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„ Candida albicans‘ key mediators in the PKC pathway as novel targets of bisindolylmaleimides“

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Manon Barsa

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Mémoire

Titre du mémoire

„ Candida albicans‘ key mediators in the PKC pathway as novel targets of bisindolylmaleimides“

Auteure

Manon Barsa

Grade académique

Magistra der Pharmazie (Mag.pharm.)

Professeur Patrice le Pape
Université Nantes

Prof. Dr. Christian R. Noe
Universität Wien

> Les amours sont comme les champignons. On ne sait si elles appartiennent à la bonne ou à la mauvaise espèce que lorsqu'il est trop tard.
>
> Tristan Bernard

Abstract

Nowadays fungal pathogens are causal agents of various infections in the human body. Among this life threatening fungi, *Candida albicans* stands out due to high mortality worldwide. Especially for immunocompromised patients candiasis has turned out to be a serious health concern. Under normal physiological conditions they are innocuous inhabitants of the human's normal flora, but only minimal variations from normal conditions can precipitate an attack.

The widespread usage of antifungal therapeutics has lost efficacy because of the resistance that *Candida albicans* has developed by various mechanisms. The exposure of *Candida albicans* to antifungal drugs creates stress responses which activate parallel signal pathways. Two major pathways (PKC and Calcineurin p.) display a major contribution to *Candida albicans'* barrier. Thereby the key regulators of these pathways (Hsp90, GSK3 and PKC) show a link between the transcriptional events which make *Candida albicans* invulnerable against therapeutic treatment. The investigation of different expressions of these key regulators confirms their presence in *Candida albicans*.

An attractive inhibitor would have the capability to stop the cascade which contributes to the resistance of *Candida*. We expected this attribute from the group of bisindolylmaleimides which has already been described to have an effect on PKC. The inhibition of PKC by BisIII was studied in carcinogenic cells but still unknown in *Candida albicans*.

The affinity micro assay should help to elucidate a possible interaction between PKCη and BisIII. Finally the affinity micro assay has confirmed an effect that BisIII exercises on *Candida albicans'* mechanism of resistance by interfering with the PKC pathway.

The knowledge about a possible interaction could be used as a starting point for further investigations with BisIII which may restore the sensitivity to azoles and furthermore increase the efficacy of the antifungal treatment.

Zusammenfassung

Zahlreiche Infektionen in unserem Körper werden heutzutage von Pilzen hervorgerufen. Unter einer großen Vielfalt verschiedener Pilzarten befindet sich *Candida albicans*, der als einer der Haupterreger gilt. Welweit wird er gefürchtet, da er sich durch eine hohe Todesrate auszeichnet. Besonders immunsuprimierte Patienten sind von der Candidose betroffen, in deren Körper sich der Erreger sehr schnell ausbreitet. Unter normalen physiologischen Bedingungen ist dieser Pilz ein harmloser Bewohner unserer Flora, doch minimale Abweichungen dieser Lebensverhältnisse können eine Infektion auslösen.

Die herkömmlichen Behandlungen mit antimykotischen Therapeutika stoßen meist auf eine Blockade, da der Erreger ausgeklügelte Mechanismen entwickelt hat, um eine Resistenz aufzubauen. Die Behandlung von *Candida albicans* mit herkömmlichen Antimykotika induziert die Bildung von reaktiven Sauerstoffradikalen, welche parallel zahlreiche Signalwege aktivieren. Besonders der PKC- und Calcineurin Signalweg scheinen maßgeblich an *Candida albicans* Abwehrsystem beteiligt zu sein. Dabei treten die Schlüsselenzyme (Hsp90, PKC und GSK3) in den Vordergrund, die eine Reihe von Transkriptionen im Körper codieren. Die Erforschung der verschiedenen Proteinexpressionen dieses Hefepilzes bestätigte das Vorhandensein dieser Schlüsselenzyme.

Nun musste ein passender Inhibitor gefunden werden, der diese Signaltransduktionskette zu unterbrechen vermochte. Von der Gruppe der Bisindolymaleimides war bereits bekannt, dass sie PKC angreifen und inhibieren. Jedoch wurden diese Studien nur mit krebsbefallenen menschlichen Zellen durchgeführt und nicht mit *Candida albicans*. Mit Hilfe der Micro-Affinitäts-Studie wurden die Interaktionen zwischen PKCη und BisIII beobachtet, wobei sich die Tatsache zu bewahrheiten erschien , dass BisIII genau auf den PKC Signalweg wirkt.

Diese Erkenntnis könnte nun als Ansatzpunkt für weitere Forschungen mit BisIII verwendet werden, um so die Resistenz von *Candida albicans* gegen Antimykotika effizient zu eliminieren und dabei den Erfolg einer antimykotischen Therapie zu erhöhen.

Table of contents

I. List of figures

II. List of reagents

1. Tris HCl pH 8,8 (1,5M)

Tris Base	181,71g
H_20	800ml
HCl 37%	Until pH 8,8 (67ml)
H_20	Ad 1000ml

2. LB4X

Tris pH 6,6 1M	1,15ml
SDS 10%	1,67ml
Glycerol 100%	1,25ml
β-Mercaptoethanol	0,165 ml
H_20	Ad 5ml

3. Decoloration Solution

Acetic Acid	7%
Absolute Ethanol	10%

4. Staining Solution (COOMASSIE BRILLIANT BLUE)

H_20	100ml
MeOH	125ml
Acetic Acid	25ml
Coomassie Brilliant Blue G-250	0,25g

5. Transfer Buffer (classic)

40 mM Glycine	29,3g
48mM TrisBase	58g
20% SDS	18,8ml
H_20	Ad 1000ml

6. Ponceau Staining Solution

Acetic Acid	3%
Ponceau S	0,6%

7. Transfer Buffer (semidry)

48mM Tris	5,82g
39mM Glycine	2,93g
20% Methanol	200ml
10% SDS	3,75ml
H_20	Ad 1000ml

8. YPD (Yeast Extract Peptone Dextrose Medium)

Yeast Extract	1%
Peptone	2%
Dextrose	2%

9. Stacking Gel

H_20	2,7ml
30% Acrylamide gel	670µl
1,5M Tris ph6,8	500µl
10% SDS	40µl
10% APS	40µl
Temed	4µl

10. Migration Buffer

TrisHCl	30,275g
Glycine	144g
H_20	100ml
20% SDS	5ml
H_20	Ad1000ml

11. Protein Extraction Reagent

MPER	1ml
PMSF	10µl
PIC	1µl

12. Acid Solution (ph=4)

(0,1M acetate buffer + 0,5M NaCl)

Acetic acid	84,7ml
Sodiumacetate	15,3ml
NaCl	2,922g

13. Basic Solution (ph=8)

(0,1M TrisHcl Buffer +0,5M NaCl)

TrisHCl	15,76g
NaCl	2,92g

14. Lysis Buffer 10X

Hepes 1M	5ml
NaCl 5M	3ml
Triton X-100	500µl
EDTA 100mM	1ml
H_20	ad 10ml

15. Lysis Buffer 1X/ 1M NaCl

Lysis Buffer 10X	1ml
NaCl 5M	2ml
H_20	7ml

16. 1,5X SDS Buffer

1M TrisHCl ph=6,8	937,5µl
Glycerin	1,5ml
SDS 10%	3ml
H_20	ad 10ml

III. Abbreviations

ATP	Adenosine triphosphate
APS	Ammonium persulfate
BCA	Bichinoninic assay
BSA	Bovine serum albumin
CHS	Chitin synthase
DMF	Dimethylformamide
EDTA	Ethylendiaminetetraacetic acid
FBS	Fetal bovine serum
GSK	Glycogen synthase kinase
HCl	Hydrocholoric acid
HeLa	Henrietta Lack
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
MAPK	Mitogen activated protein kinase
MDR	Multi drug resistance
MeOH	Methanol
M-PER	Mammalian protein extraction reagent
PBS (T)	Phosphate buffered saline (tween)
PIC	Protease inhibitor coctail

PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene fluoride
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SDS (PAGE)	Sodium dodecyl sulfate (polyacrylamide gel electrophoresis)
TBS (T)	Tris buffered saline (tween)
TEMED	Tetramethylethylenediamine
YPD	Yeast extract peptone dextrose medium

1 Introduction

Fungal pathogens are a significant cause of human mortality worldwide, especially among immunocompromised individuals [29]. They have designed an elegant repertoire of mechanisms to survive the cellular stress exerted by antifungal drugs [30]. This fact turns them into a genuine risk for the human health.

Among the different kinds of fungal pathogens *Candida albicans* represents one of the leading human fungal pathogens which is responsible for various hospital acquired infectious diseases with a mortality rate of 50% [1].

1.1 Objectives of the research

The potency of *Candida albicans* to establish a persistent resistance to antifungal drugs turns it into a serious menace for human health. Thus weakening its resistance to antifungal drugs poses an attractive target in the clinical development of an efficient treatment against this threat.

One aim of the laboratory involves the discovery of an efficient alternative to affect this fungal pathogen regarding to stop their potential of developing resistance against the antifungal drugs. The ability of *Candida albicans* to establish a persistent infection is namely based on its cellular signals that regulate release of factors from target cells responsible for the replication of pathogen [25].

Thereby the studies are focused mainly on the PKC and Calcineurin transduction pathways. Both pathways are well known for regulating drug resistance turning the *Candida* cells invulnerable by various mechanisms. Important mediators (PKC, Hsp90 and GSK3) of the two pathways are studied since. As key mediators of stress response in *Candida albicans* they are implicated in various signal pathways. Inhibition of PKC, Hsp90 and GSK3 could be utilized as a novel mechanism of antifungal treatment. Moreover it can be used as a starting point to develop novel therapeutics which nowadays present a necessity due to increased drug resistance in clinical isolates.

1.2 Working steps

1. Validation of antibodies in *Candida albicans*

The target was to study exactly the key regulators in these pathways, regarding to degrade their contribution to *Candida albicans'* virulence. Therefore the different protein expressions in the eukaryotic model were investigated to get a better understanding of our target. The technique of Western blotting was used to gain insight in the inner life of *Candida albicans*. The proteins GSK3α, PKC and Hsp90 were focus of interest, assumed to play an important role in *Candid*a*'s* transduction network. The incoming results have been validated using HeLa protein expressions as a reference.

2. Affinity Micro Assay

As a strong signal of each of these proteins was detectable, the research continued on the synthesized substance BisindolylmaleimideIII (BisIII) (synthesized by the group of IICiMED) from which we supposed that the mechanism of action in carcinogenic cells is related to its interaction with the key proteins in *Candida albicans*.

The group of bisindolylmaleimides has been chosen which is a potent inhibitor of protein kinase C. But they are not entirely selective for PKC, known to inhibit a few other protein kinases in carcinogenic human cells [34]. Furthermore they are capable of restoring sensitivity to azoles. So the effects of BisIII are ascertained on the key proteins in *Candida albicans*.

3. Affinity Chromatography and Mass Spectrometry

The affinity micro assay is chosen as the method to prepare the experiment for larger dimension like the affinity chromatography. It is more likely to assert the important parameters in micro dimension than working on larger scales. In the end it is only a preparative method for the affinity chromatography where the whole repertoire of interaction between the bisindolylmaleimides and *Candida albicans'* proteins can be described. In common with the mass spectrometry, the affinity chromatography can give a notion of the extent of interplays that can occur between the bisindolylmaleimides and the fungal pathogens.

1.3 The opportunistic fungus-*Candida albicans*

1.3.1 Model organism

The polymorphic fungus *Candida albicans* is an outstanding eukaryotic model for studying cellular processes [23]. Nowadays the prognosis of *Candida albicans'* infection is hampered by the reduced efficacy of currently available drugs, the development of antifungal resistance and the lack of rapid and specific diagnostic tests [24].

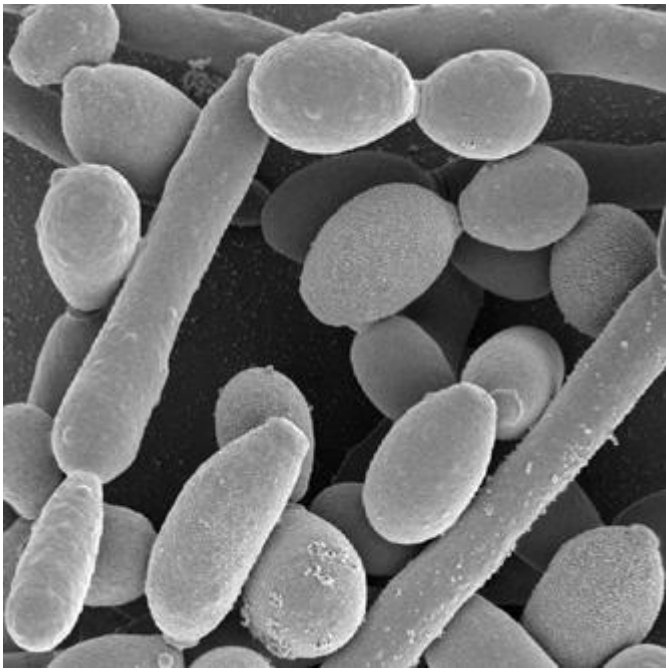


Figure 1. Candida albicans [GRAPHICSHUNT.com]

1.3.2 The fungal skills

Under normal physiologic conditions the opportunistic pathogen is an inactive component of human's normal flora in the oral cavity, gastrointestinal tract and vaginal micro flora [22].
But only minimal variations from normal conditions could provoke the *Candida* attack. The fungus disseminates through blood and lymphatic and induces a strong inflammatory response, mostly in the target organs [31]. Diseases due to *Candida* show a variety of clinical manifestations, ranging from mucutaneous infections of the mouth, esophagus, and vagina to life threatening systemic infections [28].

