Anti-neoplastic effect of MLo-1302 in Testicular Germ Cell Tumors: comparation with classical differentiating and demethylating agents, ATRA and DAC

Ana Rita Almeida Cardoso

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

U.PORTO

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ANA RITA ALMEIDA CARDOSO

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Universidade do Porto

Orientadora:

Professora Doutora Carmen de Lurdes Fonseca Jerónimo

Professora Catedrática Convidada do Departamento de
Patologia e Imunologia Molecular do Instituto de Ciências
Biomédicas Abel Salazar da
Universidade do Porto
Investigadora Auxiliar e Coordenadora do Grupo de
Epigenética e Biologia do Cancro - Centro de Investigação
do Instituto Português de Oncologia do Porto Francisco
Gentil, E.P.E.

Coorientadora:

Doutora Vera Mónica Miranda Gonçalves

Investigadora Júnior do Grupo de Epigenética e Biologia do
Cancro - Centro de Investigação do Instituto Português de
Oncologia do Porto Francisco Gentil, E.P.E.

“Adoramos a perfeição, porque não a podemos ter; repugná-la-íamos se a tivéssemos.
O perfeito é o desumano porque o humano é imperfeito.”

Fernando Pessoa

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RESUMO

Os tumores de células germinativas do testículo (TCGTs) são um grupo heterogéneo de tumores que afetam essencialmente jovens adultos entre os 14 e 44 anos, representando a neoplasia sólida mais comum nesta faixa etária, em todo o mundo. Com a introdução da cisplatina como método terapêutico, a taxa de sobrevida destes pacientes aumentou para 95% aos 5 anos, tornando os TCGT num modelo de doença curável. Contudo, alguns pacientes com doença avançada e/ou metastática adquirem resistência à cisplatina, não existindo, na atualidade, opções terapêuticas.

As alterações epigenéticas constituem uma importante característica dos TCGT, as quais podem estar envolvidas nos mecanismos de resistência à cisplatina. Adicionalmente, estas alterações podem ser usadas como potenciais alvos terapêuticos. De facto, muitos agentes epigenéticos têm sido avaliados no que concerne à sua eficácia terapêutica. 5-azacitidina e 5-aza-2'-deoxicitidina/Dacitabina (DAC) são inibidores das metiltransferases do DNA (DNMTs) e os compostos mais estudos no que diz respeito aos TCGT e, apesar dos estudos *in vitro* e com modelos animais demonstrarem propriedades anti-neoplásicas efetivas, os ensaios clínicos não têm resultados satisfatórios. Desta forma, torna-se imprescindível o investimento em novos compostos. Assim, o principal objetivo desta Dissertação foi avaliar o potencial terapêutico do MLo-1302 (novo composto desenhado para inibir as DNMTs) em linhas celulares de TCGTs e comparar com o efeito do agente desmetilante DAC e do agente diferenciador ácido retinóico (ATRA).

Para isso, linhas celulares de TCGT (NCCIT, NTERA-2 e 2102EP) foram tratadas com ATRA, DAC e MLo-1302. Inicialmente, o ensaio da resazurina foi realizado para avaliar a viabilidade celular. Após o tratamento, a proteína e o DNA foram extraídos para a realização de *western blot*, PCR específico de metilação quantitativo, *dotblot* e imunofluorescência para avaliar a expressão de marcadores associados à pluripotência/diferenciação (NANOG, OCT3/4 e SOX2/PAX6) e das DNMTs (DNMT1, DNMT3A e DNMT3B), bem como avaliar o padrão de metilação a nível global (5mC) e loco-específico (promotor do *RASSF1A*). RNA foi igualmente extraído para a realização do *RT^2 profiler array* em células tratadas com o composto para verificar a existência de alterações em genes relacionados com a apoptose, ciclo celular, metabolismo e danos no DNA. Foram ainda recolhidos os sobrenadantes após 72h de tratamento com MLo-1302 para a realização do ensaio de citotoxicidade da libertação de LDH. O efeito na apoptose e na proliferação foi também verificado através do *APOPercentage^{TM} kit* e do ensaio de BrdU, respetivamente.

Um efeito parcial do MLo-1302 na diferenciação foi verificado, uma vez que este composto apenas provocou uma diminuição significativa de NANOG em todas as linhas. O mesmo se verificou na metilação, com uma diminuição significativa apenas na DNMT1 e um efeito *minor* no padrão de metilação. Contrariamente, genes associados à promoção da apoptose encontravam-se sobre-expressos enquanto que genes anti-apoptóticos encontravam-se sub-expressos. De facto, MLo-1302 tem efeito na apoptose (e também na proliferação), igualmente demonstrado pelo aumento da caspase clivada 8.

Concluímos então que o MLo-1302 tem um efeito parcial na diferenciação e na metilação em linhas celulares de TCGT, promovendo a apoptose celular mediada por caspases.

ABSTRACT

Testicular germ cell tumors (TGCTs) are heterogeneous tumors that affect mostly young-adult aged between 14 and 44 years, representing the solid neoplasm most common in this age group, worldwide. With the introduction of cisplatin as a therapeutic method, the survival rate of these patients increased to 95% at 5 years, making TGCTs a curable disease model. However, some patients with disseminated and/or metastatic disease acquire cisplatin resistance, currently, there are no therapeutic options.

Epigenetic alterations constitute an important feature of TGCTs, which are implicated in resistance mechanisms to cisplatin. Additionally, these alterations might be used as potential therapeutic targets. Indeed, several epigenetic agents have been evaluated regarding therapeutic efficacy. 5-azacytidine and 5-aza-2'-deoxycytidine/Dacitabine (DAC) are DNA methyltransferases (DNMTs) inhibitors and the compounds more studied with regard to TGCTs and, although *in vitro* studies and with animal models demonstrate effective anti-neoplastic properties, clinical trials have not had satisfactory results. So, investment in new compounds becomes essential. Thus, the main goal of this Dissertation was to evaluate the therapeutic potential of MLo-1302 (a new compound designed to inhibit DNMTs) in TGCT cell lines and compare with the effect of the demethylating agent DAC and the differentiating agent retinoic acid (ATRA).

For this, cell lines of TGCTs (NCCIT, NTERA-2, and 2102EP) were treated with ATRA, DAC, and MLo-1302. First, resazurin assay was performed to evaluate cell viability. After treatment, protein and DNA were extracted to perform western blot, quantitative methylation-specific PCR, dotblot, and immunofluorescence to evaluate the pluripotency/differentiation-related markers (NANOG, OCT3/4, and SOX2/PAX6) and DNMTs (DNMT1, DNMT3A, and DNMT3B) expression, as well as to assess global (5mC) and loci-specific (*RASSF1A* promoter) methylation status. RNA was also extracted to perform RT^2 profiler array in cells treated with the compound that addressed genes related to apoptosis, cell cycle, metabolism, and DNA damage. Supernatants were also collected after 72h of MLo-1302 treatment to perform the LDH release cytotoxicity assay. The effect on apoptosis and proliferation was also verified using the APOPercentage^{TM} kit and the BrdU assay, respectively.

A partial effect of MLo-1302 in differentiation was verified since this compound only caused a significant decrease in NANOG in all lines. The same was true for methylation, with a significant decrease only in DNMT1 expression and a minor effect in global and loci-specific methylation. Contrarily, genes associated with the apoptosis promotion were overexpressed, while anti-apoptotic genes were downregulated. Indeed, MLo-1302

influences apoptosis (and also proliferation), as well as there is a tendency for an increase in cleaved caspase 8.

