% Isopropanol

10% Glacial acetic acid

0.25% Coomassie brilliant (R250)

Adjust the volume to 800ml with H_2O

Destaining solution

7% glacial acetic acid

2x loading buffer

15 g Tris-base
72 g Glycine
0.25 mg Bromophenol blue
2.0 ml Glycerol
0.5 ml ß-mercaptoethanol
2.0 ml 10% SDS
2.5 ml 4x stacking gel buffer

Adjust the volume to10 ml with deionized H_2O. Store at room temperature.

Transfer buffer

14.41 g Glycine
3.025 g Tris-base
200 ml Methanol

Adjust the pH to 8.3 and made up to 1l.

10x TBS buffer

100 mM Tris-base
1.5 M NaCl

Adjust the volume to 1l with H_2O and the pH to 8.3.

Blot solution A

5% BSA
100 ml 10x TBS

Adjust the volume to 1l with deionized H_2O.

Blot solution B

5% BSA
100 ml 10x TBS
0.10% Triton X-100
0.05% Tween 20

Adjust the volume to 1l with deionized H_2O.

Washing solution

0.10% Triton X-100

0.05% Tween 20

100 ml 10x TBS

Adjust the volume to 1l with deionized H_2O.

2.1.9 Equipment and applications

- Centrifuge 5415 D (Eppendorf),
- Centrifuge 5414 R (Eppendorf),
- Biofuge 28 RS (Heraeus, Sepatech)
- DNA gel electrophoresis apparatus: wide mini and mini cells for DNA agarose electrophoresis and power supplies (Bio-Rad).
- Light-Microscop (Zeiss)
- FACS Calibur (Becton Dickinson Bioscences)
- Microwave supratronic 750 (Miele)
- PCR T3-Thermocyclers (Biometra)
- Thermomixer 5437 (Eppendorf)
- Photometer: Spectrophotometer ultraspec 3000 (Pharmacia Biotech)
- Heidolph Polymax 1040 shaker
- MS1 Minishaker (Ika)
- Trans-Blot SD Semi-Dry Tranfer cell (Biometra)
- UV-Transilluminators: wavelength 302 nm and UVT-20M (Herolab)
- UV-chamber (Bio-Rad)
- Air incubator (Bachofer)
- ELISA Reader MR 5000 (Dynatech)
- Microcentrifuge tube 2 ml, 1,5 ml, 0,5 ml, 0,2 ml (Eppendorf)
- Cover glass (Roth)
- Tissues cultur flasks 75 ml (Welabo)

2.2 Methods

2.2.1 Extraction of total RNA from cell lines

The total RNA was isolated from cell lines A375 and BLM using Rneasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, the cell were washed with PBS and then lysed with 500 µl RTL Buffer. The cell lysate were applied into QIA-shredder Column and allowed to centrifuge at 10.000 rpm for 1min.After mixing with equal volume of 70% ethanol. The lysate were mixed well by pipeting and then allowed to apply into Rneasy mini spin column and centrifuged at 10.000 rpm for 1min. The spin column was washing with 500 µl of RW1 buffer by centrifugation at 10.000 rpm for 1min. After washing with RPE buffer the spin column put in new collection tube. At the end the spin column was allowed to centrifuge further for 2 min at 10.000 rpm. The total RNA was eluted with 40 µl of RNAase-free water, after the incubation at room temperature for 5 min the total RNA was collected by centrifugation for 2 min at 10.000 rpm. The concentration of total RNA was determined and stored at –80C until use.

2.2.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

2.2.2.1 Synthesis of the first strand cDNA

The synthesis of the first strand was performed by using reverse transcriptase according to the manufacturer's instruction (Roche). Briefly, the total RNA (~ 1-2 µg) was mixed with (~ 10 pmol) oligo dT, RT buffer, enzyme RT in a total volume of 20 µl. The reverse transcription was carried in a PCR machine (Biometra) using the following conditions:

30 min	16°C
30 min	42°C
5 min	85°C
5min	4°C

At the end, the first strand cDNA products were either stored at -20°c or used as template for the amplification of the TLRs or GAPDH.

2.2.2.2 Second strand cDNA Synthesis

The second strand cDNA synthesis assay was performed by using the first strand cDNA as template. Following the first strand cDNA synthesis, the second strand cDNA amplification was made by mixing of 1x PCR Reaction buffer (100 mM Tris-HCl, pH 8.4, 500 mM KCl, 10 mM dithiothreitol and 1.5 mM MgCl2), 0.2 mM Deoxynucleotide Mix, 20 pmol upstream primer, 20 pmol Downstream primer; 2.5 U/100µl Taq DNA Polymerase (Sigma, St.Louis, USA); and 5 µl first strand cDNA. The amplification process was made in PCR Cycler (T3 thermocycler, Biometra). The PCR reaction mixture was denatured for 5 min at 94°C and then subjected to 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min (annealing temperature is variable), and polymerisation for 1 min at 72°C. The PCR products were then analysed on a 2% agarose gel.

2.2.3 Amplification of cDNA of TLR7, TLR9

To amplify TLR7, TLR9 and GAPDH cDNAs, the following primer pairs were designed so that TLR7, TLR9 and GAPDH encoding region can be completely amplified using oligo dT (25 nt)

TLR7-Sense: 5‘-CAATGTGGACACTGAAGAGAC-3’
TLR7-Antisense: 5’-TAGTAGCTGGTTTCCATCCAG-3’

TLR9-Sense: 5‘-TTCCTATTCATGGACGGCAAC-3’
TLR9-Antisense: 5’-ATTCAGCCAGGAGAGAGAACT-3’

GAPDH-Sense: 5‘-TGCCATCAACGACCCCTTCA-3’
GAPDH-Antisense: 5’-TGACCTTGCCCACAGCCTTG-3’

The thermal cyler was programmed according to the following:

Reverse transcription	30 min 50 °C	
Initial PCR activation	15 min 95 °C	
Denaturation	1min 94 °C	
Annealing	1min 60 °C	35 cycle
Extension	1min 72 °C	
Final extension	10min 72 °C	

The PCR products were then analyzed on a 2% agarose gel stained with ethidium bromide as DNA marker. The 100 bp and 1kbp DNA leaders were used.

2.2.4 Treatment of melanoma cell lines with imiquimod and its derivates

The melanoma cell lines A375 and BLM were seeded in a petri dishes for 24 h under normal conditions, so that the confluency of the seeded cells was between 60 and 70% before the exposure of the cells to imiquimod or its agonists

2.2.5 Preparation of total cell lysates

After the treatment of the melanoma cells with imiquimod or with its analogues for the indicated time periods, the cells were washed once with ice-cold PBS. The cells were lysed on ice by the addition of 500 µl of RL Buffer and collected by scraping off from 25 cm^2 cell culture flasks with a plastic scraper. The cell lysate were centrifuged at 1400 rpm for 5 minutes and were stored at -20°c until use.

2.2.6 Preparation of nuclear extracts

The nuclear extracts were prepared from treated and untreated cells as described (Hassan et al., 2008; 2007; 2005a; 2004). Briefly, cells were washed with ice-cold PBS buffer and harvested by adding 500 µl of buffer A (20 mM HEPES, pH 7.9; 10 mM NaCl, 0.2 mM EDTA; and 2 mM DTT) containing protease inhibitor and incubated on ice for 10 min. The supernatant was discarded after centrifugation at 14.000 rpm for 3 min. The pellet was resuspended in 50 µl of buffer C (20 mM HEPES, pH 7.9; 420 mM NaCl, 0.2 mM EDTA; 2 mM DTT; 1 mM Na vanadate 25 % glycerol) containing protease inhibitor and incubated for 20 min at 4°C and then centrifuged at 14.000 rpm for 3 min. The supernatant was collected without disturbing the pellet and stored at -80°C until use.

2.2.7 Assessment of cell survival using MTT assay

The percentage of viable cells was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (Hassan et al., 2008; 2005a; 2004). Briefly, the cells were plated at the recommended density in microtiter plates, 30 min before the end of the incubation, a 20 µl of MTT solution (5mg/ml) was added to each well containing the cells. After the

incubation of the plate in a CO_2 incubator at 37°C for a further 5 h, the media was removed and a 200 µl of DMSO was added to each well. The plate was allowed to shake gently at RT for 5 min to dissolve the crystals. Following the incubation for 5 min at 37°C, the plate was transferred to ELISA reader and the absorbance was measured at 550 nm.

2.2.8 H&E Staining

Treated and untreated Melanoma cell lines A375 and BLM were washed twice with PBS, fixed with acetone stored at -20°C for 30 sec, then washed with PBS and stained with Hematoxylin and Eosin (H&E). This kind of staining permitted a reliable visual quantification of apoptosis using classical morphological criteria of apoptosis (Morphological alteration of Melanoma cell lines after treatment with imiquimod and its analogues).

2.2.9 Detection of apoptosis using Annexin V/PI Method

The appearance of phosphatidyleserine (PS) on the extracellular side of membrane was evaluated with annexin V/PI method. 24h later, melanoma cell lines A375 and BLM cells, either untreated or treated with imiquimod or its analogues 3M-001, 3M-007 or 3M-029 were trypsinized and washed twice in ice-cold PBS and resuspended in 1X binding buffer (Invitrogen). Thereafter, 5 µl of Annexin V-FITC (Vybrant; Invitrogen) and 5µl propidium iodide (100µg/ml) were added to 100 µl of cell suspension and incubated for 15 min at room temperature protected from light. Finally, 400 µl of binding buffer were added to the samples and handled ice-cold until analysis. The fluorescent signals of FITC and PI were detected by FL1 at 518 nm and FL2 at 620 nm, respectively, on a FACSCalibur (Becton Dickinson Biosciences) and apoptotic cells resulted from treatment of melanoma cell lines A375 and BLM with imiquimod or its analogues 3M-001, 3M-007 or 3M-029 (annexin V–positive/PI-negative) were quantified.

2.2.10 Measurement of mitochondrial membrane potential (ΔΨm) Using JC-1

The measurement of mitochondrial membrane potential was performed according to the manufacture's protocol (Invitrogen). Briefly, the melanoma cell lines A375 and BLM cells were either untreated or treated with imiquimod or its analogues 3M-001, 3M-007 or 3M-029. 24h later, the cells were trypsinized and washed twice in ice-cold

PBS and resuspended in PBS. The cells were stained with 10 µM JC-1 for 30 min at room temperature in the dark. The intensities of green fluorescence at 520–530 nm (PMT 2) and of red fluorescence at more than 550 nm (PMT 3) of 50.000 individual cells were analyzed by using a flow cytometer. The intensity voltages of the photomultipliers of detector 2 (PMT 2) and detector 3 (PMT 3) were set at 470 V. The value of ΔΨm in response to the test compound was expressed as a ratio of PMT 3 to PMT 2.

2.2.11 Western Blot Analysis

2.2.11.1 SDS-PAGE

10 to 15% polyacrylamide gels were prepared as described (Laemmli *et al*.1970). Full Range Rainbow molecular weight marker (Amersham Biosciences) was used as a protein standard. Samples were mixed with 5 x SDS sample buffer and boiled for 5 min at 95°C. After brief centrifugation samples were loaded onto SDS-PAGE gels. Minigels were run at 100 V to 200 V in a Mini-Protean II electrophoresis cell (Bio-Rad).

2.2.11.2 Protein transfer and detection of the specific signal (western blot)

Transfer of protein from SDS-PAGE (Biorad, Munich, Germany) was accomplished in a Biometra unit by using a single transfer buffer for 7 h at 5V (0.8 A per cm^2 of gel). Protan (PVDF, 0.45µm, Amersham, Braunschweig, Germany) transfer membranes were pre-incubated with methanol. The western blots were blocked in Blot solution A over night at 4°C with constant agitation. The blots were washed three times for 15 min at RT in western blot washing buffer. The washed blots were allowed to incubate with the primary antibody diluted in blot solution B (monoclonal antibody 1:5000 and polyclonal antibody 1:1000) with constant agitation over night at 4°C. The blots were washed three times at RT in TBST, 15 min each. The western blots were allowed to incubate for 60 min at RT with the secondary antibody diluted in blot solution B (1:2000). After the washing of western blots three times in TBST, 15 min each, the specific signal was detected by using ECL western blotting detection reagents (Amersham Pharmacia biotech, Braunschweig, Germany).

2.2.11.3 Stripping of Western blots

Bound antibody was removed from Western blots by incubation for 30 min at 60°C in western blot stripping buffer. After washing the Western blots three times for 10 min with TBS-T, Western blots were ready for another immunodetection of protein.

2.2.12 *In vitro* kinase assays

In vitro kinase assays were performed as described (Hassan et al., 2008, 2007; 2005a). Briefly, cell lysates were prepared from BLM cells exposed to imiquimod for 24 h using buffer L (20 mM HEPES [pH 7.9], 10 mM EGTA, 40 mM ß-glycerophosphate, 25 mM $MgCL_2$, 2 mM Na_3VO_4, 1 mM DTT, 1% NP-40, 5 µg apoprotinin, 1 mM Leupeptin, 1 µg/ml, pepstatin, and 1 mM benzamidine). Insoluble material was removed by centrifugation, and the cell lysate was incubated with specific antibody of interest to immune precipitate the kinase of interest for 1 h at 4°C. The immune complexes were allowed to bind to A-sepharose (5 mg/ml in lysis buffer) overnight at 4°C. After centrifugation, the sepharose beads were washed three times with kinase reaction buffer (80 mM HEPES [pH 7.9], 80 mM $MgCL_2$, 0.1 mM ATP, 2 mM Na_3VO4 and 20 mM NaF). Kinase activity was determined by the incubation with the recomanded amount of the corresponding kinase substrate 10µCu (γ-dATP) and immune complex in 15 µl of kinase reaction buffer and then was allowed to incubate for 30 min at 37°C. Following the termination of the reactions the addition of 15 µl of sample buffer, the kinase activity was analyzed by SDS-polyacrylamide gel electrophoresis. The dried gel was visualized via exposure to high performance autoradiography film.

2.2.13 Electrophoretic mobility shift assay (EMSA)

The details of EMSA were performed as described (Hassan et al., 2004; 2005a; 2007). The double stranded synthetic oligonucleotides carrying a binding site for the transcription factor of interest were end-labeled with [γ-^{32}P] ATP in the presence of T4 polynucleotide kinase. For binding, 4 µg nuclear extract was allowed to bind to a labeled probe in a total volume of 30 µl for 30 min at room temperature in binding buffer (10 mM Tris, pH 7.5; 50 mM NaCl, 1mM EDTA; 1 mM MgCl2; 0.5 mM DTT and 4 % glycerol). The competition assay was performed in the same manner, except that unlabeled probes containing the sequence of the binding site of interest was allowed to incubate with nuclear extracts for 20 min at room temperature before adding the

labeled probes. Electrophoresis was performed for 3 h at 100 V in 0.5 X Tris-borate-EDTA running buffer at room temperature. The dried gel was visualized via exposure to high performance autoradiography film.

2.2.14 Measurement of intracellular calcium release using flow cytometry

The cytosolic Ca^{2+} signals of imiquimod-treated BLM cells were monitored using Ca^{2+} -sensitive dye Fluo3-AM (Invitrogen, Karlsruhe, Germany) at a concentration of 4 µM for 30 min at room temperature by flow cytometry analysis as described (Hassan et al., 2008).

2.2.15 Measurement of ROS

The measurement of ROS accumulation was performed as described (Hassan et al., 2008) using DHR 123 staining (Sigma) and analysed by fluorescence-activated cell sorting (FACS) as described (Hassan et al., 2008). After the stimulation of the cells with imiquimod for the indicated time, the cells were load to incubate with 10 mM of DHR 123 (dihydrorhodamine) for 1 hour at 37°c. After the incubation, the cells were collected from culture tissues using trypsin. The collected cells were washed tree times with PBS and resuspendet in 500 µl PBS bevor the measurement of reactif oxygen species using flow-cytometry.

3. Results

3.1 Effect of imiquimod and its analogues 3M-001, 3M-007 and 3M-029 on the cell viability of melanoma cell lines

To assess and to confirm imiquimod and its analogues 3M-001, 3M-007 and 3M-029 induced-effects on the viability of melanoma cells, the cell lines A375 and BLM were subjected to different concentration of imiquimod (10, 20, and 50 µg/ml), or its analogues 3M-001, 3M-007 and 3M-029 (5, 15, 25 µg/ml) for regulated time intervals up to 120 h. Data obtained from cell viability assay demonstrated that the optimal concentration of imiquimod, which is necessary to inhibit the cell growth rate of melanoma cell lines A375 and BLM to 50% in a time period of 24 h is 50 µg/ml (Fig 3). Whereas, the optimal concentration of 25 µg/ml for a time period of 24h were noted for the imiquimod analogue 3M-001 (Fig 4) and the imiquimod analogue 3M-029 (Fig 5), respectively. In contrast, the treatment of the same cell lines with different concentrations of imiquimod analogue 3M-007 for the indicated time periods (Fig 6) demonstrated no effect on the cell viability of the melanoma cell lines A375 or BLM.

