# Immune Expression and Inhibition of Heat Shock Protein 90 in Uveal Melanoma

*by*

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*Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.*
*Albert Einstein*

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# *Abstract*

## Immune expression and inhibition of Hsp90 in Uveal Melanoma

Uveal melanoma (UM) is the most common intraocular tumor in adults. Despite the increase in diagnostic accuracy over the past several decades and the development of eye saving treatments such as plaque radiotherapy and photon beam therapy, there was no significant change in the prognosis in uveal melanoma due to metastatic disease. Therefore, we started to search for novel therapeutic targets in UM, such as heat shock protein 90 (Hsp90). This protein is a chaperone for several proteins (clients) involved in signal transduction and cell cycle control like kinases and transcription factors and plays a key role in regulating their stability and function. Hsp90 is expressed by a variety of tumor types and its inhibition results in the down regulation and proteasomal degradation of its client proteins.

The objective of this study was to examine the immunohistochemical profile of Hsp90 and its association with prognostic features in uveal melanoma. In addition, we studied the role of Hsp90 in promoting growth and survival of uveal melanoma and the molecular consequences of inhibiting Hsp90 function by the small-molecule 17-allylamino-17-demethoxy-geldanamycin (17-AAG).

Hsp90 expression was studied in five primary human uveal melanoma cell lines (92.1, OCM-1, MKTBR, SP6.5 and UW-1) by Western blot analysis and immunofluorescence methods. Using immunohistochemical methods, we assessed Hsp90 expression in 44 specimens of human UM, 14 specimens from an animal model of UM and also in metastatic specimens from patients and an animal model.

In addition we determined the cytotoxicity profile of five primary UM cell lines and one metastatic derived cell line (OMM-1.5) after exposure to Hsp90 inhibitor 17-AAG. Sulfurhodamine-B based proliferation assay was used to compare UM cell growth with a range of concentrations of 17-AAG from 100μM to 0.001μM. Further we studied changes in cell migration and invasion in the presence or absence of 17-AAG. Apoptosis and cell cycle fractions were determined by flow cytometry. The expression of intracellular proteins was determined using Western blot analysis.

Hsp90 was expressed in all five UM cell lines. Immunohistochemical expression of Hsp90 was identified in 68 % of the paraffin embedded sections from patients and 100% of paraffin embedded sections from primary tumors of an animal model of UM. Also Hsp90 was expressed in 87% of 8 metastatic specimens from patients with UM and in 77% of 9 specimens of an animal model of UM. The expression of the Hsp90 in primary tumor in UM patients was significantly associated with largest tumor dimension, in the Hsp90 positive tumors.

Inhibition of Hsp90 function by 17-AAG caused a dose-dependent growth inhibition of primary and metastatic UM cell lines and a statistically significant reduction on the proliferation rate with concentrations of 100 μM to 1 μM. 17-AAG induced cell cycle arrest with accumulations of cells in $G_1$ and loss of cells in S phase which was associated with a decrease in cyclin-dependent kinase CDK4. Also, 17-AAG induced apoptosis by caspase-dependent mechanisms with a significant increase in caspase 3-protease activity after drug exposure. The cytotoxic effect of 17-AAG was associated with decreased levels of Akt and phospho-Akt after 24 h exposure to the drug. Exposure to 17-AAG significantly reduced the migratory and invasive capabilities of all 5 uveal melanoma cell lines. 17-AAG down regulated c-Met and IGF-1R receptor transmembrane tyrosine kinases and also inhibited activation of focal adhesion kinase (FAK).

For the first time in UM, we demonstrate that over expression of Hsp90 in patients is linked to an indicator of poor prognosis.

To the best of our knowledge, this is the first report showing the effect of Hsp90 inhibitor 17-AAG, on proliferation of UM cells which induced cell cycle arrest and apoptosis. Downregulation of p-Akt in response to Hsp90 inhibition could represent one possible mechanism of modulation of the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway in uveal melanoma.

Also, we demonstrated for the first time that inhibition of Hsp90 function had an effect on UM cells motility and invasive potential and in cellular processes involved in metastasis in uveal melanoma.

This data indicates that UM is a suitable target for modulation of Hsp90 activity and that Hsp90 inhibitors are possible therapeutic modalities for UM patients. Prospective studies are needed to confirm the prognostic role of Hsp90 in UM and further trials in uveal melanoma models should be undertaken to study the effect of inhibition of Hsp90 *in vivo*.

# L'expression immunitaire et inhibition de Hsp90 dans le mélanome uvéal

Le mélanome uveal (UM) est la tumeur intraoculaire primitive la plus fréquente chez les adultes. Malgré de l'augmentation de la précision du diagnostic au cours des dernières décennies et le progrès dans le traitement local du mélanome uveal telles que des traitements de radiothérapie et l'irradiation par faisceau de protons le taux de mortalité de UM n'a pas beaucoup changé, et plus de 50% des patients atteints de UM décèdent de métastases tardives. Par conséquent, nous avons commencé à la recherche de nouvelles cibles thérapeutiques dans l'UM, tels que les protéines de choc thermique 90 (Hsp90). Hsp90 est une protéine chaperon indispensable à l'activation et la régulation d'un ensemble important de protéines de la signalisation et de la régulation cellulaire comme les protéines kinases et les facteurs de transcription. Elle et est abondamment exprimé par une grande variété de types de tumeurs et l'utilisation d'inhibiteur spécifique de Hsp90 entraîne une baisse de l'activité des protéines clientes ainsi que leur dégradation par le protéasome.

L'objectif de cette étude était d'examiner le profil immunohistochimique des Hsp90 et son association avec les caractéristiques pronostiques dans mélanome de l'uvée. En outre, nous avons étudié le rôle qu'elle joue dans les mécanismes cellulaires de prolifération et la survie de mélanome de l'uvée et les conséquences de l'inhibition de la fonction de Hsp90 par 17-allylamino-17-demethoxy-geldanamycin (17-AAG).

L'expression immunohistochimique des Hsp90 a été étudiée dans cinq lignées de cellules tumorales in-vitro (92,1, BGC-1, MKTBR, SP6.5 et UW-1) par Western blot et méthodes d'immunofluorescence. L'expression de Hsp90 a été évaluée dans 44 spécimens l'UM humaine et 14 spécimens d'un modèle animal de l'UM et dans des spécimens provenant de lésions métastatiques humaines et animales. En outre, notre étude a visé à comparer les effets antiprolifératifs du 17-AAG sur les lignées cellulaire cancéreuse d'UM humaine et dans une lignée de cellules tumorales dérivées d'une lésion métastatique (OMM-1,5). La prolifération cellulaire a été évaluée par le test à la sulforhodamine B sur les cellules exposées à une gamme de concentrations de 17-AAG entre 0,001μM au 100 μM.

En outre, nous avons étudié les changements dans la migration et l'invasion cellulaire en présence ou l'absence de la 17-AAG. Le processus apoptotique et les fractions de cycle cellulaire ont été déterminés par cytométrie en flux. Analyse de l'expression de Hsp90 et l'expression de protéines intracellulaires a été déterminée par l'analyse Western blot.

Hsp90 a été exprimé dans les cinq lignées de cellules d'UM. L'expression immunohistochimique des Hsp90 a été identifiée dans 68% des coupes en paraffine des patients et dans 100% de spécimens d'un modèle animal d'UM. Aussi Hsp90 a été exprimée dans 87 % des spécimens métastatique chez des patients avec UM et dans 77% des spécimens d'un modèle animal de l'UM. L'expression de l'Hsp90 dans la tumeur primaire a été significativement associés à la plus grande dimension de la tumeur ($p = 0,03$).

L'inhibition de la fonction Hsp90 par 17-AAG a entraîné une inhibition de croissance dose-dépendante dans les lignées de cellules primaire et métastatiques d'UM avec une réduction statistiquement significative entre 100 μM to 1 μM. 17-AAG induit l'accumulation des cellules en G1 et la perte de cellules en phase S, qui a été associée à une diminution de la kinase CDK4. En outre, 17-AAG induisent l'apoptose dans les cellules d'UM et une augmentation de l'activité de la caspase-3. L'effet cytotoxique de la 17-AAG a été associé à une baisse des niveaux d'Akt et phospho-Akt, 24 h après l'exposition à la drogue.

L'exposition à la 17-AAG a considérablement réduit les capacités migratoires et invasives des les cinq lignées de cellules d'UM. L'évaluation de Hsp90 inhibiteur sur les processus cellulaires impliqués dans l'invasion a montré que 17-AAG provoquent une baisse des niveaux de récepteurs tyrosine kinases transmembranaires c-Met et IGF-1R et aussi a également réduit l'expression de la kinase d'adhérence focale (FAK).

Pour la première fois en UM nous démontrer que l'expression de Hsp90 chez des patients est liée à un indicateur de pronostic défavorable et la modulation de cette protéine chaperon par agents inhibiteurs tels que la 17-AAG, devrait être considérée comme une cible de choix pour développer des agents anti-tumoraux dans UM. Nos résultats apportent des informations originales sur les effets cytotoxiques de 17-AAG sur la prolifération des cellules d'UM qui induit un arrêt du cycle cellulaire et l'apoptose. Le diminution de p-Akt en réponse à Hsp90 inhibition pourrait constituer un mécanisme possible de la modulation de la phosphatidylinositol 3'-

kinase (PI3K) - Akt signalisation de mélanome de l'uvée.

L'expression de Hsp90 dans métastases et l'évaluation des processus cellulaires impliqués dans l'invasion après l'inhibition de la fonction de Hsp90 suggéré l'utilité des inhibiteurs de Hsp90 dans la prévention de la progression tumorale et les métastases.

L’ensemble de nos résultats montre que le ciblage Hsp90 mai constituer une nouvelle approche thérapeutique pour les patients avec le mélanome de l'uvée. Des études prospectives sont nécessaires pour confirmer le rôle pronostique de l'Hsp90 dans UM et des essais supplémentaires visent à prouver l'efficacité de l'inhibition de Hsp90 in vivo.

# List of Abbreviations

AHA1: Activator of HSP90 ATPase homologue 1

BCL2: B-cell leukemia/lymphoma 2

BCL-X-L: B-cell leukemia/lymphoma x

BRAF: V-raf murine sarcoma viral oncogene homolog B1

BOLD: (dacarbazine, lomustine, vincristine and bleomycin)

CDK: Cyclin-dependent kinases

CDC37: cell-division-cycle 37

CHIP: carboxyl-terminal HSP70-interacting protein

COMS: Collaborative Ocular Melanoma Study Group

COX: Cyclooxygenase

CXCR4: Chemokine C-X-C motif receptor 4

CXCL12 (Scattered Derived Factor-1, SDF-1)

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinase

pERK: phosphorylated ERK

FAK: Focal adhesion kinase

FRNK: FAK-related nonkinase

GA: geldanamycin

GRP94: glucose related protein 94

HGF: Hepatocyte growth factor

HDAC: Histone deacetylase

HER2: Human epidermal growth factor receptor 2

HOP: Hsp70 / Hsp90-organizing protein

HSF1: heat shock factor 1

Hsps: heat shock proteins

Hsp70: Heat shock protein70

Hsp90: Heat shock protein 90

HIF: hypoxia inducible factor

IGF-1: Insulin like growth factor 1

IGF-R: Insulin growth factor receptor

IL-2: interleukin 2

INF-α: interferon-alpha

KDa: kilodalton

KISS1: Kisspeptin-1

MAPK: Mitogen-activated protein kinase

MDM2: Mouse double minute 2, also termed HDM2

MMP: Matrix metalloproteinases

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NQO1: NADH quinone oxidoreductase 1

PPP: Picropodophyllin

PI3K: phosphatidylinositol-3-kinase

RAF: Recombinant activated factor

Rb: retinoblastoma

RT-PCR: real time – polymerase chain rection

SF: scatter factor

Si-RNA: small interfering RNA

TIMP: Tissue inhibitors of MMPs

TRAP1: TNF receptor-associated protein 1

TTT: Transpupillary thermotherapy

uPA (urokinase plasminogen activator)

17AAG: 17-allylaminogeldanamycin

# Table of Contents

# *Chapter 1*

## Introduction

Uveal melanoma is the most common primary intraocular malignancy. Despite earlier diagnosis and treatment of the primary tumor, systemic prognosis has not improved due to already disseminated disease (Egan, Seddon et al. 1988). Although only 2% to 3% of patients present with detectable metastases at the time of diagnosis, early micrometastatic dissemination may occur several years before diagnosis and treatment of primary tumor (Eskelin, Pyrhonen et al. 2000). The development of metastatic disease represents the major challenge in uveal melanoma. Median survival after clinical detection of metastases is approximately 9 to 12 months in 10% to 15% of cases (Kath, Hayungs et al. 1993; Kivela, Eskelin et al. 2006). However, systemic therapy did not show significant influence on survival due to the high resistance to chemotherapeutic agents (Bedikian 2006). Therefore, development of an effective treatment of metastatic uveal melanoma is essential.

Current anticancer drug development involves the detection of novel molecular targets which are significant for the development of the tumor. One promising target intensely researched in other malignancies is heat shock protein 90 (Hsp90). Heat shock protein 90 is an intracellular chaperone that contributes to protein homeostasis through protective mechanisms such as anti-aggregation, intracellular translocation and protein conformational maturation (Buchner 1999; Workman, Burrows et al. 2007). In tumors, Hsp90 stabilizes many mutated proteins that mediate cell transformation, facilitating the evolution of the neoplastic process (Neckers and Ivy 2003).

This molecular chaperone has been shown to be over-expressed in a number of malignancies and cumulative data suggest that it is part of the mechanism that allows neoplastic cells

to escape normal regulation (Whitesell and Lindquist 2005). Hsp90 client proteins include tyrosine kinases receptors, components of signaling transduction pathways kinases such as Src, Akt and Raf-1 or cell-cycle regulators (CDK4, CDK6) and transcription factors, (Burrows, Zhang et al. 2004). Many of the Hsp90 clients are mutated or deregulated proteins relevant for uveal melanoma cell proliferation and survival.

In the present thesis, we wanted to understand the physiological significance of Hsp90 in uveal melanoma as well some of the consequences of Hsp90 inhibition with respect to substrates involved in the progression of primary tumor and development of metastases. Understanding the roles of Hsp90 in regulating the function and stability of uveal melanoma provides a unique opportunity for the development of therapeutic strategies based on Hsp90 inhibition.

# Literature Review

## *1.1 Uveal melanoma*

### 1.1.1 Overview of Uveal Melanoma

The uveal tract (Lat. *uva*, grape), the pigmented middle layer of the eye, is comprised of the iris, ciliary body and choroid. These layers contain melanocytes which are derived from the-neural crest. The most common intraocular tumors are the melanocyte derived nevi, which are benign lesions, or metastases from other malignancies in the body. Uveal melanoma represents the malignant transformation of the melanocytes or of a primary dysplastic nevus in the uveal tract. The choroid is highly vascularized and is the most common site for the development of uveal melanoma. About 80% of uveal melanomas arise in the choroid, 15% involve the choroid and the ciliary body, while 5% affect the iris (Li, Judge et al. 2000). Because the interior part of the eye lacks lymphatics, uveal melanoma can progress from the eye to systemic metastatic disease exclusively through hematogenous dissemination.

### 1.1.2 Epidemiology

Uveal melanoma is the primary intraocular malignancy in adults and has an increased incidence particularly in Caucasian population with 1200–1500 new cases per year. Uveal melanoma has revealed a lower incidence, between 15 to 50% , in individuals of African and Asian descent (Singh and Topham 2003).

A meta analysis study found that risk factors include light skin color, blonde hair and blue eyes. There is also a slight male predominance (Weis, Shah et al. 2006). This disease is very rare in childhood but the incidence increases in people 50 to 60 years old. Uveal melanoma cases represent approximately 5–12% of all melanomas but in comparison with cutaneous melanoma the incidence of uveal melanoma did not increase in the last two decades (Egan, Seddon et al. 1988; Singh and Topham 2003).

### 1.1.3 Host and environmental factors in uveal melanoma

Choroidal nevi are benign lesions derived from melanocytes and are relatively common being found in 3 to 20% of the population. A small number of choroidal nevi (one in 15000 per year) transform to malignant uveal melanoma. Some conditions such as dysplastic nevus syndrome and congenital oculodermal melanocytosis, a condition in which the number of melanocytes is congenitally increased, could predispose individuals to the development of uveal melanoma (Egan, Seddon et al. 1988) and (Hammer, Olah et al. 1996). Rare familial cases have been reported, but there is no evidence of a single gene causing the disease or familial inheritance in these cases. As opposed to cutaneous melanoma, ultra-violet (UV) light exposure as a risk factor remains controversial (Shah, Weis et al. 2005). Recently, in vitro studies introduced blue light (UVB) exposure as a new factor involved in the proliferation of uveal melanoma cell lines (Marshall, Gordon et al. 2006; Di Cesare, Maloney et al. 2009).

### 1.1.4 Genetic aspects

Uveal melanoma usually occurs sporadically. Although familial uveal melanoma has been described in 0.6% of patients, there is no established inherited predisposition. However, 1% of all uveal melanomas can occur in patients less than 20 years old and rare bilateral or multifocal cases have been reported, suggesting a genetic predisposition in these cases (Singh, Damato et al. 2005).

Different phenotypic associations have been linked to uveal melanoma, for example oculodermal melanocytosis, a condition that appears 35 times more frequently in uveal melanoma patients than in general population (Gonder, Shields et al. 1982). It is characterized by congenital hyperpigmentation of the periocular skin, and uvea and may represent a genetic predisposition to uveal melanoma (Egan, Seddon et al. 1988). Recently, GNAQ mutations were discovered in both blue nevi (83%) and uveal melanomas (46%), representing the first detected oncogenic mutation in this cancer (Van Raamsdonk, Bezrookove et al. 2009).

Other phenotypic associations have been described, for example with the "Familial Atypical Mole and Melanoma syndrome", (FAMMM), where dysplastic nevus syndrome has been associated with a predisposition to both cutaneous and ocular melanoma (Rodriguez-Sains 1994).

Another association is neurofibromatosis type 1 (NF1), an autosomal dominant neurocutaneous disorder a co in which 18 cases of uveal melanoma have been reported.

### 1.1.5 Diagnostic of uveal melanoma

Uveal melanoma can grow from the choroid towards the center of the eye and gain access to the subretinal space in a pathognomonic "mushroom shape" (Fig 1.1 and 1.2). Extra-ocular extension is less common because of the scleral resistance to local invasion. Invasion in the orbit can occur near the optic nerve or through intrascleral vessels. Another type of growth which is uncommon is represented by diffuse uveal melanoma which has a flat shape and could invade large parts of the choroid. It has been hypothesized that the estimated doubling time in uveal melanoma is between six months to more than four years, with a median of 1.4 years (Eskelin, Pyrhonen et al. 2000).

At early stages, uveal melanoma is usually asymptomatic and could be discovered during a routine clinical examination through direct or indirect ophthalmoscopy appearing as a solid elevated, usually pigmented tumor. In some of the cases, if the tumor does not involve the sub-macular area, the patient could be asymptomatic. If the tumors are associated with a secondary retinal detachment the patient may experience visual symptoms like blurred vision, photopsia or visual field loss. Routine follow-up of patients with choroidal nevi is important for early identification of uveal melanoma (Shields, Furuta et al. 2008) but early differentiation between large nevi and small choroidal melanomas could be challenging (McLean, Burnier et al. 1944). In some cases, an orange-colored lipofuscin pigment can be associated with the growth of suspicious nevi.

**Fig. 1.1** Clinical picture of uveal melanoma showing a well circumscribed elevated lesion

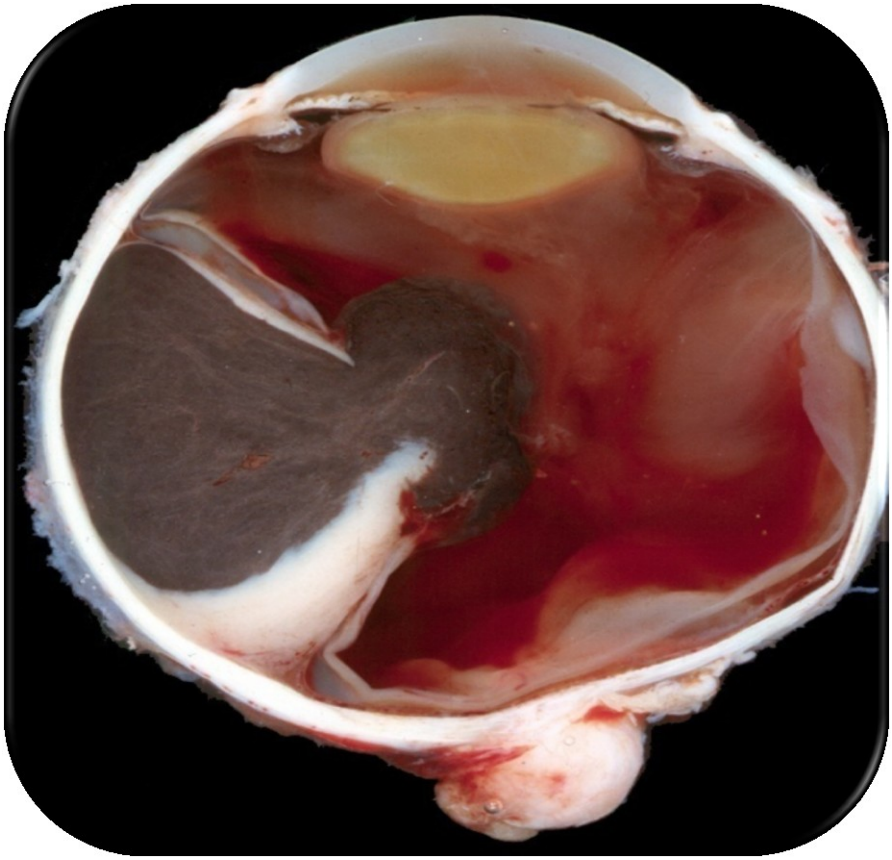


**Fig.1.2** Macroscopic view of "mushroom shape" uveal melanoma

Up to 99% of large and medium sized uveal melanomas are diagnosed by indirect ophthalmoscopy. Ultrasonography is the gold standard method for measuring tumor dimensions and detecting extraocular extension (Char, Stone et al. 1980) and high frequency ultrasonography detects ciliary body tumors. Fluorescein angiography, computerized tomography, and magnetic resonance imaging could also be used for further tumor assessment. Fine needle aspiration biopsy, through an intravitreal or transscleral route, could be performed in challenging cases for diagnostic purposes (Augsburger, Correa et al. 2002).

### 1.1.6 Melanoma progression; circulating malignant cells and metastases

At the time of diagnosis, the majority of patients are identified as having limited intraocular disease but despite advances in the treatment of the primary tumor, the mortality rates have not significantly changed due to metastatic disease.

Early micrometastases could disseminate several years before diagnosis and treatment of primary tumor (Eskelin, Pyrhonen et al. 2000). Therefore, patients with uveal melanoma are followed routinely with liver function tests and chest x-ray examinations (Diener-West, Reynolds et al. 2004). Approximately 60% of patients with metastasis are asymptomatic, and 30% have normal liver function tests. Such patients can be screened adequately by liver imaging, abdominal ultrasonography and computerized tomography (CT) which were introduced as sensitive methods in screening for metastatic disease.

Based on tumor doubling time studies, it has been estimated that micrometastases from uveal melanoma could disseminate approximately 5 years before the intraocular tumor is detected and treated and metastatic lesions could manifest even 10 years after enucleation (Eskelin, Pyrhonen et al. 2000). Logan et al (Logan, Fernandes et al. 2008), established the feasibility of single-cell

dormancy in an animal model, showing the viability of injected cells 6 weeks after injection. In uveal melanoma patients, Callejo et al (Callejo, Antecka et al. 2007), determined that using reverse transcription-PCR (RT-PCR) for tyrosinase and MelanA/MART1 in multiple blood samples, detection of disseminated tumor cells was possible in all 30 uveal melanoma patients analyzed. Further analysis of individual RT-PCR results revealed both tyrosinase and MelanA/MART1 transcripts as independent prognostic factors (Schuster, Bechrakis et al. 2007).

Metastatic disease occurs in up to 50% of patients, exclusively by vascular spread and the liver is the first site of systemic metastasis in approximately 71.4% to 87% of cases (Kujala, Makitie et al. 2003). Systemic disease occurs commonly 2 to 5 years after treatment of the primary tumor (Gragoudas, Egan et al. 1991; Sato, Babazono et al. 1997) , one-third of the metastases developing after 5 years and some after several decades (Shields, Augsburger et al. 1985). Thirty percent of uveal melanoma patients will die of systemic metastases at 5 years of follow-up and 45% at 15 years (Singh and Topham 2003).

The clinical course of patients with liver metastases is unfavorable with limited treatment options. Ten to 15% of the patients with metastatic disease have an estimated survival time of one year (Kath, Hayungs et al. 1993; Kivela, Eskelin et al. 2006). A slightly different course is reported for patients with extrahepatic metastatic melanoma with a median survival of approximately 19 to 28 months (Hsueh, Essner et al. 2004; Bedikian 2006).

There is a critical need to define risk factors for systemic metastasis to evaluate novel therapeutic modalities for uveal melanoma patients.

### 1.1.7 Prognostic factors

Several clinical and histopathological prognostic factors have been identified for uveal melanoma and the most important include: tumor location, tumor dimension, ciliary body involvement, the presence of extrascleral extension, and epithelioid cell type.

TUMOR LOCATION

Ciliary body involvement carries a poor prognosis and was found to predict a 1.6 to 2.3 higher chance of metastasis compared with posterior choroidal involvement (Li, Gragoudas et al. 2000; Kujala, Makitie et al. 2003). Choroidal tumors with an anterior location within the ciliary body are linked to decreased survival and could involve the anterior chamber angle of the eye, spread into subconjunctival tissue through an emissary canal or the trabecular meshwork and cause lymph node involvement (Dithmar, Diaz et al. 2000) (Fig.1.3 and1.4). Iris melanomas have a better prognosis and rarely metastasize, having a 10 year mortality rate of 5% (Shields, Shields et al. 2001).

Extension through the sclera can occur along ocular vessels such as the posterior ciliary arteries and vortex veins. Among the posterior tumors, those located in a juxtapapillary location frequently invade the orbit adjacent to the optic disc and carry a worse prognosis (McLean, Burnier et al. 1984).

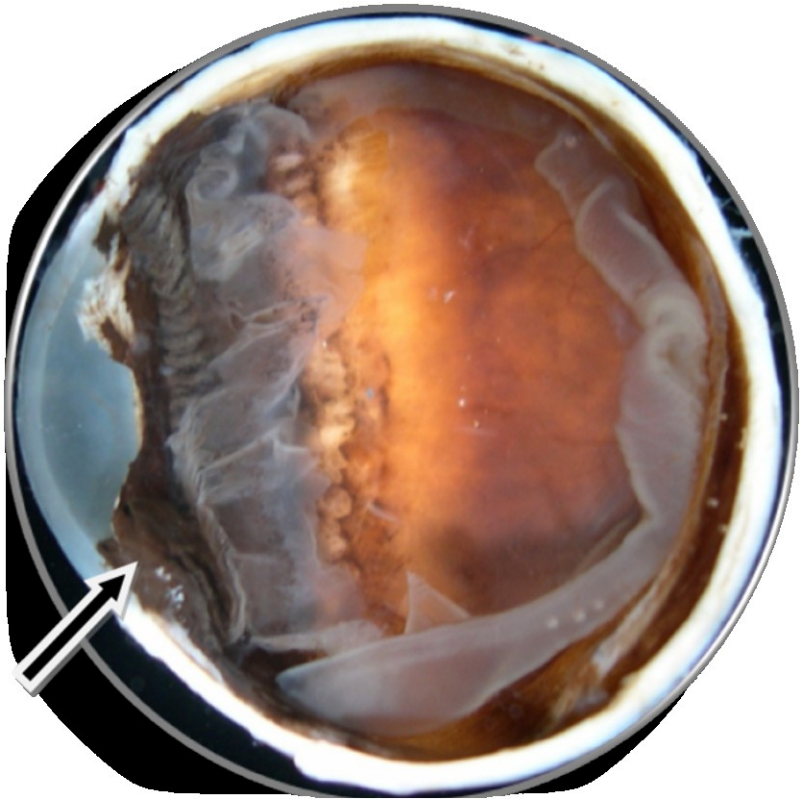


**Fig. 1.3.** Ciliary body melanoma with anterior chamber involvement (arrow).

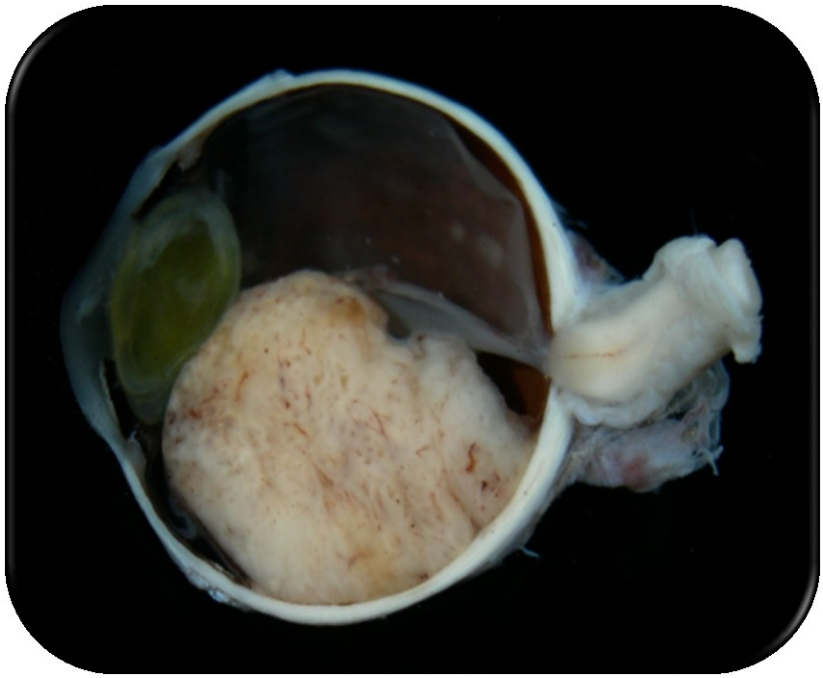


**Fig.1.4.** Gross pathology of amelanotic choroidal melanoma

## TUMOR SIZE

The size of the tumor has been identified as one of the most consistent prognostic factor for metastasis in uveal melanoma (McLean, Foster et al. 1982; COMS 1997). Tumor size is determined

by indirect ophthalmoscopy and B-scan ultrasonography and takes into consideration the largest basal tumor diameter and tumor height (Fig.1.5). Taking the largest basal diameter in consideration, melanomas can be classified as small when less than 10 mm, medium between 10 and 15 mm and large when more than 15 mm. The 10-year mortality rate is 60% among patients with large tumors and between 10 to 15% in small melanoma cases. Choroidal melanomas thickness also correlates with prognosis with a 5-year survival rate of 47% when the tumor is more than 7-8 mm in thickness (Singh, Shields et al. 2001). Clinical features, such as largest basal tumor diameter, ciliary body involvement and extraocular growth are important risk factors for increased tumor recurrence and dissemination (COMS 1997).

