Downregulation of the FOXM1 transcription factor by dual nitric oxide-/ hydrogen sulfide-donors: preliminary structure-activity relationships

by

Alexandra Rodriguez Dimitrescu

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Abstract

In recent decades, the Forkhead Box (FOX) family of transcription factors (e.g. **FOXM1**) has attracted the attention of the scientific community due to its significant role in cancer initiation and progression. Furthermore, the abnormal or deregulated expression of FOXM1 has been associated with chemotherapeutic resistance. FOXM1 plays important roles in the induction of cell cycle progression, differentiation, proliferation, DNA repair, induction of angiogenesis and suppression of apoptosis.

FOXM1 has been regarded as the "Achilles' heel of cancer", and it represents one of the most promising therapeutic targets for the development of novel anticancer agents. Inactivation of the FOXM1 signaling pathway is emerging as a novel approach in cancer therapy, because by targeting its expression and its transcriptional activity it may significantly impact on multiple facets of tumorigenesis and drug resistance.

Several therapeutic strategies have been reported in the literature to downregulate FOXM1 in cancer cells. These include; (a) siRNA, (b) inhibition of the proteasome system (stabilizes an endogenous FOXM1 inhibitor), (c) cyclin-dependent kinase inhibitors (inhibit FOXM1 phosphorylation and translocation from the cytoplasm to the nucleus), (d) modulators of the structurally related FOXO3a transcription factor (upstream modulator of FOXM1), (e) modulation of the NF-κB pathway, and, more recently, (f) dual nitric oxide-/hydrogen sulfide-releasing salicylate known as NOSH compounds.

Literature reports have shown that NOSH compounds have potent cell proliferation inhibitory properties, by promoting cell cycle arrest and apoptosis in eleven different cancer cell lines from six different tissue origins. These compounds have been shown to be potent inhibitors of cell proliferation (IC_{50}: 48-280 nM range). The observed chemical similarities

between the NOSH compounds and some thiazole antibiotics, known to downregulate the expression of FOXM1, have provided a rationale for the unexpected and highly significant *in vitro* potency of NOSH compounds. Preliminary studies from our collaborators (Dr. Khosrow Kashfi, at the City University of New York, NY, USA), have shown that NOSH compounds are able to inhibit the expression of FOXM1 *in vitro*, and have provided the basis for the current study.

The NOSH compounds possess three main chemical moieties: (1) a salicylic acid group, (2) a nitric oxide-releasing group, and (3) a hydrogen sulfide-releasing group. It is not known if these three moieties are essential for NOSH compounds to exert downregulation of FOXM1, or if only one or two of them are needed to modulate this transcription factor.

Consequently, the objectives of this thesis were:

1. To design and synthesize a series of NOSH derivatives containing two out of three components present in the parent NOSH compound; namely
    a) Salicylate and a H_2S-releasing moiety.
    b) Salicylate and a NO-releasing moiety.
    c) NO- and a H_2S-releasing moiety.
    d) H_2S-releasing moiety alone.
2. To determine the specific role of each of the three components present in the NOSH scaffold in:

a) Cell viability assays in three cancer lines of different tissue origin [colon (HT-29), liver (HepG2) and breast (SKBR-3)].

b) The downregulation of the FOXM1 protein in HT-29 and SKBR-3 cells.

Based on the results obtained during the course of this investigation, it is possible to conclude that:

a) The inhibitory effect exerted by NOSH derivatives on cancer cell viability is largely due to the presence of the 1,2-dithiole-3-thione **(ADT-OH)** group.
b) The second H_2S-releasing group tested, thiobenzamide **(TBZ)**, seems to be ***not essential*** to inhibit viability of cancer cells.
c) The organic nitrate ($-ONO_2$) moiety present in the parent NOSH-1 compound ***is not essential*** to inhibit cancer cell viability.
d) The salicylate moiety present in the parent NOSH-compounds ***seems to be not essential*** for the inhibition of cancer cell viability or the inhibition of FOXM1 expression.
e) The FOXM1 protein levels determined in HT-29 cells were not modulated by the parent drug **NOSH-1**, or any NOSH-derivative tested. This effect was only observed with **ADT-OH**.
f) The protein levels of FOXM1 in SKBR-3 cells were decreased, in a concentration-dependent manner, only by compounds containing the **ADT-OH** moiety.
g) The protein levels of FOXM1 in SKBR-3 cells were not affected by any derivative possessing the $–ONO_2$ or the thiobenzamide group.
h) <u>The design of future FOXM1 modulators derived from NOSH-compounds will have to be based on the **ADT-OH** moiety, and not on the other two groups reported for these molecules</u>.

Preface

The research work presented in this thesis is part of an ongoing international research collaboration between Professor Khosrow Kashfi (City University of New York, NY, USA), and Professor Carlos A. Velázquez (University of Alberta). Nevertheless, Professor Carlos A. Velázquez was the lead author for all intents and purposes of this thesis. The received technical training in cell biology was from former University of Alberta Professor Lars-Oliver Klotz, and training in Western blot protein analysis by current U. of A. associate Professor Paul Jurasz (results presented in Chapter 4). The literature review (background) presented in Chapter 1, the chemical synthesis of all test drugs, the statistical analysis described in Chapter 5, as well as the concluding remarks in the corresponding Chapter in this thesis, represent the original work carried out by myself.

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I would like to thank to the University of Alberta for all the laboratory facilities and the material given that made possible my research. As well as to my supervisor Carlos Velazquez for giving me the chance to be part of his team and for all his help and support.

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Thanks to my mom for all the times she gave me her support and love, all the nice talks by phone and all the help that she gave me to make my student life easier. Thanks to my dad for listening to me every time that I need it, and for all his advices that helped me to make decisions when I was confused. Mom and dad, you are the best parents that I could get, because you are more than just parents, you are my friends and my biggest support. I am the luckiest person in the world for having you as my parents. To have you on my side makes me the happiest person in this world.

I would like to thank to my sister, my best friend forever, the reason why I came here. Little sister, you are the person that helped me to take the biggest decision in my academic life, and I would like to thank you for that. I also want to thank you for been there for me every time that I needed. Thank you for all the positive talks and videos; you helped me to be a stronger person and to go thru my postgraduate studies.

I would like to thank to my loving fiancé, the person who since we started to be together has been giving me the best of his support, thank you for all the love and the advices, thank you for being the one that gives me the hand every time that I feel that I am drowning, for being the first person that listens to me and thank you for being the person

that always makes me smile and a happy person. Thank you for everything; you are a big piece of the puzzle of my life.

For all the persons that mean a lot to me, I could say WE DID IT!!, and now it is time to turn this page and start with something new in my life and to move on.

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List of Abbreviations

FOXM1	Forkhead Box M1
ASA	Aspirin (Acetylsalicylic Acid)
NO	Nitric Oxide
H_2S	Hydrogen Sulfide
ADT-OH	5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione
WHO	World Health Organization
HIF	Hypoxia-inducible Factor 1-alpha
pRB	Retinoblastoma Tumor suppressor protein
PRXD3	Peroxiredoxin 3
DMEM	Dulbecco's Modified Eagle Medium
MnSOD	Manganese Superoxide Dismutase
CCND1	Cyclin D1
CCNB1	Cyclin B1
MAPK	Mitogen-activated Protein Kinase
JNK1	c-Jun N-terminal Kinase 1
LOX	Lysyl Oxidase
LOXL2	Lysyl Oxidase-like 2
mTOR	Mamalian Target of Rapamycin
HCC	Hepatocellular Carcinoma
EXO1	Exonuclease 1
RFC4	Replication Factor C 4
POLE2	DNA Polymerase Epsilon subunit 2
EMT	Epithelial-to-mesenchymal Transition
EGF	Epidermal Growth Factor
TGFβ	Transforming Growth Factor beta

ERK	Extracellular-signal-regulated Kinase
KLP	Krüppel-like Factor 4
SRCs	Steroid Receptor Co-activators
BMDC	Bone Marrow-derivative Cells
ccRCC	Clear cell Renal Cell Carcinoma
MB	Medulloblastoma
NSCLC	Non-small Cell Lung Cancer
SCLC	Small Cell Lung Cancer
EOC	Epithelial Ovarian Cancer
GC	Gallbladder Carcinoma
PLAUR	Urokinase-type Plasminogen Activator Receptor
PEI	Polyethylimine
NPM	Nucleophosmin
NAC	N-acetyl-L-cysteine
CML	Chronic Myeloid Leukemia
HER	Human Epidermal Growth Factor Receptor
ErbB2	Receptor Tyrosine-protein Kinase
HB-EGF	Hepatic-binding EGF
TKI	Tyrosine Kinase Inhibitors
DBD	DNA-binding Domain
NRD	N-terminal Auto-repressor Domain
Cdk	Cyclin-dependent Kinase
Plk1	Polo-like Kinase 1
SKP1	S-phase Kinase Associated Protein 1
Cks	Cyclin-dependent Kinase subunit
Nek2	NINA-Related Kinase 2
CENP	Centromere protein

Cdc25	Cell Division Cycle 25
AURKB	Aurora kinase B
MMP2	Matrix Metalloprotease
VEGF	Vascular Endothelial Growth Factor
BCC	Basal Cell Carcinoma
Shh	Sonic Hedgehog
RNAi	RNA Interference
siRNA	Short Interference RNA
NFκB	Nuclear Factor kappa B
ROS	Reactive Oxygen Species
DSB	Double-strand Break
EGFR	Epidermal Growth Factor Receptor
PI3K	Phosphoinositide 3-kinase
HER2	Human Epidermal Growth Factor Receptor Type 2
TKI	Tyrosine Kinase Inhibitors
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
FHRE	Forkhead Response Element
COX	Cyclooxygenase
NSAIDS	Non-steroidal Anti-inflammatory Drugs
PIG2	Prostacyclin
LDH	Lactase Dehydrogenase
NOSH-1	(4-(3-thioxo-3H-1,2-dithiol-5-yl) phenyl 2-((4-(nitrooxy)-butanoyl) oxy) benzoate
SAR	Structure-activity Relationship
DMA	Dimethylacetamide
S_2	Sulfur

Rf	Retention Factor
RT	Room Temperature
MW	Microwave
HCl	Hydrochloric Acid
H-NMR	Hydrogen-Nuclear Magnetic Resonance
C-NMR	Carbon-Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
CH_3OH	Methanol
DCM	Dichloromethane
DCC	Dicyclohexyl Carbodiimide
DMAP	4-(Dimiethylamino) Pyridine
$CDCl_3$	Deuterated Chloroform
$KMnO_4$	Potassium Permanganate
T	Triplet NMR
S	Singlet NMR
D	Doublet NMR
Dd	Double doublet NMR
Td	Triplet doublet NMR
DMSO-d_6	Deuterated Dimethyl Sulfoxide
MS (EI)	Mass Spectrometry (Electron Ionization)
+Na	Sodium
TBZ	4-Hydroxybenzothioamide
Rf	Retention Factor
Dt	Doublet Triplet NMR
CH_3CN	Acetonitrile
$AgNO_3$	Silver Nitrate
NaCl	Sodium Chloride

M	Multiplet
M	Molar
MeOD (MeOH-d_6)	Deuterated Methanol
Q	Quintet
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
NEAA	Non-essential Amino Acid
PBS	Phosphate Buffered Saline
TBST	Tris Buffered Saline & Tween
IC_{50}	Half Maximal Inhibitory Concentration

1.0 Introduction

1.1. Background

Cancer is one of the most prevalent diseases worldwide, affecting millions of people every year. According to the World Health Organization (WHO), in 2012 there were an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths worldwide. In Canada, epidemiological data reported in 2013 showed an estimated 187,600 new cancer patients and 75,500 cancer-related deaths [1, 2].

Cancer is characterized by the transformation of normal cells to tumorous cells, due to the inactivation of normal cellular death mechanisms, unregulated replicative potential, promotion of angiogenesis, invasion, and metastasis from the original tissue (or organ) to another [3-5]. Although the causes by which cancer is initially "triggered" are not well understood, statistics have shown that most cancer cases are related to age [6], genetics [7], dietary habits [7, 8], exposure to mutagenic materials, viral infections [9-11], UV light [12, 13], and other factors which promote normal cells to undergo DNA transformation and, consequently, cell cycle dysregulations [5, 14].

In recent decades, the forkhead box (FOX) transcription factor family has attracted researchers' attention, and among these proteins, FOXM1 occupies a special place. The relevance of FOXM1 relies on its ability to induce and accelerate cell cycle progression, differentiation, proliferation, and DNA repair. This transcription factor has been shown to be highly expressed in actively dividing cells and during embryonic development. Although in adult tissues its expression is significantly decreased, and it is only re-activated either during tissue restoration or after tissue injury; a few examples would be the activation of FOXM1 expression in hepatocytes and pancreatic endocrine cells after partial hepatectomy or pancreatectomy or the activation of FOXM1b transgene protein after lung injury with butylated hydroxytoluene [15-18].

Consistent with the importance of FOXM1 in cancer cell cycle progression and proliferation, increased levels of FOXM1 at the mRNA and protein levels has been shown to be associated with cancer initiation, progression, metastasis, and the development of

drug resistance [15]. In fact, it has been observed that increased expression of FOXM1 causes accelerated mitosis in normal cells, promoting the transformation to cancer in a wide variety of cell types [19-21].

FOXM1 has been implicated in cell processes such as:

a) Cancer cell migration, by activating the transcriptional expression of HER-2, topoisomerase-2α and c-Myc [22].

b) Angiogenesis, by increasing the expression of HIF-α and VEGF [23].

c) Regulation of oxidative stress, through the expression of anti-oxidant enzymes such as catalase, peroxiredoxin 3 (PRXD3), and manganese superoxide dismutase (MnSOD) [24].

d) Suppression of apoptosis, by downregulating Bax and p53 [20, 21, 25].

1.2. FOXM1 structure

The FOXM1 protein, previously known as Trident, MPP2, HFH-11B, or Win, is a member of the FOX transcription factor family, which is composed of about fifty different FOX proteins, sub-classified from FOXA to FOXS [19, 26].

All members of the FOX family are structured by three main regions; the initial domain known as the *N*-terminal auto-repressor domain (NRD); the intermediate section constituted by a conserved winged helix known as the DNA-binding domain (DBD); and the last section known as the C-terminal domain [15, 19, 26, 27]. All FOX members share the same winged helix DBD, but each FOX member recognizes a different DNA sequence [19, 26, 27].

Each FOX member has its own distinct amino and carboxyl-terminal domains (each FOX member varies in amino acid content), and this diversity reflects the wide variety of functions exerted by these proteins [27]. A few representative examples of this diversity of action would be the role of FOXL2, which is required for female sex determination; the role of FOXP3 which is essential for normal immune function [15, 27]; the effects of FOXO and its subfamily members known to have roles in the control of the cell cycle, apoptosis, DNA repair and glucose metabolism [28]; and the crucial role of FOXE1 for the normal

function of the thyroid gland [15]. These examples reflect the wide range of biological processes in which the FOX family members are implicated [15, 22, 27].

In FOXM1, the initial domain NRD is contained from the amino acids 1 to 232, and it is known to be the inhibitory domain, which regulates the transcription activity of FOXM1. This domain is followed by an intermediate domain, DBD, known to be the smallest domain of FOXM1 and constituted by 90 amino acid residues (233 to 323), where the FOXM1 protein adopts its forkhead winged-helix form, and it is distinguished by three α-helices and three β-helices (αβααββ). The last (and longest) domain of FOXM1 is the C-terminal region, which is located between amino acids 365 and 748, containing in its structure a cyclin-Cdk phosphorylation site, which is responsible for the execution of FOXM1's transcriptional activity (See figure 1) [29].

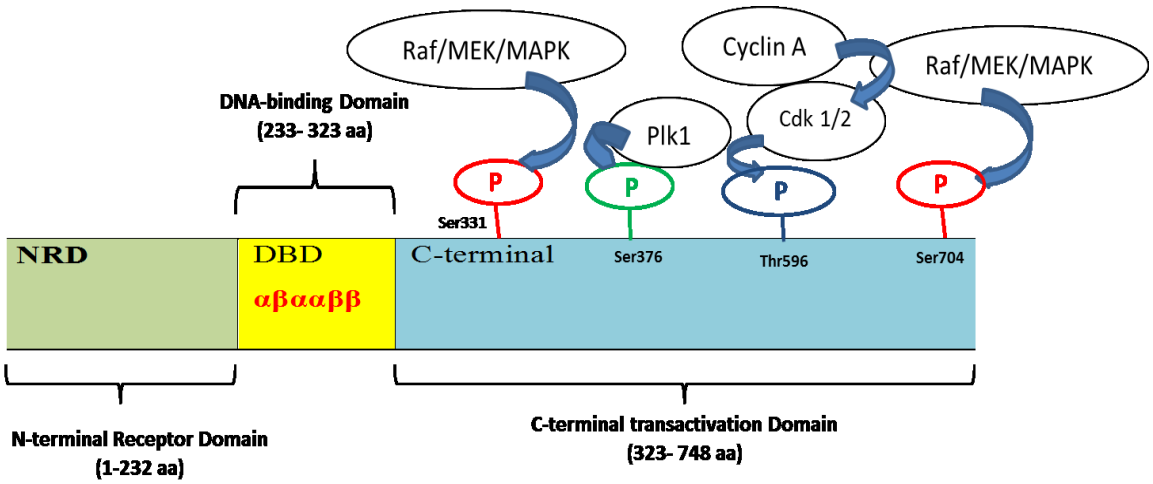


Figure 1. FOXM1 protein structure and its phosphorylation sites. FOXM1 protein contains 3 domains; the *N*-terminal auto-repressor domain (NRD) between the amino acids 1-232, followed by a conserved "middle" section DNA binding domain (DBD) between amino acids 233-323, and a C-terminal transactivation domain between amino acids 323-748. Phosphorylation sites are found in the C-terminal domain. P in red represents the Raf/MEK/MAPK phosphorylation site in FOXM1, P in blue represents a second phosphorylation site by cyclin A-Cdk 1/2, and P in green represents the third phosphorylation site in FOXM1 protein done by Plk1. This figure was modified from Park H. J. et.al, 2008 and Major M.L. et.al, 2004 [29, 30].

In the C-terminal domain is where the phosphorylation sequences of FOXM1 are located and phosphorylation takes place (further mentioned in the introduction), allowing

both the translocation of FOXM1 to the nucleus, and then the initiation of FOXM1's transcriptional activity [29, 30].

Three types of isoforms of FOXM1 protein have been characterized so far: FOXM1a, FOXM1b, and FOXM1c [15, 31, 32]. These three isoforms are formed by different splicing of exons (I-VIII, Va, VIIa) which gives to each isoform its characteristic structure [31]. FOXM1a contains exons Va and VIIa, located at the end of the C-terminal region [31, 32]; exon Va is not capable of altering DNA binding activity, whereas exon VIIa plays an important role as "disruptor" of the function of the C-terminal domain [32]. FOXM1b lacks both exons Va and VIIa, and FOXM1c only lacks exon VIIa and not exon Va [32]. Due to the presence of exon VIIa in FOXM1a, this isoform is transcriptionally inactive, whereas FOXM1b and FOXM1c lack exon VIIa and, therefore, are transcriptional activators [31]. These two isoforms are frequently observed in carcinogenic cells [31].

1.3. How does FOXM1 transcriptional activity works?

The cell cycle is essential for cell proliferation; it is necessary for the reproduction of a cell to form two daughter cells. The cell cycle has four sequential phases; the first phase, known as "first gap" (G1), followed by a S phase (where DNA replication occurs), a "second gap" (G2) and a M phase (cell division) [5].

In the adult human body the majority of the cells are not cycling. In fact, they are found to be in a quiescent or resting state (G0) residing "out-of-cycle", and only a minority of the cells are actively proliferating (cycling), principally those found in self-renewing tissues such as epithelia and bone marrow [33].

Before entering to G1, cells can enter to the resting state G0, in which growth and proliferation do not take place [5, 34]. However, growth factors or mitogens are capable of inducing cells found in G0 to reenter the cell cycle, G1 and prepare for DNA synthesis [5, 35].

The progression of the cell cycle through its different phases is controlled by a set of proteins known as cyclins and cyclin-dependent kinases (CDK), which are known to be regulated by the cyclins [5, 33].

The role of FOXM1 in the regulation of cell cycle progression is critical. In normal cells, FOXM1 participates as a regulator of the G1/S and G2/M transitions [20, 36]. During the G1/S transition, FOXM1 upregulates several genes including S-phase kinase-associated protein 1 *(SKP1*), Cyclin D1 (*CCND1*) and Cyclin-dependent kinase regulatory subunit 1 (*Cks1*), which are needed for the ubiquitination of proteins necessary for cell cycle progression, signal transduction and transcription [19, 36, 37]. During the G2/M transition, FOXM1 upregulates Polo-like kinase 1 (*Plk1*), *cyclin B2*, NIMA-related kinase 2 (*Nek2*), centromere protein A, B, and F, (CENP-A, CENP-B & CENP-F), *Aurora B*, Cell division cycle 25 (*Cdc25*) and Cyclin B1 (*CCNB1*); all of these genes are required for chromosomal stability [19, 31, 36, 37]. Finally, at the M phase, FOXM1 increases protein expression of Aurora kinase B (*AURKB)* [37].

Both FOXM1 mRNA and protein levels are barely detectable in quiescent cells, although they increase in cells stimulated to reenter the cell cycle [38]. This increase in FOXM1 expression is initiated in late G1 and early S-phase and continues through the G2-phase and mitosis where it reaches the maximum level of expression [38].

During G1, FOXM1 transcriptional activity is negligible and found to be inhibited through two mechanisms; **1)** the inhibitory action of the pRB, and **2)** an "intra"-molecular auto-inhibitory interaction between the C-terminal and the *N*-terminal domains of FOXM1 [29, 39]. However, as the cell cycle progresses, the transcriptional activity of FOXM1 increases progressively [36]. This is caused by control mechanisms that influence FOXM1 interdomain interactions and the control of the FOXM1 C-terminal region phosphorylation (late G1/ early S-phase) [16, 36, 37, 40]. During late S-phase, the FOXM1 C-terminal region is first phosphorylated by the mitogen-activated protein kinase (MAPK) on Ser331 and Ser704 in the Pro-Gly-Ser-Pro (PGSP) motif; inactive FOXM1 (unphosphorylated) is located in the cytoplasm, and once FOXM1 is phosphorylated it translocates into the nucleus, where a second phosphorylation is carried out by the Cdk1/2 (phosphorylation site at Thr 596), followed by a final phosphorylation by the Polo-like Kinase 1 (Plk1), allowing the initiation of FOXM1 transcriptional activity (Figure 2) [29, 31, 39].

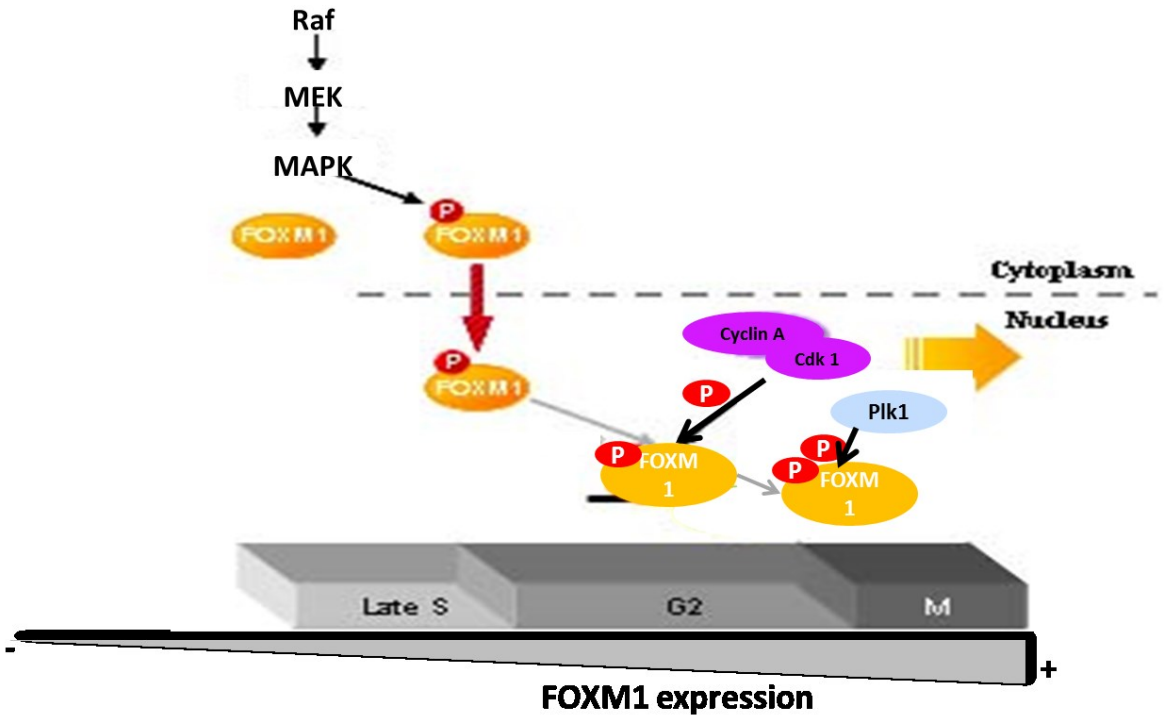


Figure 2. Phosphorylation of FOXM1 through cell cycle. Before going through its first phosphorylation, FOXM1 is found to be inactive in the cytoplasm (G1/S early phase). At late S phase, FOXM1 is activated and phosphorylated by Raf/MEK/MAPK, which allows FOXM1 to translocate from the cytoplasm into the nucleus, and binds to the DNA inducing its transcriptional activity. At the G2 phase, FOXM1 goes through two more phosphorylations, one by Cyclin A-Cdk1/2, and another by Plk1, allowing FOXM1 to trigger the expression of genes necessary for cell cycle transition. This action allows the cell cycle to proceed through G2/M without being checked (in cancer cells).

In the absence of FOXM1 expression, it has been shown that cells stop at G2 phase, experiencing chromosome disaggregation and increased DNA breakage, which causes mitosis failure [32, 37]. In fact, one study done by Kalin et al. suggested the importance of FOXM1 during cell division and cell cycle progression [15], exhibiting that FOXM1 null mice had embryonic lethal phenotype caused by abnormal formation of vital organs, e.g. liver and heart [15]. In this regard, they observed in utero embryonic death due to abnormal mitosis, which was linked to the lack of FOXM1 expression [15]. This finding suggests that FOXM1 is required for proper organogenesis during embryonic development [15]. On the other hand, overexpression of FOXM1 in normal cells results in accumulation of cells in the G2/M phase, leading to tumorigenesis [37]. Consequently, downregulation of FOXM1

results in a significant reduction of mitosis, whereas an overexpression of FOXM1 is correlated with an increased mitosis [19, 37].

1.4. Role of FOXM1 in cancer

1.4.1. FOXM1 in tumorigenesis

Genome-wide gene expression profiling of cancers has consistently identified *FOXM1* as one of the most commonly upregulated genes in the early stages of carcinogenesis [41-45]. *FOXM1* abnormal activation is regarded as one of the hallmarks of human malignancies [43]. FOXM1 plays an essential role in the regulation of a wide spectrum of biological processes including cell proliferation, cell cycle progression, cell differentiation, DNA damage repair, tissue homeostasis, angiogenesis, and apoptosis. Elevated expression of the FOXM1 protein has been observed in a multitude of malignancies involving liver [46, 47], prostate [48], brain [49], breast [50], lung [51], colon [44, 52], pancreas [53], skin [54-56], cervix [57], ovary [58], mouth [59], blood [60], and nervous system [61].

Recent studies suggest that FOXM1 contributes to cell transformation by at least three different mechanisms:

1. Antagonizing cellular senescence. In gastric cancer, the *FOXM1* gene is often abnormally activated, and *FOXM1* depletion results in the onset of p53- and $p16^{INK4A}$-independent senescence of cancer cells by inducing $p27^{Kip1}$ expression [62].
2. Stimulating the expression of anti-oxidant enzymes. Recent evidence suggests that increased levels of FOXM1 expression protect cancer cells from ROS-induced senescence [24]. This implies that cancer cells adapt to ROS by increasing the expression of FOXM1[24]. As is known, tumor cells accumulate high levels of ROS because they are metabolically more active, compared to normal cells [24, 63]. Elevated ROS increase tumorigenesis but also render cancer cells to be more vulnerable to oxidative stress [24, 63]. FOXM1 has shown be a sensor of ROS induced by oncogenic Ras and to protect cancer cells from adverse effects of oxidative stress [24]. In fact, FOXM1 has been shown to have the ability to stimulate the expression of ROS scavenger genes e.g. mitochondrial superoxide dismutase (SOD2), catalase

and peroxiredoxin 3 (PRX3) when FOXO3 is off duty (target genes of FOXO3 transcription factor) [24]. Human solid tumors are representative in this regard [44, 47, 64].

3. Stimulating stem cell-like characteristics. Cancer and stem cells share a number of properties, including a sustained proliferative capacity, immortality and the ability to self-renew and to differentiate. Recent studies from human and mouse models have revealed that FOXM1 promotes cancer initiation through the induction of "stem cell-like" attributes [42]. Furthermore, Gemenetzidis et al. have reported that FOXM1 expands the pool of human epithelial stem/progenitor cells and facilitates the conversion of differentiated epithelial cancer cells into migratory mesenchymal cancer cells[65, 66].

These findings point to a principal role for FOXM1 in tumorigenesis.