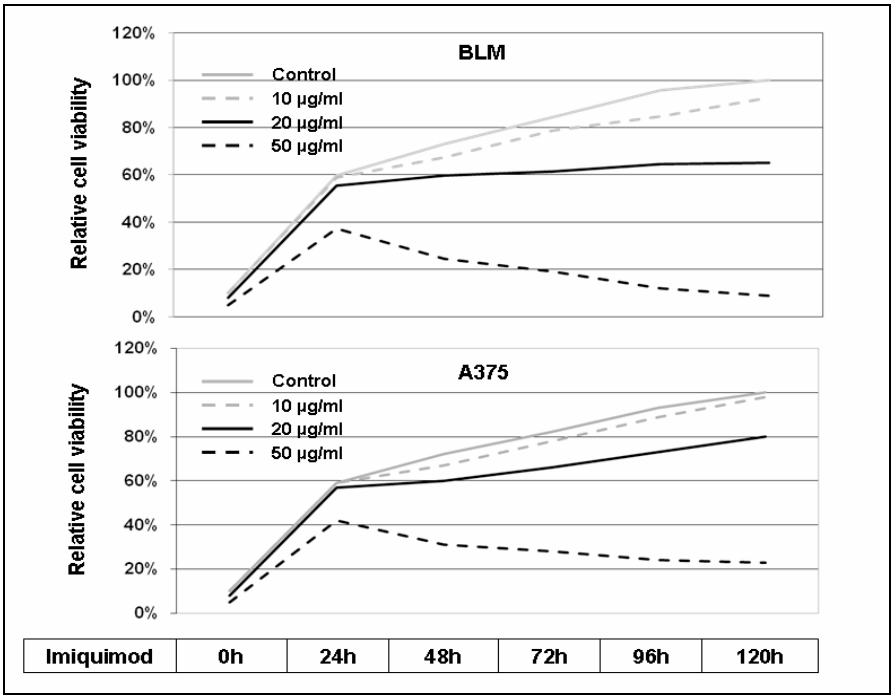


Fig 3: Effect of imiquimod on the cell viability of A375 and BLM melanoma cell line. The cells were treated with imiquimod to different concentrations for regulated time intervals up to 120 h and the cell viability was determined by MTT assay. The results are the mean of three independent experiments performed in duplicate.

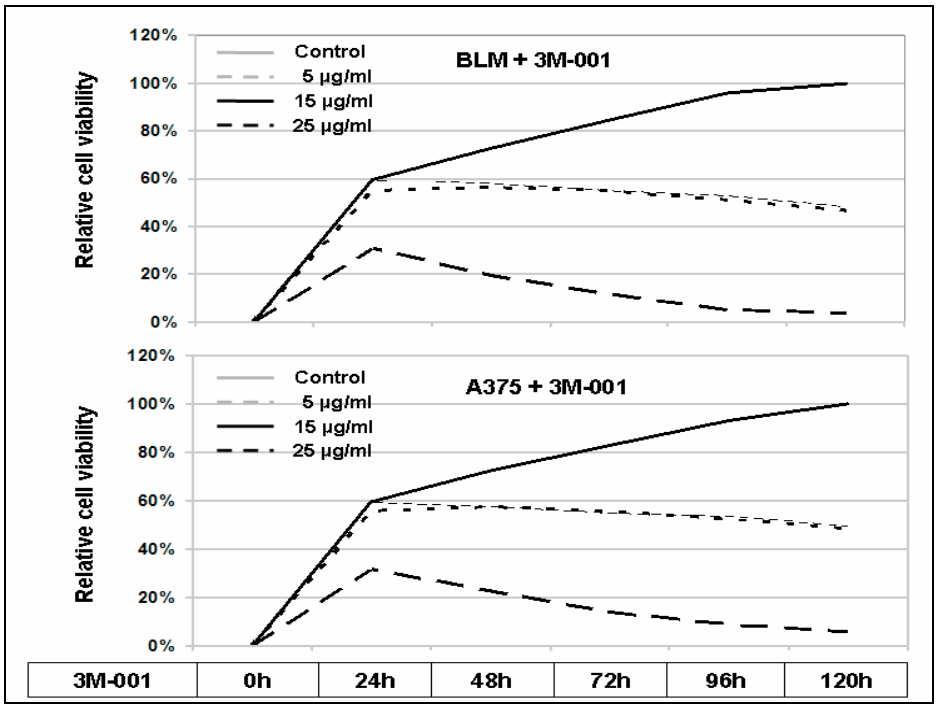


Fig 4: Effect of 3M-001 on the cell viability of A375 and BLM melanoma cell line. The cells were treated with the imiquimod analogue 3M-001 to different concentrations for regulated time intervals up to 120 h and the cell viability was determined by MTT assay. The results are the mean of three independent experiments performed in duplicate.

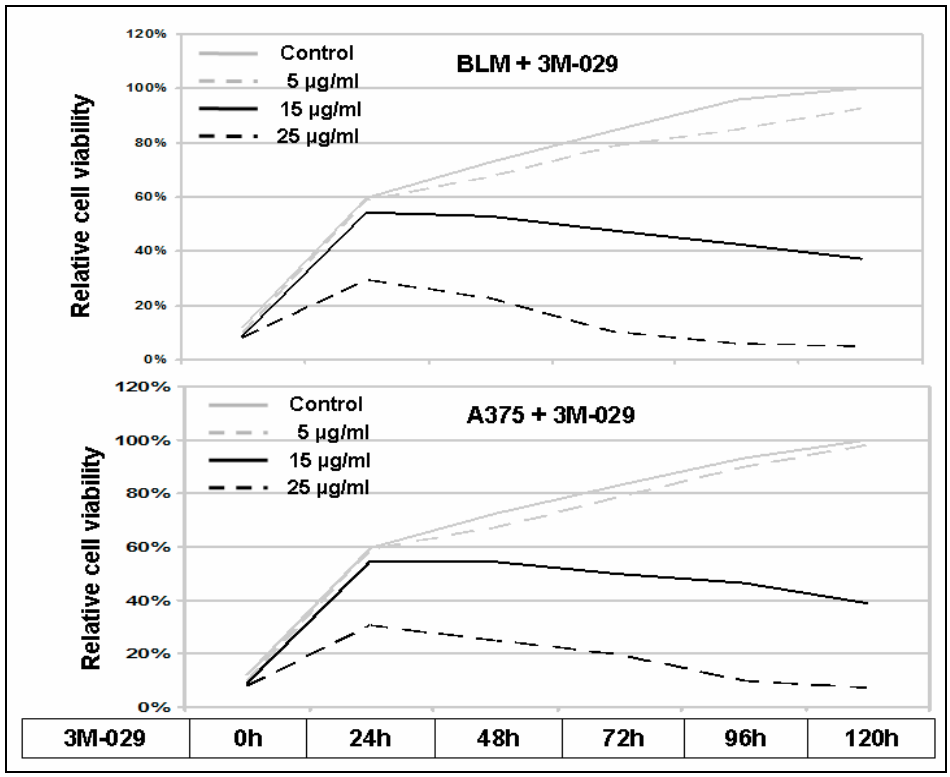


Fig 5: Effect of 3M-029 on the cell viability of A375 and BLM melanoma cell line. The cells were treated with the imiquimod analogue 3M-029 to different concentrations for regulated time intervals up to 120 h and the cell viability was determined by MTT assay. The results are the mean of three independent experiments performed in duplicate.

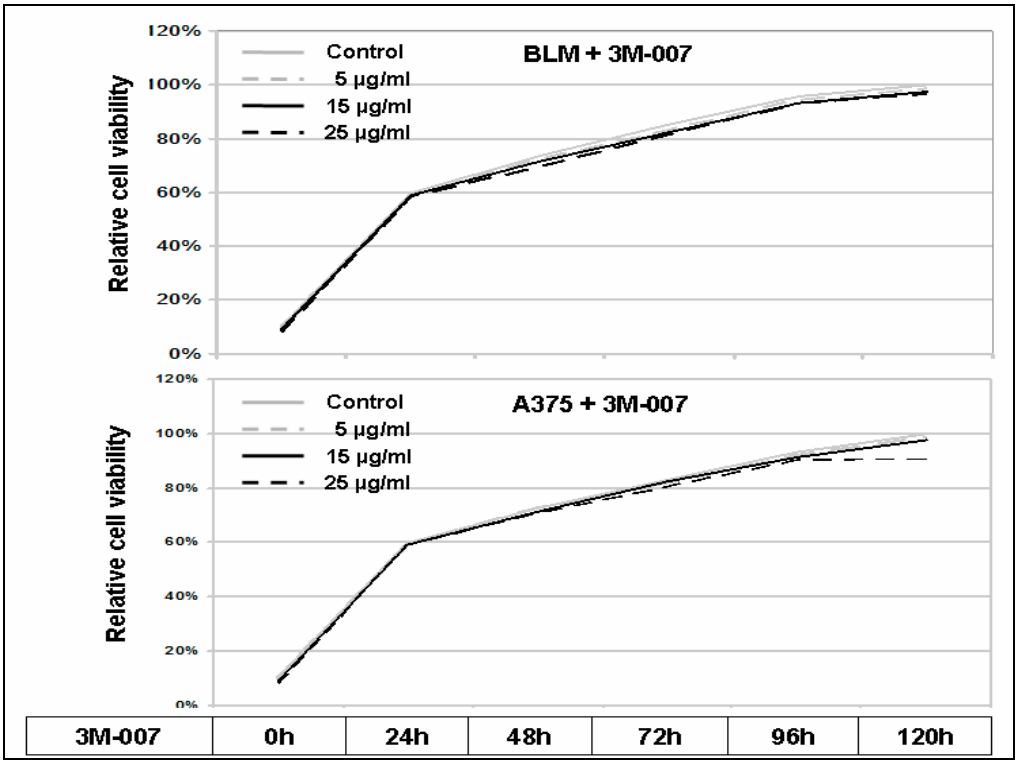


Fig 6: Effect of 3M-007 on the cell viability of A375 and BLM melanoma cell line. The cells were treated with the imiquimod analogue 3M-007 to different concentrations for regulated time intervals up to 120 h and the cell viability was determined by MTT assay. The results are the mean of three independent experiments performed in duplicate.

Based on the data obtained from kinetics as well as from time course experiments, the melanoma cell lines A375 and BLM were allowed to grow for 24 h under normal conditions before the exposure to either imiquimod or its analogues 3M-001, 3M-007 or 3M-029 for regulated time intervals up to 24 h. The treatment with the small molecule imidazoquinoline TLR7 agonists imiquimod, 3M-001 and 3M-029 was found to reduce the cell growth rate after 12 h to 90% and increased thereafter to reach 36%, 39% and 58% in response to the exposure of A375 to 3M-001, 3M-029 and imiquimod for 24 h, respectively (Fig 7). Also the exposure of BLM cells to same substances showed similar results (Fig 7). Whereas, the exposure of the melanoma cell lines to 3M-007 was not found to exert any effect on the growth rate.

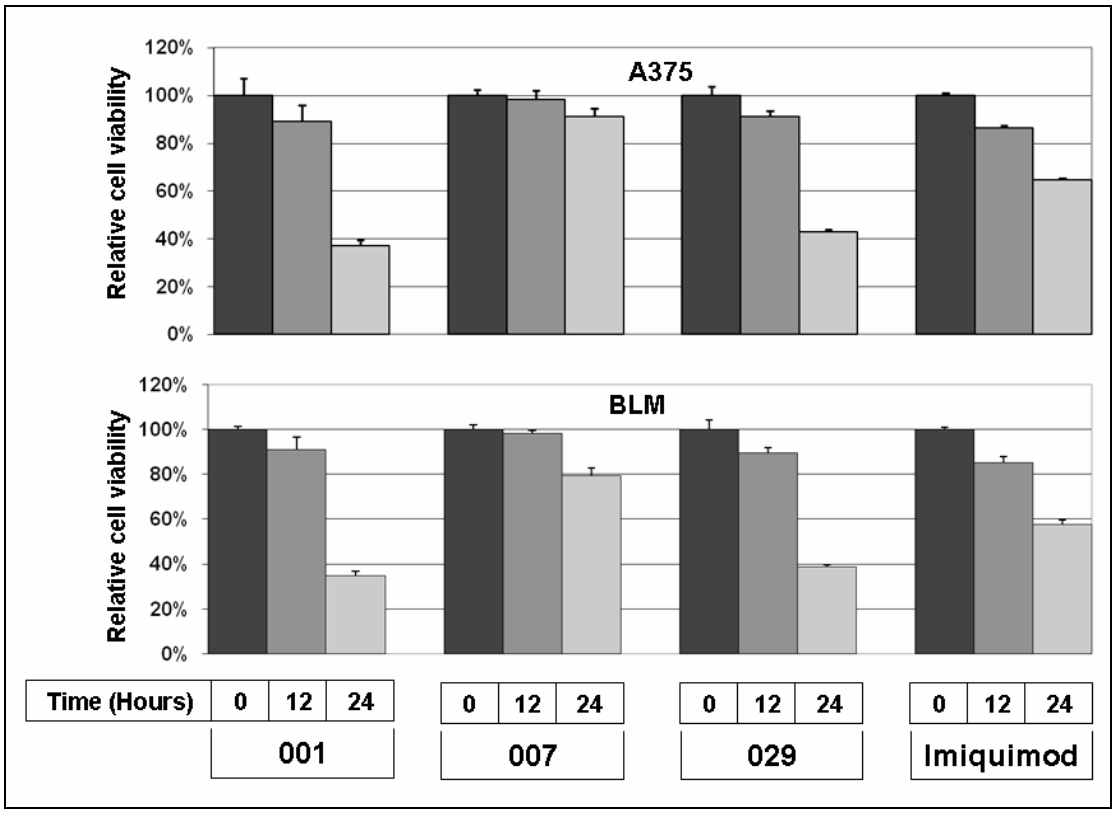


Fig 7: Effect of imiquimod, 3M-001, 3M-007 and 3M-029 on the cell viability of A375 and BLM melanoma cell line. The cells were treated with 3M-001, 3M-007, 3M-029 or imiquimod up to 24 h and the cell viability was determined by MTT assay. The results are the mean of three independent experiments performed in duplicate.

To show, whether imiquimod and its analogues 3M-001, 3M-007, 3M-029 induced cell death in melanoma cells is mediated through an apoptotic mechanism, the cell lines A375 and BLM were treated with imiquimod, 3M-001, 3M-007 or 3M-029 before the staining with Hematoxylin and Eosin (H&E staining). Data obtained from microscopic analysis of melanoma cell lines (Fig 8A, 8B) showed morphological changes in response to the treatment with imiquimod and its analogues 3M-001 and 3M-029 including rounding up and detachment from the dish as well as nuclear condensation and fragmentation. In addition, flow cytometry analysis using FITC annexin-V and propidium iodide (PI) staining demonstrated that imiquimod and its analogues 3M-001 and 3M-029 but not the analogue 3M-007 (Fig 9) are able to trigger apoptosis of both melanoma cell lines A375 and BLM.

The exposure of A375 cells to imiquimod, 3M-001 and 3M-029 was found to trigger apoptosis in 7%, 50% and 80% of treated cells respectively, when compared to control cells. Whereas the exposure of BLM cells to imiquimod, 3M-002 and 3M-029 enforced more cells into apoptosis. More than 20% of the cells showed apoptosis by the treatment with imiquimod, 3M-001 or 3M-029. These data provide evidence for

the involvement of an apoptotic mechanism in the modulation of imiquimod and its analogues induced cell death of melanoma cells.

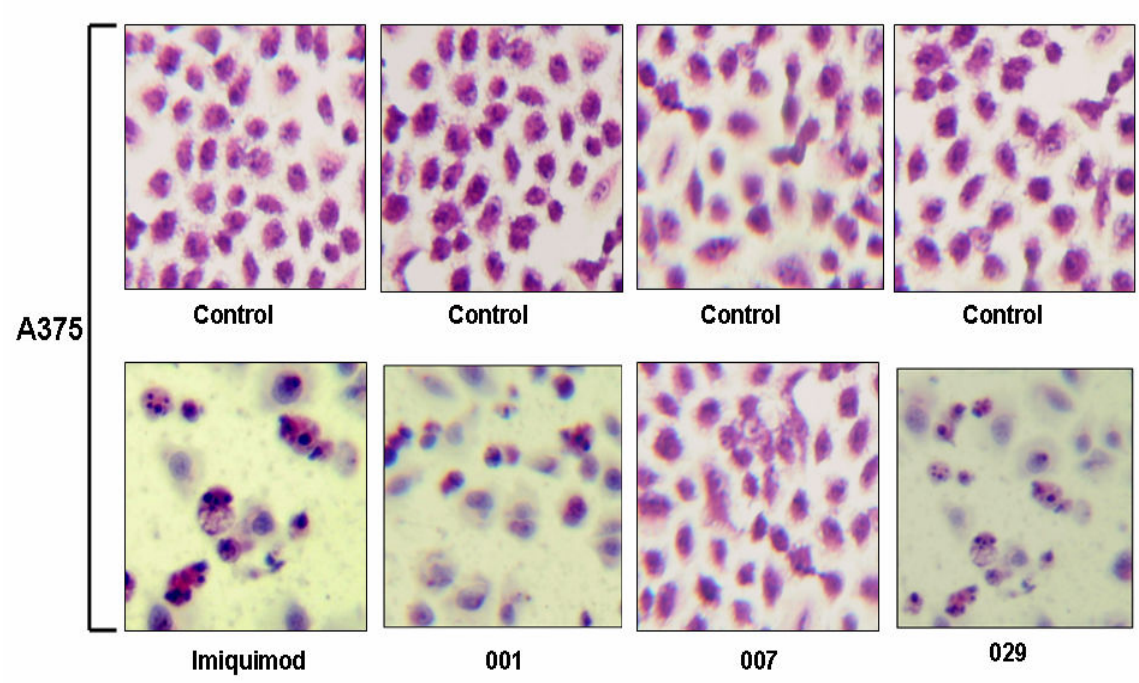


Fig.8A: Detection of apoptosis in A375 melanoma cells. Melanoma cell line A375 were treated with imiquimod, 3M-001, 3M-007 or 3M-029 up to 48h and the extention of apoptosis was determined using H&E staining as described under material and methods. Data are representative of three independent experiments.

