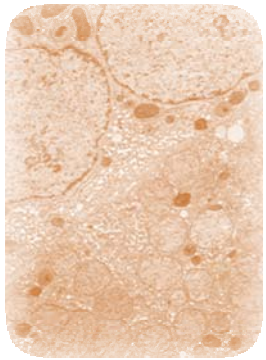
Elucidation of the anti-tumor effect of the synthetic retinoid, CD437, in malignant melanoma

Gry Irene Magnussen

2010



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Elucidation of the anti-tumor effect of the synthetic retinoid, CD437, in malignant melanoma

By

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Gry Irene Magnussen

Abstract

The synthetic retinoid 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid (CD437/AHPN) has been shown to have an antiproliferative effect, as well as being a potent inducer of apoptosis, in many cancer cell lines. The mechanisms behind CD437s activities have been reported as cell-line dependent. In this master thesis, the anti-tumor effect of CD437 was elucidated in the two human malignant melanoma cell lines, FEMX1 and WM239.

- CD437 was shown to have an antiproliferative effect in both cell lines. A reduction in cells relative to the control was first visually observed, and later confirmed by a cell count. As revealed by MTS, the reduction of viable cells was duration- and dosage dependent.
- Treatment with CD437 caused a cell-line dependent cycle arrest, yielding an S phase- and a G_1 phase arrest in FEMX1 and WM239 respectively, as demonstrated by flow cytometry. Western blot analysis revealed that the arrests were accompanied by up-regulations of p53, p21 and E2F-1 protein levels. A down-regulation of cyclin D1 was found in FEMX1 cells.
- Apoptosis was observed in both cell lines following treatment with CD437; Morphological features associated with apoptosis were seen in both cell lines. A cell count using trypan blue revealed an increase of dead cells after exposure to CD437. A TUNEL analysis demonstrated DNA fragmentation in WM239, but not in FEMX1. In both cell lines, cleavage of the initiator caspases 8 and -9 were observed, in addition to PARP cleavage. Cleavage of caspase 3 was found in FEMX1 cells.
- An increase of the orphan nuclear receptor Nur77 was found using the western blot analysis, in treated the cells. In addition, activation of c-jun was observed in both cell lines following exposure to CD437.
- Treatment with CD437 resulted in an activation of the p38/MAPK signaling pathway in both cell lines, as assessed by western blotting. Additionally, in the exposed FEMX1 cells the PI3K signaling pathway was activated.
- Preliminary results suggest that an up-regulation of p53 caused by CD437 may be regulated by Nur77. The increase of p21 did not appear to be controlled by Nur77, which may suggest a p53-independent expression of p21.

Malignant melanomas are notoriously resistant to chemotherapy, making the prognosis for patients with metastasis very poor. CD437 represent a potential new treatment option, however many more studies are needed to evaluate its effect both *in vitro* and *in vivo*.

Sammendrag

Det har tidligere vært vist at den syntetiske retinoiden ”6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid” (CD437/AHPN) har en antiprolifererende og apotose induserende effekt i mange kreft cellelinjer. De intracellulære mekanismene som forklarer effekten av CD437 har imidlertid vist seg å variere mellom ulike cellenlinjer. Hensikten med denne masteroppgaven er å vurdere CD437s anti-tumor effekt i to humane maligne melanomcellelinjer; FEMX1 og WM239.

- Det ble funnet at CD437 har en antiprolifererende virkning i begge cellelinjene. Virkningen viste seg som en reduksjon i antall celler i forhold til kontrollen. Dette ble først observert visuelt, og siden bekreftet ved celletelling. Ved bruk av en MTS analyse ble det funnet at nedgangen i levedyktige celler både var avhengig av konsentrasjon og varighet av behandling.
- Flowcytometriske analyser viste at CD437 forårsaket ulik cellesyklus arrest; en S- og G_1 fase arrest ble observert i henholdsvis FEMX1 og WM239. Det ble videre påvist at cellesyklusarresten var assosiert med oppregulering av p53, p21 og E2F-1, som vist med western blotting. En nedgang i cyclin D1 ble funnet etter behandling i FEMX1 cellene.
- Apoptose ble observert i begge cellelinjene etter behandling med CD437. En celletelling med trypanblått påviste økt dødelighet etter behandilng i forhold til kontrollcellene. TUNEL analyse viste videre at CD437 forårsaket DNA fragmentering i WM239, men ikke i FEMX1 cellene. Kløyving av initiator caspase 8 og -9, samt PARP, ble funnet ved western blotting, i begge cellelinjene. Det ble i tillegg observert en kløyving av effektor caspase 3 i FEMX1 cellene.
- Western blot analyser tyder på en oppgang i protein nivåene til kjernereseptor Nur77 og aktivering av transkripsjonsfaktor p-c-jun, etter behandling med CD437.
- CD437 behanling førte i begge cellelinjene til aktivering av p38/MAPK signalveien, mens PI3K veien kun ble aktivert i FEMX1 cellene.
- Preliminære forsøk viste videre at oppregulering av p53, som følge av CD437 behandling, er regulert av Nur77. Det økte p21 protein nivået synes imidlertid ikke å være styrt av Nur77, noe som videre tyder på at p21 ekspresjonen er uavhengig av p53.

Malignt melanom har vist seg å være særs motstandsdyktig til kjemoterapi, og prognosen for pasienter med spredning er derfor svært dårlig. I lys av både våre - og tidligere funn, representerer CD437 et potensielt nytt behandlingstilbud. Det er dog nødvendig med flere undersøkelser for å evaluere dets effekt både *in vitro* og *in vivo*.

Abbreviations

AIF	Apoptosis Inducing Factor
AJCC	American Joint Committee on Cancer
APAF-1	Apoptotic Protease-activating Factor-1
APL	Acute promyelocytic leukaemia
ATP	Adenosine Triphosphate
ATRA	All-trans retinoic acid
Bak	Bcl-2 antagonist killer
Bax	Bcl-2–associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-xl	B cell leukemia/lymphoma xL
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 like 11
B-RAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine Serum Albumin
BTB	β-mercapto bromophenol blue
Caspase	Cysteine-dependent Aspartate-directed Proteases
CD437	6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid
CDC25	Cell division cycle 25
CDK	Cyclin Dependent Kinase
CHOP/GADD153	C/EBP homologous protein
CICD	Caspase Independent Cell Death
DISC	Death inducing Signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR4	Death Receptor 4
DR5	Death Receptor 5
dsRNA	Doublestranded Ribonucleic acid
dUTP	Deoxyuridine-triphosphatase
E2F	E2F transcription factor
ECLplus	Enhanced Chemiluminescence plus
EDTA	Ethylendiaminetetra-acetic acid disodium salt
ER	Endoplasmatic Reticulumn
ERK ½	Extracellular signal.Regulated Kinases 1 and 2
FADD	Fas-associated death domain protein
FAS	TNF receptor superfamily, member 6
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
IAP	Inhibitor of apoptosis
INK4	Inhibitor of cyclin dependent kinase 4
LDH	Lactic Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
Mcl-1	Myeloid cell leukemia-1

MOMP	Mitochondrial Outer Membrane Permeabilization
MTS	3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NR4A1	Nuclear receptor subfamily 4, group A, member 1
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
Nur77/TR3/NGFB-1	Nuclear ophan receptor protein
$p14^{INK4d}$	Inhibitor of cyclin-dependent kinase 4d
$p15^{INK4b}$	Inhibitor of cyclin-dependent kinase 4b
$p16^{INK4a}$	Inhibitor of cyclin-dependent kinase 4a
$p18^{INK4c}$	Inhibitor of cyclin-dependent kinase 4c
$p21^{Cip1/WAF1}$	Cyclin-dependent kinase inhibitor 1a/ Wild-type Activating Fragment
$p27^{Kip1}$	Cyclin-dependent kinase inhibitor 1b
$p57^{Kip2}$	Cyclin-dependent kinase inhibitor 2
PARP	Poly(ADP-ribose) polymers
PBS	Phosphate buffered saline
pRB	Retinoblastoma protein
PS	Phosphatidyl Serine
PVDF membrane	Polyvinylidene Fluoride membrane
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid receptor element
RISC	RNA-induced signaling complex
RNA	Ribonucleic acid
RNAi	RNA interference
RXR	Retinoic X receptor
RXRE	Retinoic X receptor
SAPK/JNK	Stress-Activated Protein Kinase
SDS-PAGE	Sodium Dodecyl Sulphate - polyacrylamide gel
SiRNA	Small/short interfering RNA
SMAC/DIABLO	Second mitochondria-derived activator of caspases/direct IAP-binding protein
TBS-T	Tris-Buffered Saline and Tween 20
Tdt	Terminal deoxynucleotidyl transferase
TE-buffer	Trypsin/EDTA- buffer
TNF-α	Tumor Necrosis Factor-α
TNM	Tumor-Node-Metastasis
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal Transferase dUTP Nick End Labeling

INDEX

1.Introduction

1.1 Aim of the study

Much effort has been put into finding efficient treatments for human malignant melanoma, yet this form of cancer seems to be notoriously resistant to chemotherapy both *in vivo* and *in vitro* (1). There are per today, few good options for treatment of malignant melanoma that has reached an advanced stage, hence the prognosis is poor; only 14 percent of patients diagnosed with metastatic melanoma survive for five years (2). It is therefore a precarious need to gain more knowledge about the disease in order to find more suitable treatments.

Retinoids, small natural and synthetic molecules derived from vitamin A, have in the recent years evoked interest in the fields of cancer research due to their efficiency in treating certain types of cancer, both *in vitro* and in clinical trials (3;4). Due to the toxicity associated with the natural retinoids, focus has been cast on developing synthetic analogues, such as 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid (CD437). This synthetic retinoid has so far shown promising results by selectively inducing apoptosis and cell cycle arrest in several cell-lines, including melanomas (1;5-8). The mechanisms behind the anti-tumor effects of CD437 remain largely unknown, its ability to induce apoptosis and cell cycle arrests can, unlike the natural retinoids, be mediated independent of the retinoid receptors in the cell (8-10).

The goal of this master study was to evaluate the effects of CD437 on two metastatic malignant melanoma cell lines (FEMX1 and WM239) grown *in vitro*, with respect to cell death, proliferation and cell cycle arrest, and to investigate possible mechanisms involved in these processes.

1.2 Background

1.2.1 Cancer

Most people living in the developed world have some sort of relation to the word 'cancer'. Being the third most common death-causing disease worldwide, cancer kills nearly 6 million people every year (11).

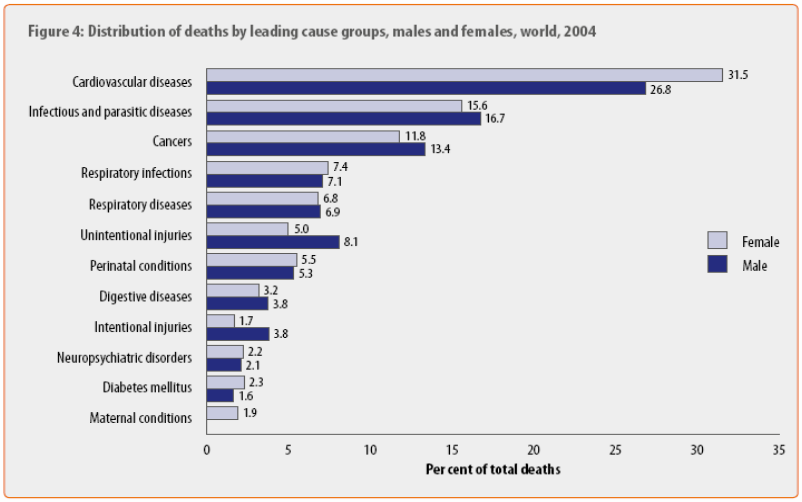


Figure 1. ***Distribution of deaths caused by disease, 2004 (11).***

Cancer can affect living cells in every part of the human body, making it a variable and complex disease. Some traits, however, are thought to be shared by most human tumors. These hallmarks include; resistance to apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, and tissue invasion (metastasis) and growth (12).

The current understanding of cancer is as a multi-step process, where a mutation in the cell can give it certain advantages to survive and accumulate further mutations. The genetic alterations will stepwise transform the cell from normal to cancerous, comparable to the Darwinian evolution, though on a much smaller scale (12).

In the human cell, a series of intracellular systems co-operate to ensure that the DNA sequence information remains unaltered (13). Despite these careful monitoring systems, mutations in certain genes can potentially lead to cancer.

Mutations occurring in both alleles of a tumor suppressor gene, lead to its loss of function, rendering the cell less capable of detecting and repairing other mutations. Mutation happening in a proto-oncogene, encoding proteins involved in regulation of cell growth and differentiation, can on the other hand augment its activity, making the cell more susceptible to uncontrolled cell division. MicroRNA genes encode short single-strand RNA sequences that regulate the expression of other genes, either by causing their mRNA to be degraded, or by blocking the protein translation. Mutations in microRNA genes can hence affect the expression of both tumor suppressor genes and oncogenes (14).

1.2.2 Malignant melanomas

Malignant melanoma is the most aggressive form of skin cancer, arising in melanocytes, the pigment-forming cells of the skin. The disease is relatively rare, and accounts for only four percent of all dermatological cancers (2). There is, however, an increase of new cases, especially in Norway and Sweden (15).

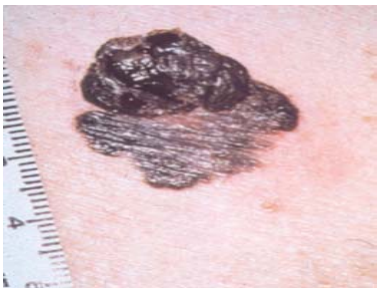


Figure 2. ***Malignant melanoma***

Several factors are thought to increase the risk of developing melanomas, such as; over-exposure to the sun, fair skin, presence of multiple birthmarks or freckles and a family history of melanoma (15).

The American Joint Committee on Cancer (AJCC) recommends using a grading system to set an appropriate prognosis when dealing with melanomas, currently used is the Tumor-Node-Metastasis (TNM) criteria. In the TNM staging system, T represents tumor thickness and ulceration, N considers the amount of metastatic lymph nodes, and the M evaluates the sites of distant metastasis as well as the level of serum Lactic Dehydrogenase (LDH) (16).

Both the Clark model and Breslow's depth are used in order to grade a melanoma according to the TNM criteria. The Clark model is a model showing the stepwise transformation of a melanocyte into a melanoma (17), and Breslow's depth is used to describe how deeply tumor cells have invaded the skin (18).

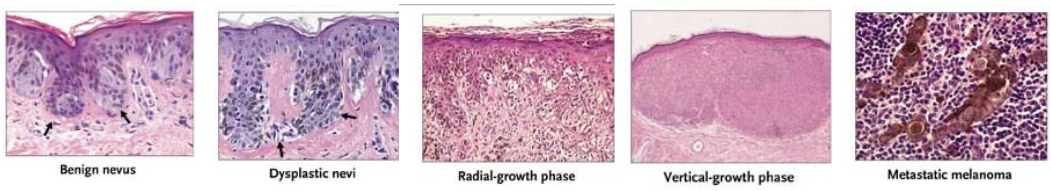


Figure 3. ***The Clark model; grading the invasion of the melanoma in the skin (17).***

The transformation of a melanocyte into a malignant metastatic melanoma is thought to involve a sequential accumulation of mutations. Although much remains unknown, some main molecular changes leading to aggressive types of melanoma have been found (19).

- Hyperactivation of the MAPK/ERK1/2 signaling pathway is common in melanomas, and is often due to mutations in genes coding for proteins such as NRAS or B-raf , thus leading to increased proliferation in these cells(20).
- The PI3K/Akt signaling pathway is also frequently altered in melanomas, causing an increased survival. Common mutation associated with this pathway are found in genes coding for Akt and PTEN (21).

- Mutations causing the transcription factor MITF to be up-regulated is commonly found in melanomas, leading to increased proliferation and survival (22).
- In many aggressive melanomas, mutations in genes coding for inhibitors of the cell cycle, such as $p14^{INK4d}$ and $p16^{INK4a}$, have been found, causing the cell to avoid apoptosis and senescence (23).

1.2.3 Signaling

In a well functioning organism, each cell must adapt to its environment. In order to do so, the cell has to monitor its surrounding environment, as well as communicate with other cells. Communication between cells involves receiving and transmitting signals, which can trigger specific short- or long term changes in the receiving cell. On a short term basis, cellular changes may include alterations in cellular metabolism, function or movement, whereas the long term changes usually involves modifications in gene expression and development (24).
Binding of the ligand will either occur to certain receptors located on the cell surface, or the ligand may penetrate the cell membrane and bind to an intracellular receptor. Ligands that bind to cell surface receptors may cause a conformational change in the receptor, which can lead to an activation of a signal transduction pathway. In eukaryotic cells, the intracellular signal transduction pathways are highly conserved, and function in essentially the same way in cells from different species (24).

The mitogen-activated protein kinase (MAPK) is a family of serine-threonine kinases, important in conveying extracellular signals to the nucleus, where they can alter the cells gen expression. The family consists of several members, where the best understood classes of MAPKs are; Extracellular signal.regulated kinases 1 and 2 (ERK1/2) -, the stress-activated protein kinases (SAPK/JNK) - and the p38/MAPK pathways (25).

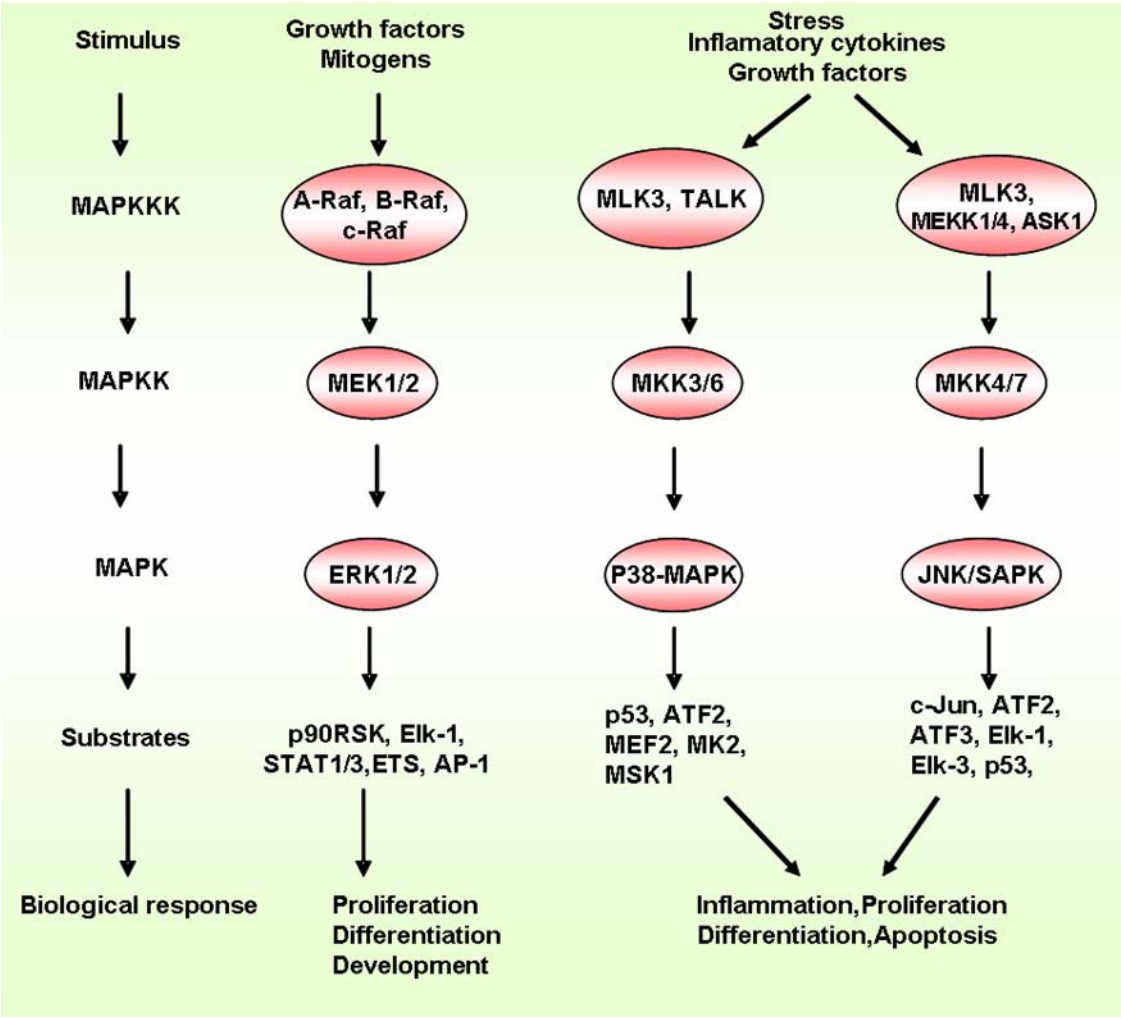


Figure 4. ***Major MAPK signaling cascades in mammalian cells (26)***

The ERK1/2/MAPK signal transduction pathway is central in several processes such as proliferation, growth arrest, cell survival, apoptosis, oncogenesis and differentiation, and can be triggered by a variety of signals such as hormones and growth factors (27). The effects of the ERK1/2 kinases, have in some studies been described as variable and sometimes opposing, depending on the strength and duration of the ERK1/2 activation (28). As previously described, proteins involved in this signaling pathway are often mutated in melanomas, leading to its hyperactivation.