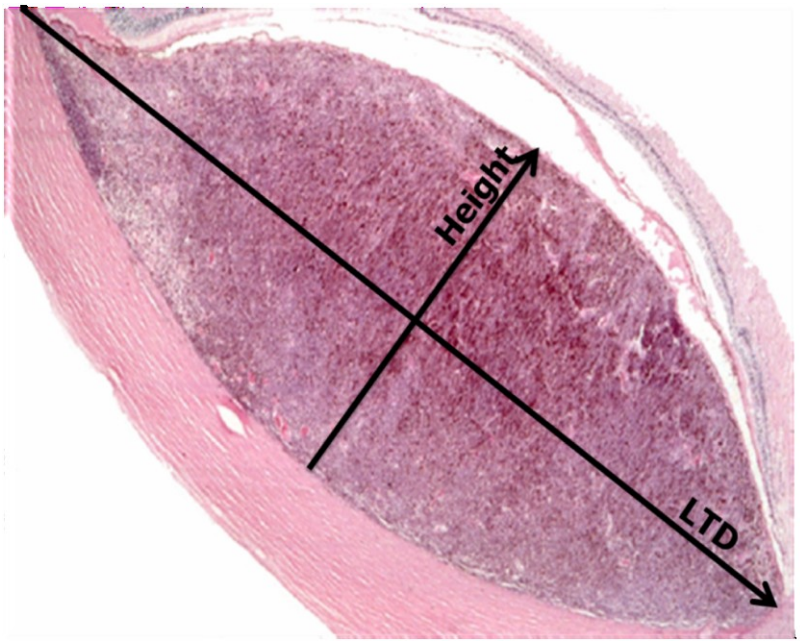



**Fig. 1.5** Tumor size: height and largest tumor dimension

## CELL TYPE

Uveal melanoma derives from the malignant transformation of the melanocytes present in the uvea. One of the most important histological prognostic factors is tumor cell type. Based on the shape and cytological characteristics, the cells are classified as spindle or epithelioid (McLean, Foster et al. 1983). The spindle cells in tumors grow in a cohesive, compact form and have elongated nuclei (Fig.1.6). These cells were originally classified by Callender as of two types -A and B (Gamel and McLean 1977). Spindle A cells, have small nuclei with a chromatin stripe along the nucleus. The spindle B cells present more cytoplasm and a single round nucleolus. Another type of cells found in uveal melanoma tumors are larger, polygonal cells with a round or oval nucleus and abundant cytoplasm. Because they resemble epithelial cells, they are called epithelioid cells (Fig 1.7). Epithelioid tumors are less cohesive and carry worse prognostic potential compared to predominantly spindle tumors (McLean, Foster et al. 1982). Tumors are classified as of mixed type when composed of spindle and epithelioid cells (McLean, Keefe et al. 1997). The majority of uveal melanoma tumors present a mixed type of cellularity, being composed of spindle and epithelioid cells. Recent studies using micro-array technology offered the ability to study the epithelioid tumors and found that this cell type is a predictor of metastasis (Chang, Worley et al. 2008).

Other cytomorphometric factors such as the mean of the largest ten nucleoli are of prognostic importance in different multivariate analyses (McLean, Keefe et al. 1997).

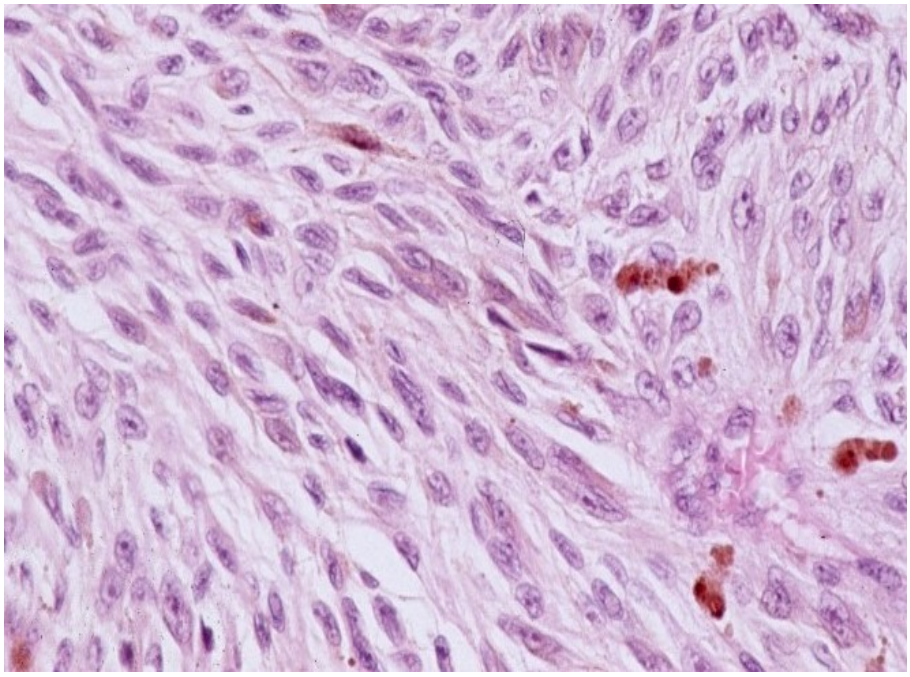


**Fig. 1.6.**
Spindle uveal melanoma cells

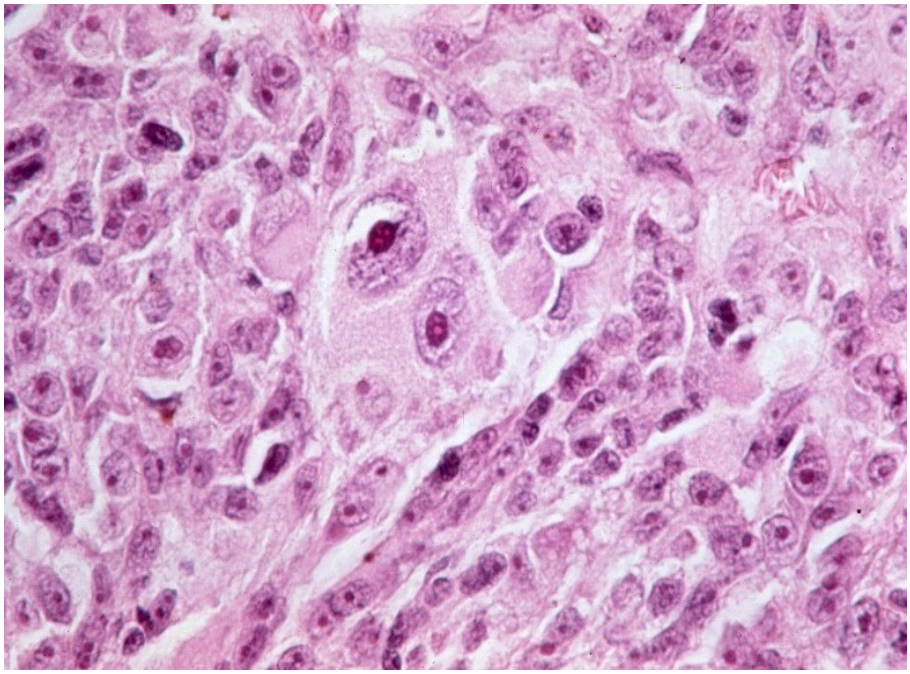


**Fig. 1.7.**
Epithelioid uveal melanoma cells

## Index of Proliferation

The evaluation of tumor histology identified that a high number of mitoses predicts decreased survival in uveal melanoma (McLean, Burnier et al. 1984). High rate of proliferative activity of the tumor cells is indicated by cells positive for PC-10 and Ki-67, and has been associated with increased 10-year melanoma specific mortality (Seregard, Oskarsson et al. 1996).

## Vascular density

The presence of high microvessel density or invasion of tumor cells into the lumen of tumor blood vessels is associated with decreased survival (Al-Jamal, Makitie et al. 2003). Some tumors are characterized by a particular vascular loop architecture, or "vascular mimicry", consisting of intratumoral channels or loops with PAS-positive basement membranes but lacking endothelial cells. The histological characterization of the microcirculation patterns led to the association of six pattern types with poor prognosis (Folberg, Rummelt et al. 1993).

## Lymphocytic and macrophage infiltration

Spontaneous regression of uveal malignant melanoma is rarely reported and the presence of an immune response in this tumor (Lambert, Char et al. 1986). The presence of infiltrating lymphocytes may modulate the immune activity in uveal melanoma and have been associated with decreased survival rates (de Waard-Siebinga, Hilders et al. 1996). Infiltration by macrophages has been associated with an increase in 10-year mortality rate (Makitie, Summanen et al. 2001), but other studies report contradictory conclusions regarding their biological role in tumor growth (Toivonen, Makitie et al. 2004).

## CYTOGENETIC FACTORS AND CHROMOSOMAL ABNORMALITIES

Uveal melanoma is characterized by genetic instability and deregulation of the cell cycle and both aneuploidy and accumulation of cells in the S-phase of the cell cycle have been correlated with poor prognosis in uveal melanoma (Hodge, Duclos et al. 1995). Chromosomal changes were found to play an important role in the progression of uveal melanoma. Uveal melanoma is characterized by genetic instability and deregulation of the cell cycle and both aneuploidy and accumulation of cells in the S-phase of the cell cycle have been correlated with poor prognosis in uveal melanoma (Naus, van Drunen et al. 2001; Worley, Onken et al. 2007). Several recurrent chromosomal changes are observed in uveal melanoma. The most frequent abnormalities, loss of one copy of chromosome 3, gain of the long arm of chromosome 8, or chromosome 6 abnormalities, are of prognostic value (Prescher, Bornfeld et al. 1992; Singh, Damato et al. 2005). Less frequent changes were reported by other groups, including anomalies in chromosomes 1p, 7q, 9p and 13q (Gordon, Thompson et al. 1994; Marshall, Nantel et al. 2007). The most frequent finding is monosomy 3 which has been observed in approximately 50% of cases (Prescher, Bornfeld et al. 1990; Lohmann, Hausler et al. 2005). Monosomy 3 appears frequently in tumors with gain of chromosome 8 and both changes have a higher incidence in epithelioid tumors arising from the ciliay body. In different studies, monosomy 3 has been shown to be a better predictive factor than clinical and histopathological factors (Sisley, Rennie et al. 1997; White, Chambers et al. 1998). Also, the metastatic lesions show partial deletions and loss of heterozygosity of chromosome 3 indicating the possible involvement of a tumor suppressor gene on this chromosome (Tschentscher, Prescher et al. 2001). One gene on chromosome 3, TGF-β R2 located at 3p21-22, was linked to progression of uveal melanoma (Myatt, Aristodemou et al.

2000). The genes involved in chromosome 8 amplifications could be present in the amplified regions 8q21.1-21.2 or 8q23-24, and one possible candidate is the MYC oncogene. However, the extent of amplification of this gene is unknown and the prognostic role of MYC in uveal melanoma is controversial (Royds, Sharrard et al. 1992; Chana, Cree et al. 1998). However, the extent of amplification of this gene is unknown and the prognostic role of MYC in uveal melanoma is controversial (Ehlers, Worley et al. 2005).

### 1.1.8 Treatment of uveal melanoma

TREATMENT OF PRIMARY TUMOR

The treatment of the primary tumor prevents further growth of the tumor and is employed in order to prevent metastatic disease. Until the late 1960's, enucleation, the removal of the affected eye, was the only treatment for uveal melanoma. This procedure is indicated for large tumors, anterior segment invasion, optic nerve involvement and extrascleral extension.

With the development of modern techniques, plaque radiotherapy (brachytherapy) became the most common procedure for local treatment of uveal melanoma. This modality involves high-dose radiation, delivered locally through a shielded plaque over the tumor (Shields, Naseripour et al. 2002). Although the rates of local recurrences are low, visual acuity is affected in 25% to 35% of cases and some of the therapeutic complications include neovascular glaucoma, cataract, radiation retinopathy and optic neuropathy (Puusaari, Heikkonen et al. 2004).

Another treatment modality is transpupillary thermotherapy (TTT), in which tumor cell necrosis is induced using an infrared diode laser through the pupil (Oosterhuis, Journee-de Korver et al. 1998). Visual acuity after this treatment is comparable with brachytherapy but the reported failure rate is 30% or more after 3 years of follow-up. TTT is commonly used as adju-

vant to radiotherapy or for the treatment of small tumors (Loercher and Harbour 2003). Other globe-sparing modalities have been developed including charged particle radiotherapy, stereotactic radiotherapy and resection.

Local tumor resection may be performed by external approaches, including: partial eye wall resection, choroidectomy, iridocyclectomy and internal approaches (endoresection) (Damato and Jones 2005).

The Collaborative Ocular Melanoma Study (COMS) conducted a prospective, multicenter clinical trial comparing plaque radiotherapy to enucleation for medium-sized posterior uveal melanomas and found no difference in survival (Diener-West, Earle et al. 2001). However, due to numerous complications and recurrences, enucleation is still required in a significant number of patients. Several factors could influence the local treatment including patient age, health, visual acuity, tumor size and location.

Despite recent progress in tumor diagnosis and treatment of the primary tumor, the prognosis remained unchanged in the last several decades and 15-30% of treated patients die within 5 years following the diagnosis, possibly due to pre-treatment dissemination of metastatic disease (Singh and Topham 2003).

## TREATMENT OF METASTATIC DISEASE

The interior of the eye lacks lymphatics and uveal melanoma cells disseminate through a hematogenous route (Woll, Bedikian et al. 1999). Due to the lack of lymphatics in the eye, uveal melanoma cells disseminate through a hematogenous route (Albert, Ryan et al. 1996).

## Regional therapies

Regional intrahepatic therapies include arterial chemotherapy with cisplatin or fotemustine delivered via a catheter into the hepatic artery (Becker, Terheyden et al. 2002). Chemotherapy can be administered together with an embolizing agent in an intrahepatic chemoembolization approach. These therapeutic modalities provide minor beneficial effects due to the high resistance to chemotherapy. Local immunotherapy with granulocyte-macrophage colony-stimulating factor (GM-CSF) or immunoembolization, have shown minor improvements in patient prognoses (Sato, Eschelman et al. 2008).

Other modalities of regional therapy are surgical resection (Aoyama, Mastrangelo et al. 2000) , radiofrequency ablation (Pawlik, Izzo et al. 2003) and hyperthermic isolated hepatic perfusion (Alexander, Libutti et al. 2003). Local surgical treatments are employed in less than 10% of uveal melanoma patients and are limited by the presence of multiple metastatic foci, large masses invading the blood vessels and hepatic failure (Curley, Marra et al. 2004).

## Systemic therapy

Clinical trials assessing potential systemic chemotherapy did not show significant efficacy of systemic therapeutic agents. Chemotherapeutic regimens such as BOLD (dacarbazine, lomustine, vincristine and bleomycin) and immunomodulatory agents, such as interferon-alpha (INF-α) or interleukin -2 (IL-2), have shown objective tumor responses of 10% to 14% in a small number of patients (Pyrhonen, Hahka-Kemppinen et al. 2002; Kivela, Suciu et al. 2003).

The survival rate of patients after diagnosis of metastatic disease ranges between 2 to 9 months with current therapeutic modalities (Bedikian 2006). Patients with extrahepatic metastatic dis-

ease have a median survival of approximately 19 to 28 months with a better 1-year survival rate of 76% (Hsueh, Essner et al. 2004).

### *1.1.9 Summary*

Uveal melanoma is a rare malignancy representing the most common primary malignant intraocular tumor. It usually has a sporadic occurrence although several phenotypic associations are considered risk factors, for example congenital ocular melanocytosis and dysplastic nevus syndrome. Metastatic uveal melanoma spreads through a vascular route preferentially to the liver. Several clinical and histologic factors are identified as risk factors for tumor progression and development of metastasis such as largest basal tumor diameter, ciliary body involvement, extraocular growth, presence of epithelioid cells and microvascular loops. Cytogenetic studies have established the presence of chromosomal aberrations in uveal melanoma; changes in chromosomes 1 and 6, monosomy 3 and gain of the long arm of chromosome 8 are linked to progression towards metastasis.

With the development of new therapeutic tools, plaque radiotherapy (brachytherapy) has become the most common procedure for local treatment of uveal melanoma. However, despite earlier diagnosis and successful treatment of the primary tumor, prognosis has not improved due to metastatic dissemination. The development of metastatic disease represents the major challenge in uveal melanoma patients and it can occur in 40% of the cases even after successful local treatment. Local therapies such as surgical resection, regional chemotherapy and chemoembolization, have shown minor improvements. Systemic therapy did not show significant influence on survival due to the high resistance to chemotherapeutic agents. The survival rate for the patients with metastasis is from a few months to one year despite of local and systemic therapy.

# *Chapter 2*

# Potential therapeutic targets in uveal melanoma

*Literature review*

## 2.1 **Molecular targets in uveal melanoma**

Targeted therapy is defined as "*A type of treatment that uses drugs or other substances, such as monoclonal antibodies, to identify and attack specific cancer cells. Targeted therapy may have fewer side effects than other types of cancer treatments*" (NCI 2009). Understanding molecular pathogenesis of uveal melanoma leads to identification of appropriate therapeutic targets. A number of molecular changes have been identified in pathways critical for progression of uveal melanoma (Ehlers and Harbour 2006; Bakalian, Marshall et al. 2008; Economou 2008). The development of molecularly targeted agents that modify these pathways led to clinical trials in patients with uveal melanoma (Triozzi, Eng et al. 2008). In this literature review, we focus on the knowledge gained about the molecular pathogenesis of uveal melanoma and the opportunities for targeted molecular therapies.

### 2.1.1 Upregulation of survival mechanisms

The retinoblastoma (Rb) protein hyperphosphorylated and functionally inactivated in uveal melanoma. The inactive state of Rb is maintained by downregulation of upstream p16INK4 (van der Velden, Metzelaar-Blok et al. 2001) and by upregulation of cyclin D, which

interacts with cyclin-dependent kinases (CDKs) and phosphorylates Rb (Brantley and Harbour 2000).

The p53 tumor suppressor pathway is functionally disrupted by upregulation of the p53 inhibitor MDM2 (mouse double minute 2) and it has been shown that disrupting the interaction of MDM2 with p53 induced apoptosis in uveal melanoma cells (Harbour, Worley et al. 2002). Inhibitors of histone deacetylase (HDAC) were shown to have a potential effect in up-regulating tumor suppressor genes, including those in the p53 and Rb pathways (Roy, Packman et al. 2005). Vorinostat, an inhibitor of HDAC, is being studied in a phase II clinical trial of metastatic melanoma including patients with metastatic uveal melanoma. Major somatic mutations in metastasis suppressor genes have not been observed in uveal melanoma; however, studies done in our laboratory by Martins et al, showed that low expression of KISS1, a metastasis suppressor gene, was associated with a higher risk of metastatic disease (Martins, Fernandes et al. 2008) . Furthermore, Bakalian et al (Bakalian, Marshall et al. 2007), showed that the expression of the metastatic suppressor gene NM23 in uveal melanoma was positively correlated with a favourable prognosis.

### 2.1.2 Apoptosis and proliferation

Antiapoptotic mechanisms are upregulated in uveal melanoma due to overexpression of anti-apoptotic proteins (BCL-2, BCL-XL) or focal adhesion kinase (FAK).

The anti apoptotic protein Bcl-2, which prevents the activation of pro-apoptotic caspase proteins, was found to be overexpressed in this tumor (McGill, Horstmann et al. 2002). Inactivation of Bcl-2 has been shown to induce apoptosis of uveal melanoma cells and to increase their sensitivity to chemotherapy (Guo, Wu et al. 2003).

## 2.1.3 Signaling pathways

PROLIFERATION, MIGRATION AND APOPTOTIC PROCESSES

Among the mechanisms involved in proliferation, activation of the mitogen-activated protein kinase (MAPK) signaling pathway occurs frequently in uveal melanoma. Mutations of BRAF or RAS kinases were not detected in this tumor; however, wild type B-Raf, was found to activate phosphorylated ERK (pERK) and promote proliferation of uveal melanoma cells (Zuidervaart, van Nieuwpoort et al. 2005). Inhibition of RAS and RAF protein kinases with Sorafenib has been shown to inhibit ERK-mediated uveal melanoma cell proliferation and also to have antiangiogenic effects (Wilhelm, Carter et al. 2004; Calipel, Mouriaux et al. 2006).

There is also evidence that the phosphatidylinositol-3-kinase (PI3K)-Akt pathway is implicated in cell-cycle, proliferation, migration and apoptotic processes (Eccles and Welch 2007). Indirect activation of AKT oncogene can occur through loss of the tumor suppressor gene PTEN that blocks activation of the Akt pathway (Abdel-Rahman, Yang et al. 2006). The immunohistological expression of p-Akt was associated with development of metastatic uveal melanoma (Saraiva, Caissie et al. 2005). PI3K-Akt pathway could be modulated through inhibition of downstream receptor tyrosine kinases.

Activation of the transcription factor NF-kB has been directly implicated in tumorigenesis including cutaneous melanoma (Ueda and Richmond 2006) and microarray analysis identified the presence of NF-kB in uveal melanoma metastasis (Meir, Dror et al. 2007). Unpublished data from our laboratory suggest that in vitro inhibition of NF-kB with Bortezomib, a proteasome inhibitor that targets NF-kB, reduced the proliferative and invasive ability of uveal mela-

noma cells (Godeiro 2008). A phase II trial is currently underway studying the effect of Bortezomib together with paclitaxel and carboplatin in patients with metastatic melanoma, including uveal melanoma.

C-Kit receptor, which signals through the MAPK and PI3K-Akt pathways, was found to be expressed in approximately 78% of primary uveal melanomas and targeting of c-kit with imatinib mesylate decreased the proliferation and invasion rates of five uveal melanoma cell lines (Pereira, Odashiro et al. 2005). However, a phase II clinical trial of imatinib mesylate as a single agent in patients with metastatic uveal melanoma did not render clinically objective responses (Penel, Delcambre et al. 2008).

Indirect alteration of the PI3K-Akt pathway could be achieved with COX-2 inhibitors. COX-2 expression has been found in approximately 60% of uveal melanomas, and correlated with poor prognosis (Figueiredo, Caissie et al. 2003). Topical administration of a COX-2 inhibitor (Nepafenac) had an effect on progression of the disseminated metastatic disease in an animal model (Di Cesare, Marshall et al. 2007). One possible explanation is the influence of the treatment on the metastatic suppressor gene NM23. Bakalian et al (Bakalian 2007), showed that treatment of the uveal melanoma cell lines with the COX- 2 inhibitor increased the expression levels of Nm23-H1 and decreased the expression levels of c-Met in *vitro* in uveal melanoma cell lines and *in vivo* in an experimental rabbit model.

Activation of the epidermal growth factor receptor (EGFR) signals through the MAPK and PI3K-Akt pathways and is implicated in tumor survival and growth. The expression of the EGFR receptor was correlated with a higher metastatic potential of the uveal melanoma cells and in a nude mouse model (Ma and Niederkorn 1998). Immunohistochemical expression of EGFR in primary uveal melanomas was correlated with poor prognosis (Hurks, Metzelaar-Blok et al.

2000). However, other studies showed that overexpression of EGFR could be attributed to macrophage infiltration (Scholes, Hagan et al. 2001). The EGFR inhibitor, Gefitinib, did not show a therapeutic effect in *in vitro* studies of uveal melanoma cell lines.

In the last decade advances have been made in understanding the molecular abnormalities involving the metastatic cascade of uveal melanoma cells. Insulin like growth factor-1 (IGF-1) and its receptor IGF-1R are mainly produced by the liver. Among the downstream pathways that can be stimulated by IGF-1 are PI3K-Akt pathway, via Akt phosphorylation, and MAPK pathway, through activation of Ras (Saltiel and Kahn 2001).The IGF-1R expression was associated with a higher mortality and thus identified as a potential therapeutic target in uveal melanoma (All-Ericsson, Girnita et al. 2002; Economou, All-Ericsson et al. 2008). Specific inhibition of IGF-1R with cyclolignan picropodophyllin (PPP), was shown to cause regression of tumor growth in a xenograft mouse model, and also antiangiogenic effects (Girnita, All-Ericsson et al. 2006; Economou, Wu et al. 2008).

A recent study by Economou et al. described the interrelation between IGF-1R and C-Met in uveal melanoma showing that co-expression of these two factors may play a role in the affinity of uveal melanoma for the liver. The c-Met and its ligand, hepatocyte growth factor (HGF), are involved in motility, invasion, and metastatic processes of the transformed cells and c-Met expression correlates with poor prognosis in uveal melanoma (Xiao, Jeffers et al. 2001) and (Mallikarjuna, Pushparaj et al. 2007; Economou, All-Ericsson et al. 2008). HGF, is expressed by primary uveal melanomas and metastatic lesions to the liver and the homing of uveal melanoma cells expressing c-Met to form hepatic metastases could be explained by the presence of their ligand, HGF, in the liver (Hendrix, Seftor et al. 1998). Different studies showed an increase in migration and invasion of uveal melanoma cells when HGF was used as a chemoattrac-

tant (Woodward, Elshaw et al. 2002; Di Cesare, Marshall et al. 2007). Met activation by HGF leads to multiple downstream effects including phosphorylation of FAK (focal adhesion kinase) and signaling through Ras–MAPK and PI3K–Akt pathways (Eccles 2005; Peruzzi and Bottaro 2006). Recently, Ye and colleagues, demonstrated the involvement of PI3K-Akt signaling pathway in HGF induced migration of uveal melanoma cells (Ye, Hu et al. 2008). c-Met peptide inhibitors were demonstrated to reduce migratory and invasive abilities of the tumor cells in animal models (Christensen, Burrows et al. 2005).

### 2.1.4 Cell motility, invasion

FAK is a protein tyrosine kinase involved in motility and invasion linking integrin signaling in the regulation of cell proliferation and survival (Hauck, Hsia et al. 2002). FAK is activated by a variety of cell surface receptors and mediates signal transduction through (MAP) kinases and the PI3K-Akt pathway (Hess and Hendrix 2006). Overexpression of FAK has been correlated with metastasis (Schlaepfer, Mitra et al. 2004). In a study by Hess and Hendrix, the expression of phosphorylated FAK was found in uveal and cutaneous melanoma cells which acquired an invasive phenotype and developed vasculogenic mimicry. Inhibition of FAK resulted in interference with ERK1/2 phosphorylation and a decrease in the metastatic potential of uveal melanoma cells (Hess, Postovit et al. 2005). The FAK pathway also contributes to the secretion of matrix-metalloproteinases and matrix remodeling (Wu, Gan et al. 2005).

Matrix - metalloproteinases (MMPs) are proteolytic enzymes regulated by their tissue inhibitors (TIMPs). They participate in invasive processes of the tumor cells through modeling of the extracellular matrix and angiogenesis. Uveal melanoma cells express MMP-2 and MMP-9, which have been associated with tumor progression (Baker, Elshaw et al. 2001). In one study the

expression of MMP-2 was associated with reduced survival in uveal melanoma (Vaisanen, Kallioinen et al. 1999). In *in vitro* experiments, depsipeptide, an inhibitor of MMPs, has been shown to decrease the migration of the primary and metastatic uveal melanoma cells. One of the pathways implicated in the matrix metalloproteinase activity is PI3K pathway. PI3K inhibitors have been shown to reduce the formation of vascular networks and to down regulate MMP-2 activity in aggressive uveal melanoma (Hendrix, Seftor et al. 2003; Hess, Seftor et al. 2003).

Chemokines, are signaling molecules that induce the migration of cells toward a gradient, have recently been implicated in tumor progression and metastasis (Payne and Cornelius 2002). Chemokine CXCR4 and its ligand CXCL12, which is expressed in lungs, liver and bone marrow, may play an important role in guiding disseminating cells to specific locations (Zlotnik and Yoshie 2000). Activation of CXCR4 by CXCL12 leads stimulation of a variety of intracellular signal transduction pathways and regulation of cellular survival, including phosphorylation of PI3K, FAK and upregulation of MMP-2 and MMP-9 (Fernandis, Prasad et al. 2004). Hypoxia induced CXCR4 is also upregulated by MET, and EGFR (Eccles and Welch 2007).

Uveal melanoma cells express the chemokine receptors CCR7 and CXCR4 and overexpression of CXCR4 in primary human uveal melanoma correlates with epithelioid cell type (Scala, Ierano et al. 2007). It has been previously established in our laboratory that migration of uveal melanoma cells can be induced when cell lines are exposed to CXCL12 (Di Cesare, Marshall et al. 2007).

Furthermore, Di Cesare et al (Di Cesare, Marshall et al. 2007) showed that inhibition of CXCR4 using TN14003, a CXCR4 peptide antagonist, affected *in vitro* migration of uveal tumor cells.

### 2.1.5 Angiogenesis and hypoxia

Hypoxia promotes endothelial cell migration and new vessel formation through PI3K and Ras-ERK1/2 MAPK. Moreover, uveal melanoma tumors express VEGF and hypoxia-inducible factor 1α (HIF-1α) (Hendrix, Seftor et al. 2003; Notting, Missotten et al. 2006).

In tumors, VEGF-R signaling contributes to cell survival, proliferation and migration (Eccles 2005). The anti-VEGF monoclonal antibody bevacizumab can be administered intravitreally in neovascular diseases; however, its role in uveal melanoma has not yet been established. Recently, sorafenib and picropodophyllin were demonstrated to have anti-VEGF activity in uveal melanoma (Economou, Wu et al. 2008). Several inhibitors of VEGF, including Sorafenib, Sunitinib, Lenalidomide, are being studied in phase II clinical trials of metastatic melanoma that includes patients with metastatic uveal melanoma (Triozzi, Eng et al. 2008).

Uveal melanoma tumors have the ability to form "vasculogenic mimicry", a network of vascular like channels capable of transporting blood in the absence of endothelial cells which may be lined by tumor cells (Maniotis, Folberg et al. 1999). Phosphoinositide 3-kinase is essential in regulation of vasculogenic mimicry in uveal melanomas and inhibition of this pathway abrogated the formation of these networks (Hendrix, Seftor et al. 2003).

Hypoxia also promotes neoangiogenesis by the activity of hypoxia-inducible factor 1α (HIF-1α) which signals through PI3K and MAPK pathways. Hypoxia induces transcriptional activation of uPA (urokinase plasminogen activator) and CXCR4 and influences adhesion, matrix degradation and invasion (Eccles 2005). In one study, exposure of uveal melanoma cells to hypoxia resulted in a significant increase in invasion of cells (Victor, Ivy et al. 2006).

### 2.1.6 Transcriptional analysis

The development of molecular profiling in uveal melanoma identified new targets involved in the tumorigenesis and the metastatic cascade in this tumor. Several factors that were linked with metastasis in a xenograft rabbit model were shown to be upregulated in a genetic transcriptional analysis in uveal melanoma and included upregulation of insulin receptor substrate 2, fibronectin 1, and cytokeratin 18 (Marshall, Nantel et al. 2007). Cytokeratin-18 expression has been previously linked to the interconverted phenotype (co-expressing vimentin and keratin intermediate filaments) and indicates poor prognosis (Hendrix, Seftor et al. 1998).

Genetic assays such as microarray analysis of patient tumor samples of uveal melanoma were able to classify the tumors in two groups: class 1 tumors, which rarely present monosomy 3 and have a better prognosis and class 2 tumors associated with lower survival and chromosomal abnormalities such as monosomy 3 (Onken, Worley et al. 2004). Among the biomarkers most strongly associated with the class 2 signatures are epithelioid cell type and upregulation of beta-catenin, E-cadherin, and HIF-1α (Chang, Worley et al. 2008).

### *2.1.7* ***Summary***

Different molecular abnormalities are involved in proliferation, apoptosis, angiogenesis and invasion in uveal melanoma. The retinoblastoma protein and p53 tumor suppressor pathways, the c-Kit, c-Met, IGF-1R signal pathways and PI3K-Akt pathway are functionally disrupted in uveal melanoma and could potentially be targeted. Proteomic studies have also suggested potential therapeutic targets and recent gene expression profiling has demonstrated molecular differences between non-metastasizing and metastasizing uveal melanoma. Several ongoing clinical trials use therapeutic agents alone or in combination with systemic chemotherapy in an effort to delay tumor progression and to affect metastatic disease in uveal melanoma. These include drugs that can modify pathways that regulate the cell cycle, apoptosis or proliferation and inhibit molecules involved in angiogenesis and invasion.