1.3.3 Combat of *Candida* infections

Despite intensive drug discovery efforts, there are only five major classes of antifungal drugs, which combat fungal infection [28]. Three of the five classes of antifungal drugs in clinical use target function or biosynthesis of ergosterol, the main sterol of fungal cell membrane. Thereby allylamines affect the squalen epoxidase, polyenes work against ergosterol and azoles attack the lanosterol demethylase. Despite their widespread usage the azoles are fungistatic rather than fungicidal. Working against the activity of 14α-demethylase, the antifungal treatment blocks the production of ergosterol, which results in an accumulation of toxic sterol intermediates [30].

In contrast antimetabolites and echinocadins try to turn fungi vulnerable targeting the fungal nucleic acid or their cell wall (Figure 2).

Compound class	Example drug	Target
Allylamines	Terbinafine	Ergosterol synthesis, squalene epoxidase
Antimetabolites	Flucytosine	Fungal nucleic acid (RNA and DNA)
Azoles	Fluconazole	Ergosterol synthesis, lanosterol demethylase
Echinocandins	Caspofungin	Cell wall, β-1-3-glucan synthesis
Polyenes	Amphotericin B	Ergosterol
Other	Griseofulvin	Fungal mitotic apparatus

Figure 2. Antifungal drugs and their targets [ANDERSON, 2010]

Classic mechanisms of getting rid of antifungal drugs, involve the over-expression of multidrug transporters that efflux azoles and mutations in Erg11 that causes Erg11 over-expression. Lanosterol 14α-demethylase is encoded by Erg11 gene. These mechanisms prevent the azoles from inhibiting their target. Additionally, the close evolutionary relationship that fungi share with the human host aggravates the search of fungal selective drug targets [30].

1.3.4 Morphogenetic plasticity

To achieve adaptability to a range of physiological extremes, the fungus has developed diverse mechanisms of sensing and responding to environmental challenges by activating developmental alterations that result in coordinated changes in cell physiology, morphology and adherence [42]. *Candida albicans'* virulence is intimately coupled with morphogenetic plasticity. It demonstrated its ability to switch between budding yeast form to a hyphal composed of filaments. This metamorphosis is implicated in virulence since the mutants defective in the yeast to filamentous transition are more attenuated in virulence [29]. The dimorphic transition causes a change in immune response because of epitope change [31].
The protein Hsp90 governs the key transition from yeast to filamentous growth in the absence of external elicitors by repressing Ras-PKA signalling. But Hsp90 capacity of orchestrating *Candida albicans'* morphogenesis is mainly a response to temperature change, because it requires an elevated temperature of 37ºC [29].

1.3.5 Stress response network

Increased ROS formation is a common consequence of many pathologies, including infection and inflammation. It provides a link between signaling pathways and transcriptional events that in turn regulate a large number of genes [32].

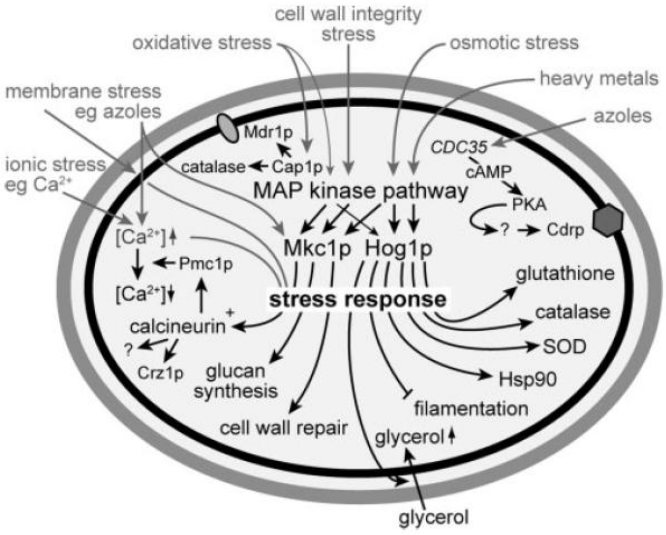


Figure 3. Stress Response Network in Candida albicans [ANDERSON; 2010]

The exposure of *Candida albicans* to antifungal drugs creates stress responses, which are transduced through a variety of membrane receptors. The intensity of the signal transmission is basically dependent on the dose and the nature of the drug. As a result, parallel signaling pathways are activated in *Candida albicans*. One of the most important is the MAP-kinase signal transduction which induces the Mkc1 and Hog1p pathways. Hsp90, Calcineurin and PKC are important actors in this network and as stress response components they pose targets in the search of antifungal treatments [38]. As a consequence they lead to an upregulation of glycerol- , glucan- , filamentation- , Hsp90- , glutathione- and SOD production (Figure 3). The deletion of these proteins would confer hypersensitivity to azoles [39].

1.4 Signaling pathway in *Candida albicans*

PKC, a serine/threonine kinase, governs a range of biological processes in *Candida albicans*. The cell wall integrity, morphogenesis, and also the response to cell wall stress undergo its regulation [1]. The contribution of the PKC- and Calcineurin pathway to antifungal resistance are transduced through a variety of substrates. Signalling through Rlm1 regulates at least 25 different genes, which are responsible for the cell wall biogenesis [46]. Inhibiting the key regulators would stop the cascade (Figure 4).

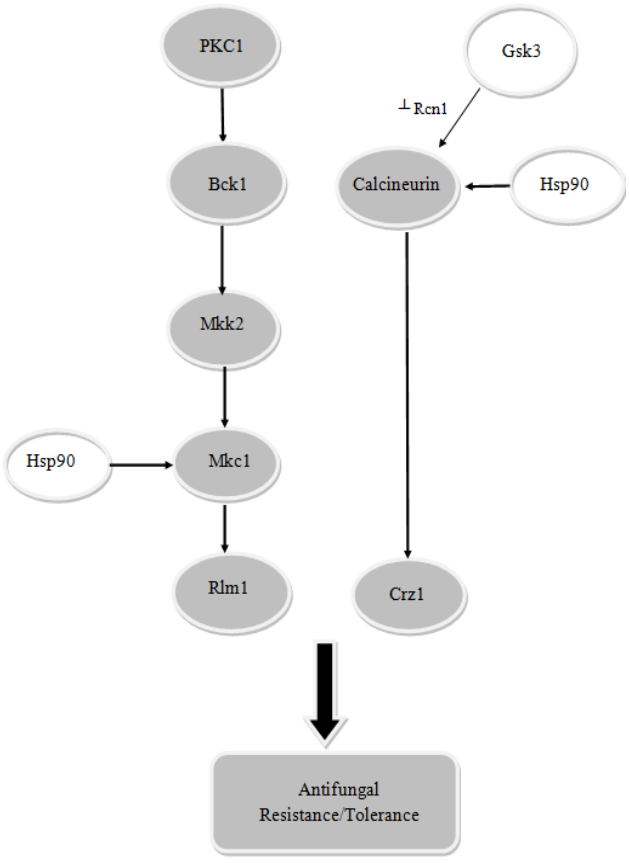


Figure 4. PKC- and Calcineurin Pathway

1.4.1 Architect of the fungal cell wall

The cell wall represents a scaffold, an armour and an environmental gate. Fungal cell walls have a dynamic structure which consists of glycoproteins and polysaccharides that are able to withstand the cytoplasmic turgor pressure

and external injuries. It acts as a mediator between the inner life and environment outside [36]. Chitin represents an essential part of the fungal cell wall while CHS are promoters of transcriptional regulation of chitin synthesis. The regulation of both, CHS gene expressions as well as chitin synthesis are under the control of PKC, HOG MAP kinase and Ca^{2+} Calcineurin signaling pathways [35].

1.4.2 PKC isoforms

Different isoforms of PKC are known and divided into three groups: the classical PKC isoforms α, βI, βII, γ (cPKC), and the novel PKC isoforms δ, ε, θ, η, μ (nPKC) and the atypical PKC isoforms λ and ζ (aPKC). The classical PKCs are characterized by four conserved domains named C1-C4. The C3 domain is responsible for ATP binding where bisindolylmaleimides are attracted to [44].

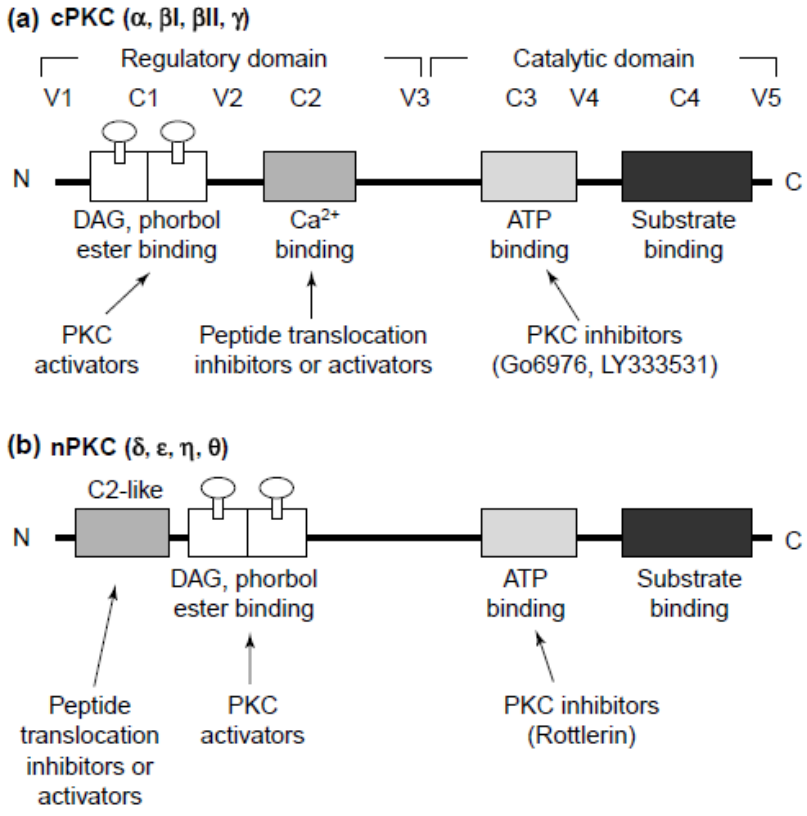


Figure 5. Structure of PKC [WAY, CHOU, KING, 2000]

1.4.3 Heat Shock Protein

Hsp90 is part of the group of the heat shock proteins that regulate the function of diverse client proteins in all eukaryotes [29]. It uses a complex mechanism of ATP-ase coupled conformational changes to establish the possibility of interaction with a bride range of unstable or metastable proteins [39, 40].

The function of Hsp90 is the enhancement of calcineurin dependent stress response, while interacting with it causes poisoning for activation. Calcineurin is considered as the key mediator of Hsp90 dependent azole resistance. Indeed Hsp90 and calcineurin are suspected to be one of the main factors causing resistance in *Candida albicans* [1].

While Hsp90 regulates the calcineurin activity, it acts also as an agent between MAPK and Mkc1. Hsp90 stabilizes Mkc1 independently of its activation status and thereby regulates PKC signaling. Genetic depletion of Hsp90 ends in decreasing stability of *C. albicans* Mkc1, which indicates another functional connection between Hsp90 and PKC (Figure 4) [1].

Hsp90 may influence evolution by releasing previously silent genetic variations in response to environmental change. The molecular chaperone pushes the development of drug resistance and enhances new mutations which have a visible effect on the phenotype. It seems that Hsp90 might affect the evolution in two different ways. On one side Hsp90 can create, as a preserver of cryptic genetic variation, new traits. On the other side Hsp90 can control mutated cell regulators with an oncogenic potential. Thus Hsp90 may play an important role as a capacitor for the storage and release of genetic variations [37].

1.4.4 GSK3

GSK3 is a ubiquitously distributed serine threonine kinase encoded by two different genes, alpha (α) and beta (β) [27]. The protein GSK3 is implicated in multiple cellular processes and linked with the pathogenesis of several diseases which makes it attractive as a key target in drug discovery [28]. A calcineurin inhibitor can be seen as a potent killer of *Candida albicans*. The protein Rcn1 works on this base, then it binds Calcineurin and inhibits so its function (Figure 4). But the effect of Rcn1 is abolished by the intervention of GSK3. Upon the phosphorylation by GSK, Rcn1 is degraded and inhibited in its interaction with calcineurin [28].

1.5 Inhibitors of the eukaryotes-Bisindolylmaleimides

The group of bisindolylmaleimides is originally isolated from the culture of Nacardiopsis. They target several other signaling molecules apart from PKC in mammalian cells [33].

Targets of bisindolylmaleimides:

- Inhibition of PKC and/or MDR activity
- Modulation of Wnt signaling pathway
- β-Catenin stabilization
- Elimination of antiapoptotic elements (cFLIP)
- Activation of intrinsic apoptotic pathways [33]

The variety of targets of bisindolylmaleimides contributes to their possible potency to disable the resistance mechanism of *Candida albicans*. They could repress uncontrolled proliferation and restore the sensitivity to antifungal treatment. Bisindolylmaleimides are inhibitors of the catalytic domain, therefore directed either to the substrate site or to the ATP binding site. Their structure resembles the staurosporine indolocarbazole structure. Although the staurosporine are more potent, the bisindolylmaleimides show a greater selectivity for PKC isoforms [45].

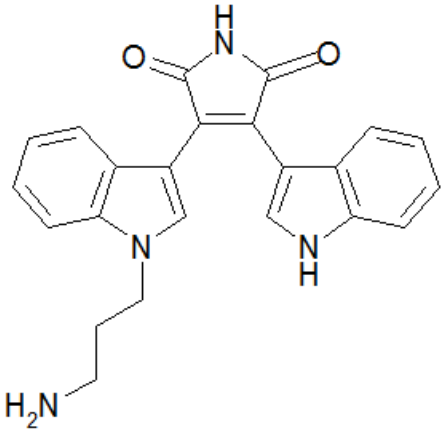


Figure 6. Structure of BisIII

2 Protein preparation

Many different proteins participate in the composition of a single cell. So the separation and isolation, or purification of proteins constitute an essential first step to further experimentation. Thereby it is important to avoid contamination to obtain a pure sample of the proteins of interest [41].