The JNK - and p38/MAPK pathways can be triggered by a variety of environmental and genotoxic stress factors, and may cause an alteration in the expression of transcription factors, cytokines and cell cycle regulators, amongst others the Activator protein-1 (AP-1), leading to a variety of cellular responses dependent on the cell type, duaration and strength of the signal (29;30). Both the JNK- and p38/MAPK pathways have been suggested to play a role in apoptosis (31). In addition, activated p38/MAPK has been shown to influence the expression of p53, an important regulator of both the cell cycle and apoptosis (32).

Another well known signaling pathway involves the Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NFκB) transcription factors. This signal transduction cascade can be activated as a response to inflammation, UV radiation and other types of stress factors, ultimately leading to the transcription of more than 100 different genes, some important in proliferation and apoptosis (19;33).

The Phosphoinositide 3-kinases (PI3Ks) signaling pathways, involve the activation of Akt, a serine/threonine kinase involved in the regulation of multiple components of the cell cycle, as well as promoting cell survival (19). The pathway is negatively regulated by a tumor suppressor protein, PTEN, which is commonly mutated in melanomas. In the absence of PTEN, the signaling pathway is constitutively active, thus leading to increased cell survival. PTEN is also a positive regulator of the pro-apoptotic protein p53, hence melanomas lacking PTEN may avoid apoptosis and cell cycle arrests (34).

1.2.4 Cell death

A cell can die in several ways, triggered by a variety of mechanisms. Different types of cell deaths are classified based on a variety of criteria; morphological-, enzymological-, functional- or immunological characteristics (35). Based on morphological changes, cell death can be divided into; Apoptosis, Autophagy, Necrosis, Mitotic Catastrophe (36).

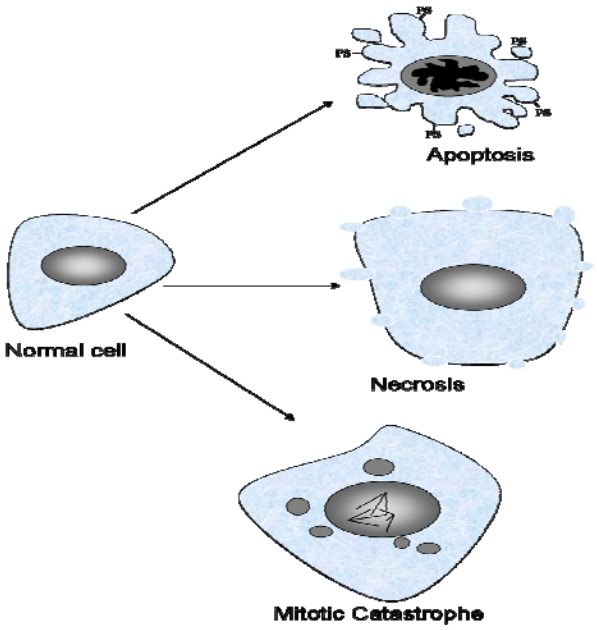


Figure 5. ***Morphological changes associated with apoptosis, necrosis and mitotic catastrophe***

1.2.4 Apoptosis

The size of a population of cells is determined by the balance between cell division and cell death. Central in this is apoptosis, an active process of programmed cell death, important in development, differentiation, proliferation, tissue homeostasis and in the removal of defect cells (37).

Apoptosis can be distinguished from other forms of cell death by its morphological characteristics, such as; cell shrinkage, membrane blebbing, chromatin condensation, cytoplasmic shrinkage, and at later stages, formation of apoptotic bodies (Figure 3). In addition, phosphatidylserine (PS) is exposed on the cell surface, sending out a signal to macrophages to engulf the dying cell, a mechanism designed to protect the nearby tissue from being affected by the cell death (38).

Several mechanisms can lead to the activation of apoptosis in the cell; binding of death ligands, intracellular stress, loss of cell anchorage, lack of growth factors, and DNA

damage. Depending on the trigger, apoptosis will be initiated through an intrinsic- or/and an extrinsic pathway (38) (Figure 5).

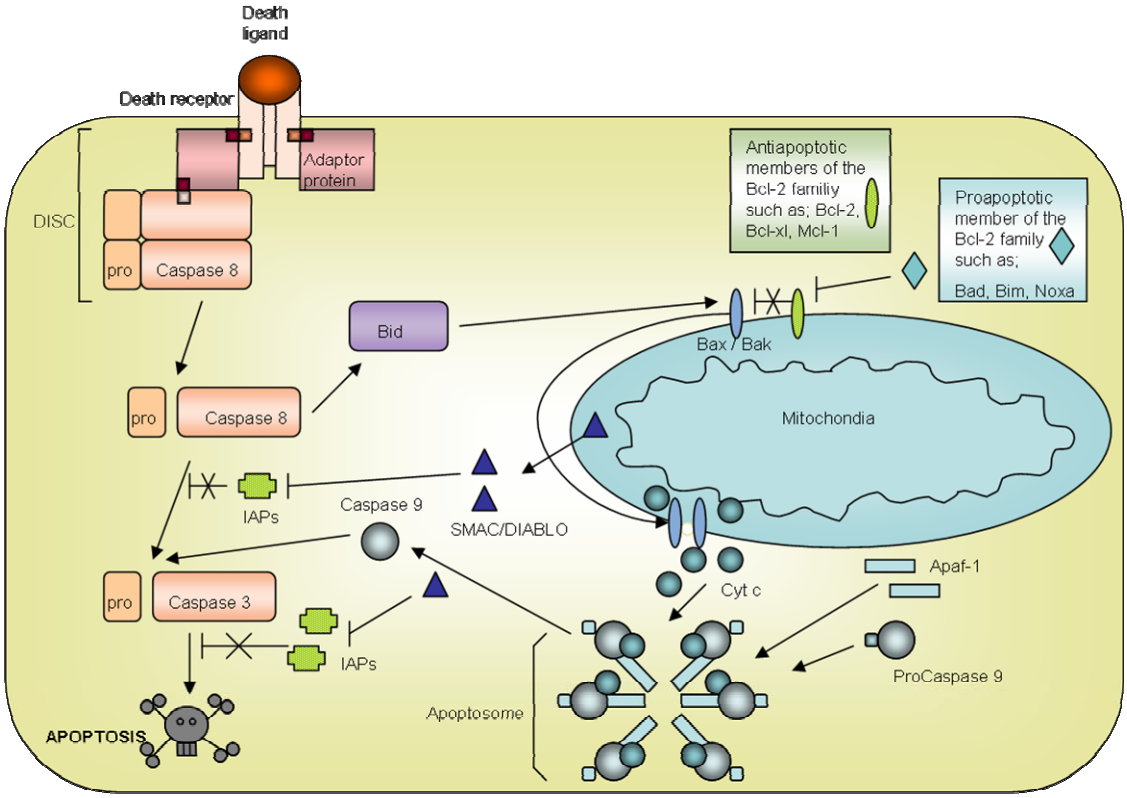


Figure 6. ***An overview of some of the components of apoptosis.***

The intrinsic pathway

Intracellular stress signals can initiate apoptosis through the mitochondrial outer membrane permeabilization (MOMP), a process carefully regulated by the Bcl-2 protein family.

The Bcl-2 proteins all share a sequence homology within conserved regions; the Bcl-2 homology (BH) domains. The Bcl-2 proteins contain 1-5 BH domains, which are required for binding to other members of the family. Most (but not all) of the pro-apoptotic Bcl-2 proteins (e.g. Bim, Bad, Bid) contain only one BH domain, these proteins are essential in sensing apoptotic signals, and will, if evoked, work to shift the balance between the multi BH domain anti-apoptotic proteins (e.g. Bcl-2, Bcl-xl, Mcl-1) and the pro-apoptotic proteins towards apoptosis (39). Although the exact mechanisms of cytochrome c release by the Bcl-2 family remain somewhat debated, it is thought that the

pro-apoptotic members of the Bcl-2 family activate Bak and Bax, which upon several conformational changes and oligomerization, can form pores in the mitochondrial membrane (40). The following drop in mitochondrial membrane potential ($\Delta\Psi_m$), leads to the release of pro-apoptotic proteins, such as cytochrome c and SMAC/DIABLO.
Once released in the cytoplasm, cytochrome c forms a complex (apoptosome) with Apaf-1 and pro-caspase 9. The apoptosome can, through cleavage, activate caspase 9 from its zymogen form (41).Caspase 9 is an initiator caspase which is capable of activating the effector caspases (caspase 3 and caspase 7), enzymes important in executing apoptotic changes in the cell.
The release of SMAC/DIABLO from the mitochondria further boosts the apoptotic machinery by antagonizing the members of the Inhibitors of Apoptosis (IAP) family, and thereby inhibiting them. The IAPs (e.g. survivin, livin, xiap) are anti-apoptotic proteins which are thought to inhibit apoptosis by a direct binding to effector caspases, thus preventing their activations (42).

Many stress signals can regulate the members of the Bcl-2 family, causing an activation of the intrinsic pathway of apoptosis.
Oncogenic stress, such as DNA damage, can lead to stabilization and accumulation of p53, a tumor suppressor protein that can act as a transcription factor causing an increase of pro-apoptotic bcl-2 members, as well as mediating repression of anti-apoptotic bcl-2 proteins. p53 may also bind directly to the mitochondria causing MOMP (43). Depending on the inducer of p53, a variety of responses have been detected, such as apoptosis, cell cycle arrest, diffentiation, senescence and DNA-repair (44).
The transcription factor Nuclear ophan receptor protein (Nur77/TR3/NR4A1) can, if induced by certain death stimuli, translocate to the mitochondria where it transform Bcl-2 to a pro-apoptotic protein, thereby causing release of cytochrome c, or it can target the endoplasmatic reticulumn (ER) thus promoting release of Ca^{2+}, in both cases initiating apoptosis (43;45).

The Bcl-2 members have also been reported to play a role in the induction of apoptosis by releasing Ca^{2+} from the ER, which in turn can lead to the release of pro-apoptotic

proteins from the mitochondria, as well as activating cytosolic kinases important in apoptosis (46).
ER stress has, in addition, been shown to induce apoptosis and cell cycle arrest through the release of C/EBP homologous protein (CHOP/GADD153) (47).

The extrinsic pathway

Apoptosis can also be initiated through signals from the outside of the cell. Death ligands (such as TRAIL, Fas and TNFα) may bind to certain receptors on the cell surface, thus activating them. Activated death receptors will cluster together, and recruit adaptor proteins (e.g. FADD and TRADD), as well as procaspase- 8 or/and 10, forming a so called Death Inducing Signaling Complex (DISC) (48). Due to the close proximity of the proteins at the DISC, the initiator caspases (caspase 8 and caspase 10) get activated through autoprotolytic cleavage. The caspases can now detach from the DISC, and perform their proteolytic activities by activating the effector caspases and Bid. The cleaved pro-apoptotic Bcl-2 member Bid can crosstalk with the intrinsic pathway by activating Bax and/or Bak, thus enhancing the apoptotic effect (49).

Caspase-independent apoptosis

Apoptosis-like cell death has been reported to occur, independent of caspase activation. In the case of Caspase Independent Cell Death (CICD), the morphology of the dying cell will be similar to the apoptotic cell, although some of the characteristics may be slightly deviating, depending on the cell type and the initiating stimulus (50). Initiation of CICD can follow the activation of a death receptor (necroptosis), or occur through the intrinsic pathway by the action of proteins such as SMAC/DIABLO and Apoptosis inducing factor (AIF) (50;51).

1.2.5 Necrosis

Necrosis is a form of cell death thought to be less controlled than apoptosis. It is often associated with an inflammatory response in the nearby tissue as a reaction to the release of intracellular material from the dying cell. Death by necrosis can occur due to cellular

trauma, such as infection or ischemia, but has also been seen as a consequence of a death signal (52).

The morphological changes seen in necrotic cells can be described as; swelling of the cell, loss of membrane integrity, and as a response to this; leakage of intracellular material to the surroundings (Figure 3).

1.2.6 Mitotic catastrophe

It is debatable whether mitotic catastrophe can be classified as a separate form of cell death, or if it is just a process leading to death, either through apoptosis or necrosis. Mitotic catastrophe is associated with deficiencies in cell cycle checkpoints, resulting in a divergent mitosis and thus aberrant chromosome segregation and excess amounts of DNA (53).

The changes in morphology in the cell undergoing mitotic catastrophe will usually include; enlargement of the cells size, uncondensed chromosomes with deviations in the mitotic spindle, as well as the occurrence of micro-nuclei (Figure 3).

1.2.7 Cell cycle

The cell cycle is a series of events leading to division of a cell. Cell division is important both during development, as well as to balance up for dying cells in a tissue.

In eukaryotic cells, the cell cycle consists of four distinct phases; Gap 1 (G_1), Synthesis of DNA(S), Gap 2 (G_2) and Mitosis (M) (Figure 5).The directional, ordered events of the cell cycle start in G_1, a phase where the cell produces proteins and grows in size. In the following S phase, the cell synthesizes a new set of DNA. During the proceeding gap phase, G_2, the cell expands and produces new proteins needed for mitosis, in particular proteins that will work as building blocks for the microtubules. The mitosis can itself be divided into an ordered series of events, ultimately leading to the physical division of the cell into two identical daughter cells. The new cells can proceed to a new round of the cell cycle, or they can enter the non-proliferative state G_0. Cells can remain in the G_0 state indefinitely, or they can re-enter the cell cycle at a later stage if needed (24).

The cell cycle is a tightly regulated process where each transition between phases requires the hetrodimerization of two molecules; cyclin (regulatory unit) and cyclin-dependent-kinase (CDK) (catalytic unit). The levels of CDKs remain relatively constant throughout the cell cycle; their activity is regulated through phosphorylations and dephosphorylations. The concentration of the different types of cyclins, on the other hand, will vary in response to molecular signals that causes their synthesis and destruction (54). The cyclin-CDK complex formation thus have a timed appearance, giving rise to three main groups of complexes, G_1 cyclin-CDK, S cyclin-CDK and Mitotic cyclin-CDK, specific to the phase they occur (55). The transitions between different phases of the cell cycle are regulated by the synthesis and destruction of cyclins, as well as through the activation of CDKs.

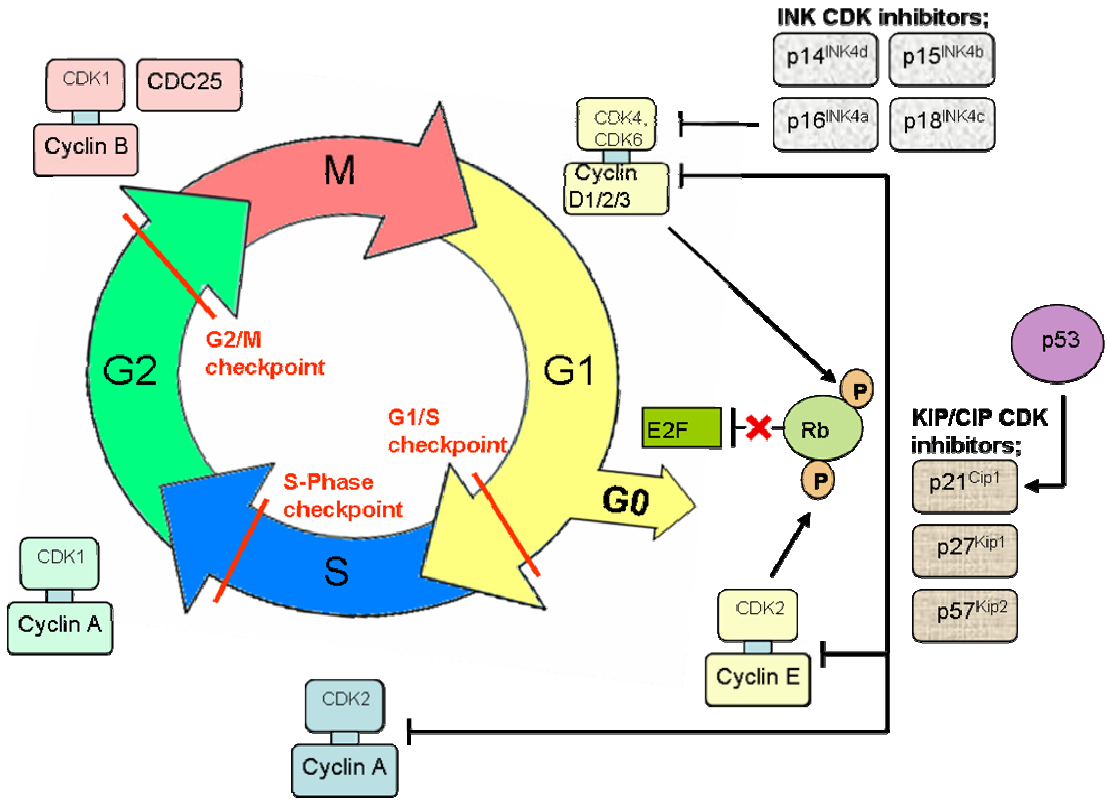


Figure 7. ***A simplified overview of the eukaryotic cell cycle, its check points and some of its key regulators, with emphasis on the G_1/S transition.***

The cell cycle is further regulated by a series of checkpoints dispersed throughout the cycle, its three main control points being the G_1/S-, S- and G_2/M checkpoints (Figure 5). At each checkpoint, surveillance mechanisms in the cell make sure that a transition is not initiated before the previous stage is completed (24). The G_1/S checkpoint is also known as the restriction point (R), a transition site where the cell evaluates both its external and internal environment before committing to a new round of the cell cycle.

Transition through the G_1/S checkpoint requires the inactivation of the retinoblastoma protein (pRb). Whilst in a hypophoshorylated state, pRb is active and bind E2F, a transcription factor necessary for the expression of genes needed for entry to the S phase (56). Cyclin D/CDK4/6 and Cyclin E/CDK2 act cooperatively to inactivate pRb through phosphorylation, by so releasing E2F, allowing the cell cycle to progress (57).

