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Anti-Neoplastic Activity of Newly Synthetized DNMTi in Urological Tumors

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Anti-Neoplastic Activity of Newly Synthetized DNMTi in Urological Tumors

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“You never know how strong
you are, until being strong is
your only choice.”

Bob Marley

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RESUMO

Os tumores genito-urinários incluindo os cancros da bexiga, rim e próstata estão entre as dez neoplasias malignas mais incidentes em todo o mundo. A elevada morbilidade e mortalidade associada a estas doenças, bem como a falta de ferramentas eficientes de deteção precoce, torna-os importantes alvos de investigação. O carcinoma de células renais (CCR) é o tumor urológico com a taxa de mortalidade mais elevada. Embora a maioria dos doentes com CCR apresentem doença localizada no momento do diagnóstico, uma percentagem destes doentes irá progressivamente metastizar. Além disso, cerca de um terço dos doentes com CCR são diagnosticados já com doença avançada. Embora, os inibidores das *TKs* (*tyrosine kinases*) apresentem uma melhor taxa de sobrevivência aos 5 anos, esta não ultrapassa os 10%, não existindo ainda terapias com fins curativos para estes estádios.

Atualmente, é geralmente aceite que as alterações ao nível da maquinaria epigenética, especialmente a hipermetilação de genes supressores tumorais, contribui para o desenvolvimento e progressão do cancro. É de realçar que ao contrário das modificações genéticas, as alterações epigenéticas são reversíveis, podendo os inibidores das *DNMTs* (*DNA methyltransferases*) constituir uma ferramenta terapêutica útil para doentes com CCR metastático.

Neste estudo foi inicialmente avaliada a utilidade de quatro novos compostos, derivados das flavanonas, como agentes antineoplásicos em linhas celulares dos três tumores urológicos mais incidentes (da bexiga, rim e próstata), bem como o seu papel na inibição das *DNMTs*. Neste sentido, o perfil global da metilação do DNA de linhas celulares de tumores urológicos foi igualmente avaliado. As linhas celulares T24, ACHN e LNCaP apresentaram os maiores níveis de 5-metilcitosina. Após calcular o valor de IC50 para cada composto, o MLo-1302 obteve o valor de IC50 médio mais baixo (1.5 μM) e foi escolhido para o ensaio de viabilidade nas linhas celulares referidas. Uma vez que a ACHN mostrou ser a linha celular mais sensível, o cancro do rim foi o modelo tumoral escolhido para validar o papel destes compostos.

Deste modo, o objetivo principal desta dissertação foi avaliar a atividade desmetilante e anti-tumoral de quatro novos compostos derivados das flavanonas em linhas celulares de CCR. Curiosamente, todos os compostos induziram inibição da viabilidade celular dependente da dose e do tempo, bem como um aumento significativo da apoptose em todas as linhas celulares testadas, tendo os compostos DD880 e MLo-1302 mostrado ser os mais eficazes. A diminuição da expressão de *KI67* acompanhada do aumento da expressão dos genes *CDKN1A* e *CASP3* permitiu corroborar os dados

fenotípicos. Adicionalmente, e de uma forma geral, todos os compostos induziram alterações morfológicas e dano no DNA nas linhas celulares de CCR. A indução de dano genómico pode ser de elevada importância uma vez que a reparação do DNA é considerada um mecanismo de resistência à terapia. Considerando o potencial desmetilante dos compostos, o maior efeito inibitório na expressão das *DNMTs*, reduzindo a *DNMT1* na Caki-2 e a *DNMT3a* em todas as linhas avaliadas foi obtido com o MLo-1302. Além disso, este composto também diminuiu significativamente a atividade da *DNMT3A* na linha celular Caki-2. Embora os outros três compostos também tenham induzido efeitos ao nível da expressão das *DNMTs*, esse efeito não foi tão pronunciado e consistente como na exposição ao composto MLo-1302. A expressão do gene *TET1* (*Ten-eleven translocation*) aumentou após exposição aos compostos DD880 e MLo-1302 em quase todas as linhas celulares. Para além disso, estes mesmos agentes induziram a expressão dos genes *TET2* e *TET3* em algumas linhas. Globalmente, o composto MLo-1302 induziu uma redução acentuada no conteúdo de 5-metilcitosina em todas as linhas celulares testadas. O composto MLo-1302 reduziu, igualmente, a metilação do promotor do gene *RASSF1A* com concomitante re-expressão na linha ACHN.

Em conclusão, demonstramos pela primeira vez que novos compostos sintetizados derivados das flavanonas atenuam o fenótipo maligno das células de CCR. De uma forma geral, o composto MLo-1302 mostrou ser o mais eficaz quer a nível fenotípico, quer ao nível molecular. Deste modo, constitui um composto promissor para a terapia do CCR avançado, embora estudos adicionais sejam ainda necessários para melhor caracterização da eficácia e perfil de toxicidade em ensaios *in vitro* e *in vivo*.

ABSTRACT

Genitourinary tumors, comprising bladder, kidney and prostate cancers, are among the ten most incident malignancies worldwide. The high morbidity and mortality associated with these diseases as well as the lack of efficient early detection tools render them as an important field to be investigated. Interestingly, Renal Cell Carcinoma (RCC) is the urological tumor with highest mortality rate. Although most RCC patients present with localized disease at diagnosis, a significant proportion eventually progresses through metastization, whereas about one third are diagnosed with advanced and poor prognosis disease. At this stage, there are no available curative therapies and targeted therapy with tyrosine kinase inhibitors (TKIs) endows 5-year survival rate lower than 10%.

Currently, it is widely accepted that aberrations in epigenetic machinery, especially hypermethylation of tumor suppressor genes (TSGs), contribute to RCC development and progression. Importantly, unlike genetic alterations, epigenetic aberrations are reversible, and thus DNA methyltransferase inhibitors (DNMTi) might be a useful therapeutic tool for RCC patients with metastatic disease.

In a preliminary approach, the anti-neoplastic and DNMTs inhibition activity of four newly synthetized flavanones-derived compounds was tested in cell lines of the three most incident urological tumors (bladder, kidney and prostate cancers). In parallel, global DNA methylation profile of urological tumors cell lines was also assessed. T24, ACHN and LNCaP were those from each tumor model that displayed the highest levels of 5-metylcytosine. Following IC50 value calculation for each compound, MLo-1302 demonstrated the lowest IC50 mean value (1.5 µM) and was chosen to perform the viability assay in the previously selected cell lines. Since ACHN cells were the most sensitive, kidney cancer was the selected tumor model to validate the role of these flavanone-derived compounds.

Thus, considering the preliminary data, the major objective of this master dissertation was to evaluate the demethylating and anti-tumoral activity of the four new flavanones-derived compounds in RCC cell lines. Interestingly, all compounds induced a time- and dose-dependent inhibition of cell viability, as well as a significant increase in apoptosis in all tested cell lines, with DD880 and MLo-1302 being the most effective compounds. Decrease in *KI67* expression along with increased *CDKN1A* and *CASP3* expression levels corroborated the phenotypic data. Moreover, in general, all four compounds induced morphological alterations and DNA damage in all cell lines. The induction of DNA damage might be extremely important since DNA repair is considered a mechanism of tumor resistance to therapy. Concerning their demethylation potential, MLo-

1302 induced the higher inhibitory effect on DNMTs expression, reducing *DNMT1* in Caki-2 and *DNMT3a* in all tested cell lines. Additionally, this compound also significantly decreased DNMT3A activity in Caki-2 cell line. Although the other three compounds also affected DNMTs expression, it was less impressive and consistent than MLo-1302. Furthermore, DD880 and MLo-1302 significantly incremented *TET1* transcript levels in almost all treated cell lines. Likewise, *TET2* and *TET3* expression was also increased in some cell lines treated with these agents. Globally, MLo-1302 induced a significant 5-methylcytosine content reduction in all RCC cell lines, particularly in ACHN. The MLo-1302 demethylating effect reduced loci-specific promoter methylation of *RASSF1A* with concomitant gene re-expression in ACHN cell line.

We demonstrated for the first time that new synthetized flavanones-derived compounds attenuate RCC cells malignant phenotype. Globally, MLo-1302 was the most effective compound both at phenotypic and molecular levels. Thus, it constitutes a promising compound for treatment of advanced renal cancer, requiring further testing to determine its efficacy and toxicity *in vitro* and *in vivo* assays.

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µM	Micromolar
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ABCB1	*ATP binding cassette subfamily B member*
ACTβ	*Actin beta*
ADT	Androgen Deprivation Therapy
AKT	Protein kinase B
AML	Acute Myeloid Leukemia
APAF-1	*Apoptotic protease activating factor 1*
APC	*Adenomatosis Polyposis Coli*
AR	*Androgen Receptor*
ATCC	American Type Culture Collection
ATM	Ataxia-Telangiectasia Mutated serine/threonine protein kinase
Bax	*BCL-2-like protein 4*
BCL2	*B-cell lymphoma 2*
BlCa	Bladder Cancer
BRCA1	*Breast cancer type 1, DNA repair associated*
BTG3	*BTG anti-proliferation factor 3*
CAB	Combined Androgen Blockade
CALCA	*Calcitonin related polypeptide alpha*
CASP3	*Caspase 3*
CCNA1	*Cyclin A1*
CCND2	*Cyclin D2*
ccRCC	Clear Cell Renal Cell Carcinoma
CD44	*Cluster of Differentiation 44*, encodes for cell-surface glycoprotein
CDH1	*Cadherin 1*
CDKN2A/ ARF	*Cyclin-dependent kinase Inhibitor 2A*
chRCC	Chromophobe Renal Cell Carcinoma
CIMP	CpG island methylator phenotype
CpG	Cytosine-phosphate-Guanine
CRPC	Castration-resistant Prostate Cancer
CTR1	Copper transporter CTR1
Cx32	*Gap junction protein beta 1*
DACH1	*Dachshund homolog 1*

DAPK	*Death-associated protein kinase 1*
DHAC	5,6-dihydro-5-azacytidine
DKK	*Dickkopf WNT signaling pathway inhibitor*
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyltransferases
DT	Doubling-Time
EC	Embryonal carcinoma
EGCG	Epigallocathechin-3-gallate
EGFR	*Epidermal growth factor receptor*
ER	*Estrogen receptor*
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FdCyd	5-Fluoro-2'-deoxycytidine
GADD45	*Growth Arrest and DNA Damage*
GATA-3	GATA3 transcriptional factor
GDF15	*Growth differentiation factor 15*
GDF3	*Growth differentiation factor 3*
GLIR1	*GLI family zinc finger repressor isoform 1*
GPER	*G-protein coupled estrogen receptor 1*
GREM1	*Gremlin 1*
GSTP1	*Glutathione S-transferase pi 1*
GUSB	*Glucuronidase beta*
H19	Imprinted maternally expressed transcript
H2AX	H2A histone family member X
HDACs	Histones deacetylases
hENT1	*Equilibrative nucleoside transporter 1*
hepaCAM	*Hepatic and glial cell adhesion molecule*
hMLH1	*MutL homolog 1*, encodes for a DNA mismatch repair protein
HOXA9	*Homeobox A9*
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
IFN-α	*Interferon alpha 1*
IGF2	*Insulin-like growth factor 2*
IGFBP3	*Insulin like growth factor binding protein 3*
IU	International Unit
JNK	*Jun N-terminus kinase*

KAI1	*CD82, Cluster of Differentiation 82,* metastatic suppressor
KI67	Marker of proliferation Ki-67
KILLIN	*Killin, p53-regulated DNA replication inhibitor*
KRT19	*Keratin 19*
LAM-5	*Membrane-anchored lipid-binding protein LAM5*
LINE-1	*Long Interspersed Element–1, transposable element*
MAGE	Melanoma-associated antigen 3
MBDs	Methyl-CpG binding protein domains
MDS	Myelodysplastic syndrome
MGMT	*O-6-methylguanine-DNA methyltransferase*
MIBC	Muscle invasive bladder cancer
miRNAs	MicroRNAs
MMP-2	*Matrix metallopeptidase 2*
MMP-9	*Matrix metallopeptidase 9*
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
Myc	*MYC proto-oncogene*, bHLH transcription factor
NANOG	*Nanog homeobox*
Neurog1	*Neurogenin 1*
NFkβ	Nuclear factor kappa B
nM	Nanomolar
NMIBC	Nonmuscle invasive bladder cancer
OS	Overall survival
p16^{INKA}	Cyclin-dependent kinase inhibitor 2A, tumor suppressor protein p16
p21	*Cyclin-dependent kinase inhibitor 1,* encoded by CDKN1A
PAI-1	*Plasminogen activator inhibitor-1*
PBMCs	Peripheral blood mononuclear cells
PCa	Prostate Cancer
PCDH17	*Protocadherin 17*
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
PEITC	Phenethyl isothiocyanate
P-gp	*P-glycoprotein*
p-GSK3β	*Phospho-Glycogen synthase kinase 3 beta*
POU5F1	*POU class 5 homeobox 1*
pRCC	Papillary Renal Cell Carcinoma
PRSS21	*Protease, serine 21*

PSA	Prostate-Specific Antigen
PTGS2	*Prostaglandin-endoperoxide synthase 2*
PTX	Paclitaxel
PYCARD/ ASC/TMS1	*PYRIN-PAAD-DAPIN and caspase-recruitment domain containing*
qMSP	Quantitative methylation-specific PCR
RAGE	Advanced glycosylation end-product specific receptor
Rap1GAP	*RAP1 GTPase activating protein*
RARβ	*Retinoic acid receptor beta*
RASSF1A	*Ras association domain family 1 isoform A*
RCC	Renal Cell Carcinoma
RIN1	*Ras and Rab interactor 1*
RPRM	*Reprimo*, TP53 dependent G2 arrest mediator
RR	Ribonucleotide reductase
SAHA	Suberoylanilide hydroxamic acid
SAM	S-adenosyl-L-methionine
SCGB3A1	*Secretoglobin family 3A member 1*
SERPINB5	*Serpin family B member 5*
SFN	*Stratifin*, encodes a cell cycle checkpoint protein
SFRP	Secreted frizzled-related proteins
SLC	Solute carrier
SLC22A2	Solute carrier family 22 member 2
SMAD2	SMAD family member 2 protein
SOX15	*SRY-box 15*
SOX2	*SRY-box 2*
Sp1	Sp1 transcriptional factor
TCF21	TCF21 transcription factor 21
TET	Ten-eleven translocation
TGCT	Testicular germ cell tumor
TGF-β	*Transforming growth factor beta 1*
THU	Tetrahydrouridine
TIMP3	*TIMP metallopeptidase inhibitor 3*
TKIs	Tyrosine kinase inhibitors
TLR4	*Toll like receptor 4*
TMEFF2	*Transmembrane protein with EGF-like and two follistatin like domains 2*
TP53	*Tumor protein p53*

TSA	Trichostatin A
TSG	Tumor suppressor gene
TU3A	*FAM107A, family with sequence similarity 107 member A*
TβR-II	*Transforming growth factor, beta receptor II*
UCC	Urothelial cell carcinoma
UCHL1	*ubiquitin C-terminal hydrolase L1*
UCK	uridine-cytidine kinase
UTR	Untranslated Region
VASA	*Germ cell-specific marker*
VBL	Vinblastine
VEGF-C	*Vascular endothelial growth factor C*
VHL	*von Hippel-Lindau*
VIM	*Vimentin*
WIF1	*WNT inhibitory factor 1*
XAF1	*XIAP associated factor 1*
XIST	*X inactive specific transcript* (non-protein coding)

I. INTRODUCTION

Urological Tumors

Urological tumors, comprising bladder, kidney, prostate and testicular tumors, are an heterogeneous class of malignancies associated with significant morbidity and mortality rates worldwide [1]. The first entities are among the 10 most frequent cancers in men, being testicular germ cell tumors (TGCTs) the less frequent. However, TGCTs represent the most common cancers in young men [2]. For 2012, it was estimated 14.1 million new diagnosed cases and 8.2 million cancer deaths from all urological cancers worldwide. Importantly, the majority of these tumors are asymptomatic at early disease stage and there are only few blood biomarkers for disease detection [3, 4]. Due to their higher incidence and mortality rates, it is imperative to improve not only early detection approaches but also new and more effective therapeutic strategies.

Epigenetics Concept

Epigenetics is defined as the study of heritable modifications of DNA or associated proteins that carries information related to gene expression during cell division. Unlike genetic abnormalities, epigenetic changes do not alter the DNA sequence and are potentially reversible [5]. In mammals, epigenetic inheritance is important for pre-implantation and fetal development, as well as, cell and tissue differentiation [6-9]. Epigenetic regulation comprises four major mechanisms: DNA methylation, histone post-translation modifications or chromatin remodeling, histone variants and non-coding RNAs' regulation. Thus far, DNA methylation is the best studied epigenetic mechanism [10]. All cancer types, harbor several epigenetic aberrations that directly contribute to malignant transformation and tumor progression [11]. Due to the reversibility of these alterations, modulation of the epigenetic machinery might provide new attractive therapeutic approaches in cancer [12].

The introduction of this dissertation summarizes the recent advances of epigenetic therapy, namely DNA methylation inhibitors (DNMTi), in the treatment of urological tumors.

DNA Methylation

DNA methylation consists in a covalent addition of a methyl group to the 5' carbon of a cytosine ring, mainly within a CpG dinucleotide, resulting on the formation of a new DNA base, 5-methylcytosine (5mC). This process is catalyzed by DNA methyltransferases (DNMTs) being S-adenosyl-L-methionine (SAM) the donor of the methyl group (Figure 1) [13, 14].

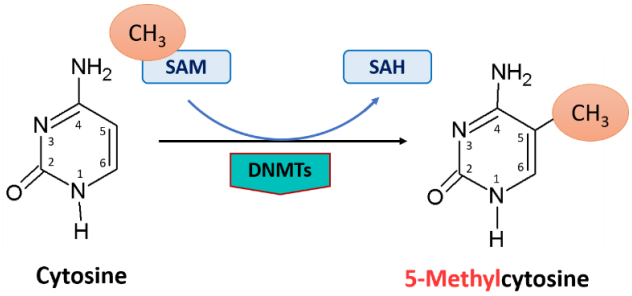


Figure 1 - DNA methylation reaction catalyzed by DNMTs using SAM as a methyl group donor.

There are three well known DNMTs enzymes that catalyze DNA methylation: DNMT1, DNMT3A and DNMT3B. DNMT1 is mainly responsible for the maintenance of parental cell DNA methylation within the newly synthesized DNA strand during cell division [15-17], while DNMT3A and DNMT3B have both *de novo* methylation activity [18, 19]. Importantly, the addition of the methyl group to cytosine does not interfere with the Watson-Crick base paring of the nucleotide. This group is inserted in the major groove of DNA, where it can be efficiently recognized by DNA-interacting proteins [20]. DNA methylation is closely linked to control of gene expression either by inhibiting the binding of transcription factors through direct methylation of CpGs dinucleotides within their binding sites and/or by acting as binding sites for methyl-CpG binding protein domains (MBDs). MBDs associated with other factors such as histones deacetylases (HDACs) can establish repressive chromatin structures (Figure 2) [21-23].

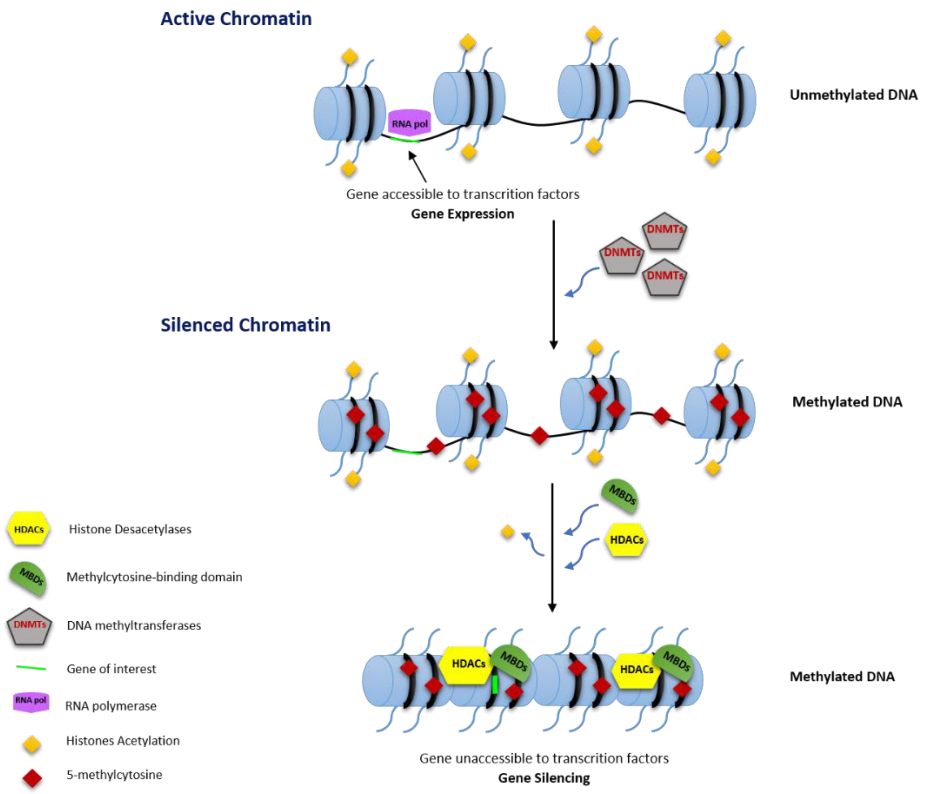


Figure 2 - Transcription regulation by DNA methylation. RNA pol: RNA polymerase; DNMTs: DNA methyltransferases; HDACs: Histone Deacetylases; MBDs: Methylcytosine-binding protein domain.

Methylation patterns are clonally inherited and preserved in daughter cells through replicative DNA methylation by DNMTs enzymes [24]. DNA methylation can be reverted through an active or a passive mechanism, or by a combination of both [25, 26]. Both maternal and paternal genome are active and passive demethylated just before the first mitotic division following fertilization [26-29]. Active demethylation comprises oxidation mediated by TET (ten-eleven translocation) proteins, and entrance into BER (Base Excision Repair) pathway [25]. TET family includes TET1, TET2 and TET3 proteins identified as dioxygenases which catalyzes the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) [30, 31]. On the other hand, passive demethylation consists in gradual methylation loss through lack of maintenance during DNA replication, such as predominant exclusion of DNMT1 from the nucleus [32]. Changes in DNA methylation pattern have been described in several human diseases, including cancer [21]. In fact, in cancer cells, gain in DNA methylation at gene promoters (hypermethylation), that are normally unmethylated, leads to gene inactivation (Figure 3A). Concomitantly, demethylation of normally methylated regions have been associated with chromosomal instability and activation of proto-oncogenes (hypomethylation) (Figure 3B) [11, 33]. Although, the mechanisms underlying

these aberrant DNA methylation patterns are largely unknown, several studies demonstrated that they arise early in tumor development [34, 35].

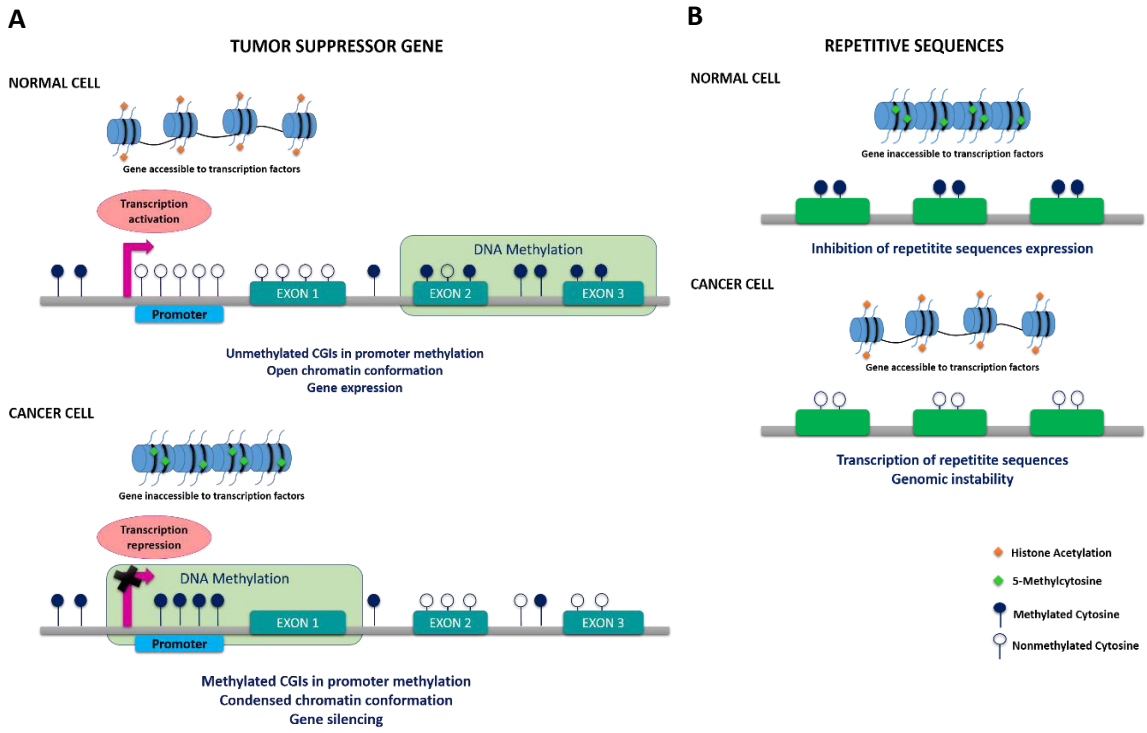


Figure 3 - DNA methylation patterns and chromatin conformation in normal and cancer cells in **(A)** CpG island (CGIs) in promoter region of TSGs and **(B)** repetitive sequences.

DNMTs inhibitors

During the last decades several compounds were described as being able to restore the normal methylation patterns by irreversibly inhibiting the DNMTs enzymatic activity and triggering their proteasomal degradation [36, 37]. This in turn, actively contributes to tumor cell reversion phenotype which might translate into significant clinical benefits for patients [38]. Indeed, two epigenetic compounds that target DNA methylation and four HDAC inhibitors were already approved by Food and Drug Administration (FDA) for cancer treatment (Table 1) [39-41].