2.1 Cell disruption

The sample preparation for running on a gel implies the lysis of the cells to provoke the release of the proteins of interest. The cells are broken, releasing soluble proteins. This process also breaks many of the sub cellular organelles, such as mitochondria, peroxisomes, and endoplasmic reticulum [21]. There are three main methods for release of intracellular proteins from microorganisms: enzymic, chemical or physical [41]. Thereby diverse detergents, salts, and buffers could be used to encourage lysis of cells and to solubilize proteins. A protease inhibitor cocktail is often added to prevent the digestion of the sample by its own enzymes. The whole tissue preparation should be carried out at cold temperatures (4°C) to avoid protein denaturing and degradation.

2.2 Materials

- Bicinchoninic Acid Protein Assay Kit, SIGMA-ALDRICH
- Sterile Nuclon Surface- 96 well-plate, SIGMA-ALDRICH
- DensiCHECK™ plus, bio-Merieux
- Shaking Incubator SI500, stuart
- Centrifuge 5417C/R, Eppendorf
- Centrifuge BR 4i, Jovan
- MM400 MixerMill, Retsch

2.3 *Candida* cultivation

To assure a strict sterile condition in order to handle with cells, it was necessary to work under the laminar air flow. CA98001 cells were applied with a loop on the culture ground (Sabouraud Gentamicin Chloramphenicol 2-Agar) in a test tube, so that they could grow fast to a visible culture after 24h incubation at 37ºC. An inoculum from the grown *Candida albicans* culture was taken and immerged in a new YPD[8] medium. After an overnight stay in a shaking incubator at a temperature of 30ºC the amount of cells was sufficient for the following protein extraction.

2.3.1 *Candida* extraction

The nutrient medium containing the *Candida* cells was divided equally into two Falcon tubes. The tubes of 50ml were centrifuged for 5min at 3500 rpm at 4ºC. Only the obtained pellets were kept.

The first Falcon tube was treated with 10ml PBS 1X to transfer the liquid afterwards into the second Falcon tube. For this reason, only the second tube was centrifuged again for 5min at 3500rpm at 4ºC. The liquid was poured away and replaced by 10ml PBS 1X. Aliquots of 250µl were created in Eppendorf tubes (2ml), which were centrifuged for 5min at 5000rpm at 4ºC, thus the formed pellets were frozen quickly in liquid nitrogen. Thereupon 5 glass marbles were added to each tube before crushing cells for 6min at high speed. After the crushing an extraction buffer[11] containing M-PER, an Anti protease cocktail (PIC:M-PER…1:1000) and PMSF (PMSF:M-PER…1:100) was added, whereupon the chosen concentration depended on the volume of the pellet, and placed for a further centrifugation at 5000rpm in the apparatuses.

The supernatants were collected in one tube to separate them afterwards into aliquots of 300µl which were stored at -80ºC.

2.4 Recuperation of Henrietta Lack's Cells (HeLa)

The HeLa cells were cultivated on the nutrient media RPMI (+10% FBS) in a 75ml flask. Before all, RPMI media had to be eliminated with a bulb pipette under the flue and the culture flask was washed with enough PBS 1X (stored at 4ºC) and removed afterwards. 6ml of the PBS 1X were used to cover the protein ground. By using a scrapper the cells were collected while the cell flask remained on ice. The liquid containing PBS and cells was taken before another washing with PBS 1X. All the PBS containing the cells was collected accurately in a test tube, which was centrifuged at 15000rpm for 10min at 4ºC afterwards. The formed pellet was kept while getting rid of the PBS 1X. 1ml extraction buffer[11] was added to each 100µl of wet cell pellet. The mix was prepared in a tube and vortexed. The pellets were well washed with the extraction buffer. The tube containing the pellet was placed at 4ºC for half an hour and then centrifuged at 15000rpm at 4ºC. Aliquots were created and kept at -80ºC.

2.5 Protein quantification

To work in a reproducible way, it was necessary to quantify the amount of proteins that had been extracted. Although many assays are suited, the bicinchoninic acid protein assay kit (BCA) was chosen to check the proteins concentration of the cell-lysates.

2.5.1 Bicinchoninic Acid Protein Assay Kit

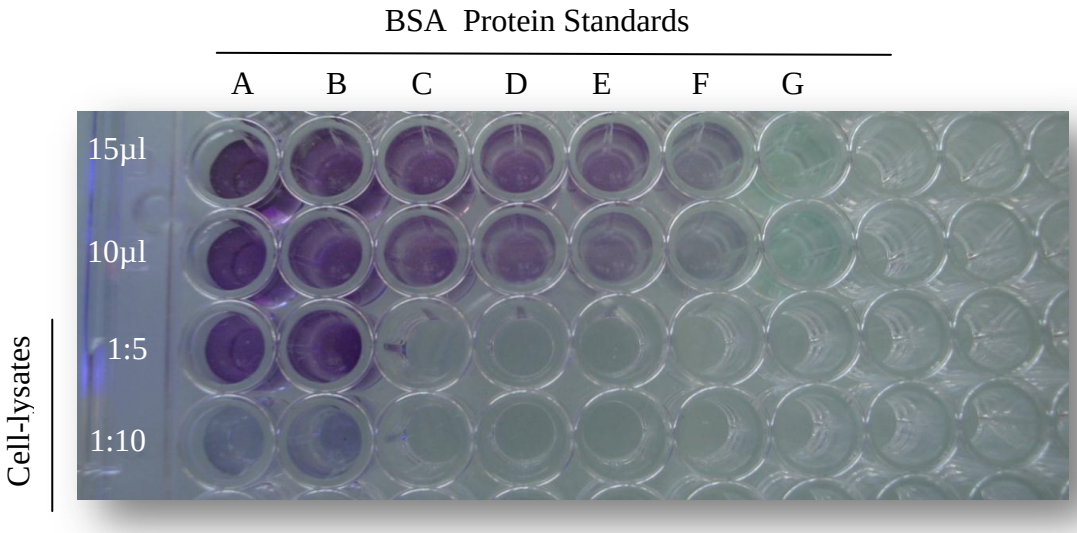
The principle of the bicinchoninic acid (BCA) assay is similar to the Lowry procedure, because both rely on the formation of a Cu^{2+} protein complex under alkaline conditions, followed by the reduction of the Cu^{2+} to Cu^{1+} [5]. The amount of reduction is proportional to the proteins present. It has been proved that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+} [6]. BCA forms a purple-blue complex with Cu^{1+} in alkine environment, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins [7].

2.5.2 Working Procedure

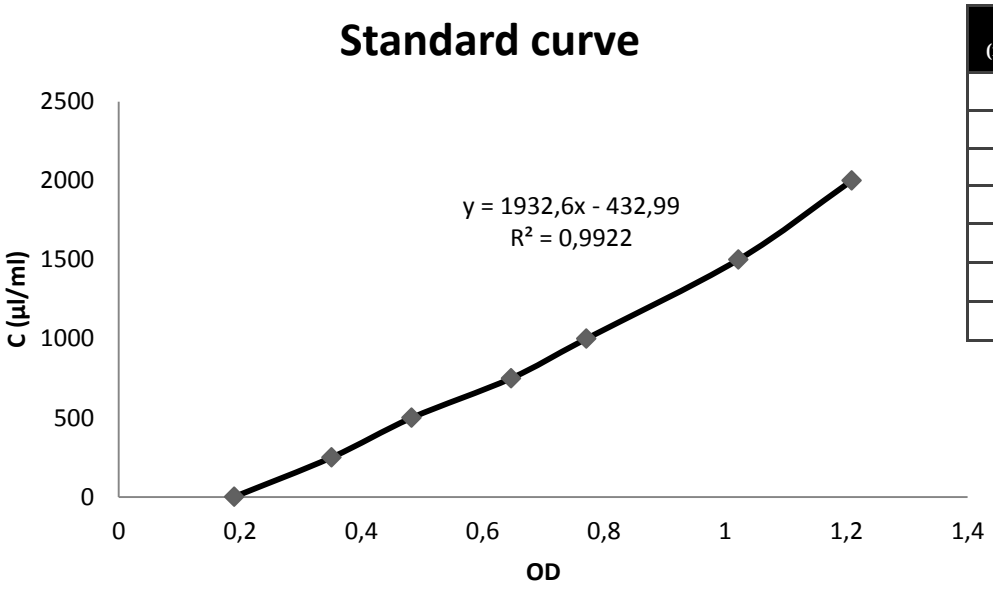
At the beginning BSA protein standards of different concentrations have to be prepared. This is accomplished by making serial dilutions.

	c	V (BSA)	V (H_20)
A.	2mg/ml	400µl	/
B.	1,5mg/ml	285µl	75µl
C.	1mg/ml	150µl	150µl
D.	0,75mg/ml	112,5µl	187,5µl
E.	0,5mg/ml	75µl	225µl
F.	0,25mg/ml	37,5µl	262,5µl
G.	0mg/ml	0	300µl

200µl of the BCA working reagent (containing Cooper Solution and acid solution in a ratio 1:50) are applied to 10µl and 15µl BSA protein standard and cell-lysate samples on a 96-well plate followed by an incubation for 30 min at 37ºC. The cell-lysates are previously diluted to achieve a more reproducible result.



The absorbance of the solution was measured at 570nm. Thereby the absorbance of the samples was compared to the standard curve so that the concentration of the unknown samples could be determined.



BSA (reference)	OD	C (µg/ml)
A	1,209	2000
B	1,022	1500
C	0,77	1000
D	0,647	750
E	0,483	500
F	0,351	250
G	0,190	0

3 Immunodetection of *Candida albicans'* signal proteins

3.1 Methods

3.1.1 Western Blotting

Immunoblotting or Western blotting is met with success as the technique, where proteins are transferred from an electrophoresis gel to a support membrane and probed with antibodies [4]. It is a powerful tool to detect and characterize a multitude of proteins.
The transferred proteins are immobilized on the surface of the membrane in a manner that is an exact replica of the gel. Unoccupied protein-binding sites on the membrane are saturated to prevent non specific binding of antibodies [4]. The blot is probed first with a specific primary antibody. Then the blot is probed a second time. The second probe is an antibody that is specific for the primary antibody type and is in addition conjugated to a detectable enzyme. The site of the protein of interest is thus tagged with an enzyme through the specifities of both antibodies [4].

3.1.2 Gel electrophoresis

"The popularity of gel electrophoresis is based on its high resolving power, its ability to analyze many samples simultaneously, the micrograms amount of proteins that it requires, the possibility it offers to recover the proteins after electrophoresis, and the modest cost of the method [19]."

Gel electrophoresis can provide information about the molecular weights and charge of proteins, the subunit structures of proteins and the purity of a particular protein preparation. It is relatively simple to use and it is highly reproducible. Polypeptides differing in molecular weight by as little as a few hundreds of Daltons and proteins differing by less than 0.1 pH unit in their isoelectrical points are routinely resolved in gels [4].

3.1.3 SDS Page [4]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly practiced gel electrophoresis technique used for proteins. It requires the denaturation of proteins to their consistent polypeptide chains.

When SDS is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. SDS denatures proteins by wrapping around the polypeptide backbone and SDS binds to protein fairly specifically in a mass ratio 1:4:1. It is usually necessary to reduce disulphite bridges in proteins before they adopt the random-coil configuration necessary for separation by size by using β-mercaptoethanol or dithiothreitol (DTT). In denaturizing SDS-PAGE separation, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

Buffers supply current carrying ions, maintain desired pH and provide a medium for heat dissipation. Continuous systems use the same buffer at constant pH, in the gel, sample, and electrode tanks. With continuous systems, the sample is loaded directly on the gel in which separation will occur.

3.1.4 Polyacrylamide-gels

Polyacrylamide-gels are formed from the polymerization of two compounds, acrylamide and N, N-methylene bisacrylamide (Bis). Bis is a cross-linking agent for the gels. The polymerization is initiated by addition of ammonium persulfate along with either DMAP or TEMED. The gels are neutral, hydrophilic, three dimensional networks of long hydrocarbons, crosslinked by methylene groups. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors, the total amount of acrylamide present and the amount of cross linker. As the total amount of acrylamide increases, the pore decreases.

The smaller the size of the protein of interest is, the higher is the percentage of mono/bis. The bigger is the size of the protein of interest is, the lower is the percentage of mono/bis.

Protein size (kDa)	*Gel percentage (%)*
4-40	***20***
12-45	***15***
10-70	***12,5***
15-100	***10***
25-200	***8***

3.2 Materials

- Block Heater, Grant
- Mini Protean® Comb, BIO-RAD
- Mini Protean Tetra System, BIO-RAD
- Power Pac-Basic, BIO-RAD
- Minipulse3, Gilson
- Yellow Squid Magnetic stirrer, IKA
- Optitran reinforced Nitrocellulose Transfer Membranes-45µm, Whatmann
- Trans Blot SD/ Semi-dry Transfer Cell, BIO-RAD
- Mini Protean 3-Cell Serial No 675, BIO-RAD
- Extra Thick-Blot Paper, xi size, BIO-RAD
- Gyro Rocker SSL3, Stuart
- Labroller Rotator, Labnet
- Minilab Roller Rotator, Labnet
- G Box, Syngene

3.3 Working Conditions

A continuous buffer-, denaturing-, one dimensional- SDS gel was considered as the best way to let the proteins migrate.

First of all the running gel solution had to be prepared. Since the concentration of the acrylamide gel influences the running condition, the percentages of 12% and 15% acrylamide were chosen. The gel volume had to be determined by calculation or measuring the amount of acrylamide gel needed to fill a cassette.