1.4.2. FOXM1 in cancer diagnostics

Koo et al. recently proposed that by studying the role and regulation of FOXM1 in cancer initiation and cancer development, it may be possible to identify reliable molecular markers that will allow early detection of pre-malignant lesions. In this context, FOXM1 seems to be a key molecule and a potential biomarker useful in the identification of patients who may respond better to current treatment modalities [42].

1.4.3. FOXM1 in cancer progression

In addition to its initial role in tumorigenesis, FOXM1 has been implicated in promoting multiple steps of cancer progression. These steps include the induction of tumor invasion, migration, and angiogenesis [67]. Due to these features, FOXM1 has been regarded as a reliable marker for early cancer detection and a target for arresting cancer cell growth and progression, which are ideal targets for an effective cancer chemopreventive or chemotherapeutic drug [42].

There is significant *in vivo* evidence implicating FOXM1 in cancer cell proliferation and growth following initial tumorigenesis. For example, the depletion of FOXM1 by siRNA in various cancer cell lines (lung, prostate, liver, breast, colon and cervix) results in

a significant reduction of cell proliferation *in vitro* [48, 51, 68], and several *in vivo* studies have shown that cancer cell proliferation and tumor growth are significantly reduced in lung adenomas and colon adenocarcinoma mouse models when FOXM1 is deleted [51-53, 69].

Suppression of FOXM1 inhibits the transcription of genes associated with proliferation and tumor growth [70]. It has been shown that suppression of FOXM1 leads to a decrease in matrix metalloproteinase 2 (MMP-2) and MMP-9 expression in pancreatic cancer cells, which is associated with an overall decrease in cancer cell migration, invasion and angiogenesis [53]. A similar role for FOXM1 in the regulation of MMP-2 and MMP-9 expression has been documented in other malignancies, such as glioblastoma, colorectal carcinoma, and breast carcinoma [44, 68, 71]. It has been found that FOXM1 regulates the expression of MMP-2 at the transcriptional level through a "forkhead consensus site" on the MMP-2 promoter [71], while MMP-9 expression is modulated indirectly via its downstream target JNK1 [72].

An increased expression of FOXM1 correlates with clinically aggressive behavior and poor prognosis for a wide variety of human cancers including cervical [57], squamous cell carcinoma [73, 74], hepatocellular carcinoma [75], medulloblastoma [76], and pancreatic cancer [77]. Genomic studies have provided complementary evidence to support the role of FOXM1 in tumor progression in a wide range of human tumors [44, 48, 64, 78, 79].

FOXM1 is ubiquitously expressed in all proliferating cells, including many tumor-derived cell lines. In normal tissues, FOXM1 is detectable only in progenitors with extensive proliferating capacity, but not in differentiated or resting cells [80, 81]. Additionally, FOXM1 has been proposed to be a master activator of metastasis. In fact, it has been observed that overexpression of FOXM1 stimulates the expression of lysyl oxidase (LOX) and LOXL2 mRNA by two ways; **1)** by directly binding to the promoters of *LOX* and *LOXL2* genes and **2)** by enhancing PI3K-Akt-mTOR signaling [82]. The induction of *LOX* and *LOXL2* expression by FOXM1 promotes collagen crosslinking at pre-metastatic sites and the attraction of CD11b+ myeloid cells in order to form receptive niches for arriving tumor cells; this formation of pre-metastatic niches facilitates tumor cell metastasis [82, 83]. FOXM1 also promotes metastasis by increasing the flexibility of the

cytoskeleton, enhancing cell migration by the microtubule destabilizing protein Stathmin [82].

Finally, cell-cell contact is known to be a critical regulator of cellular proliferation, differentiation, and motility [84]. The inhibition of proliferation by cell-cell contact is generally known as contact inhibition [84]. Cancer cells typically lose this property, allowing the uncontrolled growth of cells even if they are in contact with their neighboring cells [84].

Recently it has been shown that FOXM1 plays a key role in contact inhibition. In fact, research done by Faust et al. showed that when FOXM1 expression is diminished, there is also a decrease in cyclin A and in Plk1; consequently, a loss of contact inhibition takes place when there is an overexpression of FOXM1, and consequently, an upregulation of cyclin A and Plk1 proteins [85].

1.4.4. Organ-specific expression of FOXM1 in cancer

1.4.4.1. FOXM1 in liver cancer

FOXM1 plays a key role in the proliferation of hepatocellular carcinomas (HCC). In fact, research done in an HCC mouse model confirmed that elevated expression of FOXM1 inhibits senescence and promotes liver cancer proliferation [54, 86]. Interestingly, the suppression of FOXM1 expression delayed liver tumor growth in mice [86, 87] and rodents with a conditional deletion of FOXM1 in hepatocytes are resistant to develop HCC following exposure to a mixture of two carcinogens, namely diethylnitrosamine and phenobarbital [86].

It has been shown using oxiliplatin, a platinum-based drug, that it is possible to promote repression on FOXM1 expression via a p53-dependent pathway, which in turn results in induced senescence [88]. These results demonstrate that the modulation of p53 regulates its downstream cell-cycle related proteins by downregulating FOXM1 protein expression and upregulating p27 and p21 [88].

1.4.4.2. FOXM1 in breast cancer

The role of FOXM1 in breast cancer development is supported by the observation that infiltrating ductal carcinoma cells showed considerable levels of FOXM1, whereas the

adjacent normal tissue did not express significant levels of this transcription factor [89]. Microarray data from breast cancers revealed that FOXM1 expression is increased in ductal carcinoma, which is known to be the most commonly diagnosed form of breast cancer [90], and correlates with poor prognosis [91].

Statistical data have shown that high FOXM1 expression occurs in approximately 20% of patients with ER+ breast cancer, and is also overexpressed in stages II/III in human breast carcinomas [92, 93] In patients with triple negative cancer, 75% have been shown to have high expression of FOXM1 [92].

Breast cancer cells exhibit different expression profiles for FOXM1. Table1 shows a list of some of the most commonly used breast cell lines to study breast cancer and anticancer therapies [93].

Cell line	FOXM1 expression	Cell line	FOXM1 expression
MCF10A	Negative	SK-BR-3	High
MCF12A	Negative	BT-20	High
MCF-7	Moderate	MDA-468	High
BT-474	Moderate	ZR-75-30	High
MDA-MB-231	High		

Table 1. Expression of FOXM1 in breast cell lines. MCF10A and MCF12A are non-tumorigenic cell lines while the other 7 cell lines show the FOXM1 protein expression relative to the two non-tumorigenic cells [93, 94].

One of the main mechanisms by which FOXM1 promotes breast cancer is via the VEGF-FOXO3a-FOXM1 pathway [95]. Activation (dephosphorylation) and translocation of FOXO3a to the nucleus, results in the reduction of VEGF expression, both at protein and gene promoter levels, suggesting that FOXO3a negatively regulates VEGF expression [95]. Both FOXM1 and FOXO3a bind directly to the forkhead response element (FHRE) of the *VEGF* promoter, and this means that under FOXM1 activation, there is a displacement of DNA-bound FOXO3a from the FHRE, suggesting that FOXM1 can stimulate VEGF expression through displacement of the transcriptional activator FOXO3a [95]. In summary, FOXM1 expression promotes VEGF expression, and consequently, the activation of downstream signaling cascades such as PI3K-Akt and Raf-Ras-MAPK pathway, resulting in cell survival, cell proliferation, angiogenesis and migration [95].

Another mechanism of action exerted by FOXM1 in breast cancer involves the repression of ERβ, and the upregulation of ERα. In fact, it has been observed that FOXM1 and ERα are strongly correlated so that FOXM1 regulates ERα transcription [94, 96]. Likewise, ERα regulates FOXM1 at the transcriptional and gene promoter level [94, 96].

For patients with triple-negative breast cancer, chemotherapy is the only therapeutic alternative [89]. However, there are a considerable number of patients who develop drug resistance due to the high *FOXM1* mRNA expression, which complicates clinical interventions and lowers survival rates [92, 97]. In fact, it has been shown, that drug resistance in breast cancer has a correlation with the interaction between NF-κB and FOXM1 [89]. It has been observed that NF-κB is one of the proteins that is significantly upregulated when it interacts with FOXM1 [89], and this observation was confirmed by using *In silco* analysis, in which it was observed that the FOXM1 promoter has a binding site for NF-κB gene [98]. However, the precise molecular regulation of FOXM1 and NF-κB and their cross-talk action is not yet clear [99] .

Gene expression data obtained by Park et.al. suggested that FOXM1 directly upregulates DNA repair genes such as EXO1, RFC4, POLE2 and PLK4 by interacting and consequently upregulating NF-κB expression, which finally will lead to the promotion of cancer cell protection from DNA double-strand breaks (DSBs) induced by doxorubicin (a DNA topoisomerase IIα inhibitor) [89]. Data also showed that silencing *FOXM1* expression significantly sensitized doxorubicin-resistant MDA-MB-231 breast cancer cells and decreased cell proliferation [89].

Finally, Yang et al have recently reported that FOXM1 promotes the epithelial-to-mesenchymal transition (EMT), leading to cancer invasion and metastasis in breast cancer cells [66]. EMT in breast cancer is controlled by signaling pathways from a variety of growth factors, such as EGF and TGFβ, that are capable of inducing the expression of transcriptional factors (i.e. Slug) necessary for EMT and which are in charge of repressing the expression of the E-cadherin, an epithelial marker, and activate mesenchymal transcriptomes [66].

EGF and TGFβ pathways have been shown to be implicated in FOXM1 expression and regulation; in fact it has been observed that ERK1/2, a downstream MAPK in EGF

signaling, phosphorylates the FOXM1 protein, leading to FOXM1 nuclear accumulation and consequently the increase of its transcriptional activity [66, 100]. Yang et al. demonstrated that FOXM1 binds to the Slug promoter and activates it. They concluded that this might be the mechanism by which FOXM1 induces EMT and contributes to breast cancer metastasis [66].

1.4.4.3. FOXM1 in gastric cancer

Li et al. proposed that gastric cancer development is particularly susceptible to the loss of the epithelial zinc-finger transcription factor Krüppel-like factor 4 (KLF4). This transcription factor has shown to be a strong regulator of cellular proliferation and differentiation and tumor suppressors in gastric cancer, by inducing CDK inhibitors such as p21. Loss of KLF4 expression has been implicated in increased FOXM1 expression. [101-103]. This observation was supported by the work of Katz et al., who reported that deletion of the *Klf4* gene in stomach tissue resulted in; **a)** observable preneoplastic changes, **b)** overexpression of FOXM1 and **c)** decreased $p21^{WAF1/CIP1}$ [103]. In this regard, recent evidence supports the existence of gastric stem cells, which are considered prime candidates as the cells of origin for cancer, in which KLF4 activity is lost [104].

Feng et al. reported that there is a strong correlation between gastric chronic inflammation by bacterial infection (*H. pylori*) and the progression to gastric cancer, in which FOXM1 overexpression is implicated [105]. Their results revealed that *H. pylori*-induced FOXM1 expression *in vivo* and *in vitro*, suggesting that FOXM1 might take part in the early stages of gastric cancer [105].

H. pylori's virulent factor, CagA, inhibits $p27^{Kip1}$ mRNA and protein expression (a key tumor suppressor gene in gastric cancer) via the PI3K-Akt pathway and reduces the expression of hsa-miR-370, which results in the overexpression of FOXM1 [105]. By using FOXM1 silencing treatment it was possible to show that an overexpression of hsa-miR-370 inhibits the gastric cell proliferation induced by *H. pylori* infection, which suggested a tumor-suppressive role of miR-370 in *H. pylori*-induced gastritis toward gastric cancer [105]. This study showed that the miR-370–FOXM1 pathway is involved in the progression of

gastritis to gastric cancer induced by *H. pylori* infection by downregulating the expression of $p27^{Kip1}$, which suggests potential application in treatment of gastric cancer [105].

1.4.4.4. FOXM1 in bone cancer

Alterations in transcription factors have been shown to be implicated in the initiation and progression of osteosarcoma [106]. Overexpression of the family of steroid receptor co-activators (SRCs), novel transcriptional regulators, principally the SCR-3 gene, has shown to have a relation with the development of osteosarcoma. In fact, research done in bone osteosarcoma epithelial cells (U2OS) showed that overexpression of SCR-3 seems to upregulate mRNA and protein levels of FOXM1 [106], suggesting that FOXM1 might be a transcriptional target of SRC-3 [106].

Moreover, in U2OS cells, FOXM1 was shown to upregulate the expression of MMP-2 and MMP-9 through two different mechanisms [106, 107]. For MMP-2 expression, FOXM1 binds directly to the MMP-2 promoter site and activates its transcription [106, 107]. On the other hand, FOXM1 upregulates MMP-9 through a c-Jun N-terminal kinase (JNK1)–dependent pathway [106, 107].

Overexpression of *JNK1* allows the cell to be rescued from G1/S cell cycle block; consequently, when there is a depletion of FOXM1 protein there is a deficiency in *JNK1* expression, resulting in the accumulation of p53 and CDK inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$ which leads to mitotic block and premature senescence [106, 107].

It has also been proposed that bone marrow-derivative cells (BMDC) overexpressing VEGF-receptor 1 can promote the expression of the integrin VLA-4 and FOXM1, facilitating the initiation and development of "premetastatic niches", which are known to be clusters that precede the arrival of single metastatic tumor cells from bone, and are responsible for metastasis of cancer from bone to other organs in the body [108].

1.4.4.5. FOXM1 in renal cancer

Xue et al. demonstrated that FOXM1 expression is upregulated in most clear cell renal cell carcinoma (ccRCC) tissue specimens, at mRNA and protein levels. Furthermore, FOXM1 expression significantly correlated with primary tumor stage, lymph node metastasis, distant metastasis, and histological grade [109]. Consequently, an overexpression of FOXM1 has been associated with poor prognosis and overall patient survival in ccRCC

patients [109]. Therefore, the same authors proposed that FOXM1 would be a prognostic biomarker and a promising therapeutic target for ccRCC [109].

1.4.4.6. FOXM1 in pancreatic cancer

Human pancreatic cells predominantly express the isoform FOXM1c, which correlates directly with increased malignancy of pancreatic cancer. Research by Chen et al. showed that the *FOXM1* gene is amplified in pancreatic cancer development, as was described previously in other types of cancer. This upregulation of FOXM1 is responsible for the promotion of angiogenesis, invasion, and metastasis through induction of MMP-2, MMP-9, and VEGF [53, 110].

Another pancreatic cancer study demonstrated that transcription factor Sp1, known to be involved in processes such as cell differentiation, cell growth and response to DNA damage, positively regulated the expression of FOXM1 [102]. KLF4 did the opposite, suggesting that dysregulation of the KLF4/Sp1/FOXM1c pathway causes abnormal expression of genes downstream of FOXM1 and contributes to the pathogenesis of pancreatic cancer [102].

1.4.4.7. FOXM1 in brain cancer

The expression of FOXM1, at the mRNA and protein level, has been reported to be considerably higher in primary glioblastoma multiform cells than in their normal counterparts [71]. In this context, FOXM1 directly upregulates the expression of the DNA-damage-repair gene *Rad51* at the transcriptional level through FOXM1's binding site [71]. Additionally, FOXM1 also promotes angiogenesis, invasion and metastasis through upregulation of MMP-2, MMP-9, and VEGF [71]. All these factors contribute to the development of chemotherapeutic resistance to temozolomide in glioblastoma cells, and interestingly, negative modulation of FOXM1 sensitized glioblastoma multiform cells to the drug [111].

In neuroblastoma, it has been observed that CDK4/6 are found to be overexpressed via upregulation of the FOXM1 transcription factor, promoting tumorigenesis, disease progression and senescence suppression [112]. Studies performed with LEE011 (Novartis), a

highly potent CDK4/6 inhibitor, showed reduced FOXM1 mRNA and protein levels as early as 6 hours in sensitive neuroblastoma cell lines. However, cells resistant to LEE011 showed no reduction of FOXM1 mRNA or protein levels, and these cells did not senesce [112].

Inhibition of FOXM1 by using thiostrepton (IC_{50} = 1.69 μM) in Daoy medulloblastoma (MB) cells restored cisplatin-induced apoptosis and decreased cell viability [113]. Thiostrepton also decreased cell invasion and migration, which are crucial steps for tumor progression, suggesting that targeting FOXM1 with small-molecule inhibitors results in potent antitumor activity and chemosensitizing effects in human medulloblastoma cells [113].

1.4.4.8. FOXM1 in lung cancer

FOXM1 overexpression has been also documented in lung cancer. In non-small cell lung carcinomas (NSCLC), a close correlation between FOXM1 overexpression and the upregulation of *Plk1, survivin, CDC25A, cyclin D1* and *CDC6* has been observed. This upregulation is done by FOXM1 through three different ways; **1)** via RAS activation pathway (observed in *CDC25A* up-regulation), **2)** by direct binding to their promoter sites (for *Plk1* and *CDC6* upregulation) and **3)** via the E2F/Rb pathway (for *cyclin D1* upregulation) [114, 115]. Results have also shown that as FOXM1 protein expression increases more inactivation of tumor suppressor genes *p53* and *pRb* takes place [114]. Inactivated p53 and pRb allows damaged cells to proliferate, which is an important step for cancer initiation [114].

FOXM1 genes and protein are found to be amplified in malignant pleural mesothelioma development [116]. Xu et al. showed two different patterns of FOXM1 expression in two different NSCLC cell lines, SPC-A-1 and H292, depending on their sensitivity to the tyrosine receptor kinase inhibitor gefitinib (See Figure 3). [117]. The SPC-A-1 cell line was shown to be resistant to gefitinib due to the high protein expression of FOXM1 [117]. On the other hand, the H292 lung cell line was shown to be sensitive to gefitinib [117]. Interestingly, this sensitivity was correlated to low protein and mRNA levels of FOXM1, indicating that FOXM1 has different expression patterns between the cell lines that are resistant to gefitinib and the ones that are sensitive in NSCLC [117]. Based on this

finding, Xu et al. proposed that targeting FOXM1 could sensitize cells resistant to the drug, as represented in Figure 3 [117].

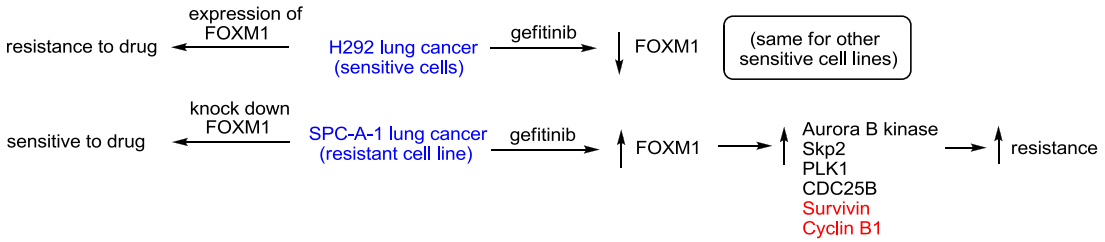


Figure 3. Proposed mechanism by which lung cancer cells are sensitized to gefitinib. Sensitive cells, H292, were shown to have low FOXM1 protein expression was correlated to their sensitivity to gefitinib, although if this cell overexpresses FOXM1 the response would be of resistance to the drug. A gefitinib resistant cell line, SPC-A-1 was shown to have overexpression of FOXM1 which was shown to be correlated with the upregulation of Aurora B kinase, Skp2, Plk11, survivin and cyclin B1, promoting the resistance of the cells to the treatment and consequently the progression of cancer [117].

In small cell lung cancer (SCLC) an elevation of FOXM1 expression has been found as well; in fact, research done by using Genistein, a soy flavonoid, has shown that this compound induces G2/M cell cycle arrest and apoptosis in the SCLC H446 cell line by inhibiting the activity of *FOXM1* gene [115].

Ha et al. suggested that FOXM1 could have some diagnostic utility by distinguishing different subtypes of pulmonary neuroendocrine tumors, based on the observation that FOXM1 is expressed more frequently in high-grade neuroendocrine tumors than in carcinoid tumors [118].

1.4.4.9. FOXM1 in laryngeal cancer

Chen et al. showed that by using Hep-2 cells (laryngeal squamous cell carcinoma) transfected with FOXM1-targeted small interfering RNA (siRNA) it was possible to downregulate FOXM1 protein signaling as well as tumour proliferation, and decrease nuclear NF-κB translocation and MMP-2 and VEGF expression [119].

Chen's group showed a decreased invasive profile in FOXM1-siRNA transfected cells compared to FOXM1-expressing cells, and a decreased expression of cyclin B1 and an increase in the expression of p27 [119]. This supports the idea that modulation of FOXM1 may represent a novel strategy to disrupt angiogenesis and tumor growth [119].

1.4.14. FOXM1 in cervical cancer

FOXM1 protein and mRNA are amplified in cervical cancer, and their overexpression is an independent negative prognostic factor (poor survival rates) in patients with early-stage cervical cancer [79].

Cervical cancer aggressiveness is associated with MMP-2 and MMP-9 overexpression [120]. Interestingly, increased expression of FOXM1 seems to promote MMP-2 and MMP-9 expression by activating the expression of the Akt/GSK-3β/Snail pathway, resulting in the increase of cell migration and invasion by cervical cancer cells [120]. He et al. have recently shown that FOXM1 knock-down abrogated the migration and invasive profile of cervical cancer cells by inhibiting the expression of phosphorylated Akt, GSK-3β and Snail [120]. These observations suggest that FOXM1 may represent a promising target for the treatment of cervical cancer.

1.4.4.11. FOXM1 in ovarian cancer

Using an immunohistochemical method, Wen et al. determined the expression of FOXM1 in epithelial ovarian cancer (EOC) specimens obtained from 158 Chinese patients and found a statistically significant relationship between FOXM1 overexpression and lymph node metastasis and poor patient survival [121]. *In vitro* studies done by the same authors in pcDNA 3.1-FOXM1 transfected HO-8910 cells (ovarian cancer cells) showed that up-regulation of FOXM1 increased MMP-2, MMP-9, and VEGF-A expression, whereas FOXM1 shRNA-transfected HO-8910 cells showed the opposite. In addition, it was shown that cell migration and invasion involved the induction of FOXM1-dependent expression of MMP-2, MMP-9, and VEGF-A. Therefore, the authors suggested that elevated FOXM1 may be a prognostic marker of EOC and that FOXM1 may serve as a promising therapeutic target for inhibition of ovarian cancer progression [121].

1.4.4.12. FOXM1 in gallbladder cancer

FOXM1 protein expression is upregulated in gallbladder cancer, and it's correlated with Nevin stage progression, metastasis and poor prognosis [122]. In fact, a recent study suggested that the high expression levels of Plk1, Nek7 and FOXM1 could be related to the occurrence and malignant evolution of gallbladder carcinoma (GC) [122].

Interestingly, research done by Tao et al. showed that it is possible to downregulate FOXM1 expression in gallbladder cancer cells (GBC-SD) by using RNA interference resulting in cellular senescence and inhibition of cell proliferation and invasion, suggesting that FOXM1 is a potential therapeutic target for the treatment of gallbladder cancer [123].

1.4.4.13. FOXM1 in colorectal cancer

Cancer cell migration, invasion, and growth are modulated by the transcriptional effects of FOXM1. As proposed by Li et al., there is a strong correlation between FOXM1 overexpression and unfavorable clinical variables such as advanced clinical stage, lymph node metastasis and metastasis of colon cancer [124]. The authors suggest that FOXM1 is directly correlated with the concomitant overexpression of urokinase-type plasminogen activator receptor (PLAUR), known to be a facilitator of migration and metastasis in cancer cells, which is highly significant, considering that nearly 50% of colon cancer patients show metastasis after colectomy, the major cause of death in these patients [124, 125].

In fact, overexpressed FOXM1 leads to increased PLAUR gene expression at the transcriptional level which is done via binding to the PLAUR promoter [124].

It has been also observed in an *in vivo* model of colon cancer that overexpression of FOXM1enhances tumorigenicity and metastasis and that when this expression is reduced, no metastasis takes place [124], suggesting that FOXM1 may be important clinically to predict metastasis after colectomy.

1.5. FOXM1 as a therapeutic target

FOXM1 has been regarded as the "Achilles' heel of cancer" [126], and it represents one of the most promising therapeutic targets for the development of novel anticancer agents [119, 126-128]. Wang et al. have proposed that the inactivation of the FOXM1 signaling pathway by novel approaches could have a significant impact on cancer therapy [129] because modulation of this single transcription factor should impact multiple facets of tumorigenesis [127]. Additionally, considering the overwhelming evidence pointing to FOXM1 as an essential modulator of tumorigenesis and drug resistance, it is reasonable to

suggest that targeting FOXM1 expression and its transcriptional activity in malignant cells constitutes a promising strategy for cancer treatment [130].

However, from a medicinal chemistry perspective, the task of targeting FOXM1 is not an easy one. Gartel et al. have described this transcription factor as "undruggable" because traditional drug discovery approaches have yielded grim results so far [20].

The long-term effectiveness of most chemotherapeutic drugs is considerably decreased by the development of acquired drug resistance [131], and it constitutes a major cause of cancer treatment failure. Researchers have shown a strong correlation between FOXM1 overexpression and the development of drug resistance [67, 130]. In this regard, the molecular mechanisms involved in FOXM1-mediated resistance are not completely understood yet, but preliminary reports suggest that FOXO proteins, which are members of the same family of FOX transcription factors, are involved.

The FOXO transcription factor family (FOXO1, FOXO3a, FOXO4 and FOXO5), functions downstream of the PI3K-Akt pathway; however, FOXO3a also functions downstream through another two pathways; ERK-MAPK and IKK [132].

Anticancer drugs such as paclitaxel [133, 134], doxorubicin [135], lapatinib [45], gefitinib [136, 137], imatinib [138, 139], and cisplatin [67, 140], depend on the transcriptional activation of FOXO, particularly the FOXO3a protein [132]. In fact, it has been observed that cancer cells possess a hyperactive PI3K-AKT pathway, which ultimately inactivates FOXO (FOXO1, FOXO3a, FOXO4, and FOXO5) proteins [132]. FOXM1 and FOXO3a have antagonistic functions and they compete with each other for binding to the regulation sites of their target genes [132]. And as FOXM1 is a vital downstream effector of the PI3K-AKT-FOXO signaling axis, its overexpression would be implicated in the lack of sensitivity of cancer cells to some chemotherapy [132, 136, 141].

Consequently, several research groups [20, 21, 93, 128, 132] have suggested the need to establish comprehensive and multidisciplinary programs dedicated to studying the role and regulation of FOXM1 by novel chemical compounds. According to these groups, this will be a prerequisite before this transcription factor can be rationally exploited as a target for pharmacological intervention and biomarkers predictive of the subsequent clinical responses [20, 21, 93, 128, 132]. Furthermore, Wonsey et al. point out that inhibition of FOXM1 would represent an attractive target for cancer therapy, indicating that "therapeutic

intervention on FOXM1 expression should have minimal toxicity in normal cells" due to the selectivity that FOXM1 has on transformed cells [93].

1.5.1. Pharmacological inhibition

1.5.1.1. Background

Despite the inherent difficulties of using pharmacological interventions to modulate the rate and the extent of FOXM1's transcriptional activity, there is convincing evidence to suggest that it is a promising strategy to counteract early carcinogenic events, as well as biochemical pathways involved in cancer promotion, invasion, and metastasis. Importantly, accumulating evidence also suggests that targeting FOXM1 can be a useful tool to decrease cancer resistance to several chemotherapeutic agents, including (but not limited to) temozolomide (glioblastoma) [111], DNA-damaging agents (platinum-based drugs and anthracyclines) [142], cisplatin (breast cancer) [132], tamoxifen (breast cancer) [143], doxorubicin (breast cancer) [89] and gefitinib (non-small cell lung cancer) [117]. These data strongly suggest that FOXM1 modulators may be clinically useful drugs for combined treatment of cancer in a wide variety of cancer types [90, 144].

1.5.2. Mechanisms of action by which drugs modulate FOXM1

1.5.2.1. siRNA

Small interfering ribonucleic acid (siRNA), a gene therapy using oligonucleotides administrated into tumors in experimental models, is one of the most commonly reported methods to modulate the expression and transcriptional activity of FOXM1, by decreasing it [23, 70].

Specific examples:

a) Inhibition of FOXM1 transcriptional activity by siRNA sensitizes breast cancer cells to cell death upon stimulation with conventional chemotherapeutic drugs and inhibits cell proliferation by increasing the population of cells in the G_2-M phase [143, 145, 146]. This suggests that it may be an effective mechanism for eliminating transformed cells in breast tumors [93]. However, this approach requires intratumoral

injection of the oligonucleotide sequence which needs to be encapsulated in a protective delivery agent such as polyethylimine-based cationic polymer (PEI) [70].

b) Xue et al. reported that downregulation of FOXM1 by siRNA inhibited cell proliferation and induced cell cycle arrest with reduced expression of FOXM1 transcriptional targets such as cyclin B1, cyclin D1, and Cdk2, and increased the expression of p21 and p27 [109]. Furthermore, down-regulation of FOXM1 reduced the expression and, consequently, the net activity of matrix metalloproteinase-2 (MMP-2), MMP-9 and vascular endothelial growth factor (VEGF), resulting in the inhibition of migration, invasion, and angiogenesis, in which these proteins are critically involved [109].

c) Knockdown of FOXM1 in the SPC-A-1 lung cancer cell line decreases cell proliferation and induces apoptosis [117]. Moreover research done by Li et al, reported that a microRNA (miRNA) named miR-134, directly targeted FOXM1 and downregulated its expression in non-small cell lung cancer cells (H1395, A549, Calu1, H1299) [147].