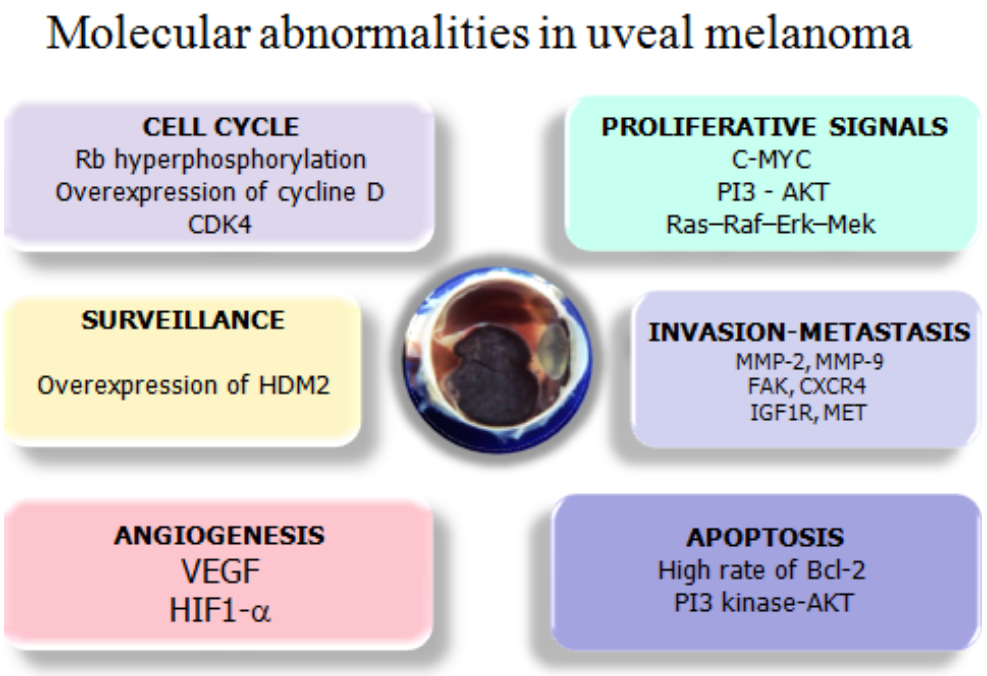



**Fig.2.1** Uveal melanoma is a result of multiple abnormalities of the proliferative and antiapoptotic mechanisms (Harbour 2007; Bakalian, Marshall et al. 2008).

# *Chapter 3*

# Heat shock protein 90

*Literature review*

## 3.1 Cellular adaptation to stress and cancer

Cells are continuously exposed to acute and chronic stress such as environmental and oxidative stresses, heat shock or other physiological and pathological demands including, ischemia, inflammation, infection and genetic mutations. The cellular response to stress maintains a delicate balance between survival and degradation (Morimoto 1993). The integrity of the cell is maintained through processes related to protein homeostasis including protein synthesis, folding, translocation and degradation (Balch, Morimoto et al. 2008). Deficiencies in protein homeostasis lead to accumulation of misfolded proteins which can lead to metabolic and oncological consequences or cell damage and death (Macario and Conway de Macario 2005).

Cellular adaptation to stress requires the ability to detect damaged proteins and results in the increased synthesis of heat shock proteins (Hsps) that function as molecular chaperones or proteases. Following exposure to stress, chaperones are required for repair of the denaturated proteins, maintenance of structural proteins or intracellular traffic from one compartment to another inside the cell. Proteases influence degradation of the denaturated proteins through the ubiquitin-dependent proteasome (Jolly and Morimoto 2000). Heat shock proteins have been classified according to their molecular size (27 to 110 kDa) into six groups: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small heat shock proteins (Schlesinger 1990). As modulators of protein homeostasis, Hsps are also expressed in normal conditions, assisting immature protein chains to attain a stable state and to translocate inside the cell. They are also involved in

cytoskeletal stabilization, receptor regulation and cell growth, signal transduction and anti-apoptotic mechanisms (Bukau, Weissman et al. 2006). For clients involved in signal transduction pathways, Hsps maintain the conformational maturation of the proteins by stabilizing them in large immobilized complexes allowing further ligand binding or phosphorylation (Helmbrecht, Zeise et al. 2000).

Cancer cells are characterized by DNA damage and genetic instability while adapting to a noxious environment and maintaining an actively proliferating state and anti-apoptotic mechanisms. Chronic adaptation to the oncogenic stress overwhelms the capacity of the normal cells to maintain homeostasis and the Hsps are integrated by the tumor cells in promoting the survival of the mutant proteins (Dai, Whitesell et al. 2007).

Tumor cells express high levels of chaperones (Jaattela 1999) and it is currently believed that Hsps are also induced by the tumor microenvironment, hypoxia, nutrient deprivation and acidosis (Workman, Burrows et al. 2007).

### 3.1.1. Hsp90: introduction and function

Heat shock proteins were initially described 1962 by Rittosa, who reported segments of chromosomal puffing in response to elevated temperatures while studying the salivary gland chromosomes of Drosophila busckii. These represented zones of increased RNA synthesis and production of a group of proteins, which were called heat shock proteins (Ritossa 1962).

Hsp90 in eukaryotic cells, comprises up to 1–2% of total cellular protein, a proportion that increases by 2–10-fold during environmental stress; The Hsp90 family includes several members: Hsp90α and Hsp90β, found mainly in the cytoplasm, share approximately 85% sequence identity at the protein level; GRP94 present in the endoplasmic reticulum, and TRAP1 in the mitochon-

dria. Hsp90α was recently found to also have an extracellular role. Hsp90N, a membrane associated variant, has been recently identified (Eustace and Jay 2004; Workman, Burrows et al. 2007). The induction of Hsps is controlled by the transcription factor heat shock factor 1 (HSF1) which is maintained in an inactive configuration complexed with Hsp90 in the cytoplasm of unstressed cells. Upon stress, misfolded proteins compete for binding with HSF1-Hsp90 complex and HSF1 promoter is free to trimerize, translocate to the nucleus, undergo phosphorylation, and activate Hsps gene expression (Zou, Guo et al. 1998). The role of Hsp90 as a chaperone in the normal cell includes protein folding and translocation between cellular compartments, thus preserving their status. During stress, Hsp90 upregulation is part of the protective mechanism that ensures protein homeostasis by repairing denaturated proteins or targeting them for ubiquitination and proteosome degradation (Wegele, Muller et al. 2004).

The function of Hsp90 requires interactions with other co- chaperones like Hsp70, Hsp40, Hsp70-interacting protein (HIP) and Hsp70/Hsp90-organizing protein (HOP), p23, cell-division-cycle 37 homologue client proteins (Cdc37) and immunophilins, to form multichaperone complexes (Pratt and Toft 2003). The Hsp90 complex ensures stability of over 100 substrate proteins and also the formation of large immobilized complexes of signal transducing molecules (scaffolding function) (Helmbrecht, Zeise et al. 2000). Heat shock protein 90 “client proteins” are involved in cell regulatory pathways and include protein kinases, nuclear hormone receptors, transcription factors, and proteins controlling cell cycle and apoptosis (Wegele, Muller et al. 2004; Bagatell, Beliakoff et al. 2005).

### 3.1.2 Hsp90 structure

Hsp90 functions as a dimer which is required for its biological activity. All the Hsp90 isoforms described above share a similar overall structure. The monomer consists of three main domains: The ATP-ase amino-$NH_2$-terminal domain (24-28 kDa), a middle region (38-44 kDa), and a carboxyl-terminal dimerisation domain (11-15 kDa) (Fig 3.1). The primary functions of these domains are ATP binding, client protein binding, and dimerization, respectively (Prodromou and Pearl 2003). The $NH_2$-terminal interface contains an adenine-nucleotide-binding pocket and the hydrolysis of ATP to ADP in this site is required for the Hsp90 activity (Prodromou and Pearl 2003). The $NH_2$-terminal domain also binds other co-chaperones, for example CDC37 and drugs. CDC37 has an important role in recruiting client protein kinases to the Hsp90 complex (Roe, Ali et al. 2004). The middle domain associates with the co-chaperone AHA1 (activator of Hsp90 ATP-ase homologue 1) to promote further the ATP-hydrolysis and also has a role in the binding of the client proteins (Panaretou, Siligardi et al. 2002). The COOH-terminal domain is involved in Hsp90 dimerization and co-chaperone binding such as the immunophilins and HOP. Interaction between this site and a co-chaperone protein called carboxyl-terminal Hsp70-interacting protein (CHIP), promotes ubiquitination and degradation of the client proteins (Xu, Marcu et al. 2002).

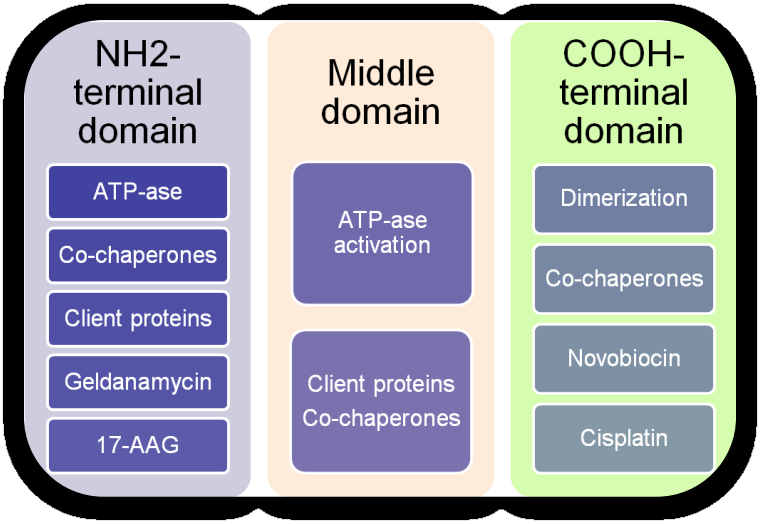



**Fig. 3.1** Hsp90 monomer has three distinct domains, N-terminal, middle domain and C-terminal regions

### 3.1.3 The Hsp90 chaperoning cycle

Hsp90 functions as a part of a multichaperone complex witch activates groups of co-chaperones and client proteins. This process is linked to the ATP-ase cycle (Fig. 3.2)

The client protein links first with an Hsp70–Hsp40/HIP complex which then is incorporated to Hsp90 via HOP/p60 adaptor forming an intermediate complex (Pratt and Toft 2003). Upon ATP binding and hydrolysis, Hsp90 dissociates from Hsp70/Hsp40/HIP and HOP and forms a second mature complex, containing a new subset of co-chaperones p23, CDC37 and immunophilins (IP). This process catalyzes the conformational maturation of the client protein, allows it to bind to its ligand and to be released from the complex to perform its function in the cell. If the ATP-dependent hydrolysis step does not take place, Hsp90 is locked in the ADP bound intermediate state (Kamal, Boehm et al. 2004). The complex including Hsp70 and HOP re-directs the client protein towards ubiquitination by recruiting an E3 - ubiquitin ligase chaperone, CHIP. This process controls the degradation of the client protein which is degraded by the ubiquitin protea-some pathway (Xu, Marcu et al. 2002; Kamal, Boehm et al. 2004) (Fig.3.2).

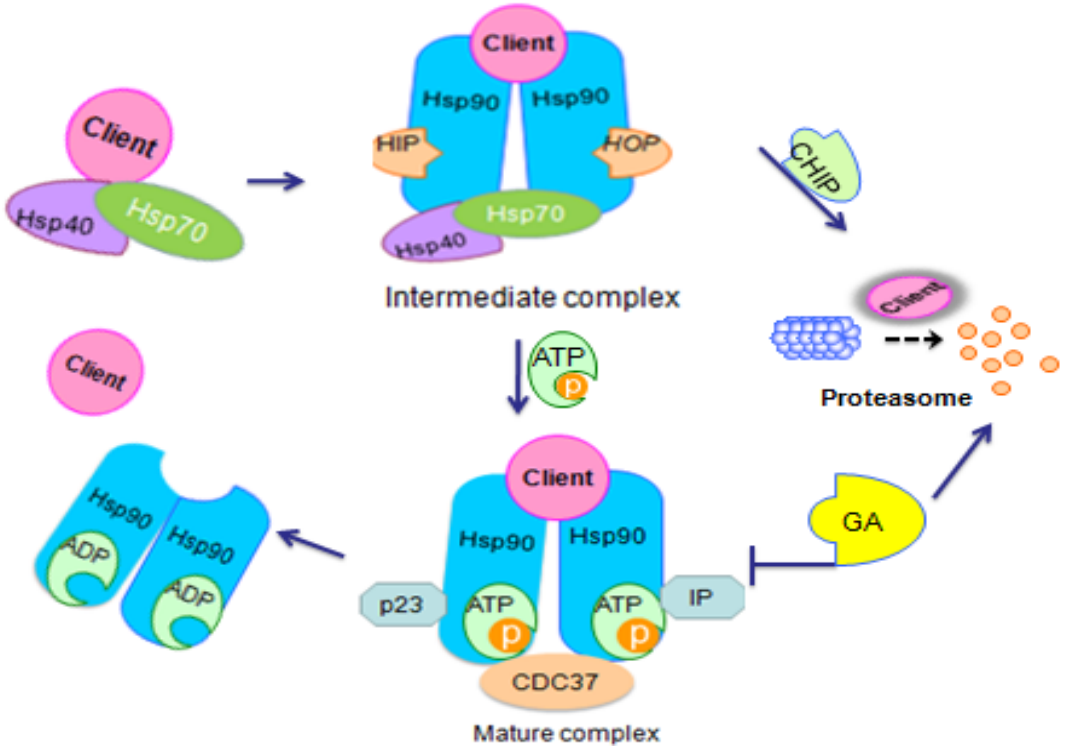



**Fig.3.2** The Hsp90 cycle.

### 3.1.4 Hsp90 as a target in cancer

Hsp90 is upregulated in many neoplastic processes (Ferrarini, Heltai et al. 1992; Faingold, Marshall et al. 2008; McCarthy, Pick et al. 2008). This may be a consequence of the increased number of deregulated instable oncoproteins which require the multichaperone Hsp90 complex for stabilization (da Rocha Dias, Friedlos et al. 2005; Whitesell and Lindquist 2005). Also they could be induced by the environment of the tumor itself, hypoxia, nutrient deprivation and acidosis (Workman, Burrows et al. 2007). The chaperone activity of Hsp90 ensures the maturation, function and intracellular traffic of a range of proteins that are involved in cellular growth, differentiation and apoptotic pathways, invasion and metastasis (Maloney and Workman 2002) and (Helmbrecht, Zeise et al. 2000; Pratt and Toft 2003).

The list of Hsp90 client proteins includes: tyrosine kinases (HER-2/neu, EGFR (Xu, Mimnaugh et al. 2001; Solit, Zheng et al. 2002), Met (Maulik, Kijima et al. 2002) and IGF-1R (Eiseman, Guo et al. 2007), signaling proteins (Akt, Raf-1 and IKK), (Schulte, Blagosklonny et al. 1995; Basso, Solit et al. 2002), mutated signaling proteins (p53, Kit, Flt3 and v-src (Schulte, Blagosklonny et al. 1995; Li, Judge et al. 2000; Isaacs, Xu et al. 2003), Bcr–Abl (Li, Judge et al. 2000; Gorre, Ellwood-Yen et al. 2002), HIF1- alpha (Isaacs, Jung et al. 2002), FAK (Schwock, Dhani et al. 2009), steroid receptors (Whitesell and Cook 1996) and cell-cycle regulators (cdk4, cyclin D) (Basso, Solit et al. 2002), survivin (Fortugno, Beltrami et al. 2003) and telomerase hTERT (Kim, Kim et al. 2008). The antineoplastic potential of Hsp90 inhibitors resides in destabilization and degradation of the client proteins (Maloney and Workman 2002) resulting in interference with multiple oncogenic signaling pathways (Whitesell and Lindquist 2005).

Hsp90 affects multiple signaling pathways including Ras–Raf-1–Mek–ERK , PI3K-Akt, because of the multiple roles of Hsp90 in modulating the activity of several components of these pathways.

Multiple Hsp90 clients are implicated in cell growth and differentiation for example (EGFR, IGF1R, HER-2 neu, Akt, RAF, BCR-ABL) and have antiapoptotic effects (Akt, p53, survivin). Additionally, Hsp90 is involved in progression of the cell cycle due to its association with CDK4 and cyclin D1, both of which are important for the G1–S cell cycle transition (Burrows, Zhang et al. 2004). Hsp90 plays an important role in angiogenesis (VEGF and HIF1$\alpha$) and tissue invasion and metastasis (Met, FAK) (Isaacs, Xu et al. 2003; Neckers and Ivy 2003; Koga, Tsutsumi et al. 2007; Wang, Pashtan et al. 2009). Consistent with the multiple roles of Hsp90 in modulating the malignant process, the inhibition of Hsp90 will have an effect on the major hallmarks of cancer (Hanahan and Weinberg 2000). Hsp90 inhibitors are under investigation as anticancer agents.

### 3.1.5 Inhibitors of Heat Shock Protein 90

The first Hsp90 inhibitors were the benzoquinone ansamycins geldanamycin and herbimycin, which are natural products discovered in *Streptomyces hygroscopicus* extracts, and radicicol, a macrocyclic antibiotic isolated from *Monosporium bonorden* (BeBoer and Dietz 1976). In 1994, Whitesell *et al.*, reported tumoricidal activity of geldanamycin and radicicol which was attributed to the pharmacological interference with Hsp90 (Whitesell, Mimnaugh et al. 1994). The drugs compete with ATP at the ATP/ADP pocket of the $NH_2$-terminal domain of Hsp90 impeding its ATPase activity and the ability to form the Hsp90-client mature complex (Roe, Prodromou et al. 1999). Geldanamycin was found to have a higher binding affinity than the nucleotide (Workman 2003). In the drug-linked conformation, the complex recruits E3 ubiquitin ligases such as CHIP to the client proteins resulting in their proteasomal degradation. Al-

though Geldanamycin exhibits multiple anti-tumor effects through depletion of the client proteins, leading to effects on cell cycle arrest and induction of apoptosis (Hostein, Robertson et al. 2001), it has been shown to be hepatotoxic in preclinical studies (Maloney and Workman 2002). Also Geldanamycin was found to be a substrate for the P-glycoprotein multidrug resistance efflux transporter and MRP efflux pump (Workman 2003).

Further analogues were developed which included 17-allylamino, 17-demethoxygeldanamycin (17-AAG). It has been well documented that 17-AAG binds Hsp90 found in tumor cells and has an improved therapeutic profile versus its precursor, being the first Hsp90 inhibitor to enter clinical trials (Kelland, Sharp et al. 1999; Burger, Fiebig et al. 2004; Banerji, O'Donnell et al. 2005; Modi, Stopeck et al. 2007). Induction of Hsp70 chaperone was observed as an effect in response to 17-AAG both in vitro and in vivo, possibly reducing the apoptotic consequences of the drug (Hostein, Robertson et al. 2001). 17-AAG is metabolized by the cytochrome P450 CYP3A4 to an equally potent metabolite 17AG (17-amino-17-demethoxygeldanamycin), (Egorin, Rosen et al. 1998) (Kelland, Sharp et al. 1999). The 17-AAG quinone is a substrate for the NADH quinone oxidoreductase 1 (DT-diaphorase, NQO1) which mediates the response to this drug in NQO1-expressing cancer cells (Kelland, Sharp et al. 1999; Workman 2003). 17-AAG and the metabolite have several limitations such as the potential for liver toxicity, adduct formation and potential to induce drug resistance as are also substrates for the P-glycoprotein efflux pump (Kelland, Sharp et al. 1999).

Hsp90 inhibitors with lower toxicity have been further developed, including17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG), which is a soluble derivative and exhibits equal or greater activity with 17-AAG (Burger, Fiebig et al. 2004; Mahalingam,

Swords et al. 2009). Other inhibitors that compete with the ATP at the amino-terminal end of Hsp90 are the hydroquinone of 17-AAG, known as IPI-504, (Pacey, Banerji et al. 2006), and KF58333, a novel oxime derivatives of radicicol (Shiotsu, Neckers et al. 2000).

Additional selective Hsp90 inhibitors have been developed; for example purine-scaffold derivatives which mimic ATP/ADP binding interface at the N-terminal domain of Hsp90, are water solubile and have been reported to have extensive antiproliferative effects in cancer cells (Chiosis, Timaul et al. 2001). Recently, the small molecule AUY922A, a novel re-sorcinylic isoxazole amide, was shown to have promising results inhibiting tumor growth and metastases in preclinical trials and entered phase I clinical trials (Eccles, Massey et al. 2008).

Different compounds that bind to the C-terminal end of Hsp90 were also identified. For example, novobiocin interferes with Hsp90 complex by binding to a site in the C-terminal do-main (Marcu, Schulte et al. 2000). Also in experimental models, the chemotherapeutic agent cisplatin was shown to also have inhibitory effects on Hsp90 at this site (Itoh, Ogura et al. 1999). Other approaches are being considered including the targeting of the Hsp90 cochaperones. SiR-NA, specifically reduced the expression of AHA1 and sensitized cancer cells to 17-AAG (Holmes, Sharp et al. 2008). The CDC37 inhibitor, celastrol, has been shown to interfere with Hsp90-Cdc37 interaction in pancreatic cancer cells (Zhang, Hamza et al. 2008).

Given the important functions of the Hsp90 in normal cells, several groups have ana-lyzed the potential cytotoxicity of the Hsp90 inhibitors on normal tissues (Kamal, Thao et al. 2003). Work from Kamal et al demonstrated that cancer cells have the potential to bind 17-AAG with a higher affinity to Hsp90 complexes leading to a preferential accumulation of this drug within tumors. This group explored the stress theory of an increased load of mutant proteins in

tumors and they were able to demonstrate that Hsp90 in tumor cells is present in a multitude of complexes multiprotein complexes with high ATPase activity while in normal cells, Hsp90 exist in a free form with low ATPase activity and lower drug accumulation (Kamal, Boehm et al. 2004; Maroney, Marugan et al. 2006). Also, information obtained from clinical trials suggested that the concentrations of 3–10 μM of 17-AAG required to achieve tumor inhibition were not toxic (Banerji, O'Donnell et al. 2005; Solit, Ivy et al. 2007). A possible explanation is that at this concentration, the Hsp90 inhibitors will bind to a small percentage (0.5–2.0%) of total cellular Hsp90, thus more than 95% of the unbound Hsp90 existing in normal cells, will not be affected by the drug (Chiosis and Neckers 2006). Another possible protective mechanism is the induction of the Hsps promoter, HSF1, process referred as the stress response. Hsp90 is associated in a multichaperone complex with its promoter, HSF1 (Zou, Guo et al. 1998). Hsp90 inhibition activates HSF1, which induces transcription of other Hsps including Hsp90 and Hsp70. Induction of the stress response can be measured by increased levels of Hsp70 and is used as a biomarker of Hsp90 inhibition in clinical trials (Bagatell, Paine-Murrieta et al. 2000). It is believed that this response may have a possible protective effect in normal tissues exposed to Hsp90 inhibitors through the antiapoptotic mechanisms of Hsp70 andHsp90.

### 3.1.6 Hsp90 inhibitors in clinical trials

Inhibition of Hsp90 in tumor cells results in alteration of the binding with client proteins and affects multiple signaling pathways inducing both cytotoxic and cytostatic effects (Hostein, Robertson et al. 2001).

Phase I clinical trials studies demonstrated that 17-AAG can be administered without significant toxicity and depletion of client protein levels Raf-1, Cdk4 and ErbB2, and induction of Hsp70 was reported in peripheral blood lymphocytes and tumor biopsies (Banerji, O'Donnell et al. 2005; Goetz, Toft et al. 2005; Wright, Morrison et al. 2005; Solit, Ivy et al. 2007). Evidence of clinical activity has been reported for 17-AAG in melanoma, multiple myeloma, prostate, and breast cancer (Pacey, Banerji et al. 2006).

Phase II clinical trials were designed based on the evidence of specific Hsp90 clients necessary for cancer development including: BCR-ABL in chronic myelogenous leukemia (Peng, Li et al. 2007), mutated FLT3 in acute myeloid leukemia (Knapper 2007), EGFR in non-small cell lung cancer (Shimamura, Lowell et al. 2005), mutant B-Raf in melanoma (Banerji, Affolter et al. 2008), HER2-overexpression in breast cancer (Citri, Kochupurakkal et al. 2004), mutant c-Kit in gastrointestinal stromal tumors (Bauer, Yu et al. 2006), activated Akt in small cell lung carcinoma (Rodina, Vilenchik et al. 2007) and HIF in renal cell carcinoma (Workman 2004; Taldone, Gozman et al. 2008).

Collectively, data obtained from phase II clinical trials investigations suggested that 17-AAG monotherapy did not achieve objective response at the schedule and dosage used.

Collectively, data obtained from phase II clinical trials investigations suggested that 17-AAG monotherapy did not achieve objective response at the schedule and dosage used.

(Heath, Hillman et al. 2008). Also, 17-AAG administration did not induce objective responses in the treatment of clear cell or papillary renal cell carcinoma patients (Ronnen, Kondagunta et al. 2006).

In cutaneous melanoma, specific NRAS and BRAF mutations and PI3Kl 3-Akt pathway are frequent. Hsp90 inhibition achieved stable disease in preliminary studies in cutaneous melanoma. In a phase II clinical trial, the effects of 17-AAG inhibition on RAF kinase expression were minimal, and no objective antimelanoma responses were seen (Solit, Osman et al. 2008). The authors concluded that the results warranted future studies of Hsp90 inhibition with a better drug formulation. Following on from 17-AAG, a variety of related analogues have entered clinical trials.

Tanespimycin a new 17-AAG analog has demonstrated promising antitumor activity and tolerability in a Phase II clinical trial in patients with HER 2-neu positive metastatic breast cancer. These results were reported for a combination of 17-AAG with Trastuzumab (Herceptin) in patients previously nonresponsive to Herceptin alone (Modi, Stopeck et al. 2007). Also results from a trial investigating the combination of tanespimycin with bortezomib in patients with multiple myeloma were reported, showing response in 47% of cases (Taldone, Gozman et al. 2008). Novel synthetic Hsp90 inhibitors have been developed, and are currently undergoing Phase I/II clinical evaluation in cancers, including purine scaffold inhibitors (CNF2024/BIIB021) (Chiosis, Timaul et al. 2001), pyrazole scaffold derivatives (AUY922) (Eccles, Massey et al. 2008) and Serenex (SNX-5422) a 6,7-dihydro-indazol-4-one scaffold Hsp90 inhibitor (Taldone, Gozman et al. 2008).

### 3.1.7 Hsp90 in uveal melanoma

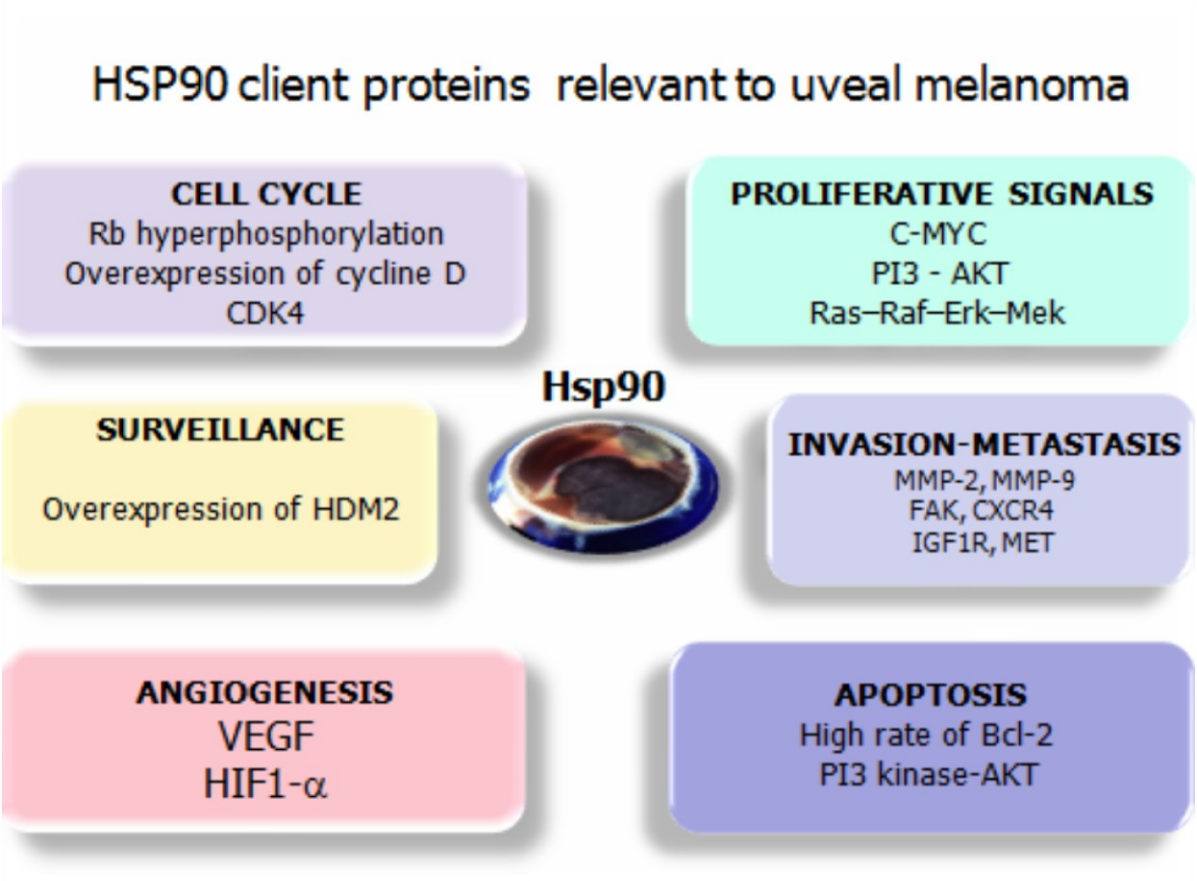
One study has been published in the literature regarding the expression of Hsps in uveal melanoma. Heat shock proteins were detected by immunohistochemistry and reported in epithelial and pigment cells of the normal eyes. The Hsp90 isoform GP96 was reported in 30 of 38 uveal melanomas cases with a mean of 53% positive cells, while Hsp70 and Hsp90 were ex-

pressed in 6% of tumor cells. The percentage of Hsps expression did not correlate with any prognostic factors (Missotten, Journee-de Korver et al. 2003).

Based on this thesis, partial data regarding the expression and inhibition of Hsp90 in uveal melanoma were published for the first time by our group, (Faingold, Marshall et al. 2006; Faingold, Marshall et al. 2007; Faingold, Abourbih et al. 2008; Faingold, Marshall et al. 2008) and the results will be presented in the following chapters.

### *3.1.8 Summary*

Hsp90 is an intracellular protein that is essential for normal proteins homeostasis. In tumors, Hsp90 plays an important role in neoplastic processes being involved in the stabilization of many signaling proteins that are deregulated in cancers. The inhibition of the Hsp90, results in the proteasomal degradation of these client proteins in multiple pathways and leading to anti-proliferative effects (Fig. 3.3). Inhibitors of Hsp90, 17-AAG and other semisynthetic derivatives, are currently under investigation in clinical trials as monotherapeutic agents as well in combination with other chemotherapeutic agents.



# *Chapter 4*

## 4.1 Thesis Hypothesis

Based on the previous literature review, given the importance of Hsp90 in malignant progression and the current development of Hsp90 targeting agents in cancer, we formulated the following hypothesis:

- Hsp90 may contribute to development and progression of uveal melanoma
- Hsp90 is a potential target for therapy in uveal melanoma

## 4.3 Purpose

- To investigate the expression of Hsp90 in uveal melanoma
- To investigate the functional roles of Hsp90 in uveal melanoma

## 4.4 Objectives

- **A.** Expression of Hsp90 in primary uveal melanoma

  **Specific objectives**

  - ***AI. In vivo:*** patient's samples - paraffin embedded tissues of primary uveal melanoma
    - Immunohistochemistry
    - Correlation with prognostic factors

- ***AII. In vivo:*** animal samples -paraffin embedded tissues of primary uveal melanoma from an experimental rabbit model.
    - Immunohistochemistry
- ***AIII. In vitro*****:** five uveal melanoma cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1)
    - Immunocytochemistry
    - Western blot

- **B.** Functional roles of Hsp90 in uveal melanoma primary tumor

  **Specific objectives**

  - ***BI. In vitro***: Investigation of functional assays after inhibition of Hsp90 in five uveal melanoma cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1) to probe the cellular functions of this chaperone.
    - Proliferation assay
    - Apoptosis Tunnel assay
    - Caspase –3 activation assay
    - Cell cycle flow cytometry assay
  - ***B.II*** Downstream molecular effects of Hsp90 inhibition in five UM cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1)
    - Western blot analysis

- **C.** Expression and functional roles of Hsp90 in metastatic uveal melanoma

- ***CI. in vivo:*** patient's samples - paraffin embedded tissues of metastatic uveal melanoma
    - Immunohistochemistry
- ***CII. In vivo:*** animal samples - paraffin embedded tissues of metastatic uveal melanoma from an experimental rabbit model.
    - Immunohistochemistry
- ***CIII. In vitro*****:** expression of Hsp90 in liver metastasis derived uveal melanoma cell line, OMM-1.5.
    - Immunocytochemistry
- **CIV.** Functional assays after inhibition of Hsp90 in one liver metastasis derived uveal melanoma cell line, after inhibition of Hsp90
    - Proliferation assay after inhibition of Hsp90 in OMM1.5 cell line
    - Functional assays after inhibition of Hsp90 in five primary uveal melanoma cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1) with different metastatic potentials
    - Migration assay
    - Invasion assay
- **CV.** Downstream effects of inhibition of Hsp90 in metastatic cascade

  Molecular effects of Hsp90 inhibition in five UM cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1) of different metastatic potential
    - Western blot analysis

# *Chapter 5*

# Materials and Methods

## 5.1 In vivo studies

The expression of Hsp90 was determined by immunohistochemistry in 44 paraffin-embedded sections of primary human uveal melanoma, in 14 sections of primary uveal melanoma from an experimental animal model of uveal melanoma and in eight specimens of uveal melanoma metastasis.