Regulation of the cell cycle is also conducted by two families of CDK inhibitory proteins (CKIs); the INK4 - and the CIP/KIP family.
The inhibitory proteins of the INK4 protein family include p14 ^{INK4d} (p14), p15 ^{INK4b} (p15), p16 ^{INK4a} (p16) and p18 ^{INK4c} (p18). These are proteins which can bind to CDK4 and/or CDK6 in the G_1 phase, thus inactivating the cyclin-CDK complexes and arresting the cell cycle (58).
The CIP/KIP family consists of three members; $p21^{Cip1/WAF}$ (p21), $p27^{Kip1}$ (p27)and $p57^{Kip1}$(p57), that can inhibit the cell cycle through binding to CDKs, by so inactivating the cyclin-CDK complex, causing a subsequent cell cycle arrest .

The tumor-suppressor protein, p53 also plays an important role in regulating the cell cycle, by being a central regulator at the G_1/S and the G_2/M checkpoints (59). p53 may increase the transcription of p21, thus arresting the cell cycle (60).

1.2.8 Retinoids

Retinoids are a family of proteins comprising synthetic and natural vitamin A derivatives and analogues, known to regulate many biological processes including growth, differentiation, metabolism, apoptosis, morphogenesis and homeostasis (61).

The effects of the natural retinoids (RA) are thought to be mediated mainly through their binding to nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each consisting of three different proteins (α, β and γ) (Figure 6) (62). The retinoid receptors can in turn dimerize and bind to specific response elements (RAREs or RXREs) found in the promoter regions of DNA, ultimately leading to a change in the cells gene expression. Whereas the RARs can only be activated through the dimerization with other RARs and RXRs, the retinoid X receptors may dimerize with other molecules, such as orphan nuclear receptors, for instance Nur77 (63).

Less is known about the pathways used by the synthetic retinoids, their effects on apoptosis and differentiation have been shown to be only partially mediated through their binding to the retinoic receptors (8).

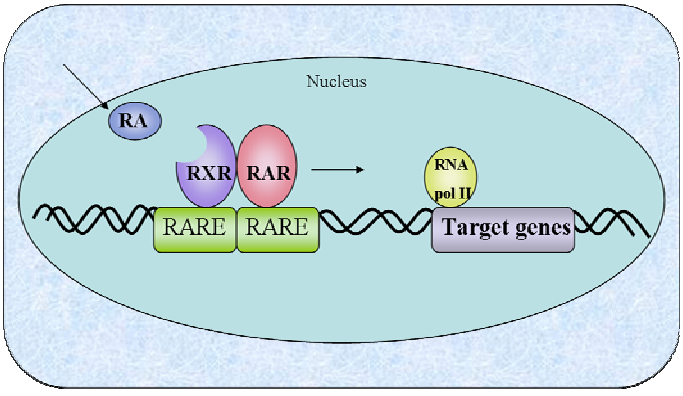


Figure 8. ***Receptor mediated effects of retinoic acids***

Studies on the effects of the natural retinoids can be dated all the way back to 1925 (64), and has over time led to the discovery of their anti-tumor qualities. Natural retinoids (such as ATRA) are now included in some cancer treatments, for instance in patients with

promyelocytic leukemia (APL) showing a >90% complete remission (3). Despite these promising results, the natural retinoids have had limited effects in other tumors, as well as causing hepatic toxicity in the treated patients due to the high doses needed for treatment (65;66).

The recent studies on the anti-tumor effects of retinoids have therefore been more concentrated on developing synthetic retinoids, associated with less toxicity and higher efficiency.

1.2.9 CD437

Synthetic Retinoid 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid (CD437/AHPN) has been proposed as a chemopreventing agent, showing promising results *in vitro*. CD437 has been reported to induce growth arrest and/or apoptosis in melanoma cells (5), as well as in ovarian- (67) -, breast (68) -, gastric (69)-, and non-small-cell-lung cancer (70), as well as leukemia (71).

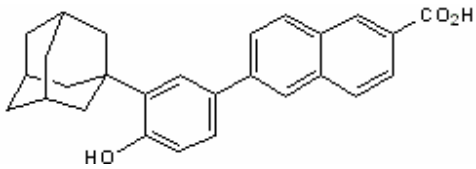


Figure 9. ***Molecular structure of CD437***

CD437 has been shown to have selective apoptotic activities. In addition, it is a strong apoptotic inducer associated with less toxicity than the natural retinoids, for instance ATRA (5;72).

1.2.10 CD437s effect on apoptosis; previous studies

The mechanisms through which CD437 induces apoptosis are diverse, and may vary a great deal between different types of cells.

It has been demonstrated that CD437 can induce apoptosis in cells lacking nucleus, indicating that transcriptional activities are not a requirement (73).

Other studies have, on the other hand, shown CD437-induced apoptosis to be directed through the activation and up-regulation of certain transcriptional factors, such as AP-1(7) and c-myc (74).

To further complicate the puzzle, CD437 can trigger apoptosis through the endoplasmatic reticulumn (ER) in some cell types (45;75), whereas in others, apoptosis may be induced through the mitochondria (73;76), or through a lysosomal pathway (71). CD437-induced apoptosis may be dependent on caspase activation (77) , or it may be induced in a caspase-independent manner (78).

Induction of apoptosis by CD437 has been reported to be partially attributable to its binding to the natural retinoid receptors (RARγ) in some cells (66), whereas it may happen in a RAR-independent mechanism in others (8;9).

Treatment with CD437 has also been shown to mediate apoptosis through the translocation of the orphan nuclear receptor Nur77 from the nucleus to either the ER (45;79) or to the mitochondria (80-83).

Many signalling pathways have been suggested to play a role in CD437 induced apoptosis in cancer cells. Studies have revealed that CD437 can trigger apoptosis through activation of the p38/MAP kinase pathway (67;84), the JNK/MAP kinase pathway (30;84), the ERK1/2/MAP kinase pathway (82), the NFκB pathway (5), or through down-regulation of the Akt signaling pathway (85).

In addition, CD437 has been shown to cause double-strand breaks in DNA, leading to p53 activation and apoptosis (86).

Other reports have pointed to the translocation of Nur77, to either the ER, mitochondria or the nucleus, as a mechanism through which the retinoid triggers apoptosis (45;82;84;87).

1.2.11 CD437s effect on the cell cycle; previous studies

Previous studies have shown that CD437 can cause cell cycle arrest, the nature of the arrest seem to be cell-line dependent (10;88-92).

A G_0/G_1 arrest was observed in human lung cancer cell lines. An elevation of the cell cycle inhibitor protein p21 was suggested to be critical for the cell cycle arrest, which occurred through a p53-independent mechanism (93). In human epidermal keratinocytes, CD437 was also reported to induce a G_1 arrest (89).

In prostate carcinoma cell lines, S-phase arrest has been observed following treatment with CD437, accompanied by elevated levels of E2F and increased degradation of cyclin B1 (88).

Studies on teratocarcinoma cell lines have shown that CD437 can cause a G_2/M cell cycle arrest, through a RAR-independent pathway (10).

2. Materials and methods

All reagents and materials described are listed under '8. Appendix'

2.1 Cell culture

The human melanoma cell lines, FEMX1 and WM239 have been used for all the experiments described in the master thesis.

The FEMX1 cell line originates from a lymph node metastasis in a patient with malignant melanoma, which was removed at the Norwegian Radium Hospital in Oslo. It was first implanted into a mouse, and grown as a xenograft cell line. These cells were later collected and grown *in vitro* as a continuous cell line; FEMX. The re-injection of FEMX cells into a nude mouse, have given rise to a new cell line called FEMX1.

The WM239 cell line was established by Meenhard Herlyn, The Wistar Institute, Philadelphia, USA, in 1981. This melanoma cell line originates from a metastasis, and is used with the kind permission from the Wistar Institute.

In order for the cells to grow *in vitro*, several factors have to be optimized to keep the conditions similar to the natural habitat of the cells *in vivo*. These are factors such as temperature, proportions of CO_2, growth factors and nutrition. The growth medium contains nutrients for the cells, ensures a correct pH balance, and provides the cells with external growth factors.
In order to keep the cell growth from becoming too dense (may cause premature aging in the cells), and to stop waste from the cells from building up, the cells must be split at regular intervals.

Both cell lines were maintained in RPMI-1640 media, containing 5% Fetal Calf Serum (FCS) and 2mM L-glutamine. The cells were incubated in a Sanyo CO_2 incubator with a temperature of 37°C, and humidified air containing 5% CO_2. The media were shifted and the cells split every 3rd day, after having reached a confluence of approximately 70-80%.

In order to split the cell culture, a mixture of Trypsin/EDTA (TE-buffer) was used to detach the cells. The old medium was first removed, and 2 ml TE-buffer added and removed, leaving a thin liquid film. The cells were then incubated at room temperature for approximately 2 minutes, whilst the cell bottle was tapped to mechanically loosen the cells.

2.1.1 Treatment with CD437

The synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) was purchased from Sigma-Aldrich™ at a stock concentration of 10mM, and diluted to 100µM in Dimethyl sulfoxide (DMSO) prior to the experiments.

When the cells were treated with CD437 for a maximum of 24 hrs, cells with a confluence of 60-70% (approximately $5x10^6$ cells) were split 1:2 a day in advance of the experiment. Prior to the longer lasting experiments (48 hrs and 72 hrs), cells were plated out a day in advance with respectively $7x10^5$ and $5x10^5$ cells.
The medium was preheated (37ºC) prior to all the experiments.

The FEMX1- and WM239 cell lines were treated with CD437 dissolved in DMSO (0.1µM or 1µM), for various time intervals (1, 3, 8, 24, 48 and/or 72 hrs). In addition, control cells, treated with DMSO alone, were given the same conditions as the cells treated with CD437, and harvested along with each experiment.

2.1.2 Preparation of protein lysate

Cells were harvested for protein analysis by gently scraping the bottle using a rubber policeman. The cell suspension was then centrifuged for 5minutes at 12000rpm in a cold centrifuge (4ºC). The medium was discarded, and cold PBS was added to wash the cells. The suspension was centrifuged, and the PBS removed. Newly prepared lysisbuffer (~100µL per sample, depending on the pellet size) (6. APPENDIX) was added to the cell pellet. The suspensions were sonicated to further lyse the cells and thus liberate the proteins.

2.1.3 Measurement of protein concentration

The protein sample (5µL) was diluted in 495µL H_2O. From this suspension 100µL and 200µL were mixed with respectively 700µL and 600µL of 1:100 lysis buffer. Bradford (200µL) was added to each of the protein dilutions. The optical density was measured at 595nm by the use of a Perkin Elmer Lambda EZ201 spectrometer. The protein concentration was then determined by the use of a pre-made standard curve.

2.2 Western blot

Protein lysates (50µg) from each sample were mixed with lysisbuffer and 6x loading dye to a total volume of 25µL each. The protein samples were further denatured by heating them for 3 minutes at 100ºC. Samples were then transferred on to a 12% Sodium Dodecyl Sulphate - polyacrylamide gel (SDS-PAGE), along with a commercial Full-range

Rainbow™ coloured molecular weight marker. The marker was added to one of the wells on the gel for the purpose of later comparing protein sizes.

The gel was set to run for approximately 3 hrs at 30mA/300V, in running buffer. The SDS-PAGE electrophoresis separates the denatured proteins based on their electrophoretic mobility. SDS binds to the proteins, giving them a negative charge. Once an electric field is applied to the gel, the proteins will migrate towards the anode. Shorter proteins will travel through the pores of the gel at a higher pace, whereas proteins with a higher mass will meet more resistance. Proteins of different sizes are thus separated.

The proteins from the gel were transferred onto a methanol-activated Immobilon-PVDF membrane, by blotting it in a wet transfercell (Bio-Rad Trans-blot DF) over night at 14mA/21V, in transfer buffer. The electric current is used to pull the proteins from the gel and on to the membrane. A solution of Naphtol-Blue- Black was used to stain the total protein content on the membrane to control for even loading.To block for unspecific bindings of antibodies, the membrane was incubated for 1 hr at room temperature, in TBS, 0.05 % - 0.25% Tween-20 with 5% w/v nonfat dry milk (TBST concentration depended on the primary antibody that was later used, see appendix). The membranes were then incubated with a primary antibody dissolved in either milk or BSA, and left on a shaker overnight at 4ºC. The membrane was washed 3x10minutes in TBST, before adding an appropriate HRP-linked secondary antibody (6.2 Antibodies used for western blot). Hybridization was allowed for ~ 45minutes at RT. The washing procedure was repeated. In order to detect proteins, the membrane was incubated at RT for 5minutes with ECLplus reagent (Amersham), and exposed using a film (Kodak). Horseradish peroxidase (HRP) from the secondary antibody catalyses light emission from the oxidation of luminol (ECLplus reagent).The emitted light was then detected on a film, and developed by the use of an AGFA Curix 60 photo developer.

2.3 Cell counting by the use of trypan blue

Dead or dying cells can be distinguished from living cells based on the uptake of trypan blue added to the medium. The cell membrane of the dying cell will be permeable to the dye, whereas uptake will not occur in the living cells.

To assay the amount of dead or dying cells, FEMX1 and WM239 cells were dyed using trypan blue, after treatment with 1μM CD437 for 24, 48 and 72 hrs. Cells not treated with CD437 were used as control. $5x10^4$cells per well were seeded in 24-well-plates, one day in advance of the treatment (control cells to be grown for 48/72 hrs were seeded $2x10^4$cells per well). After the respective treatment, cells were detached using trypsin/EDTA and collected along with the medium containing floating cells. The cell suspensions were centrifuged for 5minutes at 1200rpm, and the supernantant discarded. PBS was used to dilute the cells to a total volume of 500μL, before adding 50μL trypan blue (0.4% trypan blue in PBS). Both viable and trypan blue cells were counted, using a Bürker counting chamber.

2.4 CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS)

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)(MTS) method is based on a colorimetric principle that can be used to determine the percentage of viable cells. Viable cells contain active enzymes that can reduce MTS in the presence of phenazine methosulfate (PMS) into formazan, a water solvable molecule giving of a brown color that can be measured at 490nM. The amount of produced formazan is directly proportional to the number of living cells in the culture.
The MTS assay was used to measure a possible response in cell viability, with regards to various doses (μM) - and length of exposure (hrs) to CD437.
FEMX1 and WM239 were plated out at 5000 cells per well in 96well plates, and left to grow overnight. RPMI-1640 medium containing CD437 in DMSO was added to the cells, giving a desired concentration, DMSO alone was added to the control cells. To correct for the signal from RPMI-1640 medium was added to the empty wells on the plates. After a certain length of time, depending on the purpose of the assay, 40μL CellTiter 96® AQ_{ueous} One Solution, was added to each of the wells. The plates were set to incubate for approximately 1,5hrs, before measuring the absorbance at 490nm using ASYS UVM340 96-well plate reader.

2.5 Flow cytometry

Flow cytometry is a method where microscopic particles in suspension, such as cells and DNA, are counted and examined by passing them by an electronic detection apparatus (Figure 11). Traits such as a cell's complexity (side scattered light), size (forward scattered light) and fluorescence can be measured by the surrounding detectors, as the cell is passed through a light beam. The method can be used to measure fluorochromes attached to desired molecules in a cell (such as DNA or proteins). The fluorescens labelled molecules will emit light when excited with a laser beam with of a suitable wavelength (depending on the fluorochromes used). The emitted light will thus be proportional to the amount of labeled molecules in the cell.

The Terminal Transferase dUTP Nick End Labeling (TUNEL) is such a method for measuring fragmentation of DNA in a cell, a feature associated with apoptosis. An enzyme called Terminal deoxynucleotidyl transferase (Tdt) catalyzes the addition of dUTP where there are nicks in the DNA. A secondary marker (such as streptavidin-FITC) can again be used to label the dUTPs.

Fluorscent labelling can also be used to measure a cell's total amount of DNA, by stains such as Hoechst.

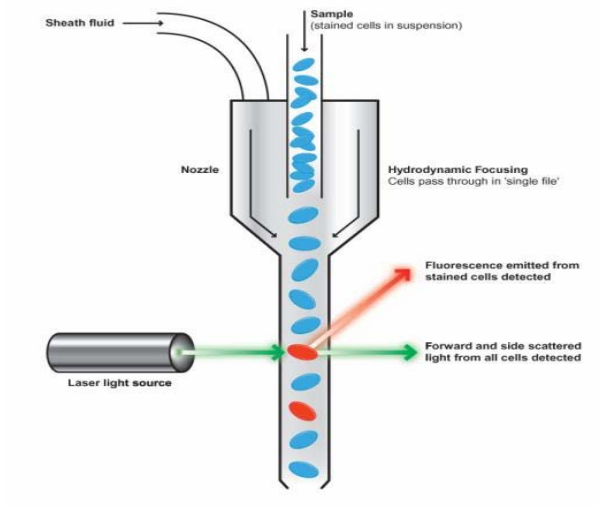


Figure 11. ***The flow cytometer***

A beam of light of a desired wavelength is sent through single cells in suspension. Surrounding detectors gathers information about the cell through detecting the scattered- and emitted light (94).

Cells were treated with 1µM CD437 or DMSO, and fixated in methanol after 24, 48 and 72 hrs of treatment. The cells were then washed 3 times in PBS, before adding 100µL newly prepared Tdt-solution to each sample (appendix). The Tdt-solution was left to work for 30 minutes, whilst the samples were incubated at 37ºC. The cells were then first washed once in PBS, before repeating the washing procedure twice using 0.1% Triton TX100 PBS. Trition TX100 in PBS was used for permeabilization of the cells prior to the immunostaining. Next, 100µL newly prepared Streptavidin-solution (appendix) was added to each sample. The cells were left to incubate at room temperature for 1 hr, whilst being sheltered for light. After the incubation, the cells were washed once in 0.1% Triton TX100 in PBS, before adding 500µL newly prepared Hoechst 33258 solution (appendix). Hoechst staining was allowed to occur for 30 minutes, whilst the cells were kept on ice (4ºC). Aggregates of cells were removed by filtration. All samples were kept on ice before being analyzed by the use of a LSR II UV laser flow cytometer.

2.6 Transient transfection with SiRNA

Transfection is a non-viral method for introducing foreign nucleic acids into a eukaryotic cell, such as small/short interfering RNA (siRNA). These are short double stranded RNA oligonucleotides consisting of 21-23bp, that for instance are involved in the RNA interference (RNAi) pathway. RNAi makes use of a natural defense mechanism in the cell, designed to protect against virus and transposons. The principle behind RNAi can be used to study the role of a protein of interest, by deliberately shutting down its expression by the use of designed sequences siRNA (95).

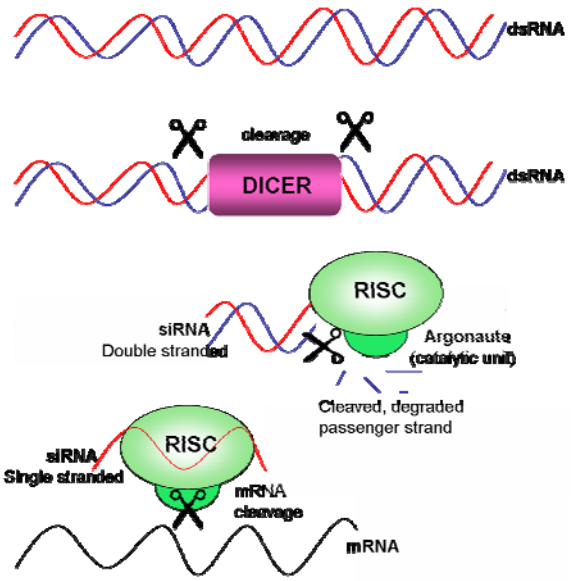


Figure 10. The main mechanisms behind RNA interference (RNAi)

A cytoplasmic ribonuclease, Dicer, cleaves a siRNA from a specific sequence of dsRNA. The siRNA attracts a complex of molecules called RISC. The catalytic compontent, Argonaute, causes the degradation of one of the strands in the siRNA. The RISC/siRNA complex can now recognise and bind to mRNA complementary to the siRNA, which the RISC can cleave (adapted from (95)) .