However, even though this approach is extremely effective in downregulating FOXM1 expression *in vitro* [37, 90, 148], it necessitates the use of very specialized lipid vesicles to allow siRNA uptake by tumor cells *in vivo*, which may represent a potential limitation in clinic.

1.5.2.2. Proteasome Inhibitors

Proteasomes are large protein complexes considered essential components of the ATP-dependent proteolytic pathway responsible for intracellular ubiquitination of most cellular proteins [149]. In cancer, proteasomes have been shown to play a key role by: **a)** controlling gene expression by degrading transcription factors such as p53, c-Jun and NF-κB [150]; **b)** contributing in cell cycle progression (turnover of p21 and p27) [149]; and **c)** regulating the levels of some proteins that have been shown to be involved in the control of apoptosis (e.g. some members of the Bcl-2 family) [150]. These large proteins are found in the nucleus and in the cytosol where they are capable of targeting polyubiquitinated proteins for degradation [16, 149, 151-154].

Multiple explanations have been postulated for the antitumor properties of proteasome inhibitors, such as NF-κB inhibition (stabilize IkB and sequester NF-kB in the cytoplasm), stabilization of p53, stabilization of p27 (a CDK inhibitor), and most recently, downregulation of FOXM1 [16, 149, 151-154].

The general mechanism of action by which proteasome inhibitors are believed to be FOXM1 modulators is represented in Figure 4 below, and it postulates that all proteasome inhibitors are FOXM1 regulators [144, 155].

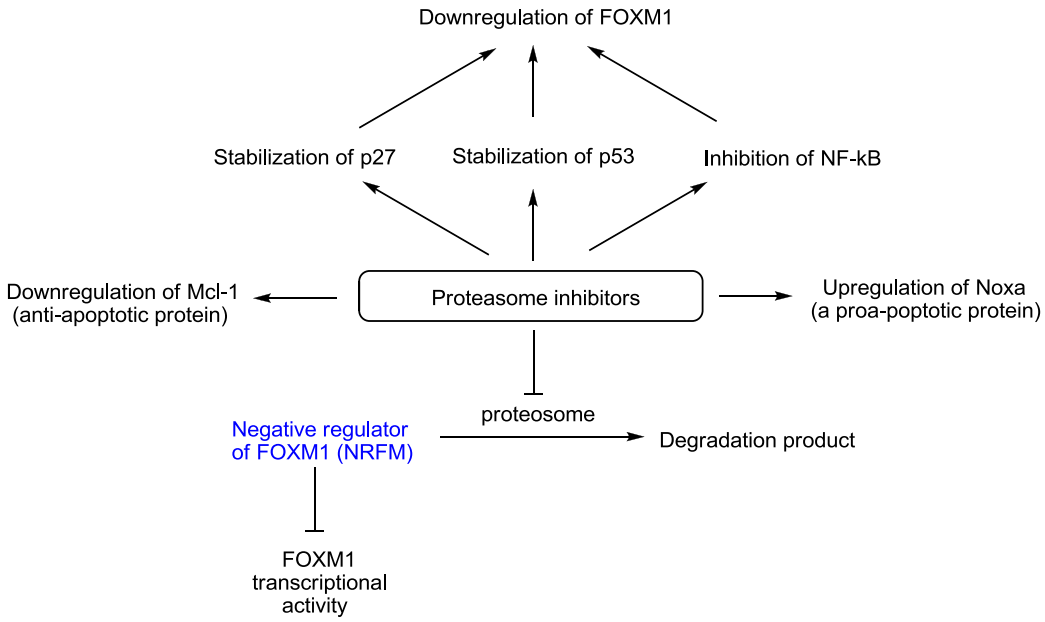


Figure 4. General hypothesis linking the activity of proteasome inhibitors to modulation of FOXM1.

Modulation of FOXM1 expression and its transcriptional activity, through inhibiting the activity of the proteasome, is one of the most promising approaches reported in the literature to target cancer initiation, progression, and metastasis [156]. This task began with a study published by Radhakrishnan et al. in which this group carried out a high-throughput screening of about 2000 compounds obtained from an NCI chemical database [127]. This assay was based on the design of a cell line (C3-Luc cell line derived from U2OS cells; which were transfected with plasmid expressing firefly luciferase under the control of FOXM1 responsive promoter) that expressed high levels of tagged FOXM1 [127]. Surprisingly, in this study, the authors identified that the antibiotic siomycin A, a known proteasome inhibitor, was capable of downregulating FOXM1 transcriptional activity, protein and mRNA response [127].

Thiazole antibiotics

The thiazole antibiotics (e.g., Siomycin A) were one of the first classes of compounds shown to inhibit the proteasome and downregulate FOXM1. Their potency (IC_{50}) ranges from 1.0 to 2.5 μM in melanoma cells, promoting apoptosis which seems to be correlated with the downregulation of FOXM1 and Mcl-1 protein [157]. In fact, Bhat et al. reported that inhibitors of the anti-apoptotic Mcl-1 protein, such as 4-amino-6-hydrazino-7-beta-D-ribofuranosyl-7-H-pyrrolo[2,3-d]-pyrimidine-5-carboxamide (ARC), exert a potent synergistic effect with thiazole antibiotics, and in general, with proteasome inhibitors, in the downregulation of FOXM1 [157].

Siomycin A

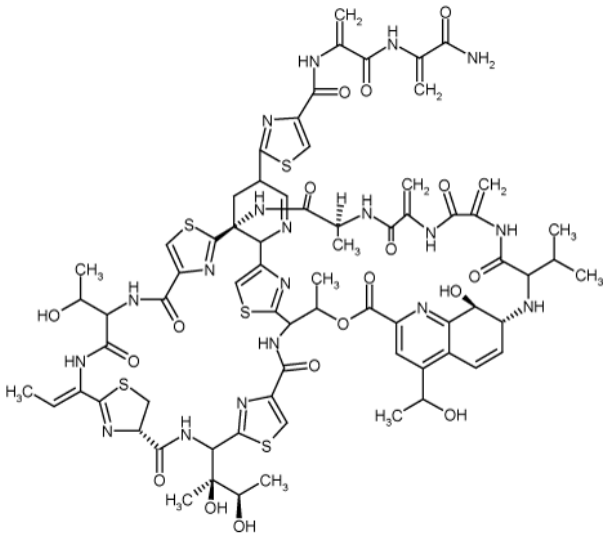


Figure 5. Chemical structure of the thiazole antibiotic Siomycin A.

In addition to the inhibition of the proteasome system, siomycin A (Figure 5) inhibits FOXM1 transcriptional activity by **a)** blocking CDK 1/2 phosphorylation on Thr 596 and **b)** by downregulating FOXM1 mRNA and protein levels required for the activation of FOXM1 in each cell cycle, causing tumor cell death *in vitro* [127, 157-159].

Thiostrepton

Thiostrepton is a thiazole antibiotic (Figure 6) known to induce cancer cell death through proteasome inhibition and also shown to inhibit the transcriptional activity and expression of FOXM1 [127, 157, 159-161]. This thiazole antibiotic has been used alone or

encapsulated into micelles; in this regard, Wang et al. reported that micelle-encapsulated thiostrepton inhibited the growth of MDA-MB-231 and HepG2 cancer xenograft models by suppressing FOXM1 expression [161]. Interestingly, this compound has been reported to induce cytotoxic effects, by down-regulating FOXM1 expression primarily at the transcriptional level on breast cancer cells, and to cause no effect on the proliferation on untransformed breast epithelial cells [160]. This difference might be due to the high levels of FOXM1 that cancer cells express, which has not been observed in untransformed cells, suggesting that thiostrepton may selectively target cancer cells [160].

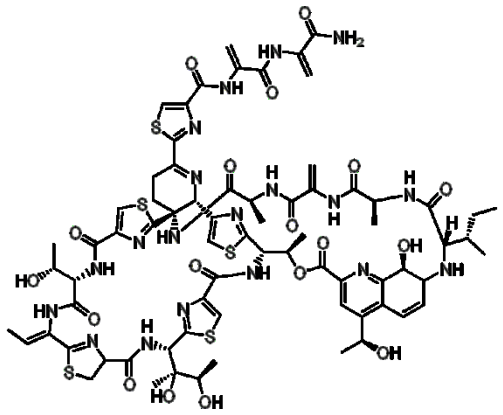


Figure 6. Chemical structure of the thiazole antibiotic thiostrepton.

It is noteworthy to mention that thiazole antibiotics do not exert any anti-growth or apoptotic effects on untransformed cells, due to its close inhibitory action on FOXM1, thus making them extremely attractive molecules for further development into specific therapeutics against cancer [158, 160].

Small molecule inhibitors of the proteasome

In this category, we describe three representative compounds, namely dacarbazine, bortezomib, and the small molecule MG-132 (Figure 7). Dacarbazine is a proteasome inhibitor (IC_{50} = 50-100 μM in melanoma cells) known to modulate melanoma cancer cells by inhibiting Mcl-1 and FOXM1 expression [157, 162]. Bortezomib was the first proteasome inhibitor proven to be effective in different types of cancer and currently it is reported to be the most used proteasome inhibitor against multiple myeloma [153, 163, 164]. The main

mechanism of action of bortezomib is its capacity to inhibit the ubiquitin-proteasome pathway by binding to the N-terminal threonine residue in the catalytic active site of the proteasome, which consequently inhibits the proteasome's activity [4, 154, 164]. Bortezomib inhibits proteasomes with a potency (IC_{50}) of approximately 20 nM in melanoma cells [165] and induces apoptosis response via upregulation of the proapoptotic protein Noxa [21].

MG-132 is another example of a small molecule inhibitor of the proteasome with high potency (IC_{50} ~200 nM in melanoma cells) [165]; it is capable of almost completely abolishing the proteasome's activity at concentrations equal to 25 μM [166].

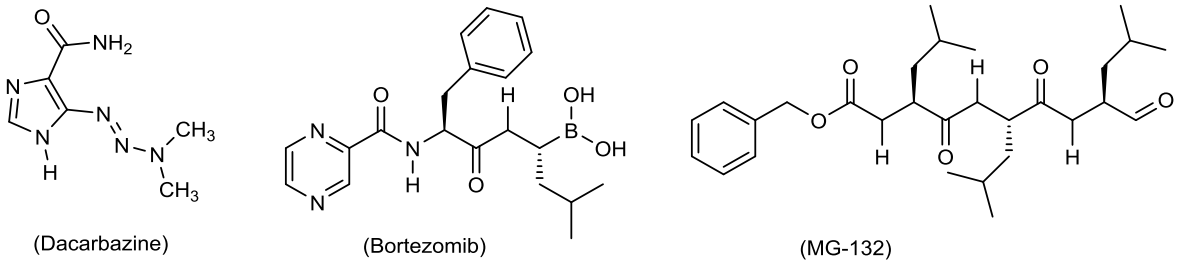


Figure 7. Chemical structures of three representative small-molecule proteasome inhibitors: dacarbazine, bortezomib, and MG-132.

Despite the promising observations suggesting that known proteasome inhibitors induce cell death rapidly and selectively in oncogene-transformed cells, but not in normal or untransformed cells [149], it has been observed (by using immunoblotting) that overexpression of FOXM1 protects cancer cells against cell death induced by proteasome inhibitors [158], suggesting that inhibition of FOXM1 may be required for the antitumor activity of proteasome inhibitors [129].

A potential drawback precluding the use of proteasome inhibitors for the systemic inhibition of FOXM1 transcriptional activity is the fact that the proteasome system modulates many other cellular functions as well. Therefore, in terms of specificity, it has been proposed by Bhat et al. that a more selective approach may involve the selective inhibition of FOXM1 and nucleophosmin (NPM), a chaperone ubiquitously expressed in mammalian cells and overexpressed in many human carcinomas[158, 166]. In fact, NPM knockdown in cancer cells leads to significant downregulation of FOXM1 [166]. Consequently, from a medicinal chemistry perspective, there is the potential for exploiting

this NPM-FOXM1 interaction to design new molecules that could modulate the level and localization of FOXM1 in tumor cells [166].

It has been discovered that *N*-acetyl-L-cysteine (NAC), which is used to identify and test ROS inducers and inhibit ROS, prevents linking to proteasome inhibitors [167]. NAC directly binds covalently to the electrophilic site of the proteasome inhibitors which possess an electrophilic group, such as bortezomib, MG132, and lactacystin, antagonizing the proteasome inhibitors' effects [167]. This same effect is observed when glutathione is present in cell culture media; therefore, it is important to consider this inhibitory effect when researchers are evaluating the activity of proteasome inhibitors on FOXM1 modulation [167].

1.5.2.3. Cyclin-dependent Kinase (CDK) inhibitors

It has been proposed that inhibitors of cyclin-dependent kinase (CDK) activity, which are either in clinical use, or currently in clinical trials, may exert their tumor-killing activity, at least in part, by inhibiting the activation (phosphorylation) of FOXM1 (previously described in Figures 1 and 2) [168].

Roscovitine is one example of a drug that could act through this mechanism; this specific CDK inhibitor has shown to arrest the cell cycle and induce apoptosis by decreasing Bcl-2 expression and by upregulating p53 [169].

Ursolic acid (Figure 8) is a pentacyclic triterpene acid that is found widely in dietary vegetables and is well known to possess a wide range of biological functions. The proapoptotic effect of ursolic acid (30 μM) on MCF-7 cells is mediated, at least in part, by inhibiting the expression of FOXM1. This effect correlates with the inactivation of CyclinD1 and CDK4 [170, 171].

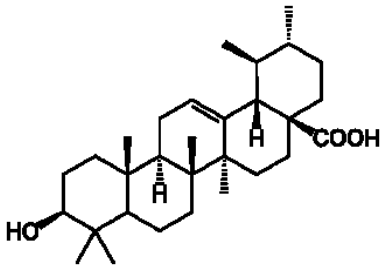


Figure 8. Chemical structure of the CDK inhibitor ursolic acid.

Finally, Rader et al. reported that administration of a highly potent CDK4/6 inhibitor, LEE011 (Novartis), reduced FOXM1 mRNA and protein levels as early as 6 hours in sensitive neuroblastoma cell lines. However, cells resistant to LEE011 showed no reduction of FOXM1 mRNA or protein levels, and these cells did not senesce [112].

1.5.2.4. Platinum-based drugs

Platinum-based chemotherapeutic drugs, i.e. cisplatin, are generally used against advanced, solid cancers such as; colon, small and non-small lung, breast, ovary, testicular and endometrial cancer [172, 173]. Their main mechanism of action is by entering into the cell and covalently attaching to the DNA, which leads to DNA-damage recognition and consequently the activation of DNA repair mechanisms, which ultimately promotes cell apoptosis response (programmed cell death) [173]. Although this mechanism is not the only mechanism by which platinum-based drugs promote their anticancer effect, it has been found in fact, that these compounds can also bind to several non-DNA target proteins, e.g. ubiquitin and cytoskeletal proteins [173].

In colon carcinoma cells, cisplatin treatment results in FOXO3a dephosphorylation at the PI3K/AKT site, nuclear translocation and its subsequent activation, leading to cyclinD2 downregulation at translational and transcriptional levels [140]. By contrast, the depletion of FOXO3a using siRNA upregulates FOXM1, which "rescues" sensitive colon carcinoma cells from cisplatin-induced cell death [140].

An additional piece of evidence showing the crosstalk between FOXO3a and its transcriptional target FOXM1, is the work done by Kwok et al., who showed that FOXM1 may be associated with resistance to cisplatin and activation of DNA damage repair mechanisms [146]. In this study, it was also shown that cisplatin-sensitive MCF-7 breast cancer cells have low levels of FOXM1, whereas their resistant counterparts have much higher levels of this transcription factor [146]. Concordantly, in a different study by Koo et al., showed that incubation of cisplatin-resistant cells with thiostrepton, a known FOXM1 modulator, induced cell death and proliferative arrest by decreasing resistance-promoting activities. These findings point to a novel mechanism of acquired cisplatin resistance in breast cancer cells, centered on the induction of FOXM1 [42].

1.5.2.5. Activation of FOXO3a

Flavonoids

Casticin (represented in 10), a natural flavonoid produced by the fruit of *Vitex rotundifolia*, has shown to inhibit the phosphorylation of the FOXO3a protein and to decrease the expression of FOXM1 (and its downstream genes), inducing cell growth inhibition and cell cycle arrest in Hep G2 and PLC/PRF/5 cells (hepatocellular carcinoma cells). The phosphorylation of FOXO3a normally takes place at the threonine-32 residue, and determines the function of this protein; therefore, He et al. recently proposed that agents that activate FOXO3a may be novel therapeutic agents that inhibit and prevent tumor growth [174]. Moreover, activation of FOXO3a could enhance the effects of current chemotherapeutic drugs such as cisplatin and paclitaxel by inhibiting FOXM1 expression that seems to be implicated in the chemoresistance of these two compounds [174].

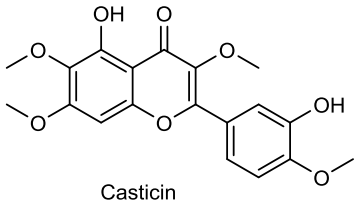
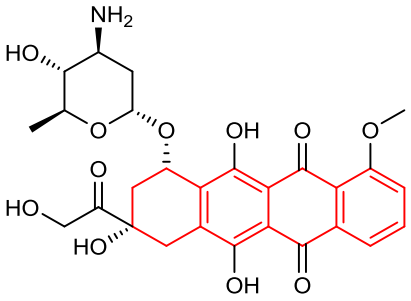


Figure 9. Chemical structure of the flavonoid casticin.

Anthracyclines

Anthracyclines are another example of FOXM1 inhibitor compounds; epirubicin (Figure 10) and doxorubicin are the two most common anthracyclines used as chemotherapeutic drugs to treat metastatic breast cancer, and they are useful in the treatment of ovarian cancer and leukemia [36, 175]. The mechanisms of action of the anthracyclines are multiple and not all of them are well established, although some of the known mechanisms are: **a)** DNA strand intercalators during cell replication [36, 175, 176]; **b)** activators of p53, cyclin-dependent kinase inhibitor $p21^{Cip1}$ and pRB [36]; **c)** downregulators of the mRNA and protein levels of FOXM1[36]; **d)** inductors of cell death by promoting

nuclear accumulation and expression of FOXO3a (a powerful transcriptional factor capable of repressing FOXM1 expression) [132, 177].



Epirubicin

Figure 10. Chemical structure of the anthracycline epirubicin. The polycyclic structure represented in red shows the functional aglycone fraction which is characteristic of this class of compounds.

Recently, Millour et al. demonstrated that FOXM1 expression levels are critical determinants of the cellular response to anthracyclines [178]. Analysis in epirubicin-sensitive and resistant MCF-7 breast carcinoma cells revealed that FOXM1 levels are higher in resistant MCF-7-EPIR cells [178]. Interestingly, they also observed that FOXM1 expression is induced by epirubicin in resistant cells, but downregulated in sensitive cells. Furthermore, by inducing the expression of FOXM1 in sensitive MCF-7 cells, they conferred epirubicin resistance to the otherwise sensitive cells, whereas depletion of FOXM1 by siRNA resulted in increased drug sensitivity in MCF-7-EPIR resistant cells [178]. The authors suggest that genotoxic chemotherapeutic agents, such as epirubicin, repress FOXM1 expression through the activation of p53 via E2F in sensitive cells, which consequently is related with an increase of pRB and a repression of E2F [178].

In chronic myeloid leukemia (CML) cells, doxorubicin promotes the expression and nuclear accumulation of FOXO3a, where it can exert a repressive mechanism on FOXM1 and induce cell death [135, 179]. Therefore, FOXM1 repression can be linked also to the action of anthracyclines via FOXO3a [24].

An additional factor involved in the response of cancer cells to anthracyclines are reactive oxygen species (ROS) [24]. These genotoxic chemotherapeutic drugs are known to induce the accumulation of ROS, which in turn activates FOXM1 in a positive feedback loop [24]. Increased levels of FOXM1 activity induce an upregulation of antioxidant proteins

including superoxide dismutase (MnSOD), catalase, and peroxiredoxin 3 (PRDX3) [24]. Consequently, the simultaneous activation of these proteins counteracts the drug-induced oxidative stress [24]. FOXM1 negatively regulates intracellular ROS levels by activating the expression of detoxifying enzymes, previously mentioned, which is one of the main mechanisms by which tumor cells evade the cytotoxic effects of chemotherapeutic drugs and promote survival of resistant clones [24].

Monteiro et al. reported that gamma H2AX (γH2AX) foci, indicative of DNA double-strand breaks (DSBs), accumulate in a time-dependent manner in drug-sensitive MCF-7 cells, but not in the resistant counterparts, in response to epirubicin [180]. FOXM1 overexpression is associated with epirubicin resistance and DSB repair [180]. Furthermore, FOXM1 knockdown was shown to significantly increase the number of γH2AX foci and to sensitize MCF-7 epirubicin resistant cells, supporting the idea that in fact FOXM1 plays a key role in epirubicin resistance [180]. In this regard, "ectopic" expression of FOXM1 can increase cell viability and abrogate DSBs sustained by MCF-7 cells following epirubicin [180].

Taxanes

Taxanes are a class of anticancer agents known to be antimicrotubule agents that mostly function by: **a)** stabilizing microtubule's dynamics, by increasing their polymerization; **b)** blocking cell cycle progression through centrosomal impairment; **c)** DNA fragmentation which leads to programmed cell death and **d)** apoptotic effects by activating Bax, a proapoptotic protein [181, 182].

Taxanes are another class of drugs that have been shown to exert their anticancer activity, at least in part, by modulation of the FOXM1 pathway by activating FOXO3a [36, 132]. In fact, taxanes have been shown to upregulate FOXO3a via JNK (c-Jun N-terminal kinase) which leads to FOXM1 downregulation [36, 132]. Paclitaxel (Figure 11) is a representative example of this class of drugs [4] and is currently used in the treatment of metastatic breast cancer [145].

Taxanes are widely used for many types of cancers such as breast, ovary, lung, bladder and esophagus, and they have shown to be very effective drugs during early stages

of cancer [182]. Unfortunately, one of the main clinical issues in cancer patients is the development of drug resistance [182]. It has been postulated that resistance to taxanes develops as a direct consequence of FOXM1 expression [36, 145].

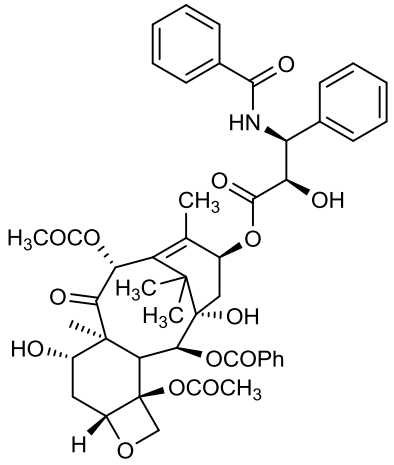


Figure 11. Chemical structure of the taxane paclitaxel.

FOXM1 stabilizes and regulates microtubules by increasing the expression and activity of the microtubule destabilizing protein Stathmin, resulting in the promotion of resistance to taxol [36, 145]. In fact, on breast cancer cell lines, it has been revealed that exits a correlation between the level of FOXM1 expression and the resistance to the taxane paclitaxel (taxol) [183]. Specifically, FOXM1 overexpression has been shown to confer resistance to paclitaxel, and depletion of FOXM1 (either by siRNA or an alternate reading frame-derived peptide [ARF26–44]) can increase the sensitivity of breast cancer cells to the drug [183].

EGFR inhibitors

The Epidermal Growth Factor Receptor (EGFR) family regulates the activation of multiple pathways implicated in cellular proliferation, differentiation, angiogenesis and survival [129, 145, 177]. The EGFR superfamily consists of a series of transmembrane receptors including HER-2/ErbB-2, and HER-3/ ErbB-3, among others. Their activation results in a homo- or hetero-dimerization following interaction with their ligands the EGF-related proteins/peptides; e.g. TGF-α, EGF, and Heparin-binding EGF (HB-EGF) [129, 145, 177].

Auto-phosphorylation of EGFR or cross phosphorylation of specific tyrosine residues results in the activation of multiple signaling cascades such as Ras/Raf/MAPK or the PI3K/Akt signaling pathways.

The result of the activation of the signaling pathways is the activation of cyclin-CDK and Plk-1, which in turn will promote cell proliferation and prevent cells from undergoing programmed cell death [129, 145]. Aberrant regulation of the EGFR pathway promotes abnormal cell proliferation and cell migration [145].

The transmembrane receptors HER2/erbB2 are upstream modulators of FOXO3a and FOXM1 [129]. In this regard, there are three drugs reported to exert inhibitory effects on the EGFR, namely herceptin, lapatinib (Figure 12) and gefitinib [36]. These drugs are described as "molecular targeted therapeutics", capable of inhibiting FOXM1 expression via the activation of FOXO3a [36].

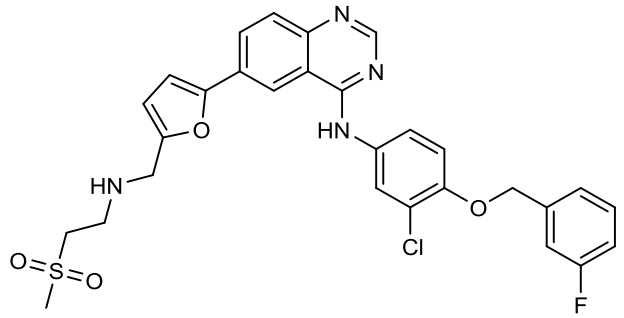


Figure 12. Chemical structure of lapatinib, one of the EGFR inhibitors exerting modulation of the FOXM1 pathway via the activation of FOXO3a.

Herceptin, which is a HER2 inhibitor, has been shown to disrupt the interaction between HER2 and its binding partner, HER3, promoting the accumulation of the Cdk inhibitor p27, hence causing cell death by inducing cell cycle arrest [145].

Lapatinib and gefitinib are tyrosine kinase inhibitors (TKI) that base their action through competitive inhibition of the ATP-binding domain of the EGFR, causing cancer cell death [132]. Lapatinib is a small 4-anilinoquinazoline kinase inhibitor known to be a EGFR/HER2 inhibitor, capable of deactivating the downstream PI3K-Akt signaling pathway, resulting in increased FOXO3a expression and consequently the downregulation of FOXM1 [21, 36, 145]. Gefitinib is more effective than lapatinib for the EGFR since gefitinib

blocks two pathways, namely the PI3K/AKT and the Ras/Raf/MAPK [36, 132, 145]. Both drugs downregulate FOXM1 at protein and mRNA levels, by activating FOXO3 expression [21, 132].

Nevertheless, it has been observed that a relatively large number of patients treated with EGFR/HER2 inhibitors develop resistant tumors [21, 36, 132]. This phenomenon could be due to the following reasons: **1)** Failure in FOXO3a activation; **2)** participation of FOXM1 in the degradation of p27, causing a promotion in tumor cell cycle progression, and the re-upregulation of some genes such as Aurora B kinase, Skp2, PLK1, CDC25B, and survivin; and **3)** dysregulation of the PI3K/AKT pathway (the latter has been mostly observed in non-small-cell lung cancer (NSCLC) [145].

Carr et al. reported that FOXM1 is responsible for the development of breast cancer cell resistance to drugs classified as EGFR receptor inhibitors, such as herceptin, lapatinib, and gefitinib [183]. Breast cancer is characterized by deregulation of EGFR and HER2, which leads to constitutive activation of the downstream PI3K-Akt signaling pathway, resulting in decreased FOXO3a and increased expression of FOXM1 [183].

1.5.2.6. NF-κB inhibitors

In cancer, it has been observed that NF-kB plays a key role in promoting the transcription of several regulators of cancer progression and invasion; e.g. cytokines (TNFα, IL6), chemokines and cell adhesion molecules (E-selectin) [98]. NF-κB is a transcription factor which has shown to be increased in a wide variety of cancers and to be related with tumor's aggressiveness [98].

In the cytoplasm, NF-κB is found to be inhibited, and this is because it is found to be bound to a group-pathway of inhibitory proteins known as IκB [98]. Once these inhibitors are phosphorylated, the translocation of NF-κB into the nucleus takes place and consequently its activation and the activation of downstream targets [98]. In cancer, active NF-κB has been reported to interfere with the transcriptional activity of p53, and to be strongly correlated with the upregulation of FOXM1, although the exact mechanism by which this occurs remains unknown [99, 184].

Emerging evidence shown by Arora et al. reported that treatment of triple negative breast cancer cells with the drug panepoxydone (an NF-κB inhibitor) caused

downregulation of FOXM1. Interestingly, reduced expression of NF-κB in the nucleus by using panepoxydone, resulted in the downregulation of FOXM1, cyclin D1, survivin and slug protein and upregulated E-cadherin [98]. In fact, the authors suggested that panepoxydone inhibited the phosphorylation of IκK which consequently promoted the cytoplasmic accumulation of NF-κB and the inhibition of FOXM1 in breast cancer cells [98].

1.5.2.7. Endocrine therapy

Breast cancer is the second leading cause of death among cancers affecting women and is the most common malignancy diagnosed in women, affecting proximately 25% of the women that develop cancer [185].

Many human malignancies are linked to the deregulation of hormone receptors. In the case of breast cancer, 70% of the diagnoses are linked to some form of estrogen receptor deregulation, which in turn has been found to be correlated with several environmental chemicals with estrogen receptor agonist activity [129, 185].