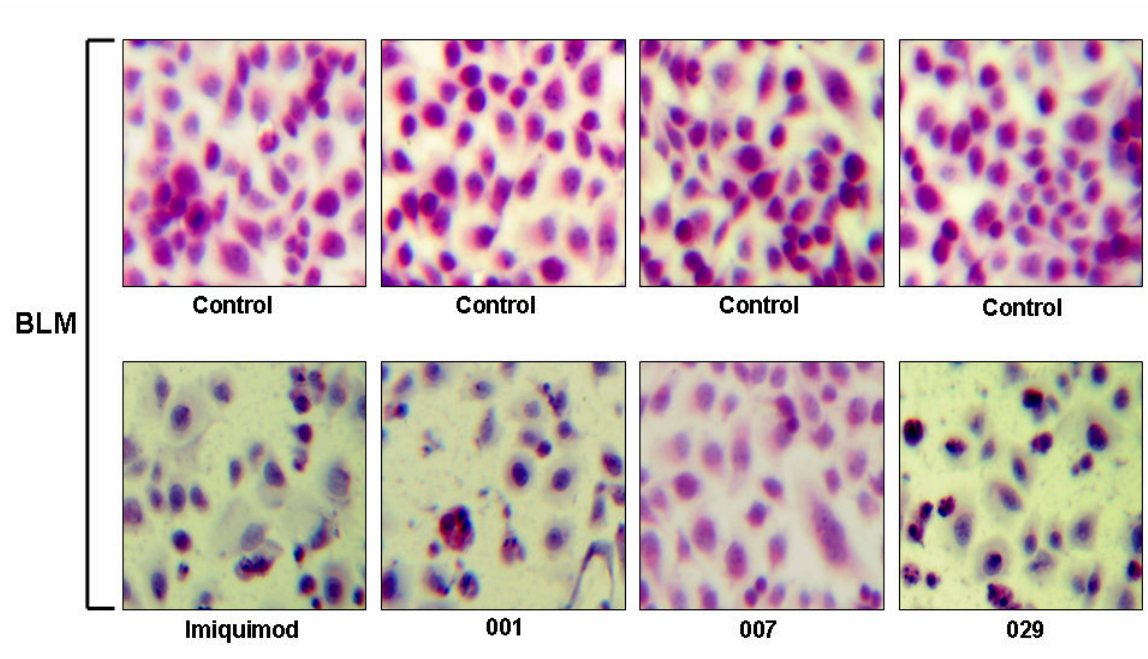


Fig.8B: Detection of apoptosis in BLM melanoma cells. Melanoma cell line BLM were treated with imiquimod, 3M-001, 3M-007 or 3M-029 up to 48h and the extention of apoptosis was determined using H&E staining as described under material and methods. Data are representative of three independent experiments.

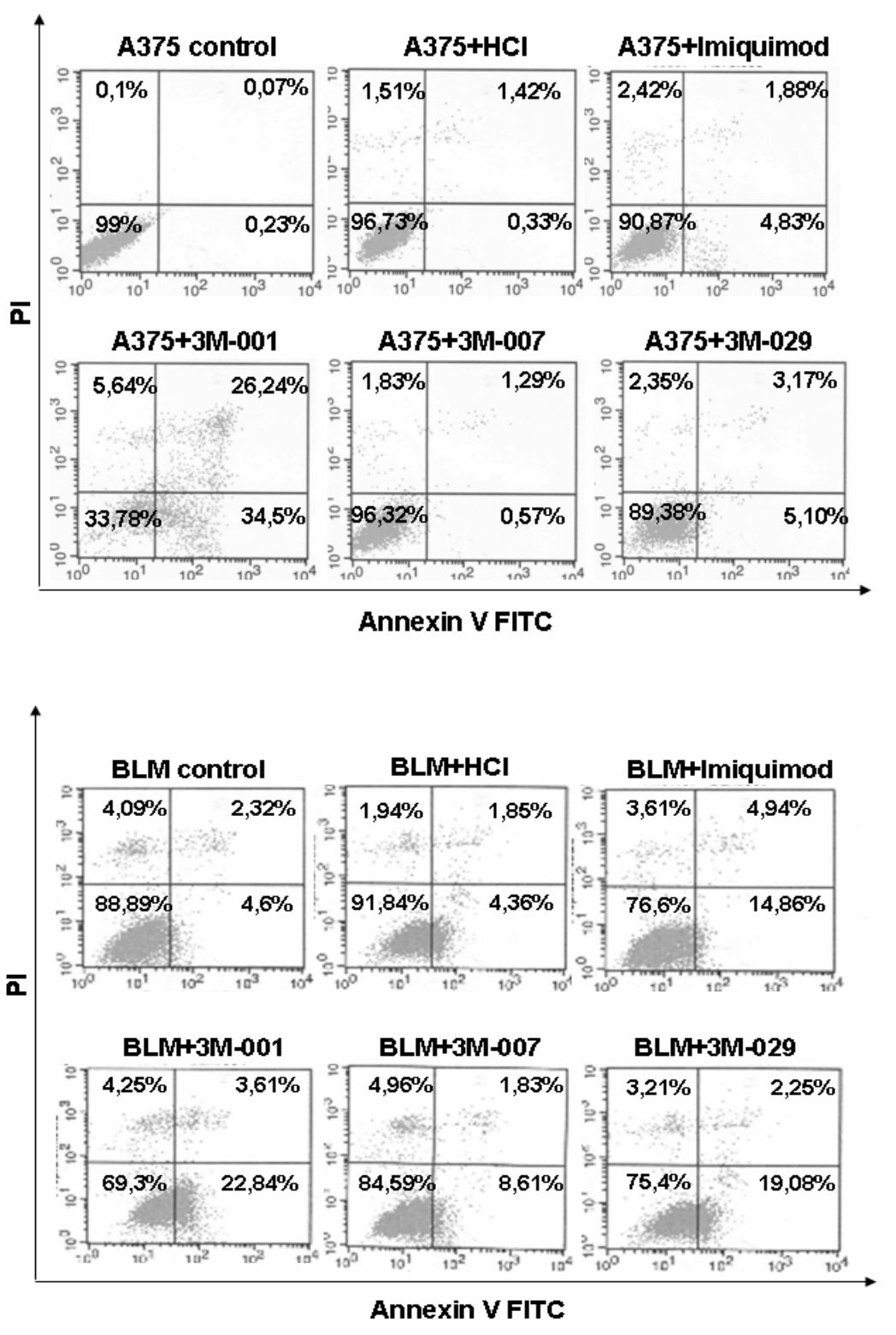


Fig 9: Detection of apoptosis in melanoma cell lines A375 and BLM following the treatment with imiquimod or its analogues 3M-001, 3M-007 or 3M-029. Melanoma cells were treated with imiquimod, 3M-001, 3M-007 or 3M-029 for 24h and the extention of apoptosis was determined by flow cytometry analysis using annexin/PI staining as described under material and methods. Data are representative of three independent experiments.

Imiquimod and its analagues 3M-001, 3M-001 and 3M-029 induced apoptosis of melanoma cells was confirmed further at the molecular level by the analysis of cleavage of PARP using immune blotting. Both melanoma cell lines A375 and BLM were exposed to imiquimod or its analogues 3M-001, 3M-007 or 3M-029 for 24h, after which the cells were lysed and the total cellular proteins were separated by SDS-PAGE. Data obtained from Western blot analysis (Fig 10A, 10B) demonstrated the cleavage of PARP in both melanoma cells in response to the exposure to imiquimod or its analogues 3M-001, 3M-029, but not by the treatment with the analogue 3M-007 when compared to the control cells. These results confirmed further the ability of 3M-001, 3M-029 and imiquimod to kill melanoma cells by an apoptotic mechanism.

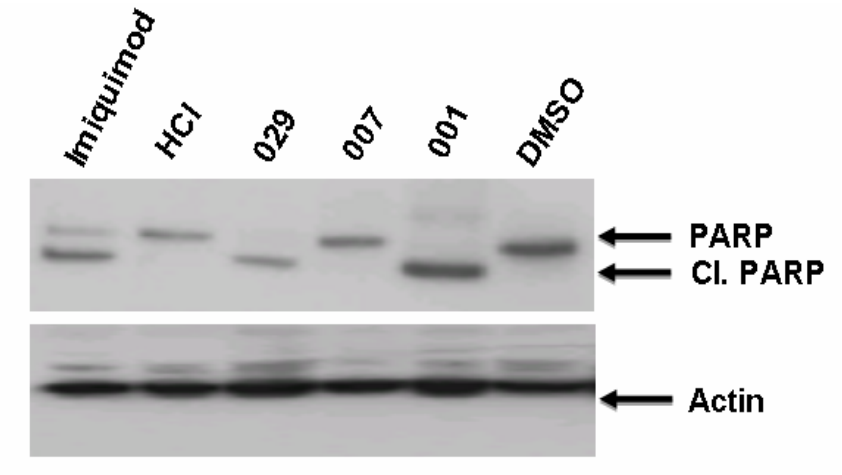


Fig 10A: Detection of PARP cleavage in A375 melanoma cell lines. The cells were treated with imiquimod or with its analogues 3M-001, 3M-007 or 3M-029 and the total cell extracts were prepared. Western blot was performed using anti-PARP antibodies as described under material and methods. Data are representative of three independent experiments

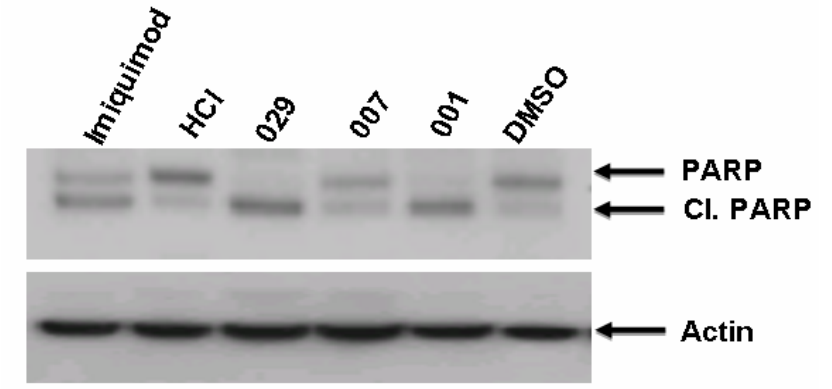


Fig.10B: Detection of PARP cleavage in BLM melanoma cell lines. The cells were treated with imiquimod or with its analogues 3M-001, 3M-007 or 3M-029 and the total cell extracts were prepared. Western blot was performed using anti-PARP antibodies as described under material and methods. Data are representative of three independent experiments

3.2 Examination of TLRs expression before and after the treatment with Imiquimod

Based on the fact that TLRs agonists can mediate its effects through recognition their corresponding receptors, and to show whether imiquimod and its analogues 3M-001, 3M-007 and 3M-029-induced cell death is associated with the expression of the corresponding TLRs, the melanoma cell lines A375 and BLM were treated with imiquimod or with HCl as control for regulated time intervals up to 48h, and the total RNAs were extracted and RT-PCR was performed. First the quality of the extracted total RNA was tested using 1.4% ethidium bromide agarose gel. The analysis of the Fig 11 demonstrates the quality of the extracted total RNA based on the detection of the transcripts of the ribosomal RNAs (1.8 kb and 4.8 kb). Whereas, Fig 12 demonstrates the expression of all examined TLRs after the analysis of cDNA products on 2% ethidium bromide agarose gel. Although the detection of both TLR-7 and TLR-9 in melanoma cells, the treatment with imiquimod did not appear to influence their expression. These results provide evidence for the expression of TLR-7 and TLR-9 in melanoma cells.

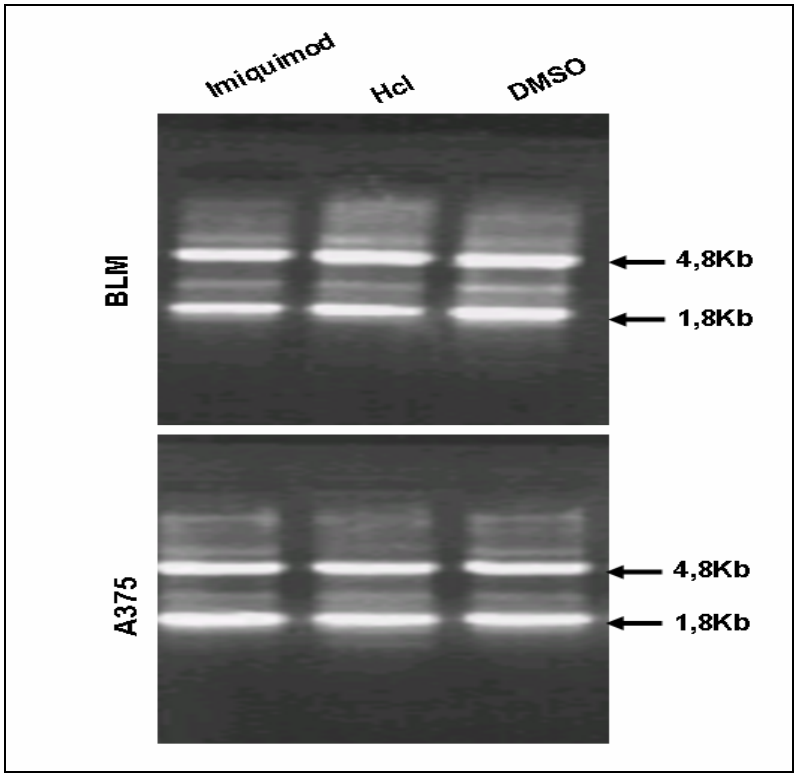


Fig 11: Examination of the quality of total RNA after the extraction from untreated and treated melanoma cells (A375 and BLM) using 1.4% ethidium bromide agarose. Rows show the both rRNA transcripts (1.8 Kb and 4.8 Kb). Data are representative of three independent experiments performed separately.

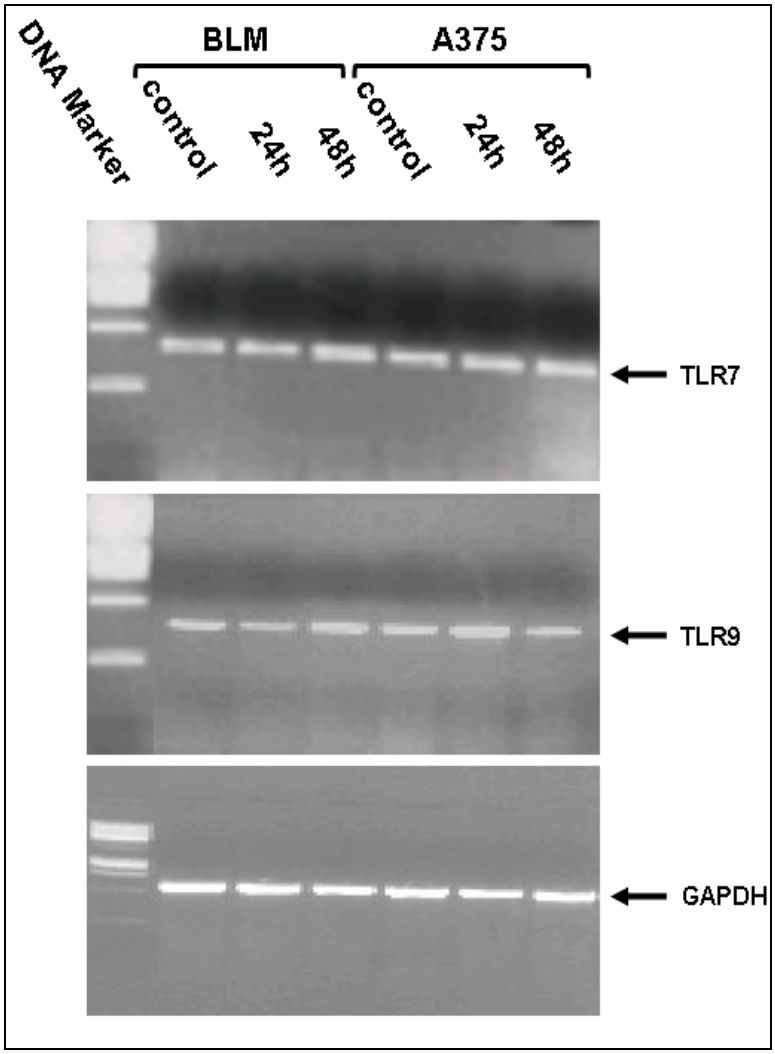


Fig 12: Expression of TLR-7, and TLR-9 mRNA in A375 and BLM melanoma cell lines before and after the treatment with imiquimod. Cells were grown in DMEM medium with 5% FCS in the presence (24h and 48h) or in the absence of imiquimod (50µg/ml). First strand cDNA synthesized from total RNA extracted from A375 and BLM cells was amplified with the specific sense and antisense primers for either TLR-7 or TLR-9 and PCR products were analysed on 2% ethidium bromide agarose gel. GAPDH was used for internal control for equal loading. Data are representative of three independent experiments performed with similar results.