Table 1 – US FDA-approved drugs that target the epigenome.

Drug	Class	Target	Date	Cancer type	Pharmaceutical Company
5-Azacytidine Azacytidine (Vidaza)	DNMTi	DNMT proteins	2004	Myelodysplastic syndrome (MDS)	Celgene Corporation (and generic)
5-Aza-2'-deoxycytidine Decitabine (Dacogen)	DNMTi	DNMT proteins	2006	Acute Myeloid Leukemia (AML) and MDS	Otsuka Pharmaceutical (and generic)
SAHA Vorinostat (Zolinza)	HDACi	HDAC class I, II and IV proteins	2006	Cutaneous T cell lymphoma	Merck & Co.
FK-228 Romidepsin (Istodax)	HDACi	HDAC class I proteins	2010	Cutaneous T cell lymphoma	Celgene
PXD101 Belinostat (Beleodaq)	HDACi	HDAC class I and II proteins	2014	Peripheral T cell lymphoma	Spectrum Pharmaceuticals
LBH-589 Panobinostat (Farydak)	HDACi	HDAC class I, II and IV proteins	2015	Multiple myeloma	Novartis

SAHA: suberoylanilide hydroxamic acid.

DNMTi can be divided in two classes depending on their mode of action, namely nucleoside and non-nucleoside analogues (Figure 4) [42].

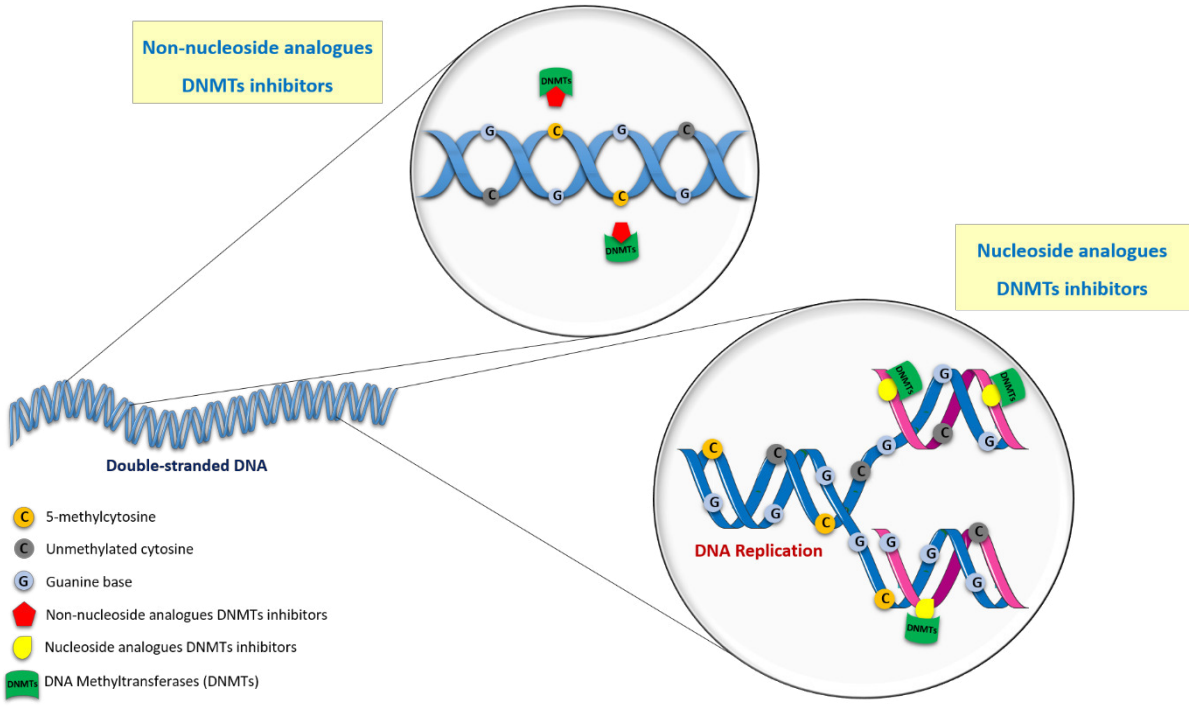


Figure 4 - Mechanism of action of nucleoside and non-nucleoside analogues DNMT inhibitors.

1. Nucleoside analogues

Nucleoside analogues comprise a modified cytosine ring which is connected to either a ribose or deoxyribose moiety, and, therefore, can be integrated into DNA or RNA, replacing cytosines. When incorporated into DNA, during S phase of the cell cycle, they covalently bind and inhibit DNMTs on DNA strand, inducing cell death or DNA damage [43,

44]. Therefore, these compounds can deplete DNMTs, which results in passive loss of cytosine methylation in daughter cells after successive DNA replications and, consequently, reactivation of epigenetically silenced genes expression [43, 45]. Moreover, lack of DNMTs activity induced by drug uptake during DNA synthesis leads to reduction of cytosine methylation in the genome [46, 47]. DNMTi can lead to activation of epigenetically silenced tumor suppressor genes (TSGs) resulting in cell death, cell cycle arrest, chromatin extension and induction of cell differentiation [46-48]. The most well characterized nucleoside analogues, 5-azacytidine (Vidaza™) and 5-aza-2'-deoxycytidine (Dacogen™), have been widely used in pre-clinical and clinical trials in a broad spectrum of cancer models due to their anti-tumorigenic activity [49, 50]. These drugs were developed in 1964 as classical cytostatic agents [51], while in the 80's it was disclosed not only their ability to induce cell differentiation *in vitro* but also their involvement in DNA methylation inhibition [52]. The clinical benefit observed in clinical trials for hematologic cancer patients, [53, 54] led to the US FDA approval of 5-azacytidine and 5-aza-2'-deoxycytidine in 2004 and 2006, respectively, for myelodysplastic syndrome (MDS) treatment [40, 41]. In addition to MDS, these compounds are also active against acute myeloid leukemia (AML) and other myeloid malignancies [55]. However, both 5-azacytidine and 5-aza-2'-deoxycytidine present several limitations in clinical practice, due to their cytotoxic effect at higher doses, instability under physiological conditions and short half-life attributable to degradation by hydrolytic cleavage and deamidation by cytidine deaminase enzyme [56, 57]. Moreover, high doses of 5-azacytidine can cause neutropenia and thrombocytopenia [58]. One of the major concerns related to these compounds is their lack of specificity which might activate the expression of normally silenced genes contributing to tumorigenesis [59]. Nevertheless, a large trial with MDS treated patients with lower doses of these agents, showed not only an increase in conversion time for leukemia but also improved overall survival (OS) [39]. To improve the stability and efficacy of azanucleosides agents, other cytidine analogues have been developed, such as zebularine, 5-Fluoro-2'-deoxycytidine, 5,6-dihydro-5-azacytidine, SGI-110, CP-4200 and Gemcitabine. Zebularine is a deoxycytidine derivate that lacks an amino group in position 4 of the pyrimidine ring [60]. This compound stabilizes the binding of DNMTs to DNA, thereby trapping the enzyme and preventing its turnover even at other sites, resulting in decreased methylation and dissociation of the enzyme-DNA complex [61]. Additionally, zebularine is stable in neutral and acidic aqueous solution, induces minimal toxic effects in animals, inhibits cytidine deaminase, and if administered continuously in cancer cells, effectively maintains gene demethylation [62]. This demethylating agent was also the first nucleoside analogue able to reactivate an epigenetically silenced gene by oral administration [62]. Importantly, this compound demonstrated a high selectivity for tumor cells [63]. 5-Fluoro-2'-deoxycytidine (FdCyd) is a fluoropyrimidine nucleoside analogue [60]

that also forms covalent bindings with DNMTs to produce a suicide complex [64, 65]. Like zeburaline, FdCyd is stable in aqueous solution, and less toxic than 5-azacytidine and 5-aza-2'-deoxycytidine [60]. However, it is rapidly metabolized *in vivo* by cytidine deaminase [66]. To overcome this obstacle, clinical studies showed that co-administration of FdCyd with a cytidine deaminase inhibitor, such as tetrahydrouridine (THU), improved its stability [67]. 5,6-dihydro-5-azacytidine (DHAC) is a hydrolytically stable 5-azacytidine nucleoside due to the saturation of 5,6-double bond which prevents the nucleophilic attack of the position 6 by water. This compound is less cytotoxic than 5-azacytidine and 5-aza-2'-deoxycytidine overcoming their main weaknesses [68]. Similarly to 5-azacytidine, DHAC can be incorporated into the RNA and inhibits its synthesis and DNA methylation in human cell lines [69]. However, clinical trials with DHAC resulted in low response rate and significant side effects, as severe chest pain, and cardiotoxicity [69, 70]. SGI-110 (guadecitabine) a dinucleotide consisting of 5-aza-2'-deoxycytidine followed by a deoxyguanosine, is a largely resistant cytidine deaminase compound that has been reported to be effective in DNA methylation inhibition *in vitro* and *in vivo*, and that can also act as an immune modulator [71, 72]. Importantly, compared to 5-aza-2'-deoxycytidine and 5-azacytidine, this second-generation hypomethylating drug with improved pharmacology and pharmacodynamic effects showed good tolerance, as well as biological activity in patients with MDS and AML [73]. It was also demonstrated that this drug protects 5-aza-2'-deoxycytidine from deamination, increasing its exposure time and metabolic stability [71, 74]. CP-4200 is an elaidic acid ester analogue of 5-azacytidine that contains a fatty acid moiety in order to turn the cellular uptake of the drug less dependent on the nucleoside transport systems. This compound had shown significantly higher efficacy comparatively to 5-azacytidine in an orthotopic mouse tumor model for acute lymphocytic leukemia, and also a strong epigenetic modulatory effect in several human cancer cell lines [75]. Gemcitabine is a pyrimidine cytosine analogue which acts as an anti-metabolite and have been used in chemotherapy [76]. This agent requires intracellular conversion in two active metabolites, gemcitabine diphosphate and gemcitabine triphosphate, which in turn can function in two ways: by binding to ribonucleotide reductase (RNR) and irreversibly inhibiting it, and/or by replacing cytosine during DNA replication [77]. Recently, a newly synthetized cytidine analogue, RX-3117 (fluorocyclopentenylcytosine), was described as able to interfere with cell division, DNA synthesis and induce cell cycle arrest at G_1 phase and apoptosis. RX-3117 cellular uptake is mediated by the human equilibrative nucleoside transporter (hENT1) and requires uridine-cytidine kinase (UCK) to be activated. RNR reduce RX-3117 diphosphate to deoxyRX-3117 diphosphate, that can be converted to deoxyRX-3117 triphosphate and subsequently incorporated into DNA molecule, inhibiting DNMTs [78, 79].

2. Non-nucleoside analogues

The cytotoxic effects inherent to nucleoside analogues derived from their direct incorporation into DNA [64, 80] gave rise to the development and discovery of new compounds that can directly bind to the catalytic site of DNMTs without requiring prior incorporation into the DNA molecule [81]. Procaine (local anesthetic) and its derivative procainamide (anti-arrhythmic drug) are two closely related small molecules able to reduce DNA methylation in cancer cells [82, 83]. These agents are thought to directly bind to CpG-rich sequences, perturbing the interactions between DNMTs proteins and its target sites (CpG islands). Procaine was able to reduce 40% of 5-methylcytosine DNA content and densely demethylate hypermethylated CpG islands, such as those located in the promoter region of the *RARβ2* gene with concomitant re-expression. Furthermore, this agent exerted growth-inhibitory effects in MCF-7 breast cancer cell line by inducing mitotic arrest [82]. Procainamide preferentially inhibits DNMT1 but not DNMT3A and 3B, suggesting a highly specific inhibitory effect of this compound [84]. IM25 is a novel and small DNMT1 inhibitor derived from procainamide, that was shown to demethylate *GSTP1* with lower toxicity comparatively with procainamide and 5-aza-2'-deoxycytidine [85]. Hydralazine is a potent arterial vasodilator agent that has been recognized as a demethylating agent [86]. However, its mechanism of action is not yet well understood. Some authors suggest that this drug inhibits DNA methylation, establishing highly stable interactions between its nitrogen atoms and the active site of DNMTs [87]. Hydralazine was shown to induce demethylation and reactivation of TSG in several cancer models, without significant cytotoxic effects [86, 88]. Interestingly, its activity is synergized when combined with valproic acid, an effective short-chain fatty acid HDAC inhibitor [87-89]. The antibiotic nanaomycin A was recently reported as a selective inhibitor of DNMT3B enzyme, with the ability to reduce DNA methylation and induce re-expression of Ras-association domain family protein 1 isoform A (*RASSF1A*) TSG in cancer cell lines [90]. Most DNMTi are not specific for a particular DNMT, which may favor toxicity. To overcome this issue, two novel small molecules were designed: MG98 and RG108. MG98 is a second-generation 20-nucleotide antisense oligonucleotide designed to hybridize with the 3'-UTR of human DNMT1 mRNA leading to enzyme downregulation [91]. A phase I clinical trial demonstrated that this compound not only inhibited DNMTs in a more selective manner but also was well tolerated being associated with lower cell toxicity [92]. Despite this DNMT1 inhibitory activity, this compound did not reach a significant response in clinical trials [92-94]. RG108 is a synthetic molecule designed to directly inhibit DNMT1 catalytic domain. This compound blocked DNMT1 without causing enzyme degradation [95] and with low cytotoxic effects [96]. Furthermore, RG108 has been shown to effectively reactivate several epigenetically silenced TSGs, without affecting the methylation state of centromeric repeats [95, 96]. Disulfiram, a compound with strong thiol-reactive functional

groups which attack the thiol group of the reactive cysteine in the active site of the aldehyde dehydrogenase enzyme [97], is used in clinical practice for alcohol abuse treatment [98]. This compound was recently described as a DNMTi since it was capable of reduce the overall levels of 5mC, demethylate and reactivate the expression of epigenetically silenced TSGs [99]. The quinoline-based compound SGI-1027 demonstrated inhibitory activity against DNMT1, DNMT3A and DNMT3B, possibly by interaction with the DNA substrate which results in demethylation and reactivation of TSGs [100, 101]. Several studies suggested that non-nucleoside inhibitors are not necessarily less genotoxic and cytotoxic than nucleosides analogues. Moreover, these compounds were reported to be less effective in the inhibition of DNA methylation and reactivation of gene expression than the nucleoside analogues inhibitors [95, 102].

3. Natural compounds

Natural products found in food appears to have antioxidant and anti-tumoral properties, as well as may represent a potent class of epigenome-targeted-drugs, including isothiocyanates (such as phenethyl isothiocyanate) and polyphenols (like epigallocatechin-3-gallate, genistein, curcumin and flavanones) [103, 104]. The role of these compounds as epigenetic modulators is now a hot topic of investigation (Table 2). Phenethyl isothiocyanate (PEITC), found in cruciferous vegetables, induces cellular growth arrest and apoptosis [105]. Epigallocathechin-3-gallate (EGCG), derived from green tea, inhibit DNA methylation by binding to and blocking the active site of human DNMT1, leading to reactivation of TSGs [106]. Genistein, one of the most common and well known isoflavones, was reported to be the major anti-cancer constituent of soybean [107]. Several molecular targets were attributable to this compound, including estrogen receptor (*ER*) [108]. Curcumin one of curry constituents, has numerous medicinal and anti-cancer properties [109]. Nowadays, Curcumin appears as a promising chemopreventive agent able to reverse, inhibit or prevent the development of several human malignancies through inhibition of specific molecular signaling pathways involved in carcinogenesis [110-112]. Curcumin was reported to induce global hypomethylation in MV4-11 leukemia cell line through molecular docking with DNMT1 enzyme, suggesting that this compound covalently blocks the catalytic thiolate of DNMT1 inhibiting DNA methylation [113]. Flavanones are a subgroup of flavonoids which are abundantly found in citrus fruits. Naringenin from grapefruit and hesperetin from oranges are the major flavanones and were reported as being able to inhibit DNMTs activity [114, 115]. These compounds also demonstrated the ability to induce apoptosis, inhibit cancer cell proliferation, invasion and migration by affecting several important signaling pathways [116].

Table 2 – DNA demethylating potential of natural compounds.

Compound	Target	Model	Results	Ref.
Curcumin	DNMT1	Leukemia cell line	Global DNA hypomethylation	[113]
	DNMT1	Docking-based virtual screening and experimental evaluation	DNA demethylation	[117]
EGCG	DNMTs	Colon cancer, esophageal cancer, and prostate cancer cell lines	DNA demethylation and reactivation of gene expression (*hMLH1*, *MGMT*, *p16^{INKA}*, and *RARβ*)	[106]
Genistein	DNMTs	Esophageal squamous cell carcinoma cell lines	DNA demethylation and reactivation of gene expression (*MGMT*, *p16^{INKA}*, and *RARβ*)	[118]
	DNMTs	Prostate cancer and RCC cell lines	DNA demethylation and reactivation of gene expression (*BTG3*)	[119]
	DNMTs	Prostate cancer cell lines	DNA demethylation and reactivation of gene expression (*p16* and *p21*)	[120]

DNMTi in urological tumors: pre-clinical and clinical trials

Urological tumors harbor several epigenetic alterations, being hypermethylation of TSGs the most common and studied one. The panel of the most relevant hypermethylated genes in each urological tumor is represented in Figure 5.

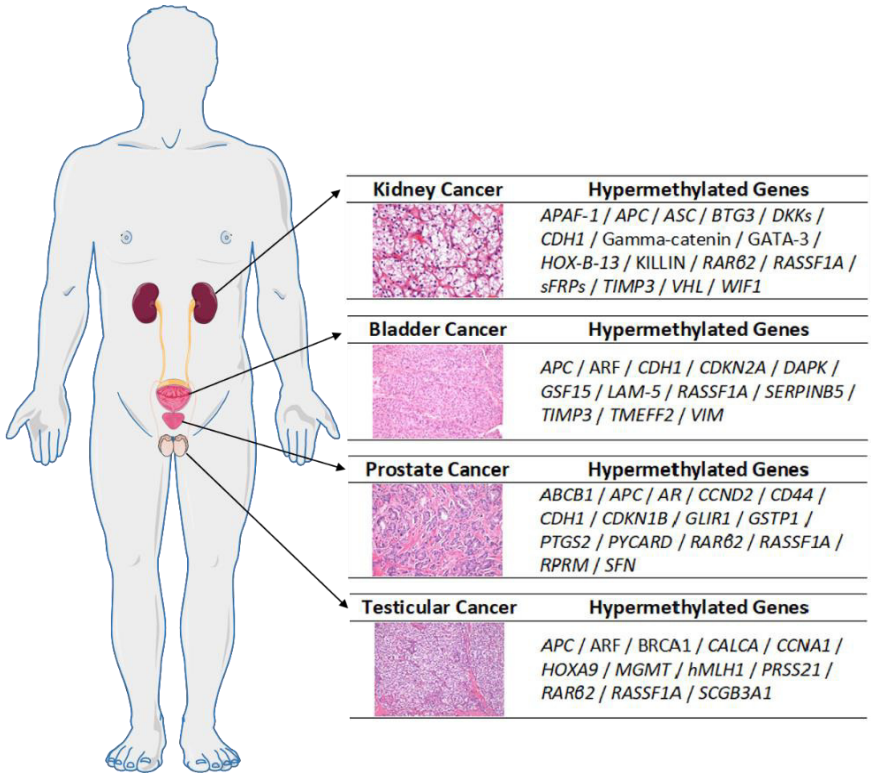


Figure 5 - An overview of the most relevant hypermethylated genes in each urological tumor, compiled from the referenced cited in the main text.

In the last decades, several studies reported the clinical usefulness of DNMTi for urological tumors therapy. Contrarily to prostate cancer, the role of these agents in the

remaining urological tumors is still rather unexplored. Pre-clinical and clinical assays performed for each urological tumor are reviewed in the next sections, being all the clinical trials (completed, terminated or ongoing) summarized in Tables 3, 4, 5 and 6 corresponding to each urological tumor model.

1. Bladder Cancer

Bladder cancer (BlCa) is the ninth most common malignant tumor worldwide and its incidence is 3 times higher among men than women, representing the 4th and the 11th most common cancer, respectively [121]. However, for unknown reasons, women have higher mortality rates than men [122]**.** Urothelial cell carcinoma (UCC) is the most common histological subtype of BlCa representing about 90% of all cases, while the remaining 10% are non-urothelial subtypes, as squamous cell carcinoma or adenocarcinoma. Depending on the degree of cancer cells histological differentiation, UCC can be classified in low-grade and high-grade. Besides, based on invasion level of muscular bladder wall, UCC can also be classified as nonmuscle invasive BlCa (NMIBC) and muscle invasive BlCa (MIBC) [123]. Despite more than 80% of UCC are superficial lesions with a favorable prognosis, these lesions tend to frequently recur. This relapsing nature of BlCa makes it one of the most expensive human malignancies to treat [124]. A deeply invasive BlCa is often correlated with fatal outcome [125]. Low-grade UCC is primarily managed by bladder-sparing techniques as endoscopic resection and intravesical chemotherapy. While in muscle-invasive phenotype, radical cystectomy with neoadjuvant chemotherapy are the first line treatment, patients with advanced disease are treated with systemic cisplatin-based chemotherapy regimens [126]. However, these therapies are not curative, and this aggressive phenotype can develop resistance to chemotherapeutic drugs resulting in treatment failure. Concerning epigenetic alterations, high-grade UCCs present higher hypermethylation levels and upregulation of several microRNAs (miRNAs), when compared with low-grade UCCs [127, 128]. The main difference between low- and high-grade UCC seems to be the quantity of aberrant hypermethylation instead of the specific targets. Epigenomic profiling has revealed that 10% of the loci in low-grade UCC displayed aberrant DNA methylation patterns, contrarily to high grade noninvasive and invasive UCC that have over 20% and 30%, respectively [129-131]. Biallelic expression of the imprinted gene *IGF2* and imprinted maternally expressed transcript *H19* were described in around 20% of BlCa [132]. Hypomethylation of *LINE-1* gene was correlated with an increased risk of BlCa and could be a potential biomarker for BlCa diagnosis and treatment [133]. Several hypermethylated genes, namely *APC*, *ARF*, *CDKN2A*, *DAPK*, *LAM-5* and *RASSF1A* have been proposed as detection markers for BlCa [134-136]. *SERPINB5* (gene that encodes for

maspin) expression is higher in normal urothelium, preserved in superficial BlCa, but is significantly diminished in invasive carcinomas. This low maspin expression is correlated to gene hypermethylation and with increased tumor cell growth *in vivo* [137, 138]. Moreover, a genome wide approach, led to identification of a panel of three genes, *GDF15*, *TMEFF2* and *VIM*, that accurately detect this neoplasm [139]. Importantly, the methylation pattern of *APC*, *CDH1* and *RASSF1A* genes are also significantly correlated with prognostic parameters [140], and disease progression [128]. The increased methylation levels of *ARF* and *RASSF1A* can be a predictor of tumor stage and/or grade with potential clinical benefit. Furthermore, *BCL2* methylation levels were significantly associated not only the with disease stage but also with tumor grade [136].

1.1. Pre-clinical Studies

Concerning pre-clinical assays in BlCa, the majority of them evaluated the efficacy of nucleoside inhibitors as potential anti-tumor agents for BlCa. Regarding exposure of BlCa cells to 5-azacytidine, it was demonstrated that this compound induced downregulation of DNMT3A/3B expression and reversed the hypermethylation with concomitant re-expression of *hepaCAM* gene. In addition, 5-azacytidine inhibited proliferation of BlCa cells and arrested cell cycle at G_0/G_1 phase. Likewise, tumor growth of nude mice was markedly reduced after treatment with this compound [141]. 5-Azacytidine combined with the histone deacetylase inhibitor Trichostatin A (TSA) decreased cell proliferation by attenuating the expression of DNMT1 in canine invasive urothelial carcinoma cells. Additionally, this combination caused a noticeable increase in p16 protein expression by demethylation of its gene promoter region [142]. A recent pre-clinical study showed a unidirectional cross-resistance of cisplatin-resistant UMUC3 cells to docetaxel. However, pre-treatment of BlCa cell lines (UMUC3, T24 and TCSSUP) with 5-azacytidine resulted in enhanced sensitivity to chemotherapeutic drugs (cisplatin and docetaxel) by demethylating and up-regulating epigenetically silenced genes involved in apoptotic pathway [143]. Exposure of T24 cells to different concentrations of 5-aza-2'-deoxycytidine resulted in increased levels of maspin mRNA and protein in a dose dependent manner. In addition, proliferation, migration and invasion of T24 cells were significantly inhibited, whereas the apoptosis was greatly increased. All these effects were associated with the activation of caspase-3, decreased ratio of Bcl-2/Bax, and reduced expression of cyclin D1, VEGF-C, MMP-2 and MMP-9 [138]. B-cell translocation gene 2 (*BTG2*) is downregulated in human BlCa samples and this appear to be due the boundary of DNMT1 to *BTG2* locus suppressing gene expression via down-regulation of its transcription factor, Sp1. 5-aza-2'-deoxycytidine induced *BTG2* demethylation and re-expression by inhibiting DNMT1

expression. Thus, this increased *BTG2* expression significantly reduced the highly malignant EJ cells tumorigenesis and invasiveness *in vitro*, together with induction of G_2/M arrest [144]. Additionally, 5-aza-2'-deoxycytidine suppressed cellular growth in several BlCa tumor cell lines. In fact, this drug induced demethylation and upregulation of *CDKN2A* gene which was associated with G_1 cell cycle arrest. Importantly, exposure of T24 cells to 5-aza-2'-deoxycytidine prior to injection into nu/nu mice decreased the rate of tumor growth and lead to reactivation of *CDKN2A* [145]. The combination of 5-aza-2'-deoxycytidine with cisplatin inhibited proliferation through inducing G_2/M cell cycle arrest and apoptosis of UCC cell lines. Moreover, 5-aza-2'-deoxycytidine enhanced not only the cisplatin-induced upregulation of caspase activity but also its anti-proliferative effect by increasing the population of cells at sub-G_1 and G_2/M phases [146]. Recently, a study involving several BlCa cell lines reported an association between *HOXA9* promoter methylation status and response to cisplatin-based chemotherapy. BlCa cells resistant to cisplatin chemotherapy with high *HOXA9* promoter methylation levels were sensitized to cisplatin by 5-aza-2'-deoxycytidine [147]. Interestingly, the combination of four drugs, namely gemcitabine, cisplatin, 5-aza-2'-deoxycytidine and TSA, inhibited the canonical Wnt/β-catenin pathway and diminished cell proliferation through repression of DNA methylation. Furthermore, the anti-apoptotic gene *BCL2L1* was significantly downregulated [148]. A comparative study with 5-aza-2'-deoxycytidine and zebularine reported that these two agents equally retarded the cell growth of BlCa cell lines (T24 and RT4), but contrarily to T24 cell line, whose exposure to zebularine resulted in the same proportion of Doubling-Time (DT) prolongation as 5-aza-2'-deoxycytidine (79.8 *vs* 79.6%), RT4 cell line DT was higher when treated with 5-aza-2'-deoxycytidine (66.4 vs 17.5%). Moreover, these two compounds diminished the methylation index and concomitantly re-expressed *APAF-1* gene [149]. Exposure of BlCa cell lines to zebularine resulted in an effective demethylation of the *CDKN2A* gene and prevented gene resilencing over extended time periods. Furthermore, zebularine induced a complete depletion of DNMT1, but not DNMT3A and 3B proteins in T24 cells. Interestingly, sequential exposure of these cells to an initial dose of 5-aza-2'-deoxycytidine (1 μM) followed by a low dose of zebularine (50 μM) prevent *CDKN2A* remethylation and resilencing [150]. Concerning SGI-110, it was reported as an *in vivo* effective demethylating drug that reduced tumor growth in human BlCa xenografted mice and induced *CDKN2A* expression by decreasing DNA methylation levels [71]. Currently, there are no available data regarding to clinical trials with demethylating drugs in BlCa (Table 3).