	Gel ***6%***	Gel ***10%***	Gel ***12%***	Gel ***15%***
H_20	8ml	5,9ml	5ml	3,5ml
30% Acrylamide-mix	3ml	5ml	6ml	7,5ml
1,5M Tris HCl (pH=8,8)	3,8ml	3,8ml	3,8ml	3,8ml
10% SDS	0,15ml	0,15ml	0,15ml	0,15ml
10% APS	0,15ml	0,15ml	0,15ml	0,15ml
TEMED	0,012ml	0,006ml	0,006ml	0,006ml

The components were mixed together and after adding TEMED and APS the gel would polymerize fairly quickly. Therefore, the transfer of the solution to the gel cassette should be carried out as fast as possible.

Before applying the acrylamide between the glass plates of the gel electrophoresis instrument, the apparatus should be assembled together. Ethanol was added to assure an uniform surface and then the gel was allowed to polymerize for about 20 min. After the polymerization the components of the stacking gel[10] were prepared. The solution was swirled gently and transferred to the top of the resolving gels using a pipette and bulb, after having eliminated the ethanol. Then the combs were aligned to its proper position by being careful not to trap bubbles under the teeth. The gel was allowed to polymerize for another 15min.
Electrophoresis cells are essentially plastic boxes with anode and cathode buffer compartments, electrodes, and jacks for making electrical contact with the electrodes. Gels are held vertically between the electrode chambers during the run. The bottom is sealed with a gasket during gel formation and the top is open to receive monomer solution. The top and

bottom ends are open and in contact with buffer for electrophoresis. High voltage direct current supplies provide electrical power for electrophoresis [4].

Meanwhile the protein samples are prepared after the following table:

Tube	Celltype	Treated with	Nber of depots	Final Volume [µl]	LB4X [µl]	Prot Sample [µl]	H_20 [µl]
1	HeLa	MPER	2	60	15	25,27	19,73
2	Candida	MPER	2	60	15	22,33	22,67

For an experiment the protein concentration of each celltype should account 40µg, which was calculated on the basis of the results obtained after the protein quantification.
In each tube the proteins LB4X[2] and H_2O were mixed together and vortexed afterwards. The samples were heated at 95ºC for 5min in a temperature block. Then they were centrifuged with 3000rpm for 3min at 4ºC.
Afterwards the rest of the electrophoresis apparatus was assembled together and as soon as the polymerization was finished, the combs were removed carefully and the gel assembly was placed into the buffer tank. The reservoir was then ready to be filled with migration buffer[11] before the samples were loaded carefully in the wells of the gels with a pipette. The tank was closed with the lid and connected to the power supply. The power supply was turned on and set for following electrical conditions: 100V for 1h, and then increased slowly at 130V for 50min.
By the time the migration of a 15% gel was set up, the apparatus was placed onto ice to provide optimal conditions. The run was then proceeded until the blue tracking dye from the sample buffer reached the end of the gel. At the end of the run the gel was removed, ready to be transferred.

3.4 Gradient Gel

During electrophoresis in gradient gels, proteins migrate until the decreasing pore size inhibits further process. The main advantages of gradient gels outclass linear gels:

1. The line of the migration protein zone is retarded more than the trailing line, thus resulting in a sharpening of proteins bands.
2. The gradient in pore size increases the regions of molecular weights that can be fractionated in a single run.
3. Proteins with close molecular weights are more likely to separate in a gradient gel than in a linear gel [9].

The usual limits of gradient gels are 3-30% acrylamide in linear or concave gradients. The choice of range depends on the size of proteins being fractionated [9].

The gradient gel advanced the resolution of our membranes in an unexpected way. Before preparing a gradient gel, the choice of the percentage had been taken. We went for the acrylamid percentage from 6%-15%. So firstly the high percentage polyacrylamide solution (15%) was loaded into the first chamber (B) of the gradient maker. The low percentage polyacrylamide solution (6%) was rapidly loaded afterwards into the second chamber (A) (Figure 7).

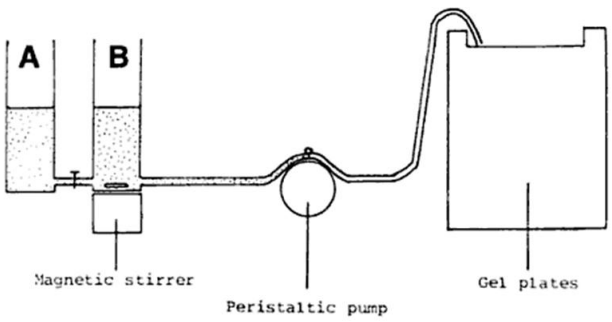


Figure 7. Gradient gel apparatus [WALKER, 1994]

The flow on the pump was initiated (25rad/min); simultaneously the magnetic stirrer was turned on. The second chamber was opened after the high percentage of the polyacrylamide solution made 1cm of the gel. The pump is stopped when the solution was approximately 3 to 4cm from the top of the glass plate. After adding the stacking, the gradient gel was ready for the migration.

3.5 Transfer

Electro transfer from a gel to a membrane is done by directing an electric field across the thickness of the gel to drive proteins out of the gel and onto the membrane. Generally electrophoretic methods assure the recovery of the targeted proteins at high purity [4].

3.5.1 Semi-dry blotting/Wet blotting

In semi-dry blotting, the gel and membrane are sandwiched horizontally between two stacks of buffered wetted filter papers in direct contact with two closely spaced solid plate electrodes. The close spacing of the semi-dry apparatus provides strengths for high fields. The term semi-dry refers to the amount of buffer that is confined to the stacks of filter paper. Under semi-dry electro transfer conditions, some low molecular weight proteins are driven trough the membranes [4].

The structure of a wet blot takes place according to the sandwich procedure, although the important filling is not jammed between two slices of bread, but two plastic grids of a gel cassette. [10].

3.5.2 Membrane

The most common membranes are constituted by nitrocellulose and PVDF. We worked only with nitrocellulose since the proteins are more likely to be on this kind of membrane. Nitrocellulose membranes are available in different pore sizes. The correct pore size depends on the size of the protein to be blotted. The blotting success for smaller proteins deteriorates with increasing pore size [10].We used a nitrocellulose membrane with a pore size of 45µm.

The capacity of nitrocellulose to bind and retain proteins ranges from 80µg/cm^2 to 250µg/cm^2, depending on the protein. Proteins bind to the nitrocellulose chiefly by hydrophobic interactions, although hydrogen bonding between amino acid side chains and the nitro group of the membrane may also be involved [11]. In most cases it makes no difference which blotting procedure is used. The advantages of a wet blot lie in the gentler transfer and the lower heat that is thus applied to the protein. The disadvantages are higher expenditure of time and the larger amount of transfer buffer that is needed for the wet transfer [10].

The selected blotting membrane, as well as six to eight filter papers (Whatmann Paper) had to be cut to gel size. This should be done fairly accurately, since overhanging filter papers and edges of membranes could cause a short circuit during blotting [10].

3.5.3 Transfer-conditions of *Candida albicans'* proteins

After the migration, the stacking gel was removed and the running gel was placed into transfer buffer[5, 9] for 20min. Meanwhile the nitrocellulose membrane was cut to the size of the gel (8x6cm) and immersed in the transfer buffer for 15min whereas the Whatman filter was only dipped into it. Then the transfer sandwich was assembled. A roller was used to roll over the sandwich gently to remove trapped air bubbles.

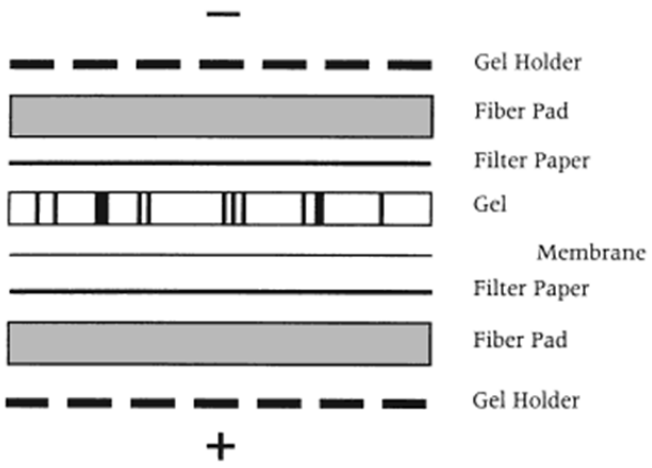


Figure 8. Gel/Filter Sandwich [ROSENBERG,2006]

In the classic transfer the gel/filter sandwich was put into the holder, so that the proteins could migrate from the gel to the membrane, in the direction of the positive electrode (anode). The whole apparatus was filled with the transfer buffer[5] and put on ice.

But the semidry transfer worked without filling a tank with buffer, placing the sandwich directly in the apparatus, only applying a few milliliters transfer buffer[9] on top of the sandwich to avoid drying out during membrane transfer.The transfer duration depended on the proteins which were going to be detected in the next step.

Timetable:

Transfer time-Semidry	Transfer time-Classic
1h…..…10V + 15min…….13V	2h…….25V

The greater the mass of the protein the more time was needed for the transfer, so the whole time was utilized when PKCη, Hsp90 and GSK3α were going to be detected later. In contrast the transfer time was reduced when the attention was turned to the Histones.

3.6 Ponceau S

Ponceau S (PS) is a negative stain that binds to the positively charged amino groups of the proteins. It also binds to non-polar regions in the protein [16]. Protein binding by this stain is readily reversible by raising the pH [17]. Ponceau S staining of blots can sometimes produce high background in the subsequent blot. This method of reversible staining of the blot gives a confidence that the migration was good and that the transfer was uniform across the membrane [15]. But the detection limit is 250ng/ml. The red color of stained proteins is difficult to photograph and the stain fades with time [17].

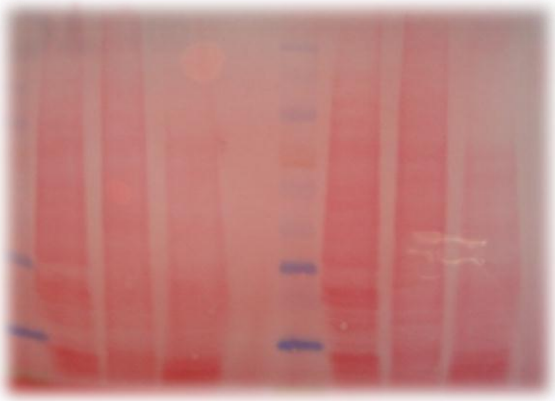


Figure 9. Ponceau S coloration

Before applying Ponceau S, the membrane was washed with PBST. A small plastic box was used for the incubation with the Ponceau Solution[7]. After two minutes small protein bands

were observable as the Ponceau S had attached to the proteins. To decolorate the membrane, it was again washed with PBST while gentle agitating on a shaking platform.

3.7 The role of Coomassie Brilliant Blue

It is more common to make protein bands in gel visible by staining them with dyes or metals. Each type of protein stain has its own characteristics and limitations regarding the sensitivity of detection and the types of proteins that take up the stain best [4]. Coomassie Brilliant Blue is the standard stain for protein detection in polyacrylamide gels. They need only to be covered with stain [4]. It is sensitive approximately down to 20µg protein per ml.

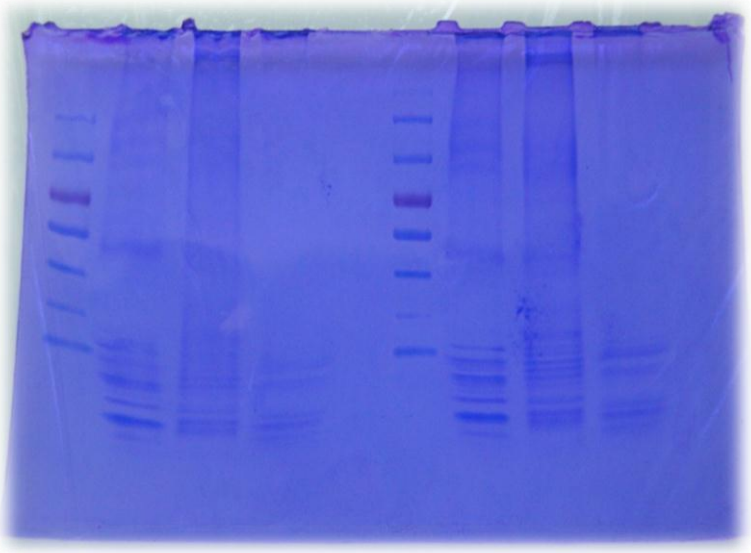


Figure 10. Gradient gel (6%-15%) after Coomassie Brilliant Blue Staining

Coomassie Brilliant Blue is an aminotriarylmethane dye that forms strong but not covalent complexes with proteins, most probably by a combination of Van der Waals forces and electrostatic interactions with NH_3^+ groups. The uptake of dye is approximately proportional to the amount of proteins, following the Beer-Lambert law [14]. Coomasssie Brilliant Blue R-250 is more soluble than the G-250 variety. In acidic solution it doesn't form colloidal particles that are too large to penetrate surface gel pores [4].

After the transfer the gel was immersed in at least 5ml staining solution[4] containing Coomassie Brilliant Blue R-250 and placed in a small plastic box on a slowly shaking

platform for 1h at room temperature. The stain was removed and saved for future use. The gel was destained without the dye by soaking it in an ethanol/acetic acid solution[3] on the shaker overnight, changing the destaining solution three or four times.

3.8 Blocking

Since not only proteins from the gel bind to the membrane but other proteins (e.g. detection antibodies) like to settle there as well, before the beginning of the actual detection reaction it is absolutely necessary to block the nonspecific protein binding sites. This happens through incubating the membrane with irrelevant proteins, which cover the non specific binding sites (block), so that the detection is reduced, and thus the sensitivity of the Western Blot is increased [10]. To minimize the loss of weakly bound proteins during blocking, a whole range of different solutions is available. Different variations of protein solutions (cattle serum albumin, horse serum) or anionic detergents (Tween 20) are often used [4].
Sometimes they are mixed. The second purpose is less known and perhaps even less understood, but blocking membrane can promote renaturation of antigenic sites [15]. Nonetheless it is crucial that the solution doesn't disturb the subsequent detection reaction.