3.3 Treatment with Imiquimod triggers apoptosis in melanoma cells

To evaluate the ability of Imiquimod to trigger apoptosis in melanoma cells, the cell lines A375 and BLM were treated with Imiquimod (50 µg/ml) and cell viability was determined at regulated time intervals up to 48h using MTT colorimetric assay as described under material and methods (Fig 13A, 13B). Imiquimod-induced cell death was observed at 12 h of exposure and increased thereafter to up to 48 h. Interestingly, the staining of A375 and BLM melanoma cells with H&E (Fig 14A, 14B) showed morphological changes including detachment from the dish, nuclear

condensation and fragmentation in response to the treatment with imiquimod. In addition, the frequency of apoptotic cells was determined by flow cytometry analysis using FITC annexin-V and propidium iodide (PI). Up to 5 and 14% apoptotic cells were detected in A375 and BLM cells respectively in response to the treatment with imiquimod (Fig 15). Moreover, the ability of imiquimod to trigger apoptosis in either A375 or BLM cells was further confirmed by the detection the hallmark of apoptosis including cytochrome c release and cleavage of caspase-3 and PARP in melanoma cells (Fig 16). Thus, these results suggest that imiquimod-induced cell death in melanoma cells is mediated by an apoptotic mechanism.

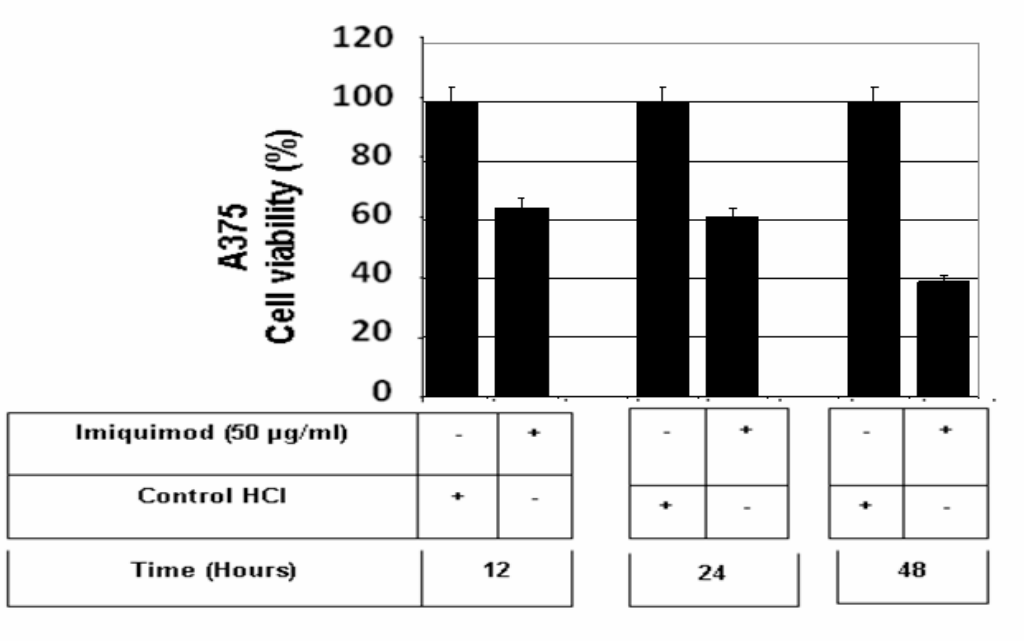


Fig 13A: Cell proliferation assays (MTT) were performed in A375 melanoma cell lines. Differential response of A375 melanoma cell lines to treatment with Imiquimod (50µg/µl). The A375 cell lines were harvested 12h, 24h and 48h after treatment with Imiquimod. The MTT assays were performed in at least two independent experiments and the data presented are the mean out of these experiments.

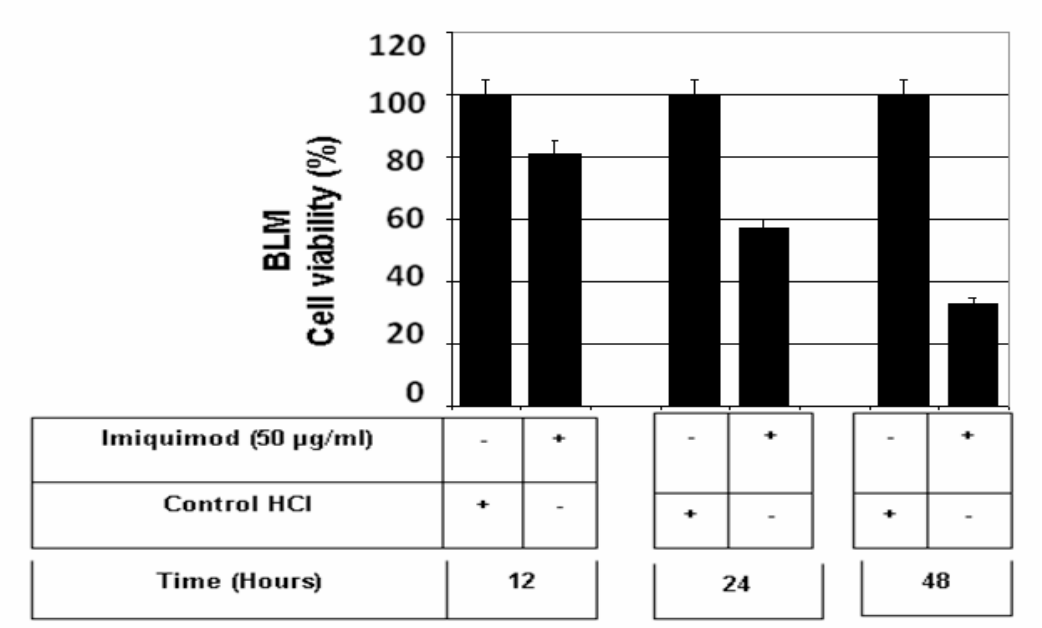


Fig 13B: Cell proliferation assays (MTT) were performed in BLM melanoma cell lines. Differential response of BLM melanoma cell lines to treatment with Imiquimod (50µg/µl). The BLM cell lines were harvested 12h, 24h and 48h after treatment with Imiquimod. The MTT assays were performed in at least two independent experiments and the data presented are the mean out of these experiments.

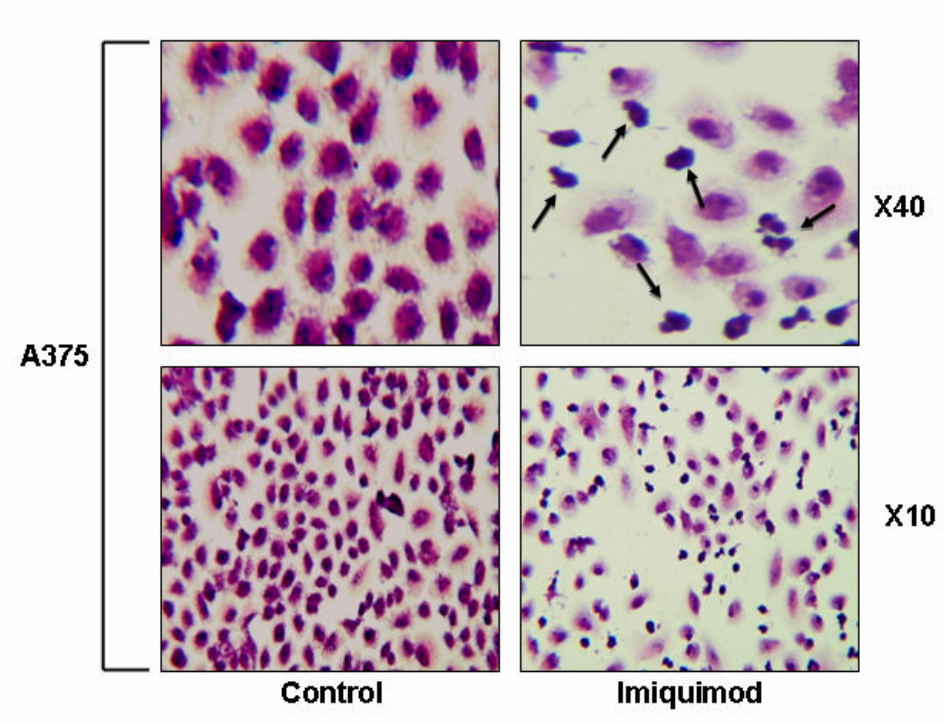


Fig 14A: Detection of apoptosis in A375 melanoma cells before and after the treatment with imiquimod (50µg/ml) using H&E staining**.**

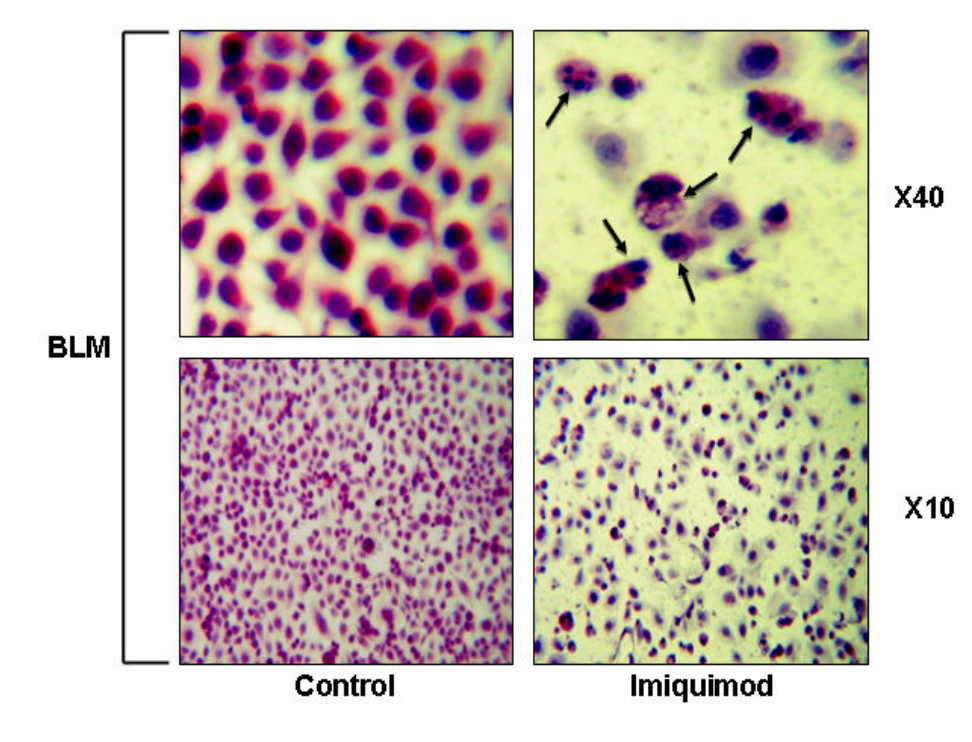


Fig 14B: Detection of apoptosis in BLM melanoma cells before and after the treatment with imiquimod (50µg/ml) using H&E staining.

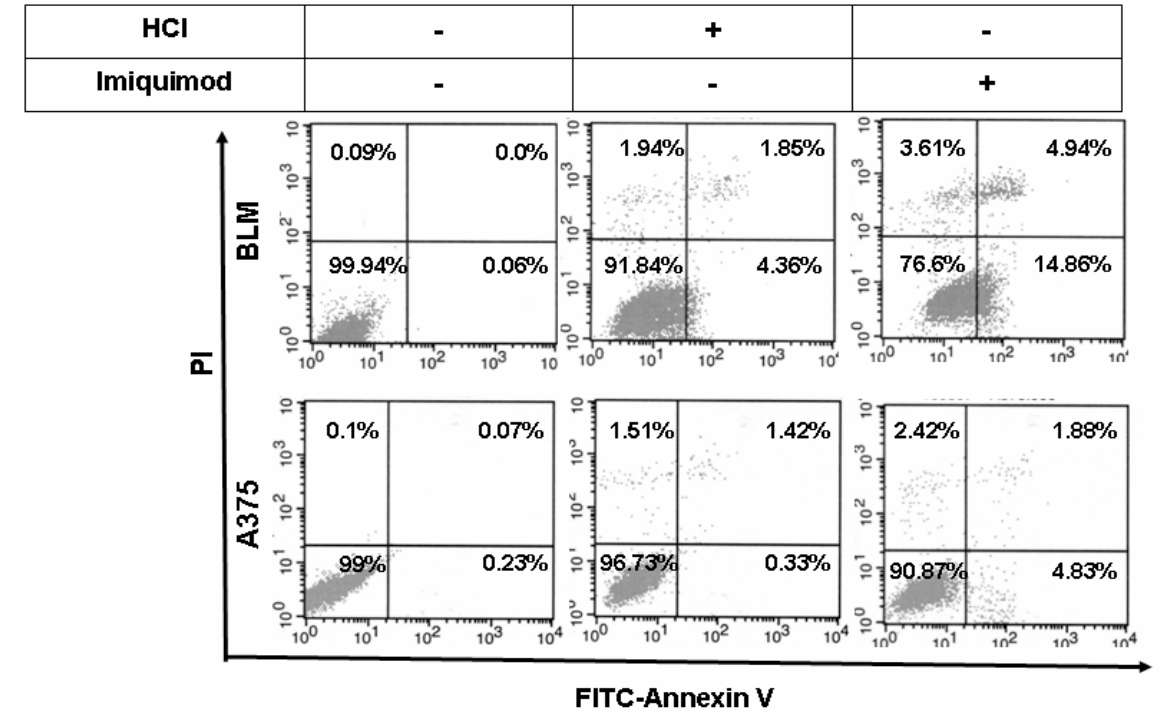


Fig 15: Detection of apoptosis in A375 and BLM melanoma cells by flow cytometry analysis using annexin/PI staining following 48 h of incubation with imiquimod**.**

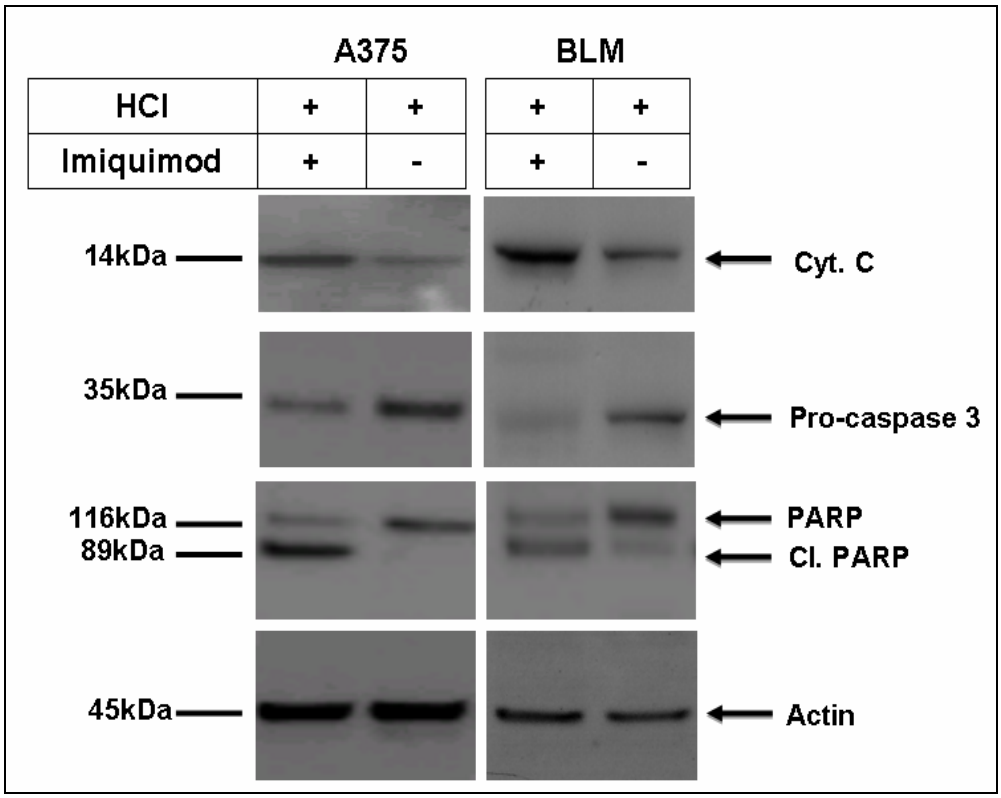


Fig 16: Detection of cytochrome c release, caspase 3 and PARP cleavage in A375 and BLM melanoma cells. BLM cells were treated with imiquimod up to 48 h and total cell extracts were prepared. Western blot was performed using anti-caspase 3, anti-cytochrome c (cyt c) and anti-PARP antibodies as described. Data are representative of three independent experiments

3.4 Imiquimod-induces apoptosis in melanoma cells is mediated by a mechanism including endoplasmic reticulum (ER) stress and mitochondrial damage

Based on the fact that Toll-like receptor 7, the target receptor of imiquimod, resides within the endoplasmic reticulum (ER), it was hypothesized whether the exposure of melanoma cells does result in the induction of the ER stress. BLM cells were treated for up to 48 h and the intracellular calcium release was assessed by staining with the Ca^{2+}-sensitive-dye-Fluo3-AM using flow cytometry. The analysis demonstrated the release of intracellular calcium following the exposure of melanoma cells to imiquimod when compared to control cells (Fig 17). In addition, imiquimod induced ER-stress was confirmed by the detection of Bap-31, a molecular marker for ER-stress in both melanoma cell lines (Fig 18). Thus, these data provide evidence for imiquimod-induced ER stress in melanoma cells by a mechanism including calcium release that is associated with Bap-31 cleavage.