The effect of natural estrogens is regulated principally by two nuclear steroid receptors, namely the ERα and ERβ, which play an important role in cell cycle regulation, acting as ligand-regulated transcription factors [96, 129, 186]. Although each one has an opposite biological effect, ERα plays a key role in breast cancer initiation and progression due to its proliferative effects [96, 129]. On the contrary, ERβ decreases growth of cancer cells and can antagonize the effects of ERα [129, 186]. It is not yet well understood how ERα is deregulated, but is it thought that its expression can be modified through epigenetic modifications (methylation at the promoter level), by a post-translational modification or by direct interaction with the co-repressor protein [185].

It has been established that FOXM1 is a transcriptional target of ERα, and upregulation of FOXM1 often leads to an increased expression of ERα, which creates a positive feedback transcriptional loop in breast cancer patients. In fact, it has been observed that FOXM1 regulates ERα at the transcriptional level by binding to the ER transcriptional complex and by promoting ERα in breast carcinoma cells [36, 96, 129, 143, 187]. Also, it has been found that ERα can also regulate FOXM1 by controlling its action at the transcriptional and

gene promoter levels, suggesting that these two proteins co-regulate each other's expression [96, 187].

Recent work published by Sanders et al. showed this transcriptional loop that exists between FOXM1 and ERα, proving that when there is a decrease in ERα-regulated gene expression, there is also a depletion in FOXM1 expression [187].

Contrary to what is thought, the expression of ERα in cancer patients generally represents good prognosis, because 2/3 of the patients will respond positively to treatment with cisplatin, trastuzumab, fulvestrant, and aromatase inhibitors [36, 96]. Sadly, when this expression occurs in conjunction with aberrantly high FOXM1 expression, the prognosis becomes poor, and patients generally develop insensitivity and resistance to therapies [96, 143, 187].

Interestingly, it has been observed that the chemoresistance to ER-related therapies may be abrogated by blocking the expression and transcriptional activity of FOXM1, which causes a significant reduction of ERα gene expression [187]. In this regard, the use of therapeutic agents capable of inactivating the PI3K-AKT pathway or having an activating response on FOXO3a can also act synergistically with anti-estrogen treatments [132]. This action can be related either to the ability of the FOXO3a transcription factor repressing the ERα-dependent expression of FOXM1 or by the direct repressing action of FOXO3a on FOXM1 [129, 132].

Tamoxifen and fulvestrant are two widely used estrogen receptor-downregulator chemotherapeutic drugs used in hormone receptor-positive breast cancer [4, 21]. Tamoxifen has been used as the anti-estrogenic gold standard therapy in breast cancer, due to its antagonistic properties on the ERα and estrogenic properties in other tissues [4, 21]. Fulvestrant is also another ERα antagonist commercially available in the United States known to bind competitively to the ERα, decreasing the binding between estrogen and ERα [4, 21, 188].

1.5.2.8. Unknown mechanism(s)

Genistein

Genistein and several of its fluorinated derivatives have been identified as effective FOXM1 modulators *in vitro*, especially the compound 7-difluoromethoxy-5,4'-octylgenistein (DFOG; see Figure 13). These compounds downregulate FOXM1 expression and its downstream genes cdc25B, CDK1, cyclin B in two gastric cancer cell lines (AGS and SGC-7901) [189] and one pancreatic cell line [21, 40], which constitutively express high levels of FOXM1. In these experiments, the authors observed a correlation between FOXM1 downregulation and $p27^{KIP1}$ upregulation.

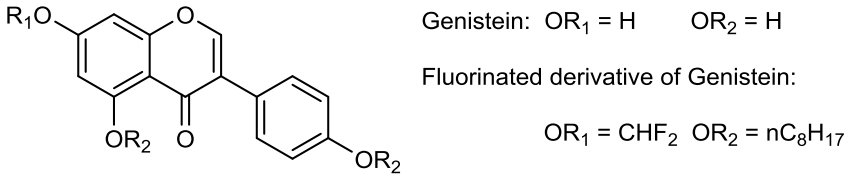


Figure 13. Chemical structures of genistein and one of its reported fluorinated derivatives (DFOG).

2-Deprenyl-rheediaxanthone B

This chemical compound (Figure 14) is a naturally occurring polyphenol extracted from the plant *Metaxya rostrate* [190]. Recently, Kainz et al. have shown that this compound at 20 μM has the ability to downregulate FOXM1 *in vitro* using SW480 and Caco2 cells. These cells differ in their expression of FOXM1; SW480 cells show high levels of FOXM1, whereas Caco2 cells express it at low levels [190].

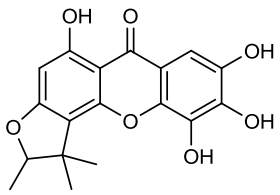


Figure 14. Chemical structure of the naturally occurring polyphenol 2-deprenyl-rheediaxanthone B.

3,3'-diindolylmethane

This heterocyclic compound (Figure 15) has been regarded as a non-toxic agent which has been isolated from natural sources, and it has been reported to modulate FOXM1 activation in breast cancer cells, leading to apoptotic cell death [21, 191]. Interestingly, Wang et al. suggested that this type of chemopreventive agent, which potentially has the ability to modulate FOXM1 expression, could be useful to decrease the incidence of cancer [129].

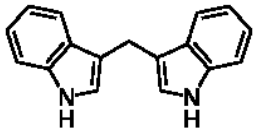


Figure 15. Chemical structure of the compound diindolylmethane.

1.5.2.9 Dual NO/H_2S-releasing non-steroidal anti-inflammatory drugs (NOSH)

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used to control fever, pain, and inflammation due to their ability to inhibit the enzymatic activity of cyclooxygenase (COX)-1 and COX-2 and there by inhibiting prostaglandin synthesis, by acetylating "strategically" located serine residues [192-194]. Nevertheless, the rationale for using NSAIDs as possible anti-cancer agents was based on the well-established relationship between inflammation and cancer, in which NSAIDs have shown to reduce cancer cell proliferation, motility, angiogenesis, and invasiveness, and are now recognized as prototypical chemopreventive agents against many forms of cancer [195, 196].

Doses of NSAIDs ranging from 81 to 325 mg taken over prolonged periods of time, have been shown to reduce the risk of developing certain types of cancers, including colon, breast, lung, prostate, and bladder [194, 197-199]. This action has been attributed to the inhibitory action that NSAIDs exert over PGE2, a major COX-2-derived modulator of cell growth [194, 196, 197]. Besides, some NSAIDs also inhibit the lipoxygenase (LOX) pathway [194, 196, 197]. As well NSAIDs have been shown to sensitize chemo-resistance phenotype tumors by inducing DNA methylation [196, 200].

COX-2 expression is induced in response to inflammatory and mitogenic stimuli, e.g. EGF, VEGF and TNF-α [196]. In fact, aberrant expression of COX-2 has been proved to be involved in the early stage of carcinogenesis and with the synthesis of prostaglandins that are involved in the inhibition of apoptosis and regulation of cell migration and invasion, all implicated in cancer development [196, 197, 201, 202]. In this regard, the COX-2 inhibitor celecoxib has been shown to inhibit NF-κB and Akt activation, as well as to reduce the proliferation of cancer cells, growth, and metastasis in colon cancer [200]. Another selective COX-2 inhibitor, known as NS-398, has been shown to have anti-proliferative, apoptotic and angiogenesis suppressor effects in many malignant cell lines, such as hepatocellular carcinoma [203].

LOX enzymes have been shown to be implicated in tumor development, cell migration and growth, although the exact role of LOX in promoting cancer growth is limited [204]. Recent studies have shown that 5-LOX is overexpressed in colon, pancreatic and in prostate cancer [204, 205]. In fact, research done by using LOX inhibitors have shown that the inhibition of LOX expression and activity promotes cancer cell apoptosis through the upregulation of E-cadherin [196, 200].

Aspirin is one of the most studied NSAIDs currently on the market, and it is used to treat pain, fever and inflammation and to prevent myocardial infarction and ischemic stroke. Lately, it has been shown to be a potential chemopreventive agent [194]. Although the exact chemopreventive mechanism of action exerted by aspirin has not been well established yet, both COX-dependent and COX-independent mechanisms have been proposed [194]. Aspirin, as well as other NSAIDs, is known to exert its anticancer action, at least in part, through the deregulation of COX enzymes, through the irreversible acetylation of COX-1 [206].

Other possible anticancer and antimetastatic mechanisms of action exerted by aspirin, include: **a)** a decrease in NF-κB activation, bv inhibiting IκB kinase (IKK) which is directly target by aspirin [194]; **b)** interference with kinase (ERK) pathway by preventing the binding between c-Raf and Ras, which leads to the induction of cell growth arrest by downregulating cyclin D and cyclin A [194]; **c)** by increasing the permeability of the mitochondrial membrane, and consequently, causing the release of cytochrome c and

activation of caspases resulting in cancer cell apoptosis [194]; and **d)** through the activation of E-cadherin [207, 208].

However, epidemiological reports have also shown a significantly higher incidence of gastrointestinal bleeding and renal disorders, which are known to be the two most common complications associated with the chronic use of NSAIDs, and more recently, a higher incidence of cardiovascular side-effects exerted by two selective COX-2 inhibitors, namely rofecoxib and valdecoxib, which resulted in the withdrawal of these two agents from the market. All these side-effects represent a serious drawback preventing their wide-spread, long-term, chemo-preventive use [209-211]. Therefore, the search for safer NSAIDs led to the design of nitric oxide-releasing NSAIDs (NO-NSAIDs), which was based on the observation that NO has some of the same properties as prostaglandins within the gastric mucosa [212]. Therefore, coupling an NO-releasing moiety to an NSAID might deliver NO to the site of NSAID-induced damage, thereby decreasing gastric toxicity [212].

Recently, a new class of NSAIDs possessing a hydrogen sulfide (H_2S)-releasing moiety (H_2S-NSAIDs) have been described in the literature. Like the NO-NSAIDs, H_2S-releasing NSAIDs are far less toxic than the parent compounds [213]. This has been attributed to the fact that H_2S, like NO, is an important bioregulatory molecule with anti-inflammatory, antioxidant, and vasodilator profiles [214-216].

Recent data obtained after *in vitro* and *in vivo* screening of a series of H_2S-releasing NSAIDs has shown that these compounds could also be used as potential anticancer agents [192, 215, 217]. In this regard, the mechanisms of action exerted by the H_2S-NSAIDs are still under investigation, but they seem to be related to the inhibition of NF-κB, in a concentration-dependent manner [192, 214]. Additional biological evaluations carried out with colon cancer cells (HT-29) and breast cancer cells (MDA MB-231) showed that H_2S-NSAIDs induced their apoptotic response by blocking the cell cycle transition at G0/G1 [214, 216]. Moreover, analysis by flow cytometry showed that H_2S-NSAIDs increase, in a dose-dependent manner, the percentage of cells in G0/G1 and reduce the percentage of cells in S and G2/M phases [217]. This could be due to the action of H_2S-NSAIDs on cyclin D1 protein expression, which has been found to be reduced [217].

Finally, it has been established that reactive oxygen species (ROS) are also involved in the mechanism of action of H_2S-NSAIDs [214]. By using molecular probes, Kashfi et al. have shown that H_2S-NSAIDs dose-dependently induced ROS (detecting intracellular levels of peroxides and superoxide anions) in MDA MB-231 breast cancer cells, which resulted in the promotion of cell death [217].

Following the trend in this field, Kashfi et al. have recently shown that H_2S-NSAIDs do, in fact, decrease cancer cell proliferation [218, 219], but *both NO-NSAIDs as well as H_2S-NSAIDs, have their own drawbacks limiting their development as pharmaceuticals*. For example, H_2S-NSAIDs have relatively high IC_{50}'s (from 2.8 to 81 μM) [218] for cell proliferation inhibition, and some NO-NSAIDs can form highly toxic quinone methide intermediates, questioning the role of NO in their biological activity [220]. Other compounds in these series display high IC_{50} values for cell proliferation inhibition [221].

Consequently, Kodela et al. postulated that a new hybrid scaffold possessing both the NO and the H_2S-releasing groups might be more potent than either one alone. This hypothesis proved to be correct, as described in the literature, and the new hybrid NO-/H_2S-relasing NSAIDs showed significant cancer cell growth inhibitory properties at the low micromolar range [222]. These new series of compounds were generically identified as the ***NOSH-compounds***.

These compounds were proven to release H_2S and NO. *In vitro* evaluations showed that NOSH compounds release NO in a time-dependent manner, reaching the highest peak at 6 hours [223]. On the other hand, the precise mechanism for H_2S release from ADT-OH structure is not yet clear [224]. Although, research done by our collaborators and other research groups, have shown by analyzing the release of H_2S in real time, that H_2S was released inside the cell [223, 224]. In fact, our collaborators used homogenized mouse liver to analyze the relation between H_2S production in media and H_2S production in tissue, in which it was observed that H_2S production in tissue is significantly higher than in media [223]. This suggests that probably intracellular enzymes are responsible for inducing H_2S release, concluding that this might minimize the loss of H_2S from the tissue [223, 224].

Recent reports from this same research group have shown that NOSH compounds had a potent cell proliferation inhibitory action on eleven different cancer cell lines from six

different tissue origins [222]. The NOSH-1 (Figure 16) compound was shown to be the most potent compound in this series, with IC_{50} values for cell proliferation inhibition in the 48-280 nM range [100,000 fold more potent compared to Aspirin (ASA)], and proved to be nontoxic (analysis done by measuring the release of lactate dehydrogenase (LDH), a measurement of cellular toxicity) [222].

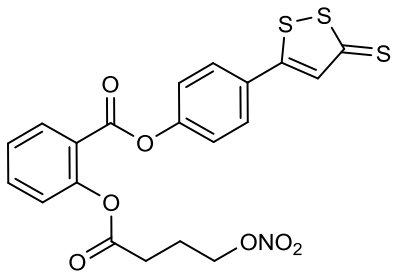


Figure 16. Chemical structure of the hybrid dual NO-/H_2S-releasing aspirin NOSH-1.

The observed chemical similarities between NOSH compounds and some thiazole antibiotics (see Figure 5 and Figure 6) provided a plausible explanation for the unexpected and highly significant increase in the *in vitro* potency of NOSH compounds compared to NSAIDs. The chemical similarities between NOSH compounds and thiazole inhibitors offered the possibility that NOSH molecules could exerted their cancer cell growth inhibitory profile, at least in part, by downregulating FOXM1. Consequently, NOSH compounds might be able to inhibit the expression of FOXM1 in cancer cell lines, and this constitutes the basis for the current thesis study.

2.0 Hypothesis

The FOXM1-dependent anticancer effect exerted by the NOSH compounds is largely due to the presence of the heterocyclic compound ADT-OH, which is the moiety that (a) resembles thiazole antibiotics, and (b) is responsible for the release of H_2S.

At the beginning of this investigation, we did not know the extent to which all the bioactive components present in the NOSH scaffold contribute to the downregulation of FOXM1, and therefore, it is also hypothesized that *derivatives possessing only two out of the three moieties in NOSH molecules are likely to exert weaker modulatory effects on FOXM1 than the parent NOSH structures*.

3.0 Objectives

Our NOSH compounds have some structural similarities to the chemical structures of the proteasome inhibitors/thiazole antibiotics, in which both our products and thiazole antibiotics contain a thio structure and a heterocyclic structure of 5 members with heteroatoms. As chemical structure similarities exist between our compounds and the thiazole antibiotics, known to be FOXM1 modulators, our compounds may modulate FOXM1 protein expression as well. Previously our collaborators (Dr. Khosrow Kashfi, etal.; City University of New York) confirmed that the NOSH-1 compound (the compound used as lead compound in evaluations in this thesis) concentration-dependently decreased FOXM1 protein expression *in-vitro*. Nevertheless, it is not known which of the three active constituents of the NOSH scaffold was responsible for this activity.

Therefore, the general objective of my thesis research was to:

1. Design and synthesize new series of NOSH derivatives containing:
    a) The aspirin and the H_2S-releasing moieties
        - Compounds **ARD-N1-109.2** and **ARD-N1-114.2**
    b) The aspirin and the NO-releasing moieties
        - Compound **ARD-N1-139**
    c) The NO- and the H_2S-releasing moieties (without aspirin)
        - Compound **ARD-N1-188**
    d) The H_2S-releasing moieties
        - Compound **ADT-OH**

2. To determine whether each of the three components present in the NOSH scaffold affects:

    a) the cell viability of three cancer lines of different origin [colon (HT-29), liver (HepG2) and breast (SKBR-3)]

    b) the expression of FOXM1 protein in two cancer cell lines of different origin (HT-29) and (SKBR-3).

4.0 Materials and Methods

4.1. Chemistry

Melting points were recorded with an Electrothermal Mel-Temp® melting point apparatus (Dubuque, IA, USA).^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AVANCE 600 NMR spectrophotometer; coupling constants (J) are reported in Hertz (Hz), and the corresponding chemical shifts are represented as δ units (ppm), using TMS as internal standard. ESI-MS spectra were recorded using a Waters micromass ZQ-4000 single quadruple mass spectrometer.

ADT **(2)** [225, 226], ADT-OH **(3)** [225, 226], 4-methoxy-4-oxobutanoic acid **(5)** [227], 2-formylphenyl methyl succinate **(7)** [222], 2-((4methoxy-4-oxobutanoyl) oxy) benzoic acid **(8)** [222], 4-hydroxybenzothioamide **(9)** [228], 2-(formyl) phenyl 4-bromobutanoate **(13)** [222], 2-(formyl) phenyl 4-nitrooxybutanoate **(14)** [222], 2-(hydroxycarbonyl) phenyl 4-nitrooxybutanoate **(15)** [222] and 4-cyanophenyl 4-bromobutanoate **(19)** [222] were prepared based on the literature with some modifications accordingly to the compounds and to improve their yield. Compounds were purified by using column chromatography on a CombiFlash Retrieve, or CombiFlash Rf system using RediSep Rf silica gel® (40-60 μm) cartridges, or prepacked RediSep Gold® columns for normal-phase and C-18 reversed-phase. Some reactions were performed on a Biotage® Initiator Microwave Reactor (Charlotte, NC, USA) and were purified by using column chromatography. TLC analysis was performed on RediSep® silica gel glass plates (UV254, 0.2 mm) and Baker Pre-Coated Hard Layer TLC Octadecyl Silica (C18) Si-C18F by using high, medium, and low polarity solvent mixture for flash chromatography. All other reagents were purchased from either Sigma Aldrich (Milwaukee, IN) or TCI America (Portland, OR) and were used without further purification.

4.2. Cell lines and culture conditions

Cell lines were chosen based on their FOXM1 expression; all three cell lines used in the investigations had to show high expression of FOXM1 protein. Human colon cancer

(HT-29) and breast cancer (SKBR-3) cell lines were obtained from the American Type Culture Collection (ATCC). The human liver cancer cell line (HepG2) was obtained from Dr. Lars-Oliver Klotz, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% non-essential amino acids (NEAA), and 1% penicillin and streptomycin. From now on, in this thesis this solution will be referred to as "*growth medium*".

Drug solutions were prepared by dissolving the drug in DMSO (primary solution), and transferring aliquots to DMEM medium to obtain final concentrations of DMSO no higher than 1%. The final drug concentrations for MTT evaluations were 100 μM, 80 μM, 60 μM, 50 μM, 40 μM, 20 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM, 0.001 μM and 0 μM (containing 1% of DMSO mixed in DMEM), which is was used as baseline, considered as 100% cell viability for comparison purposes.

In Western Blotting assay (WB), drug concentrations were 100 μM, 80 μM, 60 μM, 40 μM, 20 μM, and 0 μM (1% of DMSO in DMEM). The concentration range for WB was based on the results obtained from cell viability assays in HT-29 and SKBR-3 cell lines.

4.3. Cell Viability Assays (MTT)

Cell viability was determined by the MTT assay and all experiments were performed at least three times in quadruplicate by using different passages (starting passage 4 until passage 20). Cells' subcultures were serially propagated after harvesting the cells with 0.25% (w/v) Trypsin-0.53mM EDTA solution in growth medium.

Cells were seeded in 96-well plates [HepG2 and HT-29 ~$3x10^4$ cells/well (200 μl) and SKBR-3 ~$5x10^3$ cells/well] and incubated for 24h under standard conditions (37°C and 5% CO_2), allowing them to reach ~80-90% of confluence, prior to treatment.

After reaching the proper confluence, the growth medium was removed and the cells were incubated in Dulbecco's Phosphate Buffered Saline (PBS) for 2 h. PBS was then removed and 200 μL of media containing the appropriate dilutions of various treatments

was added and cultures were incubated at 37°C incubator for 24 h. Following the incubation period, cells were washed with PBS and 100 µL of MTT solution (0.5mg/mL) was added to each well. Cultures were incubated for 2 h or until the MTT solution turned purple. The MTT solution was extracted by vacuum and formazan crystals were then solubilized by adding 200 µL of a solution of HCl (49 µL)/isopropanol (50 mL). Absorbance was measured at 570 nM using the plate reading spectrophotometer. Cell viability for each treatment and drug concentration was calculated by normalizing the value to the control (1% DMSO in DMEM) considered as 100%.

4.4. Western Blot

The HT-29 and SKBR-3 cells were grown in 6-well plates until reaching 80-90% of confluence. Cells were treated under different concentrations of various compounds. Dilutions of 100 µM, 80 µM, 60 µM, 40 µM, 20 µM, 0 µM drug concentrations were prepared as described previously on MTT assays.

Cells were treated with various compounds for 24 h and then solubilized by 100 µL of 2X Laemmli Buffer (Sigma-Aldrich Life Science) at 95°C for 5 minutes followed by sonication.

12µL of prepared sample (~ 20 µg of protein - only measured in cells containing DMSO and not with cells under treatment) of total cell extracts from each treatment for each cell line were resolved by 8% SDS-PAGE, transferred to PVDF membranes and immunoblotted. Gels were run in triplicate or quadruplicate in parallel in the same electrophoretic tank at 150V for 1 h and 10 min. Membranes were incubated with TBST/5% milk blocking agent for 2 h at RT followed by incubation with FOXM1 antibody (A-11) mouse monoclonal IgG_2 (Santa Cruz Biotechnology, Inc.) at 1:250 overnight at 4°C with slow rocking. Membranes were then incubated at RT with goat anti-mouse HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) at 1:2000 for 1h.

Membranes were developed using Chemiluminescence ECL Prime Western Blotting solutions (Amersham). Visualisation and quantification were carried out with the Quantity One software (Imaging system VersaDoc MP5000 Bio-Rad). FOXM1 quantification was done by calculating the densitometric value obtained for FOXM1

protein divided by the densitometric value of the β-actin in the same sample. The results were analyzed by using statistical software Graph Pad Prism 5.

4.5. Statistical Analysis

All experiments were repeated two or three times. Statistical analyses were performed by using Graph Pad Prism 5.01 (Graph Pad Software, San Diego, CA, USA).

On MTT assay, values obtained from cells treated only with DMSO were considered as 100% and the percentage of viability on treated cells was calculated as the following formula.

% Viable cells= (drug treated cells/ DMSO cells) x100

Relative IC_{50} was measured by using IC_{50} evaluations with nonlinear regression analysis. To evaluate the differences between drugs and their concentrations in the MTT assays, two-way ANOVA combined with the Bonferroni test was used.

To analyze the differences between concentrations in Western Blot, one-way ANOVA was performed followed by Tukey's multiple comparison test.

Data were expressed as the means ±Standard Error of the Mean (SEM). $P<0.05$ was regarded as statistically significant, and this was represented as "+" in all graphs, whereas $p<0.01$ was represented as "°", and $p<0.001$ as "*". N= equals the number of independent experiments performed.

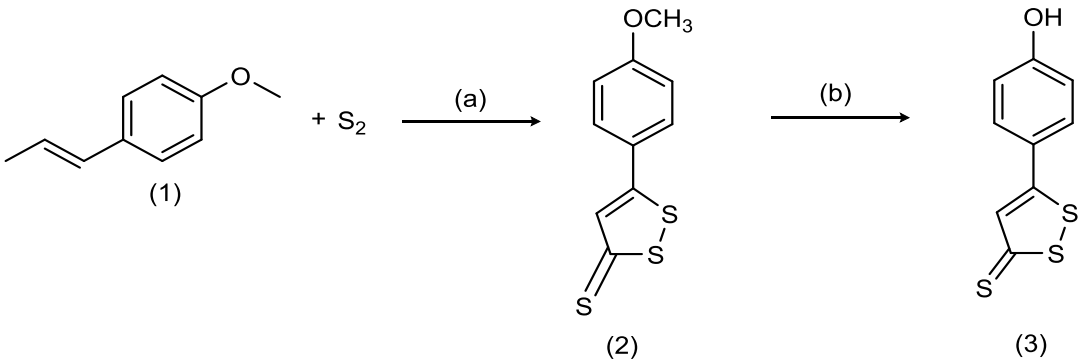
5.0 Results and discussion

5.1. Chemistry

In this project, it was possible to obtain 5 new compounds which had not been synthesized and published before. Final reactions are described in this section although various reactions, which did not succeed, were performed before getting the right reactions (those reactions are not described in this thesis).

5.1.1. Synthesis of ADT-OH

The procedure for the chemical synthesis of the hydrogen sulfide-releasing agent ADT-OH **(3)**, was based on the *International Patent WO 2008/077453 A1* protocol, with some experimental modifications (see scheme 1) which had an impact on the product's yield and in the reaction time [225, 226].



Scheme 1: Reagents and conditions: (a) DMA, 145°C, 6h; (b) Pyridine hydrochloride, microwave (MW)-based heat to generate 215°C, 20 min.

5.1.1.a. Synthesis of ADT (2)

Anethole **(1)** (11.84 g; 80 mmol; 1 eq) was dissolved in 20 mL of dimethylacetamide (DMA), and mixed with sulfur (17.92 g; 560 mmol; 7 eq). This mixture was stirred for 6 h at 145°C in a normal heating plate*.

*Note: At this stage it was not possible to use microwave irradiation because the high pressure initially resulted in the breakage of microwave glass vials.

The stirring was stopped and the mixture was cooled down overnight. Contrary to what was mentioned in the patent, in which they used ether to wash the compound, followed by crystallization with ethyl acetate, we decided to dissolve the product by using ethyl acetate. After dissolution, 100mL of HCl (1M) was added to the reaction, which allowed the precipitation of a dark orange solid, which contained ADT **(2)** along with other impurities. ADT **(2)** was purified by flash column chromatography using petroleum ether: ethyl acetate (90:10). However, some impurities were difficult to eliminate in the first column separation, so it was necessary to perform a second purification by using a second column. The yield for this reaction was 35.3%. Some physical and ^{1}H NMR spectroscopic data for compound **(2)** are listed below.

^{1}H NMR ($CDCl_3$, 600MHz): δ = 3.91 (s, 1H), 7.01 (d, J=9 Hz, 2H), 7.44 (s, 1H), 7.65 (d, J= 8.4 Hz, 2H).

Melting point: 104-106°C.

5.1.1.b. Synthesis of 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH, 3)

This demethylation reaction was also modified from the previously described patent [225, 226] although in this case a Biotage Microwave reactor was used, known to enhance the heating performance of a reaction and to decrease the formation of unwanted impurities. The use of the microwave reactor allowed us to synthesise ADT-OH in less time and with higher yield compared to conventional heating with a heating plate.

ADT (**2;** 350 mg; 1.45 mmol; 1.0 eq) obtained in the previous step, was mixed with pyridine HCl (2 g; 17.30 mmol; 11.9 eq) and stirred in a 20 mL microwave glass vial at 215°C for 20 min (no solvent). After heating, the reaction mixture was diluted with absolute ethanol and transferred to a 500 mL beaker followed by the addition of 50 mL 1M HCl solution. The addition of the acid produced an orange precipitate that was filtered and dried overnight under vacuum.

TLC analysis using a combination of solvents formed by hexane:ethyl acetate (85:15) showed that the starting material **(2)** had a Rf = 0.41, whereas the product **(3)** showed a Rf = 0.14. Some physical and ^{1}H NMR spectroscopic data for compound **(3)** are listed below.

^{1}H NMR ($CDCl_3$, 600MHz): δ = 5.21 (s, 1H), 6.89 (d, J=6.6 Hz, 2H), 7.48 (s, 1H), 7.67 (d, J= 9 Hz, 2H).

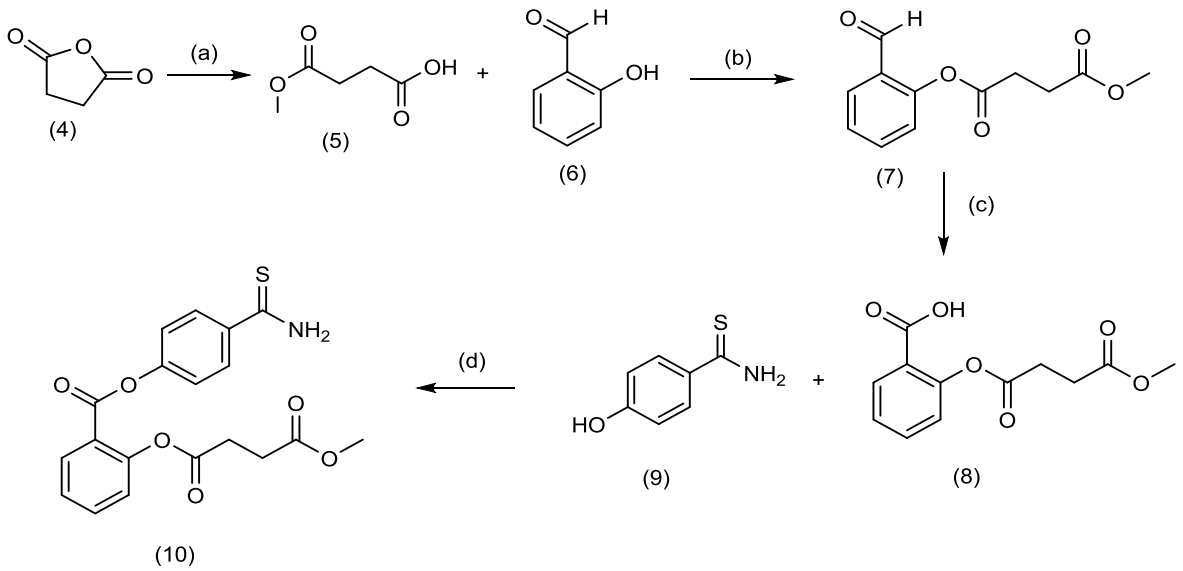
Product **3** was characterized as an orange crystal powder with a melting point of **189-191°C**.