Table 3 – Clinical Trials of DNMT inhibitors in Bladder Cancer.

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
5-Aza-2'-deoxycytidine	I (NCT00030615)	Completed	Advanced BlCa patients (n=24) received daily escalating doses of 5-aza-2'-deoxycytidine for 4 weeks. Courses repeated every 6 weeks in the absence of disease progression or unacceptable toxicity.	Data not available	[151]
CC-486 with Carboplatin or Paclitaxel protein bound particles (ABI-007)	I (NCT01478685)	Completed	CC-486 (oral azacytidine) was administered orally at doses between 100-300 mg daily for either 14 or 21 days. Carboplatin was given by intravenous (IV) infusion once every 21 days. ABI-007 was administered by intravenous (IV) infusion on two of every three weeks at a dosage of 100 mg/m^2.	Data not available	[152]
FdCyd and THU	II (NCT00978250)	Recruiting	Both drugs will be administrated over 28-day cycles through a vein for about 3 hours each day on days 1, 5 and 8, 12 of each cycle. Patients may continue to receive FdCyd and THU if their cancers does not grow, if they do not have too many side effects, and if they are willing to do so.	Data not available	[153]
RX-3117	I/II (NCT02030067)	Recruiting	Phase I study intend to determine the maximum tolerated dose of RX-3117. The enrolled subjects (n=72) will be treated in a dose expansion followed by a 2-stage Phase 2 study for over 8 cycles of therapy. Each cycle will last 4 weeks. RX-3117 will be administered 3 times each week for 3 weeks follow by 1-week rest. All subjects will be followed for at least 30 days after the last dose.	Data not available	[154]

2. Kidney Cancer

Renal Cell Carcinoma (RCC) is one of the 10 most common malignancies in the developed world, with incidence rates steadily increasing. Incidence predominates in men with a male to female ratio of 1.5:1.0 [2]. RCC is the most common type of kidney cancer (~85%) and present an heterogeneous histology, genetics and clinical behavior [155]. Benign tumors comprise papillary adenoma, oncocytoma, metanephric adenoma and adenofibroma [155]. Malignant tumors are classified in three most common subtypes namely, clear cell renal cell carcinoma (ccRCC) (65-70%), the most aggressive phenotype, papillary renal cell carcinoma types 1 and 2 (pRCC) (15-20%), and chromophobe renal cell carcinoma (chRCC) (5-10%), the less aggressive [156]. At early disease stages, RCC is clinically silent [157]. However, owing to the improved sensibility of imaging techniques, the detection of incidentally small and low-stage RCCs has increased. Nevertheless, locally advanced disease and distant metastasis are still diagnosed in a notable proportion of

patients [158, 159]. Surgery is the standard treatment for RCC patients [158]. Advanced RCC patients are highly resistant to conventional chemotherapy partly due to the overexpression of the P-glycoprotein (*P-gp*) found in 76% of the tumors [160, 161]. Therefore, targeted therapy with tyrosine kinase inhibitors (TKIs) is the first-line therapy for advanced RCC [158]. Unfortunately, this therapeutic approach is not curative and eventually all patients will become resistant to TKIs being the prognosis of metastatic RCC extremely poor, with 5-year survival rate of only 5-10% [162, 163]. Several TSGs have been reported to be inactivated by promoter hypermethylation in RCC, namely, genes encoding for *APC*, *CDH1*, *RARβ2*, *RASSF1A*, *TIMP3* and *von Hippel-Lindau* (*VHL*), [164-168]. It has been reported that in about 20-30% of sporadic ccRCC, *VHL* function is lost due to hypermethylation of a CpG island in the promoter region of the gene [169, 170]. *RASSF1A* silencing by promoter methylation was proposed as a marker not only for early detection, but also for surveillance or disease monitoring [171]. This gene appears to be methylated in about 59% of ccRCC and 75% of pRCC [172]. The *ubiquitin carboxyl-terminal esterase 1* (*UCHL1*) gene, a TSG involved in regulation of cellular differentiation, is silenced by promoter hypermethylation in RCC patients and is correlated with poor prognosis [173]. The elevated methylation frequency of Wnt antagonists family (*SFRPs*, *DKKs* and *WIF1*) in serum DNA from RCC patients was associated with a higher tumor grade, suggesting that these genes could be putative progression markers [174]. Recently, the pro-apoptotic gene *ASC/TMS1* was found downregulated by promoter hypermethylation in six RCC cell lines and in 41,1% of RCC tumors compared to normal controls. Importantly, hypermethylation of this gene was correlated with higher tumor nuclear grade. Besides that, in other cancer models, knockdown of *ASC/TMS1* reduced cancer cell sensitivity to chemotherapeutical drugs. These data suggest that this gene could be a relevant diagnostic and therapeutic biomarker [175, 176]. Several other genes were reported as frequently hypermethylated in RCC samples compared to normal tissue, namely *APAF-1*, *BTG3*, *Gamma-catenin*, *GATA-3*, *HOX-B-13*, and *KILLIN* [177].

2.1. Pre-clinical Studies

5-aza-2'-deoxycytidine suppressed the canonical Wnt/β-catenin pathway and induced apoptosis of Caki-2 cell line through demethylation and re-expression of *SFRP2* and downregulation of p-GSK3β protein [178]. This DNMTi was also able to demethylate and re-express *ABCG2* and *ASC/TMS1* genes in RCC cell lines [175, 179]. Recently, it was demonstrated that 5-aza-2'-deoxycytidine was able to decrease the proliferation of several RCC cell lines (ACHN, Caki-1, Caki-2 and A-498) mainly by inducing cell cycle arrest at G_2/M in a dose-dependent manner. This effect might be related to the suppression of p38-

NF-kB pathway phosphorylation by this demethylating agent [180]. Exposure of RCC1.18 primary cell lines to 1 µmol/L of this demethylating inhibitor during 96 hours was capable to induce *RARβ2* expression [181]. Furthermore, 5-aza-2'-deoxycytidine re-expressed *VHL* both in RCC cell lines and in xenograft murine tumors and significantly reduced tumor size of ccRCC xenograft mice [182]. A recent report, that evaluated the methylation profile of drug target genes in RCC, demonstrated that 1 µM of 5-aza-2'-deoxycytidine decreased 5-methylcytosine content in genomic DNA of Caki-2 cells leading to organic cation transporter 2 (encoded by *SLC22A2* gene) re-expression. Moreover, exposure to this compound resulted in demethylation and upregulation of 33 out of the 55 tested SLC drug transporters. As these transporters are associated with sensitivity to platinum chemotherapy, the combination of 5-Aza-2′-deoxycytidine with cisplatin was investigated. Remarkably, this drug combination was more effective in apoptosis induction than either drug alone [183]. Treatment of murine renal cell carcinoma (Renca) cells with 5-aza-2'-deoxycytidine resulted in re-expression of TβR-II at both mRNA and protein levels. This allowed the restoration of Renca cells sensitivity to TGF-β by an increase in phosphorylation of SMAD2 which is a consequence of TGF-β receptors activation [184]. The exposure of RCC cell lines to 5-aza-2'-deoxycytidine induced reactivation of the pro-apoptotic *RASSF1A* gene silenced by promoter hypermethylation [185]. Recently, it was demonstrated that 5-aza-2'-deoxycytidine significantly induced global genomic demethylation of RCC cells with restoration of *APAF-1* expression at both mRNA and protein levels. Moreover, 5-aza-2'-deoxycytidine promoted the apoptotic effect of cisplatin in ACHN cells [186]. This compound was also able to induce mRNA and protein expression of *UCHL1* gene in several RCC cell lines [187]. In fact, exposure of RCC cell lines to 5-aza-2'-deoxycytidine decreased the methylation levels with concomitant re-expression of *DACH1*, *DKK1*, *DLK1*, *GATA3*, *GREM1*, *KILLIN*, *KRT19*, *PCDH17*, *Rap1GAP*, *SFRP2*, *SFRP5*, *TCF21*, *TU3A* and *WIF-1* [188-201]. Interestingly, exposure of RCC cell lines to 5-aza-2'-deoxycytidine and zebularine effectively inhibit tumor cell growth and re-expressed *APAF-1* and *DAPK-1* mRNA transcripts. Remarkably, zebularine was more effective in achieving a DT prolongation than 5-aza-2'-deoxycytidine in a RCC p53 mutated cell line [149]. The natural compound Genistein used in combination with 5-aza-2'-deoxycytidine significantly decreased *BTG3* (TSG) promoter methylation reactivating its expression. Besides that, this combination decreased DNMTs and methyl-CpG-binding domain 2 (MBD2) activity and increased histone acetylation [119]. Exposure of human papillary ACHN cell line to 5-aza-2'-deoxycytidine synergistically augmented the anti-proliferative effects of IFN-α e IFN-β. In addition, this compound not only increased more than 10 times the expression of IFN response genes but also induced demethylation of the apoptosis-associated IFN response gene *XAF1* promoter. Interestingly, MG98 also defeated the resistance to IFN-induced

apoptosis. Either 5-aza-2'-deoxycytidine or MG98 depleted DNMT1 enzyme leading to reactivation of cancer-testis antigens MAGE and RAGE in ACHN cells which might be relevant for immune modulation [202]. In a pre-clinical assay, exposure of RCC cell lines (Caki-1, 786-O and A498) to 5-aza-2'-deoxycytidine enhanced the cytotoxicity of vinblastine (VBL), one classical cytotoxic drug against RCC. 5-aza-2'-deoxycytidine led to demethylation and re-expression of *connexin 32* (*Cx32*) which directly contributed to downregulation of P-gp by activation of c-Jun NH_2-terminal kinase (JNK). These results suggest that re-expression of *Cx32* increases RCC cells response to VBL [203]. Notably, the co-treatment of Caki-1 xenograft mice with 5-aza-2'-deoxycytidine and VBL led not only to suppression of tumor volume and weight but also reduced the expression of *P-gp*, *Bcl-2* and *cyclin B1*. This combined effect appear to be mediated by the accumulation of intracellular VBL and by apoptosis and cell cycle arrest induction [204]. Likewise, combination of 5-aza-2'-deoxycytidine with paclitaxel (PTX) synergistically inhibited RCC cell growth. Both drugs suppressed RCC cell proliferation by inducing G_2/M cell cycle arrest and PTX also enhanced tumor cell apoptosis in a dose-dependent manner. Thus, this synergistic growth suppression of RCC cells suggests that this DNMTi could remarkably increase the susceptibility of RCC to PTX [205]. A green tea extract composed by flavan-3-ols and EGCG, strongly inhibited the growth of A-498 and 769-P cell lines in a concentration-dependent manner, showing to be an effective anti-cancer agent for RCC [206]. Interestingly, oral administration of RX-3117 reduced tumor cell growth of Caki-1 xenograft mice more efficiently than gemcitabine [207].

2.2. Clinical Trials

Monotherapy with 5-aza-2'-deoxycytidine was scheduled in a phase I study at doses from 2.5 to 20 mg/m^2 on days 1-5. Even though 5-aza-2'-deoxycytidine decreased DNA methylation both in tumor and in peripheral blood mononuclear cells (PBMCs), there was no correlation between these two parameters. However, this agent induced apoptosis and increased CTR1 (copper transporter) expression through methylation-independent mechanisms [208]. A phase I clinical trial that combined 5-aza-2'-deoxycytidine, daily subcutaneously injected during 5 days at weeks 1 and 2 of a 12-week cycle, with high-dose interleukin-2 (600,000 IU/Kg), administered intravenously 14 times every 8 hours, resulted in stable disease in 3 of the 5 RCC patients enrolled. Grade 4 neutropenia was observed [209]. Another phase I trial, that enrolled 55 patients with advanced disease, from which two were RCC patients, combined 5-azacytidine subcutaneously administered with orally valproic acid. One RCC patient presented stable disease for 6 months with a significant increase in histone acetylation. Grade 3 and 4 toxicities were reported [58]. In contrast, a

phase I clinical trial with 5-azacytidine and sodium phenylbutyrate that evaluated three refractory kidney cancer patients did not reach clinical benefit or stable disease. However, one patient presented a significant decrease of DNMTs activity and the remaining two patients achieved an increase in histone acetylation [210]. Finally, in a phase I/II trial (NCT00003890) that enrolled 20 metastatic renal carcinoma patients, was assessed the anti-tumor activity of MG98. This compound was intravenously administered at a dose of 360 mg/m^2 twice weekly for three consecutive weeks out of four. The most common symptomatic toxicities were rigors, fatigue, fever, and nausea. Unfortunately, the results did not show a conclusive pattern of decreased DNMT1 activity in PBMCs post MG98 treatment [94]. However, a recent clinical study with advanced RCC patients demonstrated a great tolerance for combination of MG98 and IFN-α-2β in an intermittent schedule rather than continuous. Indeed, one patient showed a partial response, one had symptomatic improvements and eight patients achieved stable disease after combined intermittent treatment [211].

Table 4 – Clinical Trials of DNMT inhibitors in Kidney Cancer.

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
5-Aza-2'-deoxycytidine	I	Completed	Refractory RCC patients (n=3) received intravenously 5-aza-2'-deoxycytidine from 2.5 to 10 mg/m^2 on days 1-5, and from 8 to 12, or 15 to 20 mg/m^2 on days 1-5. Each cycle lasted four weeks.	Relative reduction of tumor size; Increased tumor apoptosis; Reduction of DNA methylation in both tumor and PBMC but without correlation between them; Increased expression of CTR1.	[208]
5-Aza-2'-deoxycytidine and Interleukin-2	I	Completed	Renal cancer patients (n=5) received subcutaneous 5-aza-2'-deoxycytidine daily x 5 days on weeks 1 and 2 of a 12-week cycle. High-dose IL-2, consisting of two cycles of IL-2 600,000 IU/kg intravenously every 8 hours' x 14 doses separated by a 2-week break, was administered starting on week 3. Decitabine was escalated from 0.1 to 0.25 mg/kg.	Global DNA demethylation. Up-regulation of immunomodulatory genes. Three of five evaluable patients presented stable disease. Grade 4 neutropenia was observed.	[209]
5-Aza-2'-deoxycytidine and Interferon alfa-2β	II (NCT00561912)	Terminated	Patients with advanced RCC (n=2) received 5-aza-2'-deoxycytidine 15 mg/m^2 intravenously daily over 1h for 5 days plus Interferon Alfa-2b 0.5 million units subcutaneously twice daily continuously, on day 1 cycle 3. Each cycle was 28 days long.	Terminated due to low accrual.	[212]

Table 4 - Clinical Trials of DNMT inhibitors in Kidney Cancer (cont.).

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
5-Azacytidine and Valproic Acid	I	Terminated	5-Azacytidine was administered subcutaneously daily for 10 days in patients with advanced cancers (n=55), two of them with RCC. Cycles were 28 days long and 5-azacytidine was administered at 20 mg/m^2.	One of the two treated RCC patients achieved a 6 months' stable disease. Data not available for decrease in methylation but it was observed histone acetylation. Grade 3 and 4 toxicities were observed.	[58]
5-Azacytidine and Sodium phenylbutyrate	I	Completed	5-azacytidine subcutaneously and sodium phenylbutyrate (continuous intravenous infusion) were administered in refractory kidney cancer patients (n=3) regarding three different dose regimens during 48 cycles in the 27 advanced solid tumors patients.	No clinical benefits were seen in the three tested regimens for RCC patients. Reduced tumor tissue methyltransferase activity in one patient and increased histone acetylation in two patients. Grade 3 and 4 neutropenia were observed.	[210]
5-Azacytidine and Interferon-α-2β	I (NCT00217542)	Completed	Patients (n=42) received azacytidine subcutaneously once daily on days 1 to 4 and 15 to 17 and recombinant interferon alfa-2b subcutaneously on specific days during course 1. Beginning in course 2 and for all subsequent courses, patients received azacytidine subcutaneously once daily on days 1-3 and 15-17 and interferon alfa-2b subcutaneously on specific days. Treatment repeated every 28 days for up to 12 total courses in the absence of disease progression or unacceptable toxicity.	Data not available	[213]
5-Azacytidine and Bevacizumab	I/II (NCT00934440)	Unkown	All patients (n=23) in phase I and II received bevacizumab at the standard dose of 10 mg/kg every 2 weeks and the doses were administered at specific times. 5-azacytidine was administered in different dose levels for each study phase (I/II).	Data not available	[214]
MG98	I/II (NCT00003890)	Completed	Untreated patients (n=17) with measurable metastatic renal carcinoma received MG98 360 mg/m^2 intravenous over 2 hours twice weekly for 3 weeks. Courses were repeated every 4 weeks.	No conclusive pattern of decreased DNMT1 activity was detected after MG98 treatment. Toxicities was experienced including fatigue, fever, and nausea.	[94]

Table 4 – Clinical Trials of DNMT inhibitors in Kidney Cancer (cont.).

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
MG98 and Interferon-α-2β	Study phase not provided	Completed	Patients with advanced RCC (n=19) were divided in 2 groups: 10 received a continuous regimen and 9 received an intermittent regimen twice weekly. In the first group, patients received MG98 in two 7-day continuous infusions every treatment week followed by a week of rest in each cycle. In the intermittent group, patients were treated with a 2h intravenous infusion of MG98 twice per week for three weeks with the last cycle week of rest. Additionally, both groups received interferon-α-2β subcutaneously three days per week with an initial dose of 12 MIU/day or 9MIU/day.	Interferon-α-2β 9 MIU plus MG98 125 mg/m^2 for a continuous schedule and interferon-α-2β 9 MIU plus MG98 200 mg/m^2 for an intermittent schedule were considered the maximum tolerable doses. The first showed 2 out of 7 dose-limiting toxicities, including fever and thrombocytopenia. One partial response and eight stable disease were achieved.	[211]

3. Prostate Cancer

PCa is the second most commonly diagnosed cancer and the fifth leading cause of cancer related death in men worldwide [215]. When confined to the organ, which represents early stage disease, PCa is curable by radical prostatectomy and/or radiation therapy [216]. The use of serum PSA for PCa detection lacks sensitivity and specificity, leading to a relatively high frequency of unnecessary prostate biopsies that is an expensive and invasive procedure [217]. Consequently, a substantial proportion of patients with indolent tumors is overdiagnosed and overtreated, from which several might experience side-effects, such as urinary incontinence and erectile dysfunction, without significant clinical benefit [218]. For advanced disease, the treatment of choice is Androgen Deprivation Therapy (ADT) which aims to reduce the levels of male hormones. Even though the majority of the patients initially respond to this therapy, after 18-24 months, approximately 30% of the cases progress to the lethal stage of this disease, designated as Castration-Resistant PCa (CRPC) [219]. Despite the possibility of treating these CRPC patients with secondary hormonal therapeutic agents, such as abiraterone acetate and enzalutamide, the acquired resistance inevitably occurs again after a few months [220]. For metastatic CRPC (mCRPC), chemotherapy with docetaxel represents the standard therapy, however, the median time to progression remains 6 to 8 months and OS remains less than 2 years [221, 222]. Mitoxantrone alone or in combination with prednisone, the radiopharmaceutical radium-223 and the autologous cellular immunotherapy sipuleucel T can also be used for mCRPC with a significant survival benefit [223]. Unfortunately, none of these agents are curative, strengthening the urgent need for investigation of new therapeutic approaches.

Epigenetic changes, specially DNA methylation play an important role on PCa development and progression [224, 225]. In fact, in PCa, promoter hypermethylation is directly involved in silencing of several classic TSGs, such as Androgen Receptor (*AR*), *APC*, Glutathione S-Transferase Pi 1 (*GSTP1*), *RARβ2*, *RASSF1A*, estrogen genes, cell adhesion genes (*CD44* and *CDH1*), cell cycle control genes (*CCND2*, *CDKN1B* and *SFN*) and apoptotic genes (*PYCARD*, *RPRM* and *GLIR1*) [226-233]. Methylation of the *RASSF1A* gene promoter was strongly correlated with increased risk of recurrence of PCa, aggressiveness and tumor progression [234]. Importantly, progression to CRPC has also been linked to *AR* silencing by hypermethylation which was in fact, described in 30% of the CRPC [235, 236]. *GSTP1* aberrant hypermethylation is one of the most frequent alterations in PCa since it has been observed in more than 90% of the tumors and about 75% of pre-invasive prostatic intraepithelial neoplasms [228, 237]. Therefore, it is the most promising epigenetic biomarker for detection of this malignant neoplasm [238]. Overall hypomethylation occurs either in primary PCa tumors or, most extensively, in metastatic disease [239]. Increased tumor stage (Gleason Score ≥ 7) has been associated not only with wide hypomethylation of the genome but also with promoter hypermethylation of several individual loci, including *ABCB1*, *APC*, *GSTP1*, *PTGS2*, *PYCARD*, *RARβ2* and *RASSFA1* [228, 240].