Hence, we conclude that MLo-1302 has a partial effect on TGCT cells differentiation and methylation, promoting cell apoptosis mediated by caspases.

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LIST OF ABREVIATIONS

5hmC – 5-hydroximetylcytosine
5mC – 5-methylcytosine
ACTB – Beta-actin
AFP – alpha-fetoprotein
AJCC – American Joint Committee on Cancer
ATRA – All-trans-retinoic acid
BIRC3 – Baculoviral IAD repeat containing 3
BrdU – 5-bromo-2’-deoxyuridine
BSA – Bovine serum albumin
CASP8 – Caspase 8
ChRC – Chromatin remodelling complex
CPT2 – Carnitine palmitoyltransferase 2
DAC – Dacitabine
DAPI – 4’,6’-diamidino-2-phenylindole
DDIT3 – DNA damage inducible transcript 3
DMEM – Dulbecco’s modified Eagle’s medium
DMSO –Dimethyl sulfoxide
DNMT – DNA methyltransferase
DNMTi – DNA methyltransferase inhibitor
GCNIS – Germ cell neoplasia *in situ*
GCT – Germ cell tumors
HAT – Histone acetyltransferase
HDAC – Histone deacetylase
HRP – Horseradish peroxidase
i(12p) – Isochromosome 12p
IF – Immunofluorescence
KDM – Histone demethylases
KDR – Kinase insert domain receptor
KMT – Histone methyltransferase
KRT14 – Keratin 14
LDH – lactate dehydrogenase
LINE1 – long interspersed nuclear element 1
miRNAs – microRNA
NOL3 – Nucleolar protein 3
NS – Non-seminoma

PBS – Phosphate-buffer saline
PGF – Placental growth factor
PPP1R15A – Protein phosphatase 1 regulatory subunit 15A
PROTAC – Proteolysis targeting chimeras
qMSP – Quantitative methylation-specific PCR
ROS – Reactive oxygen species
RT-PCR – Reverse transcription polymerase chain reaction
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE – Seminoma
SNAI3 – Snail homolog 3
TBST – Tris-buffered saline – tween 20%
TCGA – The Cancer Genome Atlas
TET – Ten-eleven translocation enzymes
TGCT – Testicular germ cell tumor
TNM – tumor–node–metastasis
TSG – Tumor suppressor gene
VEGFR2 – Vascular Endothelial Growth Factor Receptor 2
XIST – X-inactive specific transcript
βHCG – human chorionic gonadotropin β
λref – Reference wavelength

INTRODUCTION

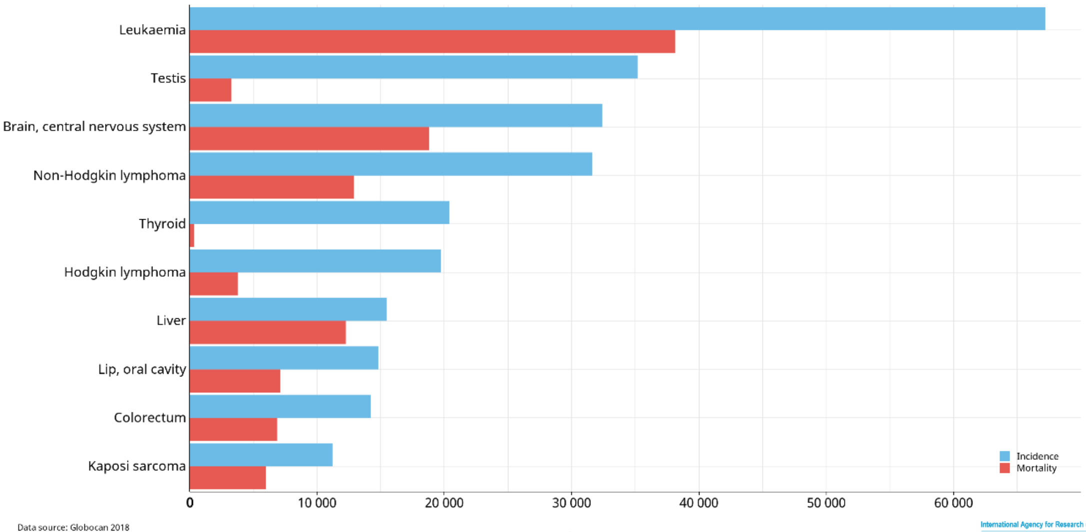
1. Testicular Germ Cell Tumors

The testis is the organ of the male genital system responsible for production of the male gametes. It is a complex structure composed of several cell types, such as germ cells, Leydig cells, Sertoli cells, mesothelial cells, mesenchymal cells, among others. Each cell type can give rise to a neoplasm, hence the wide variety of tumors within this organ. Nevertheless, around 95% of testicular cancers are originated from germ cells that fail to complete their normal differentiation – and these are called testicular germ cell tumors (TGCT) (1, 2).

TGCTs are heterogeneous cancers most frequent in young men (1). They are clinically relevant, representing the main cause of morbidity and mortality in this age group. Besides this, some diagnostic, prognostic and therapeutic challenges remain in the field (highlighted below throughout this Dissertation) (3, 4), highlighting the relevance of studying this tumor model.

1.1. Epidemiology

TGCTs are not prevalent cancers. According to Globocan 2018 (5), they represent the 21st most incident neoplasm in men worldwide, with 71,105 new cases per year. However, as already stated, they are most frequent in young men, being the second most incident neoplasm in young-adult men (aged until 34 years), where this statistic being only surpassed by the liquid tumors (leukaemias) – Figure 1. In Portugal, the numbers are similar, with our country registering 150 to 200 new cases per year.



Data source: Globocan 2018
Graph production: Global Cancer Observatory (http://gco.iarc.fr)

Figure 1: Estimated number of incident cases and deaths worldwide in men aged between 0 and 34 years. In: Globocan 2018 (5).

The incidence of these tumors is highest in northern Europe, while they are relatively low in African countries. Indeed, studies show that 1% of men population will be diagnosed with testicular cancer annually in Croatia, Slovenia and Norway (6). In the United States of America, the statistics are similar, showing that TGCTs are more common in white individuals (6.9/100,000) than in African Americans (1.2/100,000) (7, 8). Moreover, the incidence has been increasing, like in other continents (with a total of 85,635 new cases expected for 2040) (5), but the reasons for this increase are still not completely elucidated. However, it has been described that the increase of TGCTs can be associated with changes in lifestyle and environmental factors. In fact, the "genvironmental model" of understanding this disease fits both the changing epidemiology of this cancer type and the intricate association with disorders of sex development (2, 9, 10).

At initial diagnosis, approximately 70% of patients have stage I disease (11). So, one can say there is a mismatch between incidence and mortality, the former going up and the latter going down. In fact, mortality rate for TGCTs is overall very low (Figure 1) (5). The overall 5-year survival rate is approximately 95% and the cancer-specific survival rate at 15 years for patients initially diagnosed with stage I disease is greater than 99% (11). This reflects the high efficacy of current therapies based on the chemotherapy drug cisplatin.

1.2. Risk Factors

The exact aetiology of the TGCTs is still undetermined and subject to intense research. It has been demonstrated that these tumors originate from an unbalance in the complex interplay between genetic and environmental factors, with epigenetics serving as a link between these two aspects of the disease, closely related to developmental biology phenomena (2).