### 5.1.1 Patient Samples

PRIMARY UVEAL MELANOMA PATIENTS

Clinicopathological data from forty-four patients with uveal melanoma was collected from the archives of the Henry C. Witelson Ocular Pathology Laboratory, McGill University, Canada. These data included age, gender, date of diagnosis, previous treatment, development of distant metastasis and follow-up time. Patients were divided into two groups based on the treatment received: group one, primary enucleation, (n= 26) and group 2, enucleation after previous ruthenium plaque radiotherapy (n=16), or after transscleral thermotherapy (TTT) (n=2),
All data accumulation was in accordance with Canada and Province of Quebec legislation and the tenets of the Declaration of Helsinki.

### 5.1.2 Tissue Samples

Forty-four cases of enucleated UM were collected from Henry C. Witelson Ocular Pathology laboratory, McGill University, Montreal, Canada. Eight cases of metastasis and corresponding primary uveal melanoma tumor were available.

Formalin-fixed, paraffin embedded sections were hematoxylin and eosin (H&E) stained for histopathological assessment at the Henry C. Witelson Ocular Pathology Laboratory.

(McLean, Foster et al. 1983) and largest (linear) tumor dimension (McLean, Foster et al. 1982). The tumors were classified as spindle when composed of spindle cells, epithelioid when composed only of epithelioid cells and mixed when composed of spindle and epithelioid cells. The largest tumor dimension, in millimeters, was available from measurements by ultrasound prior to treatment. The follow-up time was recorded in months, between the date of diagnosis and the last available recorded data.

### 5.1.3 Immunohistochemistry

For immunohistochemistry we used an automatic process of Ventana BenchMark fully automated machine**.** The pathway Hsp90 staining module was used according to the protocol and instructions provided by Ventana Medical system Inc. Arizona. The process includes, baking, solvent free deparaffinization, followed by antigen retrieval, CC1 or (Tris/EDTA buffer ph 8.0). Slides were incubated with the primary monoclonal mouse anti-Hsp90 and mouse anti-Hsp90α (Stressgen, BC, Canada) at a dilution of 1:100 for 30 minutes at 37°C, followed by application of biotinylated secondary antibody (8 min, 37°C), and avidin / streptavidin enzyme conjugate complex (8 min, 37°C). The antibody was detected by Fast Red chromogenic substrate and contrastained with hematoxylin.

As a positive control, sections of skin melanoma were used, and for negative controls the primary antibody was omitted.

### 5.1.4 Classification of Immunohistochemistry

Immunohistochemical staining was assessed independently by two pathologists and which agreed with the classification of the slides. Samples were classified by percentage of stained tumor cells and classified as negative if none of the tumor cells showed immunostaining and positive if any of the tumor cells presented immunostaining. Specimens were further classified by a quick score method (Detre, Saclani Jotti et al. 1995; Hauser, Hanzely et al. 2006) according to the percentage of positive staining and specimens were scored (1) when less than 25% of the tumor cells displayed staining, scored (2) when 25% to 50% of tumor cells per section stained and scored (3) when more than 50% of tumor cells per section stained. The staining intensity was scored as follows: (0-1), weak staining, (2), moderate staining and (3) intense staining. A collective score, ranging from 0 to 6, was resultant by adding the above two scores. The cases were further graded as low Hsp90 expression with scores between (0–4), and high Hsp90 expression when total accumulated score was (5 and 6) representing moderate and intense staining and >50% positive cells. This division was based on previous results of quantitative analysis of immunohistochemistry of Hsp90 in urothelial carcinoma (Cappello, David et al. 2006).

### 5.1.5 Statistical analysis

Statistical analyses were based on (MedCalc Statistical Package, Version 9.2.0.2, Mariakerke, Belgium). The correlation between Hsp90 expression and clinical ad prognostic factors was determined using the correlation coefficient test and Student T test. The association between Hsp90 expression the follow-up and metastasis was assessed using the Kaplan-Meier survival analysis and log-rank test. The Student T Test and Chi square analysis were used where appropriate for the *in vitro* studies. A *p*-value of less than 0.05 was considered statistically significant.

## 5.2 In vivo studies - animal samples

For this study we used paraffin embedded blocks of primary and metastatic tumor, retrieved from the archive of Henry C. Witelson Ocular Pathology Laboratory. These samples were derived from an experimental model of uveal melanoma previously characterized (Marshall, Fernandes et al. 2007).

### 5.2.1 Animal model of uveal melanoma

This model was first described and characterized by Blanco et al in our laboratory and consistently validated in numerous studies (Blanco, Marshall et al. 2005; Marshall, Nantel et al. 2007; Di Cesare, Maloney et al. 2009).

Briefly, fourteen male New Zealand albino rabbits (Charles River Canada, St-Constant, Quebec, Canada) with a mean initial weight of 3.3 ± 0.2 kg (range: 2.9-3.6 kg) were used in this animal model.

### 5.2.2 Immunosuppression with Cyclosporin-A

Briefly, fourteen male New Zealand albino rabbits (Charles River Canada, St-Constant, Quebec, Canada) with a mean initial weight of 3.3 ± 0.2 kg (range: 2.9-3.6 kg) were used in this animal model (Blanco, Saornil et al. 2000).

### 5.2.3 Surgical Procedure

The dosage and schedule were conform with animal clinical status and recommended in previous studies (Blanco, Saornil et al. 2000). The surgical procedure consists of a tunnel scleral incision to the suprachoroidal space, followed by introduction of a cannula loaded with one million cells derived from an aggressive uveal melanoma cell line 92.1, suspended in 0.1 ml of RPMI-1640 media followed by injection of the cells in the suprachoroidal space.

### 5.2.4 Isolation of Tissues

Following autopsy of the sacrificed animals, samples were taken from the intraocular tumor and organs and observed for any macroscopically detectable metastases. The enucleated eyes were collected, macroscopically examined and preserved in 10% phosphate-buffered formalin.

### 5.2.5 Histopathological Studies of Tissue Samples

Formalin-fixed, paraffin-embedded specimens were stained with hematoxylin and eosin (H&E) for histopathological and morphological assessment and with HMB-45 monoclonal antibody (DakoCytomation, Mississauga, Ontario, Canada) which is a specific marker for melanoma cells (Burnier, McLean et al. 1991).

### 5.2.6 Immunohistochemistry for Hsp90

Formalin-fixed, paraffin-embedded sections of tumor specimens from the rabbit primary tumor and specimens of lung metastasis were cut and immunostained with monoclonal antibodies against Hsp90 (Stressgen).

## 5.3 *In Vitro* Assays

### 5.3.1 Cell culture

The cell lines used in different subsequent experiments are four cell lines (92.1, SP6.5, MKT-BR, and OCM-1) derived from patients with primary uveal melanoma and a melanocyte transformed cell line (UW-1). The original cell lines have been established as follows: the cell line 92.1 has been established by Dr. Jager (University Hospital Leiden, The Netherlands), (De Waard-Siebinga, Blom et al. 1995), SP6.5 has been established by Dr. Pelletier (Laval University, Quebec, Canada), MKT-BR has been established by Dr. Belkhou (CJF INSERM, France), and OCM-1 has been established by Dr. Albert (University of Wisconsin-Madison, USA) (Diebold, Blanco et al. 1997) and UW-1 by Dr. Saornil (University of Valladolid, Spain)(Diebold, Blanco et al. 1997).

The OMM 1.5 cell line is a uveal melanoma liver metastasis derived cell line and was established by Dr. B.R. Ksander, (Schepens Eye Institute, Boston, MA, USA), (David J. Verbik, Timothy G. Murray et al. 1997).

The cell culture procedure followed the protocol previously described in our laboratory (Marshall, Caissie et al. 2004).

Briefly, incubated cells at 37°C, enriched with 5% $CO_2$ were cultured in RPMI-1640 medium (Invitrogen, Burlington, Ontario, Canada), supplemented with fetal bovine serum (FBS), 1% fungizone, and 1% penicillin-streptomycin purchased from Invitrogen (Burlington, Ontario, Canada). Cells were cultured as a monolayer in 25 $cm^2$ flasks (Fisher, Whitby, Ontario, Canada) and the media was changed twice a week and observed for morphology. The cells were treated with 0.05% trypsin in EDTA (Fisher) and centrifuged for 10 minutes. Cells' counting was performed using the Trypan Blue dye exclusion test. The cell lines were subsequently characterized

in our laboratory and were found to be of different invasive and metastatic abilities (Marshall, Caissie et al. 2004).

### 5.3.2 Immunocytochemistry

Cytospins were prepared from the uveal melanoma cell lines 92.1 and OCM-1, MKT-BR, SP6.5, UW.1 and OMM 1.5 (200000 cells/spin) using the Cytospin 3 centrifuge (Shandon Inc. Pittsburgh, P A, USA) and fixed with 2% paraformaldehyde for 15 minutes. Cytospins were prepared from the uveal melanoma cell lines 92.1 and OCM-1, MKT-BR, SP6.5, UW.1 and OMM 1.5 (200000 cells/spin) using the Cytospin 3 centrifuge (Shandon Inc. Pittsburgh, P A, USA) and fixed with 2% paraformaldehyde for 15 minutes. Immunofluorescence technique analysed Hsp90 and Hsp90α (StressGen), using a FITC-conjugated secondary antibody (Sigma-Aldrich) and SYTO-60 red fluorescent nucleic stain (Invitrogen).

### 5.3.3 In Vitro Proliferation Assay

The Sulforhodamine-B based assay kit (TOX-6, Sigma- Aldrich) was performed as per the National Cancer Institute protocol (Skehan, Storeng et al. 1990). Briefly, the UM cell lines were seeded into wells at a concentration of $2.5x10^3$ cells per well, in a minimum of six wells per cell line. Cells with and without 17-AAG at different dilutions (ranging from 100μM to 0.001 μM), were allowed to incubate for 48 hours following cell seeding. Cells were then fixed to the bottom of the wells using a solution of 50% Trichloroacetic acid (TCA) for 1 hour at 4°C, rinsed and the Sulforhodamine-B dye was added to each well and allowed to stain for 25 minutes. The dye incorporated into the cells was solubilized in a 10 mM solution of Tris and the the solute was measured using a microplate reader at a wavelength of 565 nm.

### 5.3.4 Migration assay

A QCM 24-well colorimetric cell migration assay (Chemicon) was used for this experiment as per the manufacturer's recommendations. Briefly, uveal melanoma cell lines with and without 17-AAG (10 µmol/L) were seeded at a concentration of $1 \times 10^6$ cells/mL in serum-free RPMI 1640 in the upper well insert of a QCM 24-well colorimetric cell migration assay. In this experiment CXCL8 was used as a chemoattractant (Di Cesare, Marshall et al. 2007) after being reconstituted and diluted to appropriate predetermined optimum concentrations in serum-free RPMI 1640 (CXCL8: 300 ng/mL). The experiments were done in triplicate for all five cell lines. The plates were then incubated for 24 h in a humidified 5% $CO_2$-enriched atmosphere. The cels that protruded through the 8-µm pore membranes, located at the bottom of every well insert, were stained and eluted. The solute absorbance was read by a colorimetric plate reader.

### 5.3.5 Invasion assay

Cellular nvasive abilty was analysed in a modified Boyden chamber consisting of polyethylene terephthalate membrane with 8-µm-diameter pores precoated with Matrigel, an artificial basement membrane (Becton Dickinson Labware), (Woodward, Elshaw et al. 2002). As a control the same type of polyethylene terephthalate membrane was used without Matrigel coating.

Briefly, $1.25 \times 10^5$ cells with and without 10 µmol/L 17-AAG were added to the upper chamber in RPMI 1640 without 0.1% fetal bovine serum. As a chemoattractant we used RPMI 1640 with 10% fetal bovine serum at the bottom of the chamber. The cells were incubated at 37°C in 5%

$CO_2$-enriched atmosphere for 48 h to allow for cellular invasion through the Matrigel. Noninvading cells were removed from the upper chamber and membranes were stained using a Diff-Quick staining set. Stained cells whose nuclei had completely invaded through the membrane were counted microscopically in 20 high-power (x400) fields. Each experimental condition, including control, was done in triplicate and the average number of invading cells was then calculated for all experimental conditions.

Percentage invasion was determined for each cell line under each experimental condition using the following formula: % invasion = (mean number of cells invading through the Matrigel divided by mean number of cells migrating through control polyethylene terephthalate membrane) multiplied by a hundred.

### 5.3.6 Cell cycle analysis

After cell passage and counting, the cells were treated with (10 μmol/L) 17-AAG for 24 h. Samples were incubated on ice for 30 min, washed with PBS, and resuspended in cold staining buffer containing 0.1% sodium citrate, 0.2 mg/mL RNase, 0.3% NP40, and 50 g/mL propidium iodide. Following propidium iodide incorporation, the cells were analyzed using an Epics XL flow cytometer (Beckman Coulter) for the percentage of cells in $G_1$, the S-phase fraction, and $G_2$.

### 5.3.7 Caspase-3 protease activity assay

The caspase-3 protease activity was detected using the BD ApoAlert Caspase Colorimetric Assay (BD Biosciences Clontech) according to the manufacturer's protocol. Briefly, cells with and without 17-AAG (10 μmol/L), 1 x $10^6$ cells/mL were counted, centrifuged, and treated with a lysis buffer. After centrifugation supernatant samples of 50 μL from each cell line and controls were treated with a reaction buffer/DTT mix and 1 μL of caspase-3 inhibitor followed by 30-min incubation on ice. The Caspase-3 substrate was allowed to react with the cells, followed by a period of one hour incubation at 37°C. The samples were read in a microplate reader using a spectrophotometer at 405 nm. The experiments were repeated in triplicate.

### 5.3.8 Apoptosis assay

Apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (Apo-Direct, BD Biosciences) according to the instructions of the manufacturer. Following 48h treatment with 10 μmol/L of 17-AAG, cells were collected, washed in PBS, and fixed with 70% ethanol. This was followed by incubation with FITC-dUTP for 60 min at 37°C and data was collected and analyzed with Epics XL flow cytometer using CellQuest Pro software (BD Biosciences).

### 5.3.9 Western blot analysis

The cells were incubated with and without 10 μmol/L 17-AAG for 24, 48, and 72 h. Cells were lysed on ice in TNESV (50 mmol/L Tris-HCl, 1% NP40, 2 mmol/L EDTA, 100

mmol/L NaCl, 1 mmol/L orthovanadate) containing protease inhibitor cocktail (Sigma-Aldrich) and the lysate was cleared by centrifugation for 10 min at 12,000 x *g*. Protein aliquots of 10 µg (Bio-Rad protein assay (Bio-Rad Laboratories) were loaded onto 10% to 12% SDS-PAGE gel and then transferred to Immobilon (PVDA) transfer membranes (Millipore). The membrane was blotted for the specific antibodies, monoclonal mouse anti-Hsp90 and anti-Hsp70 (StressGen), polyclonal rabbit anti-Akt and polyclonal rabbit anti-phosphorylated Akt (p-Akt; $Ser^{473}$; Cell Signaling Technology), monoclonal anti-CDK4 anti-cyclin D1 (Cell Signaling Technology), anti IGF-1R and anti-Met (Santa-Cruz Biotechnology Inc, Santa Cruz, CA, USA) and anti-FAK[pY397], (BD Biosciences, ON, Canada). Immunoblotting with anti-actin antibodies was done to confirm equal protein loading (Santa Cruz Biotechnology Inc.). Further, horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) was applied. Detection was done using enhanced chemiluminescence with autoradiographic film (Hyperfilm ECL, GE Healthcare).

# Chapter 6

## Results

Based on this thesis, partial data regarding the expression and inhibition of Hsp90 in uveal melanoma were published for the first time by our group, (Faingold, Marshall et al. 2006; Faingold, Marshall et al. 2007; Faingold, Abourbih et al. 2008; Faingold, Marshall et al. 2008) and the results will be presented in the following chapters.

### A. Expression of Hsp90 in primary uveal melanoma

Given the importance of Hsp90 in malignant progression, we assessed its expression in a large series of primary uveal melanomas, a panel of cell lines and confirmed the expression in all primary uveal melanoma from an experimental animal model. The rationale behind these experiments was to determine whether Hsp90 is expressed in primary uveal melanoma and might be associated with survival and other clinical variables, therefore establishing it as a potential therapeutic target.

Specific objectives:

**AI. PATIENT'S SAMPLES - PARAFFIN EMBEDDED TISSUES OF PRIMARY UVEAL MELANOMA**

- **IMMUNOHISTOCHEMISTRY**
- **CORRELATION WITH PROGNOSTIC FACTORS**

# *6.1 Results*

## ➢ 6.1.1 IMMUNOHISTOCHEMISTRY

### *Hsp90 is expressed in uveal melanoma*

The clinical and histopathological data pertaining to forty-four cases of uveal melanoma are displayed in tables 1 and 2. Twenty-eight patients were males and the age of the patients ranged from 32 to 82 years (mean, 61). Seventy-seven percent of tumors ($n$ = 34) were classified as mixed cell type, 4.5% ($n$ = 2) as epithelioid cell type, and 18.8% ($n$ = 8) as spindle cell type.

Overall, 68% of tumors ($n$ = 30) were Hsp90 positive. Positive cells for Hsp90 displayed predominant intracytoplasmic staining and in a fraction of cells Hsp90 presented as nuclear staining. Hsp90 expression was observed in the normal retinal pigment epithelium, inner segments of photoreceptor layer, inner and outer plexiform layers, inner nuclear layer and the ganglion cell layer. The normal choroidal melanocytes displayed a low degree of staining (Fig.6.1). We were able to detect that in a number of cases the spindle A cells at the base of the tumor displayed low or no staining as compared with the tumor. (Fig. 6.2).

| Case | Age | Previous radiotherapy | Metastasis | Follow-up (months) | LTD* (mm) | Cell type | Hsp90 | Percent staining | Intensity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 64 | no | no | 177 | 11 | spindle | Y | 25-50 | 0 |
| 2 | 64 | no | no | 267 | 14 | spindle | Y | >50 | 3 |
| 3 | 49 | no | yes | 120 | 12 | mixed | N | 0 | 0 |
| 4 | 67 | no | yes | 36 | 15 | mixed | Y | >50 | 3 |
| 5 | 51 | no | no | 140 | 24 | mixed | Y | >50 | 3 |
| 6 | 67 | no | yes | 83 | 8.5 | mixed | Y | >50 | 3 |
| 7 | 71 | no | yes | 83 | 18.5 | mixed | Y | 25-50 | 3 |
| 8 | 60 | no | yes | 46 | 18 | mixed | N | 0 | 0 |
| 9 | 41 | no | no | 142 | 20 | spindle | Y | >50 | 3 |
| 10 | 61 | no | no | 86 | 14.5 | spindle | N | 0 | 0 |
| 11 | 62 | no | no | 82 | 16 | mixed | N | 0 | 0 |
| 12 | 80 | no | yes | 8 | 12.6 | mixed | N | 0 | 0 |
| 13 | 73 | no | yes | 12 | 12 | mixed | Y | 25-50 | 2 |
| 14 | 81 | no | yes | 2 | 19 | mixed | Y | >50 | 3 |
| 15 | 72 | no | yes | 17 | 15 | mixed | N | 0 | 0 |
| 16 | 59 | no | yes | 31 | 15 | epithelioid | Y | >50 | 3 |
| 17 | 44 | no | no | 32 | 9.2 | spindle | Y | >50 | 3 |
| 18 | 32 | no | yes | 30 | 18 | mixed | Y | <25 | 1 |
| 19 | 68 | no | yes | 52 | 11.3 | mixed | Y | >50 | 3 |
| 20 | 72 | no | yes | 44 | 11 | mixed | Y | >50 | 3 |
| 21 | 63 | no | no | 36 | 12.3 | spindle | Y | <25 | 2 |
| 22 | 65 | no | no | 38 | 11 | mixed | Y | >50 | 3 |
| 23 | 75 | no | yes | 44 | 8 | mixed | N | 0 | 0 |
| 24 | 82 | no | no | 72 | 13 | mixed | N | 0 | 0 |
| 25 | 50 | no | no | 61 | 11.5 | mixed | N | 0 | 0 |
| 26 | 76 | no | no | 62 | 18.5 | mixed | Y | 25-50 | 3 |

Table 1: Characteristics of patients and immunohistochemical staining of Hsp90 in uveal melanoma cases treated by enucleation; *LTD – largest tumor dimension

| Case | Age | Previous radiotherapy | Metastasis | Follow-up (months) | LTD* (mm) | Cell type | Hsp90 | Percent staining | Intensity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 45 | yes | no | 107 | 10 | mixed | Y | <25 | 1 |
| 2 | 63 | yes | no | 198 | 15 | mixed | Y | >50 | 3 |
| 3 | 71 | yes | yes | 22 | 18 | mixed | Y | >50 | 3 |
| 4 | 59 | yes | yes | 5 | 24 | mixed | Y | >50 | 3 |
| 5 | 58 | yes | yes | 98 | 15 | mixed | Y | >50 | 2 |
| 6 | 64 | yes | yes | 53 | 17 | mixed | Y | >50 | 3 |
| 7 | 48 | yes | yes | 25 | 14 | mixed | Y | 25-50 | 1 |
| 8 | 48 | yes | yes | 126 | 10 | mixed | Y | <10 | 2 |
| 9 | 45 | yes | no | 215 | 12 | spindle | N | 0 | 0 |
| 10 | 51 | yes | no | 197 | 15 | mixed | Y | 25-50 | 2 |
| 11 | 64 | yes | no | 181 | 12 | mixed | N | 0 | 0 |
| 12 | 50 | yes | no | 194 | 8 | mixed | N | 0 | 0 |
| 13 | 41 | yes | no | 176 | 11 | mixed | N | 0 | 0 |
| 14 | 69 | yes | no | 75 | 10.5 | mixed | Y | >50 | 3 |
| 15 | 45 | yes | no | 64 | 6.8 | spindle | N | 0 | 0 |
| 16 | 77 | yes | no | 27 | 14 | mixed | Y | >50 | 3 |
| 17 | 61 | TTT§ | no | 45 | 8.3 | mixed | Y | >50 | 1 |
| 18 | 56 | TTT | no | 45 | 6.5 | epithelioid | Y | >50 | 1 |

Table 2: Expression of Hsp90 and histopathologic parameters in uveal melanoma cases treated with radiotherapy or TTT before enucleation.

*LTD – largest tumor dimension; §- transscleral thermotherapy

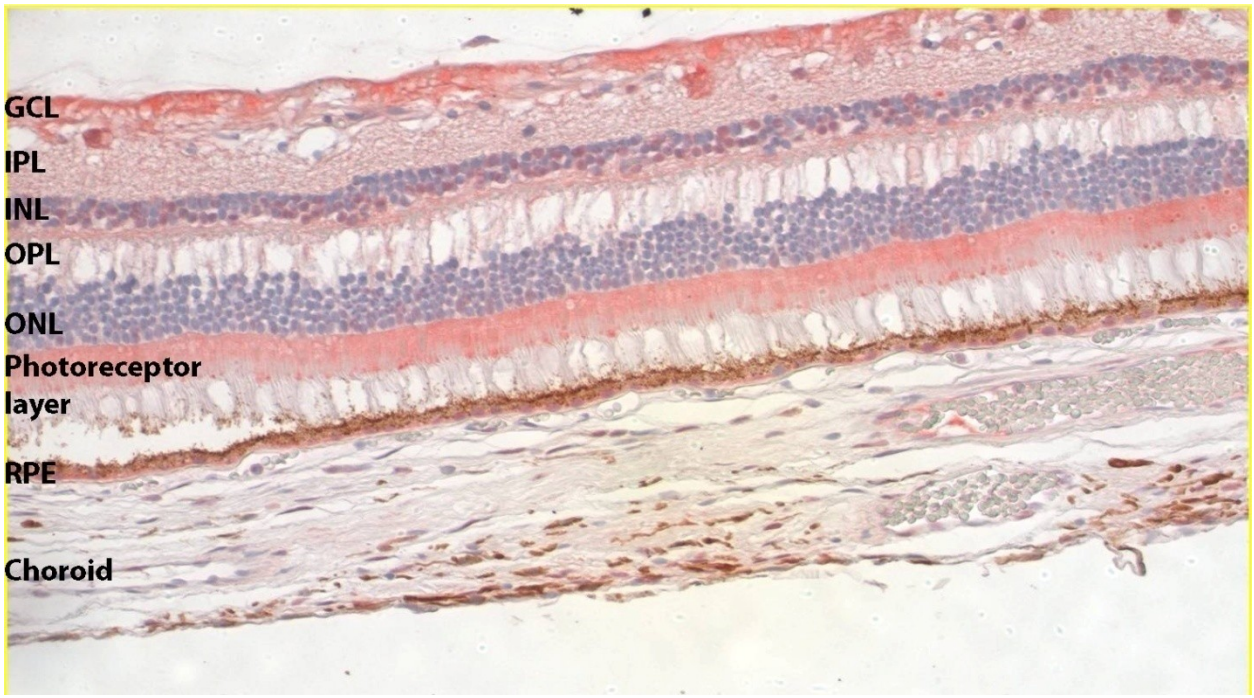



**Fig.6.1** Hsp90 staining of retinal tissue in ganglion cell layer (GCL), inner and outer plexiform layer (IPL,OPL), inner nuclear layer (INL), photoreceptor layer and a low percentage of Hsp90 staining in the retinal pigment epithelium (RPE) and choroid. Original magnification, x200.

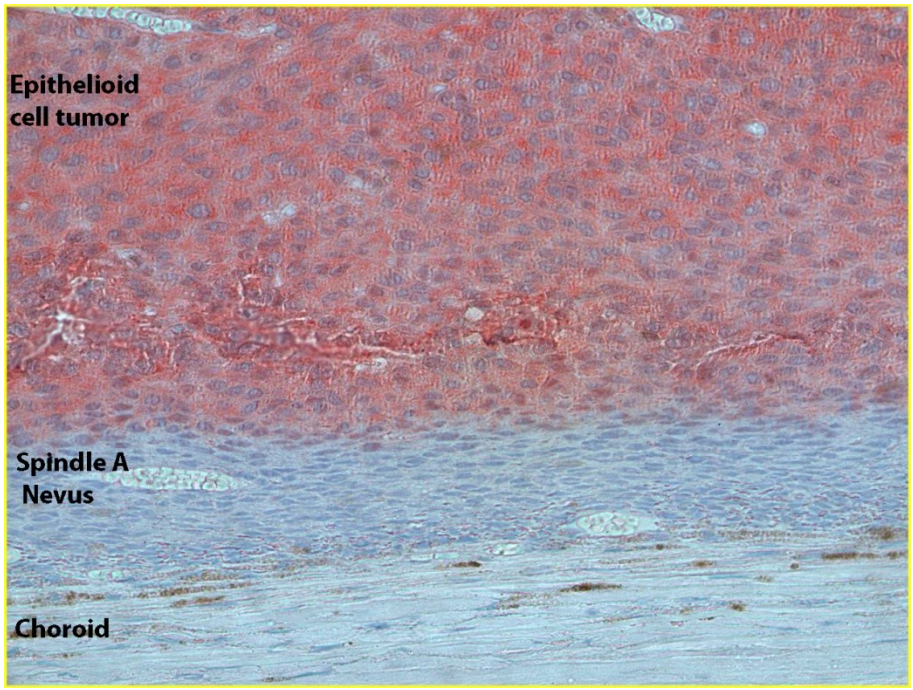



**Fig.6.2** Staining of epithelioid cell uveal melanoma for Hsp90 (red). In comparison with the tumor, the spindle A (nevus), at the base of the tumor and the choroid display low percentage of Hsp90 staining.

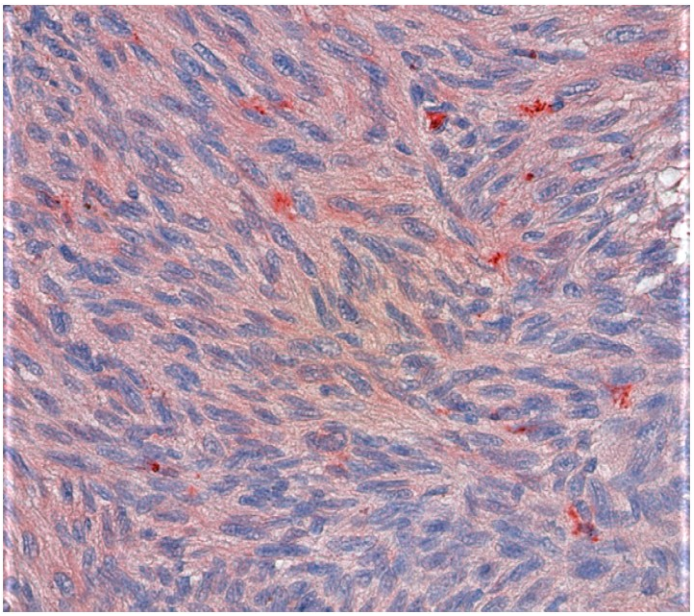


**Fig.6.3** Representative staining of tumor tissues. Spindle cell uveal melanoma stained for Hsp90 showing medium staining intensity. Original magnification, x400.

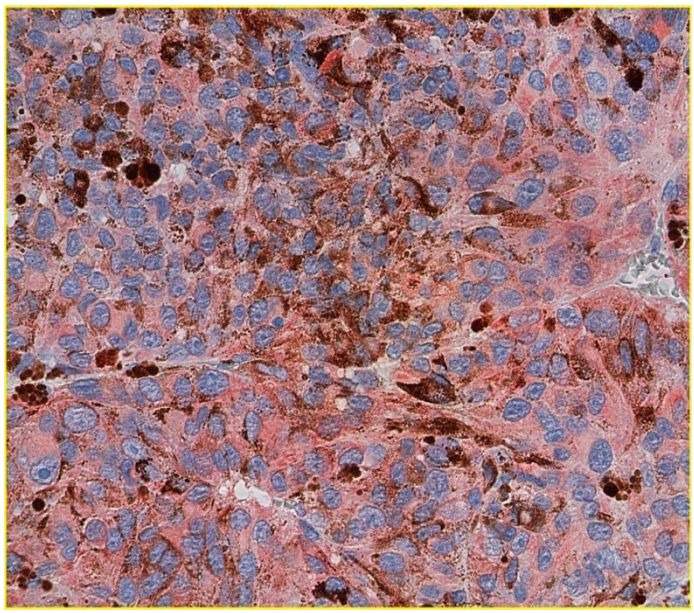


**Fig.6.4 A** Epithelioid cell uveal melanoma stained for Hsp90 showing predominant cytoplasmic staining and high intensity. Original magnification, x400.

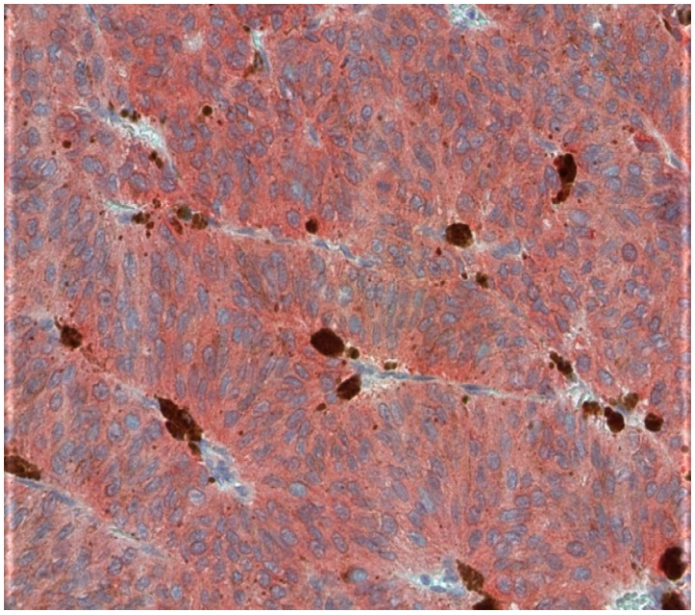


Fig **6.4 B** Mixed cell uveal melanoma stained for Hsp90 showing high staining intensity. Original magnification, x200.