3.1. Pre-clinical Studies

Numerous pre-clinical assays were performed in order to assess the utility of DNMTi for PCa treatment. Gravina et al, showed that when PCa cell lines were exposed to chronic administration (20 days) of 5-azacytidine, a significant decrease in tumor cell proliferation with significant increase in AR and PSA protein levels were achieved. Furthermore, following 5-azacytidine chronic treatment, PCa cell lines increased their sensitivity to the apoptotic effects of bicalutamide, an anti-androgen used in clinical [241]. Besides that, restoration of *AR* also sensitized xenograft models of CRPC to this anti-androgen [242]. One of the major obstacles for CRPC treatment is the development of chemoresistant tumors. Taking this into account, a pre-clinical assay evaluated the role of 5-azacytidine in association with docetaxel and cisplatin in aggressive PCa models. The results demonstrated a significant reduction of tumor cell proliferation, induction of apoptosis and sensitization of xenografts to docetaxel and cisplatin treatments [243]. PCa cells (DU145) exposure to 5-aza-2'-deoxycitidine increased the level of *plasmonigen activator inhibitor-1* (*PAI-1*) transcript in DU145 and restored the pro-inflammatory cytokines effects [244]. Moreover, 5-Aza-2'-deoxycitidine led to a significant suppression of cell proliferation, induction of cell death, and demethylation of *GSTP1* promoter, with associated protein re-

expression [245]. Recently, exposure of PCa cell lines to 5-aza-2'-deoxycytidine resulted in the re-expression of *KAI1,* a metastasis suppressor gene, found hypermethylated in PCa [246]. 5-aza-2'-deoxycitidine was able to decrease PCa cell stemness and induce a more differentiated status. *In vitro* and *in vivo* assays demonstrated that *AR* re-expression associated with the reversion of its methylation pattern led to suppression of PCa stem cells self-renewal, with a consequent decrease in tumorigenesis [247]. Likewise, in DU145 cell line, co-treatment with 5-aza-2'-deoxycytidine and TSA effectively re-expressed *AR* [248]. Contrarily, restoration of *ASC/TMS1* expression in LNCaP cells was only achieved with 5-aza-2'-deoxycytidine while TSA did not increase gene expression [249]. Fialova *et al.* combined 5-aza-2'-deoxycitidine with a histone deacetylase inhibitor (sodium butyrate, NaB) and found a significant decrease of cell viability, as well as site-specific demethylation at the *AR* promoter region followed by gene re-expression and increased acetylation of histones H3 and H4 [250]. When combined with the chemotherapeutic agent PTX, 5-aza-2'-deoxycitidine enhanced the apoptotic effects and the arrest at G_2/M cell cycle phase of this drug. This treatment strategy achieved synergistic growth suppression in all PCa cell lines, and could be an alternative for clinical management of this disease [251]. Likewise, the combination of cisplatin with 5-aza-2'-deoxycitidine resulted in a great synergy in triggering apoptotic death of DU145 cells [252]. Recently, a novel 5-aza-2'-deoxycitidine formulation based on the use of engineered erythrocyte (Erythro-Magneto-Hemmagglutinin Virosomes) drug delivery system which aims to reduce the incidence of toxicity on healthy tissues was described. This novel magnetic EMHV DDS improved the stability of the carried drug and exhibited high efficiency in confining its delivery at the site of action, *in vivo*. Moreover, it induced a significant tumor mass reduction in PCa xenografts models at a concentration which is 700-fold lower than the normal therapeutic dose. This innovative approach might be a good option for solid tumors treatment [253]. Concerning, zebularine not only was required a 100-fold higher dose than 5-aza-2'-deoxycitidine to inhibit cell proliferation but it was also less potent inducing cell death, and failed to restore GSTP1 protein expression [245]. On the other hand, zebularine was able to restore sensitivity of PCa cells to the DNA minor groove binder brostallicin, which correlated with re-expression of *GST-pi* and *GST-mu*, *in vitro* and *in vivo* [254]. Procainamide led to reversion of *GSTP1* hypermethylation with concomitant restoration of gene expression not only in LNCaP cell line but also in xenograft tumors of athymic nude mice [83]. Regarding RG108, it induced dose and time dependent growth inhibition and apoptosis of PCa cell lines. This compound repressed DNMT activity and expression and reduced global DNA methylation of androgen-responsive PCa cell lines. Furthermore, chronic treatment (14 days) with RG108 significantly decreased promoter methylation levels of *GSTP1*, *APC* and *RARβ2*, although mRNA re-expression was only succeeded for *GSTP1* and *APC* [255]. Exposure of PCa cells

to hydralazine led to cell growth and invasion inhibition and apoptosis induction. Moreover, this compound also induced cell cycle arrest and DNA damage. Additionally, the exposure of PCa cells to hydralazine decreased *DNMT1*, *DNMT3A* and *DNMT3B* mRNA levels and also DNMT1 protein levels, which might be linked to the significant decrease in *GSTP1*, *BCL2* and *CCND2* promoter methylation levels and concomitant transcript re-expression. Importantly, hydralazine restored AR expression, with upregulation of its target p21 in DU145 cell line. The attenuated malignant phenotype of PCa cells was also associated with EGFR signaling pathway disruption [256]. Disulfiram demonstrated a dose-dependent inhibition of DNMT1 activity, promoted PCa cells apoptosis and cell cycle arrest, and reduced the global 5mC content. This compound also led to re-expression of *APC* and *RARβ2* genes [99]. Besides that, it was able to inhibit PCa cell proliferation by re-expressing *estrogen receptor-β* (*ER-β*) mainly through inhibition of DNMT activity [257]. Likewise, Gemcitabine functionally inhibited DNA methyltransferase activity in both nuclear extract and recombinant protein, reactivating several epigenetically silenced genes, including *GSTP1, IGFBP3* and *RASSF1A*. This compound also destabilized DNMT1 protein in PCa cell lines (LNCaP, 22Rv1 and DU145). Moreover, it demonstrated a similar activity when compared to 5-aza-2'-deoxycitidine and at significantly lower concentrations relatively to those achieved in the treatment of patients with solid tumors [258]. SGI-1027 also depleted DNMT1 expression in LNCaP cells [100]. Furthermore, this compound and two analogues (paralmeta and metalmeta) were able to inhibit cell proliferation and viability of PC-3 cell line [259]. Recently, the DNMT inhibitory effect of 1120 small organic compounds was evaluated and the 12 most potent hits were selected for cytotoxicity tests in DU145 cell line. Notably, most of the compounds revealed inhibitory activity at low micromolar concentrations and with low cytotoxicity [260]. Mahanine, a plant-derived carbazole alkaloid, was able to restore *RASSF1A* expression in LNCaP and PC-3 cell lines. This might be related to downregulation of DNMT1 and DNMT3B enzymes activity via inactivation of AKT pathway [261, 262]. One study comparing non-nucleoside analogue inhibitors, including hydralazine and procainamide, with the nucleoside analogue 5-aza-2'-deoxycitidine demonstrated that 5-aza-2'-deoxycitidine was considerably more effective in demethylating and reactivating TSGs in PCa cell lines than the other non-nucleoside inhibitors [102]. Micromolar concentrations of natural compounds were able to inhibit DNMT, MBD and HDAC activity, reactivate methylated-silenced TSGs, induce histone acetylation, and alter nucleosome positioning in PCa cell lines [115, 263-265]. Regarding genistein, it was shown that this compound was able to reverse promoter methylation of *GSTP1, RARβ2* and *RASSF1A* with concomitant gene reactivation and protein expression in PCa cell lines (LNCaP and PC-3) [118, 266]. Likewise, this compound induced downregulation of DNMTs in PCa cells, reducing *ER-β* promoter methylation with concomitant increase in its

expression [267]. A recent study reported that the combination of genistein with daidzein, two soy isoflavones, resulted in a synergistic effect on cell proliferation inhibition and apoptosis induction of PCa cells [268]. PEITC was able to reduce DNMT protein levels and reactivated *RASSF1A* gene expression in LNCaP cells. Moreover, 5 μM of this compound promoted early apoptosis and G2/M cell cycle arrest [269]. Interestingly, the combination of PEITC with curcumin, more effectively induced cellular growth arrest and apoptosis, through inhibition of protein kinase B and NFkβ pathways, in PCa cell lines and PC-3 xenografts than either compound alone [270]. Exposure of TRAMP C1 cells to curcumin resulted in reversion of methylation status of *Nrf2* promoter, a master regulator of the cellular antioxidant defense system, with concomitant gene re-expression [271]. In addition, 5 μM of curcumin reversed CpG methylation of *Neurog1* promoter region, a cancer methylation marker usually highly methylated in PCa and whose expression is also perturbed in LNCaP cells [272].

3.2. Clinical Trials

A phase I/II clinical trial (NCT00503984) evaluated the combination of 5-azacytidine, docetaxel, and prednisone in patients with mCRPC whose disease had progressed during or after a docetaxel therapy. In phase I, 5-azacytidine and docetaxel were alternately escalated within six combinations of treatment in a standard 3+3 design, being level IV dose combination achieved (5-azacytidine 150 mg/m^2 daily for 5 days followed by docetaxel 75 mg/m^2 on day 6, every 21 days, and continuous prednisone 5 mg twice daily with growth factor support) as the recommended dose. After a safety monitoring review, the phase II dose was reduced and patients received 75 mg/m^2 daily for 5 days followed by docetaxel 75 mg/m^2 on day 6, every 21 days, and continuous prednisone 5 mg twice daily with growth factor support. The overall results demonstrated a reduction in *GADD45* methylation at day 5. This phase I/II trial showed that the combination of 5-azacytidine, docetaxel and prednisone with growth factor support is an option for mCRPC patients which provided a median progression free survival of 4.9 months for all patients and a median OS of 19.5 months for 4 of the 22 patients (18 deaths) [273]. An open label phase II study (NCT00384839) that enrolled 36 patients evaluated 5-azacytidine effects in men with progressive metastatic or non-metastatic CRPC on combined androgen blockade (CAB) with PSA-doubling time (DT) < 3 months. The primary endpoint of PSA-DT ≥ 3 months during any cycle was achieved in 19 patients. Eleven patients experienced at least one PSA-DT ≥ 6 months and nine experienced at least one PSA-DT ≥ 9 months. Twenty-four patients (70.6%) demonstrated some slowing of PSA-DT during any cycle of treatment compared with baseline, and the median overall PSA-DT during the entire duration of

therapy was 2.8 months compared with the baseline of 1.5 months. The median clinical progression-free survival for all 36 patients was 12.4 weeks. Grade 3 toxicities included fatigue and neutropenia with 4 patients discontinuing the treatment due to toxicity. After extending treatment duration, it was observed a trend in decreasing plasma DNA *LINE-1* methylation which significantly correlated with prolongation of PSA-DT [274]. Another phase I trial that combined 5-azacytidine and valproic acid enrolled 55 patients with advanced malignancies, two of each with PCa. A significant decrease in global DNA methylation and induction of histone acetylation with stable disease lasting six months in PCa patients were achieved. Neutropenic fever and thrombocytopenia were identified as dose-limiting toxicities [58]. Thibault *et al.*, conducted a phase II study with 5-aza-2'-deoxycitidine at 75 mg/m^2 in 14 men with progressive, metastatic PCa recurrent after total androgen blockade and flutamide withdrawal. Two of the 12 patients evaluable had stable disease with a time to progression of more than 10 weeks. These results disclosed that 5-aza-2'-deoxycitidine is a well-tolerated regime with modest clinical activity against CRPC [275]. Recently, a pilot clinical trial (NCT01917890) assessed the efficacy of curcumin supplementation in 40 PCa patients treated with external beam radiotherapy. Patients were divided in two groups with a random selection of those who receive 3g/day curcumin orally (n=20) and a placebo group (n=20). Interestingly, patients who received the curcumin regime presented reduced urinary symptoms, suggesting that this natural compound could offer radioprotective effects [276]. In a dose escalation phase I trial (NCT01118741) with disulfiram in 19 men non-metastatic recurrent PCa after local therapy, five patients of two different cohorts achieved a transient global demethylation response. However, disulfiram was poorly tolerated in six patients, who experienced grade 3 toxicities [277].

Table 5- Clinical Trials of DNMT inhibitors in Prostate Cancer.

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
5-Azacytidine, docetaxel and prednisone	I/II (NCT00503984)	Terminated	mCRPC cancer patients who progressed during or within 6 months of docetaxel chemotherapy, were eligible (n=22). In phase I, patients received the highest dose of azacytidine 150 mg/m^2 daily for 5 days + Docetaxel 75 mg/m^2 on day 6 every 21 days. In phase II, it was used the combination of azacytidine 75 mg/m^2 daily for 5 days followed by docetaxel 75 mg/m^2 on day 6 along with growth factor support and fixed prednisone 5 mg since day 1 to 21.	In phase I, grade 4 neutropenia was frequent. In phase II, 10 of 19 evaluable patients showed PSA response and 3 of 10 achieved an objective response. Significant demethylation of GADD45A was observed.	[273]

Table 5 - Clinical Trials of DNMT inhibitors in Prostate Cancer (cont.).

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
5-Azacytidine and Combined Androgen Blockade (CAB)	II (NCT00384839)	Completed	Chemonaïve patients with CRPC on CAB and PSA-doubling time (DT) < 3 months were eligible (n=36). CBA was continued and 5-azacytidine 75 mg/m2 was administered for 5 consecutive days of each 28-day cycle up to 12 cycles or until clinical progression or intolerable toxicities.	19 of 34 evaluable patients attained a PSA-DT ≥3 months. Overall median PSA-DT was significantly prolonged with 2.8 months. The obtained median clinical progression-free survival was 12.4 weeks. Grade 3 toxicities of fatigue and neutropenia were observed.	[274]
5-Azacytidine and Valproic Acid	I	Completed	5-Azacytidine was administered subcutaneously daily for 10 days in patients with advanced cancers (n=55), in which two of them with PCa. Cycles were 28 days ong and 5-azacytidine was administered at 75 mg/m^2.	One of the two treated PCa patients achieved a 6 months' stable disease. Data not available for decrease in methylation, neither for histone acetylation.	[58]
5-Azacytidine and Phenylbutyrate	II (NCT00006019)	Completed	Patients (n=20) received 5-azacytidine on days 1 to 7 and phenylbutyrate IV over 1-2 hours on days 8 to 12. Additional courses in these patients were repeated every 21 to 28 days in the absence of disease progression or unacceptable toxicity.	Data not available.	[278]
5-Aza-2'-deoxycytidine	II	Completed	Patients with metastatic recurrent PCa after total androgen blockade and flutamide withdrawal (n=14) received an infusion of 3 doses of 5-aza-2'-deoxycytidine 75 mg/m^2. Cycles of therapy were repeated every 5 to 8 weeks to allow for resolution of toxicity.	Two of 12 patients evaluable for response had stable disease with a time to progression of > 10 weeks. 5-Aza-2'-deoxycytidine was well tolerated with modest clinical activity against CRPC.	[275]
Curcumin and Radiotherapy	Study phase not provided (NCT01917890)	Completed	PCa patients (n=40) treated with external beam radiotherapy were separated in two groups 20 of them received 3g/day curcumin orally and 20 received placebo.	Patients treated with curcumin presented reduced urinary symptoms, showing possible radioprotective effects.	[276]
Disulfiram	I (NCT01118741)	Completed	Eligible patients (n=19) were ≥18 years old, previously treated with local therapy and subsequently developed biochemically recurrent disease. Cohort 1 (n=9) and 2 (n=10) received disulfiram treatment 250 mg and 500 mg daily, respectively. The primary endpoint was the proportion of subjects with a demethylation response. Secondary endpoints included rate of PSA progression at 6 months, changes in PSA doubling time and safety/tolerability.	Only five of the evaluable subjects were on trial for ≥ 6 months from cohort 1 and obtained a PSA progression by 6 months. Three of the responders displayed pretreatment instability in their 5-methylcytosine content. Six patients experienced grade 3 toxicities.	[277]

4. Testicular Cancer

Although testicular cancer is relatively rare, it is the most common solid tumor in young Caucasian men among 15-35 years old [215]. Testicular germ cell tumors (TGCTs) represent more than 95% of all testicular cancers and are thought to derive from primordial germ cells or early gonocytes [279, 280]. They are classified in two distinctive major histological subtypes: seminomas (40%) and non-seminomas (60%), which share the same precursor lesion, testicular intraepithelial neoplasia [281]. TGCTs patients are treated with orchiectomy with subsequent radiotherapy and chemotherapy, depending on the histology and clinical stage of the tumor [281]. Even metastatic patients can often be successfully treated with cisplatin-based chemotherapy, presenting a 5-year survival rate greater than 70% [282]. However, 15-20% of the patients are refractory to this treatment and about 15% present a later relapse and develop progressive disease [283-285]. Unfortunately, there are no effective therapies for these patients. Seminomas are largely unmethylated, while nonseminomas have a global methylation status that differ according to their degree of differentiation [286, 287]. Nonseminomas present undifferentiated and pluripotent cells, known as embryonal carcinoma (EC) cells that are proposed to be TGCTs stem cells and the malignant homologous of embryonic stem cells [279, 280, 288]. Several methylated genes have been implicated in TGCTs pathogenesis, including *APC*, *ARF*, *BRCA1*, *CALCA*, *CCNA1*, *HOXA9*, *MGMT*, *hMLH1*, *PRSS21*, *RARβ2*, *RASSF1A*, *SCGB3A* and *TP53* [289-292]. Conversely, it was recently shown that *LINE1* is extremely hypomethylated in both TGCTs subtypes [293]. Moreover, hypomethylation of the 5´-end of *XIST* gene, irrespective of gene expression, was described in plasma DNA of TGCTs patients [294]. A recent report described that an elevated methylation frequency of *CALCA* and *MGMT* was present in nonseminomas and was related with poor clinical outcome in TGCTs patients. In addition, *CALCA* hypermethylation was associated with refractory disease [295].

4.1. Pre-clinical Studies

Exposure of human EC cells to low nanomolar concentrations of 5-aza-2'-deoxycitidine resulted in a significant decrease of cell proliferation and survival. This phenotype was associated with ATM pathway activation, H2AX phosphorylation, p21 increased expression and induction of genes known to be methylated in TGCTs like *MGMT*, *RASSF1A* and *HOXA9.* Notably, not only cisplatin-resistant EC cells retain sensitivity to low concentrations of 5-aza-2'-deoxycytidine but also pretreatment with this agent re-sensitizes these cells to cisplatin-mediated toxicity. Moreover, knockdown of *DNMT3B* in EC cells reduced cell sensitivity to 5-aza-2'-deoxycytidine, supporting the role of *DNMT3B* in 5-aza-2'-deoxycytidine treatment response [296]. 5-aza-2'-deoxycytidine was able to induce

apoptosis of human teratocarcinoma stem cells, but not in differentiated cells derived from human nullipotent EC cells. Intriguingly, expression of DNMT3B was required for induction of apoptosis and differentiation of human teratocarcinoma stem cells by 5-aza-2'-deoxycytidine [297]. Exposure of NT2/D1 cells to low concentrations of 5-aza-2'-deoxycitidine resulted in DNA damage, induction of *p53* and global and gene specific promoter DNA demethylation (*RIN1*, *SOX15*, *GPER* and *TLR4*). Additionally, this treatment also led to downregulation of genes associated with pluripotency, like *NANOG*, *SOX2*, *GDF3* and *Myc* target genes [298]. A recent study with TCam-2 seminoma cell line disclosed that intrinsically high resistance to cisplatin can be decreased with a pretreatment with 5-azacytidine. Furthermore, after demethylation, the stem cell markers *NANOG* and *POU5F1*, as well as *VASA* (the germ cell-specific marker), displayed increased expression [287]. Intriguingly, low concentrations of SGI-110 were able to decrease tumor cell growth not only of cisplatin sensitive EC cells NT2/D1 and cisplatin resistant NT2/D1-R1 but also in a xenograft model of cisplatin resistant TGCT. Importantly, this compound re-sensitized refractory EC cells to cisplatin both *in vitro* and *in vivo*. The expression of *GDF15*, *CDKN1A* and *GADD45A* (p53 target genes), *RASSF1* and *SOX15* was induced after SGI-110 exposure. In xenograft models, SGI-110 increased the expression of immune pathway genes [299].

4.2. Clinical Trials

A phase II clinical trial from 1977, which enrolled 214 patients with solid cancers evaluated the effect of high doses of 5-azacytidine. Of the four evaluable testicular cancer patients, two presented partial responses with 5-azacytidine doses from 225 mg/m^2 to 150 mg/m^2 [300]. Roth and colleagues performed a phase II trial which enrolled 17 patients with refractory germ cell tumors to cisplatin treatment. Patients received 5-azacytidine at a dosage of 150 mg/m^2/day at days 1 to 5 by continuous infusion every three weeks. All patients progressed on 5-azacytidine and grade 3 and 4 toxicities were observed being the most important granulocytopenia and anemia. Therefore, the authors were unable to describe any 5-azacytidine activity on patients with germ cell tumors [301]. A single-arm phase II study (NCT00404508) demonstrated that the combination of hydralazine with magnesium valproate before chemotherapy in refractory solid tumors, including one non-seminomatous testicular cancer, resulted in decreased chemotherapy resistance with stable clinical response. Besides that, it was observed a clinical benefit concerning progression-free survival and OS of 5.6 months and 5.7 months, respectively [302].

Table 6 - Clinical Trials of DNMT inhibitors in Testicular Cancer.

DRUG	PHASE (ID)	STATUS	SCHEDULE	OUTCOME	REF.
5-Azacytidine	II	Completed	Patients with solid tumors (n=214) were enrolled and four of them were testicular cancer patients. 5-azacytidine doses varied from 225 mg/m^2 to 150 mg/m^2.	Two of the four testicular cancer patients presented partial responses.	[300]
	II	Completed	Patients (n=17) received 5-azacytidine at a dosage of 150 mg/m^2/day at days 1 to 5 by continuous infusion every three weeks.	It was not observed any 5-azacytidine activity. Grade 3 and 4 toxicities were reported.	[301]
SGI-110 and cisplatin	I (NCT02429466)	Recruiting	SGI-110 will be given subcutaneously, daily, 30 mg/m^2 on days 1-5 followed by cisplatin 100mg/m^2 on day 8, every 4 weeks. Treatment will be continued for a maximum of 6 cycles or until disease progression or unacceptable toxicity.	Data not available.	[303]
Hydralazine and Magnesium Valproate (before ChT)	II (NCT00404508)	Completed	Patients with refractory solid tumors (n=15) received hydralazine at 182 mg for rapid, or 83 mg for slow, acetylators, and magnesium valproate at 40 mg/kg, beginning a week before chemotherapy.	A decrease in chemotherapy resistance was observed. A clinical benefit was reported, namely stable clinical response a 5.6 months progression-free survival and an OS of 5.7 months.	[302]

ChT: Chemotherapy

This section is submitted to a scientific journal!

II. AIMS AND PRELIMINARY RESULTS

Preliminary Data

Urological tumors are a heterogeneous class of malignancies associated with significant morbidity and mortality worldwide. Although early stage disease can be effectively treated with surgery and/or chemo and radiotherapy, the available therapeutic options for advanced and metastatic disease are sparse and mainly not curative. Due to the high incidence and clinical aggressiveness of these malignant neoplasms, the development and investigation of new therapeutic regimens focused on disease biology is of major importance. Aberrant epigenetic alterations, especially DNA methylation, are a hallmark of these malignant tumors. However, these alterations are reversible and several efforts have been performed to design and test novel epigenetic compounds that would potentially reprogram tumor cell phenotype toward a normal state. It is widely accepted that natural compounds can modulate multiple cellular pathways, including epigenetic machinery, and therefore be used against several human diseases, as cancer. Importantly, these compounds are known not only to be safer than synthetic agents, but also present a wide availability, low toxicity, and good tolerability [104]. Despite their potential clinical utility, the field of natural compounds and their derivatives for urological cancer treatment needs to be further explored.

Thus, the main goal of this dissertation was to evaluate the usefulness of four newly synthetized compounds derived from flavanones, as anti-cancer agents in urological tumors cell lines representative of the three most incident types (Bladder, Kidney and Prostate) through DNMT inhibition.

Specifically, the preliminary goals of this study were:

1. Assess the global DNA methylation profile of a range of urological tumors cell lines to select those with the highest 5-methylcytosine content;
2. Determine the IC50 value of the four newly synthetized compounds in the previously selected cell lines to choose the most effective compound;
3. Evaluate tumor cell viability after compounds' exposure to identify the most responsive urological tumor cell line to further pursue the study.

1. MATERIALS AND METHODS

Cell Culture

Epithelial cancer cell lines (ATCC, Lockville, MD, USA) representative of the various stages of each urological cancer (bladder, kidney and prostate) were selected. The characteristics and culture conditions are displayed in Table 7. All cell lines were cultured in the recommended medium supplemented with 10% Fetal Bovine Serum (FBS) (Merck, Berlin, Germany) and 1% Antibiotic-Antimycotic (Anti-Anti (100x), GIBCO®, Life Technologies, USA) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. TrypLE™ Express (GIBCO) was used to harvest the cells for subculture. All urological cell lines were found to be negative for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Takara Bio, Shiga, Japan).

Table 7 - Growth recommended medium for each urological tumor cell line. Adapted from [304, 305].

Cancer Type	Cell Line	Cell Type	Culture Properties	Growth Medium	Disease	Expressed Genes
BLADDER	**5637**	Epithelial	Adherent	RPMI-1640	Grade II carcinoma	-
	J82	Epithelial	Adherent	Eagle's Minimum Essential (MEM)	Grade III transitional cell carcinoma	-
	T24	Epithelial	Adherent	McCoy's 5a Medium Modified	Grade III transitional cell carcinoma	PSA; HLA A1, A3, B18, Bw35, Cw4, DRw2, DRw4
	TCCSUP	Epithelial	Adherent	Eagle's Minimum Essential (MEM)	Grade IV transitional cell carcinoma	-
	SCaBER	Epithelial	Adherent	Eagle's Minimum Essential (MEM)	Squamous cell carcinoma	-
KIDNEY	**A-498**	Epithelial	Adherent	Eagle's Minimum Essential (MEM)	Primary clear cell carcinoma	-
	769-P	Epithelial	Adherent	RPMI-1640	Primary clear cell adenocarcinoma	-
	786-O	Epithelial	Adherent	RPMI-1640	Primary clear cell adenocarcinoma	Parathyroid hormone (PTH) like peptide
	Caki-2	Epithelial	Adherent	McCoy's 5a Medium Modified	Primary clear cell carcinoma/papillary carcinoma	-
	ACHN	Epithelial	Adherent	Eagle's Minimum Essential (MEM)	Adenocarcinoma; Derived from metastatic site: pleural effusion (papillary)	-

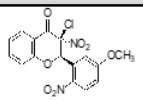
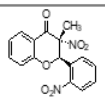
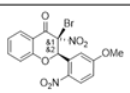
Table 7 - Growth recommended medium for each urological tumor cell line (cont.) Adapted from [304, 305].

Cancer Type	Cell Line	Cell Type	Culture Properties	Growth Medium	Disease	Expressed Genes
PROSTATE	**LNCaP**	Epithelial	Adherent, single cells and loosely attached clusters	RPMI-1640	Carcinoma; Derived from metastatic site: left supraclavicular lymph node; Androgen-responsive	Human prostatic acid phosphatase; PSA
	22Rv1	Epithelial	Adherent	RPMI-1640	Carcinoma; Derived from a xenograft that was serially propagated in mice after castration-induced regression; Androgen-responsive	PSA
	DU145	Epithelial	Adherent	Eagle's Minimum Essential (MEM)	Carcinoma; Derived from metastatic site: brain; Castration-resistant	-
	PC-3	Epithelial	Adherent	RPMI-1640 com F-12K	Grade IV adenocarcinoma; Derived from metastatic site: bone Castration-resistant	HLA A1, A9

Drug Preparation and IC50 Value

The four tested DNMTi compounds were designed, synthesized and kindly provided by Dr. Paola Arimondo's Group (ETaC: Pharmacochemistry - Cancer Epigenetic Regulation Unit group, Centre Pierre Fabre Laboratories - Research & Development, Toulouse, France). All the compounds are derived from flavanones and were synthetized to optimize their biological activity and stability as inhibitors of DNMT3A enzyme (Table 8). Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was used as drug solvent. The compounds were dissolved at 10 mM concentration and stored at -20°C until further use.

Table 8 – Characteristic of Flavanones-derived compounds.

Cpd ID	Batch Ref.	Structure	M_w (g.mol^{-1})	Molecular formula	EC_{50} DNMT3A-c (µM)	Cytotoxicity in CMV-Luc reexpression model	Stability (1/2 life time)	Quantity (mg)	Vol_{DMSO} for 10^{-2} M solution (µl)
DD880			378,72	$C_{16}H_{11}ClN_2O_7$	4.3 ± 0.9	No cytotoxicity observed at 10 µM	3.4 h	3,56	940
MLo-1302	MLo01-018		328,28	$C_{16}H_{12}N_2O_6$	> 40	Not tested	≈ 3.0 h		
MLo-1507	MLo02-040BF2		393,15	$C_{15}H_9BrN_2O_6$	3.2 ± 1.3	Cytotoxicity observed at 10 µM No cytotoxicity observed at 5 µM	1.7 h	5,06	1287
MLo-1508	MLo02-041B		423,18	$C_{16}H_{11}BrN_2O_7$	5.6 ± 0.9	A little cytotoxicity observed at 10 µM No cytotoxicity observed at 5 µM	7.0 h	1,57	371

For IC50 values calculation, cell lines were exposed to a range of compound concentrations (1 nM, 10 nM, 100 nM, 1 µM, 10 µM and 100 µM) every 24 hours, during three consecutive days. For control purposes, cell lines were also exposed to the drug vehicle (DMSO). After three days of exposure, MTT assay was performed and the IC50 values calculated using GraphPad Prism 6.