The main risk factors of TGCTs are testicular malformations, such as cryptorchidism (a birth defect in which one or both testicles are not present in the scrotum), hypospadias (a congenital disorder where the urethral opening is not at the head of the penis) and testicular atrophy, or impaired spermatogenesis (11, 12). Other reported factors include inguinal hernia, decreased gestational age, low birthweight, and low parity maternal bleeding. Many of these factors are caused by fetal exposure to high levels of estrogens and anti-androgens or xenobiotics, resulting in disruption of endogenous hormone signalling prenatally and, consequently, to undervirilization of the male embryo *in utero* (13). Evidence is much lower for environmental factors like diet, low physical exercise, some professional hazards (firefighters, metal and leather workers) and testicular trauma (14).

Next to environmental factors, genetics contribute to more than 40% of TGCTs development, representing the third highest rate among all cancer types (11, 15), being only surpassed by thyroid cancer and tumors of endocrine glands (16). Indeed, many alterations and polymorphisms have been described to enhance the tumorigenesis of TGCTs, including both sporadic and familiar tumors. However, these are very sparse, individual contributions are difficult to predict and there is no current alteration that can be truly useful in the clinic. In fact, more than 90% of patients with TGCT do not present family history of the disease (11, 15).

The isochromosome 12p (i(12p) is the most common genetic alteration in TGCTs, which can be found in about 80% of cases of TGCTs (and virtually in all type II TGCTs) (3). Additionally, about 25% of TGCTs present *gr/gr* microdeletion at Y chromosome, leading to removal of part of AZFc region, a low penetrance susceptibility allele frequently associated with male infertility (17).

Proto-oncogene *KIT* is the most commonly mutated gene, with gain of function mutations (12% of the cases) or activating mutations in exon 17 (63-93%), both associated with a high risk for development of TGCT in the contralateral testis (18). Moreover, 8 to 16% of TGCT patients have *KRAS* mutations (18). Moreover, as mentioned, several studies have been reported many susceptibility risk loci, namely single nucleotide polymorphisms in players such as *KITLG*, *SPRY4* and *BAK1* (19), which are implicated in several distinct pathways (2), for example, in sex determination or germ cell specification (*DMRT1, ZFPM1*, and *PRDM14*), centrosome organization or microtubule assembly (*TEX14, PMF1*, and *CENPE*), apoptosis or cell cycle (*GSPT1* and *CHEK2*), and DNA damage repair (*RAD51C* and *BRCA1*) (15, 20, 21).

The main factors predisposing to TGCTs and the associated risk are summarized in Table 1.

1.3. Biology and pathogenesis

Across the years, there has been progressive better and more complete understanding of the biology of these tumors. A developmental perspective over these cancers has led to an integrated classification, universal to all types of germ cell tumors (GCTs) and genders (both testicular, ovarian and extragonadal), based on the biology (including epigenetic profile) of the cell of origin. Seven subclasses (type 0 to VI) are acknowledged (22). Type II TGCTs originate from primordial germ cells or gonocytes that get arrested in differentiation, originating germ cell neoplasia *in situ* (GCNIS), while type I and III emerge from other stages of the germinative linage, and are classified separately as non-GCNIS related tumors (1) (Figure 2).

Type II TGCTs are the most frequent and, at the same time, the most clinically challenging, due to malignant behaviour. They are also histologically the most diverse group and will be the focus of this Dissertation. As mentioned before, they are derived from the precursor lesion GCNIS (1, 2). Although the initial driver in tumorigenesis of these tumors is uncertain, it is known that polyploidization is an early event in the development of GCNIS and this alteration blocks the maturation of gonocytes to prespermatogonia, which is followed by the gain or loss of chromosomal parts. Example of this is the already mentioned i(12p) (11, 23). However, in TGCTs, mutations and amplifications of oncogenes are rather rare, found in a small number of cases (2, 24, 25). The progressive proliferation of these abnormal GCNIS cells can originate seminoma (SE). On the other hand, these cells can undergo a reprogramming process and originate non-seminomas (NS), including the more undifferentiated embryonal carcinoma, and the more differentiated subtypes yolk-sac tumor, choriocarcinoma and teratoma (2, 4) – Figure 2. Mixtures of more than one TGCT component are very frequent (the second most common presentation of the disease, after pure SE), and these are called mixed tumors, and clinically handled as NS.

Table 1: Genetic and environmental risk factors for testicular germ cell tumors. Adapted from: *Lobo et al.* 2019 (2).

Factors	**Relative Risk**
Environmental	
Cryptorchidism	0.5-6x
Hypospadias	1.26-3.61
Testicular atrophy	20.5
Impaired spermatogenesis	1.16-6.72
Inguinal hernia	1.63
Low birthweight	1.28
High levels of estrogens and anti-androgens or xenobiotics	1,3
Genetic	
Familiar risk	-
Brother with TGCT	8-10x
Father with TGCT	4-6x
Twins	-
Monozigotic	76x
Dizigotic	35x
Contralateral tumors	24.8-27.6
Abbreviations: TGCT – testicular germ cell tumors	

Despite the common tumorigenic pathway, SEs and NSs are very different, specifically with very distinct clinical behaviour and morphology. Overall, both present low mutational burden and paucity of genomic aberrations (as mentioned before) (26, 27). Thus, within the developmental biology model, the epigenetic (de)regulation surely contributes to diversity. This can be misused for subtyping, as it dictates phenotypic and clinical variety

(28). Indeed, epigenetic alterations and the enzymes that establish the various epigenetic modifications have importance in the development of TGCTs. It is known that DNA methyltransferase (DNMT) 3A is relevant in proliferation of cancer cells and transformation in SE, while DNMT3B and 3L have an important role in the reprogramming process and, consequently, in embryonal carcinoma formation (29, 30) – Figure 2.