Fifty percent of the tumors (22 of 44) were classified in the high Hsp90 score group with >50% cells stained and a high degree of staining intensity. The other 50% of the cases exhibited low intensity and percentage of staining and were categorized as the group with low Hsp90 expression (Fig.6.3 and 6.4). Hsp90 stained positive in 61.5% ($n$ = 16) of the 26 patients in the primary enucleation group and 50% ($n$ = 13) of those cases were classified as high Hsp90 expression. In the group of patients treated with radiotherapy before enucleation ($n$ = 16), Hsp90 stained positive in 68% ($n$ = 11) of cases and high expression was seen in 43.75% ($n$ = 7). Two patients (4.5%) underwent transscleral thermotherapy before enucleation and both had a high degree of Hsp90 immunostaining.

## ➢ CORRELATION WITH PROGNOSTIC FACTORS

### *Hsp90 correlates with largest tumor dimension*

In the high Hsp90 expression group the LTD (mean ± SD) was 14.69 ± 4.87 mm. In the low Hsp90 expression group, the mean LTD was 12.3 ± 2.95 mm. There was a significant correlation between high expression of Hsp90 and a larger tumor dimension ≥ 15mm ($P$ = 0.03).

The immunohistopathological assessment did not correlate with other factors like cell type or previous method of treatment ($P$ > 0.05). At the end of the follow-up period (average 83 ± 66.6 months), 45.4% ($n$ = 20) of patients developed metastases. In the group of samples from the patients who developed metastases there was a significant difference between Hsp90 immunostaining: positive in (75%; $n$ = 15) of cases and negative tumors in (25%; $n$ = 5) of the tumors. There was no correlation between incidence of metastases and high versus low classification for immunostaining. There were 11 (50%) cases with metastasis in high Hsp90 and 9 (41%) in the low Hsp90 expression group. Hsp90 was not identified as a prognostic factor in uveal melanoma. In

our sample Kaplan-Meier test showed that percentage and distribution, intensity of staining were not related to survival rate ($P > 0.05$).

*A. Expression of Hsp90 in primary uveal melanoma (cont.)*

**AII. IN VIVO: IN ANIMAL SAMPLES - PARAFFIN EMBEDDED TISSUES OF PRIMARY UVEAL MELANOMA FROM AN EXPERIMENTAL RABBIT MODEL**

- **IMMUNOHISTOCHEMISTRY**

## 6.2 Results

EXPERIMENTAL RABBIT MODEL OF UVEAL MELANOMA

### 6.2.1. Gross and Histopathological Examination

After immune-histopathological examination primary tumors were found in nine out of fourteen animals (64%) which was confirmed by positive immunostaining for melanoma marker HMB-45 monoclonal antibody (Table 3).

The intraocular tumors were mainly comprised of epithelioid cells and extraocular extension could be observed in seven eyes (50%).

## 6.2.2 IMMUNOSTAINING FOR HSP90

### *Hsp90 is expressed in primary uveal melanoma tumors in an animal model*

The Hsp90 immunostaining was positive in all nine primary tumors of rabbits. The staining was intracytoplasmic and the proportion of immunostaining was always greater than 50% (+3) in all samples. Six of the tumors (66%) also displayed high intensity of staining for Hsp90 (Fig. 6.6).

| Case number | Intraocular tumor | Hsp90 percentage | Score intensity | Total score |
|---|---|---|---|---|
| 1 | yes | 3 | 3 | 6 |
| 2 | yes | 3 | 1 | 4 |
| 3 | yes | 3 | 1 | 4 |
| 4 | yes | 3 | 3 | 6 |
| 5 | yes | 3 | 3 | 6 |
| 6 | yes | 3 | 3 | 6 |
| 7 | yes | 3 | 1 | 4 |
| 8 | yes | 3 | 2 | 5 |
| 9 | yes | 3 | 3 | 6 |

Table 3. The table summarizes the presence of intraocular tumor and Hsp90 immunostaining patterns in 9 enucleated eyes from an animal experimental model of uveal melanoma.

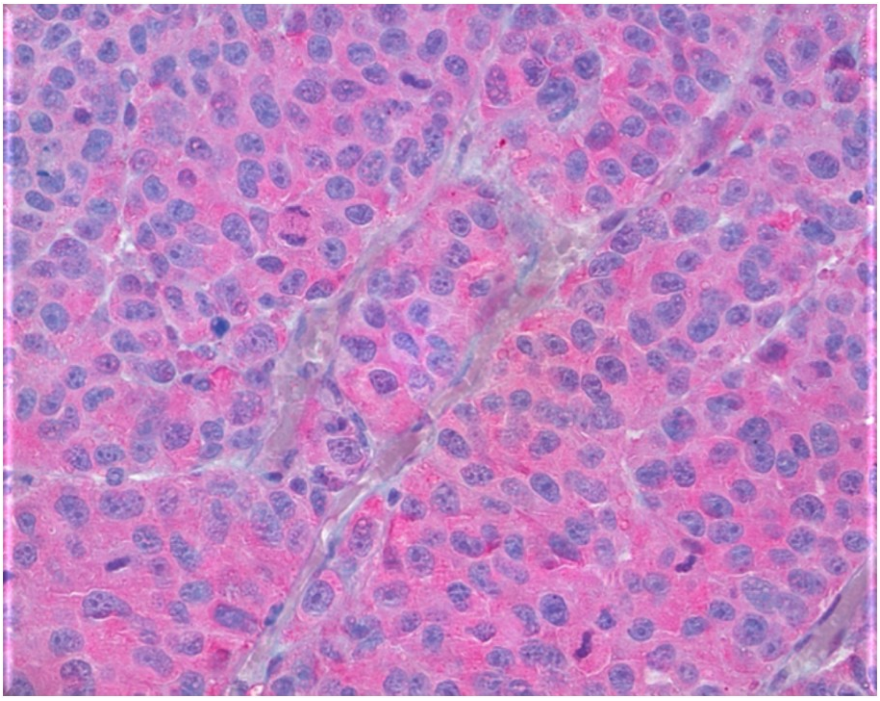


**Fig.6.6** Hsp90 staining of primary uveal melanoma section from an experimental rabbit model showing high (+3) immunostaining intensity levels.

Original magnification x400

*A. Expression of Hsp90 in primary uveal melanoma (cont.)*

***AIII. IN VITRO: IN FIVE UVEAL MELANOMA CELL LINES (92.1, MKT-BR, SP6.5, OCM, UW-1)***

- **IMMUNOCYTOCHEMISTRY**
- **WESTERN BLOT**

## 6.3 Results

### 6.3.1 IMUNOCYTOCHEMISTRY

*Hsp90 is expressed in uveal melanoma cell lines*

The expression levels of Hsp90 were studied in four human uveal melanoma cell lines (92.1, SP6.5, MKT-BR, OCM-1) and one human uveal transformed melanocyte cell line (UW-1).

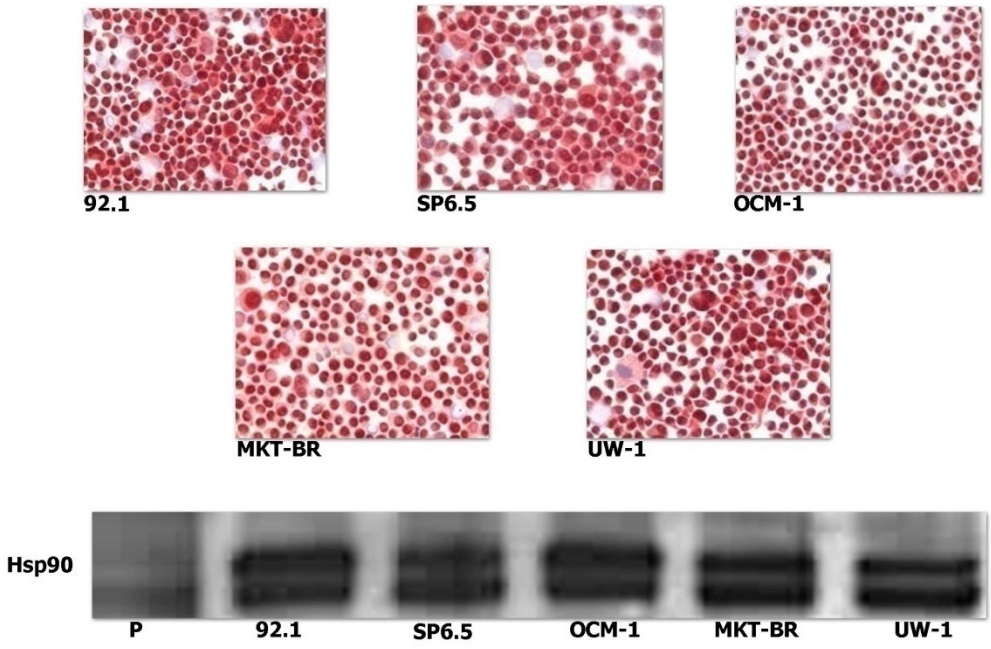
The cytospins analysis showed that Hsp90 was highly expressed in all cell lines and was found to be predominantly intracytoplasmic. A subpopulation of these positive cells showed concomitant membranous and nuclear Hsp90 expression. There was no difference between the intensity and distribution of the staining among the cell lines (Fig.6.7).

Using immunofluorescence we could detect membranous expression of Hsp90α in a subpopulation of cells which may indicate interaction between Hap90 on the cell surface and extracellular matrix (Fig 6.8).

### 6.3.2 WESTERN BLOT

Western blot analysis detected a strong expression of Hsp90 in all the cell lines (Fig.6.7).

**Fig. 6.7**:




Hsp90 immunostaining in cytospin samples of five uveal melanoma cell lines;

Western blot detection of Hsp90 in uveal melanoma cell lines. (P = positive control)

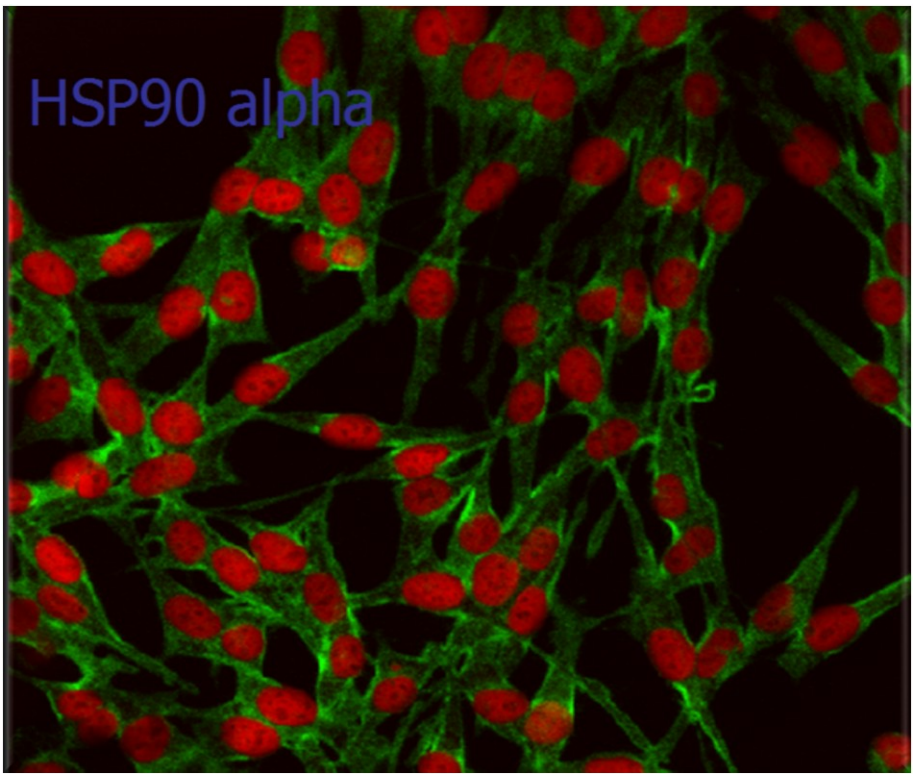



**Fig.6.8** Expression of Hsp90α in 92.1 uveal melanoma cell line visualized by confocal microscopy, showing membranous and intracytoplasmic staining (green). Original magnification, x400.

# A. Expression of Hsp90 in primary uveal melanoma

## *6.4 Summary*

- ✓ *Hsp90 was found to be highly expressed in human uveal melanoma cases. These results were confirmed in specimens from an animal model of uveal melanoma*
- ✓ *The correlation of Hsp90 with largest tumor dimension, a clinical indicator of poor prognosis, suggests that Hsp90 expression in uveal melanoma could predict aggressive behavior of these tumor cells.*
- ✓ *Hsp90 is expressed in uveal melanoma cell lines*
- ✓ *HSP90$\alpha$ was detected on the surface of tumor cells suggesting possible extracellular roles for Hsp90$\alpha$ and interaction with the extracellular matrix*

# B. Functional roles of Hsp90 in uveal melanoma primary tumor

Uveal melanoma is the result of multiple events leading to the abnormalities of cell cycle and apoptosis ensuing malignant transformation and proliferation of uveal melanocytes (Loercher and Harbour 2003). Uveal melanoma cells survive through stimulation of multiple signaling pathways, including the PI3K and the MAPK pathways (Triozzi, Eng et al. 2008). At the molecular level, Hsp90 activity might allow tumor cells survival and escape from apoptosis through interaction with multiple client proteins critical in proliferation, cell cycle control and apoptosis. Inhibition of Hsp90 arrests cell cycle and accelerates apoptosis and downregulates signaling molecules (Whitesell, Mimnaugh et al. 1994). For example, CDK4 and Akt levels were reported to decline after 17-AAG administration both in vivo and in vitro (Sato, Fujita et al. 2000).

We reported high levels of expression of Hsp90 in human uveal melanoma cell lines. Taken together, these facts represented a solid rationale for our group to conduct for the very first time *in vitro* studies addressing the effects of Hsp90 inhibitors in these cell lines with regard to proliferation, cell cycle and apoptotic effects.

Specific objectives:

**BI. *IN VITRO:* INVESTIGATION OF FUNCTIONAL ASSAYS AFTER INHIBITION OF HSP90 IN FIVE UVEAL MELANOMA CELL LINES (92.1, MKT-BR, SP6.5, OCM, AND UW-1) TO PROBE THE CELLULAR FUNCTIONS OF THIS PROTEIN**

- **PROLIFERATION ASSAY**
- **APOPTOSIS TUNNEL ASSAY**
- **CASPASE –3 ACTIVATION ASSAY**
- **CELL CYCLE FLOW CYTOMETRY ASSAY**

## 6.5 Results

### 6.5.1 Proliferation assay

*17-AAG induces antiproliferative effects in uveal melanoma cell lines*

17-AAG had a significant effect in uveal melanoma cells ($P < 0.001$) and induced a downregulation of the cellular proliferation in all uveal melanoma cell lines when compared with controls at concentrations ranging from 0.1 to 100 µmol/L (Fig. 6.9). From the dose-response data, corrected for the cell count at time zero, we calculated the percentages of growth inhibition at different drug concentrations using the formula: $GI = 100 \times (T - T_0) / (C - T_0)$. $GI_{50}$ values were extracted using the nonlinear regression analysis for a dose-response curve in a sigmoid plot, using GraphPad Prism version 3 (Banerji, O'Donnell et al. 2005).

Growth-inhibitory concentration, $GI_{50}$ values, ranged from 50 to 142 nmol/L (average, 109 nmol/L). The corresponding $GI_{50}$ values were (mean ± SD) were 123 ± 0.33, 142 ± 0.17, 93 ± 0.27, 50 ± 0.37, and 137 ± 0.3 for 92.1, SP6.5, MKT-BR, OCM-1 and UW-1, respectively (Table 4). In the following experiments, we chose the concentration of 10 µmol/L 17-AAG to obtain a maximum effect of Hsp90 inhibition. This was based on based on pharmacokinetic analysis data from patient phase I clinical trials, that demonstrated that at the highest drug dose administration , plasma 17-AAG concentrations achieved or exceeded 10 µmol/L, (Banerji, O'Donnell et al. 2005; Nowakowski, McCollum et al. 2006).

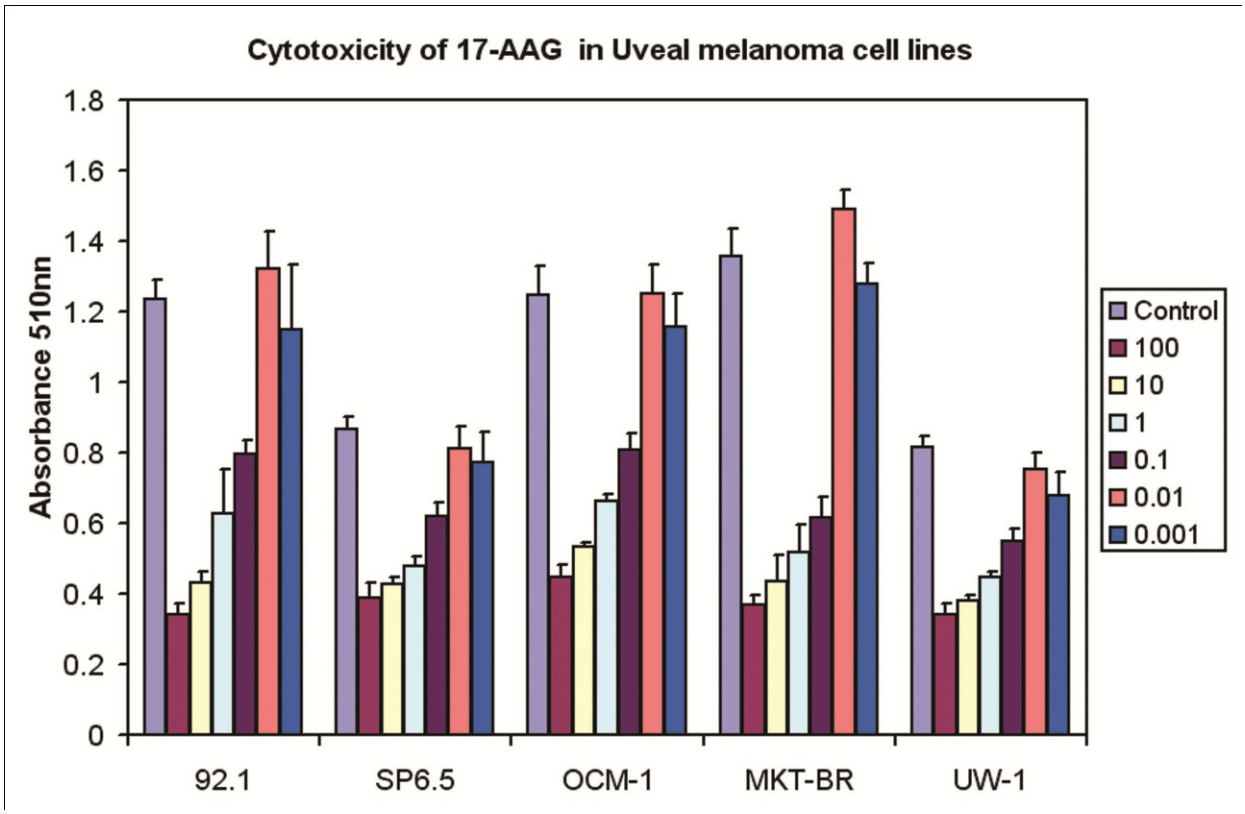



**Fig. 6.9** Sulforhodamine TOX-6 assay showing the effect of 17-AAG on proliferation of the five uveal melanoma cell lines compared with control at a concentration ranging from 100 to 0.001 µmol/L. Significant inhibition ($P < 0.001$) of cellular proliferation was seen at concentrations ranging from 0.1 to 100 µmol/L.

| Cell line | $GI_{50}$ | SD* |
|---|---|---|
| **92.1** | **123** | **0.33** |
| **SP6.5** | **142** | **0.17** |
| **OCM-1** | **93** | **0.27** |
| **MKT-BR** | **50** | **0.37** |
| **UW-1** | **137** | **0.3** |

**Tabel 4** Growth inhibitory concentration ($GI_{50}$) of the five uveal melanoma cell lines with 17-AAG.

∗ Standard Deviation.

### ➢ 6.5.2 CELL CYCLE ANALYSIS.

*Hsp90 blockade by 17-AAG induces $G_1$ cell cycle arrest*

Treatment of the five (92.1, SP6.5, MKT-BR, OCM-1, and UW-1) cell lines with 10 μmol/L 17-AAG for 24 h induced a significant increase in percentage of cells in the $G_1$ compared with baseline of 18.9% for 92.1, 56% for SP6.5, 44.7% for OCM-1, 42.2% for UW-1, and 38.7% for MKT-BR (p value <0.0001), (Table 5 and fig. 6.10).

| Cell line | G1% | S% | G2% |
|---|---|---|---|
| 92.1 w/o 17-AAG | 47.4 | 37.1 | 15.5 |
| 92.1 with 17-AAG | 66.3 | 1.02 | 33.1 |
| SP6.5 w/o 17-AAG | 29.7 | 26 | 44.5 |
| SP6.5 with 17-AAG | 85.7 | 1.25 | 11.7 |
| OCM-1 w/o 17-AAG | 37.7 | 42.4 | 19.7 |
| OCM-1 with 17-AAG | 82.4 | 4.79 | 15.5 |
| MKT-BR w/o 17-AAG | 39.2 | 29.3 | 31.9 |
| MKT-BR with 17-AAG | 77.9 | 8.58 | 15.4 |
| UW-1 w/o 17-AAG | 42.7 | 30.4 | 26.6 |
| UW-1 with 17-AAG | 84.9 | 10.1 | 29 |

**Tabel 5** Percentage of cells in different phases of the cell cycle (G1, S and G2), with and with-out 17-AAG, showing that inhibition of Hsp90 resulted in accumulation of the cells in G1 at 24 hours.

### ➢ 6.5.3 APOPTOSIS TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE–MEDIATED DUTP NICK END LABELING ASSAY

## *17-AAG induced increased rates of apoptosis*

In treated uveal melanoma cells, 17-AAG induced cell death (cells positive for FITC-dUTP) after 48 h. The percentages of FITC-dUTP–positive cells were 3.70%, 5.40%, 11.87%, 0.34%, and 6.35% in 17-AAG–treated 92.1, SP6.5, OCM-1, MKT-BR, and UW-1 whereas control cells showed 0.05%, 0.05%, 1.44%, 0.15%, and 0.26% cell death, respectively (Fig. 6.10). These results showed that 17-AAG can induce apoptotic cell death in uveal melanoma cells.

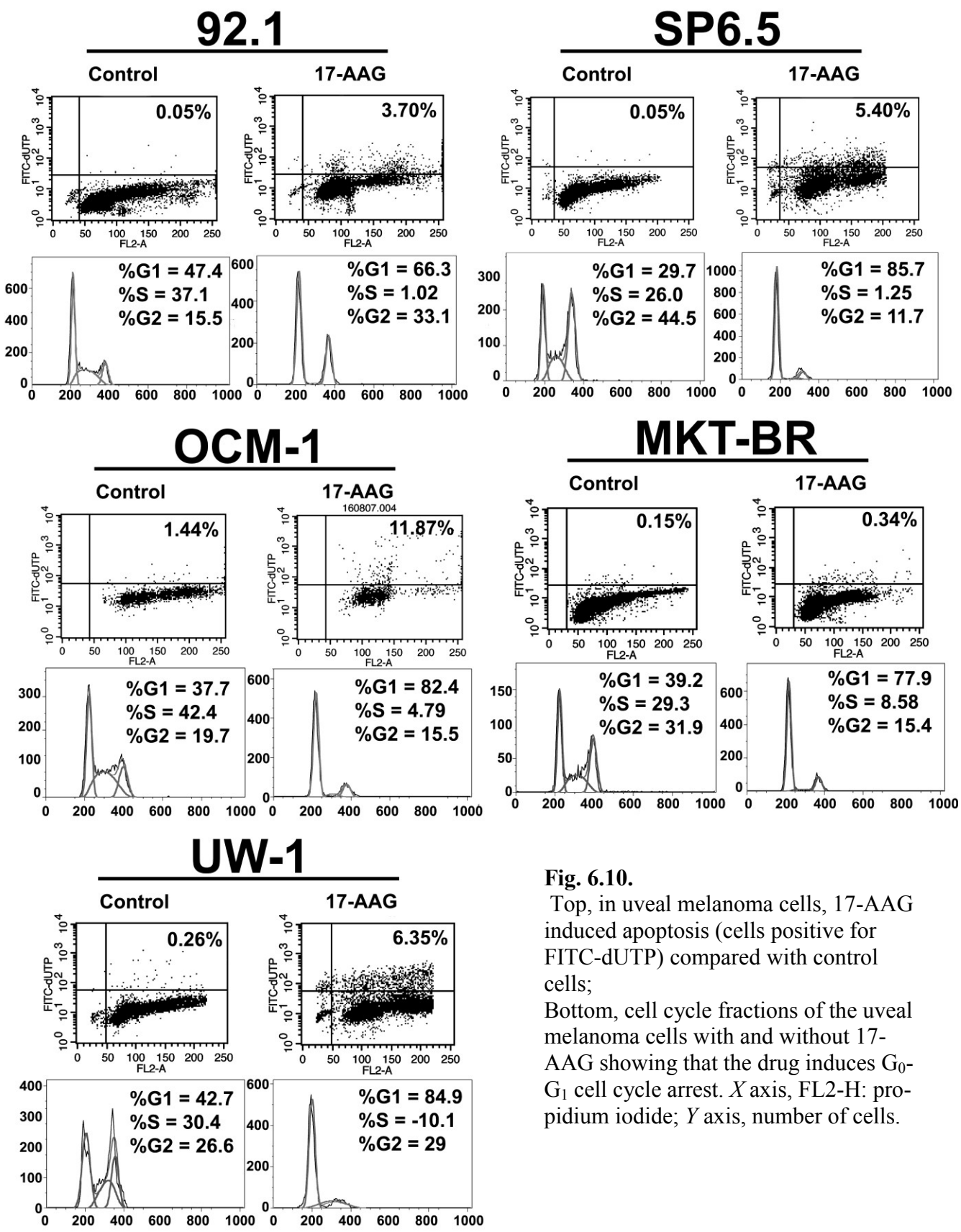



**Fig. 6.10.**
Top, in uveal melanoma cells, 17-AAG induced apoptosis (cells positive for FITC-dUTP) compared with control cells;
Bottom, cell cycle fractions of the uveal melanoma cells with and without 17-AAG showing that the drug induces $G_0$-$G_1$ cell cycle arrest. *X* axis, FL2-H: propidium iodide; *Y* axis, number of cells.

### ➢ 6.5.4 CASPASE-3 PROTEASE ACTIVITY ASSAY.

Treatment of the five cell lines with 10 µmol/L 17-AAG for 48 h showed significantly higher levels of activated caspase-3 from 0.124 ± 0.006 to 0.277 ± 0.04 for 92.1, from 0.107 ± 0.01 to 0.190 ± 0.004 for SP6.5, from 0.084 ± 0.006 to 0.120 ± 0.003 for OCM-1, from 0.182 ± 0.002 to 0.218 ± 0.010 for MKT-BR, and from 0.089 ± 0.007 to 0.129 ± 0.010 for UW-1, indicating a activation of the mitochondrial/intrinsic apoptosis pathway. The histogram figure represents average results from three separate experiments (Fig. 6.11).

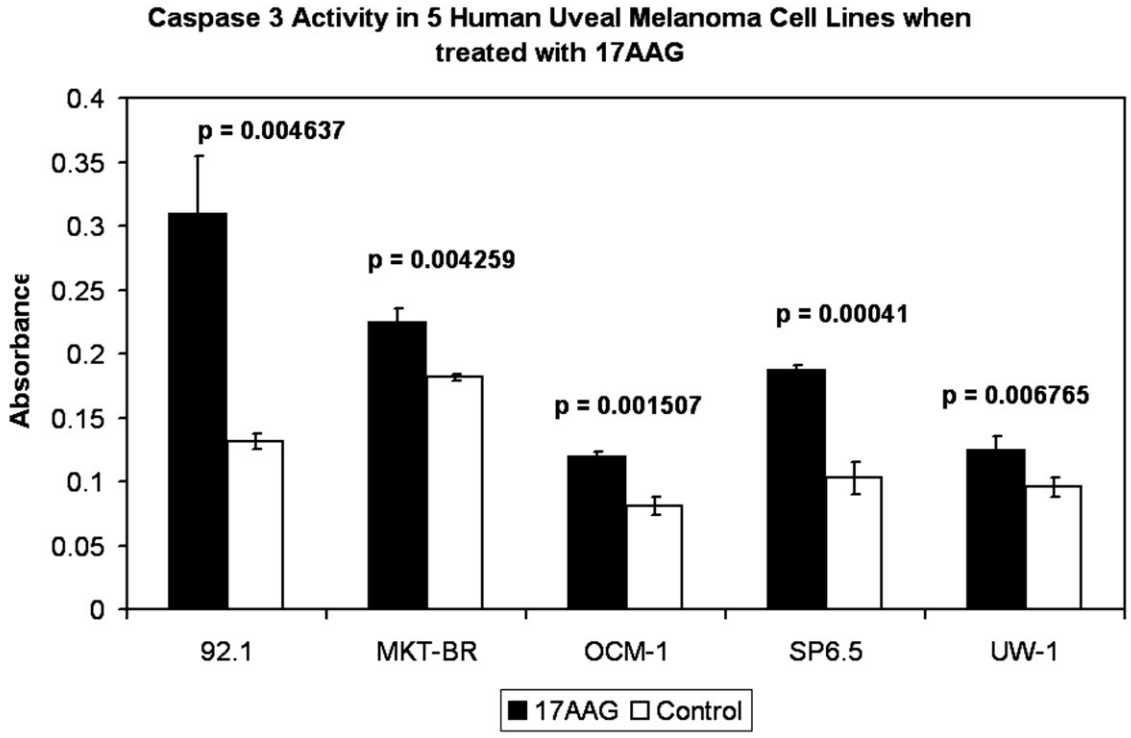



**Fig. 6.11** Caspase-3 protease activity assay showing an increase in caspase-3 on exposure of uveal melanoma cell lines (92.1, OCM-1, SP6.5, MKT-BR, and UW-1) to 17-AAG. Columns represent mean of three experiments.

## B.II Downstream molecular effects of Hsp90 inhibition in five UM cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1)

- Western blot analysis

- 6.5.5 Western blot

### *17-AAG upregulates Hsp70 and depletes P-AKT and CDK4*

Inhibition of Hsp90 was shown to influence the expression of Hsp90 and cochaperone Hsp70, CDK4, cyclin D1, Akt, and p-Akt as determined by immunobloting. The expression of Hsp90 was slightly upregulated and there was a marked increase in the expression of Hsp70 which was up-regulated after 24 h and maintained at 72h of exposure to 17-AAG. Both of these results indicate a stress response to Hsp90 inhibition and are presumably induced by HSF1 activation. The level of Akt protein was sensitive to 17-AAG treatment and in all the cell lines a marked decrease in levels of p-Akt was seen.

Hsp90 inhibition in uveal melanoma cells with 17-AAG induced $G_1$ cell cycle arrest and this result was associated with down-regulation of CDK4. Expression of cyclin D1 was modestly down-regulated by 17-AAG in SP6.5 cells and remained unchanged in 92.1, OCM-1, MKT-BR, and UW-1. The results of Western blot analysis are presented in Fig. 6.12.