Cell Viability Assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. Cells were seeded onto 96-well plates at $5x10^4$, $1.25x10^4$, $4x10^4$, $2x10^4$ and $1.5x10^4$ cells per well for LNCaP, ACHN, Caki-2, T24 and 786-O, respectively. Cells were allowed to adhere overnight and then exposed to the IC50 concentration and one above of each compound or DMSO during three consecutive days, being the media renewed every 24 hours. Afterwards, cell viability was measured at day 0, 1, 2 and 3. Briefly, 200 µL of 0.5 mg/mL MTT (Sigma-Aldrich) were added to each well and incubated in the dark at 37ºC and 5% CO_2 for 1 hour. Formazan crystals were solubilized with 100 µL of DMSO. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) at a wavelength of 540 nm with background subtraction at 630 nm. Three biological and three experimental replicates were performed for each condition, and the number of viable cells was calculated with the following formula:

$$OD\ of\ viable\ cells = \frac{OD\ experiment\ \times\ Number\ of\ cells\ at\ day\ 0}{Mean\ OD\ at\ day\ 0}$$

DNA Global Methylation Levels

Imprint® Methylated DNA Quantification kit (Sigma-Aldrich, Germany) was used to quantify 5mC global content following manufacturer's recommendations. Briefly, after DNA extraction, using the standard phenol-chloroform method (Appendix I), 200 ng of total DNA was incubated in the provided 8 wells strips that contain a specific solution to allow DNA binding and adherence to the sample well. The samples were then incubated with a 5-methylcytosine capture and detection antibody. Absorbance was measured using FLUOstar Omega microplate reader at 450 nm. DNA methylation level was compared with a synthetic fully methylated DNA positive control. Three biological and three experimental replicates were performed. The determination of the percentage of global DNA methylation was performed using the following formula:

$$\%\ Global\ DNA\ methylation = \frac{(Mean\ OD\ Sample - Mean\ OD\ Blank)}{(Mean\ OD\ Methylated\ Control - Mean\ OD\ Blank)}\ x\ 100$$

2. RESULTS

Selection of the urological tumors cell lines with the highest 5mC levels

The global DNA methylation content was determined in five BlCa cell lines (5637, J82, T24, TCCSUP and SCaBER), five RCC cell lines (A498, 769-P, 786-O, Caki-2 and ACHN) and four PCa cell lines (LNCaP, 22Rv1, DU145 and PC-3). Considering each model separately, T24, ACHN and LNCaP depicted the highest 5mC content (Table 9).

Table 9 - Quantification of global DNA methylation levels for each urological tumor cell line.

Tumor Type	Samples	Global DNA Methylation Levels (%)	Mean
Bladder Cancer	5637 (1)	18,71559633	
	5637 (2)	14,67889908	19,02140673
	5637 (3)	23,66972477	
	J82 (1)	15,77981651	
	J82 (2)	16,51376147	16,69724771
	J82 (3)	17,79816514	
	T24 (1)	43,30275229	
	T24 (2)	31,00917431	**34,98470948**
	T24 (3)	30,64220183	
	TCCSUP (1)	46,05504587	
	TCCSUP (2)	14,86238532	27,03363914
	TCCSUP (3)	20,18348624	
	SCaBER (1)	14,67889908	
	SCaBER (2)	14,31192661	14,9235474
	SCaBER (3)	15,77981651	
Kidney Cancer	A498 (1)	10,09174312	
	A498 (2)	18,34862385	15,71865443
	A498 (3)	18,71559633	
	769-P (1)	15,04587156	
	769-P (2)	12,8440367	13,57798165
	769-P (3)	12,8440367	
	786-O (1)	14,86238532	
	786-O (2)	9,724770642	16,94189602
	786-O (3)	26,23853211	
	Caki2 (1)	18,34862385	
	Caki2 (2)	28,99082569	22,93577982
	Caki2 (3)	21,46788991	
	ACHN (1)	24,7706422	
	ACHN (2)	17,6146789	**25,19877676**
	ACHN (3)	33,21100917	
Prostate Cancer	LNCaP (1)	28,25688073	
	LNCaP (2)	21,46788991	**22,62996942**
	LNCaP (3)	18,16513761	
	22Rv1 (1)	17,79816514	
	22Rv1 (2)	14,31192661	18,22629969
	22Rv1 (3)	22,56880734	
	DU145 (1)	22,3853211	
	DU145 (2)	22,75229358	21,71253823
	DU145 (3)	20	
	PC-3 (1)	15,77981651	
	PC-3 (2)	18,16513761	17,12538226
	PC-3 (3)	17,43119266	

Calculation of the IC50 Value

The half maximal inhibitory concentration (IC50) value of the four newly synthetized DNMT3A inhibitors, was calculated for each selected cell line after 72 hours of exposure (Table 10). The dose-response curves are represented in Appendix II. Overall, ACHN exhibited the lowest IC50 values for the tested compounds. Moreover, MLo-1302 was the compound with the lowest IC50 mean value (approximately 1.5 µM), while MLo-1507 presented the highest IC50 mean value (approximately 14 µM). Surprisingly, this compound, did not demonstrate any DNMT3A inhibitory activity and therefore was included as a negative control (Table 8). Considering the results obtained at this stage, compound MLo-1302 was chosen for *in vitro* experiments.

Table 10 – IC50 values obtained for each tested compound and for each selected cell line.

	DD880	MLo-1302	MLo-1507	MLo-1508
T24	5.59 µM	1.42 µM	8.39 µM	3.38 µM
ACHN	10.86 µM	1.03 µM	7.20 µM	1.84 µM
LNCaP	14.28 µM	1.85 µM	25.57 µM	1.63 µM
		1.43 µM	**13.72 µM**	

Validation of MLo-1302 growth inhibitory effect

To assess the MLo-1302 effect on tumor cell viability, the three selected cell lines were exposed to two different concentrations of this compound (1.5 and 3 µM) as well as to the vehicle (DMSO) during three consecutive days. Exposure to MLo-1302 markedly reduced cell viability of all tested cell lines, being the 3 µM concentration the most effective one (Figure 6). Whilst for T24 and LNCaP the decrease in cell viability was mainly achieved at the end of day 3 and with the highest tested concentration, ACHN suffered a greater reduction in the number of viable cells starting at day 1 and when exposed to either 1.5 µM or 3 µM concentrations. Considering these preliminary results, kidney cancer cell lines were chosen to further test the demethylation activity and the anti-cancer effects of these newly synthetized compounds. Despite MLo-1302 had been established as a negative control, its cell viability inhibitory effect and its low IC50 value, compared to the other three compounds, rendered it as an interesting compound to be investigated.

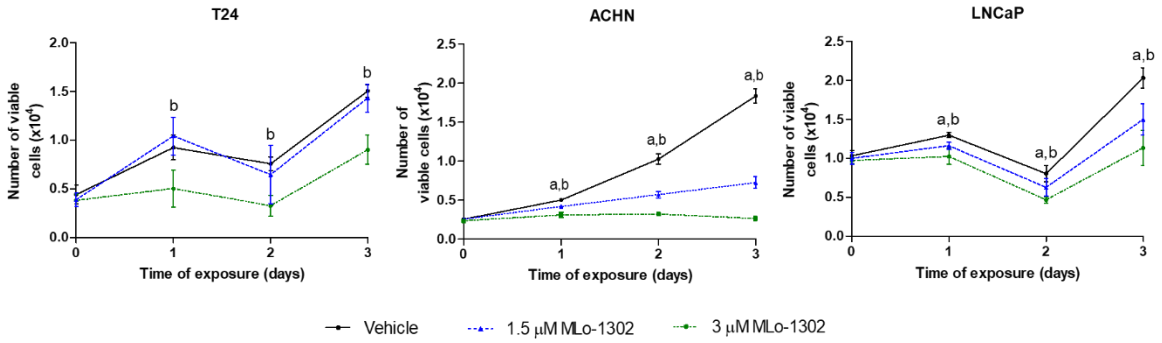


Figure 6 - T24, ACHN and LNCaP cell viability after exposure to MLo-1302 and drug vehicle, at days 0, 1, 2, and 3, measured by MTT assay. Statistically significant differences were observed between **(a)** vehicle and 1.5 µM MLo-1302, and **(b)** vehicle and 3 µM MLo-1302. All data are presented as mean of three independent experiments ± s.d.

Specific Aims

Considering the preliminary results, the major goal of this Master Dissertation was re-defined to evaluate the demethylating and anti-neoplastic properties of these newly synthetized flavanones-derived compounds in RCC cell lines.

The specific goals of this dissertation were:

1. Calculate the IC50 value of the flavanones-derived compounds in RCC cell lines to choose the two most effective concentrations;
2. Evaluate the phenotypic impact of these compounds on RCC cell lines;
3. Investigate the influence of these compounds on DNMTs and TETs expression;
4. Assess the compounds effects in global DNA methylation levels of RCC cell lines;
5. Determine the effect of these novel DNMTi on DNA demethylation along with the re-expression of genes known to be epigenetically silenced in renal cancer;
6. Study the role of MLo-1302 in DNMT3A activity.

III. MATERIALS AND METHODS

Cell culture, drug preparation, IC50 value calculation, cell viability assay and quantification of global DNA methylation levels were performed as described in the previous section.

Apoptosis Assay

Apoptosis was assessed using the APOPercentage™ apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland), according to the manufacturer's guidelines. This assay is based on the movement of the transmembrane protein phosphatidylserine from the inside to the outside of the cell membrane layer, which results in the uptake of the APOPercentage dye by the apoptotic cells. Briefly, $5x10^4$ cells/well were seeded onto 24-well plates, allowed to adhere overnight and treated as mentioned above. After 72 hours of compound exposure, 5% of the APOPercentage dye was added to the media and incubated at 37ºC during 15 to 30 minutes (depending on cell type). After PBS washing, 50 µL TrypLE™ Express was added to each well to allow cellular dissociation. Subsequently, 200 µL of Dye Releasing Agent were added and the plates were subjected to 15 minutes of intense shaking to release intracellular accumulated dye. The absorbance was determined using a microplate reader (FLUOstar Omega) at a wavelength of 550 nm with background subtraction at 620 nm. Three biological and three experimental replicates were performed for each condition. Apoptosis levels were calculated according to the following formula:

$$Apoptosis\ Levels = \frac{Apotosis\ OD}{Mean\ MTT\ OD\ at\ day\ 3}$$

The results were expressed as the OD ratio of the cells exposed to the DNMTi normalized to vehicle.

Comet Assay

After drug exposure, 50,000 cells were harvested by trypsinization, washed in PBS and re-suspended in 75 µL of low-melting point agarose (Life Technologies). This cell suspension was applied on top of a microscope slide containing normal-melting point agarose and allowed to polymerize 20 minutes at 4ºC. The slides were then, immersed in lysis solution, pH 10 (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base and Triton X-100 1%) at 4°C during 2 hours in the dark. Then, the slides were incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 40 minutes at 4ºC to allow DNA unwinding. Single cell gel electrophoresis was performed on a horizontal electrophoresis platform at 4ºC for 30 minutes at 27V. Next, slides were immersed in neutralization buffer (Tris–HCl; pH-7.5, 0.4 M Tris Base) for 10 minutes. After fixation with

100% ethanol, the slides were stained with Sybr Green® (Life Technologies) and DNA damage was evaluated under a fluorescent microscope. At least three independent and two experimental replicates were performed for each condition. The DNA damaging effect through DNA fragmentation was determined by measuring four parameters previously described including, total intensity (DNA content), tail length, tail moment (a measure of both amount of DNA in the tail and its distribution) and percentage of DNA in the tail [306]. A minimum of 50 cells were considered for each replicate.

Morphometric Analysis

Cell morphometric analysis was performed after 3 days of compound exposure. A camera incorporated into Olympus phase-contrast microscope was used to capture cell images. Cell sphericity and area were evaluated using the freehand polygon tool of the Olympus cellSens Dimension software (Olympus Corporation, Shinjuku, Japan). For each condition, at least 50 cells from every biological replicates were analyzed.

Quantitative Methylation-Specific PCR (qMSP)

After extraction and quantification (Appendix I), 1 µg of genomic DNA was submitted to sodium bisulfite modification by EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA) according to the manufacturer's instructions (Appendix III). Modified DNA was eluted with 60 µL of sterile distilled water and stored at -80°C until further use. One µg of CpGenome™ Universal Methylated DNA (Millipore, USA) was also modified and eluted in 20 µL of sterile distilled water to generate a standard curve. Bisulfite modified DNA was amplified by qMSP using TaqMan technology. Specific primers and a TaqMan probe for *RASSF1A* gene were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA, USA). *β-Actin* (*ACTB*) was used as a reference gene to normalize for DNA input. Reactions were performed in 96-well plates using Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific, USA). Briefly, per each well 1 µL of modified DNA, 9 µL of Master Mix using AmpliTaq Gold™ (Applied Biosystems) were added. The primers and probes volumes used for each gene as well as their sequences are listed in Table 11. The amplification was carried out by a period of 10 minutes at 95°C followed by 45 cycles with 15 seconds at 95°C and 1 minute at specific temperature (Table 11). All the samples were run in triplicates. The methylation levels for each sample were derived from calibration curves created using serial dilutions (1:5) of bisulfite modified CpGenome™ Universal Methylated DNA. The methylation levels for each gene were calculated after normalization to *ACTB*.

Table 11 - Primers and Probes sequences and qMSP conditions for each studied gene.

Gene	Primers Sequences (5' → 3')	Volume (µL) F+R*	Annealing Temperature (°C)
ACTB	**F:** TGG TGA TGG AGG AGG TTT AGT AAG T	0.4	60
	R: ACC AAT AAA ACC TAC TCC TCC CTT AA		
	Probe: FAM- ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA -TAMRA	0.02	
RASSF1A	**F:** GCG TTG AAG TCG GGG TTC G	0.6	62
	R: CCC GTA CTT CGC TAA CTT TAA ACG		
	Probe: FAM- ACA AAC GCG AAC CGA ACG AAA CCA -TAMRA	0.02	

***** Primers Forward **(F)** plus Reverse **(R)** at a concentration of 10 µM.

Quantitative Reverse Transcription PCR (qRT-PCR)

After compound exposure, RNA was extracted from cell lines using TRIzol® (Life Technologies) following manufacturer's instructions (Appendix IV). Complementary DNA (cDNA) synthesis was performed using the RevertAid Reverse Transcription Kit (Thermo Scientific, MA, USA) (Appendix V). Expression of target genes was quantified using NZYSpeedy qPCR Green Master Mix (2x), ROX (NZYTech) for *GUSB*, *DNMT1*, *DNNMT3a*, *DNMT3b*, *RASSF1A*, *TET1*, *TET2* and *TET3*. Expression of *CASP3*, *CDKN1A*, *GUSB* and *KI67* was quantified using Taqman expression assays (purchased as pre-developed assays from Applied Biosystems) and NZYSpeedy qPCR Probe Master Mix (2x), ROX (NZYTech). The expression of each target gene was then normalized to the expression of the housekeeping gene (*GUSB)*. Primers and PCR conditions used for each target gene are displayed in Table 12. The PCR program was performed using Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific) and all the samples were run in triplicate. Human Reference Total RNA (Agilent Technologies, USA) was used as positive control to generate a standard curve (dilutions of 1:10).

Table 12 - Primers sequences and qRT-PCR conditions for each target gene.

Gene	Primers Sequences (5' → 3') / Assay Reference	Volume (µL) F+R*	Annealing Temperature (°C)
CASP3	Hs00234387_m1	0.5	60
CDKN1A	Hs00355782_m1	0.5	60
DNMT1	**F:** TAT CCG AGG AGG GCT ACC TG **R:** ATG AGC ACC GTT CTC CAA GG	0.5	60
DNMT3a	**F:** TAT TGA TGA GCG CAC AAG A **R:** GGG TGT TCC AGG GTA ACA TTG AG	0.5	60
DNMT3b	**F:** GAA TTAC TCA CGC CCC AAG GA **R:** ACC GTG AGA TGT CCC TCT TGT C	0.5	60
GUSB	**F:** CTC ATT TGG AAT TTT GCC GAT T **R:** CCG AGT GAA GAT CCC CTT T	0.5	61
GUSB	Hs99999908_m1	0.5	60
Ki67	Hs01032427_m1	0.5	60
RASSF1A	**F:** AGC GCC CAA AGC CAG CGA AGC ACG G **R:** CCC GCA ACA GTC CAG GCA GAC GAG C	0.5	60
TET1	**F:** ACC TGC AGC TGT CTT GAT CG **R:** TTT CCC TGA CAG CAG CAA CA	0.2	62
TET2	**F:** ACG CTT GGA AGC AGG AGA T **R:** AAG GCT GCC CTC TAG TTG AA	0.3	64
TET3	**F:** CCC ACA AGG ACC AGC ATA AC **R:** CCA TCT TGT ACA GGG GGA GA	0.3	62

* Primers Forward **(F)** plus Reverse **(R)** at a concentration of 10 µM.

DNMT3A Activity Assay

Nuclear extracts from either compound treated or vehicle exposed cells were obtained using the Nuclear Extract Kit (Active Motif, Rixensart, Belgium) as instructed in the manufacturer's protocol. After protein quantification through Pierce BCA Protein Assay kit, (Thermo Scientific), 10 µg of nuclear extract obtained from each sample was used to measure DNMT3A activity with EpiQuick™ DNMT3A Assay Kit, a sensitive ELISA-based kit (Epigentek, New York, USA), according to the manufacturer's instructions. Briefly, nuclear extracts were added to a specific 96 well plate coated with CpG enriched substrate. After the enzymatic reaction, capture and detection antibodies were added to the wells. Absorbance was measured using FLUOstar Omega microplate reader, at 450 nm with background subtraction at 655 nm. Three biological and three experimental replicates were performed for each sample. The subsequent formula was used to calculate the DNMT3A activity percentage:

$$\%\ DNMT3a\ Activity = \frac{(OD\ Treated\ Sample - OD\ Blank)}{(OD\ Vehicle - OD\ Blank)} x\ 100\%$$

Statistical Analysis

One-way analysis of variance (ANOVA), with post-hoc Dunnet's multiple comparison test was used to compare the results obtained in each parameter for the different compounds concentrations and the control/vehicle, when appropriated. On the other hand, in the absence of normality, the non-parametric Kruskal-Wallis was applied. Analysis was performed with GraphPad Prism 6, and statistical significance was set at p-value < 0.05.

IV. RESULTS

IC50 value of flavanones-derived compounds in RCC cell lines

The IC50 value was calculated for each compound in three RCC cell lines, one derived from clear cell carcinoma, 786-O, and two derived from papillary carcinoma, ACHN (metastatic) and Caki-2 (primary) using MTT assay (Table 13). Compound MLo-1302 exhibited the lowest IC50 mean value in the three cell lines, approximately 1.5 μM compared to 9.5, 11 and 8.5 μM of compounds DD880, MLo-1507 and MLo-1508, respectively (dose-response curves in Appendix II).

Table 13 – IC50 values obtained for each tested compound and for each selected cell line.

	DD880	MLo-1302	MLo-1507	MLo-1508
786-O	4.77 μM	1.45 μM	12.82 μM	12.03 μM
Caki-2	12.59 μM	1.30 μM	12.30 μM	11.32 μM
ACHN	10.86 μM	1.03 μM	7.20 μM	1.84 μM

Since the IC50 mean value of all the compounds, with exception of MLo-1302, were quite similar, the phenotypic impact and the inhibitory effect on DNMT expression were assessed for all the four compounds to further select the one with the greatest anti-cancer potential. Importantly, all the cell lines were exposed to the IC50 concentration and one above, as indicated in Table 14.

Table 14 – Concentrations of the four flavanones derived compounds used for each RCC cell lines.

Compounds' Concentrations								
		DD880		MLo-1302		MLo-1507		MLo-1508	
Cell Lines	**786-O**	5 μM	10 μM	1.5 μM	3 μM	15 μM	30 μM	12 μM	24 μM
	Caki-2	10 μM	20 μM	1.5 μM	3 μM	15 μM	30 μM	12 μM	24 μM
	ACHN	10 μM	20 μM	1.5 μM	3 μM	7.5 μM	15 μM	2 μM	4 μM

Phenotypic effects

1. Flavanones-derived compounds attenuated RCC cells malignant phenotype

A time- and dose-dependent inhibition of RCC cell viability was observed for all the four compounds. Overall, the tumor cell growth suppression was evident from day 1 and with both tested concentrations (Figure 7).

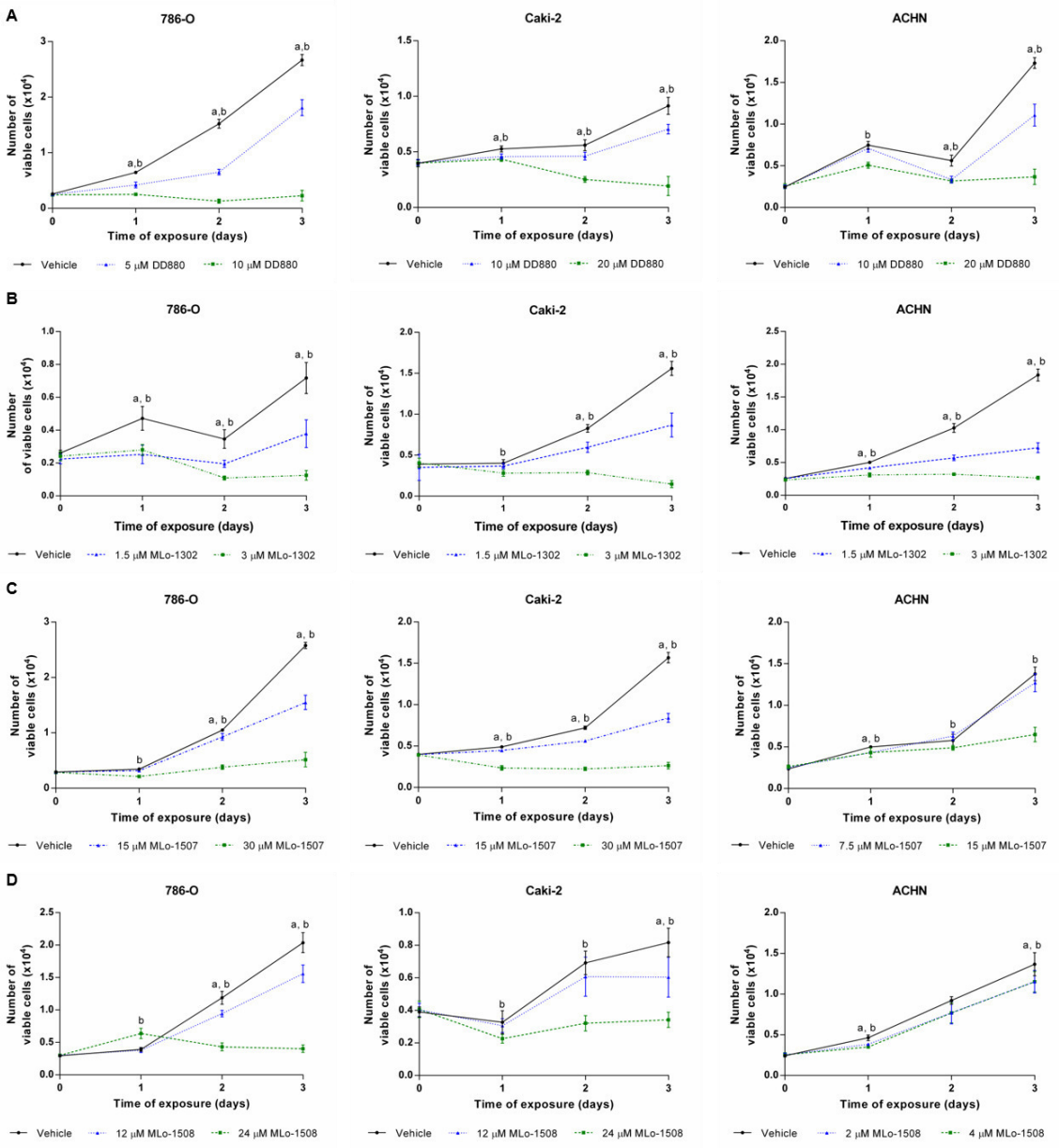


Figure 7 - Impact of the four flavanones-derived compounds, **(A)** DD880, **(B)** MLo-1302, **(C)** MLo-1507, **(D)** MLo-1508, on cell viability of RCC cell lines, 786-O, Caki-2 and ACHN, at days 0, 1, 2, and 3, measured by MTT assay. Statistically significant differences were observed between **(a)** vehicle and IC50 mean concentration, and **(b)** vehicle and the double IC50 mean concentration. All data are presented as mean of three independent experiments ± s.d.

Corroborating the preliminary results, MLo-1302 was the most effective compound by consistently reducing the cell viability of the three tested RCC cell lines in the same proportion. Moreover, MLo-1302 was the only compound in which the IC50 value really reduced RCC cell viability in 50% (Table 15). Nonetheless, similar findings were observed for compounds DD880 and MLo-1507, being the lowest reduction of tumor cell growth attained with MLo-1508, particularly in ACHN cell line.

Table 15 – Percentage of viable cells after 3 days of compound exposure in RCC cell lines.

	DD880			MLo-1302		MLo-1507			MLo-1508				
	5	**10**	**20**	**1.5**	**3**	**7.5**	**15**	**30**	**2**	**4**	**12**	**24**	**µM**
786-O	67.9	8.5	-	52.8	17.4	-	60.1	20.1	-	-	76.5	19.8	**% of Viable Cells** (day 3)
Caki-2	-	77.1	21.1	55.7	9.5	-	53.6	16.8	-	-	74.0	41.9	
ACHN	-	63.9	21.2	39.5	14.5	92.3	47.1	-	84.2	84.3	-	-	

All tested compounds induced a significant increase in apoptosis levels (Table 16). In accordance with the cell viability results, apoptosis induction was dose-dependent (Figure 8). DD880 showed the highest impact in apoptosis, especially in 786-O with 10 µM and in ACHN with 20 µM concentration (Figure 8A), whereas the lowest apoptotic levels were achieved with MLo-1508, being ACHN the less responsive cell line (Figure 8D). The remaining two compounds also displayed a pronounced effect in RCC cells death, being Caki-2 the most responsive to MLo-1302 (Figure 8B) and 786-O to MLo-1507 (Figure 8C).