Working conditions

The membrane was blocked in either 5% BSA in TBST or 5% Milk in TBST, placed on the shaker for at least 30 min, preferably 1-2h at room temperature. Meanwhile the primary antibody could be already prepared.

3.9 Detection [10]

The actual detection of the relevant protein on the membrane is reached by staining of antibodies. A suitable specific antibody serves as the tool for the immune detection. Appropriate primary antibodies can be produced in any convenient animals, such as rabbits or mice or goat .
After the binding of an antigen- specific, unlabelled primary antibody, detection with a second, species specific, labeled antibody called secondary antibody happens. It binds to the

constant part of the primary antibody that is bound to the protein. Enzymes, fluorescent dyes, or even radioactive markers can be coupled to this secondary antibody. It does have advantages compared to direct staining, in which the primary antibody is conjugated directly with the detection reagent. The fact that several secondary antibodies can simultaneously bind to the constant part of the primary antibody results in an amplification effect which increases the sensitivity .The detection reagent that is conjugated to the secondary antibody determines the type of visualization of the antibody binding to the protein. The most common secondary antibodies used in Western blotting are alkaline phosphatase and horseradish peroxydase. The coupled enzymes of alkaline phosphatase or horseradish peroxidase catalyze the conversion of an appropriately added substrate to a colored or luminescent product in the detection reaction. An advantage of this is that the enzyme can convert many substrate molecules, which results in an amplification of the signal .

Because the antigen is immobilized on a sheet, the antibody is not required to form a precipitate with the antigen. The blotting technique therefore has the potential for immunoelectrophoretic analysis of proteins by using binding of Fab fragments or binding of antibodies against a single determinant, such as monoclonal antibodies produced by hybridomas [6].

3.9.1 Luminol Reaction

Chemiluminescent substrates for horseradish peroxidase are based on oxidation of luminol. The luminol substrate provides the most sensitive signal of the blotting substrates but requires photographic exposures or specially configured imaging devices [4]. The principle is that when a substrate such as 5-amino 2,3 dihydro-1,4-phthalazinedione (luminol) is oxidized by hydrogen peroxide, light is released during the chemical reaction. The substrate is thus a limiting reagent and light production ends as the substrate is oxidized [15].

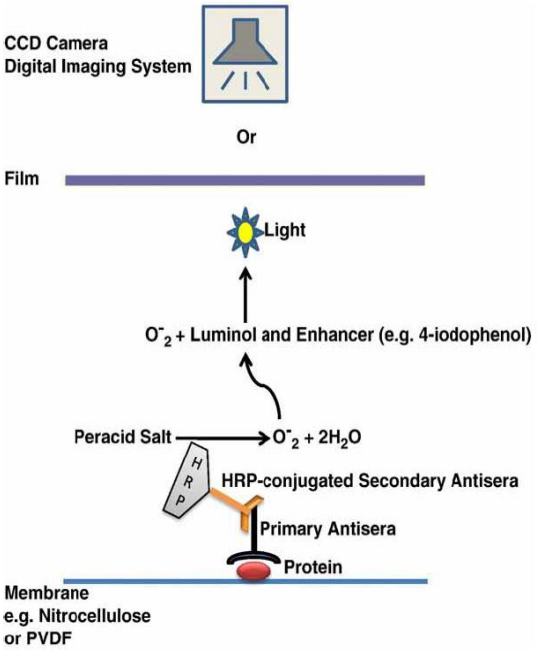


Figure 11. Visualization Reaction [MACPHEE, 2010]

3.9.2 Polyclonal vs. monoclonal

Both monoclonal and polyclonal antisera can be utilized for Western blot analyses. There are advantages and disadvantages in using either type.

An advantage of polyclonal antisera is that it can possess many antibodies that are able to detect the protein of interest. It results generally in an enhanced signal. In contrast, a major disadvantage is that it can contain antibody molecules that can bind specifically to proteins unrelated to the protein of interest. This effect can be explained by their way of production. Indeed they are obtained by immunizing animals with a synthetic peptide derived from the sequence of the Human protein. The unspecific interaction can be abolished by affinity purification [15].

Monoclonal antisera provide by their design which is directed against one epitope, an elegant tool for identifying a particular region of the antigen [18]. One particular disadvantage can arise, if the specific antigenic site is significantly affected (i.e. denatured) by electrophoresis conditions, thus limiting or preventing antisera interaction [15].

The most important ability that an antibody must have to work successfully in an immunoblot is to recognize denaturation-resistant epitopes. The epitopes that are displayed on the membrane will almost exclusively be those that are formed by linear stretches or amino acid modifications. These are the types of epitopes that remain intact following gel electrophoresis and transfer to the membrane support [18].

3.9.3 Whole Sera

Because polyclonal antibodies normally are used as whole sera, they also contain an entire repertoire of circulating antibodies found in the immunized animal at the time they were collected. Whole sera contain a wide range of antibodies that comprise the entire repertoire of antibody response presented in the animal at the time of serum harvest. This may include any auto antibodies against cellular components and antibodies against microorganisms that have infected the host animal, either at the time of collection or at any recent time, that have left circulating antibodies, or other antigens that are contaminating the immunizing preparation. These antibodies bind to the cognate, or any closely related, antigens on the blot, generating additional bands and potentially obscuring the bands of interest. Therefore, the serum may contain antibodies that specifically recognize spurious antigens [18].

Indeed the problem of whole polyclonal antibody sera can also be solved by affinity purification.

3.9.4 Titrate

The spurious bands caused by the unspecific antibodies in whole sera can be lessened by using the antibody at much lower concentrations. Then the immune antibodies have the highest concentration in serum, and thus titrating can lower the levels of the contaminating antibodies to levels that will not interfere with the analysis. Therefore dilution can be used to reduce nonspecific background problems without reducing sensitivity [18].

3.10 Visualization

The blot was soaked in a tray containing 50ml of blocking buffer (2,5g BSA dissolved in 50ml TBST) for 30min at room temperature while shaking. Afterwards the blocked membrane was incubated with 5ml of the primary antibody of interest on a rotating scroller at 4ºC overnight.

Primary Antibodies			
PKCη-Antibody	rabbit	polyclonal	whole sera
Hsp90-Antibody	rabbit	polyclonal	purified
Gsk30-Antibody	rabbit	polyclonal	purified
HistoneH3-Antibody	rabbit	polyclonal	purified

The next day the membrane was washed with TBST, the first time 15min and then 3 times for 5 min while gently agitating at room temperature. Then the incubation followed with the secondary antibody (Anti-rabbit IgG, HRP-linked Antibody) for 1h at room temperature and for another hour at 4ºC. Before visualization with the G-Box, the blot was again washed with TBST (1*15min, 3*15min). Then the immunoblots were incubated with a solution called Super Signal West Pico Chemiluminescent Substrate. Therefore two different solutions were

mixed equally together (SuperSignal West Pico Stable Peroxide Solution/ SuperSignal West Pico Luminol Enhancer Solution).

The mix was used to incubate the membrane for 5min. The apparatus G-Box took pictures of the picochemiluminescent signals on the membrane.

Figure 12. G-BOX

3.11 Stripping

The practice of stripping immunoblots and reprobing provides the detection of multiple proteins on the same blot. This is a good method to examine two antigens under identical conditions [18].

The important step that must be undertaken with any reagent to ensure proper "erasure", is to re-incubate the stripped blot in substrate and expose to film to ensure no protein detection occurs as a result of remaining antisera. Then the blots can be washed and archived or reblocked for additional blot analyses [15]. The bonds that hold antigens to nitrocellulose membranes are noncovalent, and all methods to strip the antibodies from the antigens also remove some of the polypeptides from the filter. Therefore this method is never quantitative.

Practical Parameters

The possibility to reuse the membrane with the stripping method shortened the working time. We were able to utilize the same membrane with different antibodies. Therefore the stripping solution had been heated on a magnetic stirrer. The membranes were incubated in the hot stripping solution for 30min and neutralized afterwards with Tris-buffer for another half an hour. Before blocking, they were washed with TBST as usual.
Now the wished proteins could be detected after incubation with the primary and secondary antibodies.

3.12 Antibody validation for *Candida albicans*

The first step to get a better understanding of *Candida albicans'* pathogenicity was to clear up their different protein expression since they have a contribution to the resistance of *Candida albicans* to antifungal treatment. The first step to investigate their influence implied their ways of expression, which we compared with the proteins of HeLa. The Hela proteins were just used as a reference.

3.12.1 Different ways of detection of the PKCη signal

Before we could use the proteins for the Western Blotting, they had to be extracted and prepared in the right way. The fragile work with the *Candida albicans*' cells led to different results. We accorded the right choice of lysis buffer to have a major contribution to the different results. A difference between the used MPER- and Chilov-lysis buffer efficacy could be distinguished because the signals of Chilov treated proteins were paler. The first two signals of Ca/MPER and Ca/Chilov were not even visible. The results showed that the chosen lysis buffer has finally an effect on the results. The lysis buffer MPER worked significantly better than the lysis buffer Chilov with which we treated our cells at the protein extraction step.

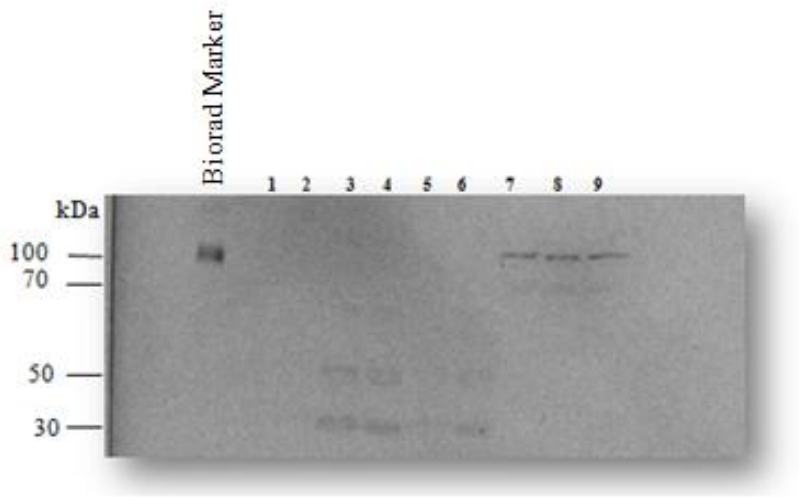


Figure 13.1. PKCη spot lightning after migration on a 12% acrylamide gel. *1. Candida alb. treated with MPER, 2. Candida alb. treated with Chilov, 3. Candida alb. treated with MPER and Chilov, 4. Second Candida alb. culture treated with MPER, 5. Second Candida alb. culture treated with Chilov, 6. Second Candida alb culture treated with Chilov and MPER, 7. HeLa treated with MPER, 8. HeLa treated with Chilov, 9. HeLa treated with MPER and Chilov*

This time the proteins had migrated on a 12% gel and were transferred then on the nitrocellulose membrane by the semidry method. The signal of PKCη had a long time coming on this picture since an admissible signal was remarkable after half an hour. The signals of *Candida albicans* are lower than the human HeLa signal.

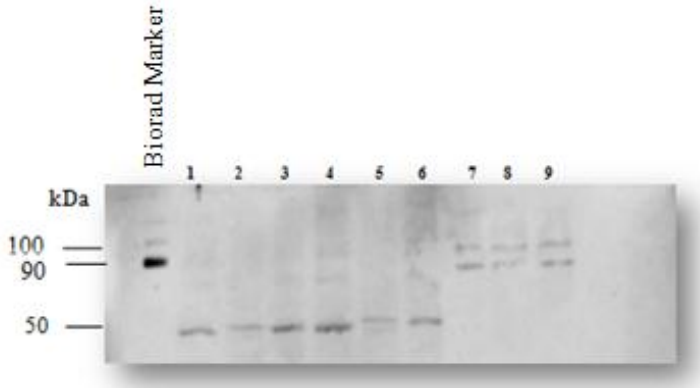


Figure 13.2. PKCη detection on a membrane transferred from a 15%gel. *1. Candida alb. treated with MPER, 2. Candida alb. treated with Chilov, 3. Candida alb. treated with MPER and Chilov, 4. Second Candida alb. culture treated with MPER, 5. Second Candida alb. culture treated with Chilov, 6. Second Candida alb. culture treated with Chilov and MPER, 7. HeLa treated with MPER, 8. HeLa treated with Chilov, 9. HeLa treated with MPER and Chilov*

The percentage of the acrylamid gel had been increased from 12% to 15%. So this change had visible effects on our result. The last time two bands of *Candida* were visible whereas this picture showed only one signal for *Candida* at 50kDa. The HeLa spots were similar to the last gel. They could be found at approxiametly 100kDa, always above the *Candida albicans'* signal. Unlike the last time PKCη already delivered a visible signal during the first minutes. The picture shows very clear, that with MPER/Chilov and even MPER alone, the signal was enhanced.