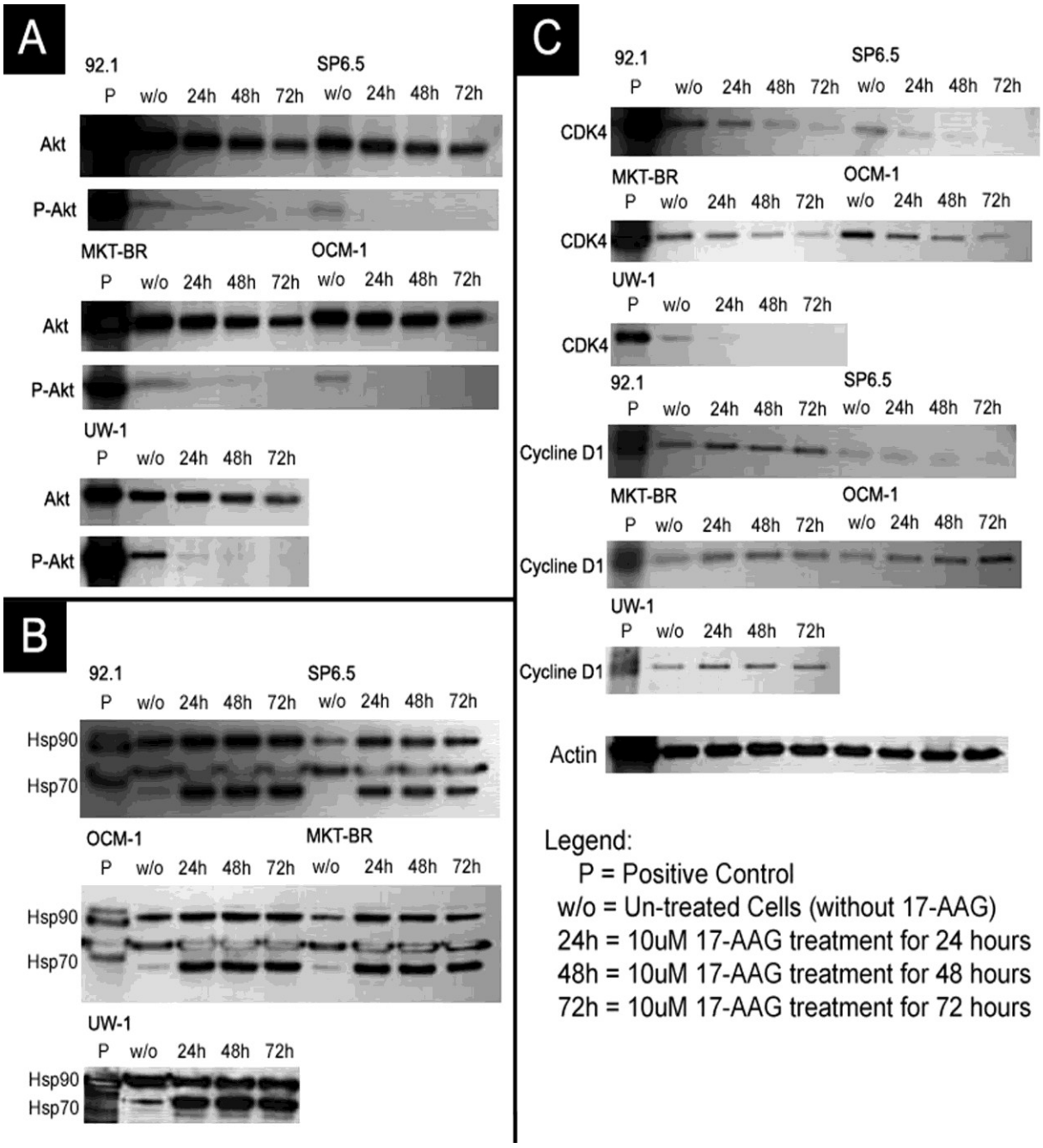



**Fig.6.12.** *A,* 17-AAG down-regulated the expression of Akt and p-Akt levels starting with 24 h of exposure. *B,* 17-AAG up-regulated the expression of Hsp70 at 24 h of exposure *C,* 17-AAG down-regulated the expression of CDK4 levels starting with 24 h of exposure. P, positive control; w/o, without 17-AAG exposure.

## B. Functional roles of Hsp90 in uveal melanoma primary tumor

### *6.6 Summary*

- ✓ *This is the first report showing the inhibitory effect of Hsp90 inhibitor, 17-AAG, on the proliferation of uveal melanoma cells.*
- ✓ *The antiproliferative activity of 17-AAG was due to both apoptosis and cell cycle arrest.*
- ✓ *The downstream molecular and cellular consequences of Hsp90 inhibition in uveal melanoma are downregulation of the Akt and p-Akt.*
- ✓ *The decrease in p-AKT levels after 17-AAG exposure in all the cell lines, is possibly contributing to induction of antiproliferative effects through modulation of the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway in uveal melanoma.*
- ✓ *In addition to the effect on pro-survival protein p-AKT, 17- AAG may also induce cell death through activation of caspase-3 mechanism.*
- ✓ *Cytostatic effect after Hsp90 inhibition in uveal melanoma is a result of downregulation of CDK4, a direct target of the drug.*

# C. Expression and functional roles of Hsp90 in metastatic uveal melanoma

The propensity of cancer cells to metastasize requires modifying cell adhesion and extracellular matrix, increase in cell motility, invasion and sustained angiogenesis (Hanahan and Weinberg 2000). The chaperoning capacity of Hsp90 facilitates the progression of tumor cells in metastatic steps such as invasion, angiogenesis and metastasis (Maloney and Workman 2002). Among Hsp90 client proteins involved in cell motility, invasion and signaling pathways leading to metastases in uveal melanoma are: IGF-1R, c-Met, FAK, signaling molecules PI3-AKT and transcription factors HIF-1α and NFKB (Neckers and Ivy 2003; Triozzi, Eng et al. 2008).

We have shown that Hsp90 inhibition in uveal melanoma led to downregulation of the AKT which could affect the migratory and invasive ability of uveal melanoma cell lines. Hsp90 expressions in metastases and roles of Hsp90 inhibition in uveal melanoma have not been explored yet.

In the present study, we wanted to assess the expression of Hsp90 in human metastatic uveal melanoma and a metastatic cell line and to confirm its expression in metastatic samples from an experimental animal model. We also studied the consequences of Hsp90 inhibition with respect to cellular processes and client proteins involved in metastases.

There is a possibility that there is an association between Hsp90 expression in metastases and the response to therapy. Inhibitors of Hsp90 could have multiple downstream effects in the metastatic process suggesting a potential benefit of Hsp90 inhibitors in metastatic uveal melanoma.

## 6.6 Results

### CI. IN VIVO: IN PATIENT'S SAMPLES - PARAFFIN EMBEDDED TISSUES OF METASTATIC UVEAL MELANOMA

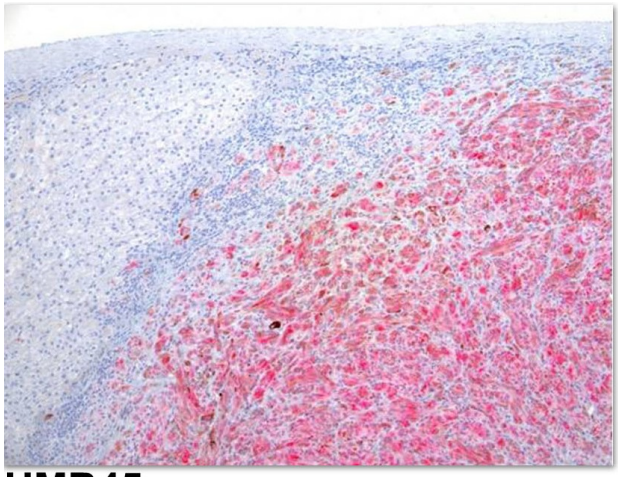
### 6.6.1 IMMUNOHISTOCHEMISTRY

#### *Hsp90 protein is expressed by uveal melanoma metastases*

Eight cases of primary uveal melanoma and corresponding metastases were available. Three of them were metastasis to the liver, one to the orbit, lung, and skin, and two to the bone (table 6). All the tumors were classified as mixed cell type. Immunohistochemistry was carried out on serial sections of each specimen with the following monoclonal antibodies: HMB-45, Hsp90, Hsp90α and representative clients of Hsp90 relevant for the metastatic process in uveal melanoma, IGF-1R and c-Met. Overall, 87% of tumors ($n$ = 7) were Hsp90 positive. In tumor cells, Hsp90 was detected both in the cytoplasm and nucleus and was classified as low, medium to high intensity scores of 1, 2 and 3 respectively (Fig.6.13, 6.14. and 6.15).

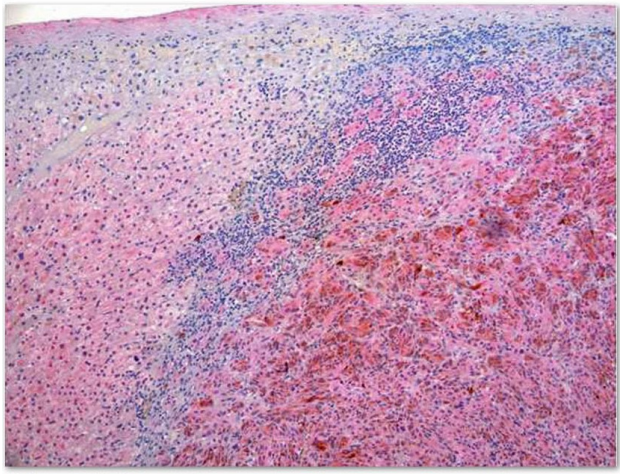
| Specimen | Cell type | HSP90 | C-Met | IGF 1R |
|---|---|---|---|---|
| **orbit** | mixed | Negative | Medium | Negative |
| **lung** | mixed | Medium | High | Low |
| **skin** | mixed | Low | High | Medium |
| **bone** | mixed | High | High | Low |
| **liver** | mixed | Medium | High | Medium |
| **bone** | mixed | High | High | Low |
| **liver core** | mixed | Medium | High | Low |
| **liver** | mixed | High | High | High |

**Table 6.** The table summarizes the patterns of HMB45,Hsp90, C-Met and IGF1-R immunostaining in eight cases of metastatic lesions of uveal melanoma.



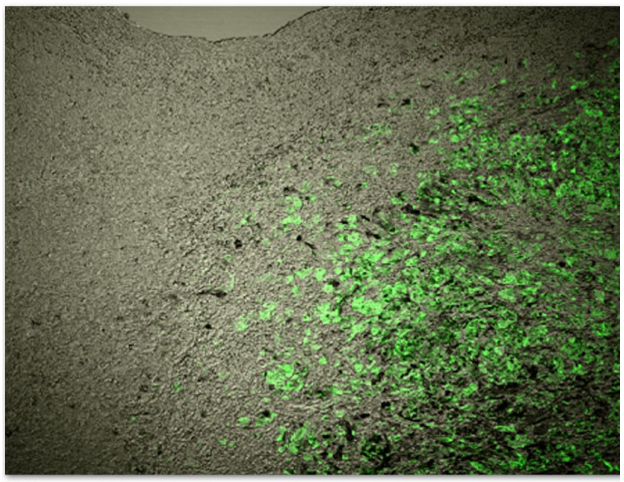
HMB45

**Fig. 6.13 a)** Immunoreactivity of HMB45 in a liver sample of metastatic uveal melanoma (original magnificationx100)



Hsp90

**b)** Hsp90 monoclonal antibody recognizes the subunits alpha and beta of Hsp90. Immunostaining for Hsp90 showing staining of the liver metastasis and also the surrounding area of inflammation.



Hsp90 alpha

**c**) Immunofluorescence picture of Hsp90α in the same area of liver metastasis showing better tumor delineation

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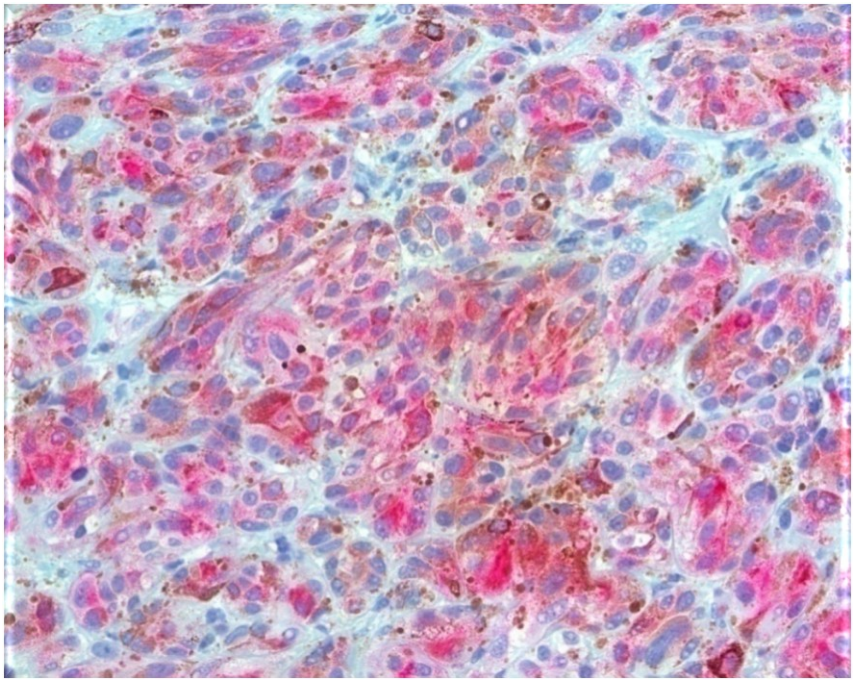
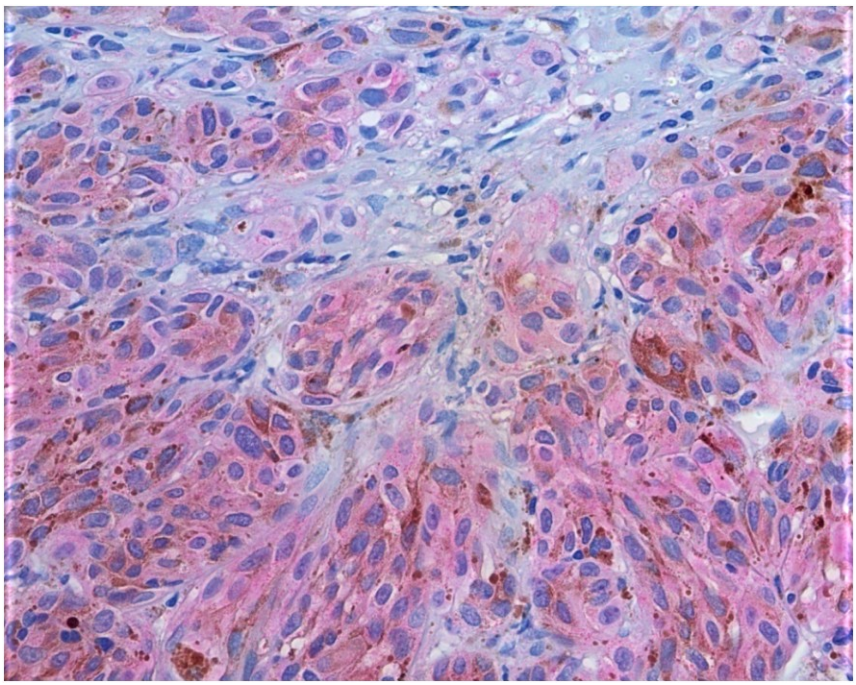


Fig.6.14 Liver metastasis staining for HMB45 (Original magnification x400)

Fig.6.15 Moderate immunostaining intensity (+2) of Hsp90 in a mixed cell type liver metastasis from uveal melanoma (original magnification ×400)



### 6.6.2 Potential targets for therapy in cell motility and invasion signaling pathways

Potential targets for therapy in cell motility and invasion signaling pathways leading to metastastatic uveal melanoma are cell membrane receptors IGF1-R, Met, signaling molecules PI3-AKT, FAK and other transcription factors. Inhibitors of Hsp90 could have multiple down-stream effects in the metastatic process.

#### IGF-1R AND C-MET

In our samples, the immunostaining intensity levels of c-Met were high (+3) while IGF-1R and Hsp90 displayed moderate immunostaining intensity (+2).

The figure below illustrates the pattern of immunostaining of Hsp90 and some of the key clients in primary uveal melanoma and corresponding liver metastasis (Fig.6.16).

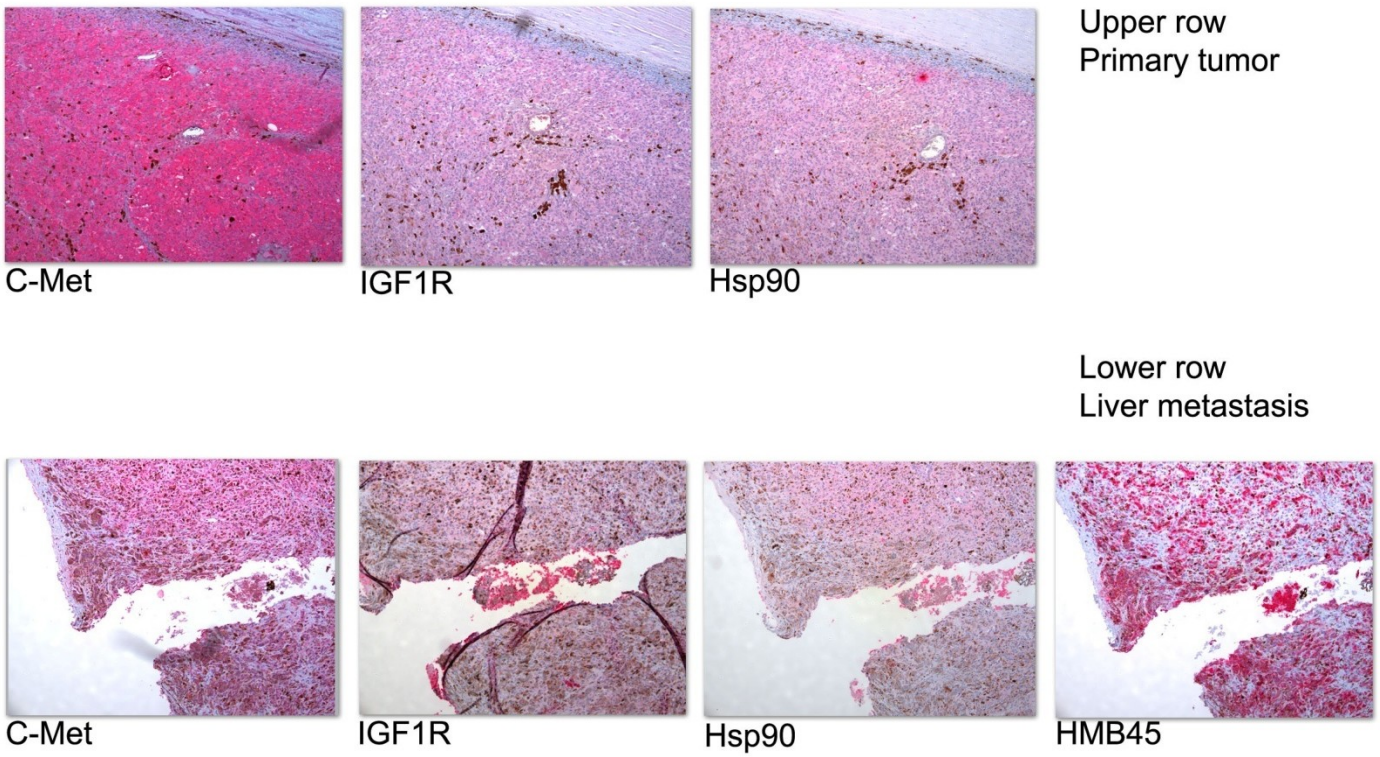



**Fig. 6.16** Immunostaining for c-Met, IGF-1R, HMB45 and Hsp90 in primary uveal melanoma (upper row) and corresponding liver metastasis (lower row) (original magnification x100)

## FOCAL ADHESION KINASE (FAK)

FAK, a cytoplasmic tyrosine kinase has multiple roles at the cellular sites of attachment during focal adhesion formation. FAK is important for many cellular processes, including cell survival, invasion, and migration. In previous studies we have shown that FAK was overexpressed in uveal melanoma and demonstrated to be present in aggressive uveal melanoma cell lines (92.1 and SP6.5, data not shown) (Figure 6.17).

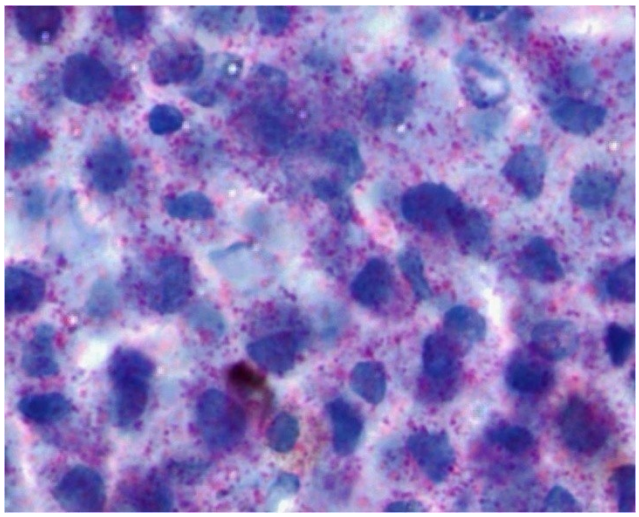


**Fig. 6.17** FAK immunostaining in cytospin samples of SP6.5 uveal melanoma cell line (red).
(original magnification x400)

## *C. Expression and functional roles of Hsp90 in metastatic uveal melanoma (cont.)*

### CII. In vivo: In animal samples - paraffin embedded tissues of metastatic uveal melanoma from an experimental rabbit model.

- Immunohistochemistry

## *6.7 Results*

Experimental Rabbit Model of Uveal Melanoma

**6.7.1 Gross and histopathological exam of metastases in the experimental rabbit model**

The metastases were observed as hard nodules in the inferior lobes of the lungs after the seventh week of the experiment, in (43%) of the animals. However, immunohistochemical assessment detected micrometastases in nine cases. Micrometastatic lesions were confirmed by positive staining for HMB-45 monoclonal antibody (Fig 6.18).

- **6.7.2 Immunostaining for Hsp90**

### *Hsp90 is expressed in lung metastases in the uveal melanoma animal model*

The Hsp90 immunostaining was positive in seven of the nine cases with metastases. The staining was predominantly intracytoplasmic with moderate to high intensity. (Fig 6.18)

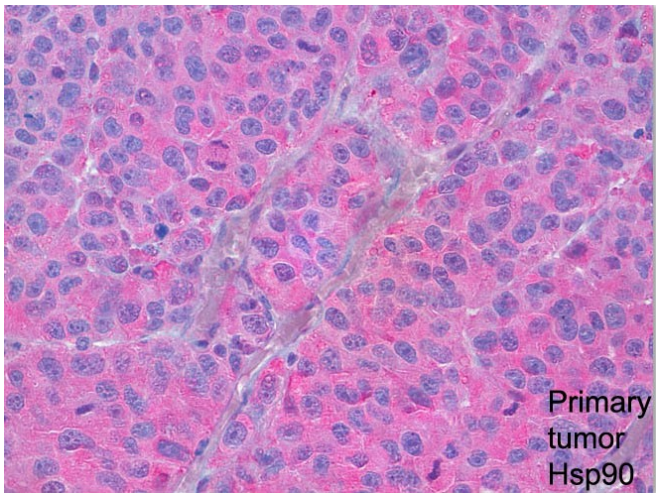
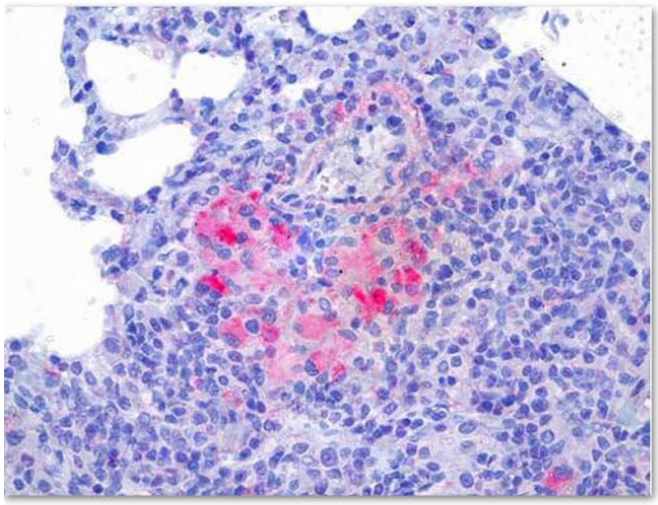
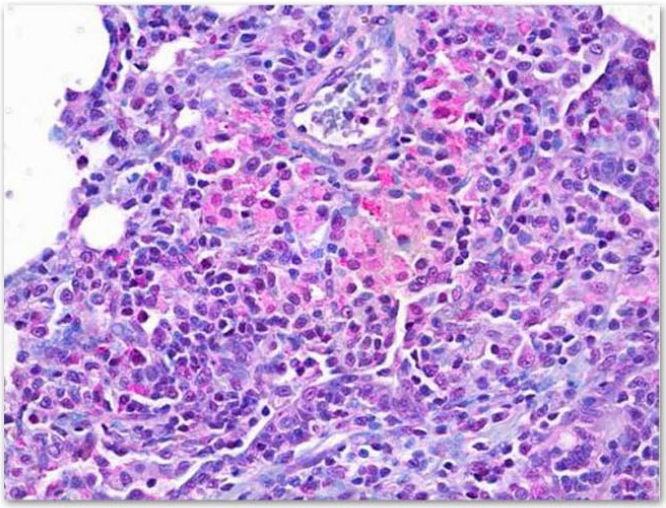



**Fig.6.18** a) Immunostaining of Hsp90 in primary tumor of an animal model




b) Corresponding lung metastasis confirmed by HMB45 staining




c) Same area of lung metastasis showing immunostaining pattern for Hsp90

## *C. Expression and functional roles of Hsp90 in metastatic uveal melanoma (cont.)*

### CIII. In vitro: Expression of Hsp90 in liver metastasis derived uveal melanoma cell line, OMM-1.5.

- Immunocytochemistry

## *6.8 Results*

- 6.8.1 Imunocytochemistry

### Hsp90 is expressed in the metastatic derived cell line

The expression levels of Hsp90 were studied in one liver metastasis derived human uveal melanoma cell lines (OMM 1.5). Cytospins of the cell line were positive for Hsp90 and Hsp90α staining. Hsp90 was predominantly cytoplasmic but in a subset of cell we were able to determine concomitant membranous expression (fig.6.19). The analysis of immunofluorescence stained samples detected expression of Hsp90α on the cell membrane of all five cell lines (Fig.6.20).

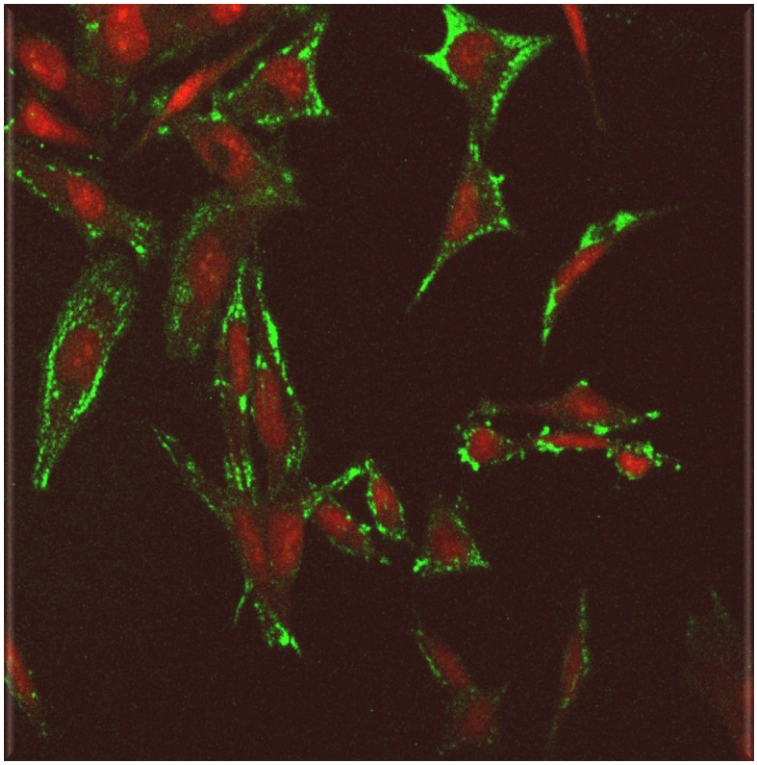


**Fig.6.19** Expression of Hsp90 in OMM1.5 cell line visualized by confocal microscopy showing membranous and intracytoplasmic staining. Original magnification, x400 (green)

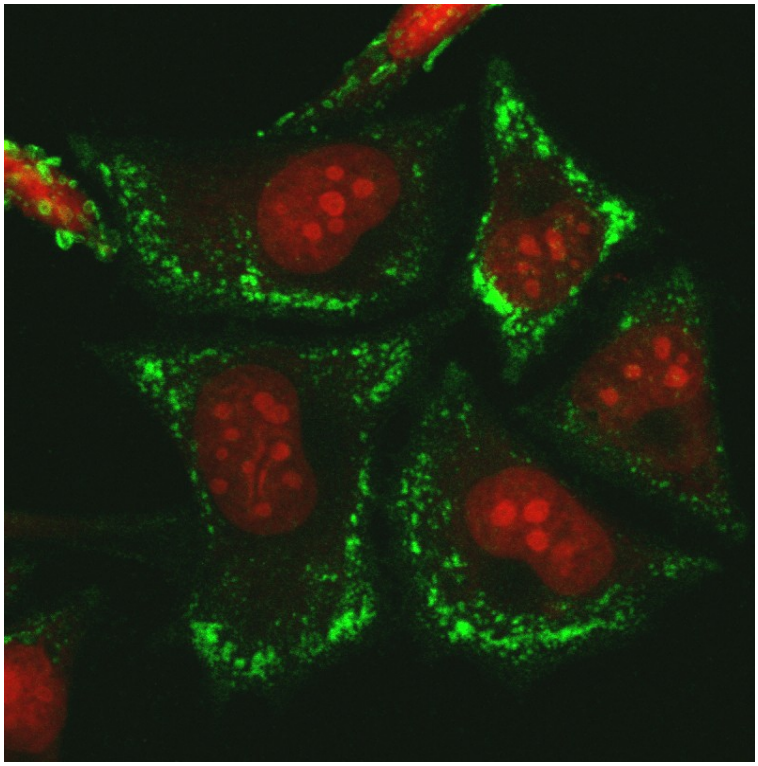


Fig. **6.20** Expression of Hsp90α in OMM1.5 cell line visualized by confocal microscopy showing membranous and intracytoplasmic staining. Original magnification, x400 (green)

*C. Expression and functional roles of Hsp90 in metastatic uveal melanoma*

## CIV. FUNCTIONAL ASSAYS AFTER INHIBITION OF HSP90 IN FIVE PRIMARY UVEAL MELANOMA CELL LINES (92.1, MKT-BR, SP6.5, OCM, AND UW-1) WITH DIFFERENT METASTATIC POTENTIALS AND IN ONE LIVER METASTASIS DERIVED UVEAL MELANOMA CELL LINE, AFTER INHIBITION OF HSP90

- PROLIFERATION ASSAY AFTER INHIBITION OF HSP90 IN OMM1.5 LINE
- MIGRATION ASSAY; INVASION ASSAY

# 6.9 Results

- 6.9.1 17-AAG REDUCED PROLIFERATION IN METASTATIC DERIVED UVEAL MELANOMA CELL LINE

Significant inhibition ($P < 0.001$) of cellular proliferation was seen in OMM1.5 cell line after exposure to 17-AAG compared with controls at concentrations ranging from 0.1 to 100 µmol/L (Fig. 6.21)

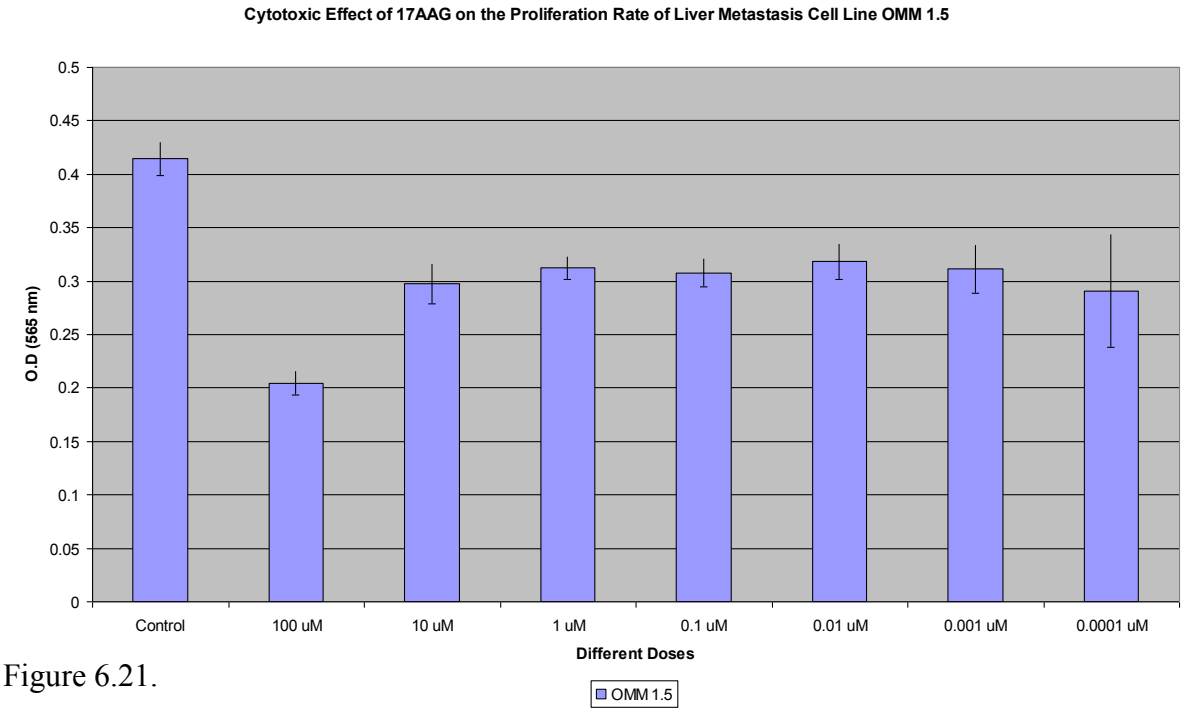



Figure 6.21.