Table 16 - Apoptosis levels after 3 days of compound exposure (normalized to respective vehicle).

	DD880			MLo-1302		MLo-1507			MLo-1508				
	5	**10**	**20**	**1.5**	**3**	**7.5**	**15**	**30**	**2**	**4**	**12**	**24**	**µM**
786-O	5.0	30.7	-	1.8	8.6	-	12.3	46.0	-	-	2.7	11.1	**Apoptosis Levels** (day 3)
Caki-2	-	3.0	8.2	1.5	13.8	-	1.8	5.5	-	-	2.9	6.6	
ACHN	-	11.7	40.2	2.4	6.3	3.3	8.6	-	2.0	2.1	-	-	

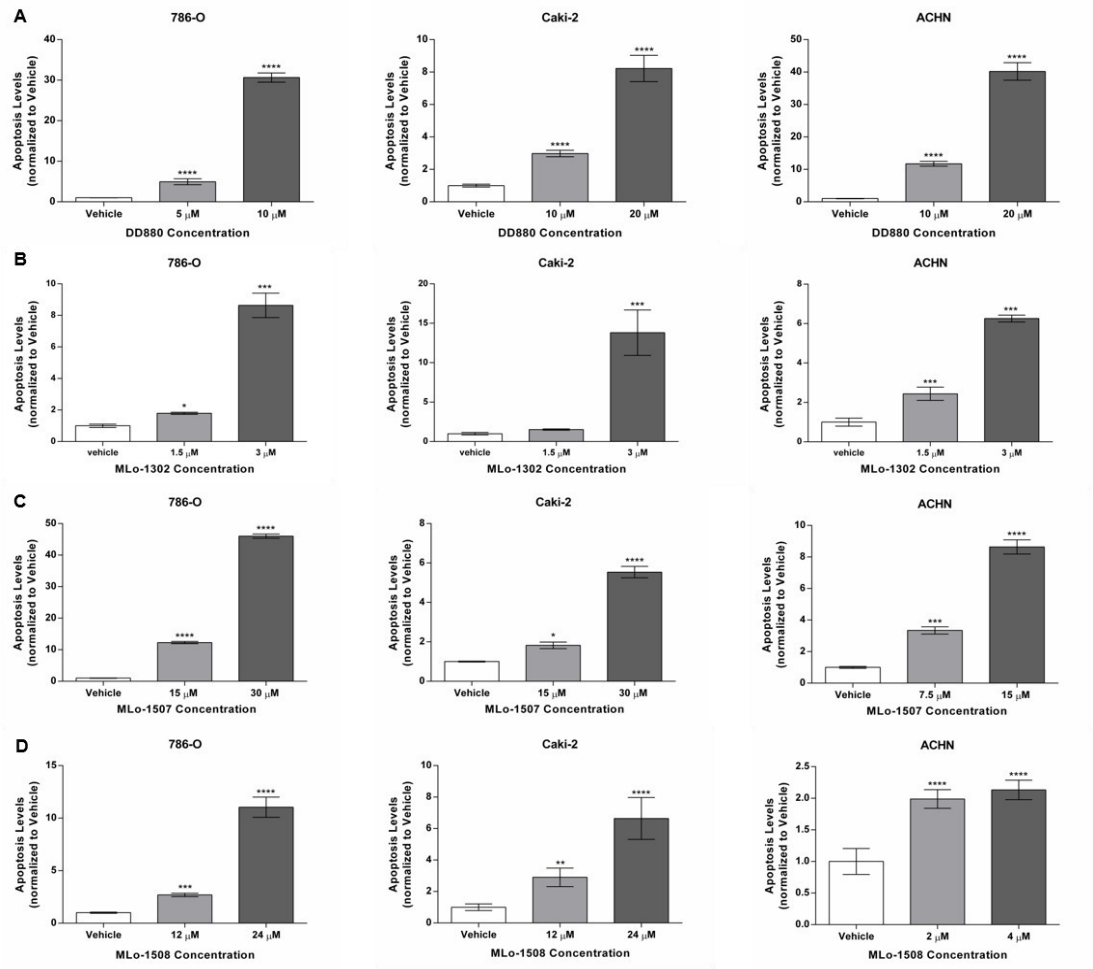


Figure 8 - Effect of the four flavanones-derived compounds, **(A)** DD880, **(B)** MLo-1302, **(C)** MLo-1507, **(D)** MLo-1508, in apoptosis levels of RCC cell lines, measured at day 3, with a phosphatidylserine-based assay. All data are presented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Molecular analysis of key genes associated with phenotypic features were performed in RCC cell lines exposed to the most effective compounds, DD880 (in apoptosis) and MLo-1302 (in cell viability). Indeed, the expression of two genes involved in cell proliferation pathway (*CDKN1A* and *KI67*) and one gene that is triggered in apoptosis (*CASP3*) were assessed. Generally, the molecular results corroborated the phenotypic data. A significant decrease in *KI67* along with a significant increase in *CDKN1A* transcript levels was depicted for all RCC lines with DD880, especially at 10 μM concentration (Figure 9A). Whereas, for MLo-1302 exposure, only a significant *Ki67* levels' reduction was found in ACHN. However, *CDKN1A* expression was increased after exposure to 3 μM MLo-1302 in Caki-2 and ACHN (Figure 9B). Regarding apoptosis, a significant augment in *CASP3* mRNA levels was found after exposure to all the compounds (Figure 9). Insufficient RNA and DNA did not allow for molecular evaluation of ACHN cell line treated with 20 μM DD880.

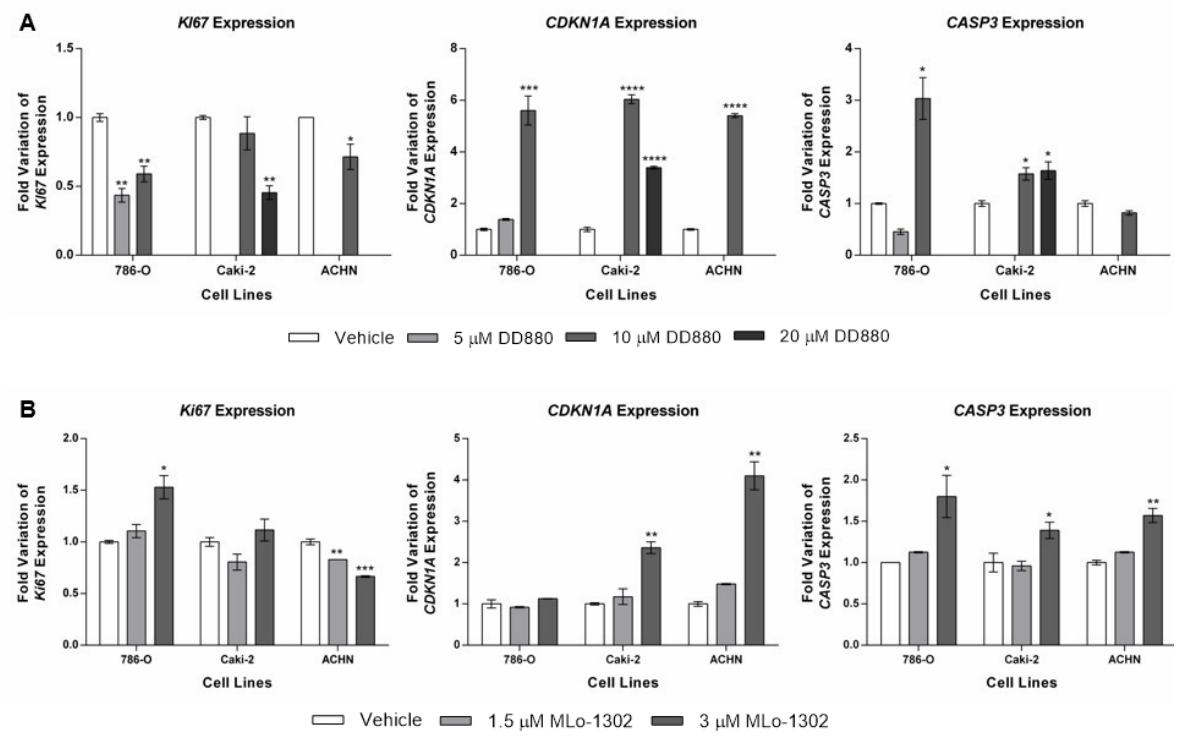


Figure 9 – mRNA expression of *KI67*, *CDKN1A* and *CASP3*, normalized to *GUSB*, in vehicle and drug exposed RCC cells after three days of exposure to **(A)** DD880 or **(B)** MLo-1302 (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

2. Flavanones-derived compounds led to alterations in RCC cell morphology

Flavanones-derived compounds induced visible alterations in cell morphology of all RCC cell lines. Although a significant cell area increase was disclosed for 786-O and ACHN exposed to MLo-1302 (Figure 10B), the remaining compounds mainly caused a significant decrease of this parameter (Figure 10A, C and D). Moreover, an increase in cell granularity and nuclei heterochromatic regions were also apparent, which combined with the small sized cell population are common features of apoptotic cells. Interestingly, cell vacuolization and debris were common at the highest compound concentration (Table 17). Globally, a significant increase in cell sphericity was observed in treated cells when compared to drug vehicle, especially for MLo-1302 (Figure 11B) and MLo-1507 (Figure 11C). Illustrations of drug-induced morphometric alterations are shown in Appendix VI.

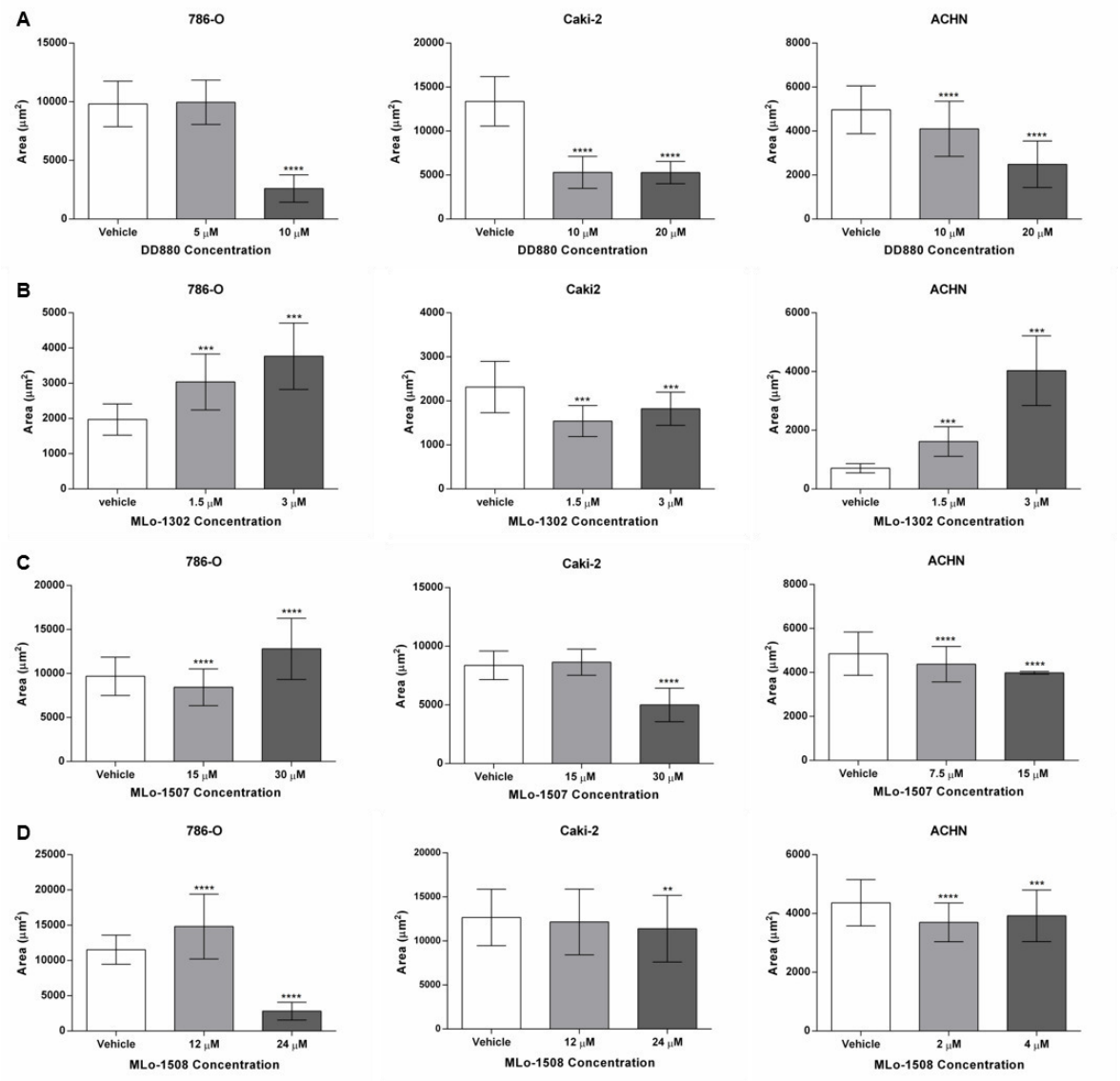


Figure 10 – Morphometric alterations induced on 786-O, Caki-2 and ACHN after exposure to the four flavanones-derived compounds, **(A)** DD880, **(B)** MLo-1302, **(C)** MLo-1507, **(D)** MLo-1508, considering variations in cell area, compared with vehicle. All data are presented as mean of three independent experiments ± s.d. (**p<0.01; ***p<0.001; ****p<0.0001).

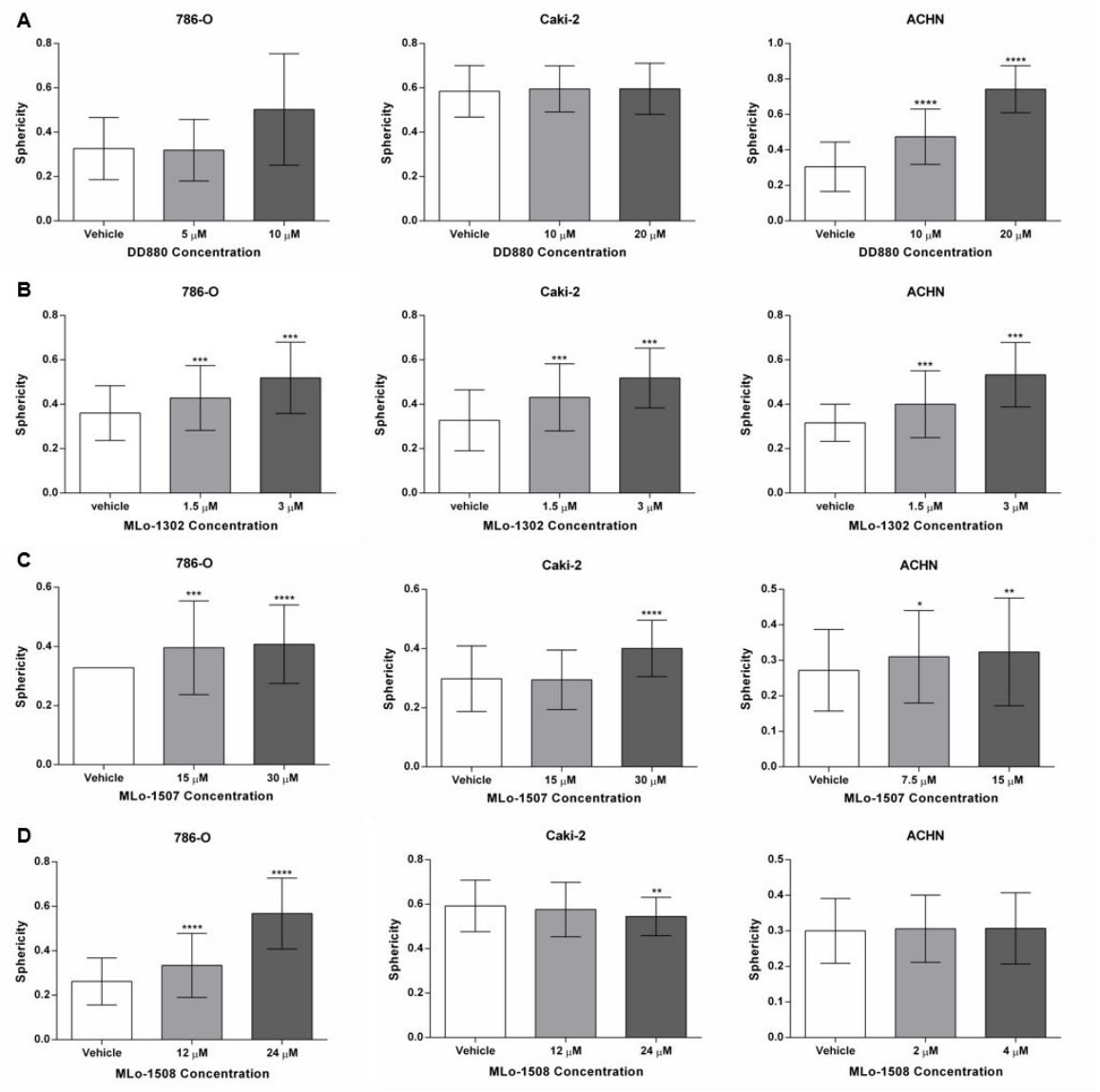


Figure 11 – Cell sphericity alterations induced in RCC cell lines after exposure to the four compounds, **(A)** DD880, **(B)** MLo-1302, **(C)** MLo-1507, **(D)** MLo-1508, compared with vehicle. All data are presented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Table 17 – Morphometric alterations induced by the four flavanones-derived compounds in RCC cell lines.

	DD880 (μM)						MLo-1302 (μM)						MLo-1507 (μM)						MLo-1508 (μM)					
	786-O		Caki-2		ACHN		786-O		Caki-2		ACHN		786-O		Caki-2		ACHN		786-O		Caki-2		ACHN	
	5	10	10	20	10	20	1.5	3	1.5	3	1.5	3	15	30	15	30	7.5	15	12	24	12	24	2	4
Area	ns	-	-	-	-	-	+	+	-	-	+	+	-	+	ns	+	-	-	+	-	ns	-	-	-
Cell Sphericity	ns	ns	ns	ns	+	+	+	+	+	+	+	+	+	+	ns	+	+	+	+	+	ns	-	ns	ns
Presence of:																								
Heterochromatic regions	ns	ns	+	+	ns	ns	ns	+	ns	+	+	+	+	+	ns	ns	+	+	ns	ns	ns	+	ns	+
Vacuolization	+	+	+	+	ns	+	ns	+	ns	ns	+	+	ns	+	ns	+	+	+	+	ns	+	+	ns	+
Cell debris	-	+	+	+	+	+	ns	+	ns	+	ns	ns	ns	+	ns	+	+	+	+	+	+	+	ns	ns

+ presence or increase relative to vehicle; **-** decrease relative to vehicle; **ns** not significant relative to vehicle

3. Flavanones-derived compounds induced DNA damage in RCC cell lines

As demonstrated by the increased comet tail length, RCC cell lines exposure to each one of the flavanones-derived compounds caused significant DNA damage, excepting for ACHN exposed to MLo-1508 (Figure 12). Furthermore, tail moment allowed quantification of DNA fragmentation which is calculated through multiplication of DNA percentage in the tail by the distance between the means of the head and tail distributions. Representative images were chosen to illustrate drug-induced DNA damage by MLo-1302 (Figure 13). The remaining images for each compound are presented in Appendix VII.

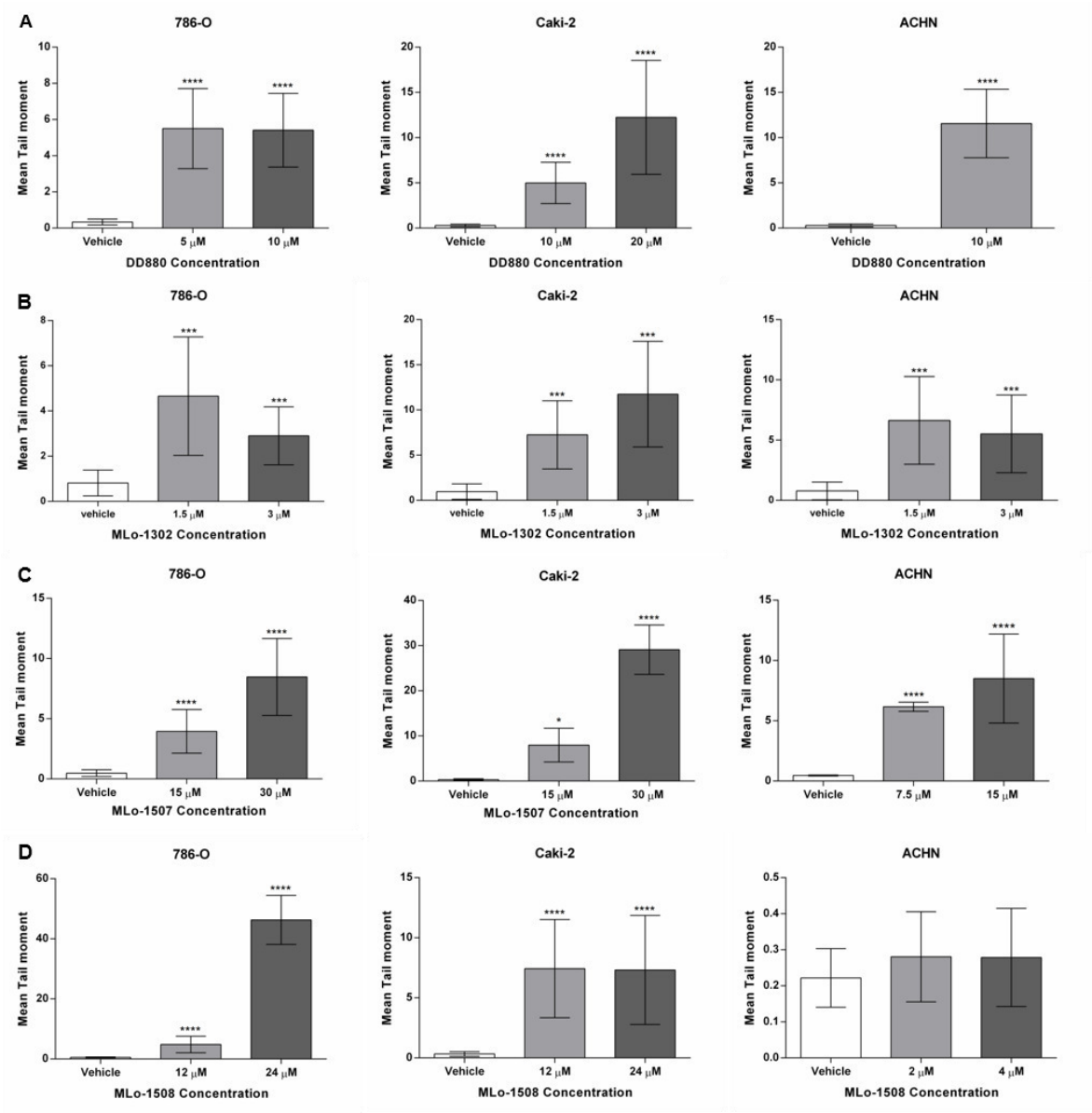


Figure 12 – Effect of the four flavanones-derived compounds, **(A)** DD880, **(B)** MLo-1302, **(C)** MLo-1507, **(D)** MLo-1508, on DNA damage of RCC cell lines measured by mean tail moment. All data are presented as mean of three independent experiments ± s.d. (*p<0.05; ***p<0.001; ****p<0.0001).

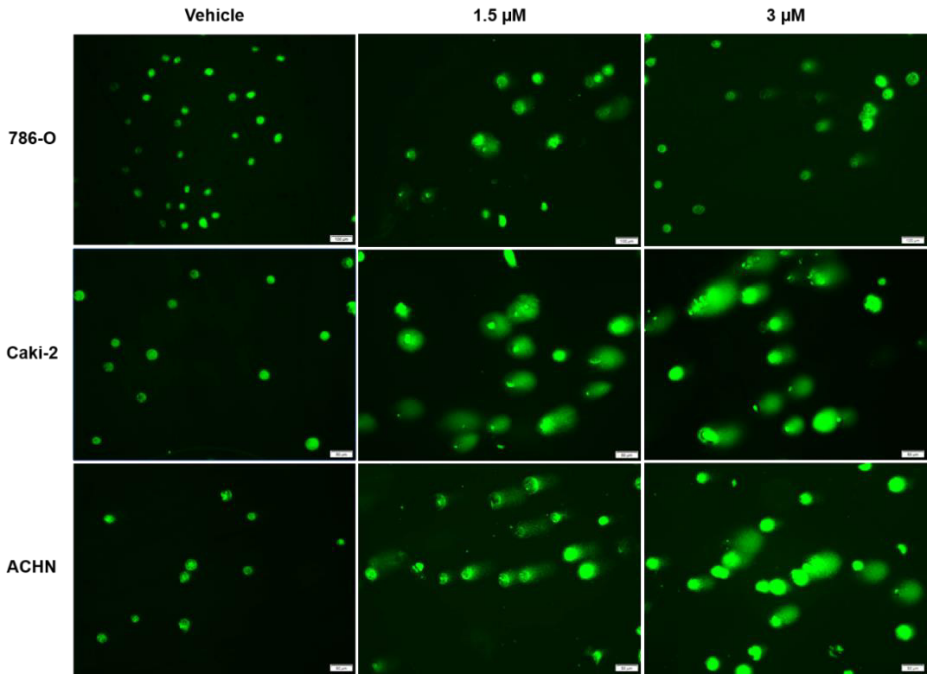


Figure 13 – MLo-1302 effect on DNA damage. Comet assay immunofluorescence images of vehicle and 1.5 µM and 3 µM MLo-1302 exposed cells counterstained with Syber Green. All data are presented as mean of three independent experiments ± s.d.