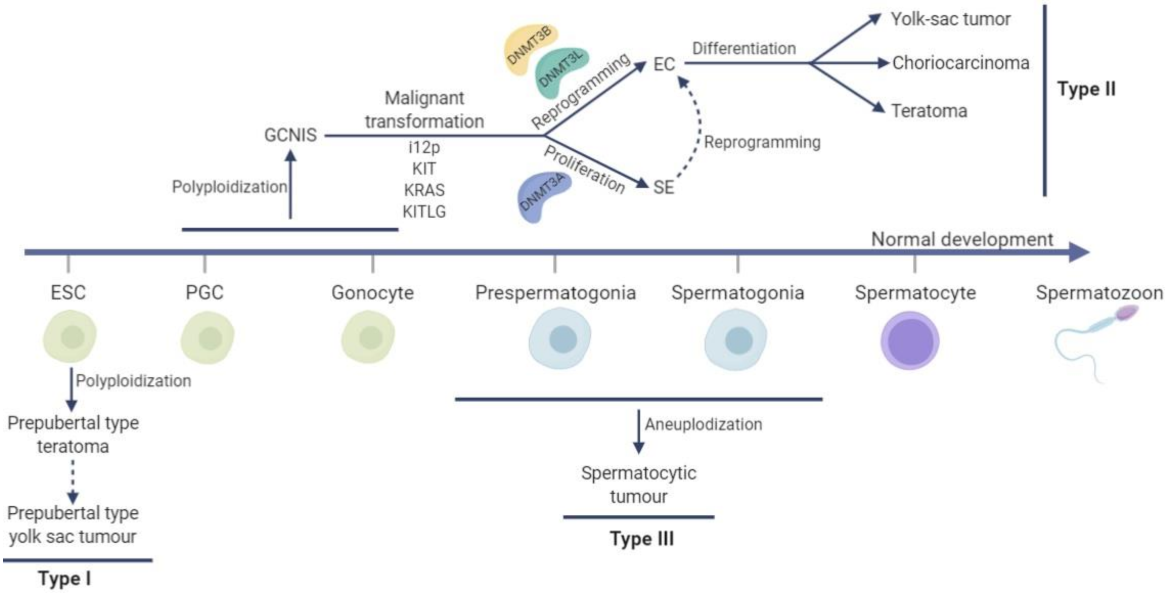

DNMT3B
DNMT3L
Reprogramming
EC
Differentiation
Yolk-sac tumor
Choriocarcinoma
Teratoma
Type II
Malignant transformation
GCNIS
i12p
KIT
KRAS
KITLG
Polyploidization
Proliferation
DNMT3A
SE
Reprogramming
Normal development
ESC
PGC
Gonocyte
Prespermatogonia
Spermatogonia
Spermatocyte
Spermatozoon
Polyploidization
Prepubertal type teratoma
Prepubertal type yolk sac tumour
Type I
Aneuplodization
Spermatocytic tumour
Type III

Figure 2: Pathogenesis of type I, II and III testicular germ cell tumors. The pathobiology of these tumors is related to developmental biology, germ cell development and epigenetic phenomena, especially within the most common type II tumors; ESC – embryonic germ cell; PGC – primordial germ cell; GCNIS – germ cell neoplasia in situ; i12p – isochromosome 12p; EC – embryonal carcinoma; SE – seminoma.

1.4. Additional details on testicular germ cell tumors related to germ cell neoplasia *in situ*

TGCTs related to GCNIS belong to the type II category in the developmental classification of the disease. Type II tumors are most frequent in the testis (although they may appear in the ovary and in extragonadal locations along the midline of the body) (1).

About 50% of patients with TGCT are diagnosed with SE, more frequent in individuals over 35 years, with median ages of 37 years. NSs are more common in younger ages, around 25-30 years (3, 31, 32).

SEs are constitutionally very similar to GCNIS, expressing pluripotency factors like *OCT3/4*, *NANOG* and *SOX17*. These do not express *SOX2*, opposite to embryonal carcinoma (4, 11). Differentiation towards other subtypes leads to switch-off of these pluripotency factors (4). *Nettersheim et al.* (33) demonstrated that *NANOG* regulatory regions can be negatively regulated by epigenetic repression mechanisms, to control pluripotency.

SEs are more sensitive to DNA damage induced by cisplatin and present an overall good prognosis, which is opposite to NSs, which usually display more aggressive course and hence deserve more aggressive adjuvant treatment. In particular, teratomas are resistant to cisplatin, which seems to be dependent on the highly differentiated program of this subtype (34).

While mixed tumors and embryonal carcinoma are rather frequent, pure forms of teratoma, choriocarcinoma or yolk-sac tumor are very rare, usually always seen as part of mixed tumors.

The main features of the different NS subtypes are summarized in Table 2.

Table 2: Main features of non-seminoma tumors. In: 2016 WHO classification (1).

NS subtypes	Description
Embryonal carcinoma	Malignant germ cell tumor composed of tumor cells similar to embryonic stem cells
Teratoma	Malignant germ cell tumor composed of mature tissues that represent one or more of the germinal layers
Yolk-sac tumor	Malignant germ cell tumor that resembles extraembryonic structures, like the yolk-sac, allantois and extraembryonic mesenchyme
Choriocarcinoma	Malignant germ cell tumor that seem like the trophoblastic cells of the extraembryonic chorion/placenta
Abbreviations: NS – non-seminomas	

1.5. Diagnosis and staging

Most men with a TGCT present with a palpable nodule that may not be painful. Physical examination can be indicative of cancer, and lead to performing a scrotal ultrasound; however, the specificity of this test for malignancy is low, with many entities simulating TGCTs, many of them being benign nodules or inflammatory masses. Testicular biopsy is not routinely performed given the risks associated with the procedure, including seeding of tumor cells in case of malignancy. This means that detection of a solid mass on ultrasound will result on the patient being proposed to removal of the testis and spermatic cord (inguinal orchiectomy) (11). The diagnosis is confirmed only by histopathology, which is essential to ensure the appropriate treatment of the disease (11, 35, 36).

Levels of the serum tumor markers, such as alpha-fetoprotein (AFP), human chorionic gonadotropin β (βHCG), and lactate dehydrogenase (LDH), should be evaluated and used for aiding in the diagnosis of TGCTs. However, these are only elevated in around 60% of these tumors and are highly dependent on histology. They are also relevant for monitoring disease progression, with elevations usually indicating metastatic events. Nevertheless, the sensitivity and specificity of these markers is far from ideal: other neoplasms can increase levels of these markers in serum (like gastrointestinal and hepatocellular carcinomas) and LDH is elevated in several benign conditions. Therefore, serum tumor markers should be careful interpreted and differential diagnosis is essential (11, 37, 38).

Staging of the disease relies on radiological examinations, including abdominal and chest computed tomography or magnetic resonance imaging, mainly to visualize the para-aortic lymph nodes, the main lymphatic drainage pathway of these tumors.

Histologically, SE is an uniform neoplasm characterized by a background of fibrous and lymphocytes (Figure 3B). NSs are very heterogeneous: embryonal carcinoma present cells with atypical cytological features and extensive necrosis (Figure 3C), choriocarcinoma is highly haemorrhagic (Figure 3D), teratoma is composed of elements derived from ectodermal, mesodermal and/or endodermal layers (Figure 3E), and yolk-sac tumor has many histological patterns, most commonly reticular and microcystic aspects within a myxoid stroma (Figure 3F) (28, 39). Histopathological examination is complemented by immunohistochemistry (11, 28). Detection of pluripotency markers like OCT3/4, SOX2, SOX17 and c-KIT are used for aiding in classifying these tumors, reflecting the developmental potential of each entity. Fluorescence *in situ* hybridization for i(12p) can be used to confirm or refute tumors with a germ cell origin (40).