The results obtained from this last reaction, showed the advantages of using the microwave reactor, in which it was possible to improve the chemical reaction for obtaining compound **(3).** The main improvements were related, time of reaction and yield improvement by decreasing the amount of impurities formed. In fact, by using the microwave reactor for 10 minutes we were able to get a yield reaction of 74.7%, but, as the time reaction was increased to 20 minutes the yield of the reaction increased as well to 86.3%; sadly, no more yield improvement was observed after 20 minutes of heating in the microwave reactor.

5.1.2 Synthesis of compound ARD-N1-109.2 (10) [Aspirin/H_2S-releasing group]

5.1.2.a. Synthesis of methyl succinate (5)

Succinic anhydride **(4)** (300 mg; 3mmol; 1 eq) was dissolved in methanol and heated under reflux for about 2 h, as described by Wheathey & Keay; 2007 [227]. The 4-methoxy-4-oxobutanoic acid **(5)** obtained was concentrated under vacuum. The product was obtained as a white crystal solid (96.2% yield); confirmation of the product's synthesis was provided by ^{1}H NMR ($CDCl_3$, 600MHz).



Scheme 2. Chemical synthesis of the hybrid ASA/H_2S-releasing compound ARD-N1-109.2 (**10**). Reagents and conditions: (a) CH_3OH, reflux, 2h; (b) DMAP, DCC, DCM, RT, overnight; (c) $KMnO_4$, acetone, 4° C, 5 min; then stir at RT for 3h; (d) DMAP, DCC, DCM, RT, 4.5 h.

5.1.2.b. Synthesis of 2-Formylphenyl methyl succinate (7)

In the second step of this synthesis, a procedure similar to the described by Kodela et al. [222] was used. Briefly, to a solution of 4-methoxy-4-oxobutanoic acid **(5)** (300 mg; 2.27 mmol; 1 eq) obtained previously, in 15 mL of dichloromethane (DCM) was added dicyclohexylcarbodiimide (DCC) (468 mg; 2.26 mmol; 1.1 eq) and DMAP (27.7 mg; 0.22 mmol; 0.1 eq) using an ice bath to cool down the reaction mixture. After stirring for 5 min, 2-hydroxybenzaldehyde was added **(6)** (277 mg; 2.26 mmol; 1 eq) and the mixture was stirred overnight at room temperature. The solution was filtered to eliminate unreacted DCC and the excess solvent was evaporated under vacuum. Product **7** was purified by silica gel column chromatography using hexane:ethyl acetate (80:20). After purification, the solvents were removed under vacuum to obtain white crystals (48.6% yield). The 2-formylphenyl methyl succinate **(7)** was analyzed by ^{1}H NMR ($CDCl_3$, 600MHz).

5.1.2.c. Synthesis of 2-Hydroxycarbonylphenyl methyl succinate (8)

The next step consisted of an oxidation reaction based on a previously reported procedure [222]. 2-Formylphenyl methyl succinate **(7)** obtained in the previous reaction was dissolved in 3 mL of acetone and the mixture cooled down in an ice bath. Then, potassium

permanganate (301 mg; 1.9 mmol; 2 eq) was added and the mixture stirred at room temperature for 3 h. The consumption of the starting material and appearance of the product were followed by TLC. After reaction completion, the mixture was poured into a beaker containing 20 mL of HCl (1M) and the solid filtered. The liquid phase was shaken with ethyl acetate (3 x 20 mL) in order to extract the carboxylic acid. Mixed organic phases were dried with sodium sulfate and the solvent was evaporated under vacuum. The 2-hydroxycarbonylphenyl methyl succinate **(8)** was present in the organic phase. On TLC, the starting material (2-formylphenylmethyl succinate (**7**) showed an Rf = 0.67 and the product (2-hydroxycarbonylphenyl methyl succinate (**8**) had an Rf = 0.48. The product (71.7% yield) was used in the next step without further purification. Confirmation of the product's synthesis was by ^{1}H NMR ($CDCl_3$, 600MHz).

5.1.2.d. Synthesis of ARD-N1-109.2

Intermediate **8** (600 mg; 2.38 mmol; 1 eq) was dissolved in about 20 mL of DCM, and mixed with DCC (491.3 mg; 2.38 mmol; 1.1 eq) and DMAP (30 mg; 0.24 mmol; 0.1 eq). This reaction mixture was cooled down in an ice bath, before the addition of thiobenzamide (**9, -**TBZ- prepared as described [228]; 364 mg; 2.37 mmol; 1 eq) and the reaction mixture stirred at room temperature for 4.5 h. The reaction was monitored by TLC using a combination of hexane:ethyl acetate (60:40). In this chromatographic system, the starting materials **8** and **9** showed Rf values = 0.80 and 0.55 respectively, whereas the product **(10)** showed a Rf = 0.70.

The solution was filtered (to eliminate the unreacted DCC), the solvent was evaporated under vacuum, and the product purified by flash silica gel column chromatography (hexane: ethyl acetate 60:40). Analysis by ^{1}H-NMR showed impurities, and therefore it was necessary to carry out additional chromatography purification, this time by reverse phase flash chromatography (C_{18} column), using a combination of water: acetonitrile (15:85). Combined organic fractions containing the spot corresponding to the product were dried under vacuum. This final product is a new product **(10)**, not found on the market or published in any patent or paper; for this reason it was necessary to perform

its characterization. Physical characterization, NMR spectroscopic and MS data for compound **(10)** are listed below.

^{1}H NMR ($CDCl_3$, 600MHz): δ = 2.74 (t, J=6.9 Hz, 2H), 2.96 (t, J=6.9 Hz, 2H), 3.71 (s, 1H), 7.21(s, 1H), 7.24 (d, J= 102 Hz, 1H), 7.26 (d, J= 9 Hz, 2H), 7.43 (td, J=7.8, 1 Hz, 1H), 7.61 (s, 1H), 7.69 (td, J=7.8, 1.8 Hz, 1H), 7.98 (dd, J= 6.6, 1.8 Hz, 2H), 8.24 (dd, J=7.8, 1.8 Hz, 1H) (Appendix Image 1)

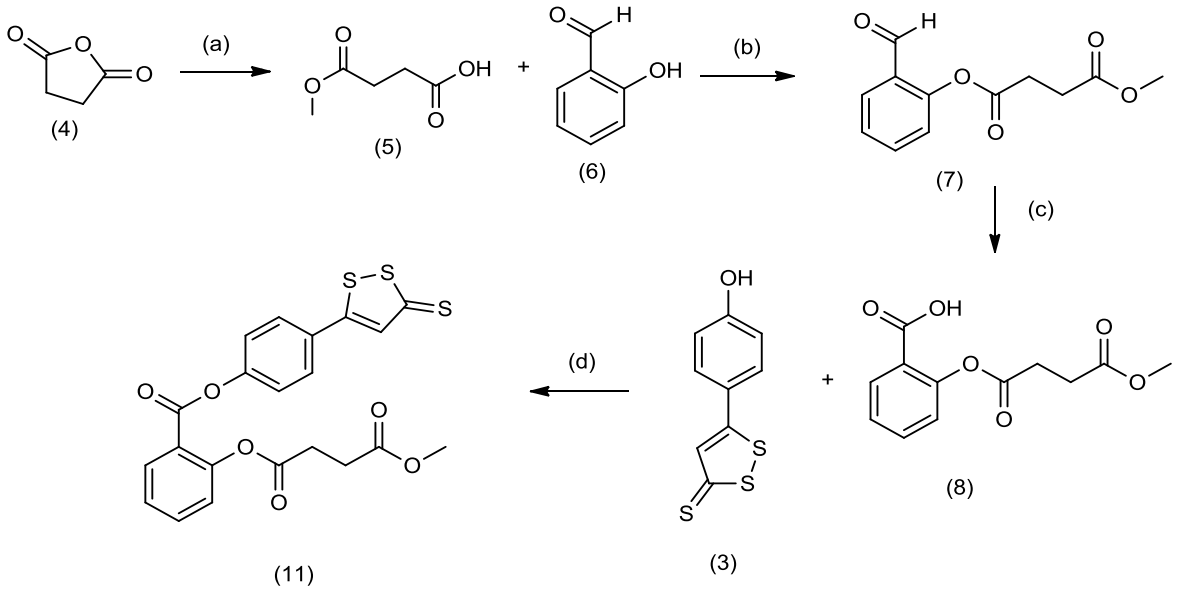
^{13}C NMR (DMSO-d_6) δ: 28.30, 28.78, 51.53, 121.19, 121.95, 124.03, 126.49, 128.99, 131.66, 135.25, 137.40, 150.21, 152.31, 162.36, 170.83, 172.08, 198.97. (Appendix Image 6)

MS (EI), m/z: 389.3, 410 (M^+ +Na).

Product obtained **(10)** was characterized as yellow small crystal, with a melting point of **120-122°C**.

5.1.3. Synthesis of compound ARD-N1-114.2 (11) [ASA/H_2S-releasing group]

Compounds 5-8 were synthesized as described in the previous section.



Scheme 3. Chemical synthesis of the hybrid ASA/H_2S-releasing NOSH derivative ARD-N1-114.2. Reagents and conditions: (a) CH_3OH, reflux, 2h; (b) DMAP, DCC, DCM, RT, overnight; (c) $KMnO_4$, Acetone, ice bath, 5 min; then stir at RT, 3h; (d) DMAP, DCC, DCM, RT, 5 h.

5.1.3.a. Synthesis of ARD-N1-114.2 (11)

The synthesis of this compound was based on a previously reported procedure by Kodela et al. with some modifications [222]. Briefly, intermediate **8** (334 mg; 1.32 mmol; 1 eq) was dissolved in 12 mL of DCM, mixed with DCC (273 mg; 1.32 mmol; 1.1 eq), and DMAP (16 mg; 0.13 mmol; 0.1 eq); this reaction mixture was cooled down in an ice bath. 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (ADT-OH, **3**; 300 mg; 1.32 mmol; 1 eq) was added to the reaction and the mixture stirred at room temperature until the starting materials were consumed (as determined by TLC), which took about 5 h. The corresponding Rf values for the starting material **(3)** and the product (**11**) were 0.41 and 0.29 respectively (using hexane:ethyl acetate 70:30).

In order to proceed with the synthesis, solids had to be filtered off, the solvent was removed under vacuum and the residue purified by flash silica gel column chromatography (hexane:ethyl acetate 70:30). Combined fractions containing the product were taken to dryness under vacuum, affording an impure product, which required a second chromatography step, this time by reverse phase column chromatography (C_{18} column) using water/acetonitrile (15:85). Combined organic fractions containing the spot of the product were dried under vacuum. This second final product **(11)** is a new product, not found on the market or published, and for this reason it was necessary to characterize it. Physical characterization, NMR spectroscopic and MS data for compound **(11)** are listed below.

Product **11** was characterized as dark metallic orange solid powder with a melting point of **110-112 °C**.

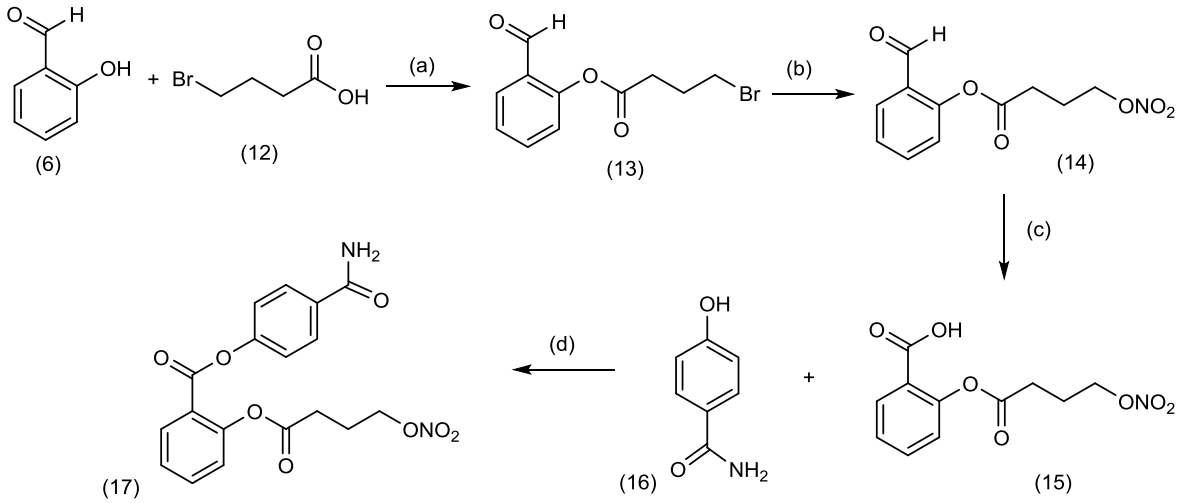
^{1}H-NMR ($CDCl_3$, 600MHz): δ = 2.72 (t, J=6.9 Hz, 2H), 2.94 (t, J=6.9 Hz, 2H), 3.69 (s, 1H), 7.22 (dd, J= 8.1, 0.9 Hz, 1H), 7.33 (dt, J= 9.6, 2.4 Hz, 2H), 7.41 (td, J=7.8, 1 Hz, 1H), 7.42 (s, 1H), 7.68 (td, J=7.8, 1.8 Hz, 1H), 7.73 (dt, J= 9, 2.4 Hz, 2H), 8.22 (dd, J=7.8, 1.8 Hz, 1H). (Appendix Image 2)

^{13}C-NMR (DMSO-d_6) δ: 28.77, 29.22, 51.95, 121.78, 123.18, 124.26, 126.40, 128.37, 129.54, 132.13, 135.16, 136.14, 151.31, 153.45, 162.26, 171.08, 171.60, 173.63, 215.56. (Appendix Image 7)

MS (EI), m/z: 461.0, 483.0 (M^+ +Na).

5.1.4. Synthesis of compound ARD-N1-139 (17) [ASA/NO-releasing drug]

The chemical synthesis of this hybrid compound (see scheme 4) was based on a published protocol by Kodela et al. with some modifications [222].



Scheme 4. Reagents and conditions: (a) DMAP, DCC, DCM, RT, 3h; (b) $AgNO_3$, CH_3CN, MW, 70°C, 2h; (c) $KMnO_4$, Acetone, ice bath, 5min; then stir at RT, 3h; (d) DMAP, DCC, DCM, RT, overnight.

5.1.4.a. Synthesis of 2-(Formyl) phenyl 4-bromobutanoate (13)

2-Hydroxybenzaldehyde **(6**, 300mg; 2.45 mmol; 1 eq) was dissolved in 20 mL of DCM, and mixed with DCC (507 mg; 2.26 mmol; 1.1 eq) and DMAP (30 mg; 0.24 mmol; 0.1 eq). The reaction mixture was cooled down in an ice bath before adding 4-bromobutyric acid (**12**, 410 mg; 2.45 mmol; 1 eq) and stirring for 3h. After the reaction was completed (Rf for **6** = 0.82; Rf for the product **13** = 0.37 using a mobile phase consisting of hexane: ethyl acetate 90:10), the suspension was filtered and the solvent evaporated under vacuum. The title product was purified by silica gel column chromatography (hexane:ethyl acetate 90:10). After purification, the solvents were removed under vacuum, affording compound **13** as a colorless liquid (53.4% yield); confirmation of the product's synthesis was by ^1H NMR ($CDCl_3$, 600MHz).

5.1.4.b. Synthesis of 2-(Formyl) phenyl 4-nitrooxybutanoate (14)

The next reaction in this sequence consisted of a nucleophilic substitution reaction in which a bromo group was replaced by a NO-releasing organic nitrate. Intermediate **13** (2-(formyl) phenyl 4-bromobutanoate, 600 mg; 2.21 mmol; 1 eq) was dissolved in 5 mL of acetonitrile followed by the addition of silver nitrate ($AgNO_3$, 564mg; 3.32 mmol; 1.5 eq). The reaction was stirred at 70°C for 2 h in a microwave reactor. The progress of this reaction was monitored by TLC using a mobile phase consisting of petroleum ether:ethyl acetate (95:05). In this system, the Rfs for the starting material 2-(formyl) phenyl 4-bromobutanoate **(13)** and the product **(14)** were 0.42 and 0.12 respectively.

After cooling to room temperature, solids were removed by filtration and the solvent was evaporated under vacuum. The crude product was purified by silica gel column chromatography using petroleum ether: ethyl acetate (95:05). Combined fractions containing the product were evaporated under vacuum, affording the title compound (**14**; 44.2% yield). Confirmation of product's synthesis was by ^{1}H NMR ($CDCl_3$, 600MHz).

5.1.4.c. Synthesis of 2-(Hydroxycarbonyl)phenyl 4-nitrooxybutanoate (15)

The next step in this synthetic procedure consisted of an oxidation reaction, in which intermediate **14** (241 mg; 0.95 mmol; 1 eq) was dissolved in 5 mL of acetone and cooled in an ice bath before adding $KMnO_4$ (301 mg; 1.90 mmol; 2 eq) and stirring at room temperature for 3h. The mixture was then transferred to a beaker containing 20 mL of 0.1M of HCl. The solids produced in this reaction were filtered off and the product was extracted with ethyl acetate. Combined organic fractions were then dried with sodium sulfate and the solvent was evaporated under vacuum to yield the title product (**15**; 74.7% yield) as a pale yellow liquid. Confirmation of the product's synthesis was by ^{1}H NMR ($CDCl_3$, 600MHz).

5.1.4.d. Synthesis of ARD-N1-139 (17)

The carboxylic acid intermediate product **15** (120 mg; 0.44 mmol; 1 eq) was dissolved in 2 mL of DCM, mixed with DCC (101.16 mg; 0.49 mmol; 1.1 eq) and DMAP (5.4 mg; 0.04 mmol; 0.1 eq) and cooled in an ice bath before adding 4-hydroxybenzamide

(**16**; 61.13 mg; 0.44 mmol; 1 eq) and stirring at room temperature overnight. The suspension was then filtered to eliminate unreacted DCC, and the solvent evaporated under vacuum. The crude product was purified by silica gel column chromatography using hexane:ethyl acetate (60:40). Combined fractions containing the product were dried under vacuum, affording the title compound (**17**; 37.8% yield).

The final product **(17)** is a new product, not found on the market or published, and for to this reason it was necessary to characterize it. Physical characterization, NMR spectroscopic and MS data for compound **(17)** are listed below.

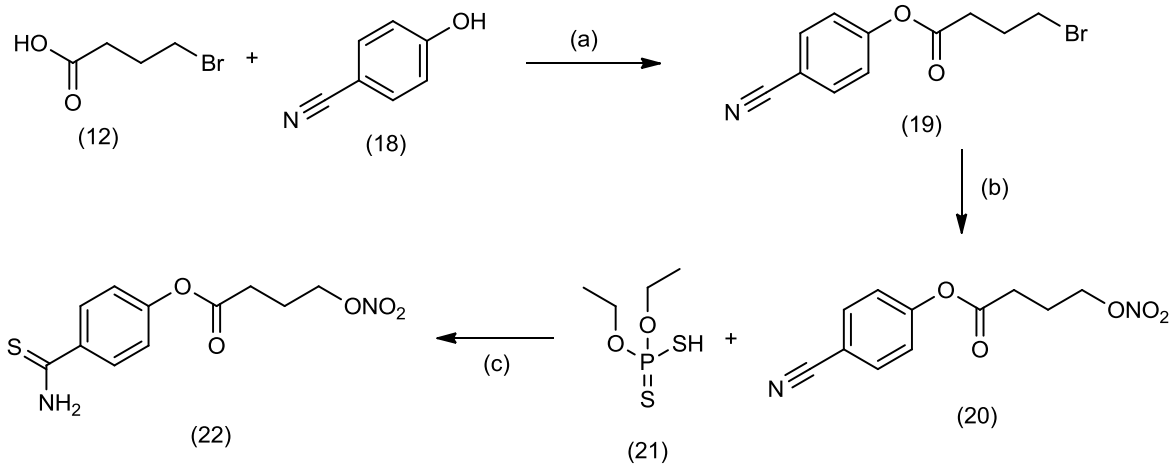
^{1}H-NMR (MeOD, 600MHz): δ = 2.11-2.07 (m, 2H), 2.74 (t, J=7.2 Hz, 2H), 4.54 (t, J=6.6 Hz, 2H), 7.27 (dd, J= 7.8, 0.9 Hz, 1H), 7.29 (dd, J= 6.6, 1.8 Hz, 2H), 7.47 (td, J=7.6, 1.2 Hz, 1H), 7.73 (td, J=7.8, 1.8 Hz, 1H), 7.89 (s,2H), 7.97 (dt, J=8.4, 2.4 Hz, 2H), 8.23 (dd, J= 7.8, 1.8 Hz, 1H). (Appendix Image 3)

^{13}C-NMR (MeOH-d_6) δ: 21.59, 29.60, 71.90, 121.50, 122.11, 123.78, 126.16, 128.95, 131.52, 131.60, 134.79, 151.10, 153.32, 162.60, 169.92, 171.47. (Appendix Image 8)

MS (EI), m/z: 389.1, 411.1 (M^+ +Na).

Product **17** was characterized as pale yellow solid as needles, with a melting point of **127-130°C**.

5.1.5. Synthesis of compound ARD-N1-188 (22) [H_2S/NO-releasing group]



Scheme 5. Chemical synthesis of the NO-/H_2S-releasing drug ARD-N1-188. Reagents and conditions: (a) DMAP, DCC, DCM, RT, 90 min; (b) $AgNO_3$, CH_3CN, MW, 70°C, 150 min; (c)H_2O, reflux, 80°C, 2h.

5.1.5.a. Synthesis of 4-Cyanophenyl 4-bromobutanoate (19)

A solution of 4-bromobutyric acid (**12,** 900 mg; 5.39 mmol; 1 eq), DCC (1.223 g; 5.92 mmol; 1.1 eq), DMAP (66 mg; 0.540 mmol; 0.1 eq), and 10 mL of DCM was stirred at 4° C for about 5 min before adding 4-cyanophenol (**18**, 642 mg; 5.39 mmol; 1 eq). This reaction mixture was stirred at room temperature for about 90 min. The reaction was completed as monitored by TLC (hexane: ethyl acetate 80: 20); the Rfs for the starting material **(18)** and the product **(19)** were 0.25 and 0.43 respectively. The suspension was then filtered off to eliminate unreacted DCC, and the solvent was evaporated under vacuum. The crude product was purified by silica gel flash column chromatography (hexane:ethyl acetate 80:20). Combined fractions containing the product were taken to dryness under vacuum to afford the title compound (**19**; 43.6% yield) as a colorless liquid. Confirmation of the product's synthesis was by ^{1}H NMR ($CDCl_3$, 600MHz).

5.1.5.b. Synthesis of 4-Cyanophenyl 4-nitrooxybutanoate (20)

The next step in this synthetic procedure consisted of a nitration reaction similar to the ones reported above. Intermediate **19** (1g; 3.73 mmol; 1 eq) was dissolved in 4 mL of acetonitrile, and mixed with $AgNO_3$ (951 mg; 5.60 mmol; 1.5 eq); this mixture was stirred

and protected from light at 70°C (microwave irradiation) for 2.5 h. At this point, we checked the reaction progress by TLC and obtained distinctive Rf values for the starting material (**19**) and product (**20**) as 0.67 and 0.44 respectively. After evaporation of the solvent under vacuum, the crude product was purified by silica gel flash column chromatography (hexane:ethyl acetate 80:20). Combined fractions containing the product were evaporated under vacuum, affording the title compound (**20**; 51.8% yield).

This intermediate product **(20)** is a new product, not found on the market or published, and for to this reason it was necessary to characterize it. Physical characterization and NMR spectroscopic data for compound **(20)** are listed below.

^{1}H-NMR ($CDCl_3$, 600MHz): δ = 2.19 (q, J=6.75 Hz, 2H), 2.75 (t, J=7.2 Hz, 2H), 4.59 (t, J=6.9 Hz, 2H), 7.25 (dt, J= 9.3, 2.4 Hz, 2H), 7.70 (dt, J=9, 2.1 Hz, 2H) (Appendix Image 4).

^{13}C-NMR ($CDCl_3$) δ: 22.20, 30.45, 71.60, 110.03, 118.14, 122.61, 122.64, 133.72, 133.75, 153.65, 170.07. (Appendix Image 9)

The product obtained **(20)** was characterized as white crystals with a melting point of **34-37°C**.

5.1.5.c. Synthesis of ARD-N1-188 (22)

The 4-cyanophenyl 4-nitrooxybutanoate **(20)** (400mg; 1.60 mmol; 1 eq) obtained in the previous step was mixed with O,O'-diethyldithiophosphate (**21**; 313mg; 1.68 mmol; 1 eq) and water (3 mL) and stirred under reflux (80°C) for about 2 h. The heating was stopped and the round bottom flask containing the reaction mixture was stored at -20°C overnight. The next morning the precipitated solids were filtered off and analyzed by TLC using hexane:ethyl acetate (70:30). The Rfs for the starting material (**20**) and product (**22**) were 0.54 and 0.19 respectively. The crude product was purified by silica gel column chromatography using hexane:ethyl acetate (70:30). Those fractions containing the product were combined, and the solvent was evaporated under vacuum.

This final product **(22)** is a new product, not found on the market or published, and for this reason it was necessary to do its characterization. Physical characterization, NMR spectroscopic and MS data for compound **(22)** are listed below.

^{1}H-NMR ($CDCl_3$, 600MHz): δ = 2.19 (q, J=6.6 Hz, 2H, CH_2), 2.75 (t, J=6.9 Hz, 2H, CH_2COO), 4.59 (t, J=6 Hz, 2H, CH_2ONO_2), 7.15 (d, J= 9 Hz, 2H, phenyl), 7.61 (s, 1H), 7.91 (d, J=8.4 Hz, 2H, phenyl). (Appendix Image 5)

^{13}C NMR (MeOH-d_6) δ: 22.21, 30.42, 71.71, 121.52, 128.44, 136.90, 153.29, 170.44, 201.53. (Appendix Image 10)

MS (EI), m/z: 285.1, 307 (M^+ +Na).

Product **22** was characterized as a yellow powder with a melting point of **93-96°C**.

Final compounds used for further analysis are listed in Figure 17. The first compound shown in this figure is known to be the control compound which it is named **NOSH-1**. This compound was used as control due to the positive results obtained from our collaborators, in which it was shown that **NOSH-1** (lead compound) inhibits cancer cell growth and proliferation by promoting apoptosis and cell cycle arrest at nM concentrations. Results also showed that this lead compound is a H_2S and a NO releaser and the ASA scaffold component of the NOSH-1 structure was shown to maintain its inhibitory activity on COX-1 and COX-2 enzymes [223]. As well, our collaborators evaluated the action of NOSH-1 on FOXM1 in which it was shown to be a downregulator at the protein level (data not published yet by our collaborators). For this reason, **NOSH-1** was used as baseline for the design of the other NOSH-derivatives presented in this thesis.

The second compound listed in figure 17 is known as **ADT-OH (3)**. This compound has been found to be one of the most common H_2S-donor compounds and interestingly it is the H_2S-donor in our **NOSH-1** compound. **ADT-OH** by itself has shown to have anti-inflammatory, chemopreventive, anticarcinogenic and antiproliferative properties [224, 229]; although its effects on FOXM1 have not been studied until now.

The third compound, illustrated in Figure 17, is the first NOSH derivative developed for this thesis project, named as **ARD-N1-109.2 (10).** This compound was

designed and synthesized in order to contain aspirin (ASA) as scaffold and as a compound that could possibly be a H_2S-releasing donor. The H_2S-releasing property could be given by the chemical structure of the TBZ **(9).**

The next compound illustrated, known as **ARD-N1-114.2 (11)**, is the second NOSH derivative that was designed and synthesized in order to be a compound containing ASA as scaffold and having a possible H_2S-releasing moiety in its structure. In this case, H_2S would be donated by the **ADT-OH (3).**

ARD-N1-139 (17) is the third NOSH derivative and the fifth compound illustrated in Figure 17. It was designed and synthesized to be a compound containing ASA as scaffold and a compound that could possibly be a NO-releasing donor. The possible NO-donor might be given by the nitrate ($-ONO_2$) structure.

ARD-N1-188 (22), the last compound listed and fourth NOSH derivative, was designed and synthesized to be a compound that contains H_2S and NO releasing moieties in its structure. The possible NO-donor would be given by the nitrate structure and the H_2S-donor would be **TBZ (9)**.