For the purpose of knocking down Nur77 (NR4A1), a transfection was performed using siRNA. Transfection was carried out using the Lipofectamine™ RNAiMAX method, a

commercially available reagent which alters the plasma membrane of the cells, making them more susceptible for taking up nucleic acids from the surrounding medium (96).
Cells (FEMX1 and WM239) were seeded ($1.5x10^4$ cells per well) in 6-well-plates a day in advance of the transfection.
The following day, master mixes were prepared containing either siNR4A1 (with the target sequence; 5'$_{GAGGAGAGAGCUUCCAUGCCUA}$3') or siCTR (containing a non-target sequence; 5'$_{GAGGAGAUCGACUUAGUACCGGCUA}$3') mixed with lipofectamine™RNAiMAX and Optimem®I. The master mixes were prepared according to the manufacturers protocol, giving a total concentration of 100nM siRNA per well. The cells were incubated for 48 hrs, and were then treated with 1μM CD437 for 24 hrs. The transfection efficiency was later tested by western blot analysis.

3. Results

3.1 Treatment with CD437 has an antiproliferative effect on FEMX1 and WM239 cells

It has previously been shown that CD437 has an antiproliferative effect in a number of cancer forms (97). To investigate whether CD437 can inhibit proliferation in FEMX1 and WM239, cells were treated with the retinoid and visually compared to control cells after 24, 48 and 72 hrs (Figue 12).

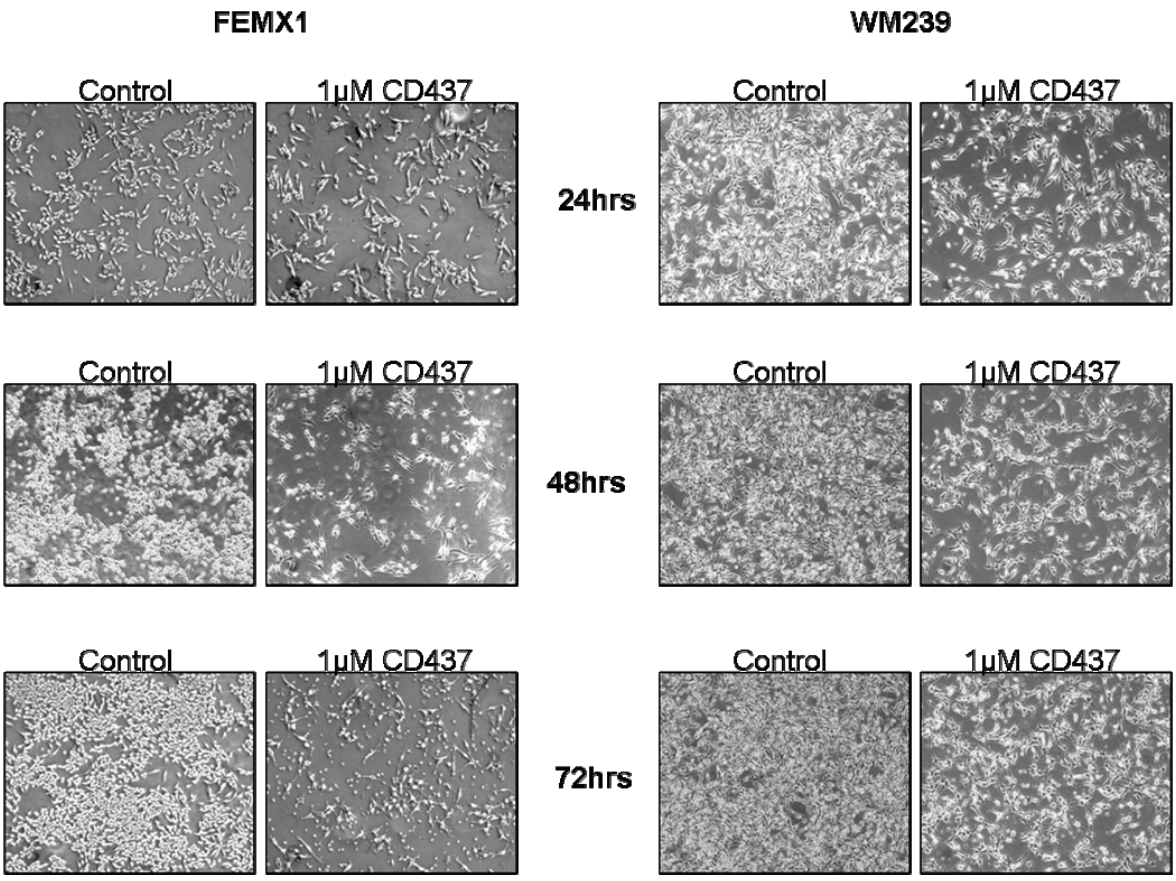


Figure 12. ***Antiproliferative effect of CD437.***

FEMX1 and WM239 cells were allowed to attach and grow for 24 hrs before treatment with 1µM CD437. The antiproliferative effects of CD437 were monitored at 24, 48 and 72 hrs of treatment, using a light microscope with 5x magnification.

Fewer cells were seen after treatment with CD437 in both cell lines, compared to the control cells. Furthermore, the difference got more pronounced after longer exposure. FEMX1 was seeded with a lower cell density than WM239 due to its rapid growth (Figure 12).

In order to quantify the effect of CD437, both treated and untreated cells were counted. To ensure that only living cells were included, dead cells were stained with trypan blue and could thus be excluded from the count.

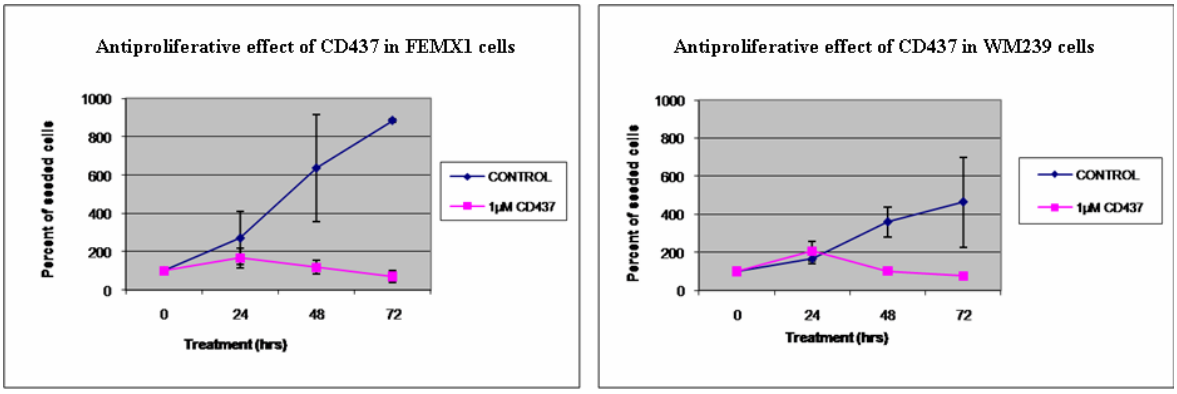


Figure 13. ***CD437s effect on proliferation in FEMX1 and WM239 cells.***

The cells were seeded in 24 well plates, and left to attach and grow for 24 hrs in advance of the treatment with 1µM CD437. Living cells were counted using a Bürker counting chamber, whilst excluding dead cells dyed with trypan blue. The amounts of living cells are plotted as percentages of seeded cells at 24, 48 and 72 hrs of treatment with CD437. The data are expressed as the mean of three independent biological experiments, with error bars showing standard deviation.

The results from the cell count were compatible with the results from the microscopical evaluation. In both FEMX1 and WM239, the control cells continued to proliferate whilst the treated cells staggered. The difference became apparent after 48- and 72 hrs, showing no overlaps in standard deviations of the control- and treated cells. After 72 hrs there were approximately 450% and 900% more control cells than originally seeded, whereas in the treated samples there were fewer cells (~70% and ~80%), in FEMX1 and WM239 respectively.

FEMX1 control cells appeared to proliferate approximately twice as fast as the WM239 control cells.

3.2 The effect of CD437 on cell viability is dosage- and duration dependent

To assess the abilities of CD437 to affect cell viability, in regards to duration and dosage of the treatment, an MTS analysis was performed.

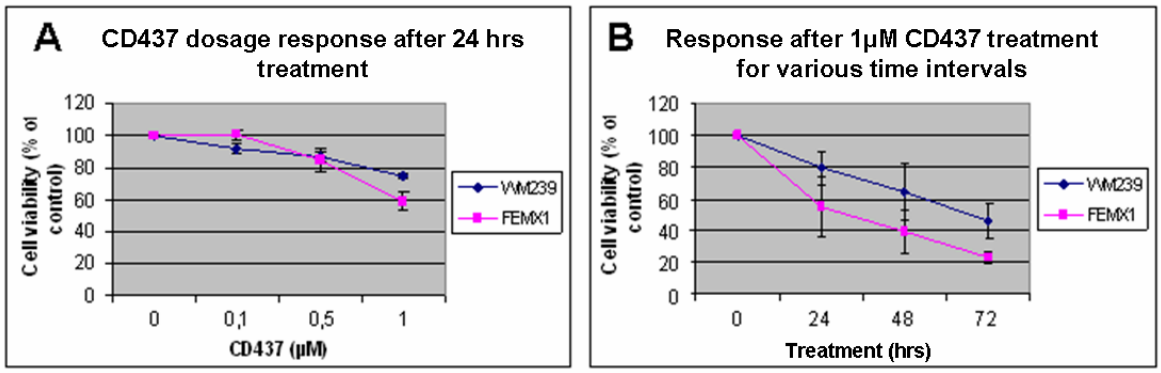


Figure 14. ***Treatment with CD437 reduces the amount of viable cells in a dosage (A) - and time (B) dependent manner.***

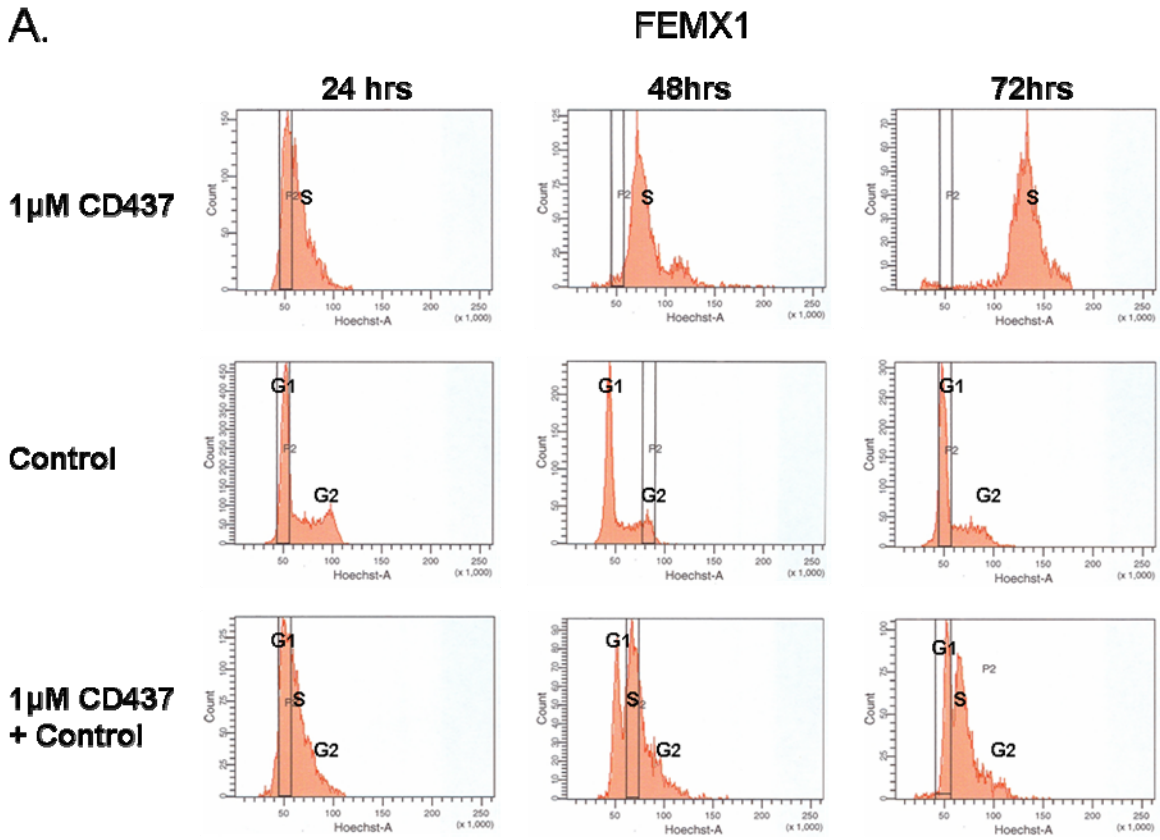
Five thousand cells were seeded per well in 96 well plates, and allowed to attach and grow for 24 hrs before treatment with CD437 or DMSO (control cells). An MTS analysis was used to determine the amount of viable cells. The changes in viability of the treated cells are presented as percentage of control cells. Data are expressed as the mean of three independent biological experiments, with error bars showing standard deviation.

Both cell lines exhibited a dosage-dependent reduction of viable cells after treatment with CD437 for 24 hrs. The FEMX1 cell line displayed a larger relative reduction of viable cells than WM239 cells, when exposed to CD437. Treatment with 0.1µM and 1µM for 24 hrs resulted in relative reductions of respectively none and 41% in FEMX1, and 8% and 25% in WM239 (Figure 14A).

When treated with 1µM CD437, both FEMX1 and WM239 showed a stronger relative reduction of viable cells when exposed for longer periods of time. The reduction was more profound in FEMX1, than in WM239. Treatment with 1µM CD437 for 24 and 72 hrs resulted in a relative reduction of respectively 45% and 77% in FEMX1, and 21% and 54% in WM239 cells (Figure 14B).

3.3 CD437 promotes G_1 and S-phase arrest

It can not be concluded from the MTS data whether the reduction in cell viability is due to an increased cell death and/or cell cycle arrest. To further investigate the effects of CD437, a flow cytometry analysis was performed to determine the impact on cell cycle distribution.



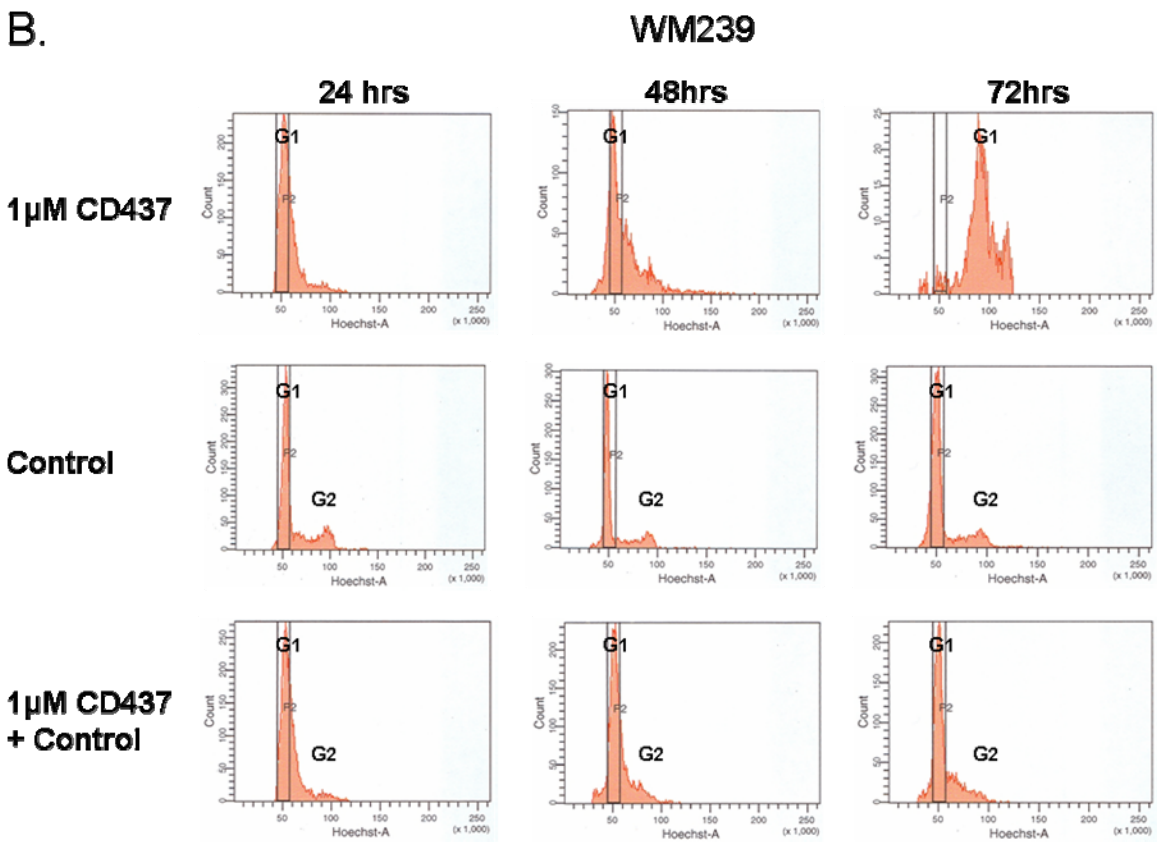


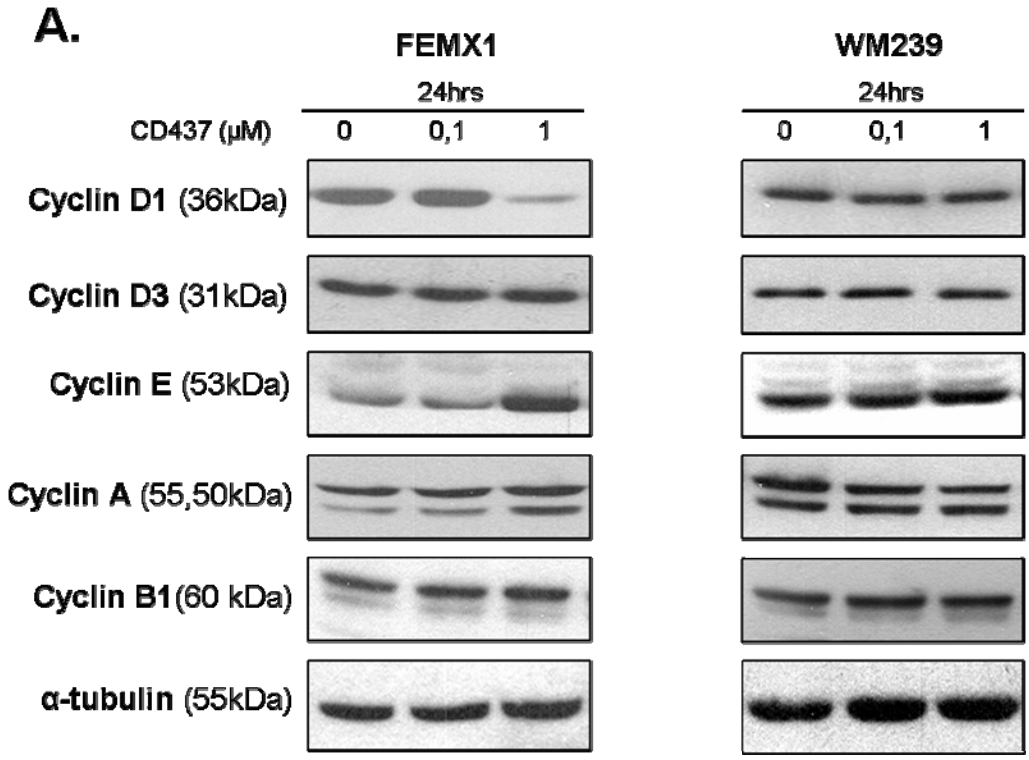
Figure 15. ***Treatment with CD437 induces S-phase arrest in FEMX1 (A) and G1 arrest in WM239 (B) cells.***

The cells were seeded in 75cm^2 bottles, and left to attach and grow for 24 hrs in advance of treatment with 1µM CD437 dissolved in DMSO, or DMSO alone (control cells). The cells were harvested after 24, 48 and 72 hrs exposure. The cells were counted (y-axis) in regards to their DNA content (labeled with Hoechst, shown on the x-axis), by the use of flow cytometry. The data presented are representative for the three individual biological experiments that have been conducted.