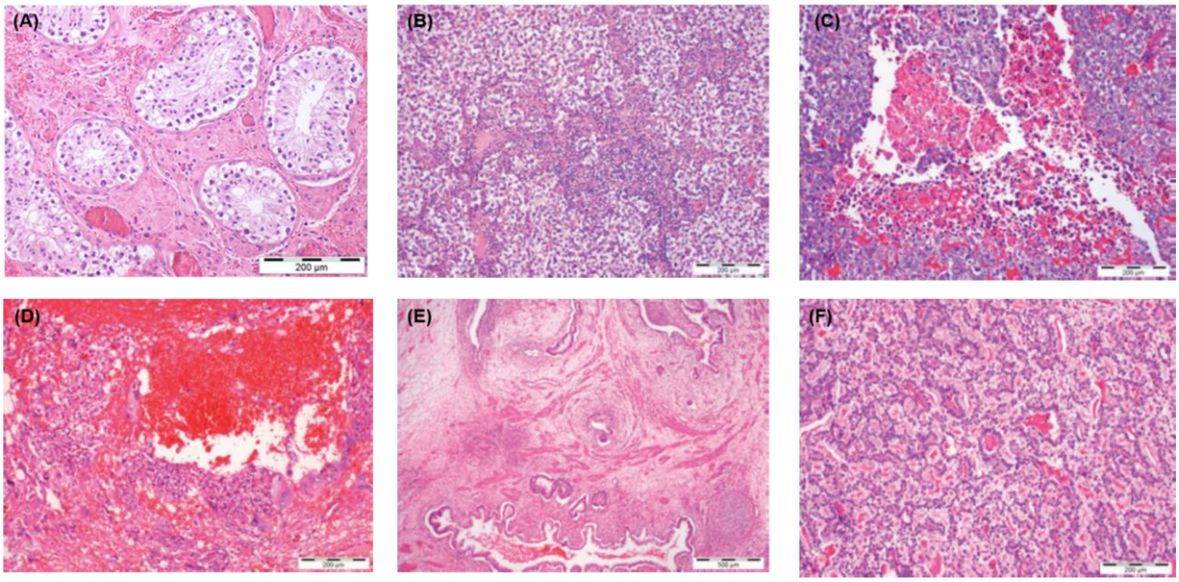


Figure 3: Histological profile of testicular germ cell tumors related to germ cell neoplasia in situ. **(A)** GCNIS; **(B)** Seminoma; **(C)** Embryonal carcinoma; **(D)** Choriocarcinoma; **(E)** Teratoma; **(F)** Yolk-sac tumor. Adapted from: (2, 28).

Final pathological staging is determined after radical orchiectomy through histopathologic evaluation, using the American Joint Committee on Cancer (AJCC) tumor–node–metastasis (TNM) staging, that it also contains an S stage, based on elevations of the serum markers LDH, AFP and βHCG (37, 41). AJCC TNM classification system for TGCTs is described in Table 3 (11, 41).

Table 3: AJCC TNM classification system for TGCTs. In: AJCC 7th edition (41).

T – Primary tumor	
pTX	Primary tumor cannot be assessed
pT0	No evidence of primary tumors
pTis	Neoplasia *in situ*
pT1	Tumor limited to testis (including rete testis invasion) without lymphovascular invasion
pT2	Tumor limited to testis (including rete testis invasion) with lymphovascular invasion; or tumor invading hilar soft tissue or epididymis or penetrating visceral mesothelial layer covering the external surface of tunica albuginea with or without lymphovascular invasion
pT3	Tumor invades spermatic cord with or without lymphovascular invasion
pT4	Tumor invades scrotum with or without lymphovascular invasion
N – Regional lymph nodes	
pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis
pN1	Metastasis with a lymph node mass ≤2cm in the greatest dimension and ≤5 nodes positive (none >2cm in the greatest dimension)
pN2	Metastasis with a lymph node mass >2cm but not >5cm in the greatest dimension; or >5 nodes positive (none >5cm); or evidence of extranodal extension of tumor
pN3	Metastasis with a lymph node mass >5cm in the greatest dimension
M – Distant metastasis	
M0	No distant metastases
M1a	Nonregional nodal or lung metastases
M1b	Distant metastases other than nonregional nodal or lung
S – Serum tumor markers	
SX	Marker studies not available or not performed
S0	Marker study levels within normal limits
S1	LDH <1.5 × ULN and HCG <5,000 mIU/ml and AFP <1,000 ng/ml
S2	LDH 1.5–10.0 × ULN or HCG 5,000–50,000 mIU/ml or AFP 1,000–10,000 ng/ml
S3	LDH >10.0 × ULN or HCG >50,000 mIU/ml or AFP >10,000 ng/ml

TNM Staging				
Stage	**T**	**N**	**M**	**S**
Stage 0	pTis	N0	M0	S0
Stage I	pT1-4	N0	M0	SX
Stage IA	pT1	N0	M0	S0
Stage IB	pT2-4	N0	M0	S0
Stage IS	Any pT/TX	N0	M0	S1-3
Stage II	Any pT/TX	N1-3	M0	SX
Stage IIA	Any pT/TX	N1	M0	S0-1
Stage IIB	Any pT/TX	N2	M0	S0-1
Stage IIC	Any pT/TX	N3	M0	S0-1
Stage III	Any pT/TX	Any N	M1	SX
Stage IIIA	Any pT/TX	Any N	M1a	S0-1
Stage IIIB	Any pT/TX	N1-3	M0, M1a	S2
Stage IIIC	Any pT/TX	Any N	Any M	Any S

Abbreviations: AFP – alpha-fetoprotein; AJCC – American Joint Committee on Cancer; HCG – Human chorionic gonadotropin; LDH – Lactate dehydrogenase; mIU – milli-international units; ULN – upper limit of normal; TGCTs – Testicular germ cell tumors

2. Epigenetics

The concept of “epigenetics” was introduced by Conrad Waddington in the 40s, and the term was used to elucidate that sometimes genetic alterations do not lead to phenotypic variations and that genes can interact with their environment. With an evolution of knowledge in this area, currently epigenetics focuses on studying reversible changes in gene expression, which occur without altering DNA sequences (42). In mammals, epigenetics is important for pre-implantation and fetal development, as well as cell and tissue differentiation (43, 44). On the other hand, disruption in these changes causes a variety of diseases, including cancer (45).

Three major epigenetic mechanisms are known: DNA methylation, histone post-translational modifications and chromatin remodelling, and regulation by non-coding RNAs. DNA methylation is the most well studied epigenetic mechanism (42). However, it is increasingly understood that all epigenetic machinery interact, changing gene expression and contributing to neoplastic transformation and progression (45) – Figure 4.