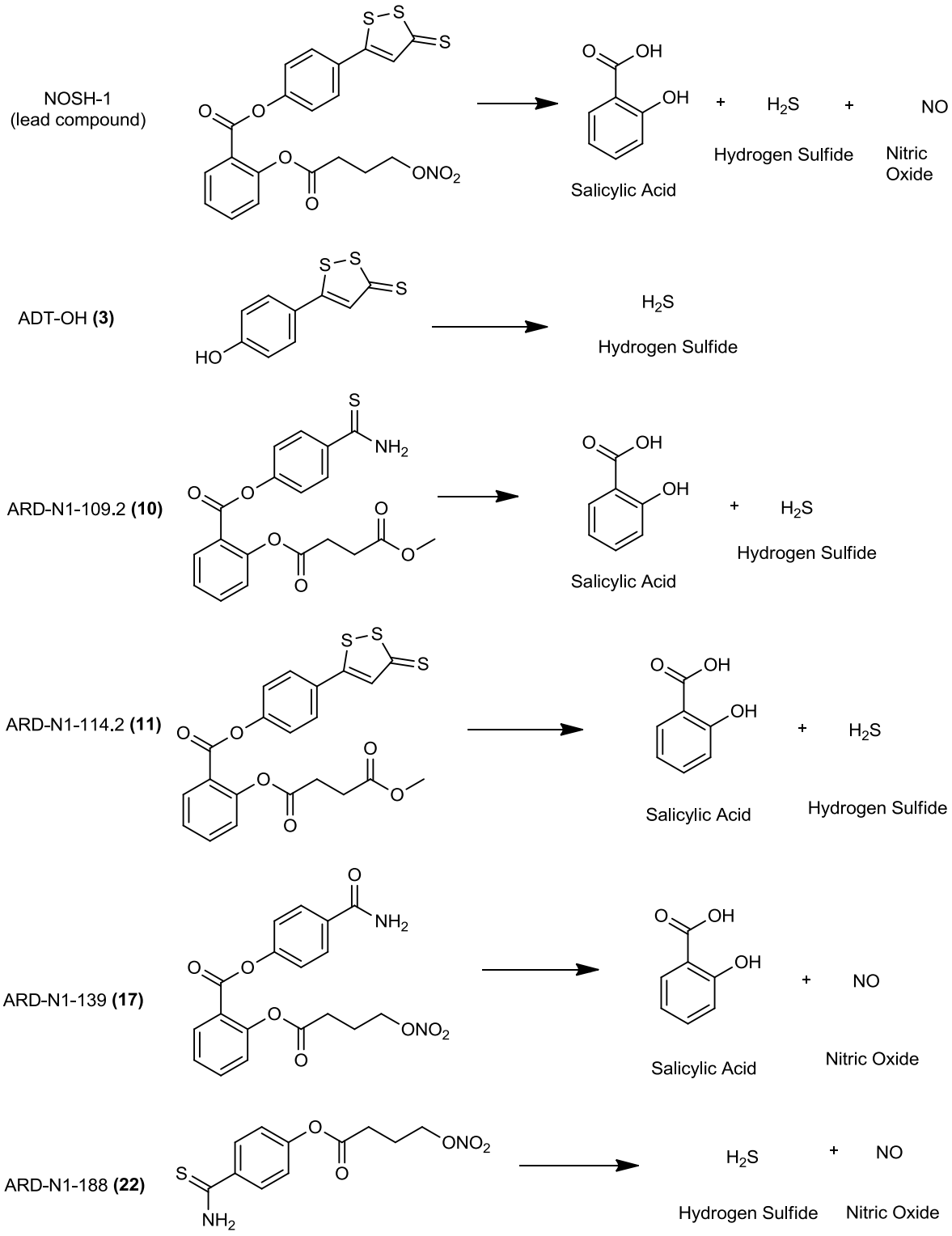


Figure 17. Chemical structures and the expected release patterns from the different NOSH derivatives

5.2. Cell Viability Assays (MTT)

Three cancer cell lines from different tissue origins were incubated in the presence of 4 new hybrid compounds designed for this project, as well as the lead **NOSH-1** compound and **ADT-OH** represented in Figure 17. The range of concentrations tested was 0.001 to 100 μM, and the effects produced by each drug were different depending on the cell line.

5.2.1. Colon cancer cells (HT-29)

As is shown in Figure 18, the lead compound (**NOSH-1**) did not show a significant inhibitory effect on HT-29 cell viability and this effect was essentially the same for all test drugs. The cell viability was not inhibited by any of the compounds at the concentrations tested, except for the H_2S-releasing compound **ADT-OH (3)**, which started to show significant inhibitory effects at 40 μM (estimated IC_{50} = 40.2 μM at 24 h incubation period).

These results are not in agreement with previous findings in the literature, where Kodela et al. [222] reported, by doing MTT on HT-29 cells, an IC_{50} = 48 nM for **NOSH-1**. In this study, NOSH-1 was not even active at the maximum tested concentration (100 μM). In this regard, none of the other NOSH derivatives showed significant cell viability inhibition.

Contrary to what was observed with the other compounds; the active compound (**ADT-OH**) showed an interesting pattern at lower concentrations, where it appears as if this molecule "promotes cell viability" in a concentration range between 0.001 and 1.0 μM in which cell viability oscillated around 150% compared to control HT-29 cells treated only with DMSO which was considered as 100%.

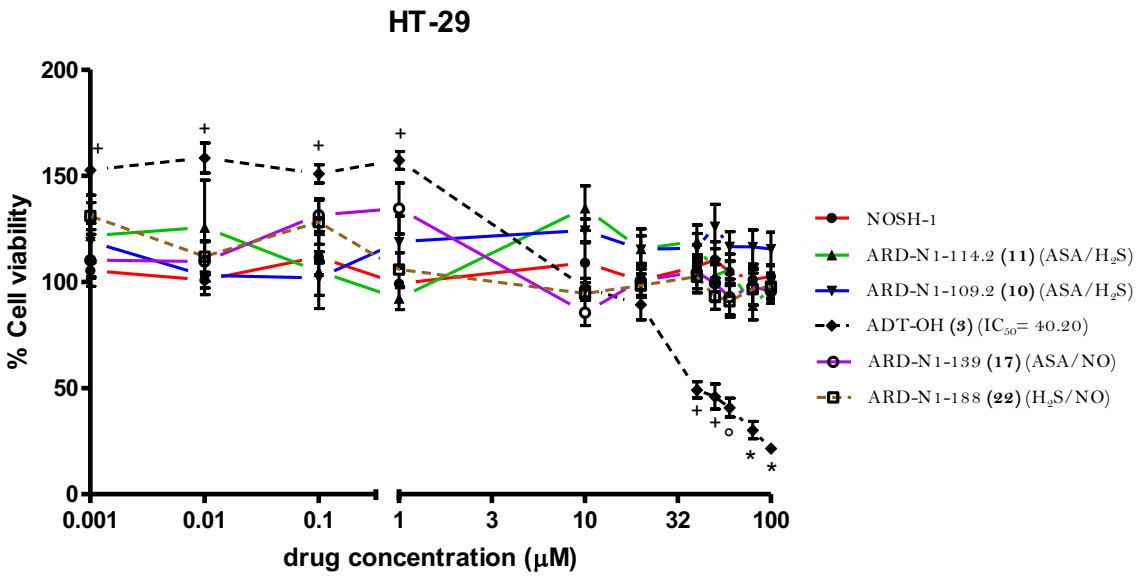


Figure 18. MTT results shown by the relationship between % cell viability and drug concentration in colon cancer cells (HT-29). Six compounds were used to test % cell viability. The drug concentrations for MTT evaluations were 100 μM, 80 μM, 60 μM, 50 μM, 40 μM, 20 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM, 0.001 μM and 0 μM (containing 1% of DMSO mixed in DMEM), which was used as baseline, considered in the statistics as 100%. Drug treatment was left for incubation for 24 h and all experiments were performed at least three times in quadruplicate (N=3) by using different passages. Statistical analysis of differences between percentages of cell viability between groups of data in MTT evaluations was performed by using two-way ANOVA followed by the Bonferroni test. Data are expressed as the means ±Standard Error of the Mean (SEM). +$p<0.05$, °$p<0.01$, *$p<0.001$ were regarded as statistically significant relative to non-treated cells (just DMSO)

5.2.2. Liver cells (Hep G2)

As is shown in Figure 19, there were three molecules that significantly inhibited viability of HepG2 cells, namely **ADT-OH**, **NOSH-1**, and **ARD-N1-114.2** (drug **11**). The estimated IC_{50} values were 13.1 μM, 28.2 μM, and 13.5 μM respectively. I am proposing that the potential anticancer activity exerted by **NOSH-1** and **11** could be largely due to the presence of the **ADT-OH** moiety because (a) none of the other compounds showed any significant effects on viability up to 100 μM, and (b) the inhibitory potency in cell viability of the active molecules (expressed in terms of IC_{50} values), was very similar to that of **ADT-OH**. Unfortunately, the Kodela et al. group did not test the potency of NOSH molecules in HepG2 cells and therefore, it was not possible to make any comparisons.

It was also observed that two compounds promoted viability of HepG2 cells at lower concentrations. In fact, it was possible to observe that the compound named as **ARD-**

N1-188 (drug **22**) significantly increased cell viability at concentrations ranging from 0.001 to 0.1 µM. Compound **ARD-N1-109.2** (drug **10**) showed a significant increase at the two lowest concentrations (0.001 to 0.01 µM). Cell viability oscillated around 150%, compared to control HepG2 cells treated only with DMSO which were considered as the 100%.

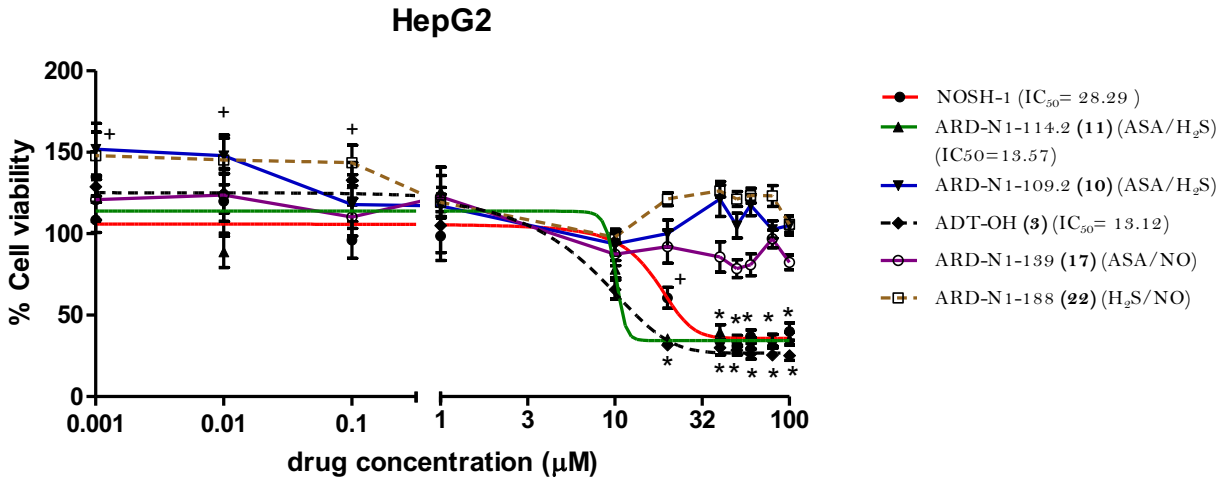


Figure 19. MTT results shown by the relationship between % cell viability and drug concentration in liver cancer cells (Hep-G2). Six compounds were used to test % cell viability. The drug concentrations for MTT evaluations were 100 µM, 80 µM, 60 µM, 50 µM, 40 µM, 20 µM, 10 µM, 1 µM, 0.1 µM, 0.01 µM, 0.001 µM and 0 µM (containing 1% of DMSO mixed in DMEM), which is was used as baseline, considered in the statistics as the 100%. Drug treatment was left for incubation for 24 h and all experiments were performed at least three times in quadruplicate (N=3) by using different passages. Statistical analysis of differences between percentages of cell viability between groups of data in MTT evaluations was performed by using two-way ANOVA followed by the Bonferroni test. Data are expressed as the means ±Standard Error of the Mean (SEM). +$p<0.05$, *$p<0.001$ were regarded as statistically significant relative to non-treated cells (just DMSO)

5.2.3. Breast cancer cell (SKBR-3)

Similar results were observed in SKBR-3 cells (figure 20) as in HepG2 cells, in which, **ADT-OH**, **NOSH-1**, and **ARD-N1-114.2** (drug **11**) were the only effective molecules, with estimated IC_{50} values of 19.7 µM, 35.7 µM, and 49.3 µM respectively (see Figure 20). These results are not in agreement with Kodela et al., who reported **NOSH-1** potency with an IC_{50} = 75 nM in the same cell line. It was also interesting to observe that the two hybrid compounds containing $–ONO_2$ in their structure had a tendency to promote cell viability at concentration range from 20 µM to 80 µM oscillating close to 150% (compared to control cells treated only with DMSO which were considered as 100%),

although at the highest concentration (100 µM) these compounds returned to control values (100%).

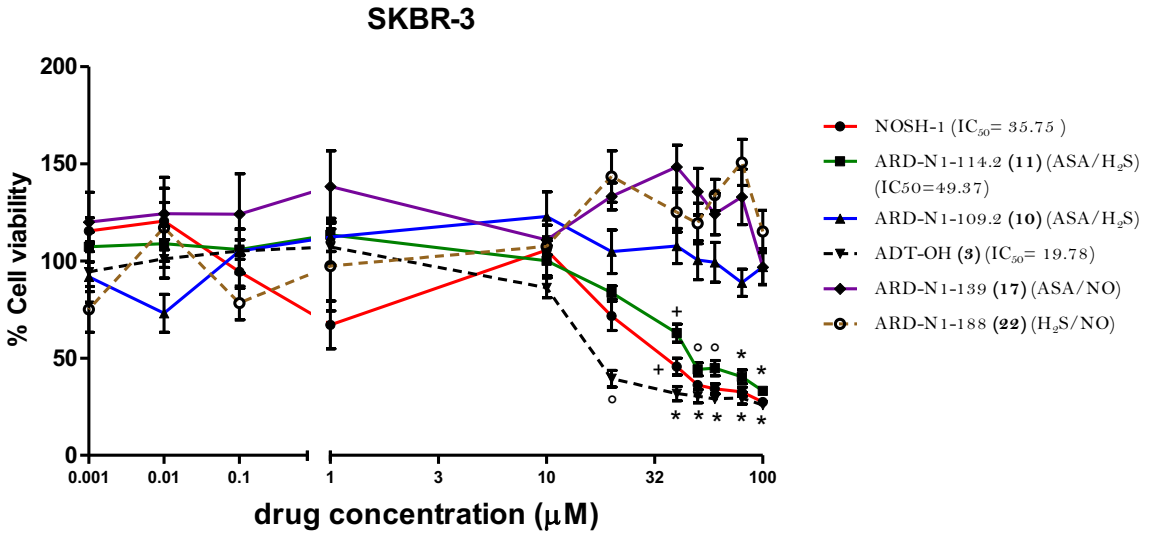


Figure 20. MTT results shown by the relationship between % cell viability and drug concentration in breast cancer cells (SKBR-3). Six compounds were used to test % cell viability. The drug concentrations for MTT evaluations were 100 µM, 80 µM, 60 µM, 50 µM, 40 µM, 20 µM, 10 µM, 1 µM, 0.1 µM, 0.01 µM, 0.001 µM and 0 µM (containing 1% of DMSO mixed in DMEM), which is was used as baseline, considered in the statistics as the 100%. Drug treatment was left for incubation for 24 h and all experiments were performed at least three times in quadruplicate N=3 by using different passages. Statistical analysis of differences between percentages of cell viability between groups of data in MTT evaluations was performed by using two-way ANOVA followed by the Bonferroni test. Data are expressed as the means ±Standard Error of the Mean (SEM). +$p<0.05$, °$p<0.01$, *$p<0.001$ were regarded as statistically significant relative to non-treated cells (just DMSO).

Taken together, these results suggest that:

a. The lead molecule (**NOSH-1**) possibly inhibits cell viability only after it is metabolized by non-specific esterases in these cancer cells, releasing the alcohol-containing moiety **ADT-OH**.

b. Compounds possessing the hydrogen-releasing group thiobenzamide [**TBZ (9)**] such as **ARD-N1-109.2** (**10**) are inactive. In this regard even though we did not measure the release of hydrogen sulfide (H_2S) from this (or any other) molecule in the series, it is possible to speculate that the release of H_2S from **TBZ** is not essential for cancer cell viability inhibition.

c. The other NOSH-derivatives (**17** and **22**), containing ASA + ONO_2, and ONO_2 + H_2S [TBZ **(9)**] respectively in their structure, showed that compounds containing - ONO_2 in their chemical structure, did not exert any cell viability inhibition at any concentration tested.

d. The differences observed between Kodela's results and the results reported in this thesis could possibly be explained due to the variations in the cells' origin (e.g. variation in cells donors, explaining the variation in the cells' response), culturing media, passaging methods and number. In order to confirm this statement it would be interesting to repeat MTT assays with Kodela's cell lines following their protocol.

e. Although the MTT assay was used in this project because it is a quick and easy assay, and is largely used in the screening of the antiproliferative effects of new compounds [230], it has been shown that it has some drawbacks as assay. The MTT assay shows the metabolic activity of viable cells [231]. Unfortunately in some cases it has been shown that its metabolic activity may be changed by different conditions which can cause variations in results [231].

   In our case, the increase in the MTT signalling could be explained by two possible reasons; 1) it is possible that the cells are increasing their metabolic activity with some of the treatment compounds at some concentrations or 2) there is an increase in cell proliferation, and not in cell viability, found to be inhibited when the concentration of some NOSH-derivatives and **ADT-OH** increases. These possible options have to be probed by the use of other methods in order to confirm that some of the compounds are in fact causing some effects on cell proliferation or a possible promotion of apoptosis under high concentrations of **ADT-OH** release.

5.3. FOXM1 Expression in the drug-treated HT-29 and SKBR-3 cells

To investigate the FOXM1 protein expression after treatment, two cancer cell lines from different tissue origin, human breast cancer cells (SKBR-3) and human colon cancer cells (HT-29) were used. Reference compound (**NOSH-1**) and NOSH-derivatives were tested at concentrations of 0 - 100 μM and left incubating for 24 h.

5.3.1. Effects of NOSH compounds on the expression of FOXM1 in colon cancer cells (HT-29).

The first molecule tested was **NOSH-1** (the lead compound). Interestingly, this compound did not exert a significant decrease in FOXM1 protein expression after 24 h of incubation. In fact, at concentrations of 60 μM and 80 μM, **NOSH-1** seemed to increase FOXM1 protein expression to almost double that observed with the control (0 μM), although this trend was not statistically significant.

This result is interesting due to the fact that the data suggest that FOXM1 protein may increase in colon cancer cells when they are treated with this compound; however the results are not significant due to the range of error (Figure 21). This result is consistent with the observation made in the cell viability assay in which **NOSH-1** had no significant effect on HT-29 cell viability (see Figure 18).

Similar results were observed with the hybrid compounds **10, 11, 17** and **22**. In this regard, compound **10** (H_2S-ASA) showed a tendency to increase the levels of FOXM1 at concentrations higher than 20 μM, with the highest level detected at 60 μM (Figure 22). The same apparent increase in FOXM1 protein levels at concentrations higher than 20 μM was observed with compound **17** (NO-ASA), although in this case the last two concentrations (80 and 100 μM) seemed to "decrease" the expression of FOXM1 protein to levels similar to those obtained with control cells (0 μM, cells treated only with DMSO) (Figure 24).

Compound **22**, a hybrid molecule with both NO and H_2S, but not ASA, did not have a statistically significant effect on FOXM1 protein levels. Nevertheless, we also observed an apparent increase of FOXM1 levels at drug concentrations higher than 20 μM (Figure 25). The protein levels of FOXM1 in all the drug-treated cells, regardless of the drug concentration, were always higher than those observed in the control cells. The same trend was observed when cells were incubated with compound **11**, a hybrid drug designed to release both ASA and H_2S. In this particular case, it was observed a gradual increase in the protein expression of FOXM1, to the point where, at 60 μM, there was a statistically significant value. This suggests that at this concentration, compound **11** seemed to promote/induce the expression of FOXM1 protein in these cells. It is noteworthy to point

out that the protein levels of FOXM1 were still higher than those observed for the control group at concentrations higher than 60 μM, but these were not statistically significant (see figure 23).

These results suggest that, rather than exerting an anticancer effect (by decreasing the protein expression of FOXM1) the drugs (including the reference **NOSH-1**) seem to promote the protein expression of this transcription factor. This is supported by the observation that some of these drugs seem to promote cell viability rather than inhibiting it (Figure 18).

When the effect of the **ADT-OH** compound was examined, a similar biphasic response was observed in which, at low concentrations (20 and 40 μM) shows a tendency to increase FOXM1 protein levels, but at higher drug concentrations (60-100 μM), these levels were significantly decreased in a concentration-dependent manner (Figure 26).

HT-29 cells + NOSH-1

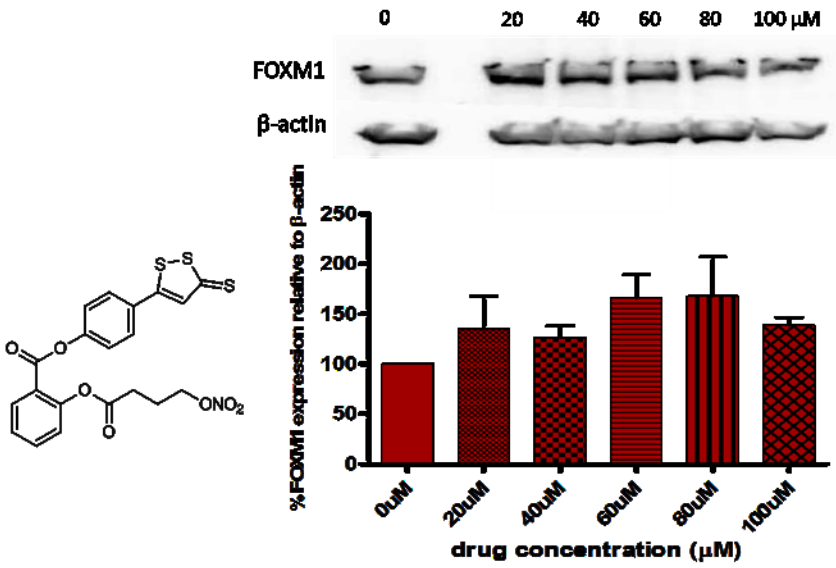


Figure 21. % FOXM1 expression relative to β-actin in HT-29 colon cancer cells under different concentrations of our lead compound NOSH-1 after 24 h of incubation Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

HT-29 cells + ARD-N1-109.2 (10)

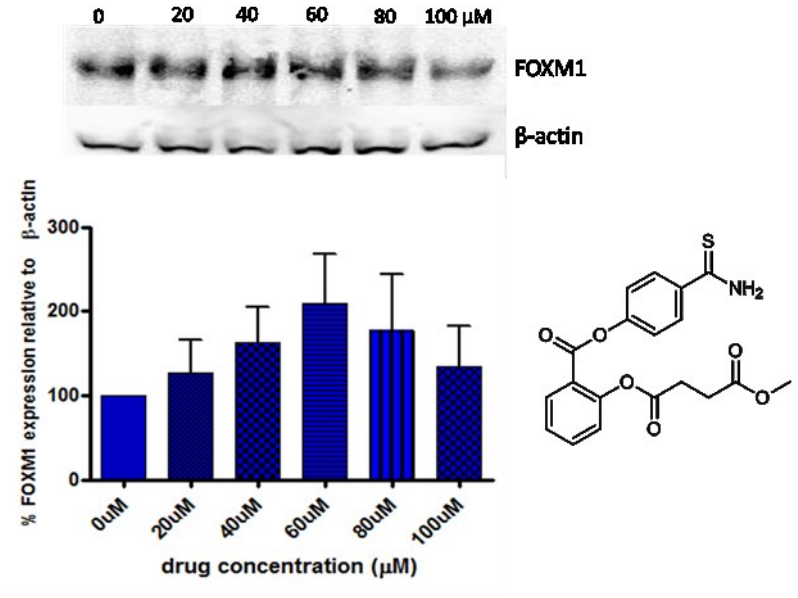


Figure 22. % FOXM1 protein expression relative to β-actin in HT-29 colon cancer cells under different concentrations of compound ARD-N1-109.2 (**10**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

HT-29 cells + ARD-N1-114.2 (11)

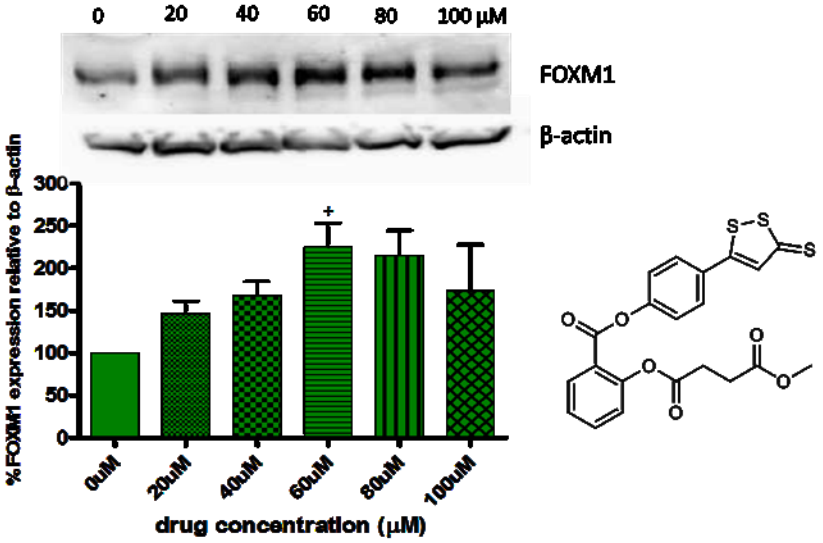


Figure 23. % FOXM1 protein expression relative to β-actin in HT-29 colon cancer cells under different concentrations of compound ARD-N1-114.2 (**11**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=4; + $p<0.05$

HT-29 cells + ARD-N1-139 (17)

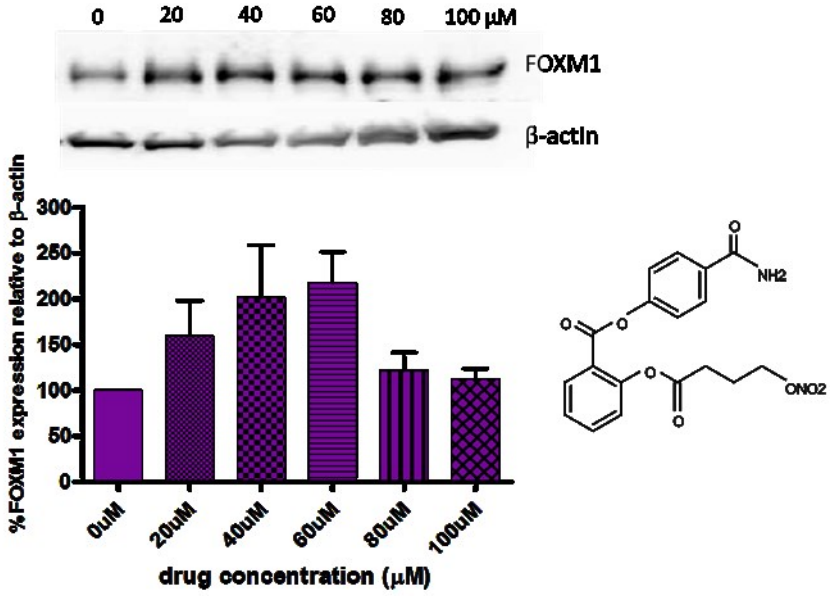


Figure 24. % FOXM1 protein expression relative to β-actin in HT-29 colon cancer cells under different concentrations of compound ARD-N1-139 (**17**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

HT-29 cells + ARD-N1-188 (22)

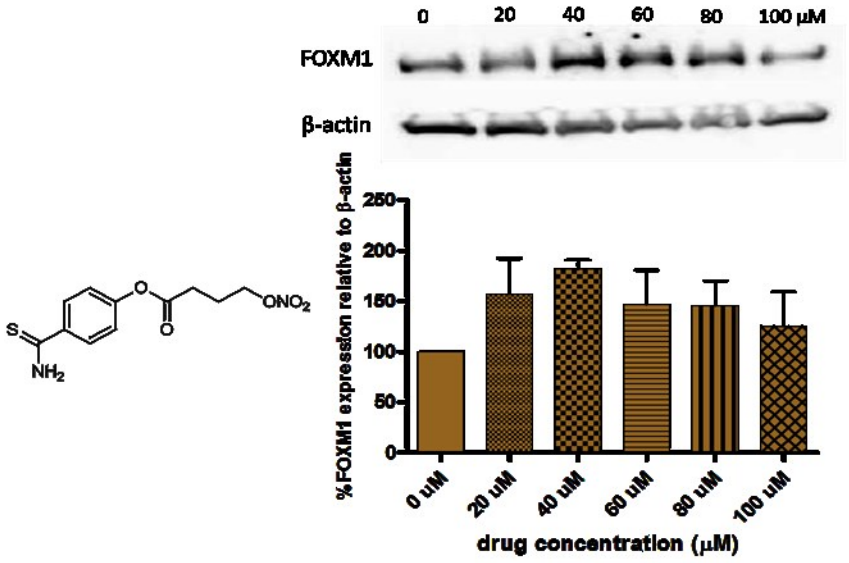


Figure 25. % FOXM1 protein expression relative to β-actin in HT-29 colon cancer cells under different concentrations of compound ARD-N1-188 (**22**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

HT-29 cells + ADT-OH (3)

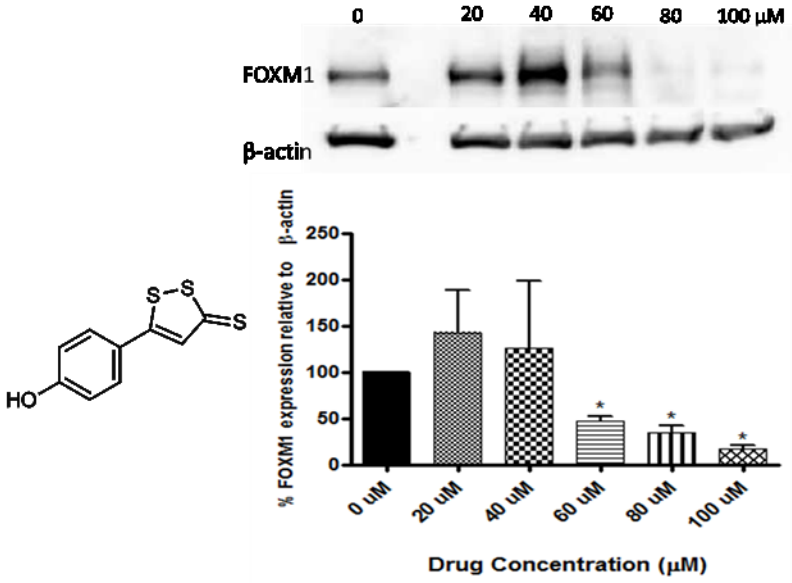


Figure 26. % FOXM1 protein expression relative to β-actin in HT-29 colon cancer cells under different concentrations of compound ADT-OH (**3**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=4; * $p<0.001$

5.3.2. Effects of NOSH compounds on the expression of FOXM1 in breast cancer cells (SKBR-3).

These experiments were carried out in the same way as those described for HT-29 colon cancer cells in section 5.3.1. The NOSH derivatives, NOSH-1 (lead compound) and ADT-OH were tested at concentrations = 0, 20, 40, 60, 80 and 100 µM. The incubation time for all drug treatments was 24 h, followed by western blot analysis FOXM1 protein.