The treated cells contained fewer cells than the control cells, a difference attributable to the antiproliferative effects of CD437 previously shown. The graphs of the cells treated with CD437 are thus more ragged than those showing the control cells. In order to find the exact location of the distribution peaks in the treated cells, mixtures containing both untreated- and treated cells were analyzed.

The results from the flow analysis showed that FEMX1 cells have been arrested in the S-phase of the cell cycle. CD437 appear to arrest FEMX1 in G1/S phase after 24 hrs of treatment, whereas the S-phase arrest became apparent after 48 and 72 hrs.
Treatment with CD437 caused a G1-arrest in WM239; the arrest was evident after 24, 48 and 72 hrs.

Western blot analysis was used to further evaluate the effect of CD437 on the cell cycle by assessing the expression of various associated proteins.



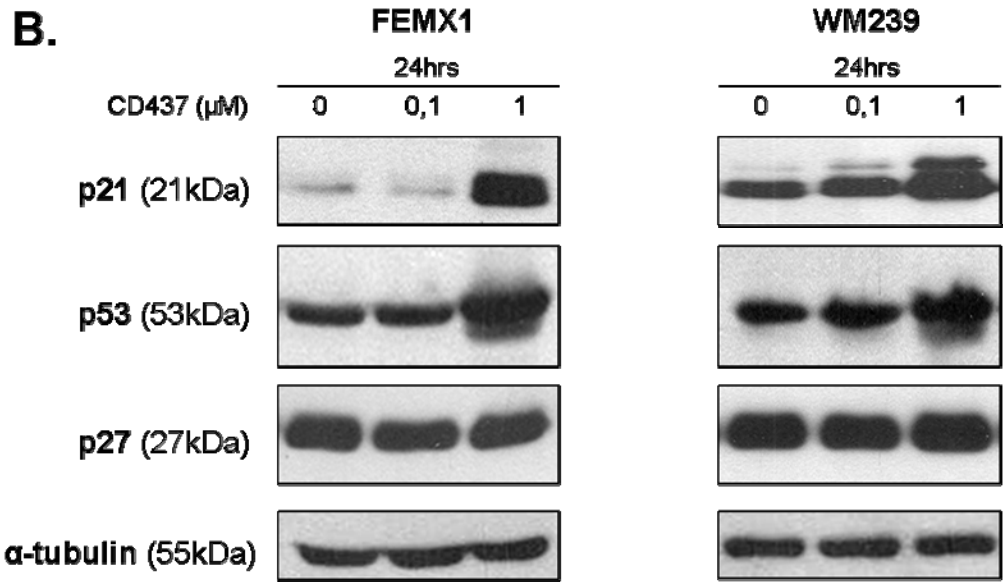


Figure 16. ***Effects of CD437 on the expression of cyclins (A) and some of the regulators of the cell cycle (B) in FEMX1 and WM239.***

The cells were seeded in 75cm^2 bottles containing RPMI 1640 media with 5%FCS / 2mM L-glutamine, and left to attach and grow for 24 hrs in advance of treatment with 0.1μM or 1μM CD437. Control cells were treated with DMSO alone, and given the same conditions as the cells exposed to CD437. Protein lysates were prepared as described under '2.0 Methods'. Western blot analysis was used to evaluate the effect of CD437 on protein expression. Representative loading controls (α-tubulin) have been enclosed. The data presented have been reproduced at least three times through independent experiments, yielding the same pattern.

Since it is well documented that the total protein level of cyclin dependent kinases (CDKs) remains relatively constant throughout the cell cycle, these proteins have not been tested (54).

In FEMX1 cells, a substantial increase of cyclin E and a marginal increase in cyclin A were seen after 24 hrs of treatment with 1μM CD437. Moreover, whereas a reduction in the cyclin D1 level was observed, the level of cyclin D3 remained unaffected. There were no changes in the expression pattern of any of the examined cyclins in WM239 cells.

Treatment with 1μM CD437 led to a prominent increase of p21 and p53 in both cell lines, whereas the protein expression of p27 was unaltered.

3.4 Treatment with CD437 causes cell death in FEMX1 and WM239 cells

Studies have shown that CD437 can cause cell death, allegedly apoptosis, in a variety of cell lines (1;5-8). To examine the retinoid's death promoting abilities, dead cells were stained with trypan blue. Both dead/dying- and living cells were counted using a Bürker counting chamber.

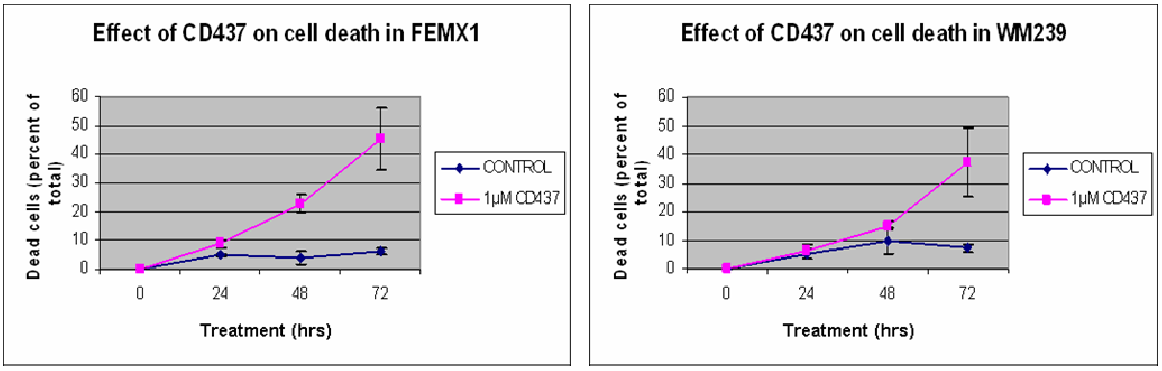


Figure 17. ***Effect of CD437 on cell death in FEMX1 and WM239 cells.***

The experiments were carried out as described under Figure (previous figure).

CD437 caused cell death in both FEMX1 and WM239, the relative percentage of dead cells increased with prolonged exposure. Seventy-two hours of treatment with 1µM CD437 resulted in 45% and 37% dead cells in FEMX1 and WM239 respectively.

3.5 Treatment with CD437 causes morphological changes in FEMX1 and WM239.

In order to monitor the effect of CD437 on morphology, treated- and control cells were viewed by the use of a light microscope.

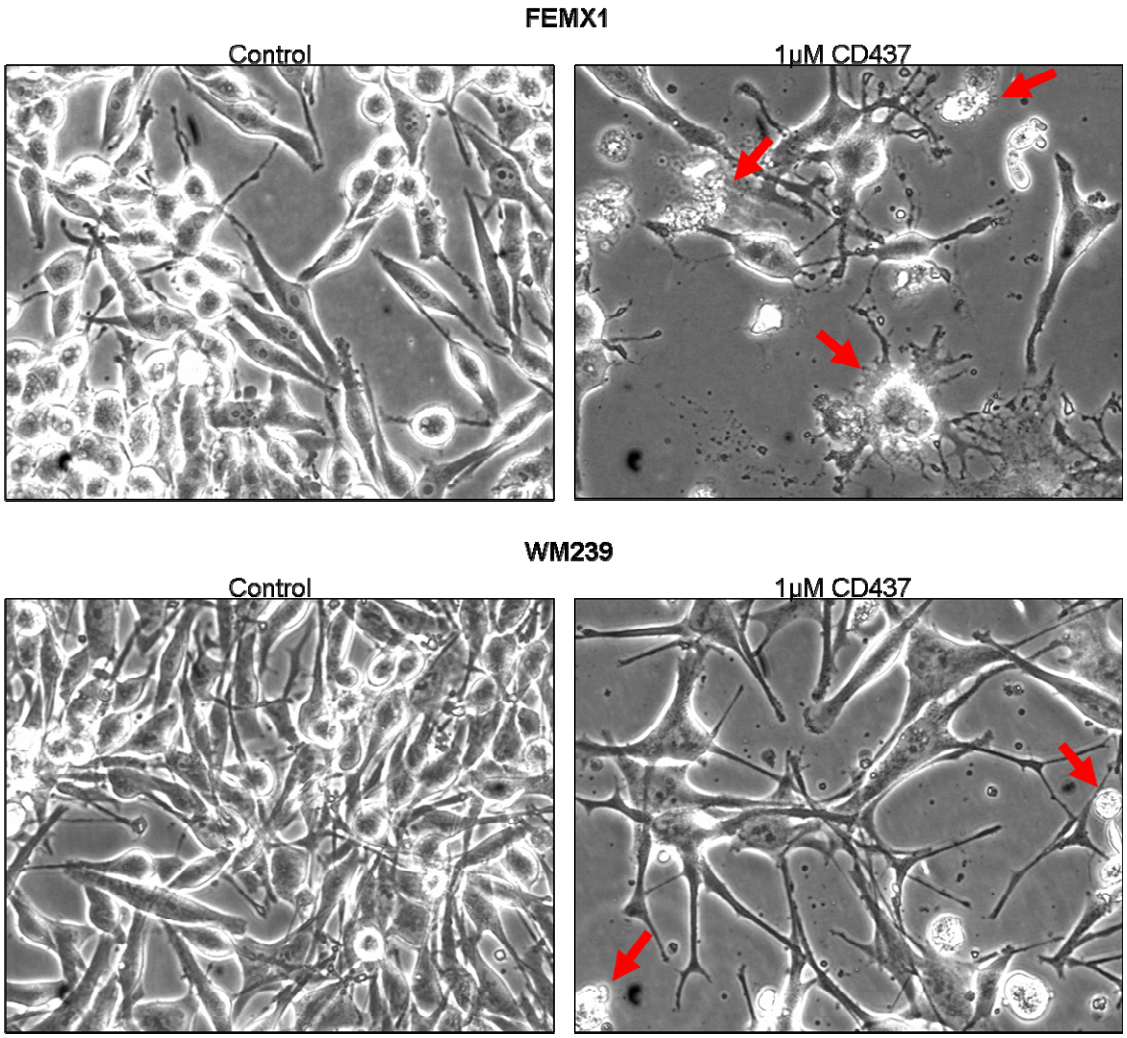


Figure 18. ***Effect of CD437 on cell morphology.***

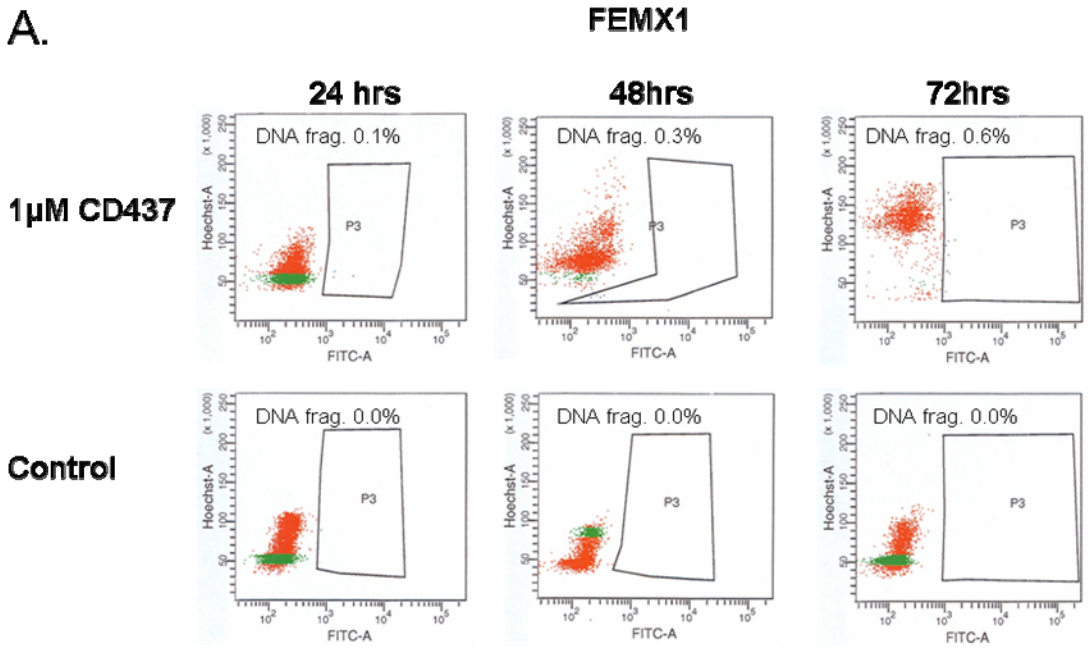
FEMX1 and WM239 cells were allowed to attach and grow for 24 hrs prior to treatment with 1µM CD437. The morphological features of the cells were visualized after 72 hrs of treatment, using a light microscope with 32x magnification.

Both cell lines showed morphological changes associated with apoptosis when treated with the retinoid. These changes included; expansion in overall cell size of some cells, an alteration in shape with an increase of pseudopodes (axon like arms going out from the cell bodies), as well as many small fragments floating around in the media. These fragments may be residues from dead cells (apoptotic bodies). Many smaller cells were

also seen (arrows), some showing signs of membrane blebbing and chromatin condensation. These cells were rounded up, and did no longer have pseudopodes.

3.6 DNA fragmentation in WM239 cells after treatment with CD437 indicates ongoing apoptosis

In order to assess whether the dead/dying cells found in the previous cell count were undergoing apoptosis, a TUNEL analysis was performed to determine DNA fragmentation. Fragmentation of DNA is a biochemical feature that can often be seen in apoptotic cells. Apoptosis might well happen without DNA fragmentation, hence this can not be used as an exclusive mean to define this type of cell death (35).



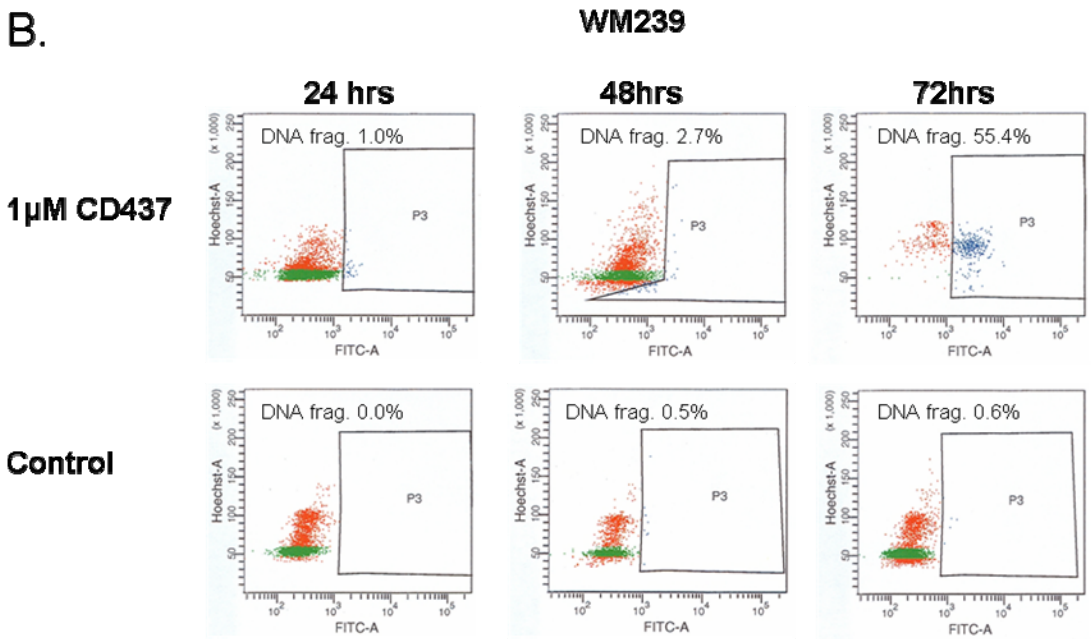


Figure 19. ***Effects of CD437 on DNA fragmentation in FEMX1 (A.) and WM239 (B.) cells.***

The cells were seeded in 75cm^2 bottles, and left to attach and grow for 24 hrs in advance of treatment with 1µM CD437 dissolved in DMSO, or DMSO alone (control cells). The cells were harvested after exposure to the retinoid for 24, 48 and 72 hrs. Potential DNA fragmentation was assessed by the use of the TUNEL technique. DNA strand breaks were determined by measuring the incorporation of fluorescein-labeled dUTP (x-axis), relative to the overall DNA content of the cells (y-axis). Percentage of cells found to have DNA fragmentation has been enclosed on the individual figures. The data presented are representative for the three individual biological experiments that have been conducted.

The TUNEL analysis gave no apparent increase of DNA fragmentation in FEMX1 cell after 24, 48, 72 hrs exposure to CD437. Fragmentation of DNA was on the other hand pronounced in WM239 after 72 hrs treatment with CD437, showing 55.4% of cells having DNA strand breaks. No prominent changes were seen in WM239 cells in regards to DNA fragmentation, when treated for shorter time intervals (24 and 48 hrs).

3.7 CD437 causes alterations in the expression of proteins involved in apoptosis

To further investigate the intracellular mechanisms behind CD437 mediated cell death in FEMX1 and WM239, expression of selected proteins involved in apoptosis was investigated using western blot analysis.

Caspases can be activated through cleavage from their inactive zymogen form, thus promoting apoptosis (98). Likewise, cleavage of PARP has also been reported as a marker for apoptosis (99).To investigate whether CD437 could cause caspase cleavage and/or PARP activation in FEMX1 and WM239, potential protein cleavage was examined using western blot analysis.

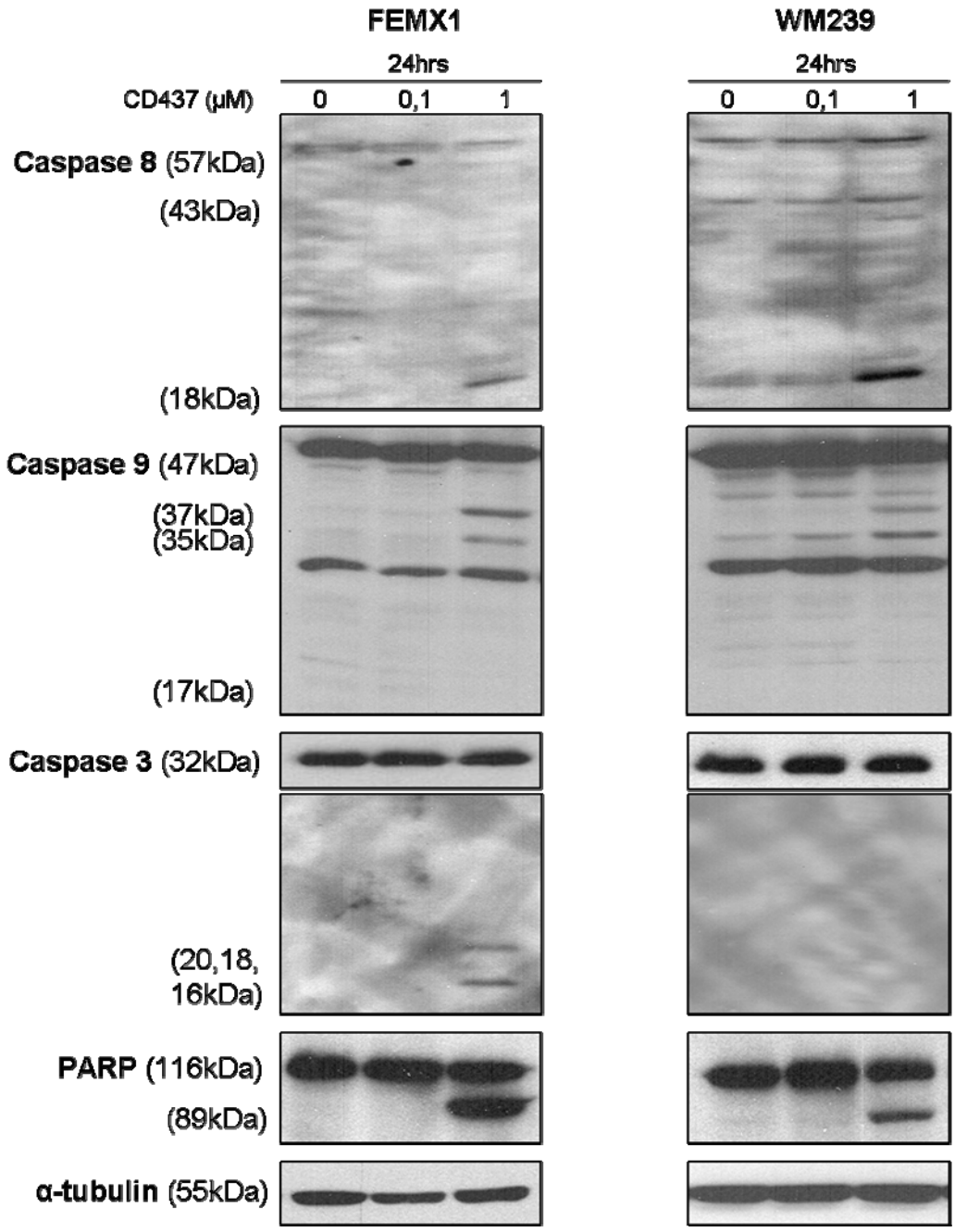


Figure 22. ***Effects of CD437 on caspases and PARP.***

The experiments were carried out as described under Figure 16).