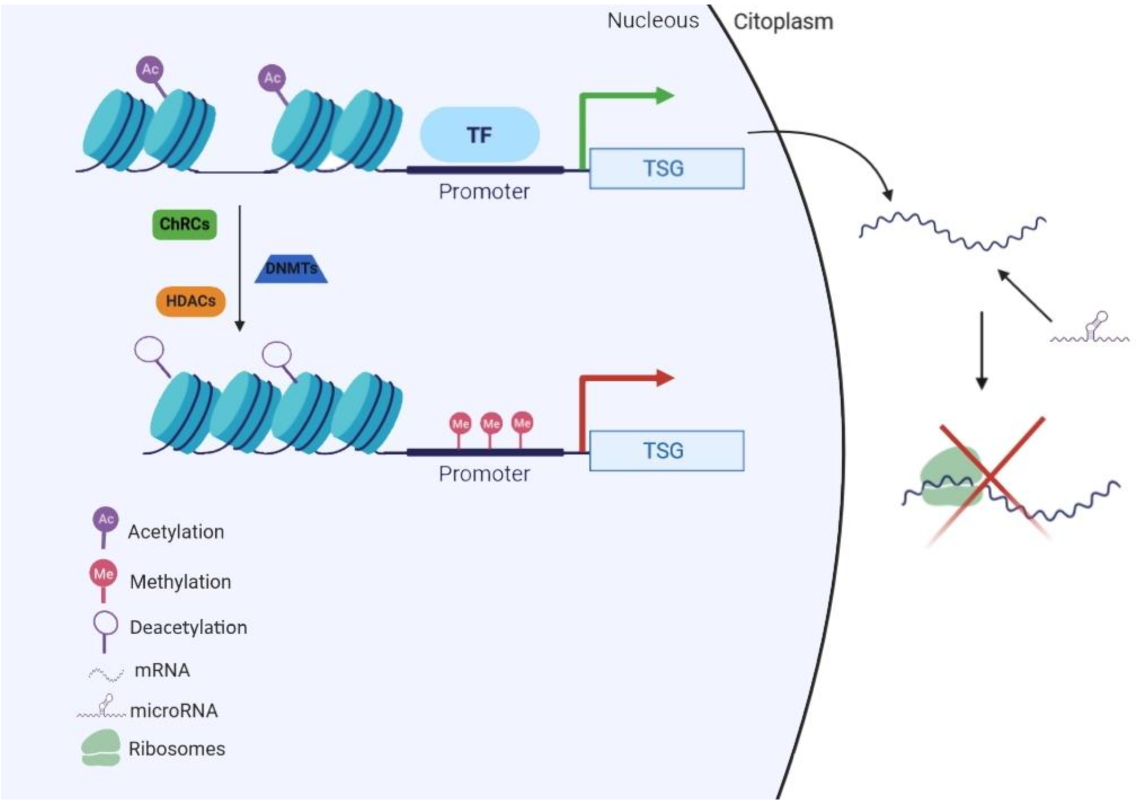


Figure 4: Transcription regulation by epigenetic mechanisms. When DNA is not methylated or histones are acetylated, chromatin has an open structure, transcription factors bind to gene promoters and there is active transcription of these genes. DNMTs and HDACs lead to DNA methylation and histone deacetylation, ChRCs further lead to condensation of chromatin, and the transcription factors do not have access to gene promoters, so transcription is repressed. On the other hand, non-coding RNAs, like microRNAs, can interact with mRNA and suppress translation. TF – transcription factor; TSG – tumor suppressor gene; ChRCs – chromatin remodelling complexes; DNMTs – DNA methytransferases; HDACs – Histone deacetylases.

DNA methylation consists in the covalent addition of a methyl group to the 5’ carbon cytosine of the DNA, originating a new DNA base, 5-methylcytosine (5mC). This process is catalysed by a family of enzymes, the DNMTs, which use S-adenosyl methionine as cofactor, the donor of the methyl group. Three main DNMTs are described: DNMT1, DNMT3A and DNMT3B; the former is responsible for the preservation of parental cell DNA methylation in a replication-dependent manner, while the latter are specifically associated with *de novo* methylation, which occurs during embryogenesis/germ cell development and assure re-establishment of parental imprinting marks (42, 46-48). Other than these, there is DNMT3L, which is expressed during embryonic development, losing its expression after birth (49).

The DNA methylation process is dynamic and can be reverted through DNA demethylation. This involves the action of ten-eleven translocation enzymes (TETs; including TET1, TET2 and TET3), which catalyse oxidation of 5mC to 5-hydroximetylcytosine (5hmC), so-called “active demethylation”. Active demethylation occurs, for instance, in the paternal genome in the zygote. On the other hand, there is a “passive demethylation” that happens in the maternal genome, due to failure of DNMTs in establishing methylation during replication.

DNA methylation occur mainly at CpG sites and is associated with transcriptional silencing. These sites are mapped in gene promoters and in regions of large repetitive sequences. In the latter, CpGs are methylated to prevent chromosome instability. Oncogenes are usually methylated while tumor suppressor genes (TSG) are hypomethylated; deregulation in this pattern contributes to cancer development (42, 45) – Figure 4.

Histone post-translational modifications, like methylation and acetylation, occur in the N-terminal tail of histones and is mediated by histone-modifying enzymes. Acetylation is controlled by the balanced activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs): while the former add acetyl groups to the lysine residues of histones, the latter remove this groups. In general, acetylation of histones is associated with chromatin unfolding and gene transcription, while HDACs are seen as transcription co-repressors (50, 51). Histone methylation (transfer of methyl groups to histone lysine or arginine residues) is mediated by histone methyltransferases (KMTs), and removal is catalysed by histone demethylases (KDMs). These can associate with transcriptional activation or repression, depending on the modified amino acid and its position, meaning that these alterations are very versatile and the net effect can vary (52-54) – Figure 5.

Non-coding RNAs, mainly microRNAs (miRNAs), have been reported as regulators of gene expression (55). Specifically, miRNAs are very appealing, since they can be easily detected in biofluids in a cost-effective manner, allowing for diagnosis and follow-up of patients (56). They modulate gene expression post-transcriptionally by targeting specific mRNA molecules (Figure 4), meaning that they function as oncomiRNAs or as tumor suppressor miRNAs (57, 58).

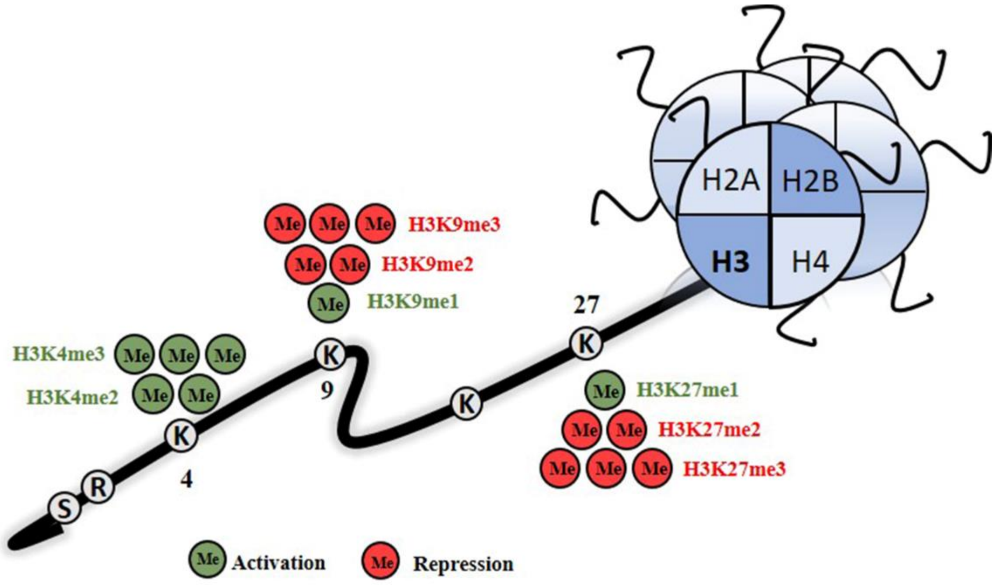


Figure 5: Repression and activation marks associated with methylation of histones. H3K4me2/3, H3K9me1 and H3K27me1 are activation marks, while H3K9me2/3 and H3K27me2/3 are repression marks. ME - methylation; H3 – histone 3. In: *Cardoso et al.* 2020 (59).

2.1. DNA methylation in testicular germ cell tumors

In general, TGCTs display global hypomethylation and locus-specific hypermethylation more pronounced in NSs than in SEs. Indeed, SEs exhibit global hypomethylation and erasure of imprinting marks, similar to their originating cell (27, 60). On the other hand, several gene promoters were shown to be hypermethylated in NSs, namely *MGMT*, *CALCA*, *HIN1* (*SCGB3A1*), *RASSF1A*, *HOXA9*, *CRIPTO*, *MCAM*, *MLH1*, *S100A2*, *SSBP2*, *APC*, *VGF,* and *PGP9.5* (61-66). Specifically, it is described that *MLH1*, *RASSF1A* and *HIC1* hypermethylation is correlated with cisplatin resistance (61, 67), and *CALCA*, *MGMT*, *HOXA-9,* and *SCGB3A1* hypermethylation was associated with poor prognosis in TGCTs patients (66, 68). Besides this, data from genome-wide studies showed different patterns among NS subtypes, for example with non-canonical methylation (CpH methylation) occurring in embryonal carcinoma (and correlating with *DNMT3A/B* expression), a pattern followed by embryonic stem cells, while more differentiated components lose such non-CpG methylation and exhibit patterns approximating somatic cancers, indicating shifts in methylation that follow tumor differentiation (27).