The first molecule analyzed was **NOSH-1** (lead compound). This reference compound significantly decreases the expression of FOXM1 at protein level in a concentration-dependent manner (see Figure 27). This effect was statistically significant at concentrations 60 µM to 100 µM and was consistent with the observations made in cell viability assays, in which NOSH-1 significantly decreased cell viability of SKBR-3 breast cancer cells (see Figure 20).

The NO-ASA derivative, **ARD-N1-139** (**17**,) showed no significant decrease at the highest test compound concentration (100 µM; see Figure 30). Nevertheless, when tested

the H_2S-releasing moiety **ADT-OH** significantly decreased the FOXM1 protein levels in this cell line (Figure 32). This effect was very similar to that observed with the reference compound **NOSH-1.** Thus, this result strongly suggests that FOXM1 expression in this cell line is downregulated by the **ADT-OH** moiety. We can speculate that the other components present in NOSH-1 are unlikely to play a significant role in the expression levels of FOXM1. This is an important observation because both cell viability and FOXM1 protein expression were decreased only after treatment with **ADT-OH**, which does not contain the ASA or the ONO_2 groups in its chemical structure.

This notion was reinforced by the following observations:

a) Compounds **22** (H_2S-NO, Figure 31) and **10** (H_2S-aspirin, Figure 28) did not significantly decrease FOXM1 protein levels at any of the drug concentrations. Considering that compound **10** possesses a potential hydrogen sulfide-releasing (thio) group in its structure (TBZ), which is different from **ADT-OH** and **NOSH-1**, it is reasonable to assume that the chemical structure of TBZ is not essential to downregulate FOXM1 protein expression.

b) Compound **11** (H_2S-ASA, Figure 29), which contains the **ADT-OH** moiety, significantly decreased FOXM1 protein levels in a concentration-dependent manner. Nevertheless, as was discussed in the experiments observed with HT-29 cells, a biphasic response was exerted by compound **11** in which, at low concentrations (20 μM) it significantly increased FOXM1 protein levels, whereas at higher concentrations, the FOXM1 protein levels decreased in a concentration-dependent manner. This decrease was significant at concentrations = 80 - 100 μM.

SKBR-3 cells + NOSH-1

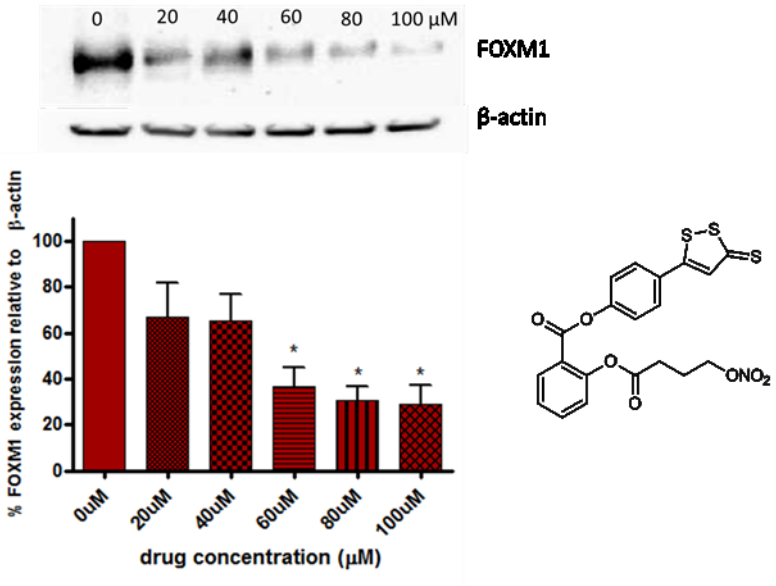


Figure 27. % FOXM1 protein expression relative to β-actin in SKBR-3 breast cancer cells under different concentrations of our lead compound **NOSH-1** after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=4; *p<0.001

SKBR-3 breast cancer cells + ARD-N1-109 (10)

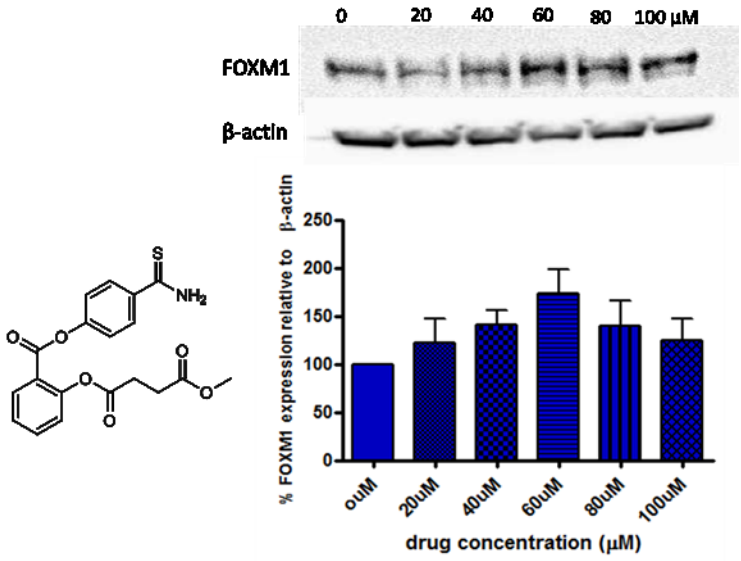


Figure 28. % FOXM1 protein expression relative to β-actin in SKBR-3 breast cancer cells under different concentrations of compound ARD-N1-109.2 (**10**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

SKBR-3 cells + ARD-N1-114.2 (11)

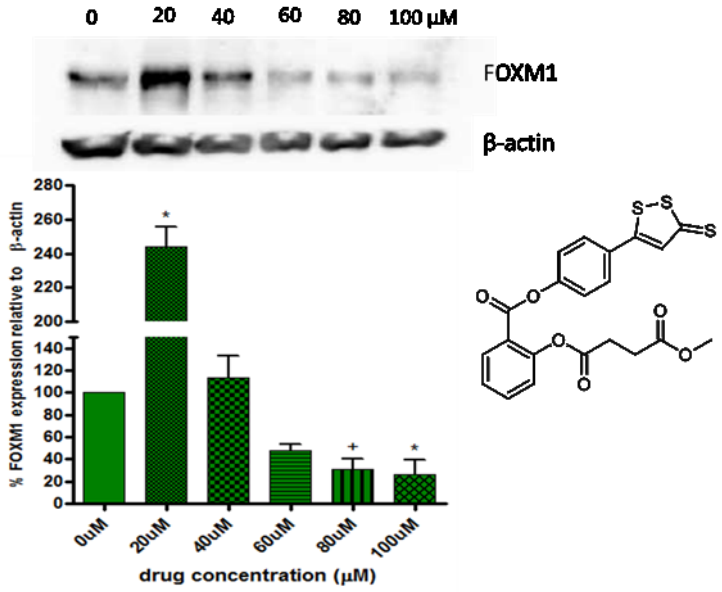


Figure 29. % FOXM1 protein expression relative to β-actin in SKBR-3 breast cancer cells under different concentrations of compound ARD-N1-114.2 (**11**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3; * $p<0.001$

SKBR-3 cells + ARD-N1-139 (17)

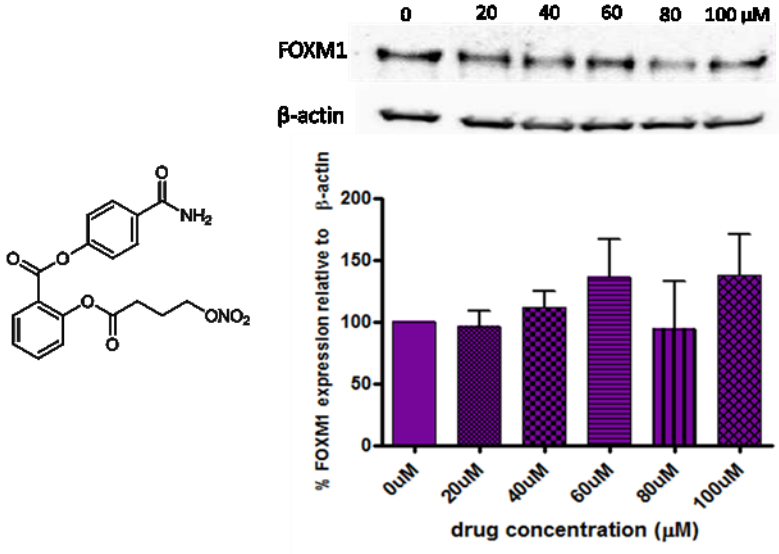


Figure 30. % FOXM1 protein expression relative to β-actin in SKBR-3 breast cancer cells under different concentrations of compound ARD-N1-139 (**17**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

SKBR-3 cells + ARD-N1-188 (22)

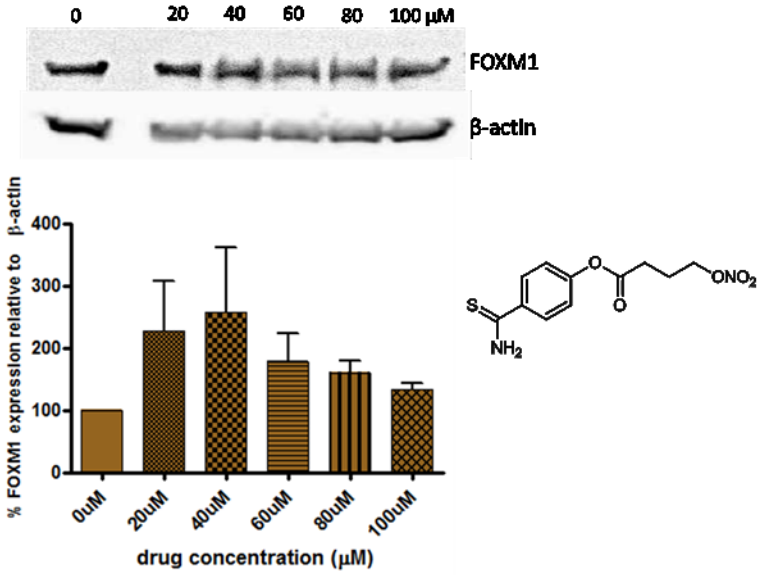


Figure 31. % FOXM1 protein expression relative to β-actin in SKBR-3 breast cancer cells under different concentrations of compound ARD-N1-188 (**22**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=3

SKBR-3 cells + ADT-OH (3)

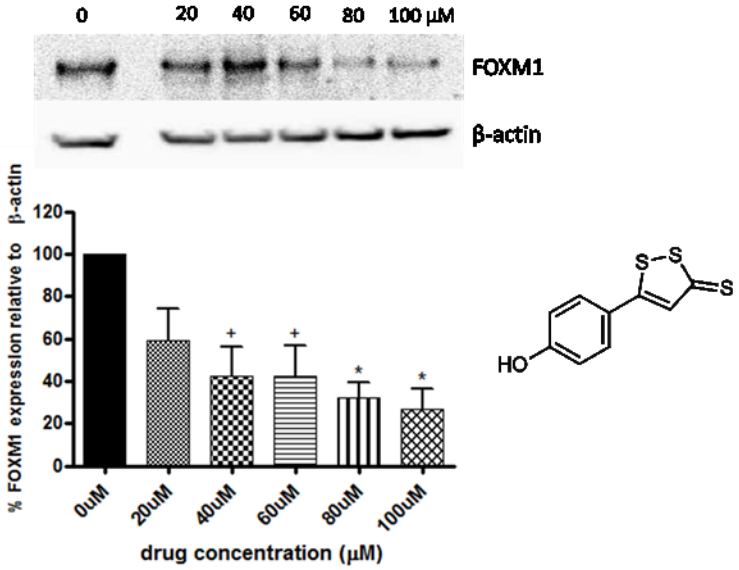


Figure 32. % FOXM1 protein expression relative to β-actin in SKBR-3 breast cancer cells under different concentrations of compound ADT-OH (**22**) after 24 h of incubation. Statistical analysis was performed by using one-way ANOVA followed by the Tukey test. Data are expressed as the means ±Standard Error of the Mean (SEM). N=4; + $p<0.05$; *$p<0.001$.

Considering the limited scope of this research study, it is not possible to provide a suitable explanation for the differential tissue responses exerted by the test compounds, or the biphasic response obtained in the cell viability and Western blot experiments. However, at this point, it is possible to make some reasonable speculations.

- The NOSH-1 compound is a weak anticancer agent in colon tissue compared to breast cancer tissue. A significant effect exerted by this drug on HT-29 cells was not observed. This compound did not reduce the cell viability of HT-29 cells and it did not decrease the expression of FOXM1 as measured by Western Blot analysis.

  On the other hand, NOSH-1 was active in SKBR-3 cells. This compound significantly decreased cell viability as well as FOXM1 protein expression. However, it is noteworthy to point out that this statement needs further validation by using additional colon and breast cancer cell lines, including primary cells from both colon and breast cancer patients.
- Several compounds in this project produced a biphasic response on FOXM1 protein expression. It is possible to speculate that it could be related to ROS.

  It is known that cancer cells accumulate higher levels of ROS compared to normal cells, due to their increased metabolic activity [24]. However, high levels of ROS in cancer cells render them to prone to the toxic effects of increased oxidative stress [24, 63]. FOXM1 directly regulates the expression of scavenger enzymes, reduces intracellular ROS levels, and protects tumor cells from oxidative stress [24].

  Low levels of ROS are required for the expression of FOXM1 and the induction of cell growth and proliferation. However, high levels of ROS might induce a downregulation instead [24].

  The literature indicates that H_2S-NSAIDs dose-dependently induce ROS, which results in the promotion of cell death in MDA-MB-231 breast cancer cells [217]. Based on all these information, it can be speculated that some of the compounds used in the project showed biphasic responses on FOXM1 protein expression probably because of ROS. We speculate that when drugs' concentrations were relatively low, there was a low formation of ROS, promoting the expression of FOXM1 (measured by Western Blot analysis) and consequently an increase of cell viability (observed in the MTT assay). However, when the concentration of the

compounds was increased to 100 μM, an abnormal increase of ROS may occur, and oxidative stress might take place, resulting in the downregulation of FOXM1 protein and consequently a decrease of cell's viability (observed in the MTT).

- NOSH-derivatives developed in this project share some chemical similarities with proteasome inhibitors/thiazole antibiotics, and probably share the same pathways of action on FOXM1 inhibition (e.g. NF-κB signaling or blockage of CDK 1/2 phosphorylation). Gartel et.al. proved that thiol molecules (e.g. thiazole antibiotics) affect NF-κB signaling as well as CDK1/2 phosphorylation [16, 232].

  If we consider that our compounds **NOSH-1, ARD-N1-114.2** (**11**) and **ADT-OH** have a thiol group in their chemical structure; the response of a decrease in FOXM1 protein, observed in the Western blot analysis, could probably be done through the inhibition of NF-κB or through the blockage of CDK 1/2 phosphorylation, which are known proteasome inhibitors/thiazole antibiotic actions.

- Another possible explanation of the results observed in this thesis project is the nature of the H_2S-releasing compound. Interesting research done by Switzer et.al. showed that the effects of H_2S-releasing molecules on NF-κB signalling and on cancer cells' inhibitory response are dependent on the nature of H_2S-releasing compound and the cell type [233]. As was previously described, FOXM1 is correlated with NF-κB expression. If there is an inhibition on NF-κB then FOXM1 will be inhibited as well.

  If the nature of the H_2S-releasing compound affects the inhibitory response on cancer cells in a different way, then it is possible to speculate that the differences observed between **ADT-OH** and **TBZ/**containing structures could be due to the origin of the H_2S-releasing nature, which will cause a different inhibitory response on cell viability and on FOXM1.

  Similarly, if the nature of the H_2S-releasing compound varies depending on the cell type, then it is possible to explain **NOSH-1** and **ARD-N1-114.2** (**11**) were able to reduce cell viability and decrease the expression of FOXM1 protein in breast cancer cells but not on colon cancer cells.

- Switzer et.al. also showed that ADT-like molecules are capable of inhibiting NF-κB activity via covalent-thiol modifications in NF-κB subunits to inhibit DNA binding

in estrogen receptor negative breast cancer cell [224, 233]. In my project, the SKBR-3 breast cancer cell line, a negative estrogen receptor cell line was used, three compounds containing the ADT molecule (**ARD-N1-114.2** (**11**), **ADT-OH** and **NOSH-1**), produced decreased cell viability and FOXM1 protein expression in the ER (-) cell line (SKBR-3). This inhibitory response could possibly be explained due to the fact that in estrogen receptor negative ER (-) breast cancer cells, ADT-molecules are known to inhibit NF-κB transcriptional factor [224], which could probably inhibit indirectly FOXM1 protein expression.

- Although FOXM1 has been found to be downregulated when NF-κB transcriptional factor is inhibited, another transcription factor may play a key role in FOXM1 inhibition [98]. As was mentioned before, FOXO3 acts downstream on the IKK pathway, and it has been found to be an NF-κB antagonist. When there is an activation of NF-κB, then FOXO3 is suppressed, allowing FOXM1 to promote gene expression; but if FOXO3 is activated, then NF-κB is inactive as well as FOXM1 protein, resulting in cancer cell growth inhibition [234]. If our compounds can possibly inhibit NF-κB, due to their possible ADT-like potency, than the activation of FOXM1 could possibly be affected through its competing action with FOXO3, which would result in the inhibition of cancer cell viability and the inactivation of FOXM1 protein.

Additional note:

- Kodela et al. reported the NO and H_2S-release profile of NOSH compounds, including **NOSH-1** [222]. The release profile of the organic nitrates, as well as the ADT-OH moiety, has been extensively reported in the literature [223, 224, 233], and consequently we considered it unnecessary to determine the release profile of the new hybrid molecules synthesized in this research. Nevertheless, we realized that the release profile to measure both, NO and H_2S, will be required to prove that these molecules maintain the release profile reported in the literature.

6.0 Conclusions

Based on the results obtained in this investigation, it is possible to conclude that:

a) The inhibitory effect on cancer cell viability exerted by the NOSH-derivative compounds SKBR-3 and HepG2 cancer cells is largely due to the presence of the **ADT-OH** group.
b) **ADT-OH (3)** compound decreased cell viability in the three cancer cell lines evaluated in this project (SKBR-3, HT-29 and HepG2) and was the only compound capable of inhibiting FOXM1 at the protein level by more than half.
c) The organic nitrate **(-ONO_2)** present in the reference NOSH-1 compound ***is not essential*** to exert inhibitory effects on the *in vitro* viability of the cancer cell lines studied in this project.
d) Similarly, the thiobenzamide (a possible H_2S-releasing group) from NOSH-derivative compounds, seems ***not be essential*** for inhibiting the viability of cancer cells studied. However, the **ADT-OH** from NOSH-derivatives (potential H_2S-releaser in NOSH-derivatives) appears to be more active than the equivalent thiobenzamide group.
e) The use of the ASA structure in the new hybrid NOSH-derivative compounds seems to not be essential for the inhibition of cancer cell viability or protein expression of FOXM1. Consequently, the design of NOSH compounds could be simplified by taking out this moiety from **NOSH-1**.
f) The expression of FOXM1 at the protein level in HT-29 cells is not modulated by **NOSH-1** or the NOSH-derivatives; in fact the only compound capable of inhibiting its expression was **ADT-OH (3)**.
g) The expression of FOXM1 at the protein level in SKBR-3 cells is decreased in a concentration-dependent manner by compounds containing **ADT-OH** in their structure [**ADT-OH (3)**, **NOSH-1** and compound **(11)**], but not by any other derivative possessing the –ONO_2 or the thiobenzamide group.

h) The design of future FOXM1 modulators derived from NOSH compounds will have to be based on the biological effects associated with the **ADT-OH** moiety, and not the other groups reported for these molecules.

7.0 Future directions

The modulation of FOXM1 by NOSH compounds needs further study. Some of the future studies envisioned as part of ongoing investigations are:

1. To analyze the response of NOSH compounds in non-cancer cells, assessing the potential cytotoxic profile of **NOSH-1** and **ADT-OH**.
2. To design and synthesize NOSH compounds substituting the thiobenzamide structure for ADT-OH.
3. To measure cell growth proliferation, cell cycle analysis and apoptosis in different cancer cell lines by using these newly synthesized NOSH-derivatives compounds.
4. To measure at different time points over 24 h, the release and determination of nitric oxide (NO), hydrogen sulfide (H_2S) and salicylate levels produced by the new synthesized NOSH-derivative compounds, as well as investigating the induction of ROS as possible part of their mechanism of action in order to understand the biphasic response observed with some NOSH-derivatives.
5. To determine if the downregulation of FOXM1, at the protein level, exerted by NOSH-1 and NOSH-derivatives is caused by decreased FOXM1 transcription. This evaluation would be done by using quantitative Real Time-PCR (qRT-PCR).
6. Due to the similarities between thiazole antibiotics/proteasome inhibitors, and the chemical structure of NOSH compounds (both contain a thio moiety and a heterocyclic structure of 5 members), it will be necessary to study the potential inhibitory effects of NOSH compounds on the proteasome system. If NOSH-derivative compounds do not inhibit the proteasome, this would indicate that NOSH-derivatives decrease FOXM1 protein levels by a different mechanism than the thiazole antibiotics (e.g. siomycin A or thiostrepton).
7. Considering that NO-NSAIDs [235] and H_2S-NSAIDs [219] are known to inhibit the NF-κB pathway, it will be necessary to study the relationship between NOSH-derivative compounds, the expression of NF-κB, and the correlation with FOXO3a.

8. In this regard, it will be necessary to investigate if NOSH-derivatives modulate the expression of other members of the FOX family, mainly the FOXO3a transcription factor, which is known to be an upstream modulator of FOXM1.
9. To investigate if the NOSH-derivatives are capable of inhibiting the phosphorylation and subsequent translocation of FOXM1 from the cytoplasm to the cell nucleus.

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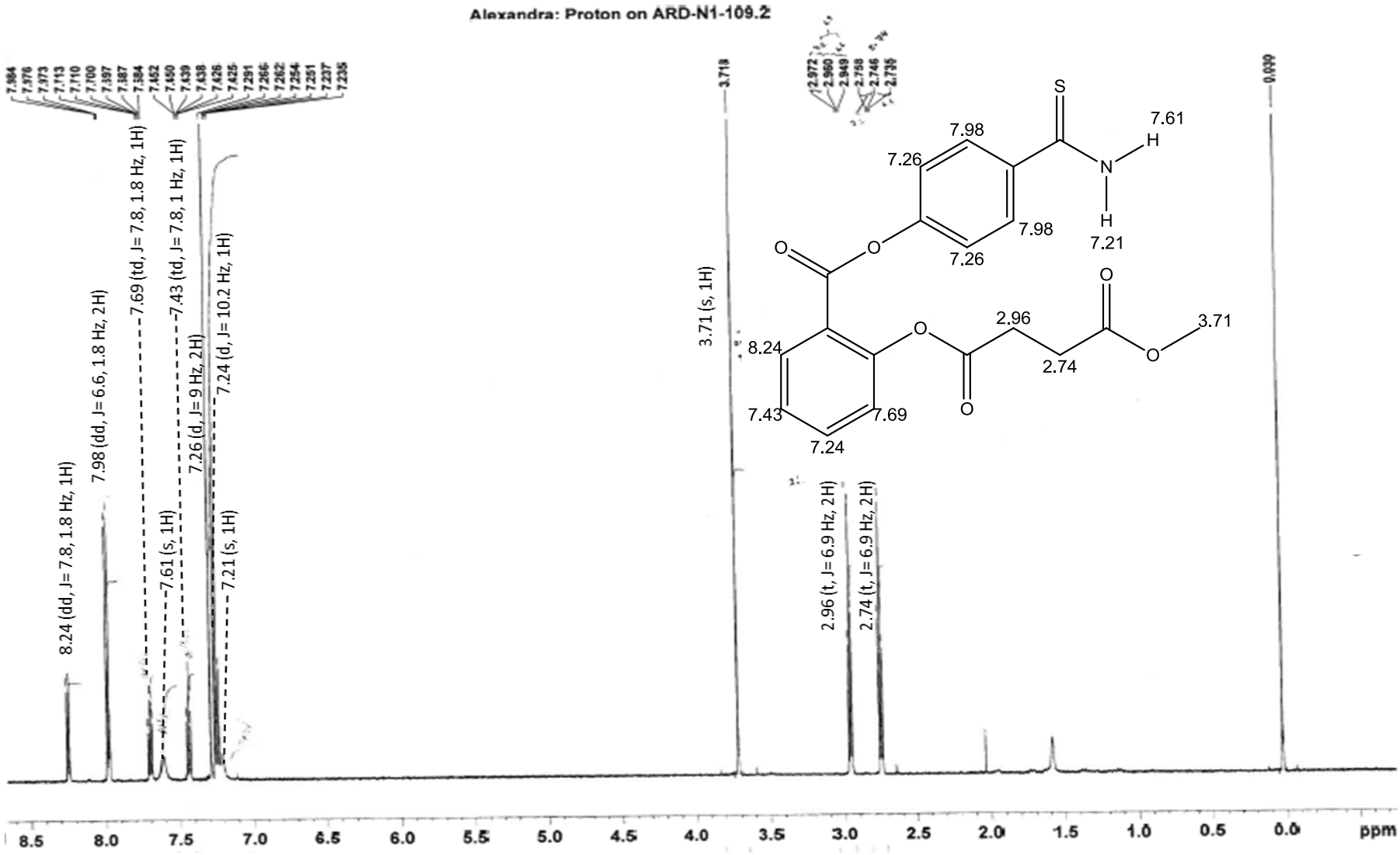
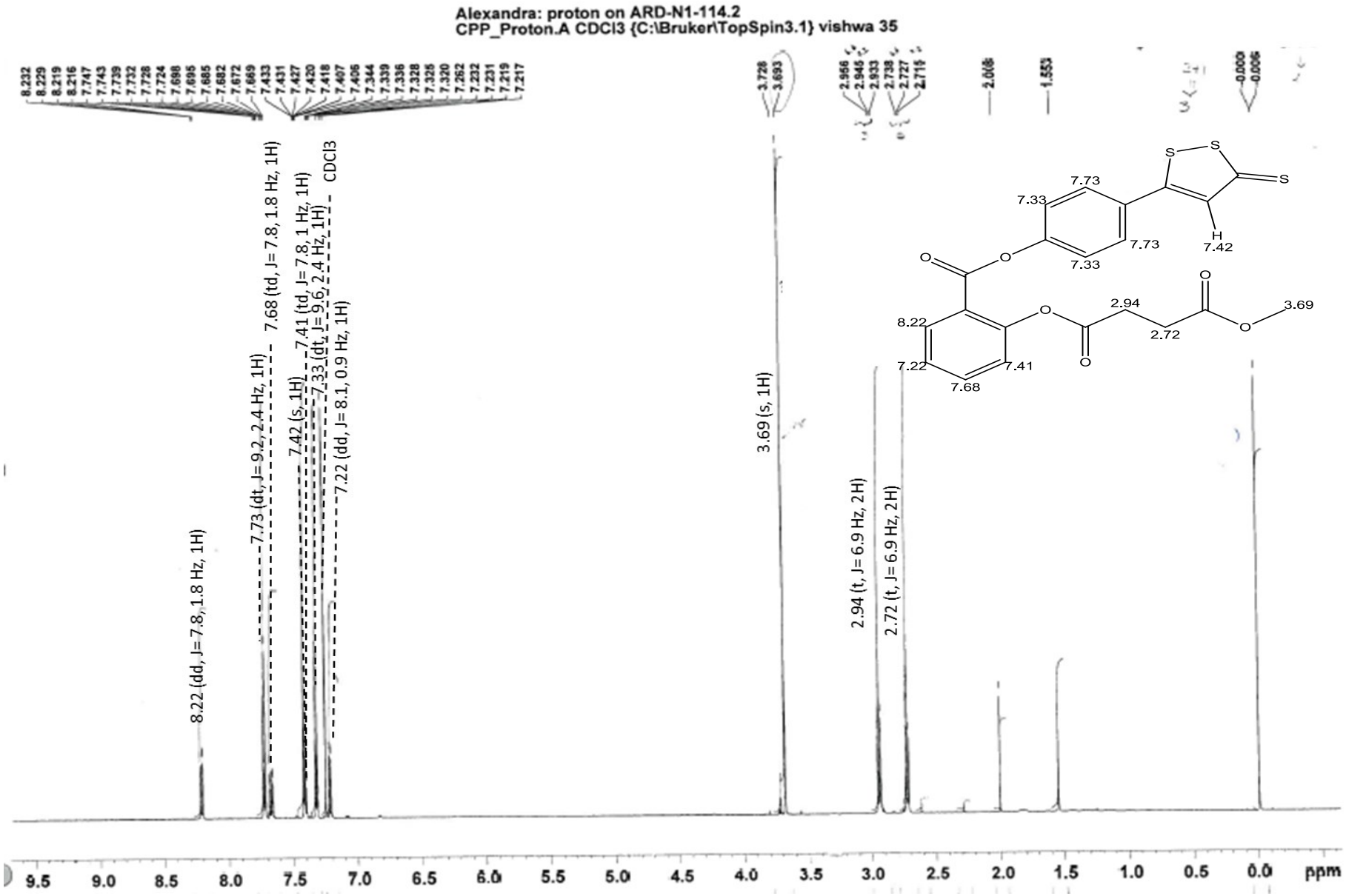
Appendix