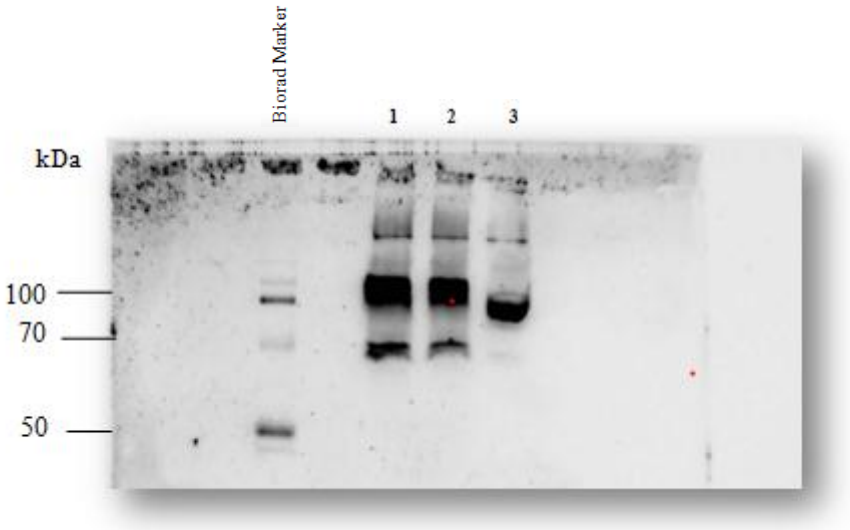


Figure 13.3. PKCη detection after the classic transfer. *1. Candida alb. treated with MPER, 2. Candida alb. treated with PMA250 (inactivated), 3. HeLa treated with MPER*

The picture above shows the membrane which was obtained after the classic transfer from a 12% gel. The semidry transfer had been replaced by the classic transfer regarding to enhance the expression of the spots. Unlike the results of the semidry transfer, the spots were now stronger expressed. Yet the characteristics of the PKCη signals differed from the signals obtained after the semidry transfer of a 12% gel. The *Candida* spots were expressed at 70kDa and 100kDa not at 50kDa. While the HeLa signal was usually above the *Candida* signal, this picture shows an inverse situation. Summing up, the modification of the transfer leads to a change in detection.

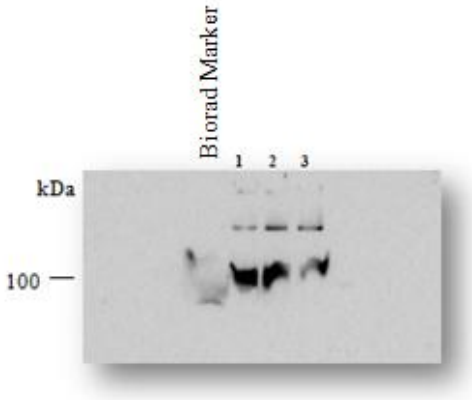


Figure 13.4. PKCη detection after the classic transfer from a 15% acrylamide gel. *1. Candida alb. treated with MPER, 2. Candida alb. treated with PMA250 (inactivated), 3. HeLa treated with MPER*

In this experiment the percentage of the acrylamide composition was increased from 12% to 15%. Even though references preferred a 12% acrylamide gel, the aim was to see Histones (17kDa) and PKCη on the same gel. The higher percentage should avoid the running out of the small proteins during the gel migration. However the results indicated an amelioration of our working parameters. The spots resembled a smile, which pointed out to too hot or too fast migration. In fact, the migration of a 15% gel heated the whole tank due to a stronger resistance, which occured as soon as a higher percentage of acrylamide was chosen.

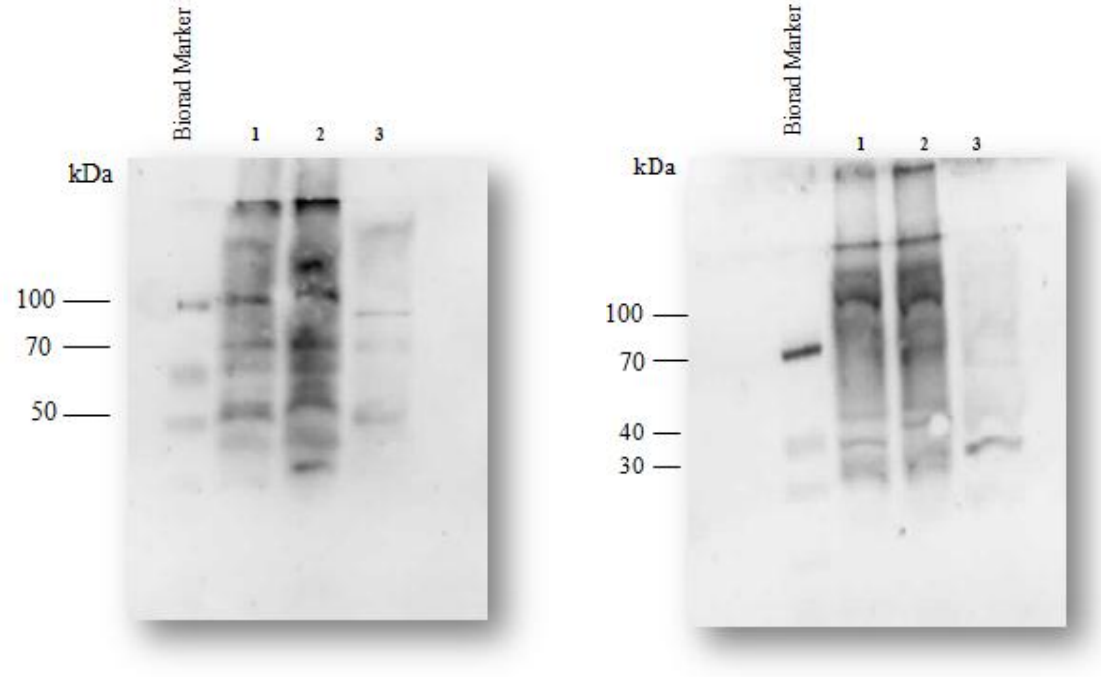
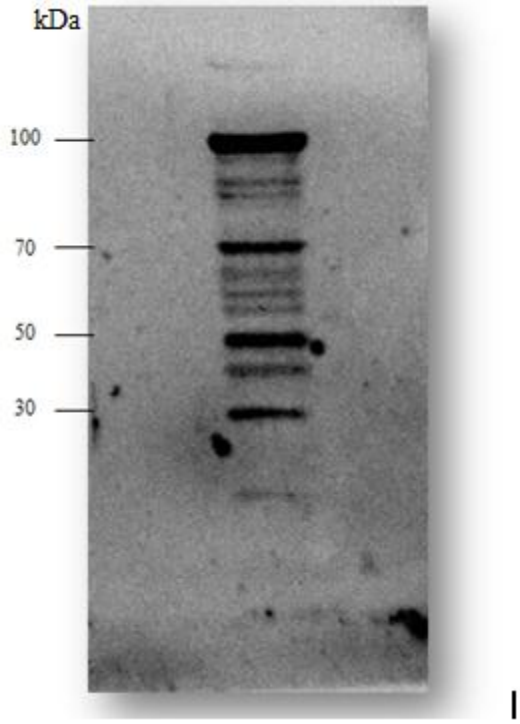


Figure 13.5. PKCη detection after transfer of a 12%gel and after transfer of a 15%gel. *1. Candida alb. treated with MPER, 2. Canida alb. treated with PMA250 (inactivated) 3. HeLa treated with MPER*

PKCη was on this picture transferred from a 12% acrylamide gel. There was a strong difference between the classic and semidry transfer. While the semidry transfer didn't result in any signal, the classic transfer made more than a spot visible. The modification of the transfer method should lead to an optimized detection, as the suspicion rised up that the semidry transfer wasn't the best solution of transferring the proteins to the membrane. The two different methods compared, the semidry version led to less detection.

The difference between the classic and semidry transfer had also been studied using a 15% gel. The result didn't differ from those of the 12% gel, since the semidry transfer also didn't result in any spots. The signals were strong, at the first regard very unspecific, but three spots stood out from the others as they were stronger expressed. The spots at 100kDa, 70kDa and 50kDa could be related to those of the PKCη.

We tried out different percentages of acrylamide to see the difference in the way of detection. As the high molecular proteins were more likely to be detected with a low percentage of acrylamide gel than the low molecular proteins with a high percentage gel, we decided to combine two different percentages. The mixing up of two different percentages could be carried out by preparing a gradient gel.



Actually we had a higher resolution using a gradient gel. The PKCη signal was detected in evidence. Thereby the quantities of dots could be distinguished at different kDa levels. As already detected the majors spots were at 100kDa, 70kDa and 50kDa. So the gradient gel resulted finally in a very clear image.

Figure 13.6. PKCη detection on a gradient gel using a Candida albicans culture

The Histones H3 had been detected successfully at 17kDa. A 15% gel was used to reach a higher expression of the Histones. In this experiment the transfer conditions were changed, then the membrane was kept overnight at 5V. The Histones act as references for the quantity measurement. They should give an idea for the intensity in which the proteins of interest are detected.

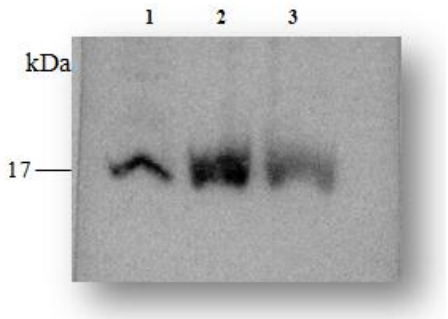


Figure 13.7. Histones H3 *1. Candida alb. treated with M-PER, 2. Candida alb. treated with PMA250 (inactivated) 3. HeLa treated with M-PER*

That's why we decided to use a gradient gel as we wanted to detect the proteins of interest and Histones on the same membrane after stripping.

3.12.2 Making Hsp90 visible

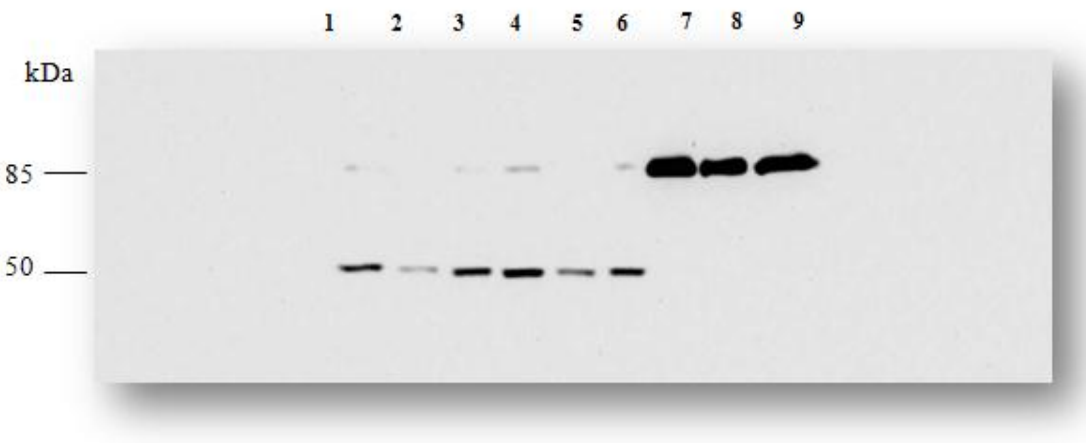


Figure 13.8. Hsp90 lightning on 12% gel. *1. Candida alb. treated with M-PER, 2. Canida alb. treated with Chilov, 3. Candida alb. treated with M-PER and Chilov, 4. Second Candida alb. culture treated with M-PER, 5. Second Candida alb. culture treated with Chilov, 6. Second Candida alb. culture treated with Chilov and M-PER, 7. HeLa treated with M-PER, 8. HeLa treated with Chilov, 9. HeLa treated with M-PER and Chilov*

This time the transfer was carried out in a semidry manner from a 12% acrylamide gel. The revelation showed many spots differing in their intensity. The signal from the human HeLa cells distinguished in their strongness from the other dots while the *Candida albicans*' cells expressed two dots, one at 90kDa at the same kDa level as the HeLa signal, and the others

much lower at 50kDa. This effect could be caused by the primary antibody, which detects endogenous levels of the total Hsp90 protein.

References explain the effect of the fragment at 47kDa as the result of the protease ycaB, which is typical in *Candida albicans*. The eliciting antigen appears to be part of the C-terminal portion of *Candida albicans* Hsp90 [21].

3.12.3 Femtoluminescence detects Gsk3α

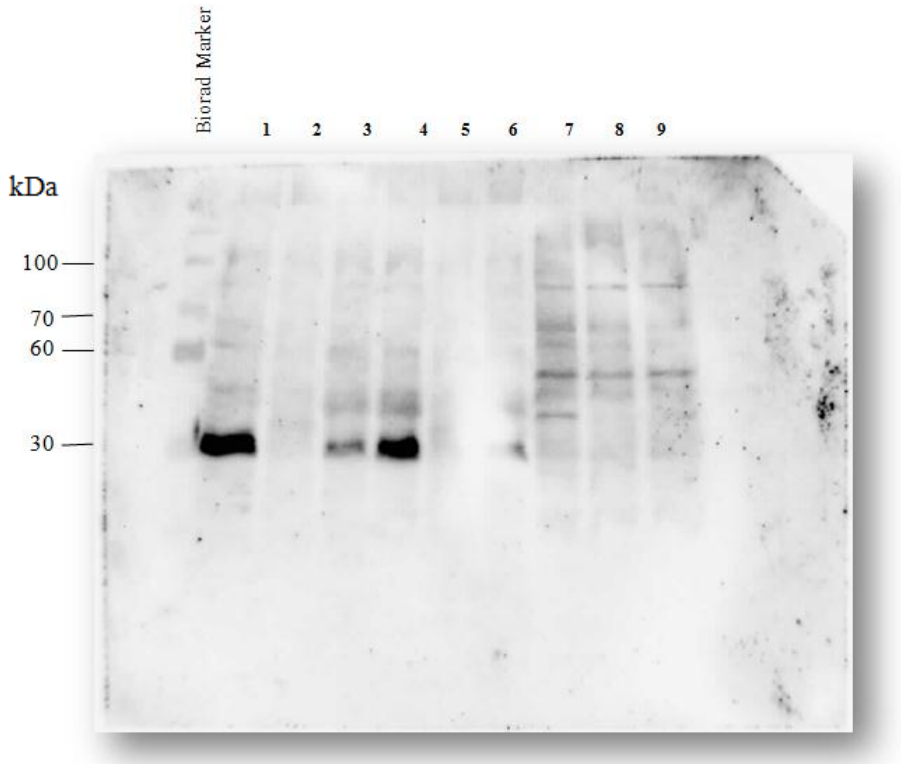


Figure 13.9. GSK3α detected on a 12%gel. *1. Candida alb .treated with M-PER, 2. Candida alb. treated with Chilov, 3. Candida alb. treated with M-PER and Chilov, 4. Second Candida alb. culture treated with M-PER, 5. Second Candida alb. culture treated with Chilov, 6. Second Candida alb. culture. treated with Chilov and M-PER, 7. HeLa treated with M-PER, 8. HeLa treated with Chilov, 9. HeLa treated with M-PER and Chilov*

The protein GSK3α was very difficult to detect, because its concentration was too low to give an adequate signal with picoluminescence. So the idea occurred to reveal the membrane under femtoluminescence. This method was sensible enough to make the dots of GSK3α visible.