To investigate whether imiquimod-induced intracellular Ca^{2+} release is associated with mitochondrial damage, the melanoma cell lines A375 and BLM were treated with imiquimod for the indicated time points before measuring the mitochondrial membrane potential by flow cytometry using JC-1 as described in the material and methods section. Interestingly, flow cytometry analysis (Fig 19) demonstrated the loss of mitochondrial membrane potential in response to the treatment with imiquimod when compared to HCl treated control cells, suggesting an important role of mitochondria in the modulation of imiquimod-induced apoptosis.

However, the pre-treatment with NF-κB-inhibitor alone was found to trigger mitochondrial membrane potential in melanoma cell. Whereas, the inhibition of NF-κB-pathway prior to the exposure of melanoma cells to imiquimod increased markedly the loss of mitochondrial membrane potential to 60% compared to 49% when melanoma cells treated alone with NF-κB-inhibitor or with imiquimod alone 37% (Fig 20). This results demonstrate the relevanz of combination therapy in melanoma treatment.

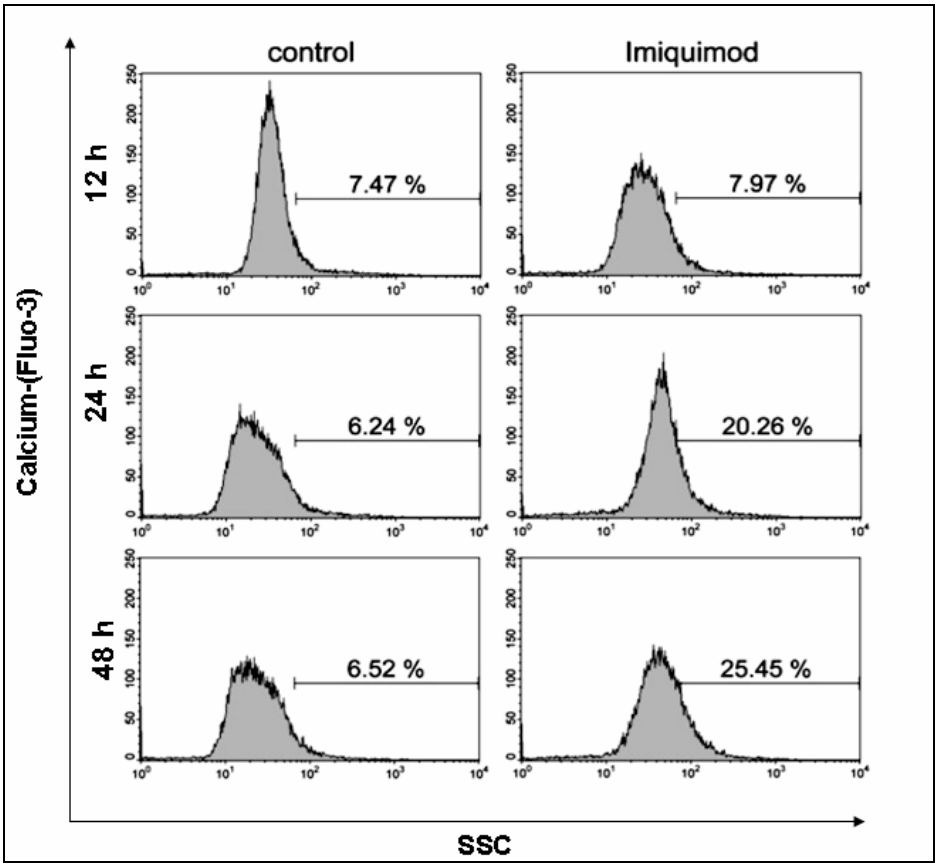


Fig 17: Release of intracellular calcium following the exposure of melanoma cell to imiquimod assessed by staining with Ca^{2+}-sensitive-dye-Fluo3-AM using flow cytometry. SSC: Side scattered cells). Data are representative of three independent experiments.

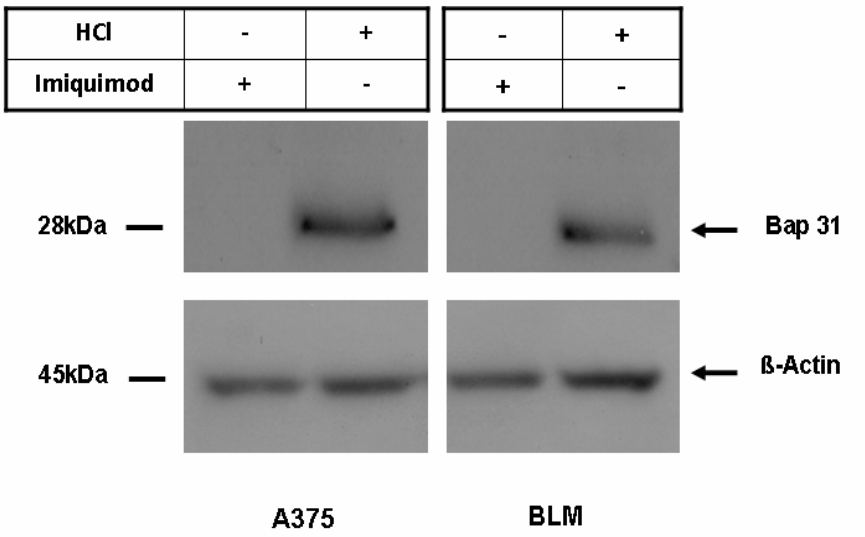


Fig 18: Detection of Bap-31 degradation in treated melanoma cells. A375 and BLM cells were treated with imiquimod up to 48 h. Western Blot was performed using anti-Bap 31 antibodies (ER marker) as described. Data are representative of three independent experiments.

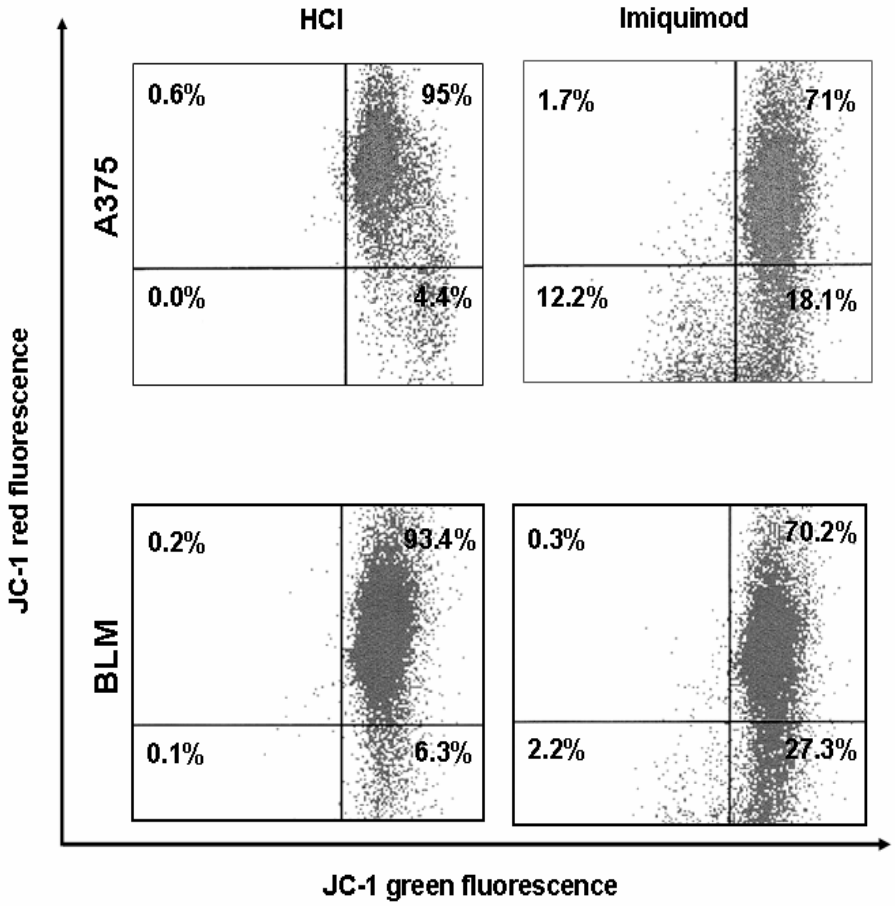


Fig 19: Loss of mitochondrial membrane potential in melanoma cells. A375 and BLM cells were treated with imiquimod and stained with JC-1 followed by flow cytometry analysis. Melanoma cells with intact mitochondria displayed high red and high green fluorescence and appeared in the upper right quadrant of the scatter plots. In contrast, cells that had lost their mitochondrial membrane potential displayed high green and low red fluorescence and appeared in the lower right quadrant.

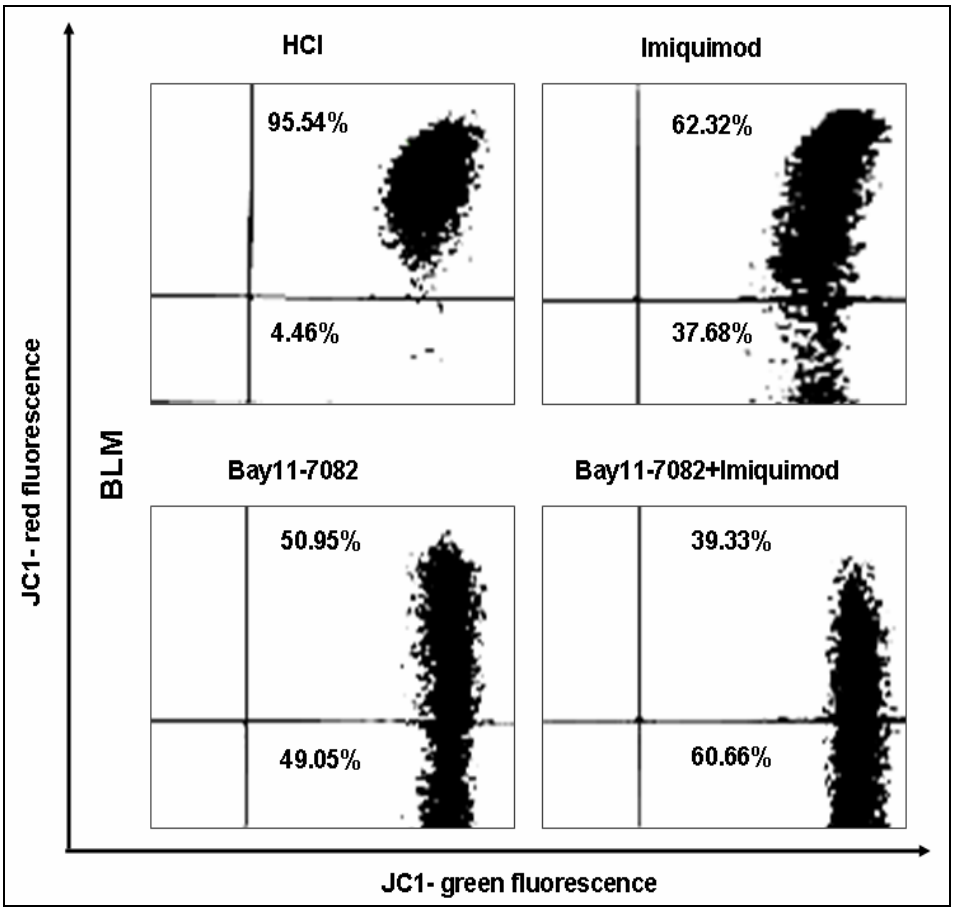


Fig 20: Loss of mitochondrial membrane potential in melanoma cells. BLM cells were treated either with imiquimod, with Bay11-7082 or with imiquimod and Bay11-7082 and stained with JC-1 followed by flow cytometry analysis. NF-κB inhibitor (Bay11-7082) enhances imiquimod-induced loss of mitochondrial membrane potential in melanoma cells. Melanoma cells with intact mitochondria displayed high red and high green fluorescence and appeared in the upper right quadrant of the scatter plots. In contrast, cells that had lost their mitochondrial membrane potential displayed high green and low red fluorescence and appeared in the lower right quadrant. Data are representative of three independent experiments.

3.5 Imiquimod-induced apoptosis is associated with ROS accumulation and activation of NF-κB pathway

Based on the fact that excessive ROS cause apoptosis through several mechanisms including disruption of mitochondrial membrane potential (Cai et al., 1998), we examined wether imiquimod-induced mitochondrial membrane damage results in accumulation of reactive oxygen species (ROS) in response to the treatment with imiquimod. The melanoma cell lines were treated with imiquimod and the accumulation of ROS (Fig 21) was assessed by flow cytometry analysis using dihydrorhodamine (DHR 123).

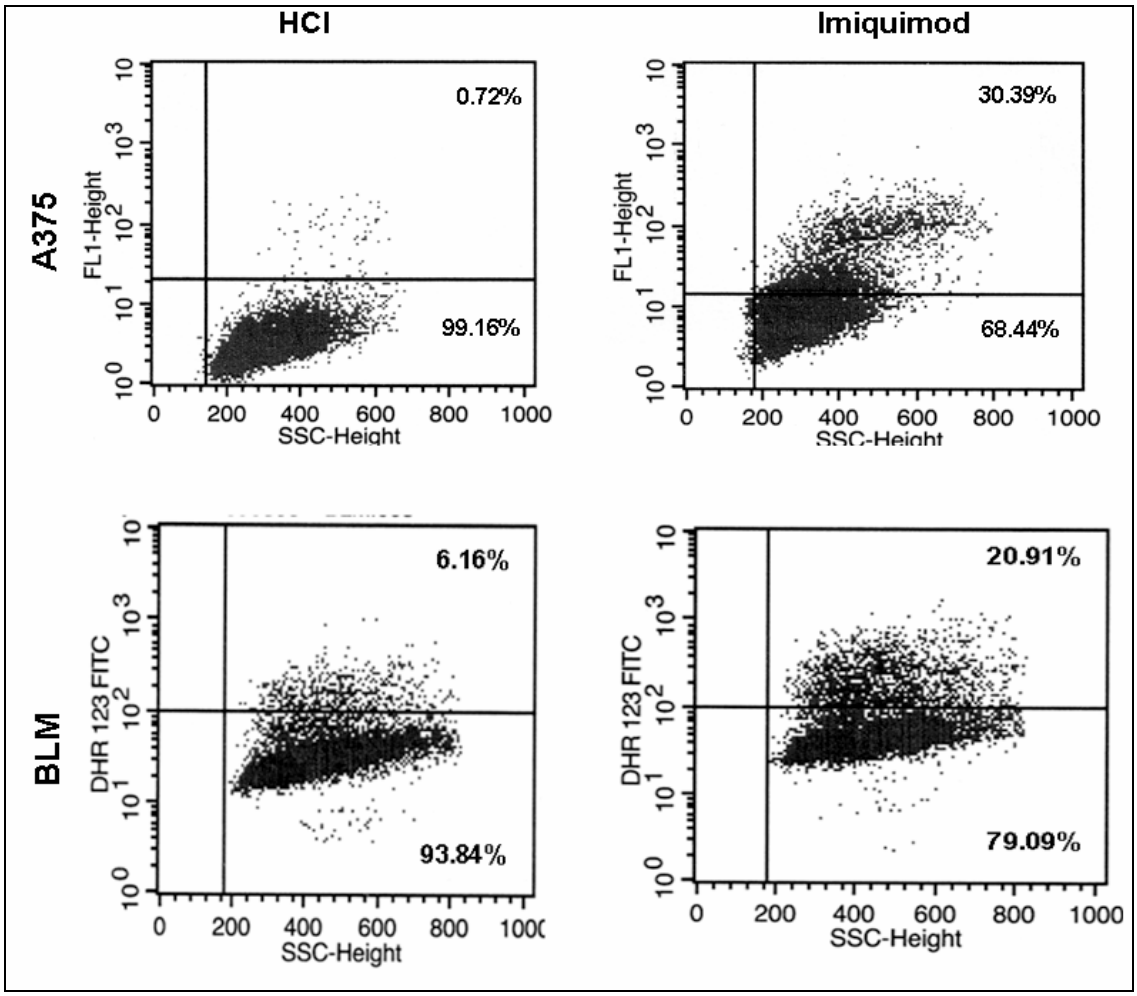


Fig 21: Imiquimod-induced accumulation of ROS in melanoma cells. A375 and BLM untreated- and treated-cells were analysed for ROS generation by flow cytometry using dihydrorhodamine (DHR 123). Data are representative of three independent experiments.