Cleavage of PARP, as well as caspase 8, -9 and -3, was found in FEMX1 cells treated with 1μM CD437. The WM239 cells showed cleavage of caspase 8, caspase 9 and PARP, after 24 hrs of treatment with1μM of the retinoid. No cleavage of caspase 3 was

detected in WM239. Treatment with the lower dosage, 0.1μM CD437, yielded no detectable changes in protein expression in either cell line (Figure 22).

Activation of caspase 9 requires the release of cytochrome c from the mitochondria. The pro-apoptotic members of the Bcl-2 family are central in mitochondrial mediated apoptosis. Previous studies have shown that the expression of these proteins may be elevated as a response to treatment with CD437(76;100).

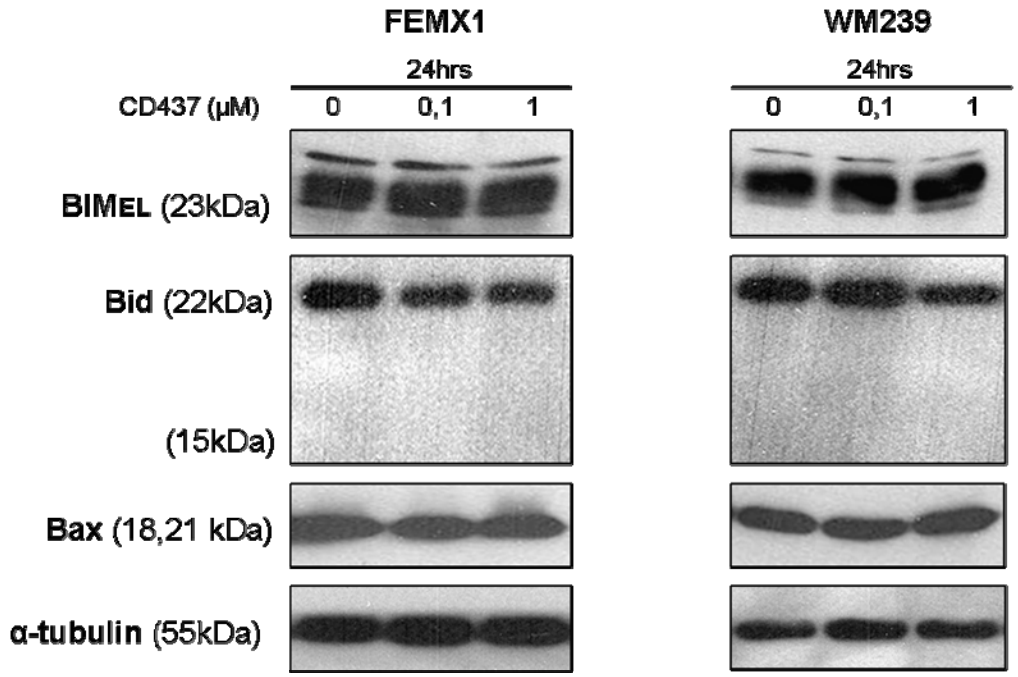


Figure 20. ***Effects of CD437 on protein levels of some of the pro-apoptotic members of the Bcl-2 family.***

The experiments were carried out as described under Figure 16.

No alterations in the overall protein expression were detected in any of the tested pro-apoptotic members of the Bcl-2 family. Cleavage of Bid was not observed in either cell line.

A reduction of the pro-apoptotic members of the Bcl-2 family may contribute to mitochondrial mediated apoptosis (39). In order to evaluate whether treatment with

CD437 could affect the expression of such proteins in FEMX1 and WM239, western blot analysis was again employed.

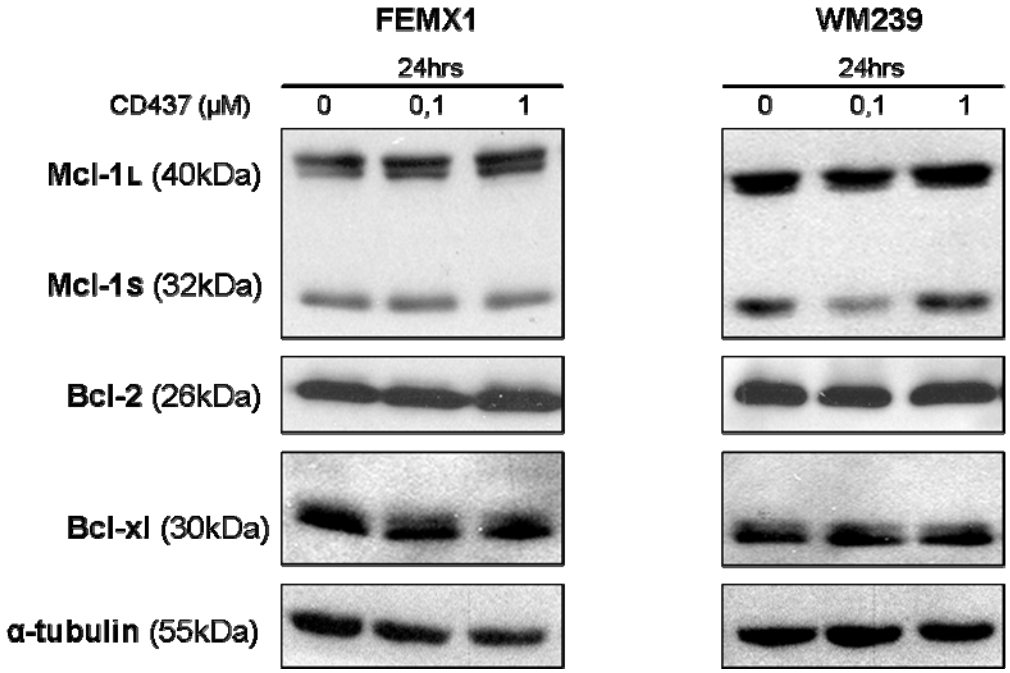


Figure 21. ***Effects of CD437 on protein levels of some of the anti-apoptotic members of the Bcl-2 family.***

The experiments were carried out as described under Figure16.

No major alterations in protein expression of the anti-apoptotic members of the Bcl-2 family were detected in either cell line, post treatment with the retinoid.

IAPs are known to inhibit both the initiator- and effector caspases in promoting apoptosis (101). Western blot analysis was conducted to determine whether treatment with CD437 could affect the protein expression of IAPs in FEMX1 and WM239 cells.

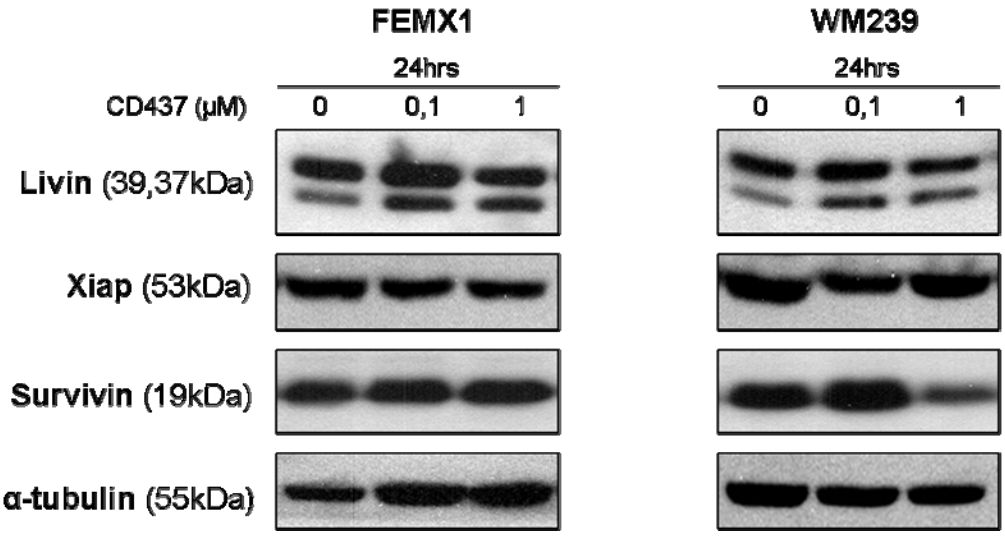


Figure 23. ***Effects of CD437 on protein levels of some of the members of the IAP family.***

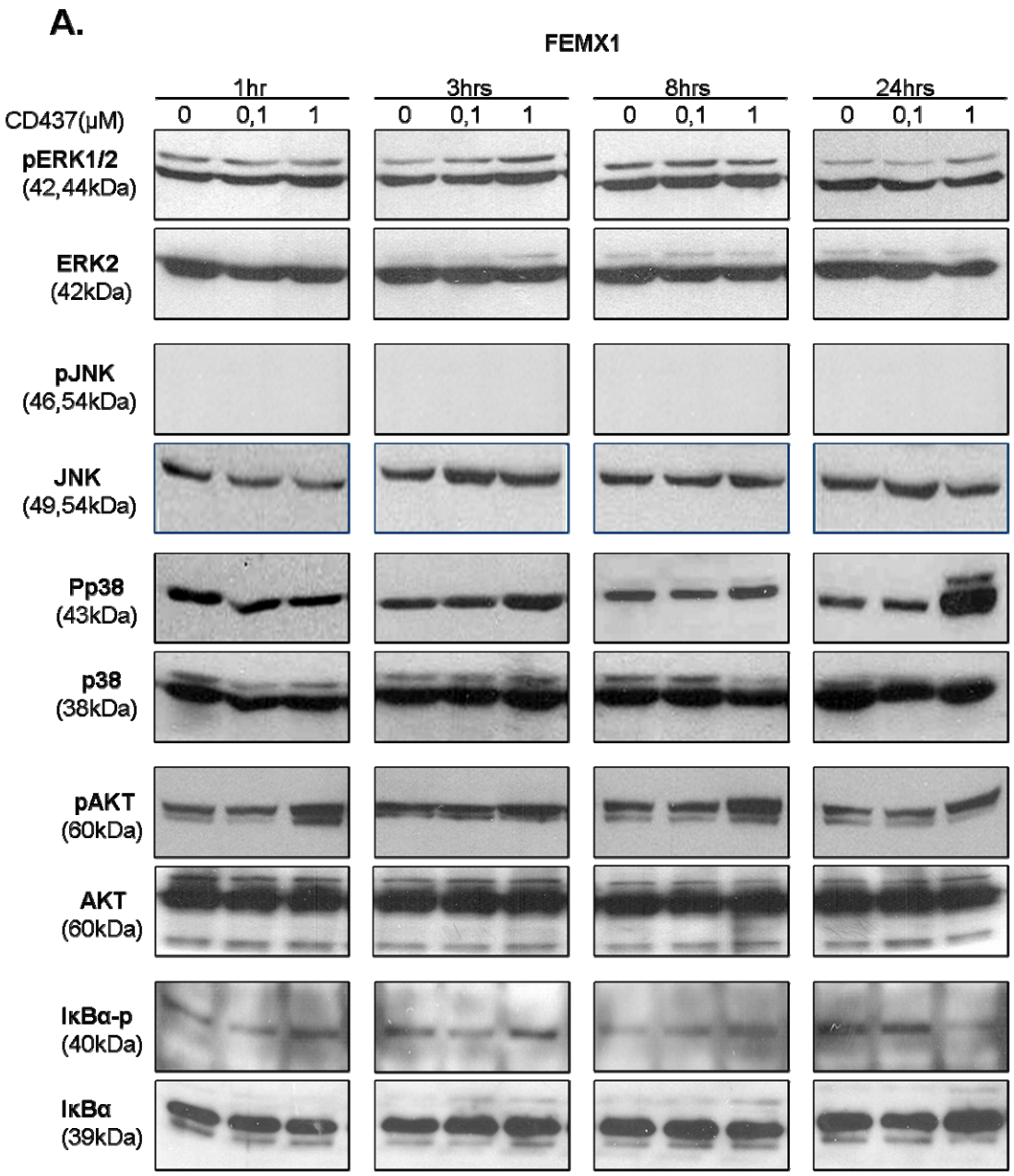
The experiments were carried out as described under Figure 16.

Exposure to 1µM CD437 for 24 hrs caused a decrease in the expression of survivin in WM239 cells. No other prominent changes were seen.

3.8 Signaling pathways are activated by CD437 in FEMX1 and WM239

As described in the introduction chapter, previous studies have shown that CD437 can trigger several intracellular signaling pathways (***1.2.10 CD437s effect on apoptosis; previous studies***) leading to apoptosis and/or cell cycle arrest. In order to elucidate whether CD437 activates any of the previously described signaling pathways, western blot analysis was performed measuring the level of both the phosphorylated and total expression of some key proteins.

Activation of signaling pathways may happen quickly after exposure to a ligand, hence the protein expression of various signaling molecules has been determined after 1, 3, 8 and 24 hrs of treatment with CD437.


A.
FEMX1
1hr
3hrs
8hrs
24hrs
CD437(μM)
0
0,1
1
pERK1/2
(42,44kDa)
ERK2
(42kDa)
pJNK
(46,54kDa)
JNK
(49,54kDa)
Pp38
(43kDa)
p38
(38kDa)
pAKT
(60kDa)
AKT
(60kDa)
IκBα-p
(40kDa)
IκBα
(39kDa)

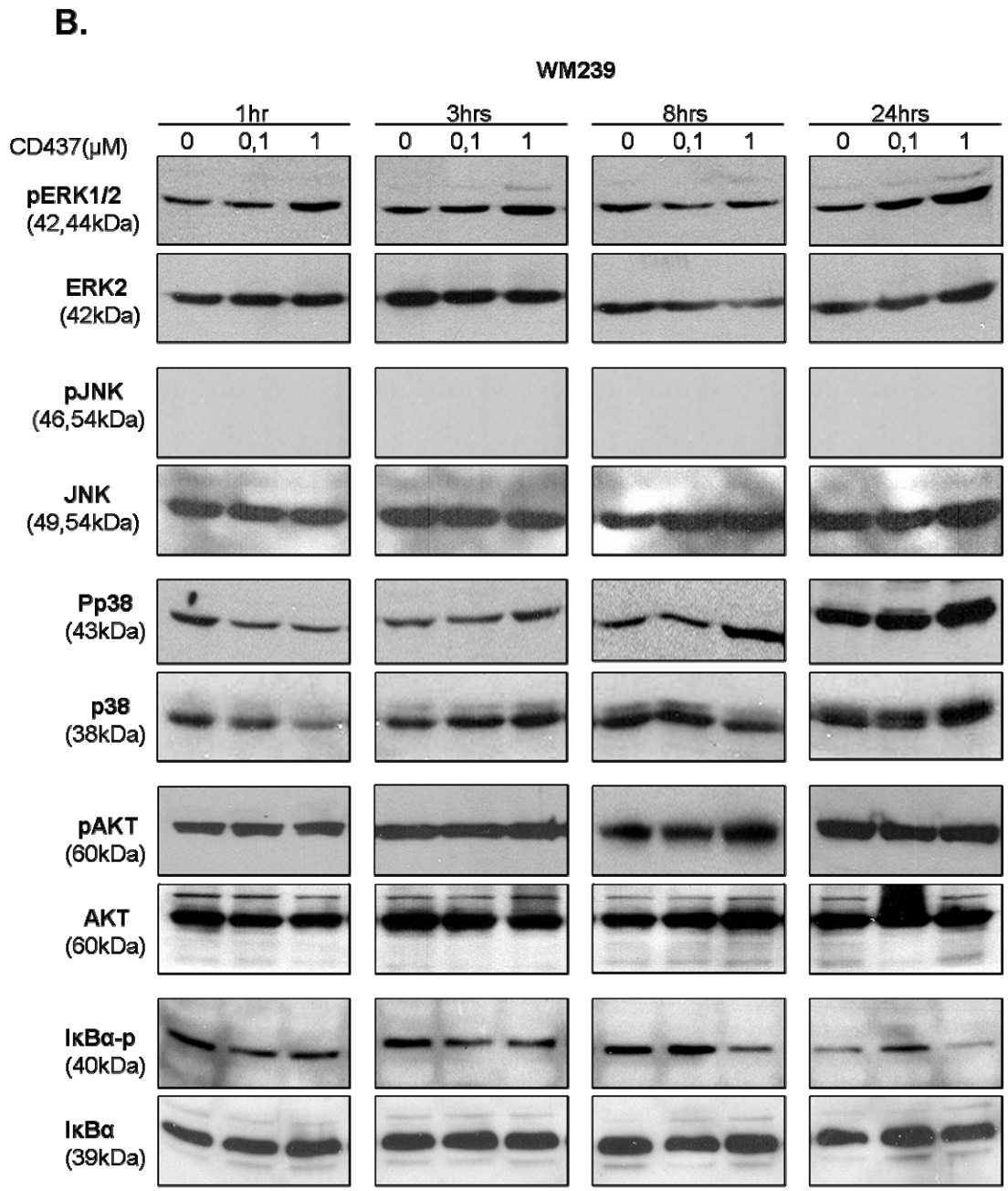


Figure 24. ***Effect of CD437 on the expression of a selection of key proteins involved in intracellular signaling in FEMX1 (A) and WM239 (B).***

The experiments were carried out as described under Figure 16. The cells were, however, harvested after 1, 3, 8 and 24 hrs treatment with CD437.

The overall protein expression of the signaling proteins has been enclosed, both as a loading control, but also to make sure CD437 does not affect the total expression of the protein.

Some of the phosphorylated proteins were weekly expressed (pJNK and IκBα-p). To ensure that this was not due to technical problems; positive controls were enclosed during the western blot analysis (not shown).

The results revealed an elevation in phosphorylated AKT after treatment with 1μM CD437 (1, 3, 8 and 24 hrs) in FEMX1. The level of activated p38 was marginally increased after 3 and 8 hrs and strongly increased after 24 hrs of exposure to 1μM CD437. No distinct alteration in protein expression could be detected in cells treated with 0.1μM CD437. These results indicate that treatment with 1μM CD437 has activated the PI3K- and the p38/MAPK signaling pathways in FEMX1 cells.

CD437 appeared to activate only the p38 signaling pathway in WM239. Thus, Pp38 expression was elevated after 3, 8 and 24 hrs of treatment with 1μM CD437. Treatment with lower concentration of CD437 (0.1μM), failed to produce any noticeable alteration in protein expression.