The initial step of polyploidization leads to the gain of X chromosomes (tumor cells have supernumerary X chromosomes). Consequently, X-inactive specific transcript (*XIST*) is found to be expressed in TGCTs, by demethylation of its promoter. Other sequences are found to be hypomethylated in these tumors, like long interspersed nuclear element 1 (*LINE1)*. ALU is hypomethylated in SEs, but hypermethylated in NSs (49, 60, 69-71). A summary of DNA methylation patterns of TGCTs is illustrated in Figure 6.

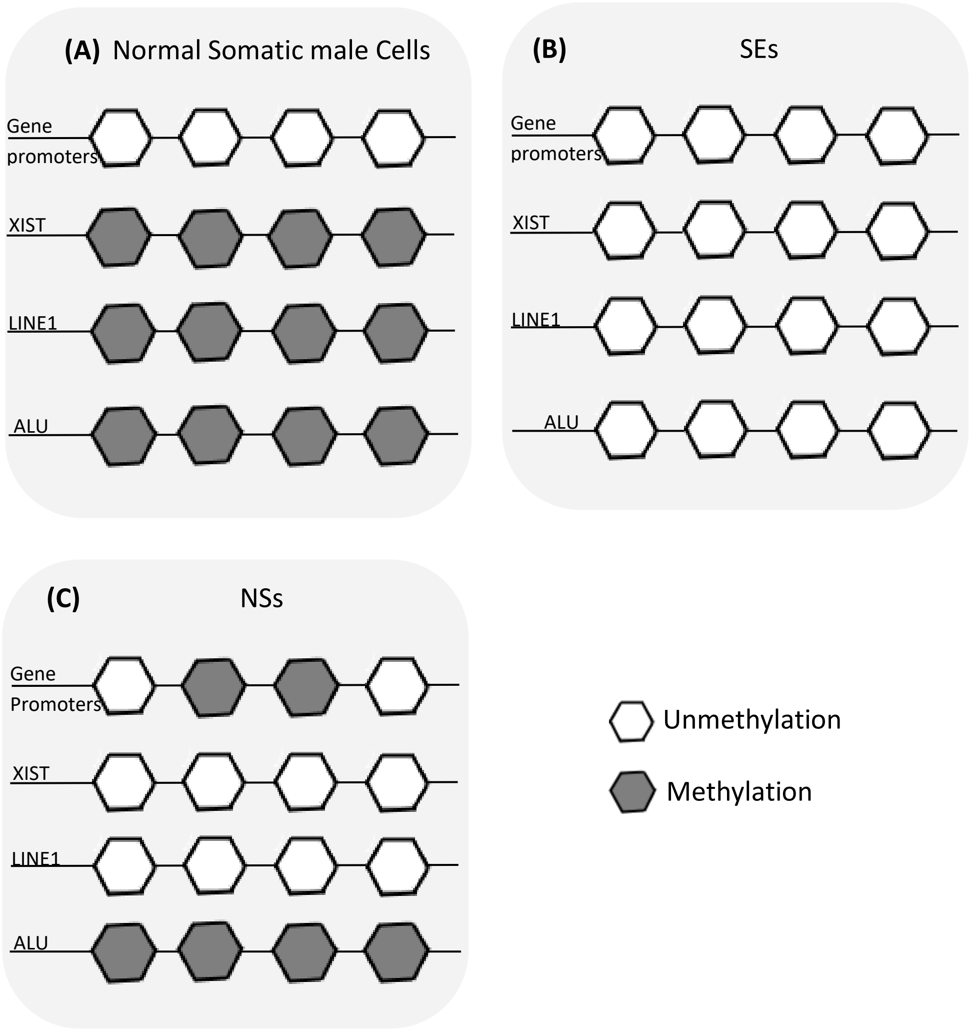


Figure 6: Methylation pattern of **(A)** normal somatic cells, **(B)** seminomas, and **(C)** non-seminomas. In normal somatic cells, gene promoters are unmethylated in general, while repetitive sequences, like LINE1 and ALU, are densely methylated. The XIST promoter (a long non-coding RNA involved in X-chromosome inactivation) is methylated in normal somatic male cells (due to XY chromosome constitution) but demethylated overall in TGCTs, both SE and NS (due to the initial step of polyploidization and universal gain of X chromosome). SEs show an overall unmethylated profile. NSs present locus-specific methylation of gene promoters and methylated ALU sequence. SEs – Seminomas; NSs – non-seminomas; XIST – X-inactive specific transcript; LINE1 – Long interspersed nuclear element 1. Adapted from*: Cardoso et al.* 2020 (59).

Overall, deregulation and specific expression patterns of DNMTs and/or TETs can be expected in TGCTs, given the shifting methylation profile across subtypes. Studies (both *in vitro*, *in vivo* and using patient samples) have showed differential expression of DNMTs and TETs in TGCTs, with NSs displaying higher expression levels of DNMTs and lower/variable expression levels of TETs (50, 72-74). Studies of mRNA profile and immunoexpression showed also that *DNMT3L* is overexpressed in NSs (75) mainly in embryonal carcinoma (76, 77).

2.2. Other epigenetic alterations in testicular germ cell tumors

Regarding histone post-translational modifications, *in silico* analysis reported that SEs present higher expression levels of HATs, while NSs display overexpression of HDACs (50). Furthermore, immunoexpression analysis showed that there are significant differences between histological subtypes of TGCTs in several HDACs isoforms. While HDAC2 and 3 was shown to be highly expressed in all types of TGCTs, HDAC1 presented low levels, except in choriocarcinomas that showed high expression of all isoforms (78). Studies on histone methylation are more conflicting. Our research team (50) and other authors (3, 15, 79) showed that SEs show overrepresentation of activation marks and, consequently a high expression levels of enzymes primordially implicated in establishment of these marks (KDM4D, KDM3A, KMT2B/C/D, SETD1A, or SMYD3), while NSs are associated with repressive modifications and overexpression of enzymes that catalyse addition of repressive marks (EHMT2 or EZH2). Oppositely, *Almstrup* and co-workers (80) with their results demonstrated that SEs display high levels of selective repressive modifications.

Chromatin remodelling complexes (ChRCs) in TGCTs are not yet much elucidated and more studies are for sure necessary. Still, *Jostes et al.* (81) described that BRD2, BRD3 and BRD4 are highly expressed in TGCTs.

Concerning non-coding RNAs, specifically miRNAs, large retrospective and prospective studies have showed that embryonic miRNAs (for example miR-371a-3p) have a clinical impact for TGCT patient management (82, 83). Overall, miR-125b, miR-302 family, miR-371, miR-372, miR-373, and miR-375 are described as important in TGCT tumorigenesis. miR-125b is considered a tumor suppressor miRNA that regulates several mechanisms, like proliferation, apoptosis and, importantly, pluripotency. It is known to be downregulated in TGCTs, and low levels of this miRNA are associated with tumor growth, inducing the recruitment of pro-tumorigenic macrophages to the microenvironment (84). One the other hand, miR-375 was recently appointed to be a promising marker of teratoma (the histology left undetected by miR-371a-3p) (27) but this has been disproved in the liquid biopsy setting (85). Besides this, it is described that miRNAs of the 371-3 cluster, miR-375, and miR-302a/b/c/d may disrupt the *TP53* pathway, leading to development of TGCTs (4). But, while miRNAs of the 371-3 cluster disrupt the p53 pathway (by targeting LATS2), miR-885-5p (a p53 activator) was shown to be highly present in mature teratomas (86). Moreover, our research team (85) proposed, then, a miRNA switch (371a-3p to 885-5p) as involved in the differentiation process. Still, it has been showed that miR-302s are highly present in TGCTs, acting as oncogenes, which induce the expression of SPRY4 and, consequently, activate MAPK/ERK pathway, leading to proliferation of tumor cells (87).