Effects on DNA Methylation

1. Flavanones-derived compounds associated with DNMTs altered expression in human RCC cell lines

A significant increase in *DNMT1* expression levels were observed in cells exposed to DD880, MLo-1507 and MLo-1508 compounds, with exception of 786-O treated with DD880 (Figure 14A), where no changes were apparent. Furthermore, a significant reduction in *DNMT3a* was verified in ACHN after exposure to DD880 (Figure 14A). Overall, MLo-1302 induced the greatest DNMTs expression inhibitory effect in the tested RCC cell lines. Indeed, this compound not only reduced *DNMT1* expression levels in Caki-2 but also *DNMT3a* in all treated cell lines. Surprisingly, all cell lines displayed a significant increase of *DNMT3b* expression levels after MLo-1302 exposure (Figure 14B). Additionally, Caki-2 exposure to MLo-1507 (Figure 14C) and MLo-1508 (Figure 14D) was associated with a significant decrease of *DNMT3a* expression levels. Nonetheless, 786-O and Caki-2 exposed to DD880 and ACHN treated with MLo-1507 displayed increased *DNMT3a* mRNA levels (Figure 14A and C). Besides that, a significant reduction of *DNMT3b* expression was depicted in both Caki-2 and ACHN cell lines exposed to MLo-1508 (Figure 14D). In view of these results conjugated with the previous described phenotypic effects, the additional experiments were conducted only with DD880 and MLo-1302.

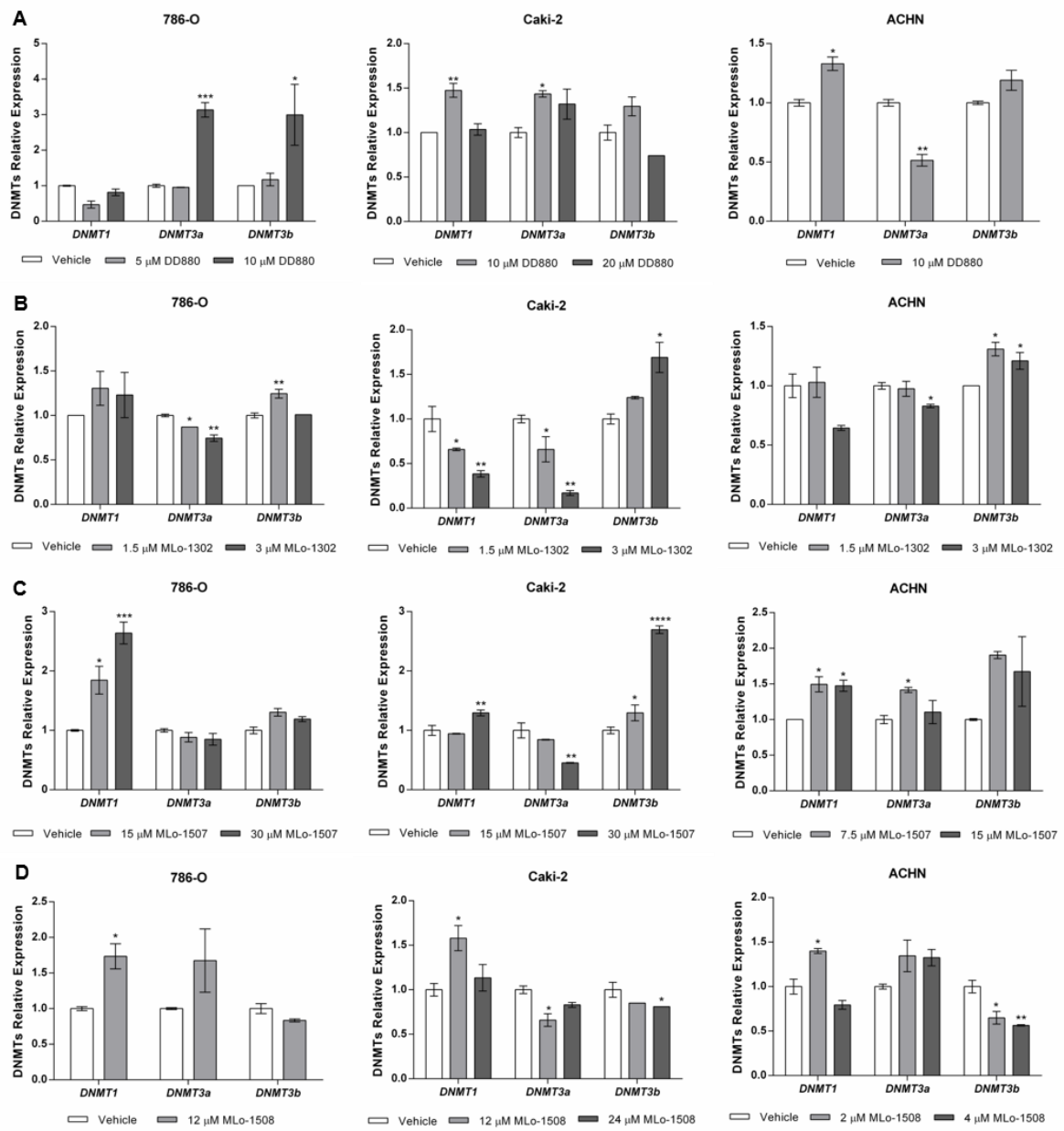


Figure 14 – Impact of the four flavanones-derived compounds **(A)** DD880, **(B)** MLo-1302, **(C)** MLo-1507, **(D)** MLo-1508, on *DNMT1*, *DNMT3a* and *DNMT3b* mRNA expression in the three tested RCC cell lines, normalized to *GUSB,* in vehicle and drug exposed cells. All data are presented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

2. Flavanones-derived compounds associates with TETs upregulation in human RCC cell lines

TETs expression was also evaluated in RCC cells exposed to the newly synthetized compounds. Interestingly, excepting ACHN treated with MLo-1032, both compounds induced *TET1* expression in the tested cell lines, (Figure 15A and B). Additionally, DD880 increased *TET2* and *TET3* transcript levels in Caki-2 and 786-O, respectively (Figure 15A),

whereas MLo-1302 significantly augmented *TET2* expression in 786-O and Caki-2, and increased *TET3* expression levels in ACHN cell line (Figure 15B).

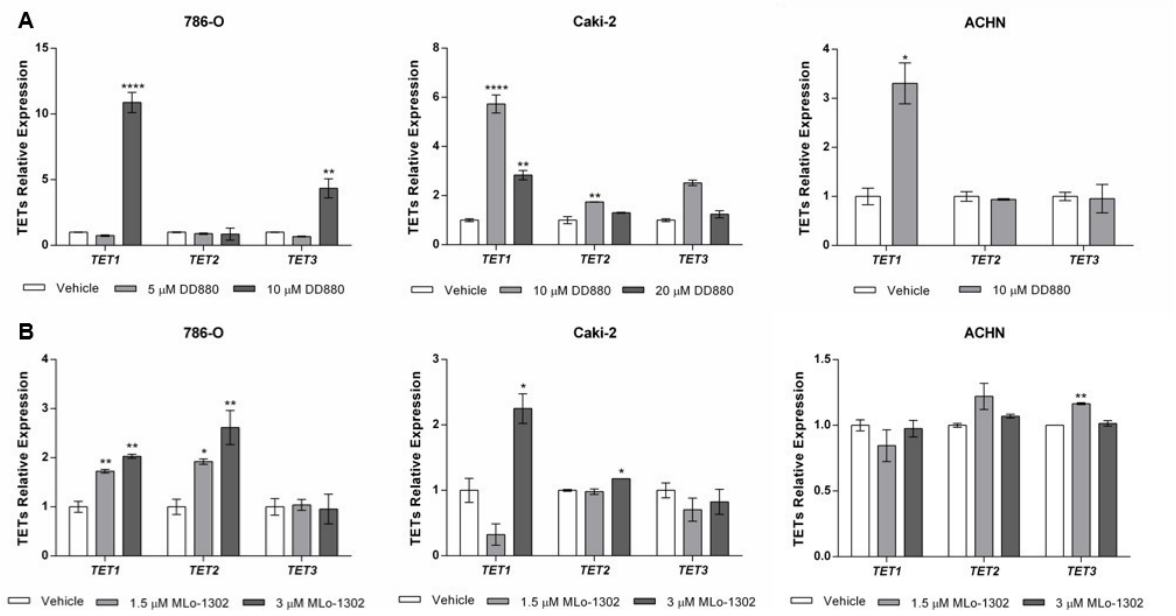


Figure 15 – Impact of **(A)** DD880 and **(B)** MLo-1302 on mRNA expression levels of *TET1*, *TET2* and *TET3* in RCC cell lines, normalized to *GUSB,* in vehicle and drug exposed cells. All data are presented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01; ****p<0.0001).

3. MLo-1302 decreased global DNA methylation levels in RCC cell lines

A slight (~6.5%), though significant, decrease in global 5-mC content was observed in 786-O exposed to DD880 (Figure 16A). In contrast, MLo-1302 significantly reduced global methylation levels of all RCC cell lines, particularly at lower concentration, being the most significant reduction found in ACHN (70%) (Figure 16B).

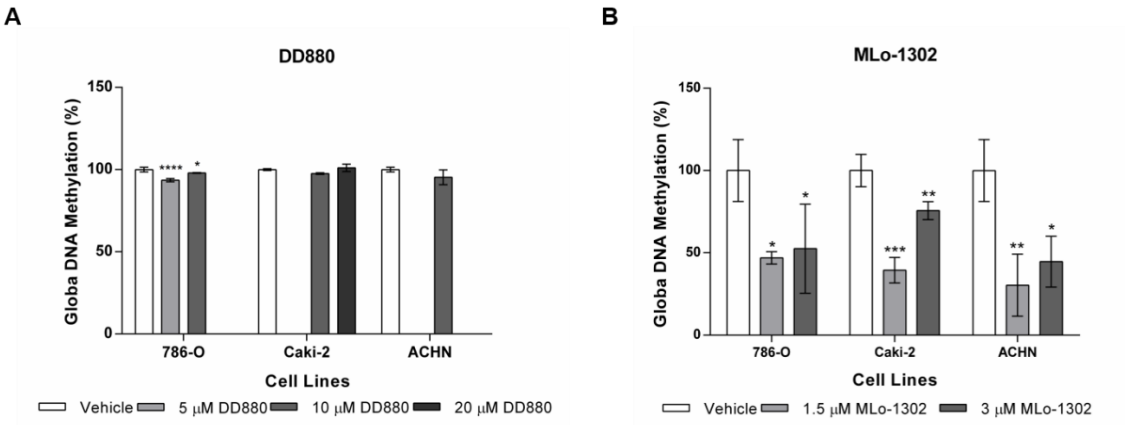


Figure 16 - Effect of **(A)** DD880 and **(B)** MLo-1302 on global DNA methylation of 786-O, Caki-2 and ACHN. Data are presented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

4. Flavanones-derived compounds induced re-expression of genes epigenetically silenced in RCC

The promoter methylation and expression levels of *RASSF1A*, a gene known to be regulated by hypermethylation in RCC, were evaluated. Although exposure to DD880 resulted in an unexpectedly increase of *RASSF1A* methylation levels in 786-O and ACHN (Figure 17A), significant increased expression levels were observed in all RCC cell lines treated with 10 µM DD880 (Figure 17A). Regarding MLo-1302, a significant *RASSF1A* promoter demethylation and concomitant re-expression was depicted in ACHN cell line exposed to 3 µM concentration (Figure 17B).

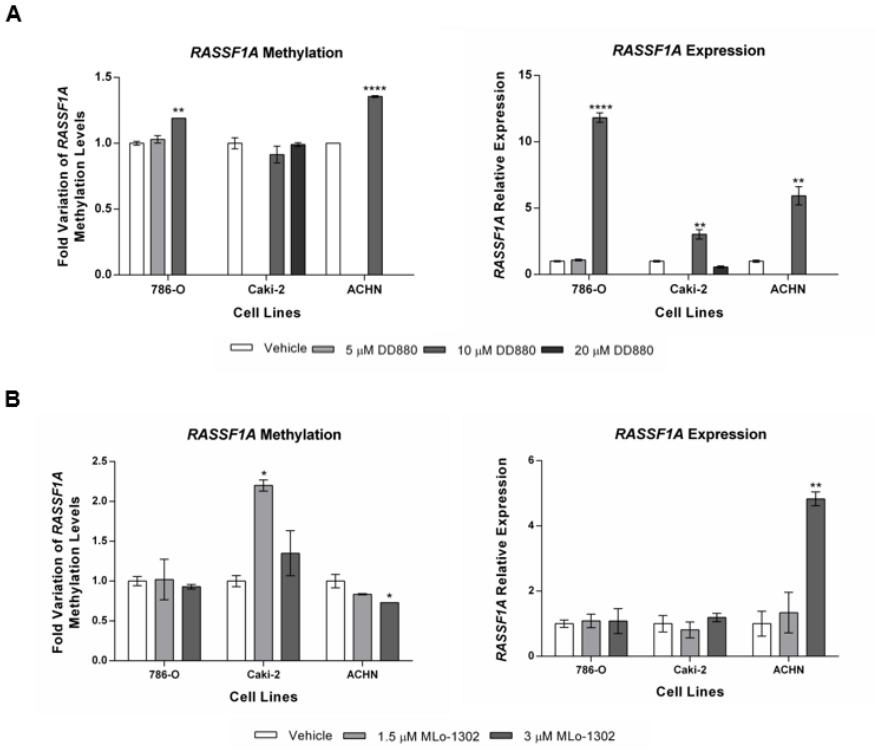


Figure 17 – Effect of **(A)** DD880 and **(B)** MLo-1302 exposure on *RASSF1A* methylation and expression levels, normalized to *ACTB* and *GUSB*, respectively, in vehicle and drug exposed cells. All data are represented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01; ****p<0.0001).

5. MLo-1302 induced alterations in DNMT3A activity in human RCC cell lines

Due to the surprisingly decrease in *DNMT3a* expression and in the global DNA 5-methylcytosine content induced by MLo-1302, the activity of this enzyme was also evaluated in RCC cells. Interestingly, a statistically significant 56% reduction of DNMT3A activity was found in Caki-2 exposed to 3 µM MLo-1302 (Figure 18). Conversely, a 24% significant increase was observed for 786-O cell line treated with 1.5 µM MLo-1302 (Figure 18). Although without reaching statistical significance a 40% decrease in DNMT3A activity

was depicted by ACHN exposed to both concentrations (p=0.075 for 1.5 µM MLo-1302 and p=0.066 for 3 µM MLo-1302) (Figure 18).

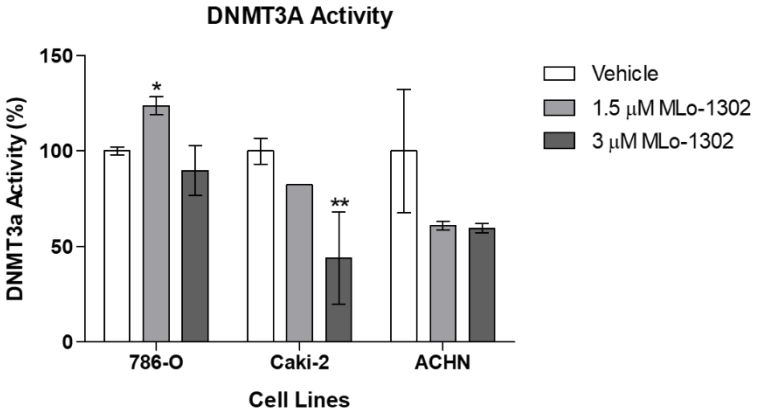


Figure 18 – Effects of MLo-1302 exposure on DNMT3A activity of RCC cell lines compared with vehicle. Data are presented as mean of three independent experiments ± s.d. (*p<0.05; **p<0.01).

V. DISCUSSION

Discussion

Cancers of the bladder, kidney and prostate rank among the ten most common malignancies worldwide, affecting mainly middle-age individuals. Currently, due to the important morbidity, they represent a heavy economic burden on the healthcare system. Moreover, among all genitourinary tumors, RCC is the most lethal [307, 308]. Despite the use of modern imaging methods that allow early detection of localized RCC, enabling curative therapeutic options, about 25-30% of the patients progressively develop metastasis and die [309, 310]. Moreover, about one third of RCC patients are already diagnosed with metastatic disease. At this disease stage, therapy regimen with TKIs allows for disease control, increasing survival for up to 9 months. This treatment, however, is not curative and eventually all metastatic RCC (mRCC) patients will develop TKI-resistance, facing an extremely poor prognosis, with a 5-year survival rate inferior to 10% [162, 163]. Therefore, the development of novel and more effective drugs for treatment of advanced disease is mandatory.

Deregulation of epigenetic machinery has been implicated in renal carcinogenesis. Until now, many efforts have been done to identify target genes dysregulated through epigenetic mechanisms [311]. Importantly, several TSGs involved in pathways that contribute to cellular homeostasis maintenance were found silenced by promoter methylation in RCC [177, 311]. In fact, TSGs hypermethylation has been reported in about 20% and 7% of ccRCC and pRCC, respectively [312, 313]. Moreover, these carcinomas also present a CpG island methylator phenotype (CIMP) which confer a more aggressive behavior, specifically in pRCCs which was associated with worse overall survival [313, 314]. DNMT1, 3A and 3B proteins were also found overexpressed in the three most frequent sporadic RCC subtypes being associated with poor prognosis [315]. Therefore, DNMT inhibitors might be a useful therapeutic tool for RCC. Indeed, the two FDA-approved nucleoside DNMTi (although for hematological neoplasia) have already been used in RCC clinical trials as monotherapy or in combination with other therapeutic agents [208-210]. However, compared with hematological malignancies, 5-azanucleosides showed reduced effectiveness in RCC, similarly to other solid tumors. This might be due to limited incorporation into DNA, since RCC cells are much less proliferative when compared to hematological neoplasms [316]. Thus, non-nucleoside DNMTi, which do not need to be incorporated into DNA, might overcome this limitation. Remarkably, most of DNMTi compounds were intended to target DNMT1, whereas DNMT3A and 3B inhibition has been less explored. Although DNMT1 is the most important DNMT enzyme due to its role in DNA methylation marks maintenance, DNMT3A and 3B, once responsible for *de novo* methylation, should also be considered.

In this study, we report for the first time, the anti-neoplastic activity of four newly synthetized flavanones-derived compounds in RCC cell lines. Interestingly, these compounds reduced cell viability and induced cell death through apoptosis in a dose- and time-dependent manner, being the most effective response achieved with the highest concentration for all the compounds. Overall, the phenotypic assays revealed that DD880 and MLo-1302 were the most effective compounds. Remarkably, a 91.5% reduction of cell viability in 786-O cell line after 72h exposure to 10 µM DD880 was observed. This result surpasses the 23.6% inhibition obtained with 20 µM EGCG, another polyphenol compound, after 6 days of treatment [317]. Moreover, in a recent study, exposure of 786-O cells to 100 µg/ml EGCG during 48h resulted in a 30-35% reduction of cell viability [318], corresponding to a 10-fold higher concentration than the highest used in our study. In fact, 30 µM MLo-1507 decreased 786-O growth in about 64% within the same exposure time. Comparing to 5-aza-2'-deoxycytidine, which reduced Caki-2 cell viability in approximately 53% and augmented apoptosis 4.38-fold after 96h of treatment with 5 µM concentration [178], 3 µM MLo-1302 decreased Caki-2 cell viability and increased apoptosis in a more expressive manner (90.5% and 13.8-fold, respectively), suggesting that MLo-1302 might be a more effective anti-neoplastic compound. Importantly, both reduction of cell viability and apoptosis increase were confirmed at the molecular level through an overall significant decrease of *KI67* transcript levels, a well-known cell proliferation marker, and increased *CDKN1A* gene expression, after exposure to either DD880 or MLo-1302 compounds. Moreover, significantly augmented *CASP3* mRNA expression also corroborates the results obtained with apoptosis assay, for both compounds.

All the four tested flavanones-derived compounds induced alterations in cell morphology of the three RCC cell lines tested. The increased cell sphericity depicted after compound exposure is, indeed, a marker of the acquisition of a more epithelial phenotype indicating attenuation of aggressiveness. This is in accordance with the previous phenotypic results regarding cell viability impairment and apoptosis induction. Interestingly, MLo-1302 induced a dose-dependent increase in 786-O and ACHN cell area. This might be due to cell swelling, suggesting cell death due to necrosis. However, since cell area increment was followed by increased heterochromatic foci, it might also be indicative of cellular senescence. Therefore, the mechanism of death in these two cell lines requires further elucidation. Conversely, the remaining compounds disclosed a significant reduction in cell area in all tested cell lines, which along with increased heterochromatic regions and cellular fragmentation, are suggestive of the apoptotic effect, supporting the results obtained with the APOPercentage™ assay. Furthermore, and except for MLo-1508 in ACHN, marked DNA damage was also depicted after exposure to the tested compounds in both

concentrations. These results might be of particularly importance, since DNA repair has been implicated in therapy-resistance [319]. Therefore, DNA repair machinery impairment induced by these compounds might be beneficial for standard cancer therapy. Moreover, DNA damage-induced phenotype might render RCC cells sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors [320], and thus, these compounds might also be useful as chemosensitizers.

Interestingly, lower MLo-1302 concentration (1.5 µM) decreased global DNA methylation content in all tested RCC cell lines. In fact, a 70% reduction was achieved in ACHN and 55-60% for the remaining cell lines. This results are in line with the 50% global 5-methylcytosine content reduction observed in Caki-2 cell line treated with 1 µM 5-aza-deoxycytidine reported elsewhere [183]. Interestingly, MLo-1302 was also the flavanones-derived compound that presented the greatest DNMTs expression inhibitory effect, with significant *DNMT1* expression reduction in Caki-2, and *DNMT3a* downregulation in all RCC cell lines. Intriguingly, this compound also led to 56% decrease in DNMT3A activity in Caki-2 cell line. Nonetheless, because MLo-1302 was synthesized to be a negative control for DNMT3A inhibition, these results are somewhat unexpected. Indeed, it is noteworthy that this compound has a IC50 value similar to 5-aza-2'-deoxycytidine and displayed a higher phenotypic impact in RCC cell lines. It is widely known that natural compounds (including curcumin, ECGC, genistein and PEITC) downregulate DNMTs expression in several cancer models, including prostate, colon, esophageal and breast cancer [104, 106, 118, 265, 321]. However, they are weak DNMTs inhibitors in RCC cell lines. In fact, in ACHN cell line, 50 µM genistein only reduced DNMT3B expression in about 27%, whereas no significant effect was observed for DNMT1 and DNMT3A [119]. Thus, the hypothesis that MLo-1302 is a new anti-neoplastic compound with impact on DNA methylation should be considered and further explored. Concerning the other three compounds, DD880 reduced *DNMT3a* transcript levels in ACHN, while MLo-1507 and MLo-1508 decreased *DNMT3a* expression in Caki-2. No significant inhibition of DNMTs expression was detected in 786-O cells. These results might be related to the fact that ACHN and Caki-2 (both pRCC), exhibit higher global DNA methylation than 786-O, a ccRCC cell line.

Furthermore, increased TETs mRNA expression levels, suggestive of an active demethylating mechanism, was observed in all RCC cell lines treated with DD880 and MLo-1302. After exposure to both compounds, the highest expression levels were observed for *TET1*. Importantly, this enzyme was previously reported to be downregulated in RCC, correlating with poor prognosis. Similarly, in ACHN cells, *TET1* ectopic overexpression significantly associated with decreased cell viability and invasion, as well as increased apoptosis [322]. Overall, these data suggest that phenotypic effect of these two novel

compounds may result, at the least partially, from induced *TET1* re-expression. Thus, *RASSF1A* re-expression might result from the combined increased TETs expression and reduced DNMTs expression and activity. *RASSF1A* silencing by promoter methylation is present in about 30-100% of RCC [172, 323]. Demethylation and respective re-expression (6-fold) was only achieved in ACHN treated with 3 µM MLo-1302. Conversely, exposure to the highest concentration of DD880 was associated with increased *RASSF1A* methylation levels in both 786-O and ACHN, whereas *RASSF1A* re-expression was observed in all DD880 treated RCC cells.

It should be recalled that epigenetic regulation comprise several interconnected and complex mechanisms, including histone post-translational modifications [10]. Indeed, natural compounds such as ECGC, genistein and curcumin also modulate HDACs and induce histone acetylation in other cancer models [324-327]. In RCC, exposure of ACHN cells to 50 µM genistein incremented histone acetylation along with repressive marks inhibition [119]. Additionally, most of the natural compounds exhibit stereogenic centers and fused ring systems which render them as higher complex molecules that simultaneously act in several targets. Their ability to interact with several protein families, like DNMTs, HDACs, HATs, hinder the precise characterization of its anti-neoplastic effects, being difficult to distinguish whether their effects result from direct modulation of one or more epigenetic targets [328]. This might explain the observed *RASSF1A* re-expression without significant promoter demethylation. Therefore, the interaction of these drugs with other epigenetic enzymes, such as histone acetylases and deacetylases should not be excluded and must be further investigated. Conversely, *RASSF1A* re-expression through promoter demethylation in ACHN cell line treated with MLo-1302 represents a pivotal result concerning the mode of action of this compound. In fact, similar results were achieved with 200 nM 5-aza-2'-deoxycytidine after 2-6 days of treatment of the same line [329]. Likewise, 786-O cells exposure to 5 µM 5-aza-2'-deoxycytidine reduced *RASSF1A* promoter methylation and a 50 to 100-fold re-expression was achieved [185].

Nonetheless, since MLo-1302 does not need to be incorporated into DNA molecule and was demonstrated to attenuate malignant features in RCC at low concentrations, it might be useful in clinical practice. Importantly, when compared to synthetic agents, natural compounds, which are present in the regular diet, have been considered safer and more advantageous regarding their tolerability and availability [104]. Although epidemiological studies have shown a correlation between increased antioxidants intake and lower tumor morbidity and mortality rates [330, 331], there is no data available regarding the anti-neoplastic activity of flavanones family in kidney cancer [116]. Thus, our study might well represent the first approach to this important issue.