3.9 CD437 causes an increased expression of transcription factors in FEMX1 and WM239

Previous studies have revealed that CD437 alter the expression of transcription factors associated with proliferation and apoptosis (7;45;82;88;102). In order to elucidate whether exposure to CD437 can alter the expression of a selection of transcription factors, western blot analysis was performed.

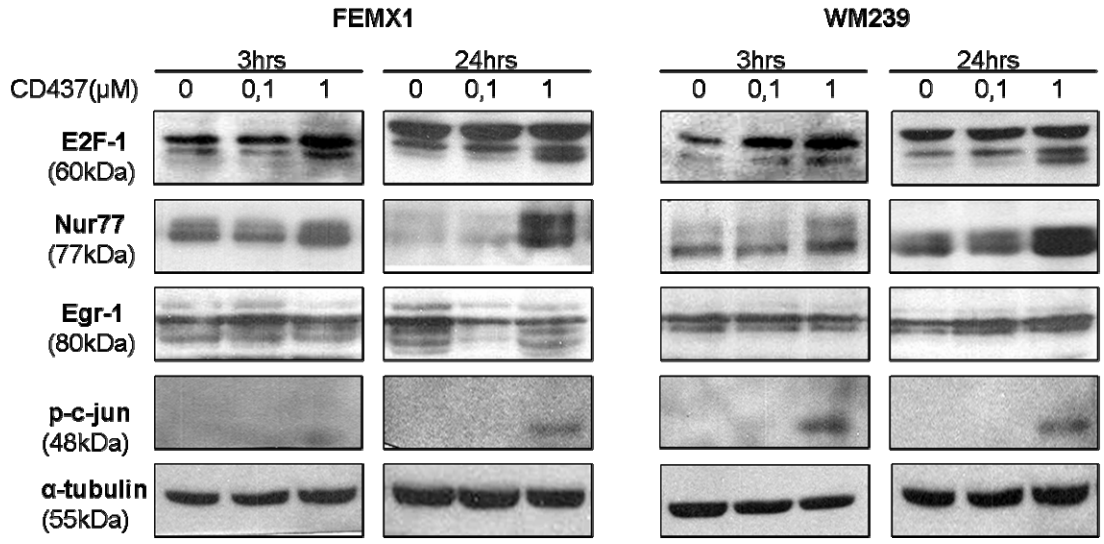


Figure 25. ***Effect of CD437 on the expression of a selection of transcription factors in FEMX1 and WM239***.

The experiments were carried out as described under Figure 16. The cells were, however, harvested after 3 and 24 hrs treatment with CD437.

In both cell lines increased protein expressions of Nur77, E2F-1 and p-c-jun were seen after 3 and 24 hrs treatment with CD437 (Figure 25).

3.10 Preliminary results suggest silencing of Nur77 may affect the expression pattern of other proteins following treatment with CD437

Treatment with CD437 resulted in an increase of the orphan receptor Nur77. A previous study suggests that CD437 induced apoptosis requires the expression of Nur77 (103). In order to study the effect of CD437 in the absence of Nur77, a siRNA transfection was performed to shut down its expression.

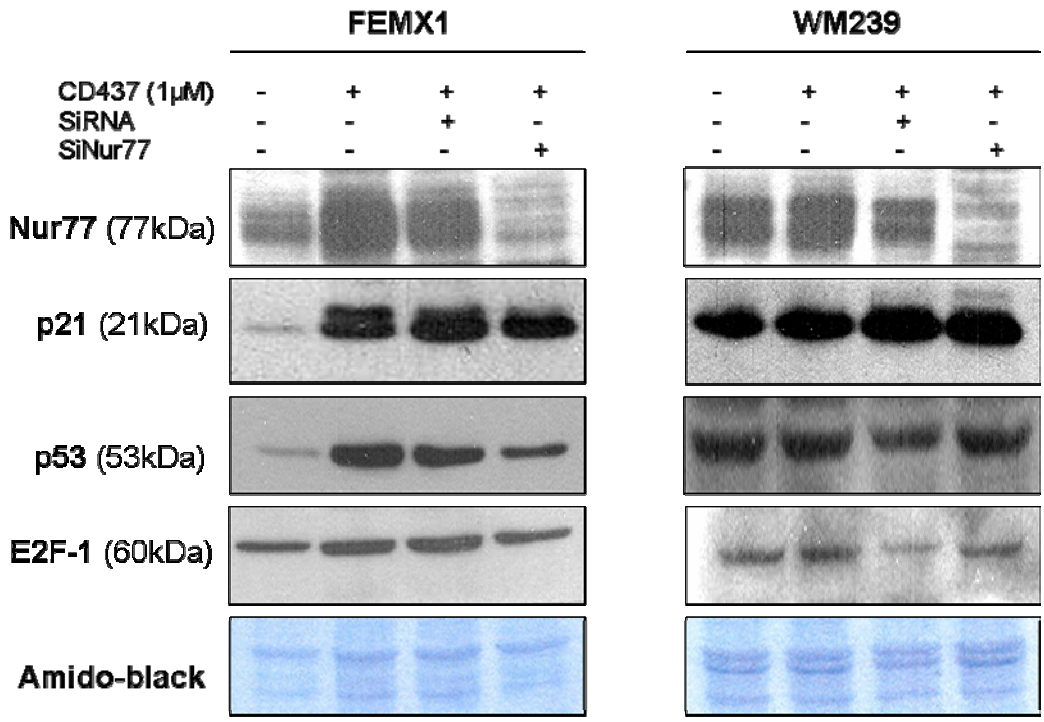


Figure 26. ***Effect of CD437 on the expression of various proteins, in the absence of Nur77.***

FEMX1 and WM239 were plated out at 5000 cells per well in 96well plates, and left to grow overnight. Transfections were carried out as described under '2.6 Transient transfection with siRNA'. Transfected- (SiNur77 or SiCtr) and control cells were allowed to transfect/grow for 48 hrs, before treatment with 1µM CD437/DMSO for 24 hrs. Protein lysates were prepared as described under '2. Methods'. Western blot analysis was used to evaluate the effect of CD437 on protein expression. Amido-Black staining has been enclosed as loading control. The data is preliminary.

Transfection with siNur77 effectively shut down the expression of Nur77. In FEMX1 cells treated with CD437, it appeared as the lack of Nur77 decreased the expression of p53 and E2F-1. No The WM239 cells, on the other hand, did not show any changes in protein expression following treatment with CD437, in regards to SiNur77. The results need to be reproduced before drawing any conclusions.

Due to lack of time, no proper loading control has been enclosed in this preliminary study, instead a copy the amido-black staining of total protein content on the membrane has been enclosed.

4. Discussion

Previous studies have shown that the synthetic retinoid, CD437, has a strong antiproliferative effect in many cell lines, an effect contributed to by both growth arrest and increased cell death (97). The results from our experiments with CD437 in the two melanoma cell lines are in accordance with these findings. In both cell lines, treatment with CD437 resulted in a marked reduction of cells relative to the control. The antiproliferative effect was first studied visually by the use of microscope. The findings were further confirmed by counting cells using trypan blue and the MTS assays. Both the MTS and the cell count showed the same tendency, with a growth inhibition relative to the control of respectively 77% and 92% in FEMX1, and 54% and 84% in WM239, after 72hrs treatment with 1μM CD437. The results from the MTS assay showed less growth inhibition than the cell count. A study comparing different methods for assaying proliferation suggests that MTT and MTS-based assays underestimates the antiproliferative effects, suggesting the importance of careful evaluation whilst using the method (104). Either way, all three proliferation assays performed showed a profound antiproliferative effect of CD437.
The growth inhibitory effect was demonstrated to be both duration and concentration dependent. Similar findings have been made in other studies (70;74;86;89;92;97;100;105;106). The effect was more pronounced in FEMX1 than in WM239 cells. FEMX1 proliferates approximately 50% faster than WM239, as seen in the control cells, and it may be speculated that CD437 is more effective in rapidly proliferating cells.

It has previously been shown that the antiproliferative effect of CD437 is, at least partially, due to cell cycle arrest (7;10;70;86;89;91;92;106-108). To further assess the antiproliferative property of CD437, in FEMX1 and WM239 cells, a cell distribution

assay was preformed using flow cytometry. Whereas FEMX1 cells gradually were arrested in S-phase, the WM239 cells were halted in G_1 upon CD437 treatment. In agreement with this, cell-dependent cycle arrests, mediated by the retinoid, have been suggested in several studies, as described in '1.2.11 CD437s effect on the cell cycle'. Treatment with lower concentration (0.1μM CD437) yielded little effect, in regards to alterations in cell cycle distribution (not shown). Similar results have also been found in human gastric cancer cell lines treated with 0.1μM CD437, suggesting a higher dosage is needed for an arrest to occur (91).

It must be stressed that there is a certain improvement potential to the technique used in our study, in regards to assessing cell cycle distribution. Due to the antiproliferative effects of CD437, a discrepancy in the relative amount of cells in the control and treated samples emerged. Prior to the dying procedure it would have been favorable to count the cells in order to make suspensions with an approximately equal amount of cells. The uneven concentration of cells in the different samples made it difficult to compare the relative location of the distribution peaks. In samples with fewer cells, peaks were shifted further to the right due to differences in voltage, compared to samples with larger quantities of cells (Trond Stokke, personal communication). To surpass this problem, approximately equal amounts of control cells and treated cells were mixed, thus making it possible to recognize the relative location of the CD437 mediated arrests.

In addition, the low concentration of cells in the treated samples caused a limited cell count, and thus more ragged curves. Never the less, the cell cycle arrests could clearly be distinguished.

To further elucidate the mechanisms explaining the observed cell cycle arrest, expression of different cyclins was assessed using western blotting.

Treatment with CD437 for 24 hrs resulted in a decrease in cyclin D1 in FEMX1, a finding suggesting G_1-arrest or delayed progression through the G_1-phase. The observed increased expression of cyclin E and A following CD437 treatment are somewhat surprising as these cyclins are generally associated with the G_1/S and S-phase progression (54). However, in accordance with our findings, CD437 mediated S-phase arrest in hepatoma cells was accompanied by an elevation in cyclin A (100). In addition, a

decrease in cyclin D has been found following treatment with CD437 in other cells arrested in S-phase (8). As opposed to our findings, CD437 has been shown to either increase/decrease the expression of cyclin B/B1 respectively, whilst simultaneously halting the cells in S-phase(88;109). Thus, the exact role of cyclins in CD437-mediated S-phase arrests has not been determined.

In contrast to FEMX1 cells, the G1-phase arrest observed in WM239 cells was not accompanied by changes in the overall protein levels of cyclins, suggesting cell line specific differences. Cell cycle arrests may have other causes than alterations in cyclin, as will be further discussed below.

In addition to changes in the expression of cyclins, an increase in the levels of the transcription factor E2F-1 has been reported to accompany CD437-mediated S-phase arrest (88;92). The tumor suppressor pRb guards the checkpoint between the G_1/S phases, by suppressing the transcription factor E2F (which comes in several isoforms, including E2F-1). E2F-1 is released from pRb in mid- to late G_1 phase, and can thus transcribe genes that contribute to the entry into S phase. Once the cells have entered S-phase, the cyclin A/CDK2 complex will bind to E2F-1, by so hindering its DNA binding function. A deregulation in the binding between cyclin A/CDK1 and E2F-1 may cause an S-phase arrest (110). Treatment with CD437 has previously been shown to increase the E2F-1 stability, thus causing an up-regulation in its overall protein expression, and a subsequent S-phase arrest (88). In accordance this, treatment with CD437 caused an increase in the expression of E2F-1 in FEMX1 cells.

It is well documented that an increased expression of CKIs may cause the cell cycle to halt. The expressions of p27 and p21 were thus evaluated following treatment with CD437. In both FEMX1 and WM239 cells, a marked elevation of p21 was detected, whereas p27 remained unaltered. The cell cycle regulator p21 can bind to CDK 2/4/6, and by so inhibit their action, causing the cell cycle to halt. An increase in p21 protein expression has previously been associated with both S-phase- and G1 arrests, mediated by CD437 (8;70;91;92;100;108;109).

p53 is another important regulator of the cell cycle, and has been shown to activate transcription of p21 (60). An elevation in p53 levels can be seen in response to DNA

damage, and has been found to arrest cells (111;112). Following treatment with CD437, a clear increase in the expression of p53 was detected in both FEMX1 and WM239, providing a possible explanation to the elevated levels of p21. In agreement with this, p53 has previously been suggested to cause cell cycle arrest in cells treated with CD437 (70). On the other hand, it should be noted that induction of p21 may also occur in a p53-independent manner, thus suggesting that other regulatory proteins may be involved (113). In accordance with this, elevation of p21 following treatment with CD437 has been reported to be independent of p53 in human lung cancer cell lines (114).

The relative reduction of cells following the exposure to CD437 can thus be, at least partially, explained by cell cycle arrest. However, studies have previously suggested that treatment with CD437 can trigger apoptosis in a variety of cancer cell lines (1;5-9;66-70;73-77;84;86;87;89;91;92;100;106;108;109;115-119). In order to elucidate whether the antiproliferative effect of CD437 could also involve increased cell death; a cell count using trypan blue was performed. Treatment with 1μM CD437 for 72 hrs resulted in respectively 45% and 37% dead cells in FEMX1 and WM239.

Trypan blue dyes cells with defective cell membranes, yet the method is also used to evaluate apoptotic cells, which per definition have intact membranes. Cells undergoing apoptosis *in vivo* will usually be engulfed by circulating phagocytes or neighboring cells, due to the exposure of phosphatidyl serine on the cell surface. When apoptotic cells take up trypan blue from the medium, it is usually due to cells undergoing a process of 'secondary necrosis', as a response to not being eaten (120). Thus both necrotic and apoptotic cells can potentially be dyed with trypan blue, however, the amount of apoptotic cells might be underestimated.

To elucidate whether the dying cells are undergoing apoptosis, measurements of its biochemical and morphological features must be employed. The term 'apoptosis' describes cells with morphologically similar features, however it should be kept in mind that different subgroups may display diverse biochemical features (35). At least two independent assays should therefore be employed to determine apoptosis, yielding a similar result.

The effect of CD437 on cell morphology was visually evaluated by the use of a light microscope, in collaboration with an experienced pathologist. Cells displaying morphological features associated with apoptosis were found in both FEMX1 and WM239, such as; rounding-up of the cell, retraction of pseudopodes, reduction in cellular volume, plasma membrane blebbing and signs of chromatin condensation. These results suggest that CD437 can induce apoptosis in our cell lines.

Oligonucleosomal DNA fragmentation is a morphological characteristic of apoptotic cells. It is, however, not an exclusive requirement for apoptosis, cells have been found to undergo apoptosis without showing signs of DNA breakage (35).
Never the less, the DNA fragmentation, as assessed by TUNEL assay, found in WM239 after 72 hrs exposure to CD437, indicates an on going apoptotic process. Fragmentation of DNA following treatment with CD437 has been reported in other cell lines (106;118). To our surprise, the TUNEL assay showed no DNA fragmentation in FEMX1. There might be several explanations to this. As discussed over, apoptosis might occur without DNA fragmentation, although this is more an exception than the rule. Interestingly, a previous study using FEMX cells (the progenitor of the FEMX1 cell line) showed no increase in FITC intensity after treatment with an immunotoxin. The cells did, however, display a range of other apoptotic features (121). Thus, it might be possible for apoptosis to occur without DNA fragmentation in this cell line. Another explanation to the missing DNA fragmentation could be technical problems, although this seems unlikely. The results have been reproduced three times, whilst simultaneously yielding an increase in FITC intensity in the treated WM239 cells. The FITC-positive WM239 cells might thus work as a positive control for the method.
Another way of detecting apoptosis in cells is to examine the expression and activity of key components involved (35). The caspases are known as the executers of apoptosis. These cystein proteases are synthesized as zymogens that upon cleavage can be activated (122). The presence of proteolytically active caspases can be used as a marker for apoptosis; however, it can not be used as an exclusive means to define it (35). To further elucidate whether CD437 could trigger the intrinsic pathway of apoptosis, western blot analysis was used to examine a potential cleavage of caspase 9. Activation of caspase 9

requires the release of cytochrome c from the mitochondria, and hence MOMP (41). In both cell lines, activation of caspase 9 was found after 24 hrs of treatment with 1μM CD437. These results suggest that cytochrome c must have been released from the mitochondria, which again indicates that CD437 can activate the intrinsic pathway in our cell lines.

A downstream target of caspase 9 is caspase 3, an effector caspase responsible for executing many of the apoptotic changes in the cell (41). Following treatment with CD437, activation of caspase 3 was found in FEMX1 cells. Surprisingly, WM239 showed no signs of caspase 3 cleavage. One of the targets of caspase 3 is the DNA repair enzyme, PARP. The activation level of PARP depends on the mechanism triggering it. Mild cases of DNA damage lead to activation of DNA repair mechanisms, whereas severe damages induce over-activation of PARP and thus necrosis. Active apoptotic proteases, such as caspase 3 and -7, can on the other hand, deactivate PARP by cleaving it, thus inhibiting its DNA-repairing abilities. Cleavage of PARP can hence be used as a marker for apoptosis (99;123). A profound cleavage of PARP was seen following treatment with CD437 in both cell lines. The PARP cleavage found in WM239 suggests that another apoptotic protease, such as caspase 7, must be active in these cells. In consistency with these results, previous studies have also reported cleavage of caspases and PARP, following treatment with CD437 (70;92).

CD437 has previously been shown to induce apoptosis through activation of caspase 8 (124). In consistency with these results, activation of caspase 8 was seen following treatment with CD437 in both FEMX1 and WM239. Cleavage of caspase 8 is associated with activation of death receptors, and thus activation of the extrinsic pathway of apoptosis (122). It has previously been found that p53 may trigger the extrinsic pathway by stimulating the transcription of Fas and DR5 (death receptors) (125). Hence, it can be speculated whether the observed increase of p53 may sensitize the cell to extracellular death signals, leading to the activation of the extrinsic pathway and a subsequent caspase 8 activation. Studies have on the other hand suggested that caspase 8 activation might be induced by ER stress, and not exclusively through the binding of a death ligand (126). Either way, the cleaved caspase 8 may now activate caspase 3/7.

Activation of caspase 8 may also activate the pro-apoptotic Bcl-2 member, Bid, which upon cleavage can trigger the intrinsic pathway of apoptosis (127). Cleavage of Bid following treatment with CD437 was not observed in either of our cell lines.

The Bcl-2 family consists of both pro- and anti-apoptotic proteins, a shift in their relative balance can lead to the activation of the intrinsic pathway of apoptosis (128). Previous studies have shown that CD437 can trigger MOMP and thus the intrinsic pathway (73;76). In order to investigate the effect of CD437 on the protein expression of various members of the Bcl-2 family, western blot analysis was performed. No marked alterations were found in either cell line, in regards to the tested pro- and anti-apoptotic Bcl-2 proteins expression. It should be noted that the Bcl-2 family consists of several other proteins that have not been examined, such as Bcl-w, Ai, Bcl-B, Bak, Bik, Bmf, Hrk, Noxa and Puma. It should be mentioned that several of these factors can be induced by p53 (43).

As mention earlier, none of the tested members of the Bcl-2 family displayed any pronounced alterations following the treatment. This might, however, not be the actual situation, not all Bcl-2 members have been examined, as well as the apoptotic shift leading to the MOMP might have occurred earlier. To present an alternative explanation; a study has shown that the orphan nuclear receptor, Nur77, may transform the Bcl-2 in to a ‘killer’, thus causing release of cytochrome c (129). An increase of Nur77 was seen in both cell lines following treatment with CD437. Yet another possibility; several ER membrane proteins have been shown to interact with the anti-apoptotic members of the Bcl-2 family, thus reducing their anti-apoptotic activities and causing a subsequent release of cytochrome c from the mitochondria (130;131).