The activation of NF-κB by reactive oxygen species (ROS) is widely documented (Kaltschmidt et al., 1999). Thus, we examined whether NF-κB is activated in response to imiquimod-induced ROS accumulation in melanoma cells. The analysis of nuclear extracts prepared from imiquimod treated and untreated cells by EMSA assay (Fig 22) demonstrates the enhancement of the DNA-binding activity of the nuclear transcription factor NF-κB. In addition, further assays of treated and untreated cells by Western blot analysis and *in vitro* kinase assays confirmed the activation of NF-κB pathways. Data obtained from kinase assays demonstrated the activation of IKKα, whereas protein levels remained unchanged. In addition, Western blot analysis on cytoplasmatic protein extracts demonstrated the degradation of IκBα, the inhibitor of IKKα (Fig 23). Taken together, these data demonstrate the ability of imiquimod to trigger the NF-κB pathway in melanoma cells.

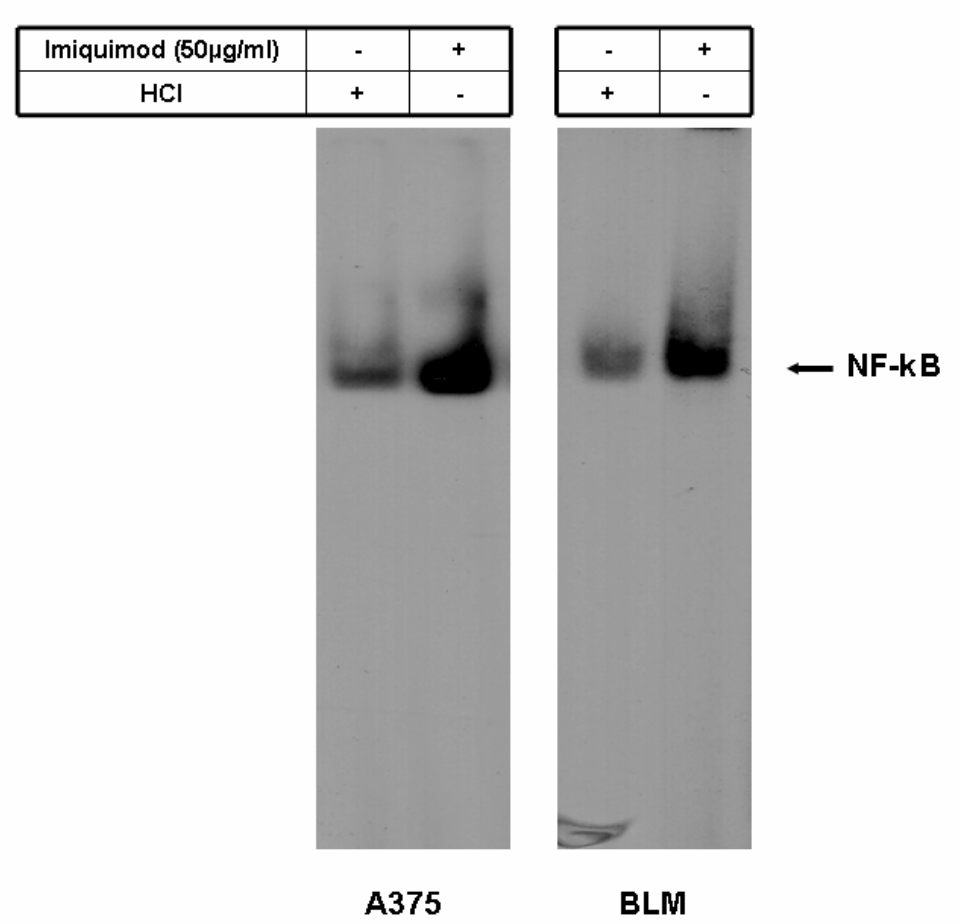


Fig 22: The analysis of nuclear extracts prepared from imiquimod treated and untreated cells by EMSA assay. EMSA demonstrates the activation of the nuclear transcription factor NF-κB by imiquimod. Data are representative of three independent experiments yielding similar results

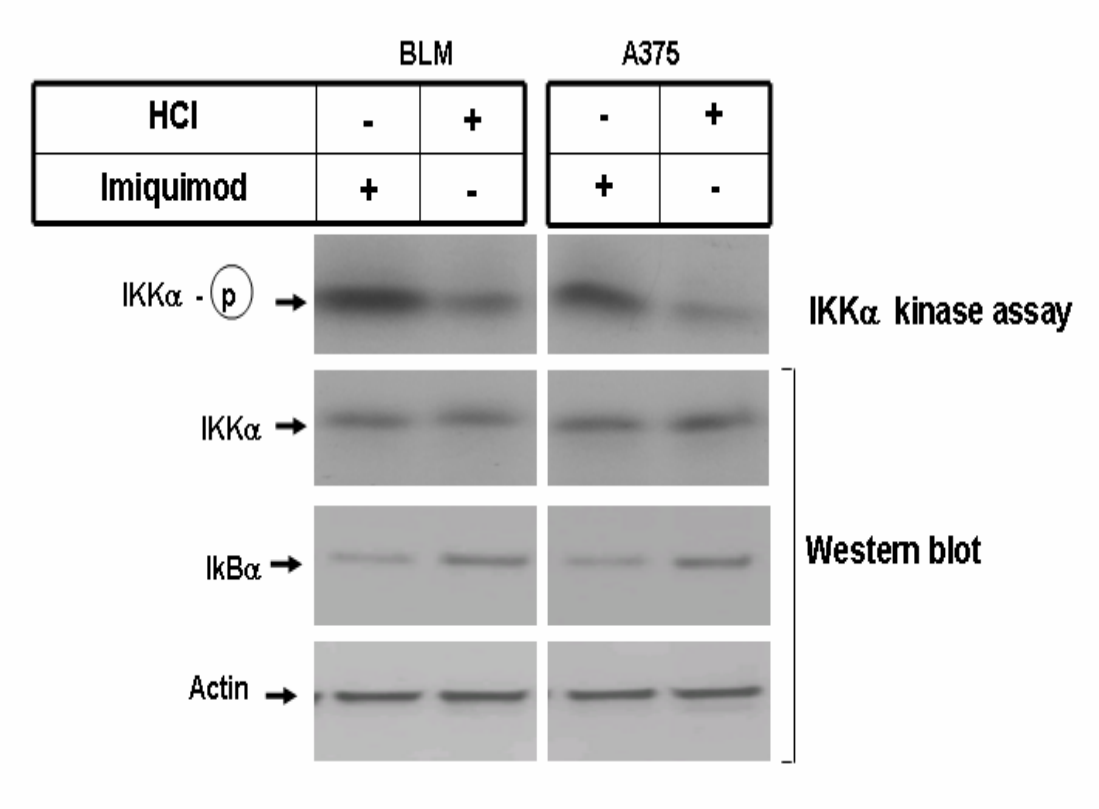


Fig 23: Western blot analysis demonstrated the expression levels of IKKα before and after the exposure to imiquimod. EMSA demonstrated the activation of IKKα and Western blot showed the degradation of IkBα.

3.6 Induction of XIAP protein in response to the treatment with imiquimod

To show whether XIAP, an inhibitor of apoptosis, is associated with imiquimod-induced activation of NF-κB pathway, the melanoma cell lines were treated with imiquimod. Western blot analysis of total cell extracts demonstrated the expression of XIAP, the target of NF-κB pathway (Fig 24). These data suggest the involvement of NF-κB activation in the regulation of XIAP protein in melanoma cells.

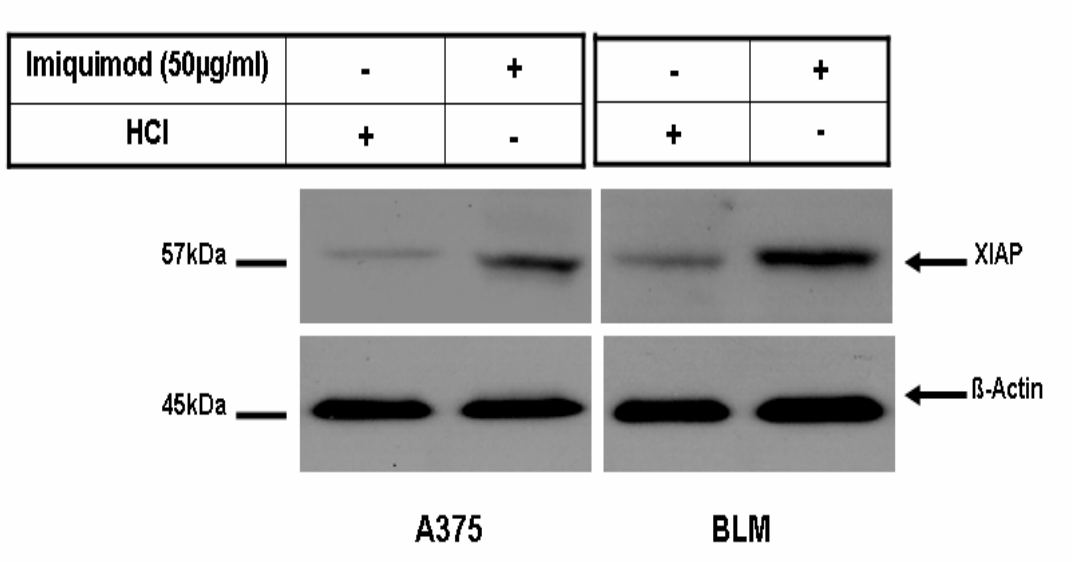


Fig 24: Imiquimod-induced effects on XIAP. Western blot analysis demonstrated the expression of XIAP in both A375 and BLM cells in response to imiquimod. Results are representative of three independent experiments with identical results.

3.7 Inhibition of ROS accumulation, NF-κB activation, or XIAP expression potentiates imiquimod-induced apoptosis in melanoma cells

To investigate, whether the inhibition of either ROS accumulation, or NF-κB pathway and subsequently its physiological substrate XIAP should influence imiquimod-induced apoptosis in melanoma cells, the melanoma cell lines were pre-treated either with Bay11-7082, the specific inhibitor of NF-κB, or with NAC, the specific inhibitors of ROS, or transfected with XIAP specific siRNA, before the exposure to imiquimod. For the indicated time (24 h), treated and control cells were subjected to either Western blot analysis, MTT assay, flow cytometry analysis using Annexin V/PI, or EMSA. Data obtained from Western blot analysis (Fig 25) demonstrated the inhibition of imiquimod-induced expression of XIAP in melanoma cells in response to the pre-treatment with Bay11-7082. Whereas, data obtained from EMSA (Fig 26) showed the inhibition of the DNA-binding activity of NF-κB in response to the pre-treatment with Bay11-7082.

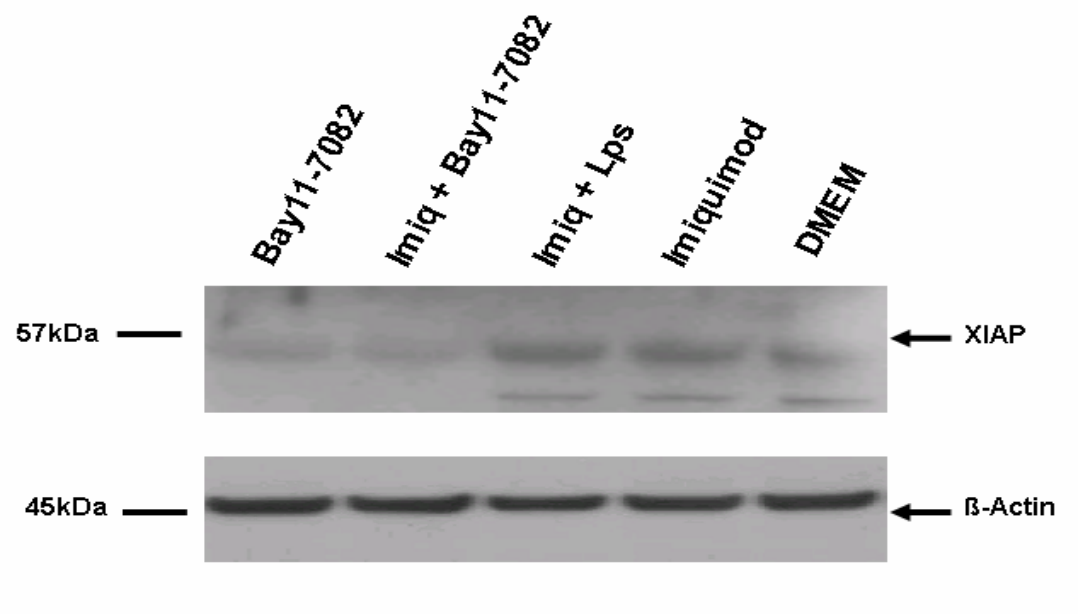


Fig 25: Inhibition of XIAP in melanoma cells in response to pre-treatment with Bay11-7082. Western blot analysis demonstrates the inhibition of XIAP in BLM melanoma cells in response to pre-treatment with Bay11-7082. Results are representative of two independent experiments with identical results.

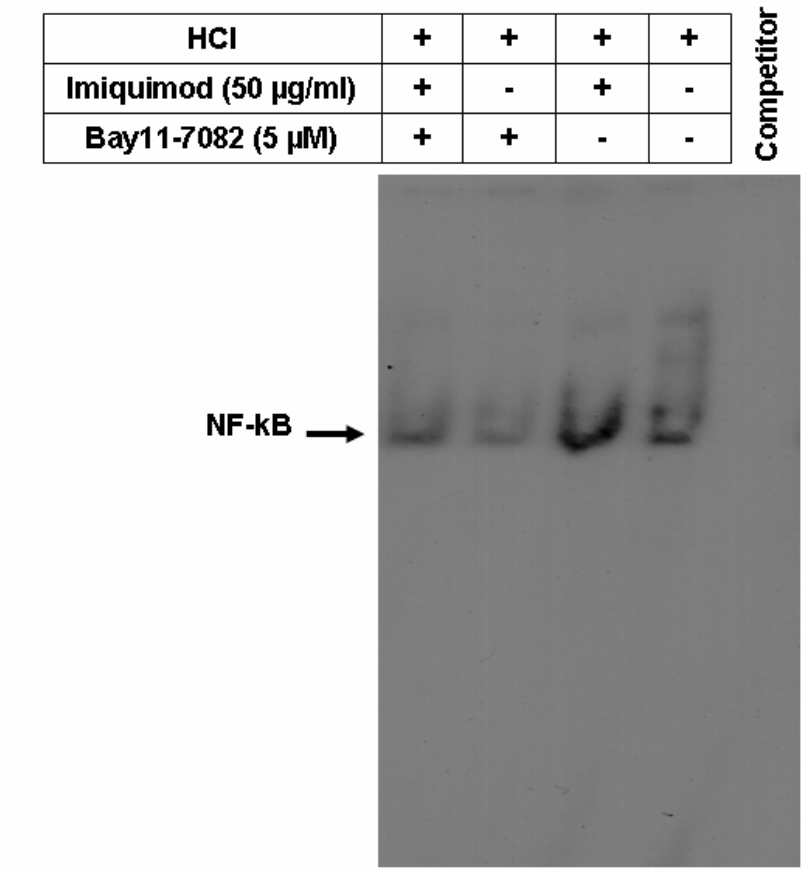


Fig 26: EMSA demonstrated the inhibition of the NF-κB when imiquimod was combined with Bay11-7082 compared with control cells. Data are representative of three independent experiments yielding similar results

In addition, the analysis of the staining with the Ca^{2+}-sensitive-dye-Fluo3-AM using flow cytometry demonstrated a significant increase of imiquimod-induced Ca^{2+} release (Fig 27), that was correlated with the enhancement of apoptosis induced (Fig 28). Furthermore, the inhibition of imiquimod-induced XIAP expression (Fig 29) together with the enhancement of cell death induced by the inhibitor of ROS accumulation (Fig 30) provides evidence for the involvement of ROS accumulation in the modulation of imiquimod-induced activation of NF-κB pathway. Also, the knockdown of imiquimod-induced-XIAP protein by its specific siRNA, as evidenced by Western blot (Fig 31), was found to enhance imiquimod-induced cell death in melanoma cells (Fig 32) suggesting the relevance of the combination of imiquimod with the inhibitor of NF-κB pathway in melanoma therapy.