### 6.9.2 Migration assay in primary uveal melanoma cell lines

## 17-AAG reduced the migration in uveal melanoma cell lines

All five human uveal melanoma cell lines showed migratory ability toward CXCL8 at a level greater than the selected negative control (serum-free RPMI 1640). After Hsp90 inhibition, the migratory potential of the uveal melanoma cells, was significantly reduced in three of the cell lines (92.1, SP6.5, and OCM-1) which were previously characterized as having an aggressive phenotype. (Fig. 6.22A).

### 6.9.3 Invasion assay in primary uveal melanoma cell lines

## *17-AAG inhibits invasion of uveal melanoma cells*

There was a marked decrease in the invasive ability of the uveal melanoma cell lines in all the experiments. The average number of cells that invaded the Matrigel baseline decreased significantly after addition of 17-AAG from 7 ± 3.6 to 0.4 ± 0.6 ($P = 1.64E^{-07}$) for 92.1, from 6.9 ± 6.5 to 0.8 ± 1.2 ($P = 0.001$) for SP6.5, from 38.6 ± 17.6 to 0.66 ± 0.89 ($P = 4.61E^{-09}$) for OCM-1, from 9.7 ± 5.4 to 1.33 ± 1.44 ($P = 3.72E^{-06}$) for MKT-BR, and from 7.33 ± 9.68 to 0 ± 0 ($P = 0.006$) for UW-1. The average results of percentage invasion from three experiments were at baseline: MKT-BR, 52.10 ± 17.04; SP6.5, 39.21 ± 15.92; OCM-1, 35.8 ± 4.20; 92.1, 18.74 ± 7.69; and UW-1, 6.92 ± 10.13. The addition of 17-AAG decreased the percentage invasion in all cell lines: MKT-BR, 6.89 ± 3.14; SP6.5, 5.37 ± 3.76; 92.1, 1.23 ± 0.26; OCM-1, 0.62 ± 0.11; and UW-1, 0 ± 0 (Fig. 6.22B).

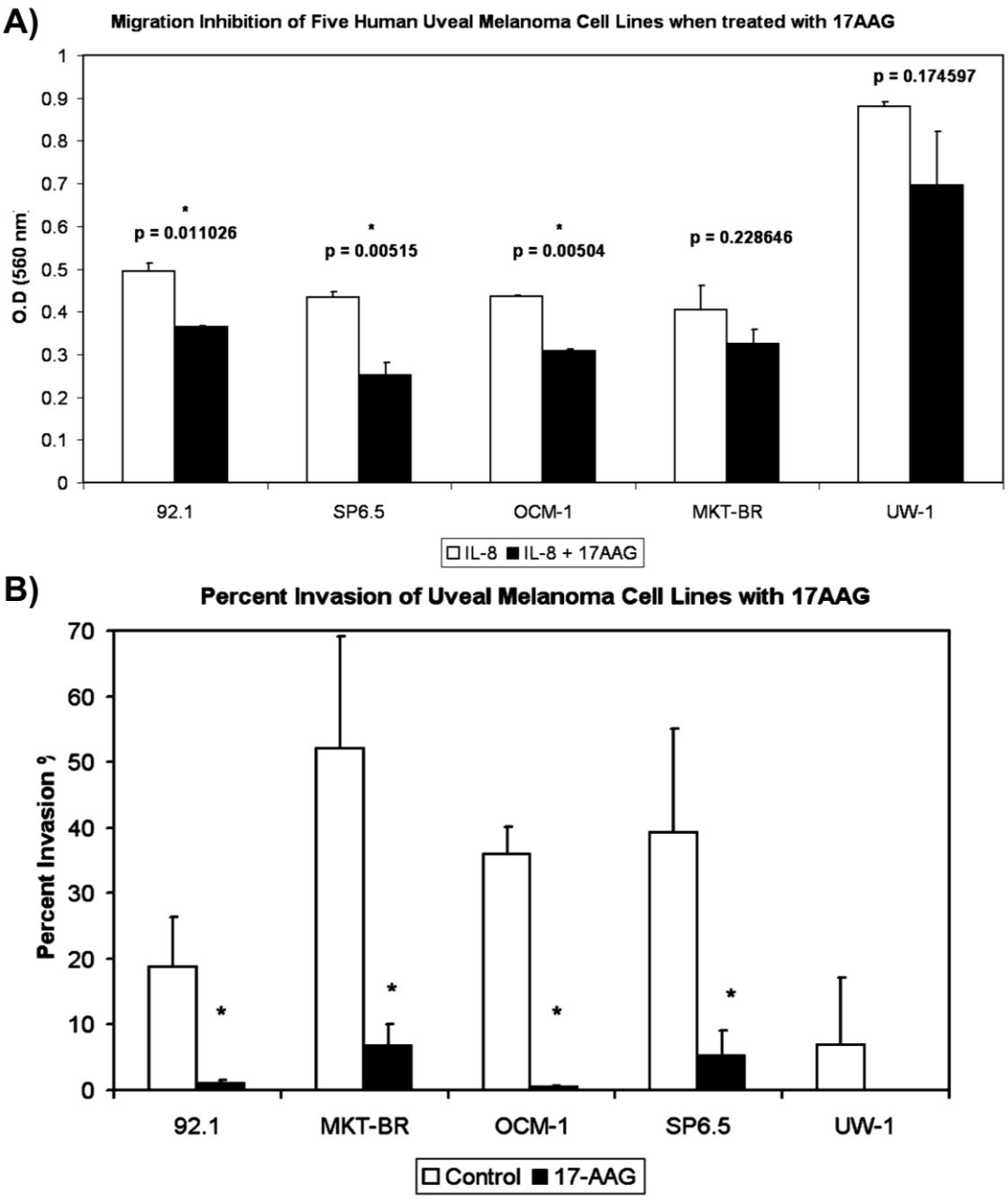



**Fig. 6.22 A:** Motility assay showing a reduction in the migratory capacity of the uveal melanoma cells upon addition of 17-AAG with statistical significance in three of the most invasive cell lines (92.5, SP6.5, OCM-1).

**Fig. 6.22 B:** Percentage of invasion of uveal melanoma cells with and without addition of 17-AAG showing a significant reduction in invasion ability of all cell lines.

## *C. Expression and functional roles of Hsp90 in metastatic uveal melanoma (cont.)*

### CV. Downstream effects of Hsp90 inhibition in five UM cell lines (92.1, MKT-BR, SP6.5, OCM, and UW-1) of different metastatic potential

- Western blot analysis

# 6.10 Results

- 6.10.1 Downstream effects of inhibition of Hsp90 affecting client proteins in metastatic cascade; Western Blot

Immunoblotting was used to examine the ability of the Hsp90 inhibitor to influence the expression of FAK, C-Met and IGF-1R. In all five cell lines we found a decrease in the expression of FAK, and downregulation of the IGF-1R. Also, 17-AAG downregulated the mature chain of Met (140 kDa) in a time-dependent manner (Fig.6.23)

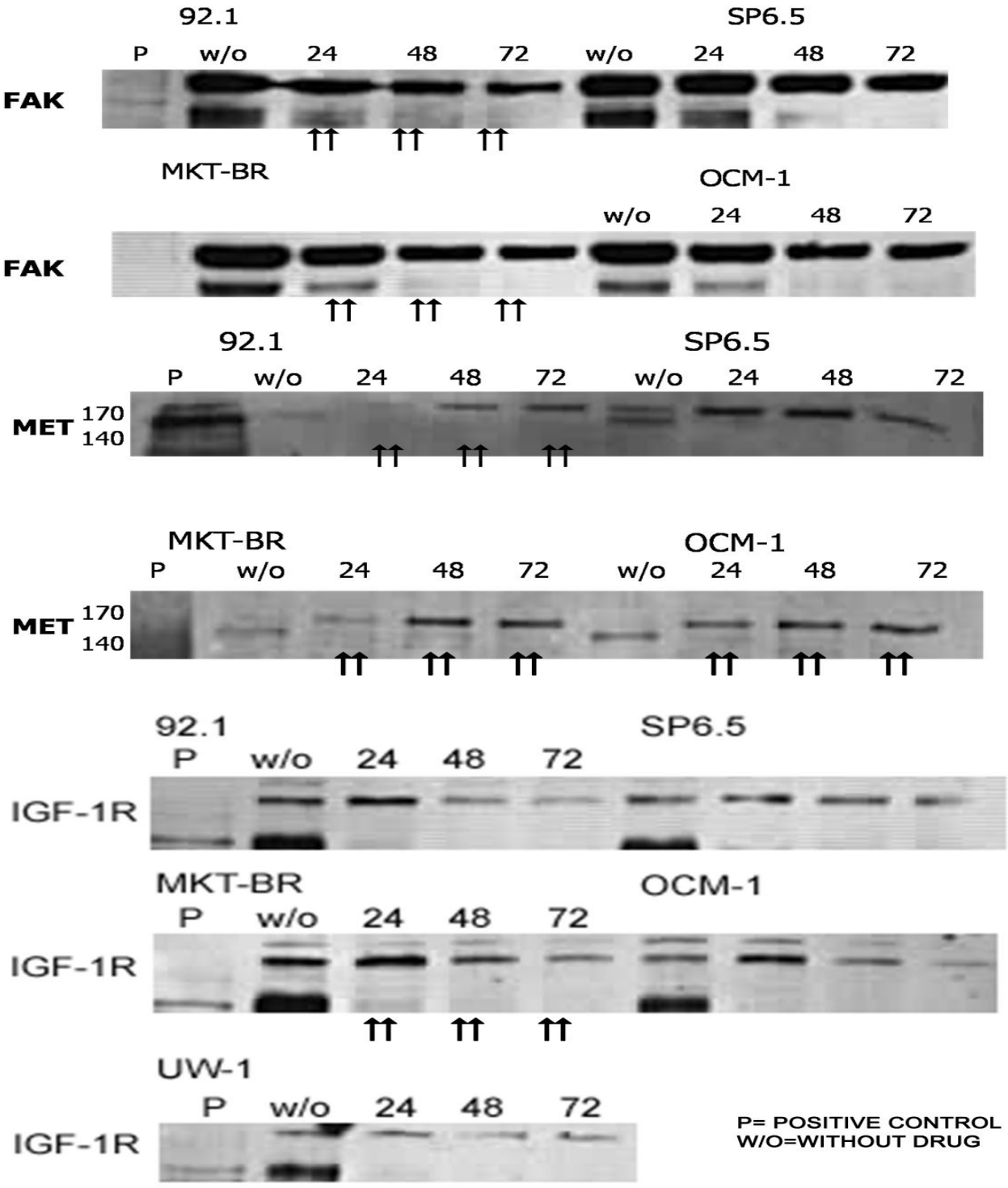



**Fig. 6.23** 17-AAG down-regulated the expression of FAK, c-Met and IGF-1R levels starting with 24 h of exposure. P, positive control; w/o, without 17-AAG exposure (arrows).

## C. Expression and functional roles of Hsp90 in metastatic uveal melanoma

*6.11 Summary*

- ✓ *Hsp90 was found to be expressed in human uveal melanoma metastases cases. These results were confirmed in specimens from an animal model of UM.*
- ✓ *Hsp90 is expressed in uveal melanoma cells from a metastatic derived cell line.*
- ✓ *HSP90$\alpha$ was detected on the surface of the tumor cells suggesting possible extracellular roles for Hsp90$\alpha$ and interaction with the extracellular matrix*
- ✓ *This is the first report showing the inhibitory effect of Hsp90 inhibitor, 17-AAG, on the proliferation of metastatic uveal melanoma cells.*
- ✓ *The evaluation of Hsp90 inhibitor on cellular processes involved in invasion showed that 17-AAG down regulated c- Met and IGF-1R receptor transmembrane tyrosine kinases and also inhibited activation of focal adhesion kinase.*
- ✓ *Inhibition of Hsp90 function had an effect on uveal melanoma cells by reducing their motility and invasive potentials.*

# *Chapter 7*

# Discussion

## A. Expression of Hsp90 in primary uveal melanoma

Uveal melanoma is a primary intraocular malignancy that can cause both visual loss and death from metastases. Metastatic disease occurs early in the development of uveal melanoma, in most of the cases even before clinical detection of the primary tumor (Kivela, Eskelin et al. 2006). Clinical management of the primary tumor can often preserve the eye and visual function, although it does not have an impact on mortality rate. The treatment options for metastatic disease are minimally effective. These facts support the shift towards other methods of treatment such as adjuvant systemic therapy in reducing uveal melanoma-associated mortality (Singh and Topham 2003). An alternate approach is to target uveal melanoma based on the molecular mechanisms that allow the tumor cells to successfully metastasize. Modern techniques including genetic and proteomic studies have identified target proteins involved in apoptosis, proliferation and metastasis in uveal melanoma (Zuidervaart, Hensbergen et al. 2006; Harbour 2007; Marshall, Nantel et al. 2007).

Heat-shock protein 90 is an abundant molecular chaperone which has been shown to be over-expressed in a number of cancers, both solid tumors and hematological malignancies. There is a large body of evidence supporting the important role of Hsp90 in regulation of growth and survival of tumor cells (Isaacs, Xu et al. 2003; Whitesell and Lindquist 2005).

We assessed the expression of Hsp90 in patient specimens, cell lines and animal model samples of uveal melanoma and evaluated the association between its expression, prognosis and other clinical variables. Heat shock protein 90 was expressed in 68% of the tumors, whereas

normal choroid melanocytes and nevi expressed none or very low levels, indicating that Hsp90 may represent a tumor-selective therapeutic target. Our results are in concordance with previous studies suggesting that tumor cells express 2- to 10-fold higher levels of intracellular Hsp90 when compared with normal cells (Ferrarini, Heltai et al. 1992). In cutaneous melanoma, Hsp90 expression was shown to be up-regulated in malignant cells in comparison to melanocytic nevi (Becker, Multhoff et al. 2004). In addition, Hsp90 expression in prostate, renal and bladder cancer was found to be higher in comparison with normal tissues, further validating Hsp90 as a target. In uveal melanoma, a single previous study has reported the expression of Hsp90, which was expressed in 12 of 20 tumors with a mean staining of 6.7% ± 2.6% of tumor cells per slide (Missotten, Journee-de Korver et al. 2003). Our results indicate a higher degree of expression as determined by immunohistochemistry in patient samples with an average of 50% of tumor cells stained and these results were confirmed in the samples from the animal model of uveal melanoma. The reason for the discrepancy between our results and others in expression is not clear. The differences might be explained by heterogeneity of the tumor, differences in the stage of the disease, treatment modalities, immunohistochemical methods, case selection, and possible differences in embedding and processing techniques.

Hsp90 expression and its therapeutic implications depend on the tumor type. Increased expression of Hsp90 was observed in cutaneous melanoma, breast tumors, lung cancer, leukemia, and Hodgkin's lymphoma (Hsu and Hsu 1998; Yano, Naito et al. 1999; Becker, Multhoff et al. 2004; Whitesell and Lindquist 2005). The overexpression of Hsp90 in cancers may be the result of an increase load of genetically instable oncogenes present in the tumor environment (Maloney and Workman 2002; Sreedhar, Kalmar et al. 2004). Many of the Hsp90 clients are mutated and are relevant for cell proliferation or antiapoptotic mechanisms in uveal melanoma, for

example p53, Bcl-2, cyclin D1, and c-myc (Coupland, Bechrakis et al. 1998; Ehlers and Harbour 2006; Triozzi, Eng et al. 2008). Moreover, c-myc proto-oncogene has been shown to activate the production of Hsp90 at transcriptional levels (Teng, Chen et al. 2004). The increase of the expression of Hsp90 in uveal melanoma is possibly the result of the oncogenic stress, thus promoting cell survival mechanisms and buffering potential lethal mutations (Jolly and Morimoto 2000).

Hsp90 has been shown to correlate with an increased malignant phenotype in breast cancer (Pick, Kluger et al. 2007), gastric cancer (Zuo, Dai et al. 2003) and lung cancer (Gallegos Ruiz, Floor et al. 2008). However, the expression of Hsp90 is associated with good prognosis in endometrial cancer (Nanbu, Konishi et al. 1998), whereas expression of Hsp90 was seen to be of no prognostic significance in ovarian cancer (Elpek, Karaveli et al. 2003) and salivary gland tumors (Vanmuylder, Evrard et al. 2000). In our study Hsp90 expression was not significantly correlated with survival or metastatic disease as determined by Kaplan Meier analysis, which might be due to either the size of our sample or the follow-up interval. However, our data showed that high expression of Hsp90 was associated with a clinical indicator of poor prognosis: the largest tumor diameter. This is the most significant clinical prognostic indicator for high risk of metastasis in uveal melanoma (McLean, Foster et al. 1982). Moreover a recent study by Augsburger and colleagues, advanced the theory that the mortality rates observed after treatment of uveal melanomas of different sizes are due to cytogenetic abnormalities which are more frequently observed in larger tumors than in smaller ones (Augsburger, Correa et al. 2007).

Similarly, Hsp90 expression in skin melanoma was also associated with histological prognostic factors such as Clarke's level and increased Breslow depth, although no association was seen between high Hsp90 expression and survival (McCarthy, Pick et al. 2008).

Although the expression of Hsps may increase after radiotherapy stress in uveal melanoma, (Blom, De Waard-Siebinga et al. 1997), we found no difference in staining between the patients treated by enucleation only, versus radiotherapy followed by enucleation, possibly due to the short induction of the Hsp90 in between the two procedures. Primary uveal melanomas showed a higher degree of variability in Hsp90 immunohistological expression with a subset of tumors that showed low expression or were negative for Hsp90. Studies in cutaneous melanoma revealed an association between the Hsp90 expression and the tumor response to 17-AAG (Burger, Fiebig et al. 2004). The potential implications of Hsp90 expression and correlation with the response to treatment should be subject of further investigation.

All of our cell lines had high expression of Hsp90 with no variability in expression between the different cell lines. Immunohistological detection of both isoforms of Hsp90 ($\alpha$ and$\beta$) was displayed predominantly intracytoplasmatic, whereas nuclear staining was detected in only a fraction of the cells. Possibly, the importance of cytoplasmic staining in uveal melanoma reflects the intracytoplasmic oncogenic stress and an increased binding of cytoplasmic Hsp90 client proteins. Nuclear localization of Hsp90 is an indicator of acute response to stress and was correlated in a study in breast cancer with a higher MHC class I expression (Gebhard, Schutz et al. 1999) and also higher levels of Hsps on the surface of the cells have been associated with an increase of MHC class I antigens and higher immunogenicity (Wells, Rai et al. 1998). In uveal melanoma, high HLA class I expression is associated with poor prognosis (Krishnakumar, Abhyankar et al.

2003) possibly due to an attenuation of the innate immune response. The surface localization of Hsp90α in uveal melanoma cells indicate possible extracellular role for Hsp90 outside the cell and interaction with matrix and immunogenic effects. In previous studies, membrane and extracellular expression of Hsp90 has been shown in other types of cancer cells and has been correlated with increased tumor invasiveness and metastatic activity (Eustace and Jay 2004).

Our findings have important implications for Hsp90 as a target for therapy in uveal melanoma. High Hsp90 expression could biological predict aggressive behavior of these tumor cells and may have implications regarding tumor selectivity upon inhibition with Hsp90 inhibitors.

## B. Functional roles of Hsp90 in uveal melanoma primary tumor

Hsp90 is overexpressed in cancer cells where it is essential for the stability and function of a range of proteins associated with cell growth, differentiation and survival. These include proteins involved in cell survival and proliferation AKT and PKB, c-RAF, p53, CDK4, hTERT, antiapoptotic mechanisms, BCR–ABL, surviving, invasion and angiogenesis HIF-1α, VEGF and MET and many others (Maloney and Workman 2002; Neckers and Ivy 2003). Hsp90 inhibitors affect conformational maturation of the complexed-clients and influence the function and degradation of its client proteins, in different oncogenic signalling pathways (Isaacs, Xu et al. 2003; Burrows, Zhang et al. 2004).

Among the mechanisms involved in tumor progression in uveal melanoma, activation of Ras–Raf-1–Mek–ERK and phosphatidyl- inositol-3 kinase – Akt pathways and abnormalities

in cell cycle control are frequently reported (Harbour, Worley et al. 2002; Zuidervaart, van Nieuwpoort et al. 2005; Triozzi, Eng et al. 2008). Inactivation of metastable signaling proteins client proteins by Hsp90 inhibitors in these pathways could affect signal transduction, cell-cycle control and apoptotic processes (Sato, Fujita et al. 2000; Kamal, Boehm et al. 2004; da Rocha Dias, Friedlos et al. 2005). Several Hsp90 inhibitors, including the benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin, (17-AAG), are currently undergoing phase I and II clinical evaluation (Pacey, Banerji et al. 2006).

We showed that Hsp90 is abundantly expressed in primary uveal melanoma sections and cell lines and we further evaluated Hsp90 as a primary target for novel therapy using 17-AAG in uveal melanoma.

We examined the antiproliferative activity of 17-AAG in a panel of human uveal melanoma cell lines and we have shown that Hsp90 inhibition caused a marked reduction in a dose-dependent manner in the proliferation of all uveal melanoma cell lines. 17-Allylamino-17-demethoxygeldanamycin effectively inhibits cell proliferation of uveal melanoma cell lines, indicating the presence of mutated or overexpressed signaling proteins that are protected from degradation by Hsp90. Although Hsp90 is expressed in both benign and malignant cells it is possible that due to the oncogenic stress and an overload of mutated, unstable proteins, tumor cells have a higher dependence on Hsp90 (Maloney and Workman 2002; Whitesell and Lindquist 2005). Kamal and colleagues demonstrated that in these cells, Hsp90 is present in multiple complexes due to an increased load of mutated proteins, which are hypersensitive to Hsp90 inhibition, leading to the accumulation of Hsp90 inhibitors seen in malignant cells. In normal cells

Hsp90 is found predominantly in a free form and is less likely to be affected by the drugs, possibly explaining the therapeutic selectivity seen in clinical trials (Kamal, Thao et al. 2003).

17-AAG downregulated the proliferation in the different cell lines, with an average growth-inhibitory concentration ($GI_{50}$) over all cell lines of 109 nmol/L, and these results were consistent with the analysis of the 17-AAG cytotoxicity in cutaneous melanoma cell lines (Burger, Fiebig et al. 2004). Two phenotypically aggressive cell lines which display a higher invasive, and metastatic potential (92.1 and SP6.5) (Marshall, Caissie et al. 2004) were relatively less sensitive to the agent ($GI_{50}$ =123 and 143 nmol/L, respectively). In accordance with our results, a subsequent study in uveal melanoma found a marked decrease in cell line proliferation at similar 17-AAG concentrations (Babchia, Calipel et al. 2008).

The expression of Hsp90and the response to the drug was similar in all uveal melanoma cell lines therefore no correlation could be made between Hsp90 expression levels and the magnitude of the response to therapy. Similar results were observed in cutaneous melanoma and breast cancer cell lines, (Pick, Kluger et al. 2007; McCarthy, Pick et al. 2008). However, Burger et al demonstrated in a preclinical study of cutaneous melanoma that there was an association between Hsp90 expression and the tumor response to 17-AAG (Burger, Fiebig et al. 2004). There is the possibility that the response to Hsp90 inhibitors could be dictated by the expression of the Hsp90 itself, so that future studies might address the effects on Hsp90 levels together with those on the Hsp90-associated client proteins.

In our study, the inhibition of Hsp90 by 17-AAG was associated with up-regulation of Hsp70 protein after 24 h of exposure, indicating presence of a stress response, which is a pharmacodynamic end point used to monitor the drug activity in clinical trials (Burger, Fiebig et al.

2004; Banerji, O'Donnell et al. 2005). Hsp70 and Hsp90 transcription is regulated by heat shock factor 1(HSF1), which is activated upon Hsp90 inhibition (Zou, Guo et al. 1998). Hsp70 and Hsp90 transcription is regulated by heat shock factor 1(HSF1), which is activated upon Hsp90 inhibition (Pratt and Toft 2003). On the other hand, both Hsp90, Hsp70 chaperones have anti-apoptotic effects (Jolly and Morimoto 2000) and their induction may influence the tumor cells sensitivity to 17-AAG and induce drug resistance. However, the same response and induction of Hsps may have protective effects in normal tissues exposed to Hsp90 inhibitors. Along with induction of Hsp70, our results showed a slight increase in Hsp90 expression, similar with the results obtained in other tumor models indicating an increase in Hsp90 after drug inhibition (e.g., in Hodgkin lymphoma cells) (Georgakis, Li et al. 2006). This response could also modify the cellular sensitivity to the 17-AAG possibly due to the antiapoptotic effects of the Hsp90 itself. Clarke et al, in profiling the human colon cancer cells following inhibition by 17-AAG, showed that the induction of the drug target (Hsp90) correlated with resistance of the tumor cell lines to 17-AAG (Clarke, Hostein et al. 2000). Other researchers showed that downregulation of Hsp70 prior to inhibition of Hsp90, significantly increases cell death in response to 17-AAG (Guo, Rocha et al. 2005). Therefore, dual inhibition of Hsp90 and Hsp70 may be of therapeutic value in uveal melanoma.

Heat shock protein 90 client proteins are involved in controlling cell cycle and apoptosis (Whitesell, Mimnaugh et al. 1994; Bagatell, Beliakoff et al. 2005). 17-AAG has been shown to induce cell cycle arrest and apoptosis in multiple malignancies (Hostein, Robertson et al. 2001; Georgakis, Li et al. 2006). Using 17-AAG as an inhibitor in this study, we demonstrated the induction of cytostasis and apoptosis in all the uveal melanoma cell lines.

In uveal melanoma, upregulation of antiapoptotic signals is a common event and uveal melanoma metastases are even more refractory to chemotherapy, than cutaneous melanoma (Albert, Ryan et al. 1996). The multiple mechanisms that reduce potency of many chemotherapeutic drugs in this disease are related to upregulation of antiapoptotic mechanisms. Increased resistance to induction of apoptosis has been correlated with a higher metastatic potential in animal models (Bagatell, Beliakoff et al. 2005). In this tumor, evasion of apoptosis is due to activation of insulin-like growth factor - phosphatidylinositol 3-kinase-Akt pathway, and overexpression of anti-apoptotic proteins (BCL-2, BCL-XL). Inactivation of Bcl-2 has been shown to induce apoptosis of uveal melanoma cells and to increase the sensitivity of uveal melanoma cells to chemotherapy (Guo, Wu et al. 2003). The phosphatidylinositol-3 kinase (PI3K)/Akt pathway induces cancer progression because it stimulates cell proliferation and suppresses apoptosis. Activation of this pathway can occur through mutation of the tumor suppressor PTEN, mutation or amplification of PI3K, amplification of Akt, and activation of receptors or oncogenes upstream of PI3K (Aziz, Davies et al. 2009). Phospho-Akt has been correlated with poor prognosis in uveal melanoma (Saraiva, Caissie et al. 2005).

The expression of Hsp90 has been implicated in the regulation of apoptosis by binding APAF-1 and Bcl-2 (Pandey, Saleh et al. 2000). Both Akt and its activating kinase 3-phosphoinositide-dependent kinase-1 rely on Hsp90 for stability. Hsp90 and its co-chaperones modulate tumor cell apoptosis through formation of Akt-Hsp90 complexes, thus stabilizing the Akt kinase activity and phospho-Akt dephosphorylation (Sato, Fujita et al. 2000). Dysregulation of the Akt pathway, may result in a reduction of tumor cell proliferation , survival or migratory and invasive capacity (Vivanco and Sawyers 2002).

Drug inhibition of uveal melanoma cell lines in our study resulted in a decrease of Akt and activated p-Akt, possibly causing to a decrease in proliferation through induction of cell growth arrest and death. Our results were confirmed in a subsequent study in uveal melanoma, where 17-AAG induced downregulation of Akt in four uveal melanoma cell lines. However further inhibition with a specific Akt inhibitor did not affect cell proliferation (Babchia, Calipel et al. 2008). Akt could promote cancer cell survival in uveal melanoma through multiple signaling pathways. Growth factor receptors like IGF-1R, EGFR, and Her2-Neu activate Akt kinase which induces prosurvival transcription factors, such as NFKB and suppresses proapoptotic factors. Inactivation of Akt by Hsp90 inhibitors leads to reduced BAD (Bcl-XL/Bcl-1-associated death promoter) phosphorylation, inducing cytochrome C release and apoptosis (Vivanco and Sawyers 2002). Several other studies indicated that targeting the PI3K-Akt pathway with 17-AAG caused inhibition of Akt phosphorylation, induction of apoptosis and downregulation of multiple Akt and Raf dependent pathways (Hostein, Robertson et al. 2001; Basso, Solit et al. 2002; Solit, Zheng et al. 2002; Georgakis, Li et al. 2006).

Inhibition of Hsp90 may induce apoptosis in uveal melanoma through different mechanisms and in this 17-AAG addition induced a marked increase in the apoptotic marker caspase-3 in all uveal melanoma cell lines, indicating activation of the mitochondrial intrinsic pathway.

Targeting Hsp90 may also prove to be beneficial in combination with new systemic targeted therapy in addition to traditional therapeutics. Because the activity and stability of Akt is dependent on the link with the Hsp90 superchaperone complex, down-regulation of Akt with 17-AAG may increase the susceptibility of the uveal melanoma cells to other forms of chemotherapy and induction of apoptosis.

Other mechanisms leading to survival and progression in uveal melanoma are related to progression of the tumor cells through cell cycle. This is explained by inappropriate signals such as inactivation of the retinoblastoma protein, which govern cell transit through the $G_1$ phase of the cell cycle. CDK4/cyclin D complexes play an essential role during progression through the $G_1$ phase of the cell cycle by phosphorylating the retinoblastoma protein (Brantley and Harbour 2000). In uveal melanoma, deregulations of the cell cycle occur possibly due to the overexpression of cyclin D1 (Coupland, Bechrakis et al. 1998) and activation of endogenous cycline- dependent CDK4 and CDK6 (Yano, Naito et al. 1999). In tumors, CDK4-cycline D complex and CDK2 and CDK 7 depend on Hsp90 for stabilization (Srethapakdi, Liu et al. 2000; Prince, Sun et al. 2005).