3.13 Discussion

Western blotting had offered us the possibility to get an insight in the protein world of *Candida albicans*. To get the best resolution, many difficulties had been resolved.
One obstacle presented the detection of small molecules on one gel. As the semidry transfer was used as the unique possibility to drive proteins out of the gel and on the membrane at the beginning, the small molecules (e.g. Histones) weren't recovered. It is well known that under semidry electro transfer conditions some low molecular weight proteins are driven through the membranes. So they got lost on the way, finally ending up in no detection. With the change of the transfer method, the small proteins had been rescued. Through the classic transfer you can see the proteins of interest. Furthermore, the spots of PKC and Hsp90 proteins were enhanced, being stronger detected with this classical transfer method. The modification of the transfer led to a change in detection, modifying the number of spots.
So the transfer problem was solved. As the low weight molecules were detectable, the problem occured to see both, high weight molecules and low weight molecules on one gel. Thus the stripping method was brought in to solve this problem. The stripping solution facilitated the detection of the proteins on the same gel but not at the same time.
The highest possible resolution was targeted, what was finally realized with the gradient gel.
So there wasn't any obstacle remaining to step forward to the essential work with the affinity micro assay.

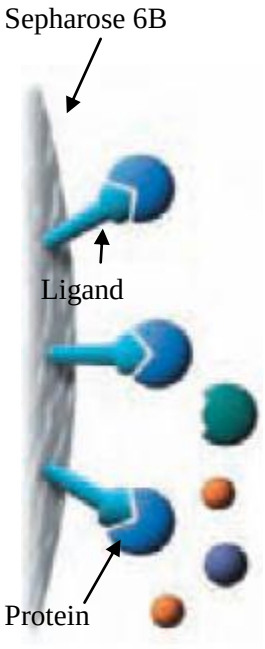
4 Affinity micro assay

The affinity micro assay uses the principles of an affinity chromatography; but in smaller dimension. As an efficient proteomic approach it allows to discover the true selectivity of an inhibitor. The small quantities which are required for the in vitro experiment render him attractive as a preparative method for the affinity chromatography. It concentrates its action only on the proteins of interest.

4.1 Technique [8]

Like the affinity chromatography it uses the reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. The technique scores with high selectivity for the proteins of interest.

It is unique in purification technology since it is the only technique that enhances the purification of a molecule on the basis of its biological function or individual chemical structure. The technique affords the separation of active biomolecules from denaturized or functionally different forms, the isolation of pure substances at low concentration in large volumes of samples and also the removal of specific contaminants.



Successful affinity purification uses a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand has to retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule has to be reversible to allow the target molecules to be removed in an active form. Any component can be used as ligand to purify its respective binding partner.

We worked with the typical biological interaction between an antibody and antigen.

Figure 14. Affinity chromatography [GE HEALTHCARE, 2007]

The affinity between ligand and target molecule is supposed to be a result of electrostatic or hydrophobic interactions, van der Waals‘forces and/or hydrogen bonding. To elute the target molecule from the affinity medium this interaction can be reversed, either specifically using a competitive ligand, or none specifically, by changing the pH, ionic strength or polarity.

4.1.1 The choice of matrix, ligand and coupling method [47]

In general the matrix should be chemically and physically inert. The particle size and porosity are designed to maximize the surface area available for coupling a ligand.
Sepharose provides a macroporus matrix with high chemical and physical stability and low non-specific adsorption to facilitate a high binding capacity and sample recovery and to ensure resistance to potentially harsh elution and washing conditions. The choice of a preactivated Sepharose matrix depends on the functional groups available on the ligand and whether a spacer arm is required.

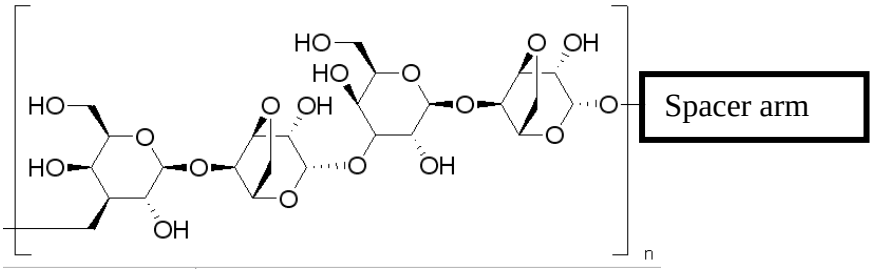


Figure 15. Epoxy activated Sepharose 6B [GE HEALTHCARE, 2007]

To avoid steric interference, a spacer arm is introduced between the matrix and the ligand. The binding site of a target protein is often located deep within the molecule, so that it has no access to the binding site. In this case the spacer arm should ameliorate the effective binding. The length plays thereby an important role because if it is too short, the arm is ineffective and the ligand fails to bind the substance. But if it is too long, the nonspecific binding of the proteins is enabled and in series the selectivity of the separation is reduced.

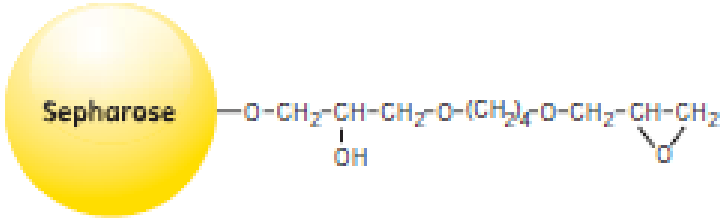
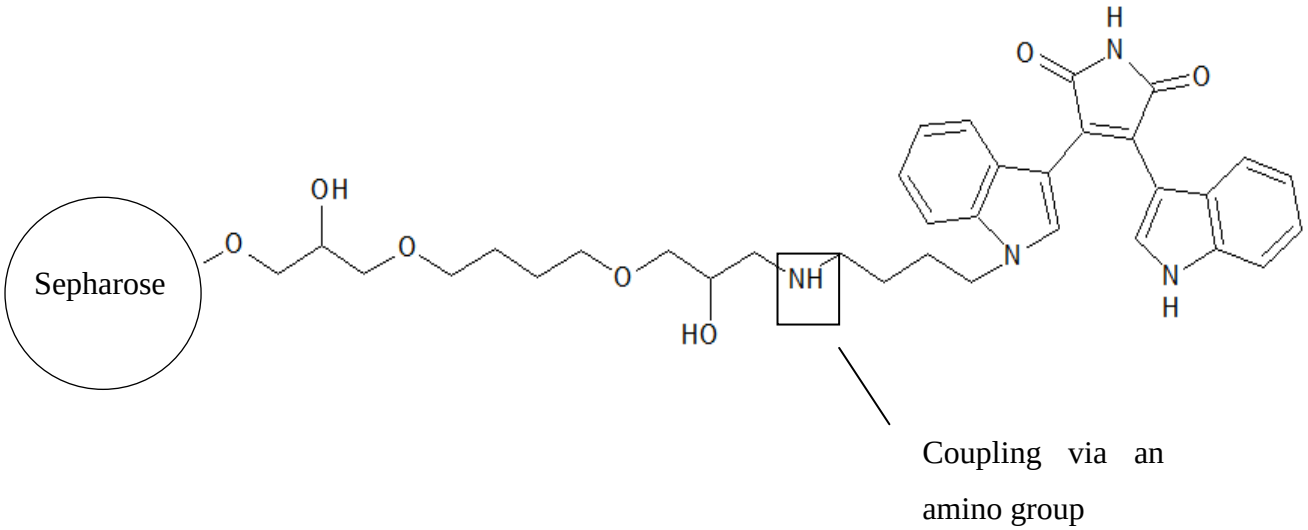


Figure 16. Spacer arm [GE HEALTHCARE, 2007]

In our case the Sepharose reacts with the epoxy group of the 12 atoms spacer arm and creates so a linkage to the hydrophilic group which is suitable for the immobilization of the ligand.
The ligand must selectively and reversibly interact with the target molecule and must be compatible with the anticipated binding and elution conditions. It must carry chemically modifiable functional groups through which it can be attached to the matrix without loss of activity. Too high affinity will result in low yields since the target molecule may not dissociate from the ligand during elution. In our experiment we worked with BisIII.
Ligands are coupled via reactive functional groups such as amino, carboxyl, hydroxyl, thiol and aldehyde moieties. The ligand should be coupled through the least critical region of the ligand to minimize interference with the normal binding reaction.



Coupling via an amino group

4.1.2 Kinetic in affinity chromatography

The binding adsorption and desorption of a target protein to and from an affinity ligand can be considered involved in terms of the binding equilibria and the kinetics of adsorption and desorption.

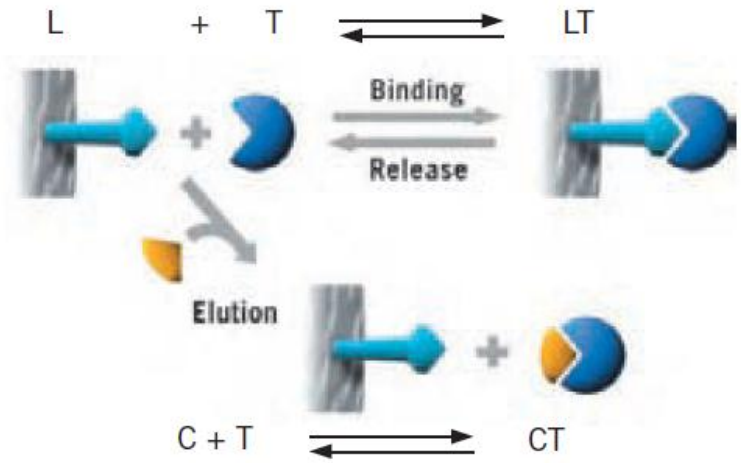


Figure 17. Kinetic [GE HEALTHCARE, 2007]

C...concentration of free competing ligand
T...concentration of free target
CT…concentration of the competing ligand/target complex

If the competitive ligand isn't very effective in capturing the target protein, the concentration of the competiting ligand has to be increased.

Agents that weaken the interactions between the ligand and the protein are expected to function as efficient eluting agents such as ATP for kinase. The eluting agents compete for binding to the target protein.

4.2 Materials

- Centrifuge 5417C/R, Eppendorf
- Rotator RO1, APELEX
- Mini lab Roller Rotatorie, Labnet
- VIVASPIN 500, Sartorius Stedium- biotech
- Shaking Incubator SI500, Stuart

— 96 well-plate, Sterile Nuclon Surface

— DensiCHECK^{TM} plus, bio-Merieux

4.3 Working Conditions

4.3.1 Preparing the matrix

The epoxy-activated sepharose 6B must be first of all washed at neutral pH before coupling the ligand. To ensure the reproducibility of the affinity micro assay, we always initiated the experiment with the same quantity of sepharose.
4,28mg freeze dried epoxy-activated sepharose were weighted in 2ml Eppendorf tube each for the matrix and the control matrix to be afterwards washed with water. About 1ml water was added to the samples and mixed manually for 5 minutes before 1min centrifugation at 2000rpm at room temperature. The supernatant was removed while the obtained pellet was kept. This procedure was repeated for an hour whereas the medium began to swell.

4.3.2 Coupling the ligand

To obtain a concentration of 0,1M for the bisindolylmaleimides, 17mg BisIII should be directly dissolved in the coupling solution containing 200µl 50% DMF and 200µl 0,2M Na_2CO_3. Then 100µl sepharose 6B were mixed with 100µl prepared BisIII. Thereby we watched out to keep one matrix as our control, which wasn't coupled to the ligand. The coupling was now carried out by adding 10mM NaOH while incubating overnight on a rotating wheel at 30°C.

4.3.3 Blocking active groups

The next day the ligand excess was washed away using the coupling buffer. Any remaining active group was blocked by incubating in 1M ethanolamine ph=8 where it stayed for at least

one day at a temperature between 40°C and 50°C. The active groups of the control matrix should also be inactivated in this step.

To ensure that there was still no active group, the matrixes were washed with four cycles of altering pH. One cycle was performed with an acid solution[12] while in next cycle the solution was replaced by a basic[13] one. Thereby we worked with caution to keep the pellets after centrifugation at 2000rpm at room temperature.

4.3.4 Protein Association

Before the association could take place, proteins should be prepared in binding buffer[14]. We mixed 100µl matrix with 50µg of our protein extract and let the solution[14] incubate 3h at 4°C on a rotating scroller. Afterwards the samples were centrifuged to obtain the supernatant which were studied later by western blotting or dot blotting.

Four washing runs with strong and less strong concentration of NaCl[15] were carried out thereby working on ice. The supernatant was always poored away after centrifugation at 2000rpm for one minute.

4.3.5 Elution

After several different attempts to elute the protein of interest, we choose the competitive elution as the most efficient method. In the process a step elution was proceeded using 10mM BisIII and 10mM ATP as a competitor. The samples were each incubated for 15min in the elution solution (100µl), the supernatants were collected after centrifugation at 2000rpm for 1 minute and a second elution using the same parameters was performed.

4.3.6 Western Blotting

The supernatants and eluates, which were collected in the course of the in vitro association, were concentrated via viva spin. They were centrifuged with 15000rcf and so concentrated to a volume of 30 µl.