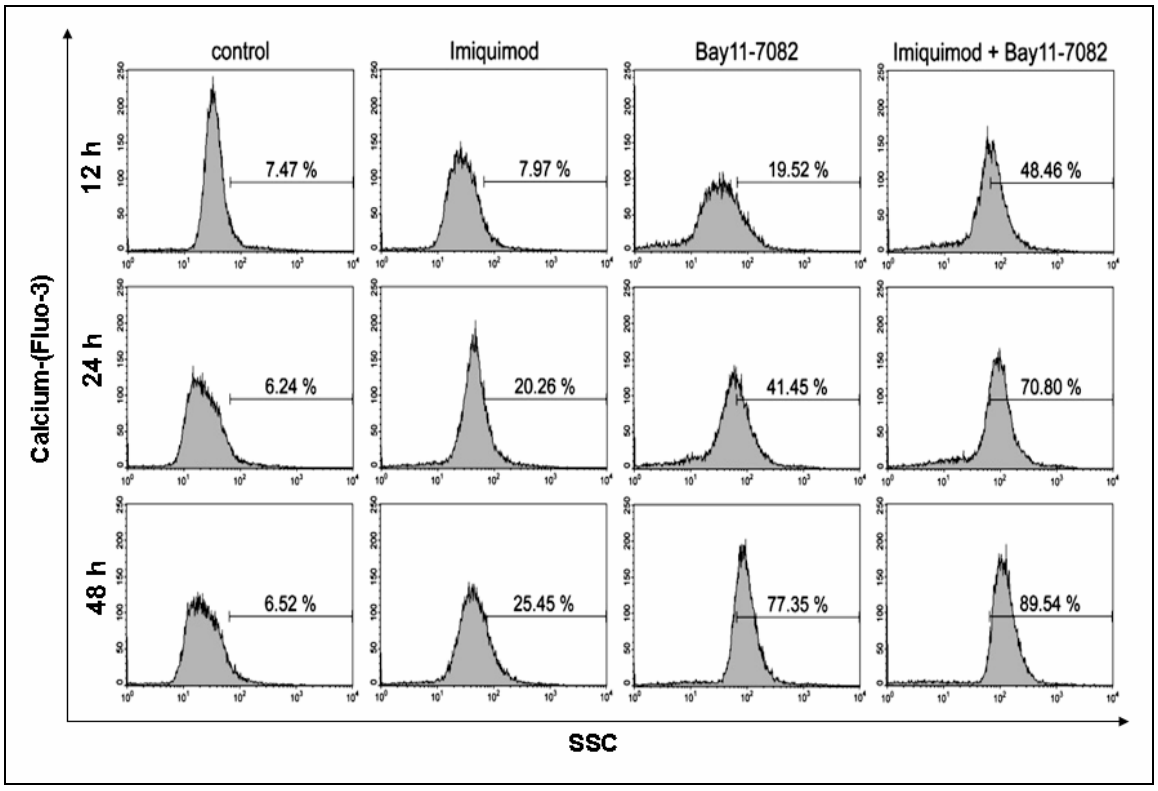


Fig 27: Release of intracellular calcium following the exposure of melanoma cell to imiquimod and to the combination of Bay11-7082 and imiquimod, assessed by staining with Ca^{2+}-sensitive-dye-Fluo3-AM using flow cytometry. SSC: side scattered cells. Data are representative of three independent experiments

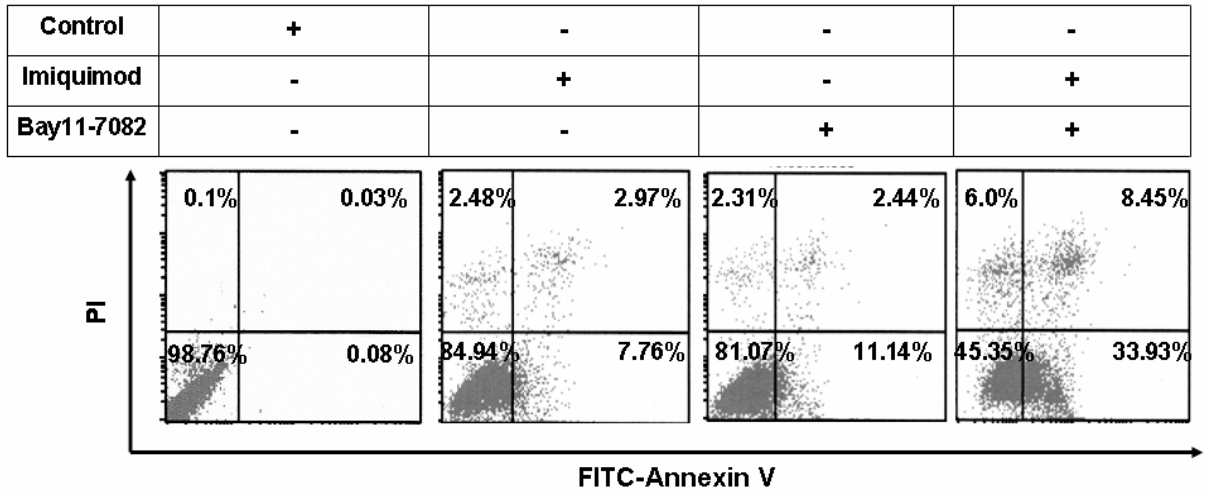


Fig 28: Detection of apoptosis in BLM melanoma cells following treatment with imiquimod and Bay11-7082. Melanoma cell lines were pre-treated with Bay11-7082 before incubation with imiquimod was performed for 24 h. The extent of apoptosis was determined by flow cytometry using annexin/PI staining. Data are representative of three independent experiments.

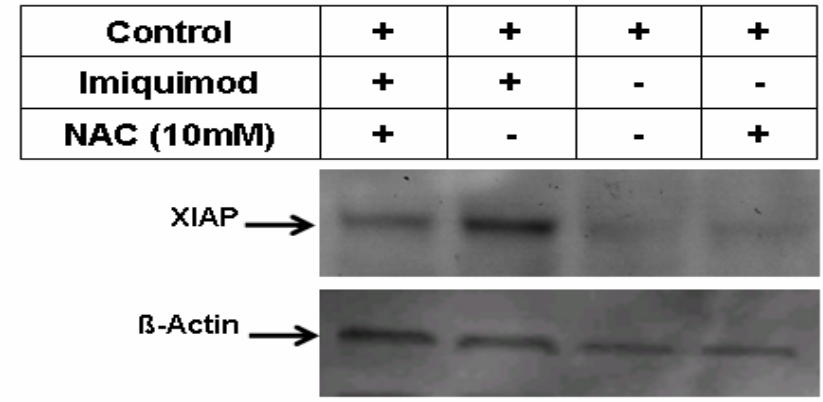
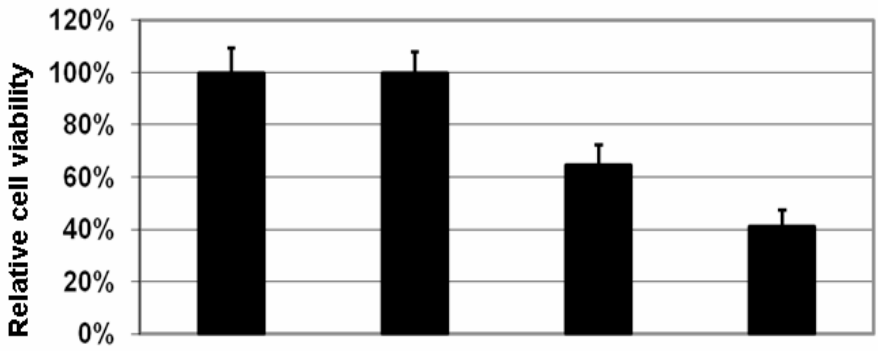


Fig 29: Inhibition of imiquimod-induced expression of XIAP by the inhibitor of ROS. ß-Actin was used as internal control for loading and transfer. Data are representative of three independent experiments performed separately



HCl	+	+	+	+
Imiquimod (50 µg/ml)	-	-	+	+
NAC (10 mM)	-	+	-	+

Fig 30: Enhancement of imiquimod-induced cell death in BLM melanoma cells in response to the pre-treatment with ROS-inhibitor (NAC). Data presented are the mean ± SD of three independent experiments performed in duplicate

Scramble	-	-	-	+
siRNA	-	+	+	-
Imiquimod	+	+	-	-

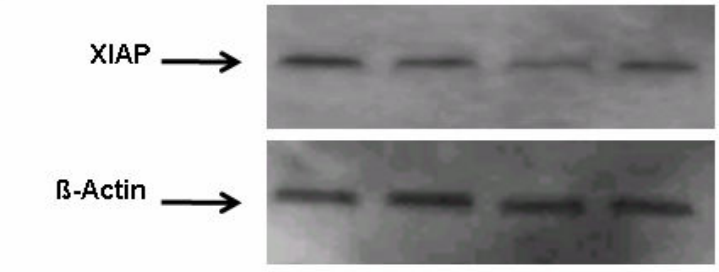


Fig 31: The abrogation of imiquimod-induced XIAP protein in BLM melanoma cells by siRNA. Actin was used as internal control for loading and transfer. Data are representative of three independent experiments performed separately.

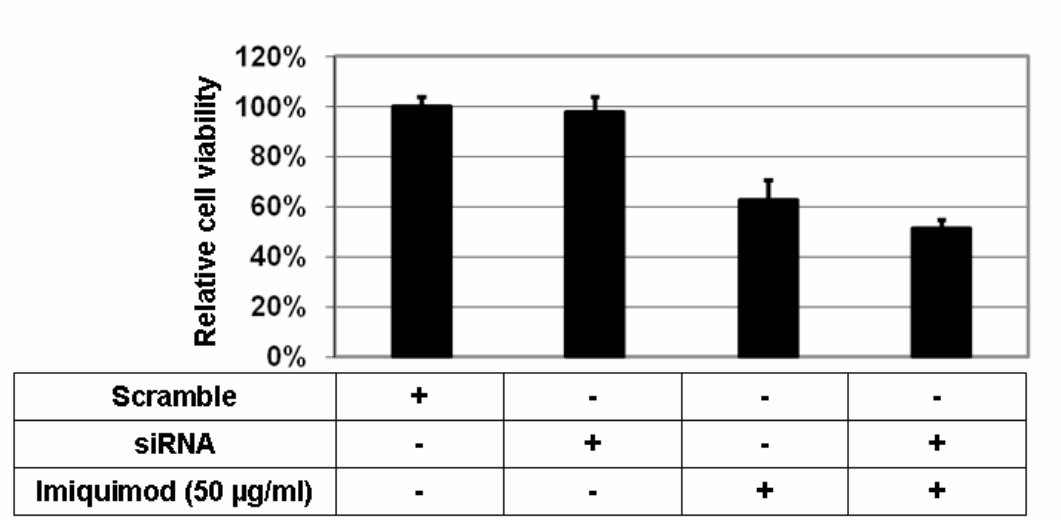
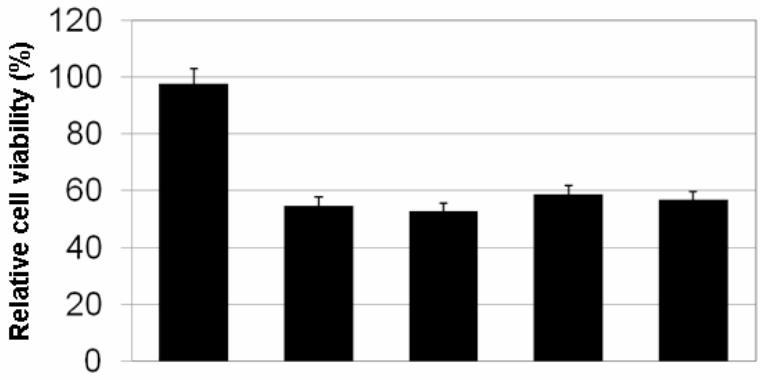


Fig 32: Enhancement of imiquimod-induced cell death in BLM melanoma cells by the knock down of XIAP protein.

3.8 Mitogen activated protein (MAP) kinase Signalling pathway is not involved in the modulation of imiquimod-induced-apoptosis

To investigate whether MAP kinase signalling pathways JNK, p38 and ERK are involved in the regulation of imiquimod-induced cell growth in melanoma cell lines, BLM cells were pre-treated with the specific inhibitors of the MAP kinase pathways JNK (SP600125), p38 (SB-203580) and ERK (U0126) 1h prior to the exposure to imiquimod for 24 h. Treated and control cells were subjected to cell viability assay. Based on the obtained data, the pre-treatment of both melanoma cell lines with the specific inhibitors of JNK (SP600125), p38 (SB-203580) or ERK (U0126) did not appear to influence imiquimod-induced cell death in melanoma cells (Fig. 33), suggesting that imiquimod-induced apoptosis in melanoma is mediated by a molecular mechanism independent from MAP kinase signalling pathway.



Control	+	-	-	-	-
Imiquimod	-	+	+	+	+
U0126 (10 µM)	-	-	+	-	-
Sp600125 (20 µM)	-	-	-	+	-
SB-203580 (20 µM)	-	-	-	-	+

Fig 33: Induction of apoptosis in melanoma cell lines BLM by imiquimod is MAP kinase signalling pathways JNK, p38 and ERK independent. MTT assay demonstrating no effect of the specific inhibitors of JNK (SP-600125), p38 (SB-203580), ERK (U0126) on the both melanoma cells viability after the treatment with imiquimod.

4. Discussion

The data obtained from this study demonstrated the ability of imiquimod and its analogues to kill melanoma cells by an apoptotic mechanism and provided insight into the molecular mechanism, which thought to be involved in the regulation of imiquimod-induced apoptotis in melanoma cells. Imiquimod-induced apoptosis of melanoma cells was found to be mediated by both mitochondrial and endoplasmic reticulum dysregulation. The mitochondrial damage was characterized by the loss of mitochondrial membrane potential ($\Delta\Psi m$), cytochrome c release and cleavage of caspase-3 and PARP. Whereas, endoplasmic reticulum dysregulation was characterized by the release of intracellular Ca^{2+} and degradation of Bap31 protein, as marker for ER stress (Hassan et al., 2008). In addition, this study demonstrated for the first time the reliability of combinatory therapy (combination of anticancer agents and pharmacological inhibitor of survival pathways), and did potentiate the use of targeted therapy in the treatment of malignant melanoma as evidenced by the combination of NF-κB inhibitor mit imiquimod.

Apoptosis is a highly conserved, innate mechanism through which eukaryotic cells undergo programmed cell-death. This mechanism permits the elimination of undesired or defective cells by an orderly cascade of cellular disintegration without inducing an inflammatory response (Schwartzman and Cidlowski, 1993). In addition to deregulated proliferation, substantial evidence indicates that proliferating tumors require anti-apoptotic mutations in order to survive and propagate (Evan et al., 1995). Therefore, the reconstitution of apoptosis in malignant cells by the inhibition of survival pathways is a consequent approach in cancer therapy.

Understanding the molecular events that contribute to apoptosis of tumor cells and how tumours evade apoptotic death might enable a more rational approach to anticancer therapy. Indeed, the ability of cells to evade apoptosis is considered an essential hallmark of cancer. One of the most important advances has been, therefore, the recognition that resistance to cell death is an important aspect of development of resistance to anticancer drugs (Soengas and Lowe, 2003). Much recent research on new cancer therapies is now focused on devising ways to overcome this resistance and to trigger apoptosis of tumor cells.

Imiquimod belongs to the class of Toll-like receptor (TLR) agonists that stimulate both adaptive-and innate immunity by interacting with TLR-7 that subsequently leads to

NF-κB-mediated transcription of pro-inflammatory genes, including TNF-α, IFN-α and IL-12 (Stary et al., 2007; Urosevic et al., 2005; Hemmi et al., 2002). Besides the activation of NF-κB pathway, imiquimod has been reported to trigger apoptosis *in vitro* und *in vivo via* a mechanism including mitochondrial pathway (Schön et al., 2004).

Toll-like receptors (TLRs), which recognize a variety of pathogen-associated molecular patterns, are centrally involved in the initiation of the innate and adaptive immune responses. Thus, TLRs play an essential role in the host defense against microbes by recognizing conserved bacterial molecules. The expression or up-regulation of TLRs has been detected in many tumour cell lines or tumors, especially epithelial derived cancers. Activation of TLRs in tumour cells promotes not only tumor cells proliferation and their resistance to apoptosis, but also enhances tumour cell invasion and metastasis by the regulation of metalloproteinases and integrins (Huang et al., 2008). Moreover, the activation of TLR signaling in tumour cells has been reported to induce the synthesis of pro-inflammatory factors and immunosuppressive molecules, which subsequently enhance the resistance of tumour cells leading to immune evasion (Huang et al., 2008). Although the TLR profile varies on different tumour cells, the current evidences indicate that the expression of TLRs is functionally associated with tumor progression (Yu and Chen., 2008). Thus, TLR expression may promote malignant transformation of epithelial cells. Currently, 13 mammalian-TLR analogues have been identified. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface; TLRs 3, 7, 8 and 9 are found almost exclusively within endosomes. Different TLRs exhibit specificity for pathogen-derived ligands; TLRs 2, 3, 4, 5, 7 and 9 recognize bacterial lipoproteins, double-stranded RNA/poly (I:C), lipopolysaccharides (LPS), flagellin, single-stranded RNA and CpG-containing DNA, respectively (Heil et al., 2004; Hayashi et al., 2001; Hemmi et al., 2000; Aliprantis et al., 1999; Poltorak et al., 1998). However, the ligands for TLRs 10, 12 and 13 remain unidentified.

In the present study, the enhancement of imiquimod-induced apoptosis of melanoma cells was demonstrated for the first time in response to the inhibition of NF-κB pathway (Bay11-7082) *via* a mechanism including both mitochondrial damage and endoplasmic stress. Imiquimod-induced apoptosis was found to be associated the

loss of mitochondrial membrane potential (ΔΨm), cytochrome c release, cleavage of PARP and intracellular Ca^{2+} release.