^{1}H-NMR of compound ARD-N1-109.2 (ASA/H_2S [TBZ]) [10]



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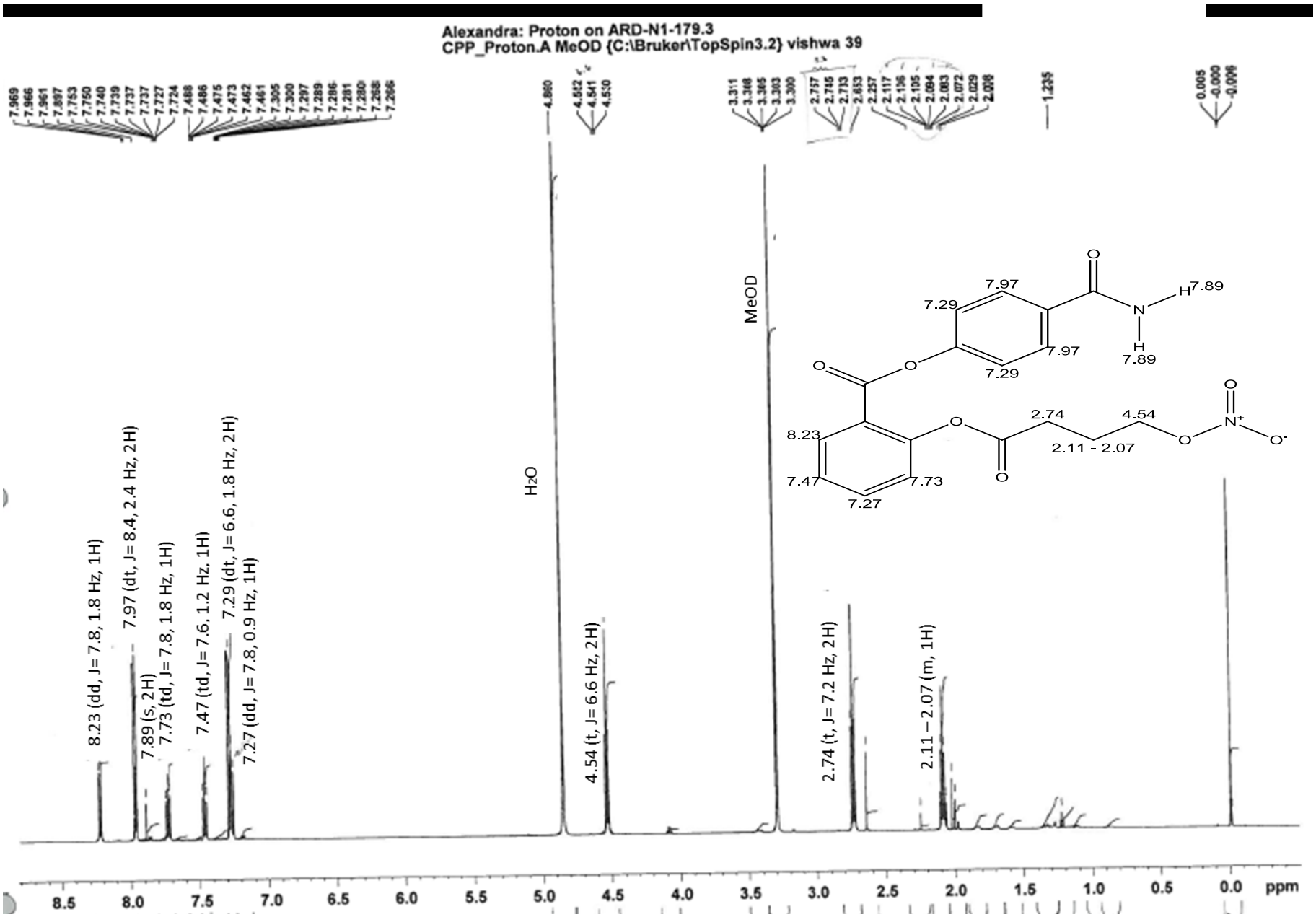
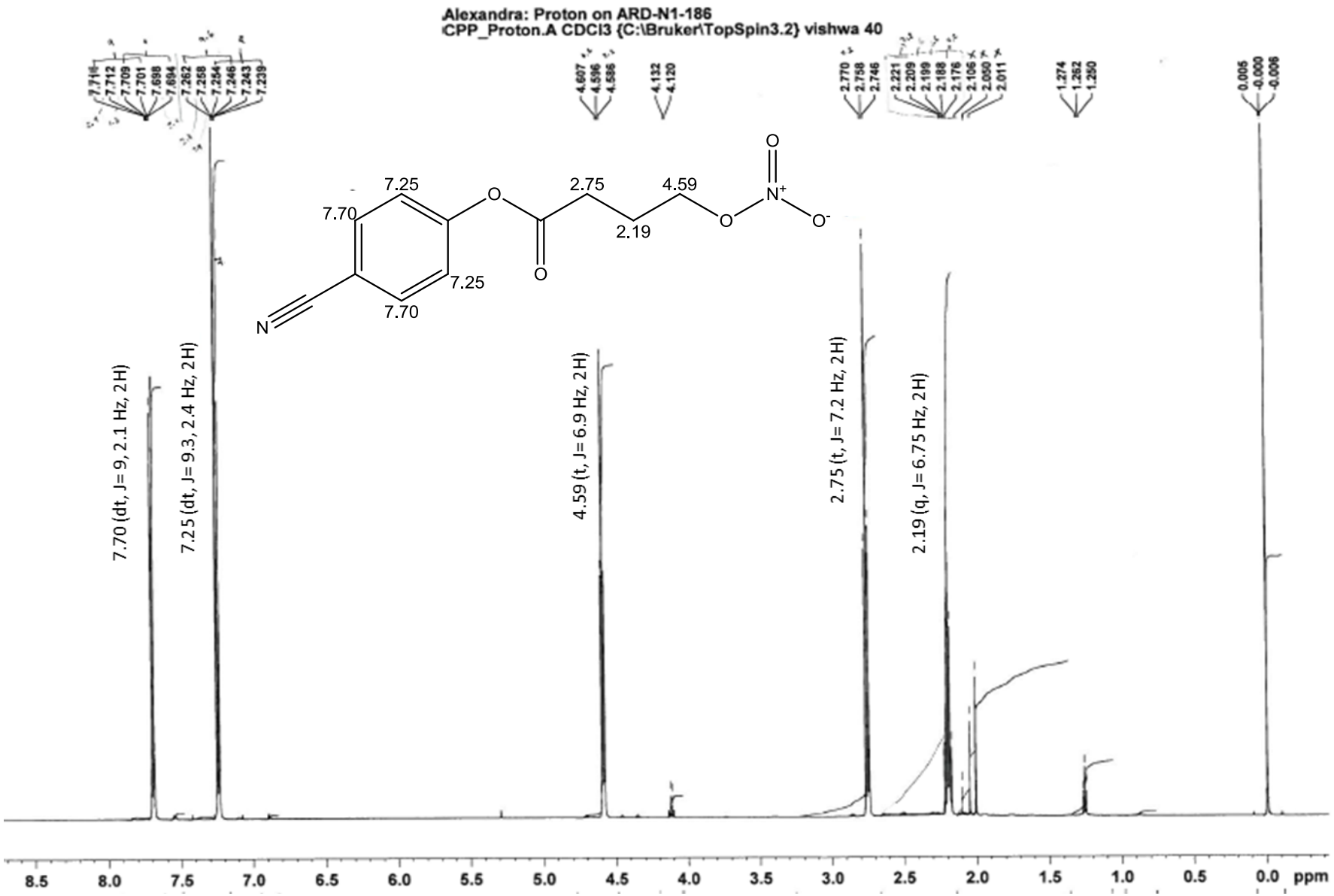
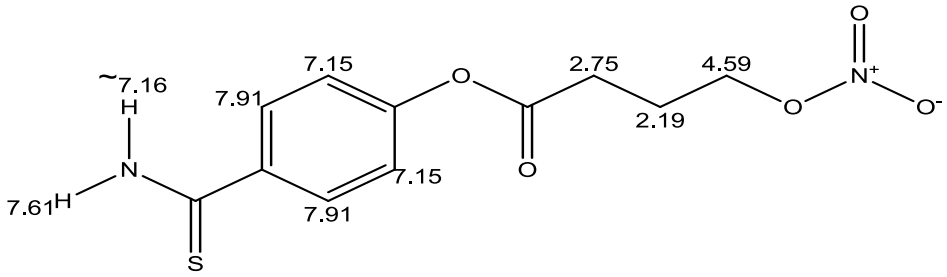
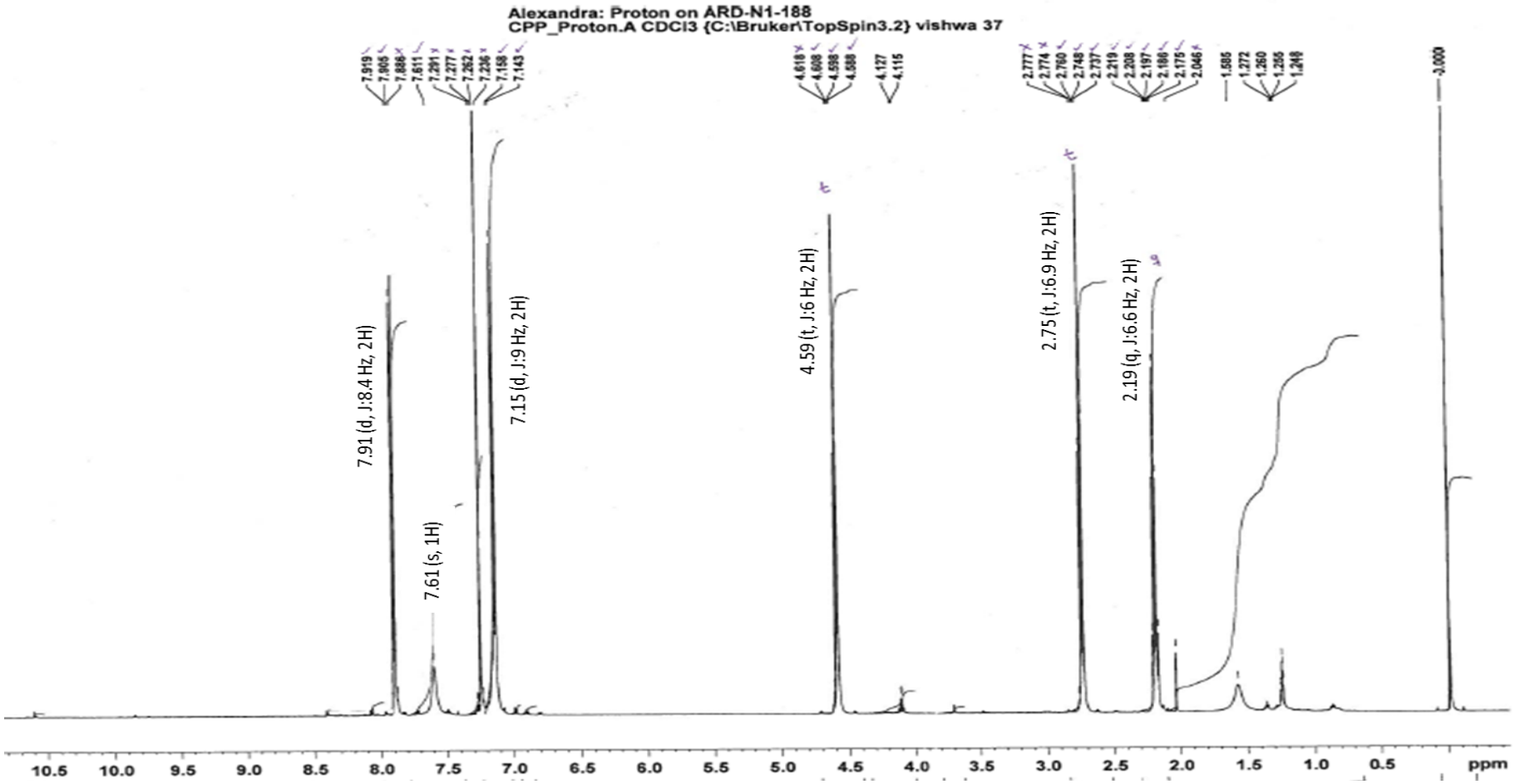


[1]H-NMR of compound ARD-N1-139 (ASA/NO) [17]



[1]H-NMR of intermediate compound ARD-N1-188 [20]





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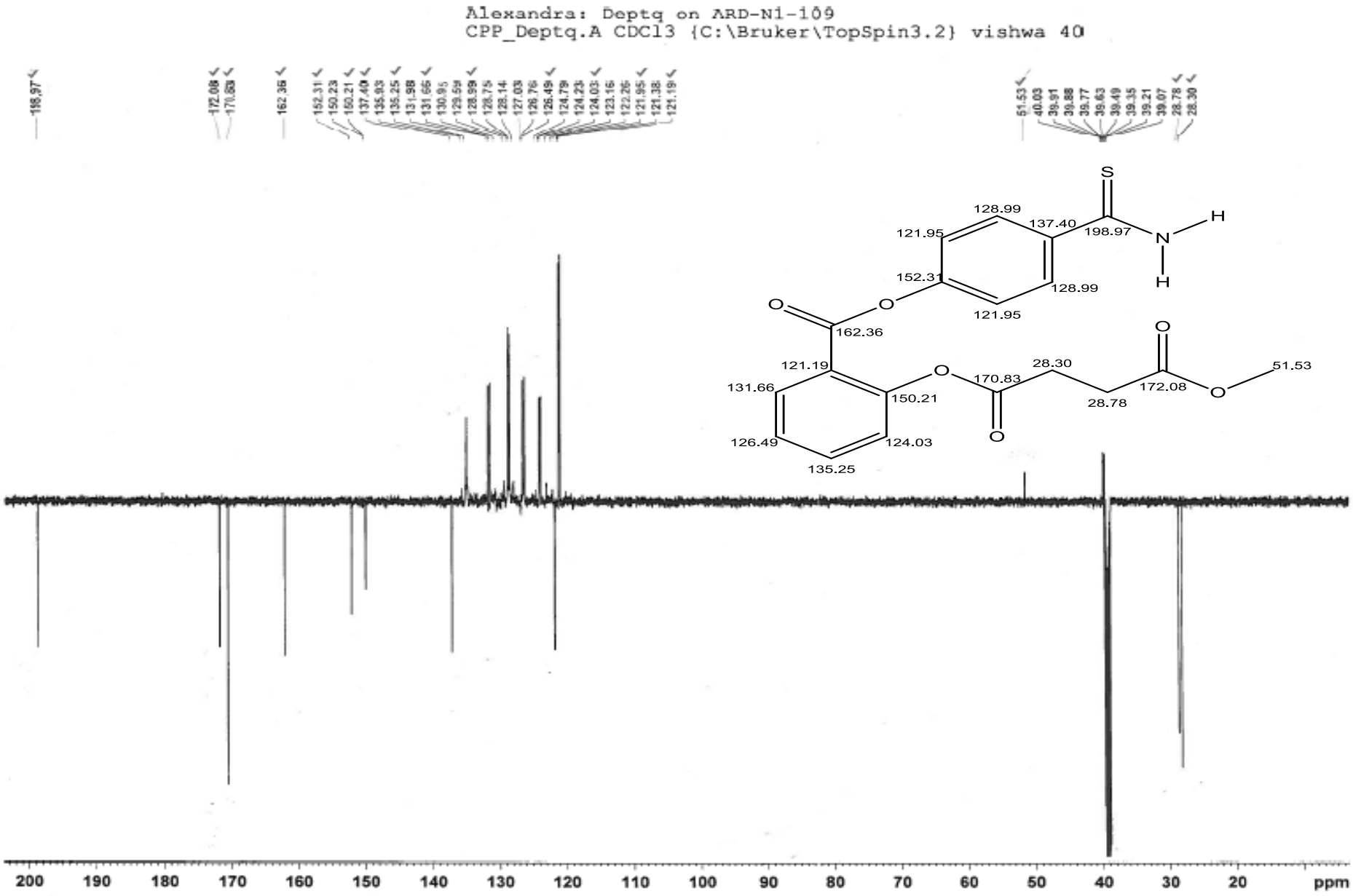
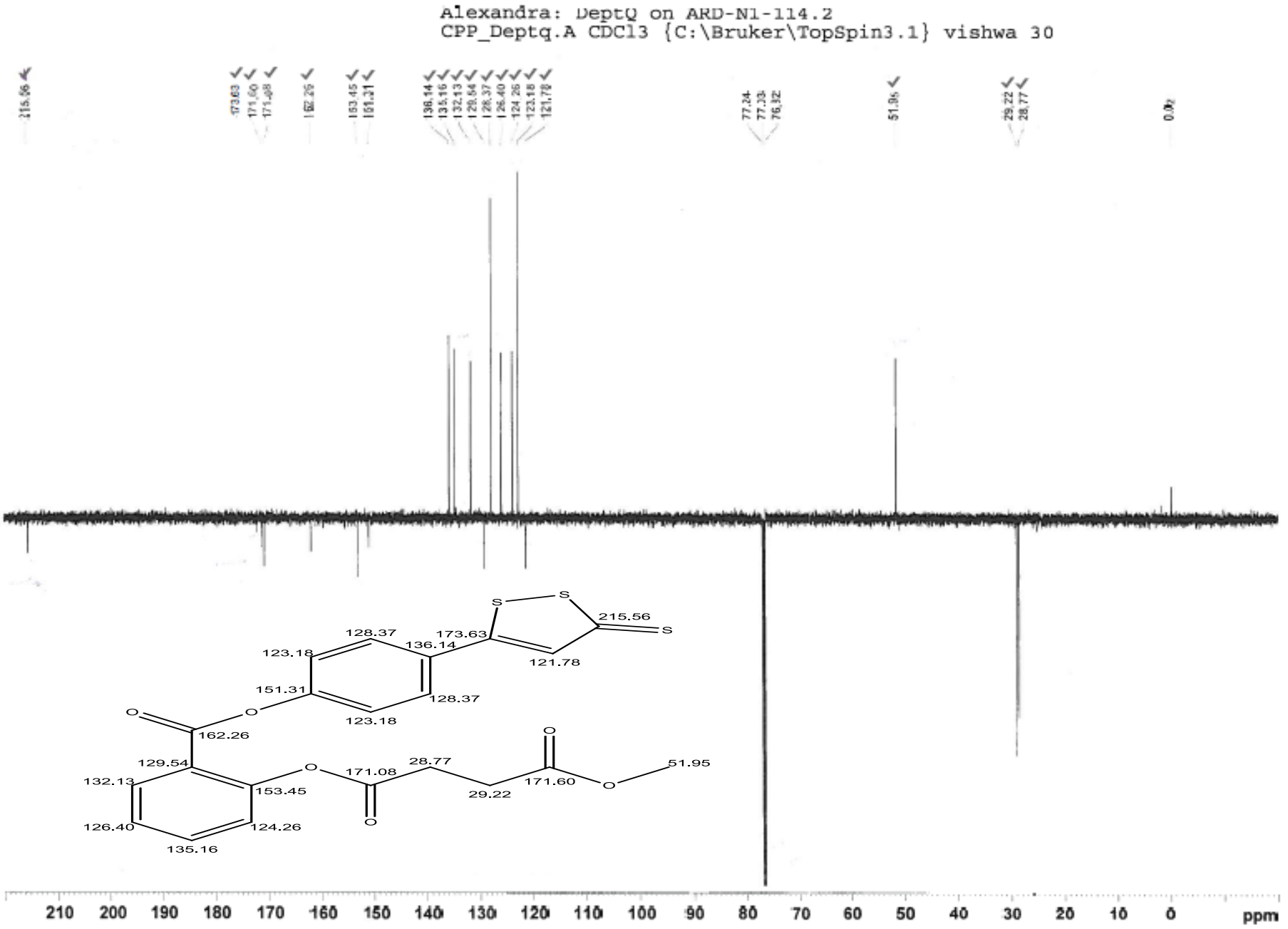


^{13}C-NMR of compound ARD-N1-109 (ASA/H_2S [TBZ]) [10]



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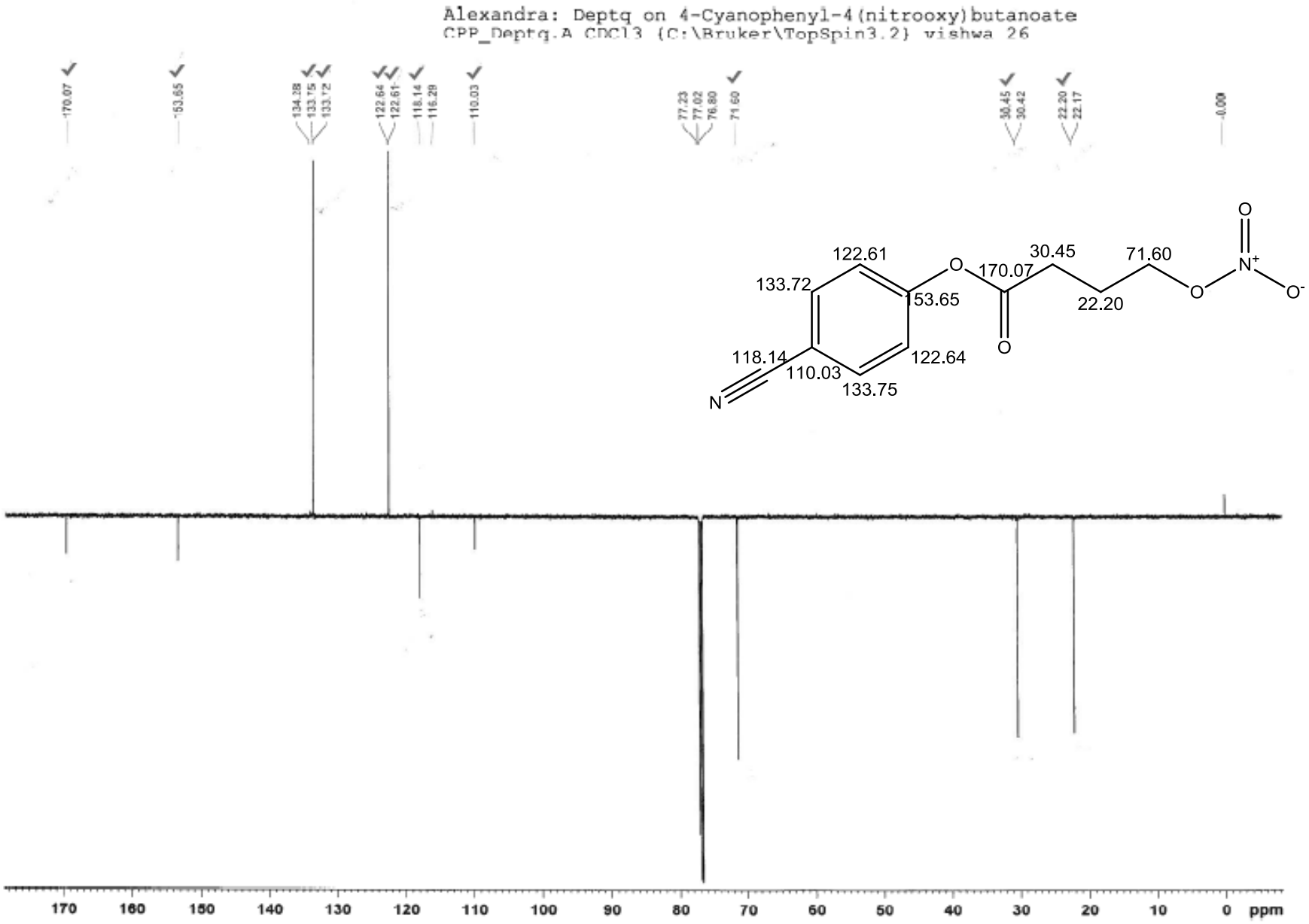
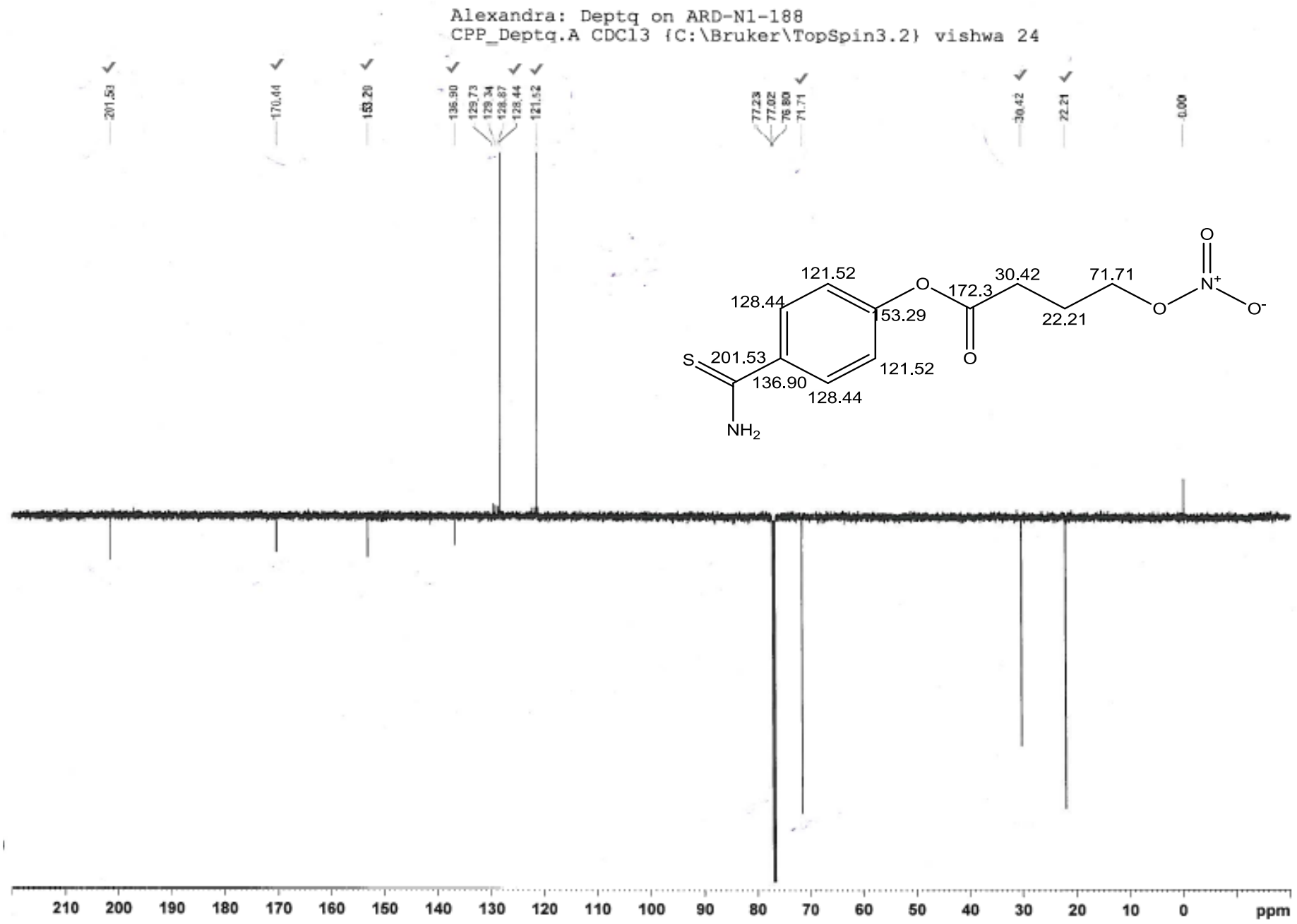
^{13}C-NMR of compound ARD-N1-139 (ASA/NO) [17]



^{13}C-NMR of intermediate compound ARD-N1-188 [20]



^{13}C-NMR of compound ARD-N1-188 (NO/H_2S [TBZ]) [22]