Following these scenarios, the protein-protein interaction would not cause any shift in the overall protein expression of the anti-apoptotic Bcl-2 members.

IAPs are anti-apoptotic proteins which may bind to caspases, thus inhibiting their activation. Members of the IAP family, such as survivin, livin and xiap, may be inhibited by apoptotic proteins released following MOMP (42). Treatment with CD437 resulted in a decrease of survivin in WM239. To our understanding, no such result has previously

been reported following treatment with CD437. However, the finding is in consistency with the on-going apoptosis in the WM239 cells.

Whilst central in the regulation of the cell cycle, the tumor suppressor protein p53 also plays an important role in triggering apoptosis (132). As previously discussed, both our cell lines displayed a marked increase of p53 following exposure to CD437. The expression of p53 is under normal conditions kept low. The protein may, however, be activated in response to several stressful situations, leading to its stabilization and accumulation in the cell (132). p53 mainly mediate apoptosis through the intrinsic pathway. It works both as a transcription factor for the pro-apoptotic genes, as well as by hindering the transcription of anti-apoptotic proteins, such as Bcl-2 and IAPs (44). Additionally, p53 alone, has been shown to directly target the mitochondria causing a MOMP (43). The increase of p53 found in our cells in response to treatment with CD437, might thus be a possible explanation to the MOMP and subsequent activation of caspase 9. Previous studies have reported that the involvement of p53 in CD437 mediated apoptosis is cell line specific (68;70;86).

Transcription factors are proteins that may bind to specific sequences of DNA and thereby control the transcription of certain genes. Depending on the target genes, transcription factors may have an influence on apoptosis (133). A previous study on melanoma cells suggests that CD437 may induce apoptosis and growth inhibition through the activation and up-regulation of the transcription factor complex AP-1 (7). Members of the ATF, c-jun and c-fos protein families are involved in formation of the dimeric AP-1 complex, giving rise to more than 20 different complexes. The composition of proteins both associated with- and directly involved in the AP-1 complex determines its effect, such as proliferation, apoptosis or terminal differentiation (134). In order to evaluate the effect of CD437 in regards to AP-1 activation, protein expression of p-c-jun (one of the three members of the c-jun family) was examined in FEMX1 and WM239 cells. An increase of p-c-jun was found in both cell lines after treatment with the retinoid. Since the transcriptional activity of c-jun is regulated by phosphorylation (135), the increase of p-c-jun thus suggests that CD437 may up-regulate its activity in our cell lines. Up-regulation of c-jun has been suggested to induce apoptosis in many cell lines (136). On the opposing

side, the transcriptional activity of AP-1 usually leads to increased proliferation. Thus the antiproliferative effect of many retinoids is thought to be mediated through their anti-AP-1 activity, which is thought to involve RARs (102). Exactly how activation of AP-1 complexes mediated by CD437, affects FEMX1 and WM239 remain uncertain, even if the retinoids overall effect is anti-proliferative.

The orphan receptor Nur77 has been reported to have a central role in CD437 mediated apoptosis (43;45;82;87;137-139). The apoptotic effect of Nur77 is determined by its subcellular localization. In addition, its ability to induce death is dependent on both the cell type and death stimuli (43). Nur77 may bind to DNA as a monomer, homodimer or heterodimer, thus increasing the transcription of genes, which are usually associated with survival (43;140). However, studies have shown that Nur77 can, upon the heterodimerization with RXR, translocate to the cytoplasma where it interacts with the mitochondria (141). Translocated Nur77 can bind to Bcl-2, causing a conformational change which turns Bcl-2 into a pro-apoptotic molecule, thus triggering the release of cytochrome c (142). As previously discussed, an increase of Nur77 was observed following treatment with CD437 in FEMX and WM239.

In order to explore the effect of Nur77 following treatment with CD437, its protein expression was shut down by the use of siRNA transfection. The lack of Nur77 did not affect the expression of p21 following treatment with CD437, which indicates that Nur77 is not responsible for the induction of p21. Interestingly, the transfected siNur77 prevented up-regulation of p53 following CD437 treatment in FEMX1. This result is preliminary; however it suggests that Nur77 may have an influence on the expression of p53. E2F-1 may provide a link between Nur77 and p53; Nur77 has been shown to induce the expression of E2F-1 (143), whereas E2F-1 indirectly affects p53 by up-regulating proteins involved in stabilizing and activating p53 (144). To further explore this possibility, the expression of E2F-1 was determined in the treated siNur77 sample, yielding a week decrease in E2F-1 in FEMX1 cells. These results may suggest a possible connection.

The treated siNur77 cells showed a simultaneous decrease of p53, whilst p21 remains unaltered, suggesting that induction of p21 is p53-independent. It should be stressed that

the results need to be reproduced. They have however been enclosed, as the findings may provide new insight in the role of Nur77 in the anti-tumor effects of CD437.

Several signaling pathways have been shown to play a role in mediating the effects of CD437 (10;71;88;89;91;145). Based on these previous findings, five different signaling pathways associated with CD437-induced apoptosis were examined. An activation in the p38/MAPK signaling pathway was found after 3, 8, 24 hrs treatment in both FEMX1 and WM239. In addition, an increase of pAKT was found after 1, 3, 8 and 24 hrs.

Up-regulation of the p38/MAPK signaling pathway has previously been seen in other cell lines following treatment with CD437 (67;84;87). The activation of p38/MAPK has, moreover, been suggested to be necessary for Nur77 relocation to the mitochondria and subsequent apoptosis, following treatment with CD437 (67;87). In contrast, another study showed that activation of p38/MAPK did not to play a direct role in apoptosis, but rather was a secondary effect (84). How p38/MAPK affects Nur77 can not be determined from our study. It would, however, be interesting to inhibit p38/MAPK and elucidate how it affects the expression of Nur77. In addition, AP-1 activation may be mediated through the MAP kinase pathways, including p38/MAPK (135), thus providing a possible explanation to the activation of AP-1 following treatment with CD437.

The activation of the PI3K signaling pathway in FEMX1 was unexpected. An increased activation of this pathway is usually associated with survival, cell growth and proliferation (146). In addition, the PI3K pathway has previous been reported to be down-regulated by CD437. This latter study suggests that pAKT may inhibit the nuclear export of Nur77, and thus hinder its pro-apoptotic activities (85). An up-regulation of pAKT may be a controversial finding in a cell population characterized by 45% dead cells. Perhaps not an adequate explanation, however it is possible; FEMX1 may be a heterogeneous cell population. The activation of the PI3K pathway can thus be the reason for survival in the other half of the population, following treatment with CD437.

5. Conclusion

In the present study, the synthetic retionid, CD437, was shown to have an anti-tumor effect on the two melanoma cell lines, FEMX1 and WM239. It was demonstrated that CD437 had an antiproliferative effect in both cell lines, contributed to by both cell cycle arrests and apoptosis. These findings are, as discussed, in accordance with previous publications on the field. Our results contribute to the growing understanding of the mechanisms behind CD437s activities, thus making the retinoid more eligible for further testing both *in vitro* and *in vivo*.

6. Future Perspectives

The master project has been limited by its time-frame of only nine months. Many ideas have arisen during the work on the thesis, but have thus not been investigated. Some of these thoughts on future perspectives might be interesting to look into, and are listed below as bullet-points;

- In order to get a better understanding of the effect of CD437 it would be useful to evaluate its effect in more melanoma cell lines, including cells with mutated p53.
- To our understanding, the effect of CD437 on normal melanocytes has not been elucidated. It would thus be interesting to investigate how these cells are affected by exposure to CD437.
- Expression of Nur77 was elevated following treatment with CD437 in FEMX1 and WM239. Studies have, as previously discussed, suggested translocation of Nur77 to both mitochondria and ER. It would thus be interesting to study CD437-mediated translocation of Nur77 in our cell lines, by the use of for instance confocal fluorescence microscopy. Other studies have suggested that both ER (75) and the lysosomes (71) may be involved in CD437-mediated apoptosis. It might thus be revealing to investigate how CD437 affects these organelles in FEMX1 and WM239, by tracking potential changes in CHOP and .

- Sun et al has previously had promising results, *in vitro*, by combining CD437 and TRAIL (118). CD437 were shown to up-regulate the expression of death receptors (DR4 and DR5), thus sensitizing the cells for treatment with TRAIL, resulting in a synergistic effect. It would be interesting to conduct a similar experiment on melanoma cells.
- A microarray analysis of CD437-induced transcripts have already been conducted on a set of melanoma cells (8). It would, however, potentially be useful to include more melanoma cells to look for a pattern.

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8. APPENDIX

8.1 Materials

8.1.1 Cell culture

Cell culture

- RPMI (Roswell Park Memorial Institute) 1640 media
  (LONZA, Verviers, Belgium)
- L-glutamine
  (LONZA, Verviers, Belgium)
- Foetal Bovine Serum (BSA)
  (PAA Laboratories, GmbH, Austria)
- Trypsin/EDTA (Ethylendiaminetetra-acetic acid disodium salt)

(LONZA, Verviers, Belgium)
- DPBS(Dulbecco's Phosphate Buffered Saline without Ca and Mg) (LONZA, Verviers, Belgium)
- NaCl (9mg/mL) (Fresenius Kabi Norge A/S, Halden, Norway)

Treatment
- CD437 (synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437)
  (Sigma-Aldrich, MO, USA)
- Dimethyl sulfoxide Hybri-max® (DMSO) (Sigma-Aldrich, MO, USA)

Harvesting of cells for protein
- sterile Phosphate buffered saline (PBS)
  (LONZA, Verviers, Belgium)

- NP-40 Lysis buffer
  - Lysis buffer (1% NP-40, 10% glycerol, 20mM Tris-HCl (pH 7,5), 137mM NaCl, 100mM NaF)
  - Aprotenin (0,02 mg/mL)
  - Phosphatase inhibitor cocktail 1 (10µL/mL)
  - Phosphatase inhibitor cocktail 2 (10µL/mL)
  - PhenylMethaneSulfonyl Fluoride (PMSF) (1mM)
  - Leupeptin (0,02 mg/mL)
  - Pepstatin (0,02 mg/mL)
  - Sodium vanadate (1mM)

Ingredients in the NP-40 lysis buffer are purchased from Sigma-Aldrich.

Harvesting of cells for flow cytometry
- Sterile Phosphate Buffered Saline (PBS)
- (LONZA, Verviers, Belgium)
- Methanol (Merck, Damstadt, Germany)

Protein measurement
- Sterile H_2O (B. Braun Medical AS, Vestskogen, Norway)
- BioRad Protein Assay (Bradford) (BioRad, (Life Science), Oslo, Norway)

8.1.2 Western blot

Founding of polyacrylamide gels

Reagent	Producer	12 % gel	Stacking gel
30% Acrylamide/BIS (29:1)	BioRad Laboratories	12mL	1,34mL
1.5M Tris-HCl (pH 8.8)	Sigma-Aldrich	7,5mL	-
0.5M Tris-HCl (pH 6.8)	Sigma-Aldrich	-	2,5mL
10% Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	0,3mL	0,1mL
Deionized H_2O		10,1mL	6mL
10% Ammonium persulfate (APS)	BioRad laboratories	150µL	50µL
Temed	BioRad laboratories	10µL	5µL
Total volume		30mL	10mL

6x loading dye

- 0.5M Tris-HCl (pH 6.8) (0.35M)
- SDS (10%)
- Glycerol (0.6M)
- Dithiothreitol (DTT) (0.6M)
- Bromphenol Blue (Sigma-Aldrich, Mo, USA) (0.012%)

Molecular weight marker

- Full-Range Rainbow Weight Marker ((Amersham), GE healthcare, Buckinhamshire, UK)

Western Running Buffer (1x) (1000mL)

- TrizmaBase (Sigma-Aldrich, MO, USA) (pH 8,3) (3,2g)
- Glycine (BioRad, (Life Science), Oslo, Norway) (14,4g)
- SDS (1g)
- H_2O (added to a total volume of 1000mL)

Western Transfer Buffer (1x) (1000mL)

- TrizmaBase (5,8g)
- Glycine (BioRad, (Life Science), Oslo, Norway) (2,9g)
- SDS (0,37g)
- Methanol (200mL)
- H_2O (added to a total volume of 1000mL)

PVDF-Membrane

- Immobilon-PVDF membrane, with pore size 0.45µm (Millipore, MA, USA).

Amido Black (1000mL)

- 1 g Naphtol-Blue-Black (Sigma-Aldrich, MO, USA)
- 450ml methanol
- 100ml Acetic Acid
- 450ml H_2O

Discoloring fluid (1000mL)

- 900mL methanol
- 80mL H_2O
- 20mL Acetic Acid

TBST

- TBS (pH 7.6) (Sigma-Aldrich, MO, USA) (1000mL)
  - NaCl (8g)
  - TrizmaBase (2.42g)
  - H_2O
- Tween®20 Sigma Ultra (Sigma-Aldrich, MO, USA) (0.05-0.25%)

Hybridization

- Dry non-fat milk (BioRad, (Life Science), Oslo, Norway)
- BSA (Sigma-Aldrich, MO, USA)

Detection of proteins

- ECL reagent (Amersham ECL plus western blotting system, GE healthcare bio-Sciences Corp. NJ, USA)
- XOMat UV plus film (Kodak)

Instruments

- Electrophoresis; Hoefer SE400 Gel Electroforesis Unit (Amersham)
- Blotting cell; Trans-blot DF (Bio-Rad laboratories)
- Developer; AGFA Curix 60 (AGFA)

8.1.3 Cell counting by the use of trypan blue dye

- Trypan blue (Merck, Stockholm, Sweden)

8.1.4 MTS

- CellTiter 96® AQ_{ueous} One Solution (Promega, WI, USA)

Plate reader

- ASYS UVM340 (Fisher Scientific, Oslo, Norway)

8.1.5 Flowcytometry

- Triton TX100 (Sigma-Aldrich, MO, USA)

- Terminal Transferase recombinant (Roche Diganostics, Mannheim, Germany)
- Tdt kit including;
    - 5x Reaction buffer (1x)
    - $CoCl_2$ (1,5mM)
    - Tdt (160U)
    - Biotin-16-dUTP (0,5nmol)
    - DTT (0,1mM)
    - Ionized H_2O

- 1/50 Streptavidin-FITC solution (volume pr. Sample: 100µL)
    - Streptavidin-FITC (Amersham, GE healthcare, Buckinhamshire, UK) (2 µL)
    - 3% w/v nonfat dry milk in 0,1% Triton HX100 1xPBS (98µL)

- 1/250 Hoechst solution (volume pr. Sample: 500µL)
    - 0,1 % Triton HX100 1xPBS (498µL)
    - Hoechst 33258 (2µL) (Sigma-Aldrich, MO, USA)

- Instrument: LSR II UV laser
- PC-based FACS DiVa software.

8.1.6 Transfection

- Lipofectamine™ RNAiMAX Cat. No. 13778-075 (Deliveredby: Invitrogen, CA, USA)
- Optimem®I (Invitrogen, CA, USA)
- SiNR4A1, Reference sequence; 'NM 002135,3' (Invitrogen Corporations, CA, USA).
- SiCtr (Invitrogen Corporations, CA, USA).

8.2 Antibodies used for Western Blots

Primary antibody (catalog number)	**Supplier**	**Size (kDa)**	**Dilution of primary antibody**	**Secondary antibody**	**Dilution of secondary antibody**
Akt #9272	Cell Signaling Technology Inc, MA, USA.	60	1/500 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA.	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
A-tubulin DM1B	Calbiochem® Merck Chemicals Ltd, Nottingham,UK.	55	1/333 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.
Bax Ab1 Clone 2D2	NeoMarkers, Thermo Fisher Scientific Inc, CA, USA.	18, 21	1/200 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.
Bcl-2 M0887, Clone 124	DakoCytomation, Glostrup, Denmark.	26	1/1000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.
Bcl-xl #2762	Cell Signaling Technology Inc, MA, USA.	30	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
Bid #2002	Cell Signaling Technology Inc, MA, USA.	22, 15	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
Bim Rbxbim/bod AB17003	Millipore Corporate, MA, USA.	23, 26	1/2500 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.

Caspase 3 AF-605-NA	R&D Systems Inc, MN, USA.	16, 18, 20, 32	1/2000 in 1X TBS, 0.05% Tween-20 with 2% w/v nonfat dry milk.	HPR-conjugated *Anti-Goat* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
Caspase 8 #9747	Cell Signaling Technology Inc, MA, USA.	18, 43, 57	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
Caspase 9 #9502	Cell Signaling Technology Inc, MA, USA.	47, 37, 35, 17	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
Cyclin A Sc-751	Santa Cruz Biotechnology Inc, CA, USA.	50, 55	1/1000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.
Cyclin B1 #4138S	Cell Signaling Technology Inc, MA, USA.	60	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
Cyclin D1 SP4	NeoMarkers, Thermo Fisher Scientific Inc, CA, USA.	36	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1 Tween-20 with 5% w/v nonfat dry milk.
Cyclin D3 #2936	Cell Signaling Technology Inc, MA, USA.	31	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1 Tween-20 with 5% w/v nonfat dry

E2F-1 Sc-251	Santa Cruz Biotechnology Inc, CA, USA.	60	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1 Tween-20 with 5% w/v nonfat dry milk.
Egr-1 Sc-189	Santa Cruz Biotechnology Inc, CA, USA.	80	1/1000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
ERK2 Sc-154	Santa Cruz Biotechnology Inc, CA, USA.	42	1/5000 in 1X TBS, 0.25% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.25 Tween-20 with 5% w/v nonfat dry milk.
IκBα #9242	Cell Signaling Technology Inc, MA, USA.	39	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
IκBα-p #9246	Cell Signaling Technology Inc, MA, USA.	40	1/1000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
Livin IMG-347	BioSite Incorporated, CA, USA.	37, 39	1/333 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
Mcl-1 Sc-819	Santa Cruz Biotechnology Inc, CA, USA.	32, 40	1/500 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.

Nur77 (p15) #35595	Cell Signaling Technology Inc, MA, USA.	77	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
p21 (DCS60) #2946	Cell Signaling Technology Inc, MA, USA.	21	1/3000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
p27/Anti-KIPI #610242	BD Biosciences Pharmingen, NJ, USA.	27	1/1000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
p38 $pTpY^{180/182}$/ 44-684G	BioSource/Invitrogen Corporation, CA, USA.	38	1/1000 in 1X TBS, 0.1% Tween-20 with 3% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
p53 Sc-126	Santa Cruz Biotechnology Inc, CA, USA.	53	1/1000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
pAKT (Ser437) #4058	Cell Signaling Technology Inc, MA, USA.	60	1/500 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
PARP #9532	Cell Signaling Technology Inc, MA, USA.	89, 116	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.

p-c-Jun #62615	Cell Signaling Technology Inc, MA, USA.	48	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
pMAPK (Anti-active® MAPK) pTEpY #V8031	Promega Corporation, WI, USA.	42, 44	1/1000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
pP38 (Thr180/Tyr185) #4631	Cell Signaling Technology Inc, MA, USA.	43	1/500 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
pSAPK/JNK #9255	Cell Signaling Technology Inc, MA, USA.	46, 54	1/1000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.	HPR-conjugated *Anti-Mouse* WM401B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
SAPK/JNK #9258	Cell Signaling Technology Inc, MA, USA.	49, 54	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
Survivin AF886	R&D Systems Inc, MN, USA.	19	1/2000 in 1X TBS, 0.05% Tween-20 with 1% w/v nonfat dry milk.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.05% Tween-20 with 5% w/v nonfat dry milk.
Xiap #2045	Cell Signaling Technology Inc, MA, USA.	53	1/1000 in 1X TBS, 0.1% Tween-20 with 5% BSA.	HPR-conjugated *Anti-Rabbit* WM402B Promega Corporation, WI, USA	1/5000 in 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.