Cell cycle analysis in our study showed that exposure to 17-AAG resulted in a $G_1$ cell cycle arrest in all of the human uveal melanoma cell lines, associated with a decrease in the levels of cell regulatory protein CDK4. Cyclin D1 was moderately reduced in SP6.5 cell line possibly due to down-regulation of the phosphatidylinositol 3-kinase-Akt–dependent pathway that is required for its expression (Muise-Helmericks, Grimes et al. 1998). However, although Akt and p-Akt were down-regulated, expression of cyclin D1 remained unchanged in the other four uveal melanoma cell lines, suggesting a different mechanism of regulation. It has been previously shown that wild type B-Raf–ERK1/2 pathway activates cyclin D1 expression and cell proliferation in uveal melanoma cell lines (Calipel, Mouriaux et al. 2006). Subsequently, 17-AAG has been shown to decrease cyclin D1 expression levels, in four uveal melanoma cell lines: wild type B-Raf cells Mel270 and 92.1 and mutated V600E-B-Raf cells OCM-1 and TP31, suggesting that cyclin D1 is involved in the 17-AAG–mediated inhibition of cell proliferation (Babchia, Calipel et al. 2008). The differences between our results might reflect changes in the patient-

derived passaged cell lines used in the experiments, raising the possibility that different client proteins will be differentially susceptible to modulation by 17-AAG.

The mechanisms behind the $G_1$ cell cycle arrest remain to be understood in detail. Srethapakdi and colleagues have suggested that the ability of 17-AAG to induce cytostasis is cell line dependent, whereas Rb-positive cell lines (like uveal melanoma cell lines) are prone to arresting in a G1 cell-cycle arrest in response to 17AAG and other Hsp90 inhibitors (Srethapakdi, Liu et al. 2000). Also, previous studies have shown that the maturation of CDK4 is dependent on Hsp90 /Cd37 complex and this kinase was shown to be a direct target of the drug (Stepanova, Leng et al. 1996; Basso, Solit et al. 2002). Our results are in concordance with other studies showing that 17-AAG induced a reduction of cyclin D1–associated CDK4 activity (Georgakis, Li et al. 2006; Babchia, Calipel et al. 2008).

Uveal melanoma cells proliferation could also be affected by Hsp90 inhibition due to interference to the Raf-1/MEK1 pathway as Raf- 1 associates with the Hsp90/Cdc37 for stabilization (Schulte, Blagosklonny et al. 1995). Wild type B-Raf, was found to activate phosphorylated ERK (pERK) and promote proliferation of uveal melanoma cells (Zuidervaart, van Nieuwpoort et al. 2005; Calipel, Mouriaux et al. 2006). Inhibition of RAS, and RAF protein kinases with 17-AAG has been shown to inhibit ERK-mediated, uveal melanoma cell proliferation and also to have antiangiogenic effects (Wilhelm, Carter et al. 2004; Calipel, Mouriaux et al. 2006). In a recent study, treatment of uveal melanoma cell lines with 17-AAG downregulated B-Raf, and deregulated MEK/ERK pathway in uveal melanoma cell lines expressing wild type B-Raf (Babchia, Calipel et al. 2008), suggesting that the 17-AAG–mediated inhibition of cell proliferation is due to the effects on B-Raf and the inhibition of ERK1/2.

Collectively these data suggest that in uveal melanoma, interference with Ras–Raf-MAPK activated pathway and PI3 kinase-PDK-Akt/PKB pathways by Hsp90 inhibitors has cytostatic results through inactivation of cyclineD-CDK4 effects on the G1–S cell cycle transition.

We have demonstrated for the first time the effects of 17-AAG on proliferation via targeting Hsp90 using a panel of uveal melanoma cell lines. Hsp90 inhibition inactivated multiple kinases, such as AKT and p-AKT and significantly reduced expression of CDK4. We showed that this was accompanied by cytostasis, and apoptosis. Our data indicate that at the very least, expression of Hsp90 and its critical clients should be assessed in uveal melanoma patients treated with Hsp90 inhibitors. Although there is no one key oncogenic protein driving uveal melanoma susceptible to depletion, there is potential for Hsp90 inhibitors in uveal melanoma.

## C. Targeting Hsp90 to Inhibit Tissue Invasion and Metastasis

The understanding and management of the metastatic process in uveal melanoma has evolved in the past decades. However, despite early diagnosis and management of the primary tumor the mortality rates have not significantly changed. Overall mortality rate remains high because of the frequent development of metastatic disease, especially hepatic metastasis (Singh and Topham 2003). Early micrometastatic dissemination may appear several years before diagnostic and treatment of the primary tumor and approximately 60% of patients with metastases are asymptomatic, and 30% have normal liver function tests (Eskelin, Pyrhonen et al. 2000). In approximately 80% of the cases hepatic metastases are refractory and less responsive to chemotherapy than liver metastases from cutaneous melanoma (Albert, Ryan et al. 1996). Minor responses to local treatment for hepatic metastases, such as immunoembolization, have been observed and

30% of uveal melanoma patients will die of systemic metastases at 5 years of follow-up and 45% at 15 years (Singh and Topham 2003).

The mechanisms behind the metastatic cascade in uveal melanoma are yet to be elucidated and our ability to predict prognosis is limited. However, cytogenetic studies have demonstrated that specific alterations in the tumor karyotype, monosomy 3, gain of chromosome 6 and gain of the long arm of chromosome 8, can be linked to the onset of metastasis (Sisley, Rennie et al. 1997). Harbour and colleagues exposed some of the evidence for a stem cell-like phenotype associated with metastasis, suggesting that early deregulation of cell cycle and apoptotic control lead to malignant transformation and proliferation of uveal melanocytes followed by the progression of the tumor in two classes with genotypic and phenotypic differences (Chang, Worley et al. 2008). Class 1 tumors which is not characterized by monosomy 3 has a better prognosis than class 2 tumors which have monosomy 3 and are liked to metastatic death (Onken, Worley et al. 2004). These two classes present a distinct gene signature and the biomarkers most strongly associated with the class 2 include epithelioid cell type, cytokeratin-18 and upregulation of beta-catenin, E-cadherin, and HIF-1 alpha (Chang, Worley et al. 2008).

A study by Marshall and colleagues demonstrated upregulation of a group of genes including insulin receptor substrate 2, fibronectin 1, and cytokeratin 18 which are linked to metastatic disease in uveal melanoma (Marshall, Nantel et al. 2007). Recent progress made in molecular approach to diagnosis has also led to the identification of several therapeutic targets involved in apoptosis, proliferation, invasion, metastasis, and angiogenesis of uveal melanoma (Zuidervaart, Hensbergen et al. 2006; Harbour 2007). A multitude of targets related to PI3K-Akt pathway and RAS-RAF- MAPK pathway, such as c-Kit, c-Met and FAK and IGF-1R are linked to metastatic cascade in uveal melanoma (Triozzi, Eng et al. 2008). Progress in the management

of uveal melanoma and the development of new targeted therapeutic strategies, alone or in combination with other chemotherapeutic could be considered for uveal melanoma patients.

Elucidation of the pathways that contribute to tumor progression and metastasis may lead to development of targeted therapies for uveal melanoma. The metastatic process is complex, requiring invasion from the primary tumor, intravasation and survival in the circulation, extravasation and colonization of a distant site (Eccles 2005). One of the factors implicated in cell motility and invasion is focal adhesion kinase (FAK) a protein tyrosine kinase that links integrin signaling and signal transduction pathways for cell proliferation and survival (Hauck, Hsia et al. 2002). In a study by Hess et al, the expression of phosphorylated FAK was found in uveal and cutaneous melanoma cells where correlated with the acquisition of an invasive phenotype and vasculogenic mimicry (Hess, Postovit et al. 2005). The FAK pathway also contributes to the secretion of matrix-metalloproteinases and matrix remodeling (Wu, Gan et al. 2005).

Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in invasion through remodeling of the extracellular matrix, in angiogenesis and the metastatic processes. The expression of MMP-2 and MMP-9 in uveal melanoma cells was associated with matrix degradation allowing tumor cells dissemination (Baker, Elshaw et al. 2001). Uveal melanoma disseminates preferentially to the liver. A selective mechanism in dissemination of melanoma cells to the liver includes signaling through CXCR4 and its ligand CXCL12 which is expressed in lungs, liver and bone marrow (Zlotnik and Yoshie 2000). Activation of CXCR4 by CXCL12 enables further signaling through PI3K-Akt pathway and proteins involved in cell adhesion and motility for example, FAK and upregulation of MMP-2 and MMP-9 (Fernandis, Prasad et al. 2004). Increased migration of uveal melanoma cells could be induced by CXCL12 *in vitro* and interfe-

rence with the CXCR4/CXCL12 axis resulted in inhibition of uveal tumor cell migration (Di Cesare, Marshall et al. 2007). CXCR4, is also upregulated by hypoxia as well as oncogenes such as MET, and EGFR (Eccles and Welch 2007).

Invasive uveal melanoma cells which acquire intermediate filaments vimentin and cytokeratin, induce expression of c-Met receptors (Hendrix, Seftor et al. 1998; Mallikarjuna, Pushparaj et al. 2007). The c-Met ligand, HGF, is expressed by primary uveal melanomas and metastatic foci to the liver and different studies showed an increase in migration and invasion of uveal melanoma cells when HGF was used as a chemoattractant (Woodward, Elshaw et al. 2002; Di Cesare, Marshall et al. 2007). Recently, Ye et al demonstrated that invasive mechanisms in uveal melanoma are regulated through PI3K-Akt signaling pathway concomitant with a loss of cell adhesion molecules E-cadherin and β-catenin (Ye, Hu et al. 2008)

A study by Economou et al. described the interrelation between IGF-1R and c-Met in uveal melanoma showing that co-expression of these two factors may play a role in the affinity of uveal melanoma for the liver. The authors demonstrated a strong correlation between IGF-1R expression and poor survival, pointing to IGF-1R as a prognostic indicator and potential therapeutic target (Economou, All-Ericsson et al. 2005).

An important role in tumor cells survival, proliferation and migration is attributed to hypoxia and angiogenesis. Uveal melanoma tumors express VEGF and have the ability to form "vasculogenic mimicry", a network of tumor-cell lined channels that may be implicated in invasion and connect with tumor vasculature in absence of endothelial cells (Maniotis, Folberg et al. 1999). Inhibition of the PI3K/ Akt pathway abrogated the formation of these networks and de-

creased MMP-2 activity in uveal melanoma (Hendrix, Seftor et al. 2003; Notting, Missotten et al. 2006).

Exposure to hypoxia accelerates invasive processes of uveal melanoma cells through upregulation of HIF-1 alpha and interference with tumor cell adhesion molecules and also by activation urokinase plasminogen (uPA) activation (Eccles 2005).

As outlined above, the activation of one or more oncogenic pathways is involved in the metastatic cascade in uveal melanoma requiring the complex coordination of multiple kinases and transcription factors many of which are stabilized by Hsp90. The chaperoning capacity of Hsp90 facilitates the progression of tumor cells in processes such as invasion, angiogenesis and metastasis (Maloney and Workman 2002) through interaction with: IGF-1R, c-Met, FAK, signaling molecules in Ras–MAPK and PI3K–Akt pathways, VEGF, MMP2 and transcription factors HIF-1α and NFKB, all of which depend on Hsp90 for activity and stability (Neckers and Ivy 2003; Triozzi, Eng et al. 2008). The potential of the targeted therapy option of Hsp90 inhibitors in uveal melanoma depends on the presence of the target and the activation mechanisms of Hsp90 in the processes involved in metastatic cascade. To the best of our knowledge there are no studies assessing the expression of Hp90 in metastatic uveal melanoma.

We found positive immunostaining for Hsp90 in eight metastatic cases, seven animal model samples of metastatic uveal melanoma and in one metastases derived uveal melanoma cell line. Heat shock protein 90 was found to be expressed in 87% of the patient samples (seven of eight). Weak staining intensity of Hsp90 was observed in areas of inflammation and normal tissue surrounding the metastatic lesions. The presence of both isoforms of Hsp90 (α andβ) was detected predominantly intracytoplasmatic whereas nuclear staining was seen in only a fraction of the cells suggesting dual roles of Hsp90 in cytoplasm and nucleus. Immunostaining for

Hsp90α subset showed a strong intracytoplasmic expression and was also detected by immunofluorescence on the surface of a fraction of the cells.

We previously showed that all of our primary cell lines expressed similar high levels of Hsp90 independent of their different metastatic potential. The expression levels of Hsp90 in one liver metastasis derived human uveal melanoma cell lines (OMM 1.5) showed that the cells were positive for Hsp90 total and Hsp90α staining. Hsp90 was mostly expressed intracytoplasmatically in all the cells, while Hsp90α showed concomitant membranous Hsp90 expressions. Our findings are consistent with the results of previous studies, where overexpression of Hsp90α was reported in pancreatic carcinoma cells and fibrosarcoma (Gress, Muller-Pillasch et al. 1994). Zuo et al reported a significant association between Hsp90α expression and lymph node metastases in gastric cancer (Zuo, Dai et al. 2003). In cutaneous melanoma, Hsp90 expression was overexpressed in tumor cells in comparison with melanocytic nevi and reported to be present in 92% (13 out of 14) of melanoma metastases. In nearly all the metastases analyzed, Hsp90 was expressed on the surface of the cells (Becker, Multhoff et al. 2004). Hsp90α and Hsp90β are isoforms of Hsp90. In cutaneous melanoma, Hsp90 expression was overexpressed in tumor cells in comparison with melanocytic nevi and reported to be present in 92% (13 out of 14) of melanoma metastases (Schmitt, Gehrmann et al. 2007).

Based on its functions involving cytoskeletal organization, and lamellipodia formation, extracellular Hsp90 might also play an important role in cell motility and metastasis. In fibrosarcoma cells, Hsp90α assists in the activation of matrix metalloproteinase-2 (MMP2), leading to increased tumor invasiveness (Eustace and Jay 2004). Due to the small sample of metastases studied we were not able to determine the biological significance of the expression of Hsp90 in me-

tastatic uveal melanoma. Further studies should be undertaken to assess the predictive role of Hsp90 expression in metastases and the response to Hsp90 inhibition.

We examined the antiproliferative activity of 17-AAG in one metastatic derived uveal melanoma cell line *in vitro*. Hsp90 inhibition led to a decrease in cell proliferation of the uveal melanoma cells. Our results suggest that Hsp90 could be involved in proliferation and survival of metastases.

As highlighted before, intracellular and extracellular heat shock protein 90 has a role in cancer cell motility, invasion and metastasis. Inhibition of cell-surface Hsp90 with drugs linked to cell-impermeable beads led to inhibition of tumor cell invasion (Eustace and Jay 2004). Migration could be experimentally inhibited with neutralizing antibodies against Hsp90 effect that has been attributed to alterations of the actin cytoskeleton (Lang, Moser et al. 2007). The effects of Hsp90 inhibition with regard to the processes involved in uveal melanoma metastasis have not been explored.

We examined the activity of 17-AAG in a panel of human uveal melanoma cell lines *in vitro* and found that the 17-AAG administration significantly decreased migratory capacity in three of the cell lines (92.1, SP6.5, and OCM-1) and decreased the invasive abilities of all the uveal melanoma cells. Our results confirm data from other studies in which Hsp90 inhibition significantly reduced tumor cell motility and invasion in vitro (Tsutsumi, Scroggins et al. 2008; Yang, Rao et al. 2008). Furthermore, Tsutsumi and colleagues showed that a cell-impermeable Hsp90 inhibitor, significantly reduced tumor cell migration and integrin -cytoskeletal reorganization and diminished colonization of the lung by melanoma cells in an animal model (Tsutsumi, Scroggins et al. 2008). Our results point to the therapeutic potential of Hsp90 inhibitors as a new approach to targeting the metastatic phenotype in uveal melanoma.

Cancer invasion is a process involving alterations in the adhesion of the cells to the extracellular matrix, followed by reorganization and cell invasion (Eccles 2005). An early step in the metastatic cascade involves adhesion of the tumor cells to the extracellular matrix.

One of the principal players involved in cell adhesion is focal-adhesion kinase (FAK) a cytosolic nonreceptor tyrosine kinase that localizes to cellular focal adhesions or cell contacts within the extracellular matrix (Hess and Hendrix 2006). FAK is a component of the integrin-mediated adhesion and in tumors, and links integrin mediated signaling to other major pathways that control cell survival, proliferation, migration, and invasion (Li and Hua 2008). In uveal melanoma, phosphorylation of FAK was found to correlate with angiogenesis and invasion (Hess, Postovit et al. 2005). FAK is stabilized by Hsp90 and is sensitive to the destabilizing effects of Hsp90 inhibitors which stimulate the proteasome-mediated degradation of FAK. These effects have been reported in breast cancer, prostate carcinoma and Ewing's sarcoma cells (Ochel, Schulte et al. 1999). We confirmed these results in our study and demonstrated downregulation of FAK in a panel of uveal melanoma cell lines after 17-AAG exposure. It is possible that in uveal melanoma, Hsp90 is involved in cell adhesion through stabilization of FAK thus enabling cell motility and invasive and metastatic potential of the tumor cells. Pharmacologic modulation of FAK protein levels with Hsp90 inhibitors is a feasible approach to alteration of FAK function in uveal melanoma.

Furthermore, the ability of tumor cells to migrate and survive within the circulation are part of the complex cascade of cellular events that result in the successful establishment of metastases (Eccles and Welch 2007).

One of the factors implicated in cell motility and invasion in uveal melanoma is the hepatocyte growth factor (HGF) and its receptor c-Met (Woodward, Elshaw et al. 2002; Di

Cesare, Marshall et al. 2007). C-met activation by HGF leads increase cellular motility, growth and invasion signaling through Ras–MAPK and PI3K–Akt pathways (Eccles 2005; Peruzzi and Bottaro 2006). Recently, Ye and colleagues revealed that the activation of PI3K-Akt pathway induced by the HGF and c-Met axis results in deregulation of the E-cadherin and beta-catenin pathway promoting motility and migration of uveal melanoma cells (Ye, Hu et al. 2008). C-Met is an Hsp90 client and is destabilized by Hsp90 inhibitors with consequences to the downstream c-Met-dependent Akt, Erk and cell motility and invasion (Webb, Hose et al. 2000; Wang, Pashtan et al. 2009). In our study, 17-AAG, downregulated c-Met in uveal melanoma cell lines. Our results are in concordance with previous studies which showed that Hsp90 inhibition resultated in downregulation of newly synthesized Met protein in small cell lung cancer cells and bladder cells (Maulik, Kijima et al. 2002; Koga, Tsutsumi et al. 2007).

Thus, the Hsp90 inhibitors might be expected to impact tumor progression towards metastasis in uveal melanoma in which Met activity contributes to the invasive/metastatic phenotype.

Insulin like growth factor 1 (IGF-1) and its receptor IGF-1R, are important for cell motility and metastasis. IGF-1 is mainly produced by the liver and in uveal melanoma, IGF-1R expression is correlated with worse prognosis. A study by Economou et al. showed that co-expression of IGF-1R and c-Met may play a role in the dissemination of uveal melanoma to the liver and IGF-1R is dependent on Hsp90 for its stabilization (Eiseman, Guo et al. 2007). It has been previously shown that Hsp90 inhibitors reduced IGF-1R expression at the transcriptional level and induce IGF-1R protein degradation (Nielsen, Andrews et al. 2004). We demonstrated that treating uveal melanoma cells with 17-AAG resulted in downregulation of IGF-IR. Our data confirm previous studies in which 17-AAG administration inhibited IGF-1R signaling by down-

regulation and impaired IGF-IR phosphorylation in pancreatic cancer cells (Lang, Moser et al. 2007) and depleted IGF-1R in pediatric solid tumor cells (Bagatell, Beliakoff et al. 2005). Our results highlight the possibility of using IGF-1R as potential target using Hsp90 inhibitors.

The activated Akt kinase modulates many processes in the metastatic pathway including downregulation of E-cadherin, and altering cellular adhesion and angiogenesis. Inhibition and dysregulation of the Akt pathway can result in reduction in signal transduction, proliferation, and invasion (Vivanco and Sawyers 2002; Qiao, Sheng et al. 2008). Both Akt and its activating kinase 3-phosphoinositide-dependent kinase-1 rely on Hsp90 for stability (Sato, Fujita et al. 2000; Basso, Solit et al. 2002). In our study, the exposure of human uveal melanoma cell lines to 17-AAG resulted in a decrease of Akt and activated p-Akt, possibly contributing to decrease in migration and invasion of uveal melanoma cells. It is possible that inhibition of PI3K/-Akt pathway could lead to antiangiogenic effects as its inhibition abrogated the formation of vascular networks in uveal melanoma (Hendrix, Seftor et al. 2003; Notting, Missotten et al. 2006). In different studies, Hsp90 inhibition has been shown to disrupt VEGF and HIF-1$\alpha$ mediated angiogenesis (Isaacs, Jung et al. 2002; Le Boeuf, Houle et al. 2004). Further investigation, especially utilizing models of *in vivo* metastasis, is required to unveil the complete mechanism of Hsp90 in uveal melanoma metastasis.

For the first time our group demonstrated the effects of Hsp90 inhibition involved in uveal melanoma metastasis. Our data support the idea that Hsp90 is an attractive target for anti-metastatic therapy as it is implicated in multiple aspects of the metastatic process, most notably cell adhesion, cell motility and invasion. Inhibition of Hsp90 could result in multiple effects in metastatic cascade in uveal melanoma.

## *Conclusion and future perspectives*

In this thesis my goal was to elucidate the expression and some of the functional roles of Hsp90 in uveal melanoma. Our principal findings showed that Hsp90 is highly expressed in uveal melanoma and has also been detected in metastases. Hsp90 expression correlated with largest tumor dimension, a clinical indicator of poor prognosis, therefore establishing Hsp90 as a target in uveal melanoma. We have also shown that Hsp90 has roles in proliferation, cell cycle progression, apoptotic mechanisms, migratory and invasive potential in this tumor. These processes could be modulated in vitro through the inhibition of Hsp90 at relative low dose levels. Key players in uveal melanoma, p-Akt, CDK4, FAK, c-Met, and IGF-1R, which depend on Hsp90 for activity and stability, could be downregulated following inhibition of Hsp90 suggesting that targeting Hsp90 may be as effective as targeting several signaling pathways. Collectively our data identified for the first time Hsp90 as a potential target for novel therapeutic approaches in patients with uveal melanoma (fig.7.1)

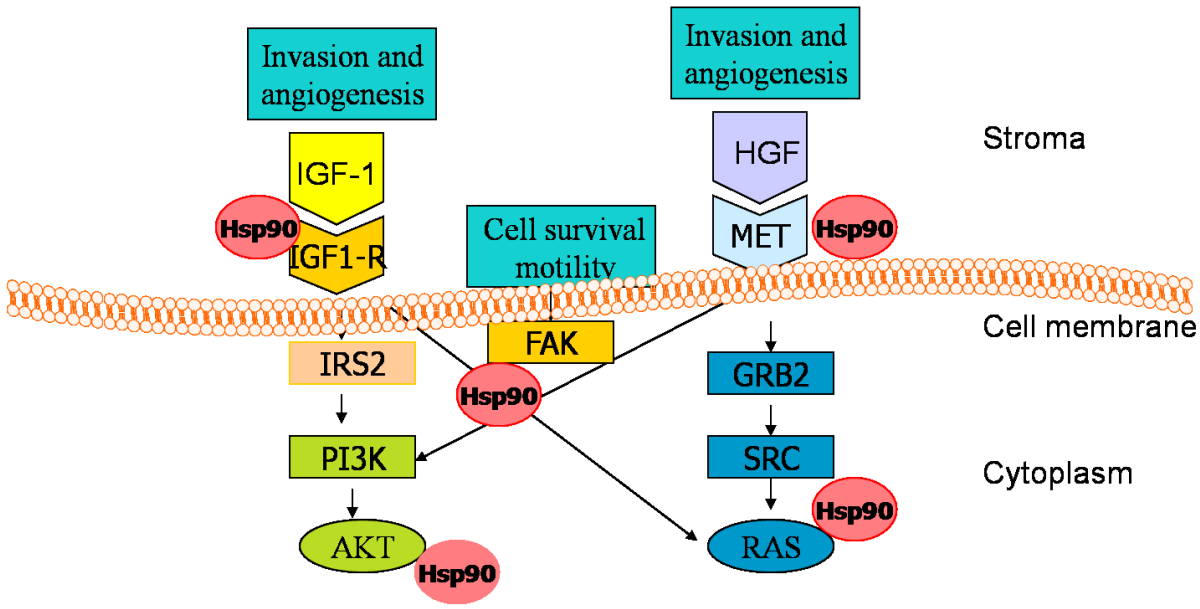



**Fig.7.1** Potential targets for therapy with Hsp90 inhibitors in uveal melanoma

Our findings could have important implications for Hsp90 as a target for therapy in uveal melanoma. Hsp90 inhibitors are researched in Phase II and III clinical trials, and depletion of client protein levels and upregulation of Hsp70 was reported in peripheral blood lymphocytes and tumor biopsies in several types of cancer. The Hsp90 inhibitor, 17-AAG, is well tolerated and evidence of results have been seen in metastatic melanoma, prostate cancer and multiple myeloma (Pacey, Banerji et al. 2006) however has some limitations, due to formulation and drug metabolism (Kelland, Sharp et al. 1999).

Novel Hsp90 inhibitors are currently being assessed *in vitro*, in animal models and in clinical trials (Chiosis, Timaul et al. 2001; Eccles, Massey et al. 2008; Taldone, Gozman et al. 2008; Okawa, Hideshima et al. 2009). It is probable that further investigation will identify Hsp90 inhibitors with better therapeutic activity than 17-AAG, that can efficiently downregulate the oncoproteins in uveal melanoma. Due to the concurrent activity against oncogenic proteins in multiple pathways, the outcomes of Hsp90 inhibition could have a broad impact. Moreover, recently introduced drug agents target particular functions of Hsp90 and other members of the Hsp90 chaperone complex like Hsp70 (Guo, Rocha et al. 2005), Cdc37 (Stepanova, Leng et al. 1996; Zhang, Hamza et al. 2008) and AHA (Holmes, Sharp et al. 2008) and it is likely that they will provide a better selectivity towards targets in uveal melanoma.

Novel techniques including genomics and proteomics have the potential to define better targets in this tumor, with modern techniques like, and improve the ability to predict whether certain tumor types or individual patients are particular sensitive or more resistant to treatment with Hsp90 inhibiting drugs. Using protein microarray techniques (Whatmann Inc.), we were able to assess the protein array profile in lysates from uveal melanoma cells before and after in-

hibition with 17-AAG, showing induction of Hsp70 and downregulation of Hsp90. This technique could allow simultaneous profile of samples from an individual patient for the presence of specific targets and evidence of client proteins downregulation and therapeutic activity after Hsp90 inhibition.

Hsp90 inhibitors have been shown to display synergistic activity when used in combination with proteasome inhibitors or kinase inhibitors (Mitsiades, Mitsiades et al. 2006). Evidence of responses to the combination of Tanespimycin with Bortezomib was reported in 47% of cases in patients with relapsed, refractory multiple myeloma (Taldone, Gozman et al. 2008). A combination of 17-AAG with Trastuzumab (Herceptin) showed potential therapeutic in breast cancer patients previously nonresponsive to Herceptin alone (Modi, Stopeck et al. 2007). In *in vitro* studies we demonstrated a synergistic activity between proteasome inhibitor Bortezomib, Imatinib and 17-AAG (Oliver, Di Cesare et al. 2007) in uveal melanoma cell lines. Future experiments, tailored to specific clients in uveal melanoma are needed to determine possible beneficial therapeutic combinations.

In this thesis we have demonstrated the promising therapeutic potential of Hsp90 as a target for uveal melanoma treatment. Understanding the roles of Hsp90 holds the hope of progress in translating these findings to further *in vitro* and *in vivo* assessment and improving the treatment of uveal melanoma patients.

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## Publications and Presentations

Partial data from the present thesis has been published in the following papers:

*Immune expression and inhibition of heat shock protein 90 in uveal melanoma*

Faingold D, Marshall JC, Antecka E, Di Cesare S, Odashiro AN, Bakalian S, Fernandes BF, Burnier MN Jr.

Clin Cancer Res. 2008 Feb 1;14(3):847-55.

Other publications in uveal melanoma submitted during the preparation of this thesis:

*Molecular pathways mediating liver metastasis in patients with uveal melanoma.*

Bakalian S, Marshall JC, Logan P, Faingold D, Maloney S, Di Cesare S, Martins C, Fernandes BF, Burnier MN Jr.

Clin Cancer Res. 2008 Feb 15;14(4):951-6.

*Expression and migratory analysis of 5 human uveal melanoma cell lines for CXCL12, CXCL8, CXCL1, and HGF.*

Di Cesare S, Marshall JC, Logan P, Antecka E, Faingold D, Maloney SC, Burnier MN Jr.J Carcinog. 2007 Jan 29; 6:2.

*Expression of nm23-H1 in uveal melanoma*

Bakalian S, Marshall JC, Faingold D, Logan P, Antecka E, Burnier MN Jr.

Melanoma Res. 2007 Oct; 17(5):284-90

*A view of VEGF in ocular melanoma: prospective implications for patient care*

Jonathan Cools-Lartigue[†], Jean-Claude Marshall, Dana Faingold and Miguel N Burnier Jr Expert Review of Ophthalmology, April 2007, Vol. 2, No. 2, Pages 161-164

Data from this thesis has been presented in the following meetings:

Association for Research in Vision and Ophthalmology (ARVO) and American Association for Research in Cancer (AACR), and European association for Vision and Research (EVER)

Dana Faingold, Jean-Claude Marshall, Silvin Bakalian, Stephanie R Cruess, Emilia Antecka, Oscar Kasner, M.N. Burnier Jr. *The effect of lens proteins on proliferation in uveal melanoma cell lines.* Invest. Ophthalmol. Vis. Sci. 46: E-Abstract 3874. ARVO, 2005

Dana Faingold, J. -C. Marshall, Silvin.Bakalian, Abdulah Al-Kandari, Chaim Edelstein, Miguel N. Burnier. *Immune Expression and Inhibition of Hsp90 in Human Uveal Melanoma* Jr. Invest. Ophthalmol. Vis. Sci. 47: E-Abstract 2247. ARVO, 2006 and AACR, 2006

Dana Faingold, Jean Claude Marshall, Sebastian Di Cesare, Emilia Antecka, Silvin Bakalian, Miguel N. Burnier JR. *Effect of the heat shock-90 inhibitor 17-AAG in uveal melanoma* Invest. Ophthalmol. Vis. Sci. 48: E-Abstract 5247 ARVO, 2007

Dana Faingold, D. A. Abourbih, S.Bakalian, E. Antecka, Miguel N. Burnier, Jr. *Immune Expression and Inhibition of Hsp90 in Metastatic Uveal Melanoma* .Invest. Ophthalmol. Vis. Sci. 49: E-Abstract 77 ARVO, 2008 and EVER, 2008

B. F. Fernandes, D. Faingold, E. Antecka, C. Martins, S. Bakalian, and M. N. Burnier, Jr. *Phosphorylated Fak Is Expressed in Primary Uveal Melanoma*
Invest. Ophthalmol. Vis. Sci. 49: E-Abstract 73. ARVO, 2008

Dana Faingold, Emilia Antecka, Silvin Bakalian, Alexandre N. Odashiro, M. N. Burnier Jr. I *Immune expression of alpha B-crystallin in uveal melanoma.* Invest. Ophthalmol. Vis. Sci. 50: E-Abstract 5757 ARVO, 2009

## Awards

The Alma Mater Travel Award McGill University, (2006) Montreal, Canada for the project: *Immune Expression and Inhibition of Hsp90 in Human Uveal Melanoma*.

The 2006 Henry R. Shibata Cedar Fellowship award, from the Cedars Cancer Institute Montreal Canada, for the project: *Immune Expression and Inhibition of Hsp90 in Human Uveal Melanoma*.

The 2007 Henry R. Shibata Cedar Fellowship award from the Cedars Cancer Institute Montreal Canada, for the project: *Hsp90 as a novel target in uveal melanoma.*

Best poster award in Pathology/Oncology section, at The European Association for Vision and Research, (EVER). Portoroz, Slovenia, October, 2007, for the project: *Immune expression and inhibition of Hsp90 in uveal melanoma.*