Figure 18. Viva Spin

They were prepared for deposing on the gel after this schema:

		nber of depots	Volume Final [µl]	LB4X [µl]	Each prot [µl]	H_20 [µl]
1	SampleControl	1	30	6	-24	
2	SampleHela	1	30	6	-24	
3	SampleCa	1	30	6	24	
4	Supernatant Control	1	26	6	20	
5	SupernatantHe	1	26	6	20	
6	Supernatant Ca	1	26	6	20	
7	HeLa 10.5	1	30	6	24	
8	Ca 11.5	1	30	7,5	12	10,5

The migration last 2 hours at 100V that followed by the classic transfer at 25V for 2h30min. Membranes were incubated with the primary antibody PKCη overnight at 4°C and with the secondary antibody (1h at room temperature) before detecting the signal with picoluminescence.

4.3.7 Dot Blot

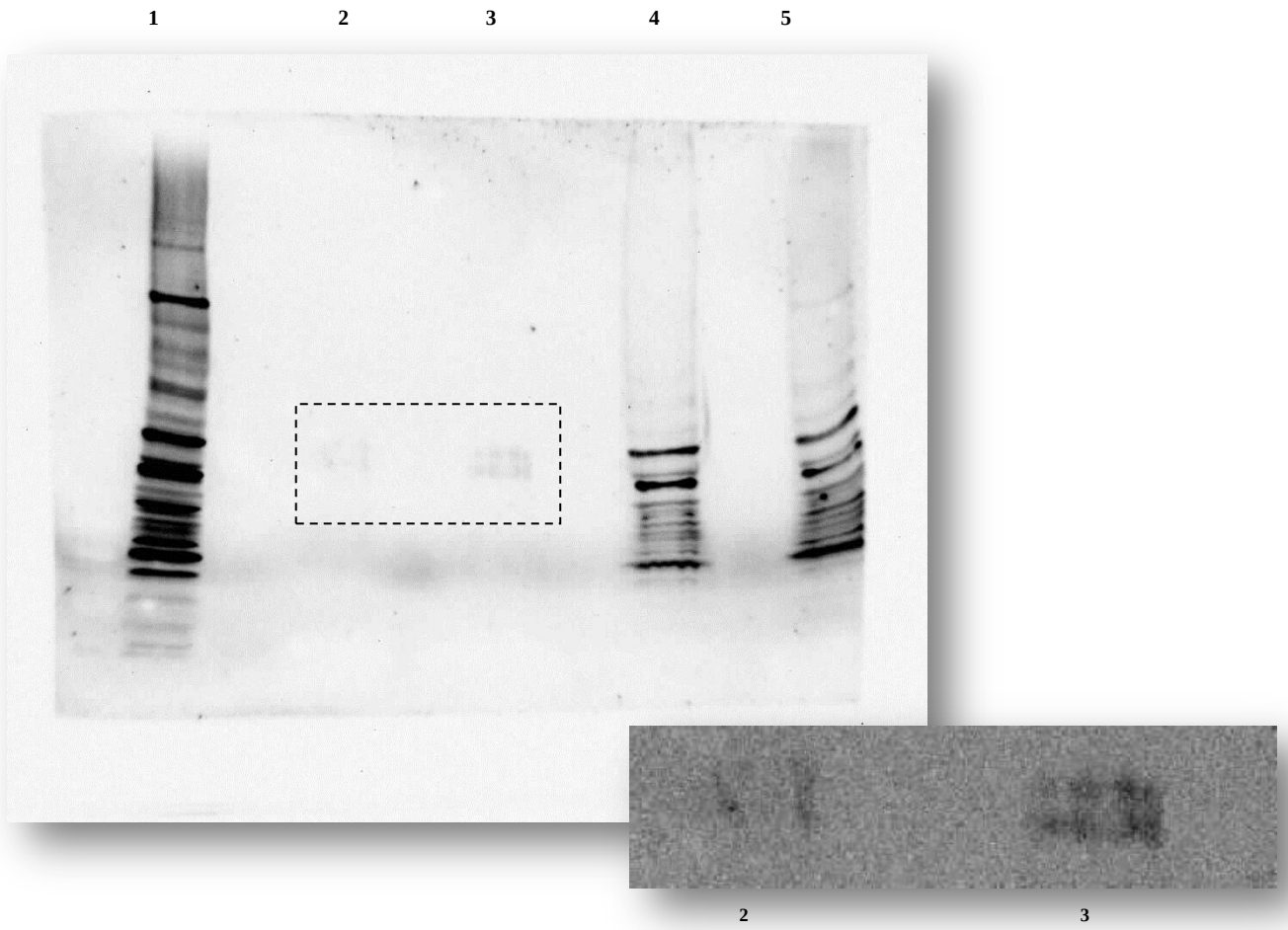
The eluates and supernatants were concentrated via vivaspin and mixed up with LB4X before denaturating at 95°C. The samples were then ready to be applied on the nitrocellulose membrane. We used a pipette to add 5µl after 5µl to form small dots of our samples. The dried membrane was blocked with milk for 30min and then incubated with the primary antibody overnight at 4°C. After incubation with the secondary antibody for one hour at room temperature, the spots on the membrane could be detected.

4.3.8 Immuno –Fluorescence

This time our interest was directed to the matrix with the ligand after we had eluted the proteins. After blocking in BSA/TBST for 39min at room temperature, we incubated the matrix with the primary antibody (1:500 dilution in BSA/PBST) for an hour at 37°C while agitating. Afterwards the matrixes were washed with PBST (0,05%Tween). Then they were incubated with the secondary antibody for an hour at 37°C and washed again with PBST. Finally they were incubated with TMB for 15min at room temperature, protected from light. We could already remark a coloration after a few minutes. After centrifugation at 2000rpm we kept the supernatant to read the optical density at 450nm.

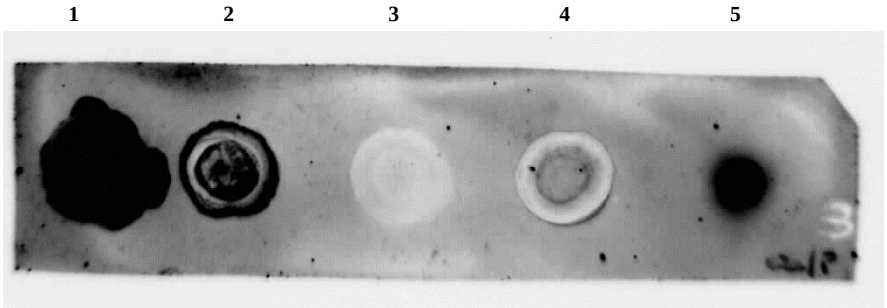
4.4 Results-Affinity micro assay

After the proteins in *Candida albicans* have been validated, the task was to see an interaction between the synthesized bisindolymaleimideIII and the PKCη of the fungal pathogen. As the affinity micro assay was already successfully documented with the human carcinogen cells (HeLa), first of all the experiment was reproduced with the given parameters. But the results were not as expected thus a few parameters of the protocol have been changed. The quantity of the matrix has been augmented to enhance the amount of interaction between the ligand and the proteins. To test the quality of the elution, different types have been tried. The competitive elution seemed the most efficient method compared to the way of elution with the Laemmli buffer[16]. The result was a very weak signal but still not satisfying. Later, the control matrix, which shouldn't give any signal, delivered the same signal as the sample of interest. So, the interest was focused on blocking the remaining groups, because they were supposed to cause an unspecific signal. Therefore the incubation with ethanolamine was carried out which had finally a visible effect on the results. The unspecific signal was minimized but not fully eliminated. The little noise, that stayed, was insignificant because the signal of the sample showed a much higher intensity. To increase the intensity, a longer association time was initiated with the protein, ending up in an inverse effect which is possibly regarding the kinetics. The balance went from one side to the starting position again. After this reflexion the association time was again reduced. At the same time we changed the mode of elution. The step elution was introduced and after adding ATP, which is known as the natural ligand of protein kinase, the expected signal in *Candida albicans* was noticeable.



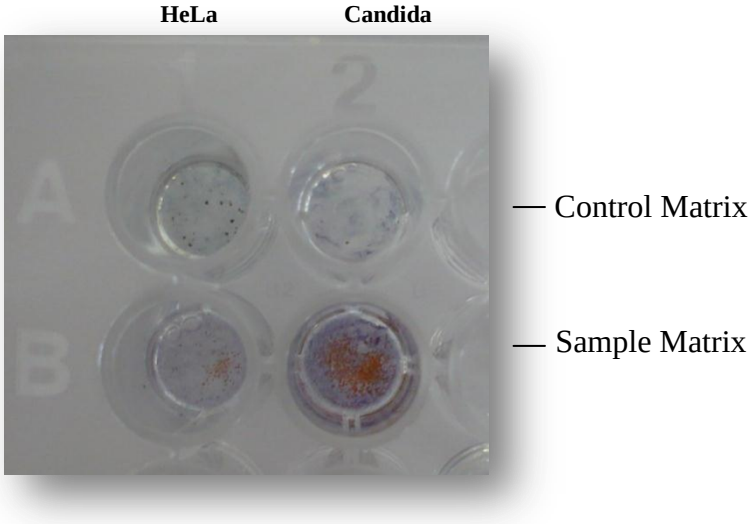
1. Candida albicans (reference),2. Eluat Control,3. Eluat Sample,4. Supernatant Control, 5. Supernatant Sample

For the first time an evidence was established that there must be an interaction between PKCη and BisIII in *Candida albicans*, validating the hypothesis of an action of this ligand through its interaction with PKC.



1.Supernatant Control, 2. Supernatant Sample, 3. Eluat Control, 4. Eluat Sample ,5. Candida albicans reference

The dot blot of *Candida albicans* is another proof that there is an interaction between the ligand and our protein of interest. The concentration of the control supernatant is observably higher than the concentration of the supernatant of the sample. This also shows that there must be some proteins linked to the ligand. But the suspect remained that we didn't manage to elute the whole amount of proteins. This fact wanted to be attested with an immune reaction. The affinity micro assay was performed with the usual parameters. To prove that the proteins last coupled to the ligand, the proteins were eluted and tested on the remaining matrix. Therefore an antibody was attached, which was specific for the protein of interest. It has been revealed with the help of a fluorescent substrate at 450nm.



The eluates of the samples HeLa and *Candida albicans* were visibly colorated, which indicated that there was PKCη. The control matrixes in contrast were clear. Indeed, a considerable amount of PKCη remained attached to the matrix like suspected.

The proof was delivered that there is an interaction between the ligand and PKCη. But the elution parameters need to be improved and so the interaction between the ligand and other proteins of *Candida albicans* (i.e. Hsp90, GSK3) has to be studied more exactly before stepping forward to the affinity chromatography.

5 Conclusion & Perspectives

As soon as the normal body balance is disturbed, the natural inhabitant *Candida albicans* can turn to an invasive infection. The candiasis infestation is located mainly in the intestinal and mucutaneous system, but known to affect many parts of the human body. The exposure to all common modern conditions like antibiotics or steroids, oral contraceptives, overly-acidic pH levels from poor diet and stress, hormone imbalances and exposure to environmental toxins sets our bodies up for the fall into fungal yeast growth. Then the harmful fungal overwhelms the beneficial flora of an immunocompromised individual. It is called "opportunistic" fungus, using the opportunity to colonize all tissues in an uncontrolled way. Large numbers of this germ can weaken the immune system since *Candida albicans* wants to gain control over this system.

Once infected with candiasis, it can cause a wide spectrum of clinical syndroms. So the treatment varies depending on the anatomic location of the fungal pathogen. The therapy includes azoles, allylamines, polyens, which interfere with the ergosterol biosynthesis. Furthermore the antifungal treatment creates stress responses, which activate a wide range of signaling pathways. Key regulators of these pathways are involved in the development of a remarkable resistance. Their contribution to establish a persistent resistance presents a serious threat for the human health, since the antifungal handling loses their efficacy due to the potency of *Candida albicans* to develop an effective method to get invulnerable to the conventional therapy in a short term.

So the decision was made to validate the proteins which are assumed to be responsible for the defence scaffold of *Candida albicans*. First of all the different amounts of protein expression were quantified, known to play an important role in the transduction network. Therefore the work focused on the key regulators PKC, Hsp90 and GSK3. It could be proofed that they are all in a considerable amount in *Candida albicans*. But their participation to the resistance has not been exactly researched yet. PKCη was chosen for further experiments, because it emerged in the majority of western blots as the strongest expressed protein in comparison to GSK3 and Hsp90.

The bisindolylmaleimides are well known inhibitors of PKC in mammalian cells. They are assumed to interfere with the PKC signaling pathway. An investigation with the affinity micro assay seemed to be reasonable to resolve the question if BisIII is also a potent inhibitor of PKC in *Candida albicans*.

For the first time an interaction between PKC and BisIII could be observed. Thus this protein could be utilized as a target to restore the sensibility of *Candida albicans* to the azoles. It has not been elucidated yet if there are also interactions with Hsp90 and GSK3.

Nevertheless this work is an approach for more detailed studies with the affinity chromatography and mass spectrometry, where the whole repertoire of interactions can be described and so used as an essential and novel step in the antifungal treatment.

6 Literature

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V. Curriculum vitae

Personal information

Name	Manon Barsa
Date of birth	June, 20th 1988
Address	Kindergartengasse 4 2212 Großengersdorf
Citizenship	Austria

Education

Since October 2006	University of Vienna, education in Pharmacy
Sept 1998-June 2006	Grammar school Konrad Lorenz Gymnasium

Working experience

Since December 2011	Part-time work at the pharmacy "apotheke-hernals"
February 2011-June 2011	Diploma thesis at the University of Nantes, Group IICiMed - EA 1155 Cibles et médicaments des infections de l'immunité et du cancer
October 2009-February 2011	Part-time work at the pharmacy "St. Franziskus Apotheke"
July 2009	Internship at the pharmacy "Apotheke Aegidius Wolkersdorf"
August 2006	Internship at the pharmacy "Stadtapotheke Gänserndorf"