The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (Saelens et al., 2004). All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens et al., 2004). The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi. These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an “apoptosome” (Hill et al., 2004; Chinnaiyan, 1999).

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). Members of the TNF receptor family share similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain” (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways.

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution caspases that begins this phase of apoptosis. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Once

caspases are initially activated, there seems to be an irreversible commitment towards cell death. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or “executioner” caspases, cleaving various substrates including cytokeratins, PARP and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee et al., 2001). Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10).

The data of the present study revealed a potential role for ER in the regulation of imiquimod-induced effects in melanoma cells. The role of ER stress in tumour therapy is relatively unexplored at present, although the ER stress response is presumably important for regulating the balance between tumour cell death and growth as well as for the sensitivity to chemotherapeutic agents. Indeed, recent developments indicate that the ER is emerging as a new focal site for the initiation of endogenous cell death. The ER plays an important role in maintenance of intracellular calcium homeostasis, protein synthesis, post-translational modifications and proper folding of proteins as well as their sorting and trafficking. Alterations in calcium homeostasis and accumulation of unfolded proteins cause ER stress. A variety of different agents including chemical toxicants, oxidative stress, inhibitors of protein glycosylation, calcium homeostasis (Lee., 2001) or disturbed ER membrane integrity caused by proapoptotic proteins (Scorrano et al., 2003; Zong et al., 2003) can induce ER stress leading to cell death. Importantly, a recent report described sustained calcium accumulation in the mitochondrial matrix induced by ER stress as a new trigger of apoptosis by causing permeability transition, dissipation of the electrochemical potential, relocalization of BH3-only proteins to mitochondria and the release of Cytochrome c (Hassan et al., 2008).

The results obtained from this study reveal that the treatment of melanoma cells evokes a rapid increase of intracellular Ca^{2+} suggesting a central role for ER stress in imiquimod-induced apoptosis of melanoma cells as evidenced by the degradation of Bap 31, a marker for ER stress (Hassan et al., 2008). The endoplasmic reticulum (ER) is primarily recognized as the site of synthesis and folding of secreted,

membrane-bound, and some organelle-targeted proteins. Several factors are required for optimum protein folding, including ATP, Ca^{2+} and an oxidizing environment to allow disulphide-bond formation (Gaut and Hendershot, 1993). As a consequence of this specialist environment, the ER is highly sensitive to stresses that perturb cellular energy levels, the redox state or Ca^{2+} concentration. Such stresses reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress. Protein aggregation is toxic to cells and, consequently, numerous pathophysiological conditions are associated with ER stress (Kaufman.et al., 2002). Elevation of intracellular calcium is also known to induce oxidative stress including the uncoupling of mitochondrial respiration and permeability transition. Reactive oxygen species (ROS), including superoxide anion, H2O2 and hydroxyl radicals are by-products of oxidative phosphorylation that are constantly generated during metabolism (Adler et al., 1999). In addition to the induction of apoptosis, ROS has been widely reported to enhance the activation of NF-κB (Kim et al., 2008). In accordance, the activation of NF-κB pathway together with XIAP, the inhibitor of apoptosis, as well as the accumulation of ROS and the loss of ($\Delta\Psi$m) have been demonstrated in imiquimod treated cells.

Dissipation of the ($\Delta\Psi$m) is a general feature of apoptosis, irrespective of the cell type (neurons, fibroblasts, thymocytes, monocytes, hepatocytes, lymphocytes, tumour cells, etc.) and of the apoptosis inducer: toxins, suboptimal culture conditions, interventions on second messenger systems, ligation of cell surface receptors (Fas/Apo-1/CD95, TNF-R, etc), glucocorticoid receptor occupancy, or absence of obligatory growth factors (Hirsch et al., 1997; Kroemer et al., 1997). The ($\Delta\Psi$m) collapse constitutes an early event *vis-à-vis* the other manifestations of apoptosis detectable at the levels of the nucleus (chromatin condensation and DNA fragmentation, PARP cleavage), of the cytoplasm (activation of CPP32, shrinkage, calcium influx, and potassium efflux), or of the plasma membrane (phosphatidylserine exposure and later increase in permeability). Nonetheless, the ($\Delta\Psi$m) collapse marks an already irreversible stage of the apoptotic process (Zamzami et al., 1995).

The combination of NF-κB inhibitor together with imiquimod is considered for promising therapy for melanoma. The enhancement of imiquimod-induced apoptosis

by the combination of NF-κB inhibitors with chemotherapeutic agents may be worthwhile to be tested as an alternative approach to overcome melanoma resistance to conventional therapy.

The transcription factor NF-κB is widely reported to play an important role in carcinogenesis as well as in the regulation of immune and inflammatory responses. The NF-κB is sequestered in the cytoplasm in a complex with IκB. Almost all NF-κB activation pathways converge on IκB kinase (IKK), which phosphorylates IκB resulting in Lys 48-linked poly-ubiquitination of IκB and its degradation. This allows migration of NF-κB to the nucleus where it regulates gene expression. NF-κB induces the expression of diverse target genes that promote cell proliferation, regulate apoptosis, facilitate angiogenesis and stimulate invasion and metastasis. Moreover, the inhibition of NF-κB pathway by its specific inhibitor (Bay11-7082) increased imiquimod-induced apoptosis and blocked imiquimod-induced activation of NF-κB and subsequently suppression of apoptosis inhibitor, XIAP.

The observation that NF-κB plays a key role in melanoma cell survival (Bharti et al., 2002) was the reason to combine the pharmacological agents to study the killing efficiency in melanoma cells *in vitro.* One such agent is Bay 11-7082, an irreversible inhibitor of IκB phosphorylation, which blocks proteasomal degradation of IκB, allowing it to sequester NF-κB in the cytoplasm in an inactive state (Pierce et al., 1997). Although interventions that interrupt the NF-κB pathway may induce cell death by themselves, particularly in cells dependent on NF-κB survival signalling, (Keller et al., 2000; Moalli et al., 1992) accumulating evidence shows that they may also sensitize neoplastic cells to the lethal actions of conventional cytotoxic agents (Patel et al., 2000; Weldon et al., 2001). The present findings suggest that interruption of IKKα-NF-κB cascade in melanoma cells may also be particularly effective in potentiating apoptotic responses to novel agents, such as imiquimod that is reported to trigger apoptosis in different tumor types including basal cell carcinoma (Schön et al., 2004). The imidazoquinoline compound imiquimod activates immune cells *via* the Toll-like receptor 7-dependent signaling pathway.

In conclusion, besides the assessment of the killing efficiency of imiquimod and its analogues in melanoma cells, the present study suggest model (Fig 8) for imiquimod-

induced apoptosis of melanoma cells by a mechanism including both endoplasmic reticulum and mitochondrial dysregulation.

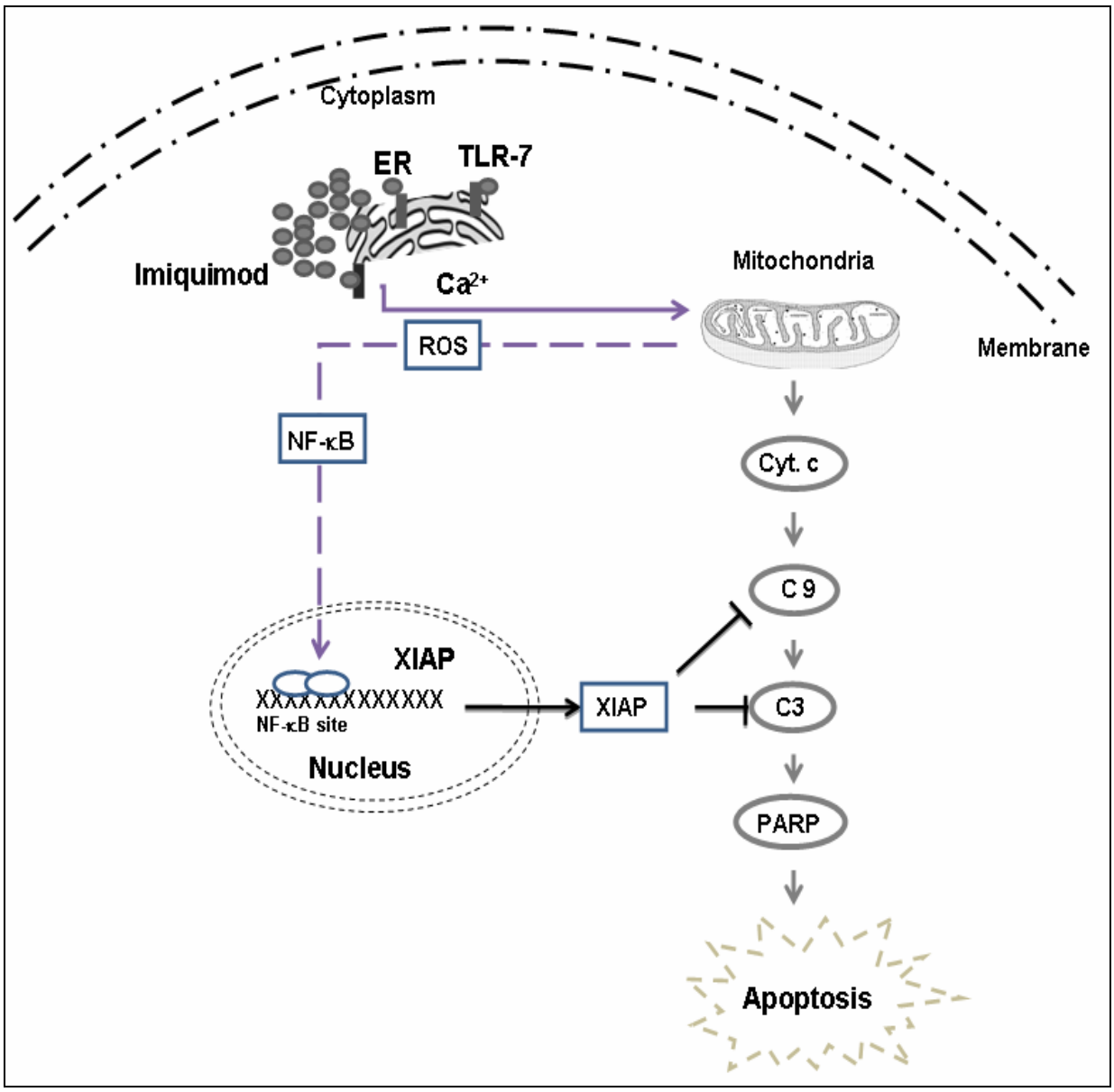


Fig 28: Model for imiquimod-induced apoptosis in melanoma cells. Upon binding of imiquimod to TLR7 results in the induction of ER stress that is characterized by Ca2+ release and Bap31 cleavage. The elevation of intracellular $Ca2^{+}$ release does trigger the loss of mitochondrial damage as evidenced by ROS accumulation, cytochrome c release and cleavage of both caspase-3 and PARP. On the other hand, the accumulation of ROS in melanoma cells mediates the activation of NF-κB pathway that subsequently does block, in part, imiquimod-induced apoptosis.

5. Summary

Melanoma is the most aggressive form of skin cancer. Patients with melanoma metastasis have 5-years survival rates of 50%-20%. This poor prognosis largely results from melanoma resistance to conventional therapy. Therefore, the aim of this study was to assess the killing efficiency of toll-like receptor agonists (imiquimod and its analogues) in melanoma cell lines and to provide insight into the mechanism, which is responsible for the regulation of imiquimod-induced cell death in melanoma cells. The data obtained from this study demonstrated the ability of imiquimod and its analogues to trigger cell death in melanoma cells by an apoptotic mechanism that is characterized by the induction of intracellular Ca^{2+} release, the loss of mitochondrial membrane damage ($\Delta\Psi$m), accumulation of reactive oxygen species (ROS), release of cytochrome c and cleavage of PARP. The characterization of imiquimod-induced effects in melanoma cells, in detail, demonstrated the ability of imiquimod to induce the activity of the IKKα-NF-κB pathway as evidenced by activation of IKKα, the kinase of NF-κB inhibitor (IκBα), as well as the enhancement of the DNA-binding activities of the transcription factors NF-κB. In addition, imiquimod was found to enhance the expression of XIAP protein, the inhibitor of apoptosis. Moreover, data obtained from inhibitor experiments using the specific inhibitor of NF-κB pathway (Bay11-7082) or XIAP-specific siRNA, suggesting a potential role for NF-κB inhibition in potentiating imiquimod-induced apoptosis of melanoma cells. Therefore, the reconstitution of apoptosis in malignant melanoma by the inhibition of survival pathways in combination with anticancer agents may have a consequent effect in melanoma therapy.

In conclusion, this study demonstrated for the first time the reliability of combinatory therapy and potentiated the application of targeted therapy in the treatment of malignant melanoma.

6. Zusammenfassung

Das maligne Melanom ist die bösartigste Form des Hautkrebs. Patienten in fortgeschrittenen Stadien mit metastierendem Melanomen haben innerhalb von 5 Jahren eine Überlebensrate von lediglich 20% bis 50%. Diese schlechte Prognose begründet sich in der Resistenz des Melanoms gegenüber herkömmlichen Therapien. Daher war das Ziel dieser Studie die Letalität des Toll-like Rezeptor-Agonisten Imiquimod und dessen Analoga in Melanom-Zellinien abzuschätzen und Erkenntnisse über den Mechanismus, der für die Regulierung des Imiquimod-induzierten Zelltods in Melanom-Zellen verantwortlich ist, zu gewinnen.

Die in der vorliegenden Studie erhobenen Daten zeigen daß Imiquimod und dessen Analoga den Zelltod in Melanom-Zellen durch einen apoptotischen Mechanismus auslösen. Dieser apoptotische Mechanismus ist durch die Freisetzung von intrazellulären Ca^{2+}-Ionen, dem Verlust des Potentials der mitochondrialen Membran ($\Delta\Psi m$), der Bildung reaktiver Sauerstoffspezies, der Freisetzung von Cytochrom-C und der Spaltung von PARP gekennzeichnet. Die weitere Analyse der Imiquimod-induzierten Effekte in Melanom-Zellen zeigt die Fähigkeit von Imiquimod, die Aktivierung des IKKα-NF-κB Signalwegs zu induzieren. Beweis dafür ist die Aktivierung von IKKα, der Kinase des NF-κB-Inhibitors (IκBα), sowie die Erhöhung der DNA-Ankopplungsaktivität des Transkriptionsfaktors NF-κB. Darüberhinaus konnte festgestellt werden, dass Imiquimod die Expression des XIAP-Proteins, dem Inhibitor der Apoptose erhöht. Außerdem zeigen die Daten, welche bei der Applikation des spezifischen Inhibitors des NF-κB-Signalwegs (Bay11-7082) sowie bei der Applikation von XIAP-spezifischer siRNA gewonnen wurden, die Notwendigkeit der Inhibition des NF-κB-Signalweges, um die von Imiquimod induzierte Apoptose zu erhöhen. Daher wird die Deregulierung der Apoptose in malignen Melanomen durch die Blockade von lebenswichtigen Signalwegen in Kombination mit Antikrebs-Agenzien einen entscheidenden Effekt in der Therapie von Melanomen darstellen.

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8. Appendix

Published articles:

Hassan M, **Alaoui A**, Feyen O, et al. (2008). The BH3-only member Noxa causes apoptosis in melanoma cells by multiple pathways. *Oncogene* Volume: 27 Issue: 33 Pages: 4557-4568

Nambiar S, Mirmohammadsadegh A, Hassan M, **Alaoui A,** et al. (2007). Identification and functional characterization of ASK/Dbf4, a novel cell survival gene in cutaneous melanoma with prognostic relevance. *Carcinogenesis* Volume: 28 Issue: 12 Pages: 2501-2510

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Alaoui A, Hassan M, Feyen O, et al. (2009). Inhibition of NF-kappa B pathway potentiates imiquimod-induced apoptosis in melanoma cell. *Exp Dermatol* Volume: 18 Issue: 3 Pages: 313-313

Mirmohammadsadegh A, Hassan M, **Alaoui A**, et al. (2009). Demethylation of Line-1 DNA in melanoma. *Exp Dermatol* Volume: 18 Issue: 3 Pages: 315-316

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Manuscripts under review:

Alaoui A, Hassan M, Feyen O, Essmann F, et al. Inhibition of NF-kB signaling potentiates imiquimod-induced apoptosis in melanoma cells *(In Review process/ J of Immunol)*

A. Alaoui, S. Santroulidis, F. Essmann, M. Hassan. Induction of indoleamine-2, 3-dioxygenase by death receptor activation in melanoma cells is regulated by IFN g-independent mechanism and triggers apoptosis by mitochondrial damage-dependent ROS accumulation *(In Review process/ Oncogene)*

EIDESSTATTLICHE ERKLÄRUNG

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbst verfasst wurde und dass ich keine anderen als die von mir angegebenen Hilfsmittel verwendet habe. Alle Stellen, die aus anderen Werken im Wortlaut oder dem Sinn entsprechend übernommen wurden, habe ich mit Quellenangaben kenntlich gemacht.

Düsseldorf, 04. Mai 2009 ----------

(Amine Alaoui)