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

Herein, we demonstrated that the four newly synthesized flavanones-derived compounds attenuated RCC malignant phenotype. Moreover, MLo-1302 was the most effective compound in tumor growth inhibition. Importantly, this agent was not only able to efficiently induce phenotypic alterations in RCC cells, but also significantly decreased DNA methylation at both global and loci specific levels. Further studies are now mandatory to disclose its role as a novel anti-neoplastic DNMTi for RCC therapy.

Future Perspectives

In this work, we have investigated four newly synthesized flavanone-derived compounds as potential anti-neoplastic agents. Although their tumor-growth inhibitory effect was proven, there is much to be unveiled about their mechanism of action. Indeed, the expression of genes, both at transcript and protein levels, representative of the cancer hallmarks, need to be evaluated to further validate the previous results and to explore their therapeutic potential for RCC management.

Additionally, since phenotypic data suggested an effect in cellular senescence and DNA repair, the assessment of key players of these mechanisms, such as *Laminin*, *E-cadherin* and *PARP1,* might also be investigated.

To validate the demethylation activity of these compounds, we plan to evaluate the methylation and expression levels of additional epigenetically regulated genes implicated in renal carcinogenesis. It would also be interesting to understand the apparently contradictory results obtained for *RASSF1A,* assessing the effect of these compounds in chromatin remodeling, namely by studying HDACs deregulation as well as alterations in histone acetylation (e.g. H3ac and H4ac).

Finally, the activity levels of DNMT1 and 3B and proteins levels of the three DNMTs implicated in CpG methylation should also be evaluated in RCC cells exposed to the flavanone-derived compounds to accurately identify their targets.

VII. REFERENCES

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VIII. APPENDICES

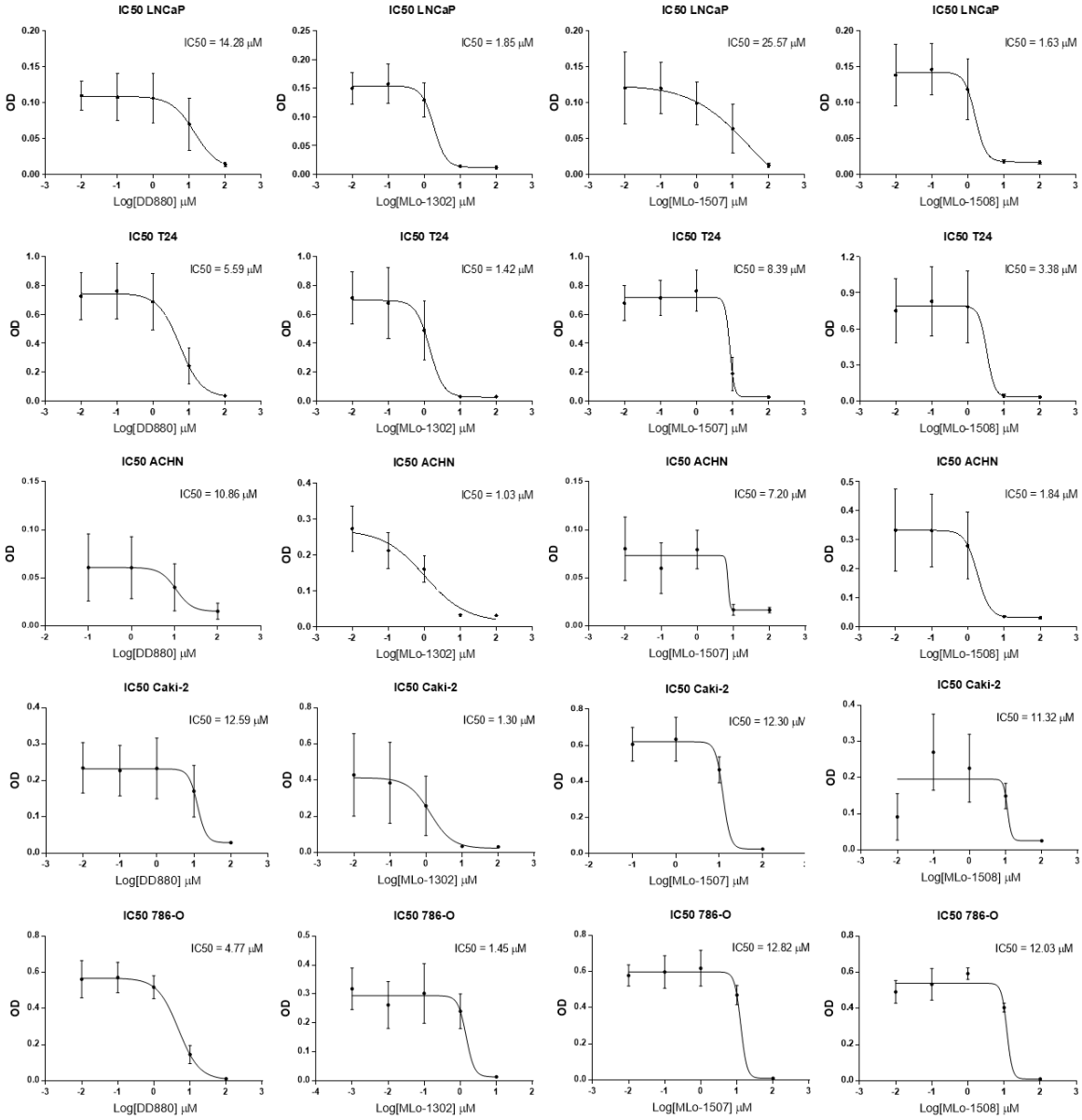
APPENDIX I – DNA Extraction: Phenol-Chloroform Method

The cell lines' pellet was centrifuged at 13 000 rpm for 5 minutes. Then, the samples were digested in 500 µL of SE solution (75 mM NaCl and 25 mM EDTA), 30 µL of 10% SDS (Sigma-Aldrich, Germany) and 15 µL of proteinase K (20 mg/mL) (NZYTech, Portugal) by incubation overnight in a bath at 55°C, by adding proteinase K (20 mg/mL) every 12h until completed digestion.

DNA extraction was performed within Phase Lock Gel Light tubes (5PRIME, Germany) previously centrifuged at 12 000 rpm for 5 minutes, at 4°C. After that, 500 µL of phenol-chloroform solution at pH=8 (Sigma-Aldrich, Germany; Merck, Germany) and samples were added to the tubes. The mixture was centrifuged at 13 000 rpm for 15 minutes, at 4°C, and the upper aqueous phase containing DNA was transferred to new 2 mL tubes together with two-fold volume of absolute ethanol (Merck, Germany) and 1/3 volume of 7.5 M Ammonium acetate (Sigma-Aldrich, Germany) of original amount of this phase. If necessary, 2 µL of glycogen may be added to help in DNA precipitation.

The samples were placed overnight at -20°C to precipitate DNA. These components were centrifuged for 20 minutes at 13 000 rpm and at 4°C. The supernatant was discarded and washed twice with cold 70% ethanol. Lastly, pellets were air dried and eluted in sterile distilled water (B. Braun, Melsungen, Germany) with a volume that depends on pellet size.

APPENDIX II – Dose-response curves



APPENDIX III – Sodium Bisulfite Modification: EZ DNA Methylation-Gold™ Kit

Genomic DNA extracted from cell lines was submitted to bisulfite sodium conversion and 1000 nanograms of DNA were used, according to DNA concentration obtained. Sterile distilled water was added depending on the DNA volume of each sample necessary to obtain 1000 nanograms, up to a total volume of 20 µL. Besides, 130 µL of CT Conversion reagent was also added to each sample. After centrifugation at 4 000 rpm for 30 seconds, the mixtures were incubated in MyCycler™ Thermal Cycler System (Bio-Rad, California, USA) for 10 minutes at 98°C and 3 hours at 64°C, for DNA denaturation and sodium bisulfite conversion.

After the incubation, samples were transferred to a Zymo-Spin IC™ column with 600 µL of M-binding buffer and centrifuged for 30 seconds at 10 000 rpm. Then 100 µL M-Wash buffer were added and another centrifugation was performed. Following, 200 µL of M-Desulphonation buffer was added to column and then incubated for 20 minutes at room temperature. An additional centrifugation, two washing steps were performed.

Finally, the column was placed in a 1.5 mL tube to collect DNA by elution with sterile distilled water, incubation for 5 minutes at room temperature and 30 seconds of centrifugation at 12 000 rpm. This step was repeated, wherein the volume eluted was dependent on the initial quantity of DNA.

APPENDIX IV – RNA Extraction: TRIzol®

The RNA extraction solution (Trizol) was added to the pellet of cells and mixed with a syringe. After 5 minutes of incubation at room temperature, 200 µL of chloroform was added, and then incubated again for 3 minutes at room temperature. Following, a 15 minutes' centrifugation at 10 600 rpm, at 4°C, was performed, and the supernatant was transferred to RNAase free tubes. To RNA precipitation, 500 µL of isopropanol were added followed by incubation at room temperature for 10 minutes. After that, the samples were centrifuged at 10 600 rpm for 10 minutes, at 4°C, and the supernatant was discarded. Lastly, 1 mL of 75% ethanol was added to wash the pellet followed by a 5 minutes' centrifugation at 8 400 rpm, at 4°C, and the supernatant was posteriorly rejected. Pellets were air dried and resuspended in 30-200 µL of RNA Storage Solution. After 30 minutes on ice, the RNA was storage at -80°C.

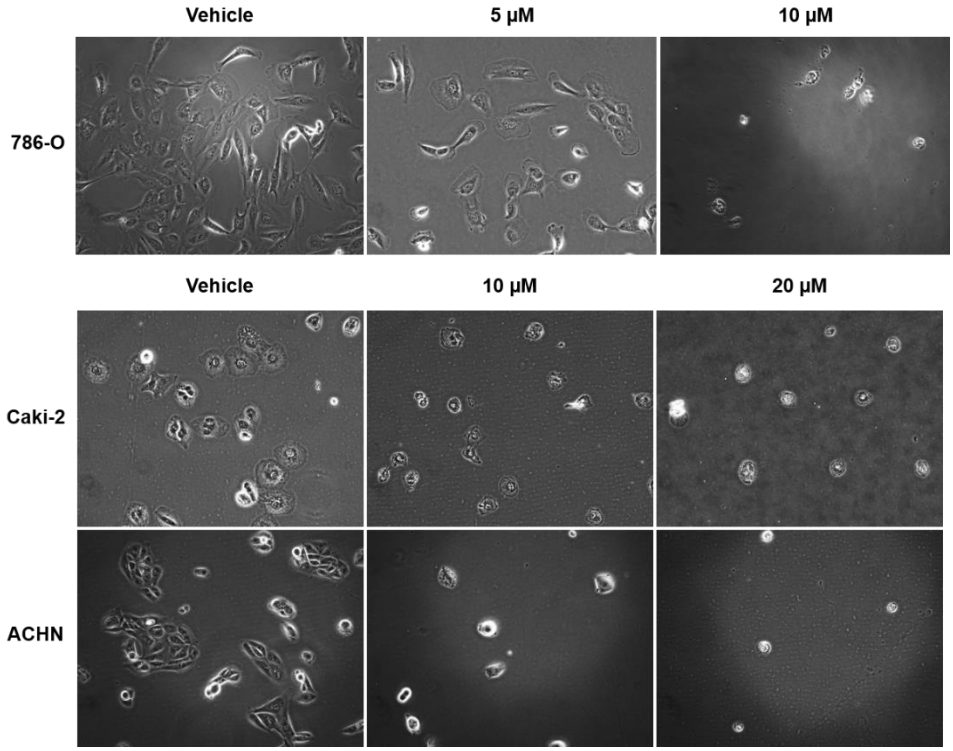
APPENDIX V – cDNA Synthesis: RevertAid Reverse Transcription Kit

The amount of RNA to synthetize 1000 nanograms was calculated for each sample, according to RNA concentration obtained, and sterile distillated water was added in order to achieve a final volume of 11 µL within PCR tubes. Additionally, 1 µL of Random Hexamer primer was added to the mixture and all the tubes were incubated at 65°C for 5 minutes in MyCycler™ Thermal Cycler System.

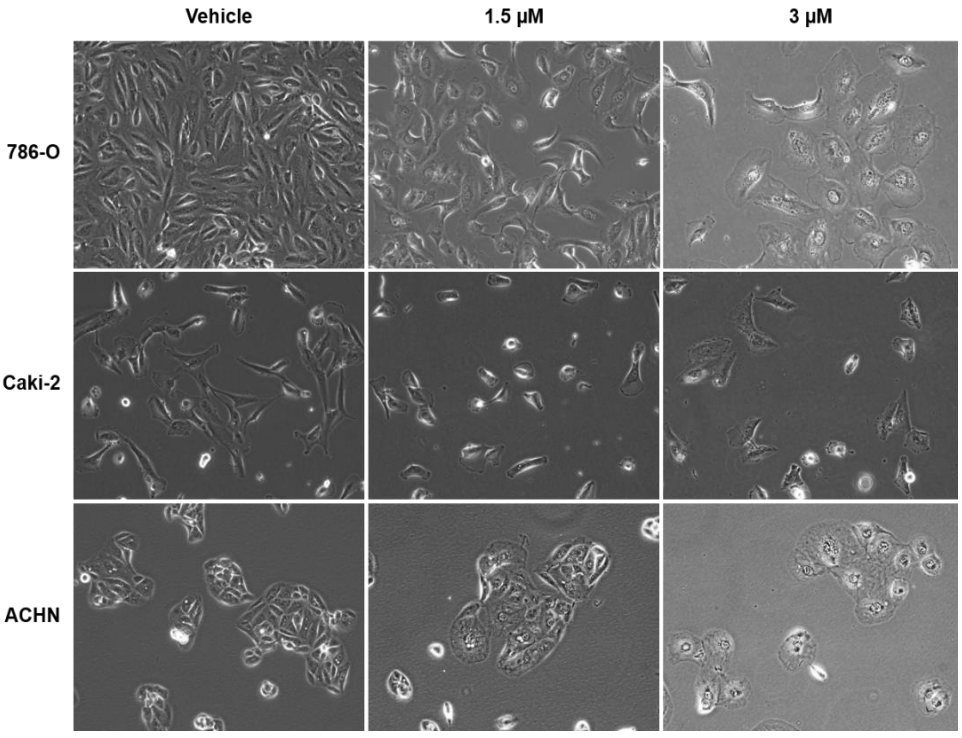
A mix containing 5x reaction buffer, ribolock RNase inhibitor (20 U/µL), 10 mM dNTPs Mix, and ReverAid RT (200 U/µL) was prepared with a total volume of 8 µL per well, and then added to the samples' tubes. Lastly, tubes were centrifuged at 1 000 rpm for 1 minute and submitted to one PCR cycle including 5 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 70°C in MyCycler™ Thermal Cycler System. All the samples were storage at -20°C.

APPENDIX VI – Illustrations of drug-induced morphometric alterations

DD880



MLo-1302



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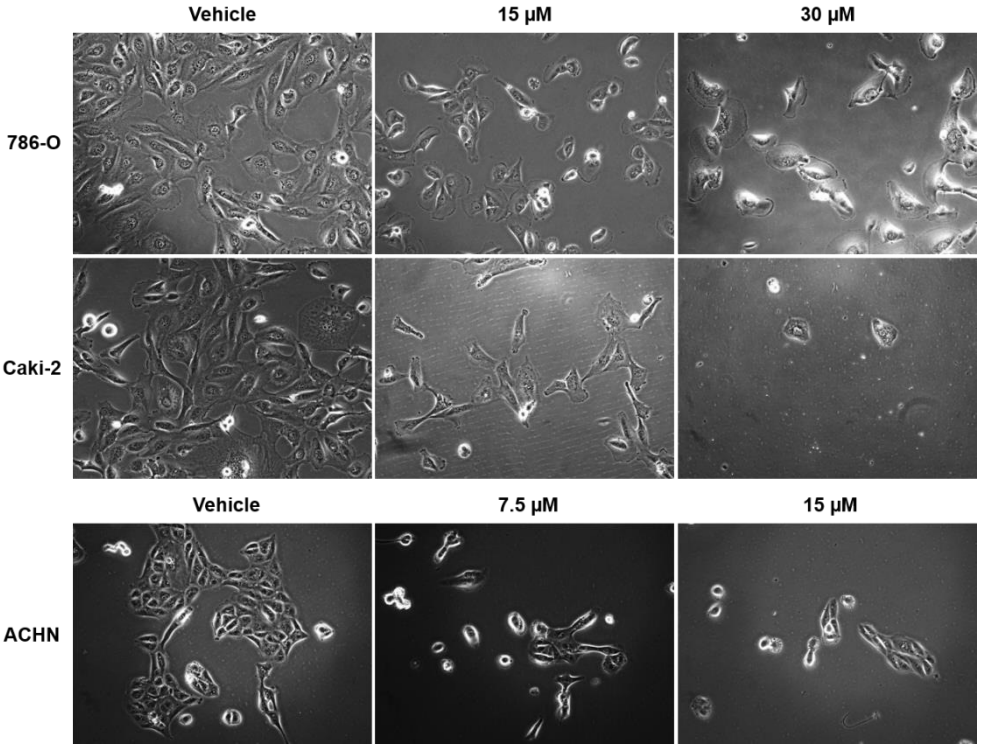
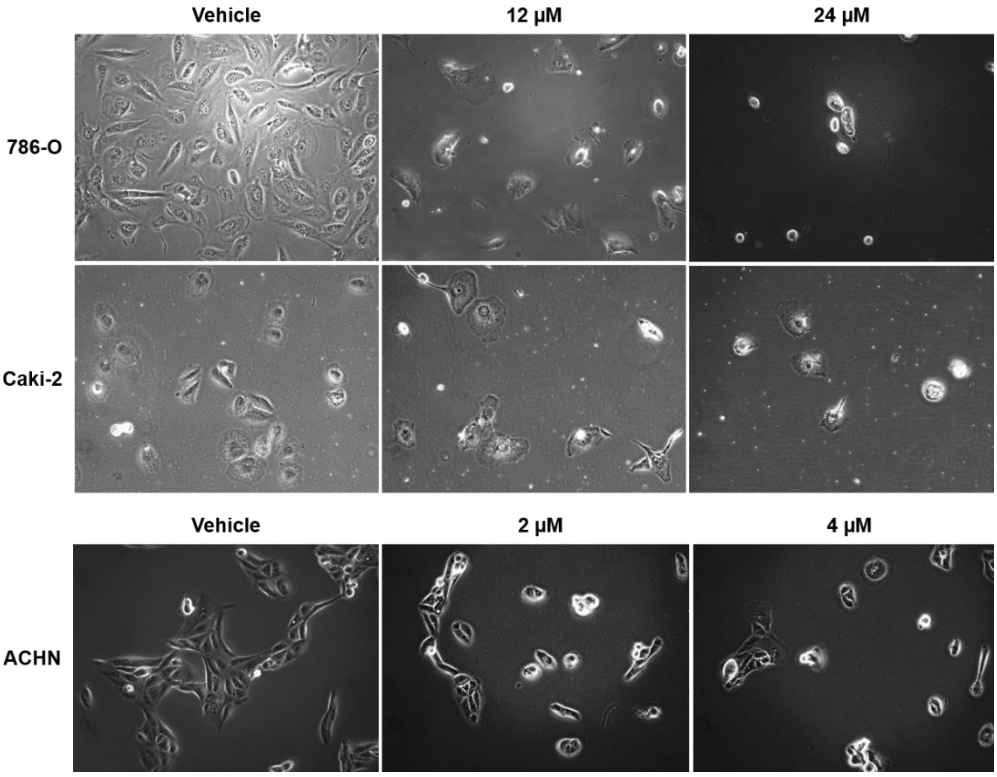
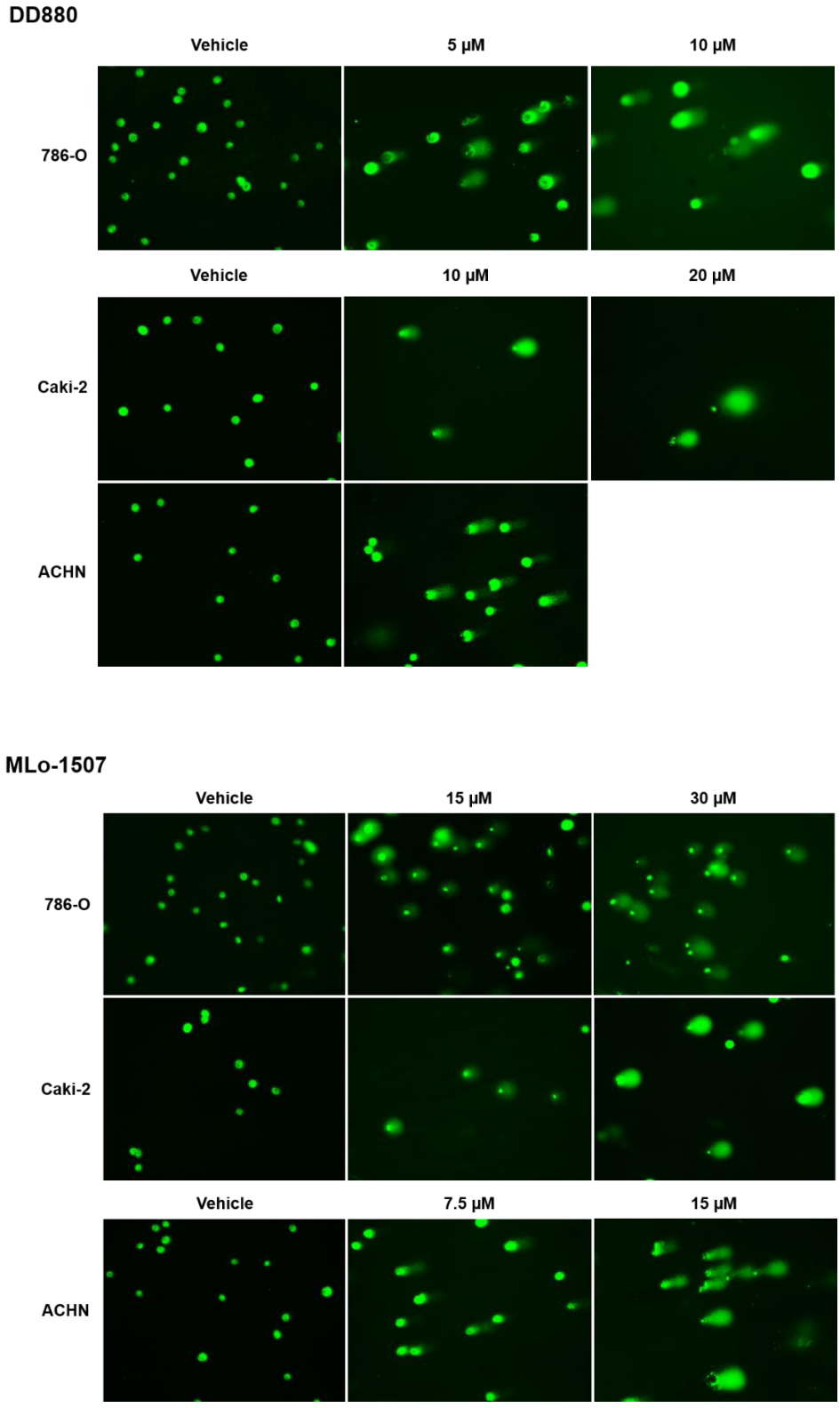
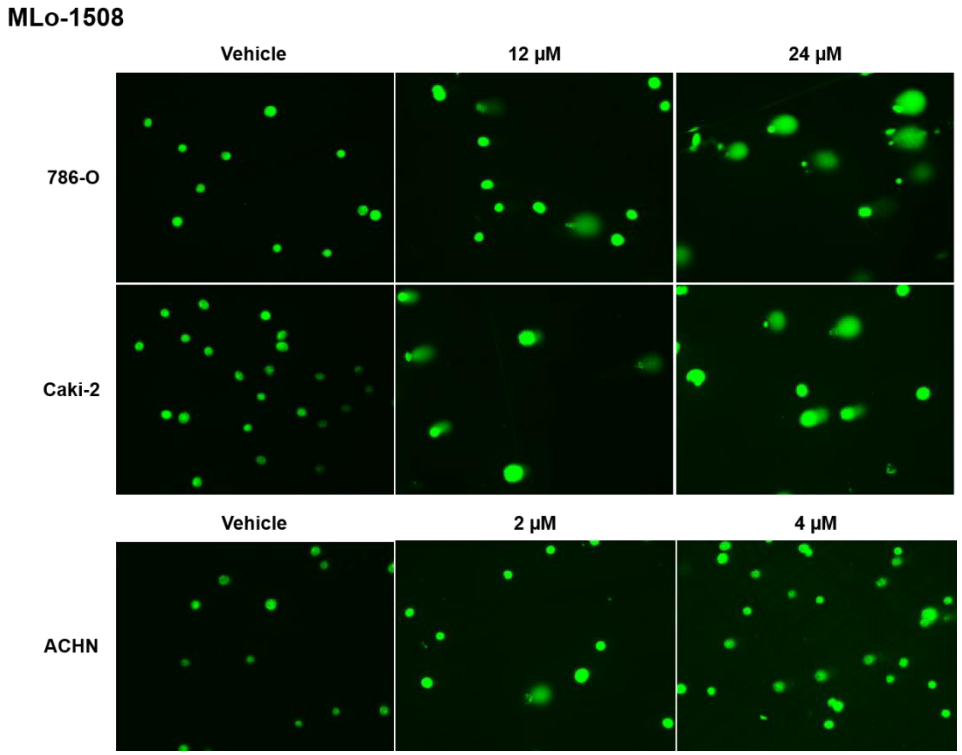


MLo-1508



APPENDIX VII – Illustrations of drug-induced DNA damage




MLo-1508
Vehicle
12 μM
24 μM
786-O
Caki-2
Vehicle
2 μM
4 μM
ACHN