3. Testicular germ cell tumors’ therapy

Overall, about 68% of patients are diagnosed with localized disease (80% and 60% of SEs and NSs, respectively) (2) and the cure rate is around 95% (3), making TGCTs a model of curable cancer even in the case of disseminated disease (88). Indeed, for a substantial amount of patients (around 75%) orchiectomy alone is curative (61, 89). However, some patients need adjuvant chemotherapy to avoid disease relapse, mainly patients with clinical stage II and III and those considered at high risk of relapse (11, 90). For this, the International Germ Cell Cancer Collaborative Group (91) grouped the patients in three categories of prognosis according to post-orchiectomy levels of serum marker and whether metastatic spread includes non-pulmonary visceral metastases or mediastinal primary metastases (Table 4). In fact, lymphovascular invasion has been shown to be predictor of recurrence of TGCTs (11, 92-94). To uncover biomarkers with high accuracy in determining the subset of patients that will never relapse is very relevant, in order to avoid that these patients are subjected to aggressive treatments (not truly benefiting from them), enduring associated long-term side effects (95).

Table 4: Prognosis grouping. In: International Germ Cell Cancer Collaborative Group (91).

Prognosis grouping (risk status)	**Tumor type**	**Metastases**	**Serum markers**			**Progression free survival (%)**	**Overall survival (%)**
			AFP (ng/ml)	**βHCG (IU/l)**	**LDH**		
Good	SE	No	Normal	Any	Any	82	86
	NS	No	<1,000	<5,000	<1.5 × ULN	89	82
Intermediate	SE	Yes	Normal	Any	Any	67	72
	NS	No	1,000-10,000	5,000–50,000	1.5–10.0×ULN	75	80
Poor	SE	No patients classified with poor prognosis					
	NS	Yes	>10,000	>50,000	>10 × ULN	41	48

Abbreviations: SE – seminomas; NS – non-seminomas; AFP – alpha fetoprotein; βHCG – human chorionic gonadotropin β; IU – international units; LDH – lactate dehydrogenase; ULN – upper limit of normal

The guideline therapy for these patients is cisplatin-based combination chemotherapy: BEP, in which “B” refers to bleomycin, “E” refers to etoposide and “P” to platinum, which is usually cisplatin (96, 97). Cisplatin was responsible for the major drop in mortality of these patients upon its incorporation around the 1970s. Despite its remarkable efficacy, a modest (but clinically meaningful) subset of patients develop resistance to cisplatin. The major relevance of this event relates to the absence of validated therapeutic options for these patients, which are the ones experiencing morbidity and mortality from disease in the present days (98, 99). Several studies have tried to explain the mechanisms of resistance to cisplatin, but a specific alteration, target and mechanism is lacking (100-103). Nevertheless, there is evidence that epigenetic deregulation can also provide insight on this matter (34, 61, 104). Cisplatin acts by forming covalent binding to the DNA, establishing platinum-DNA lesions in dividing cells. These lesions will be recognized by proteins participating in the process of DNA repair, activating p53 expression and downregulating of BCL expression, leading to apoptosis (105). It is hypothesized that if DNA is hypermethylated, the platinum access is limited. Thus, there is a decrease of cellular damage and apoptosis and, consequently, resistance to therapy (97, 98) – Figure 7. In fact, and as mentioned before, it is proposed that hypermethylation of *MLH1*, *RASSF1A* and *HIC1* promoter genes was associated with cisplatin resistance (61, 67). Because these alterations are reversible, they can be therapeutically targeted with “epidrugs”, some of them already approved for cancer treatment or under investigation in clinical trials, mainly DNMT inhibitors (DNMTis) (45, 106).

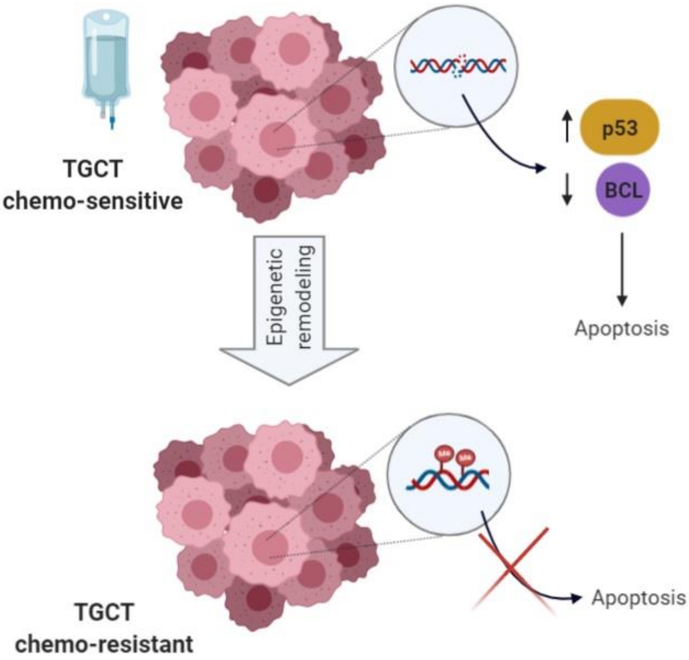


Figure 7: Possible mechanism of cisplatin resistant of testicular germ cell tumors. In a first phase, there is formation of cisplatin adducts that are recognized, leading to apoptosis of tumor cells. By tumor progression the DNA of cells is methylated, preventing that cisplatin accesses to DNA, blocking apoptosis. TGCT – testicular germ cell tumors; Me – methylation.

3.1. DNMT inhibitors in testicular germ cell tumors

In the last decade, DNMTis have been demonstrated as efficient therapeutic agents, contributing effectively to death of tumor cells (46).

Overall, DNMTis block DNMTs action, leading to global hypomethylation and, consequently, to re-expression of genes (essentially TSG), reversing the anti-neoplastic effect (107). According to mode of action, DNMTis can be divided into two main groups: nucleoside analogues and non-nucleoside analogues. The former incorporate directly into DNA, during S phase of cell cycle, disrupting replication; the latter bind to the catalytic site of DNMTs, preventing their action (46).

The exact mechanism of action of the nucleoside analogues remains unclear. However, it is proposed that these compounds are formed by a modified cytosine ring connected to a ribose or deoxyribose, replacing cytosine by incorporation into DNA during S phase of cell cycle. When completely incorporated into DNA, they covalently bind to DNMTs, inhibiting them. This causes DNA damage and cell death. Additionally, these drugs can deplete DNMTs, leading to loss of methylation pattern in the cells after successive replications and, consequently re-express genes abnormally silenced by methylation process (46, 108).

The most studied DNMTis are 5-azacytidine (Vidaza^{TM}) and 5-aza-2'-deoxycytidine (Dacogen^{TM}), the latter also called by Dacitabine (DAC). Both were developed as cytostatic agents (109), but studies *in vitro* showed that they can induce cell differentiation and inhibit DNA methylation (110). After that, they were to use for cancer treatment. This led to Food and Drug Administration and, later, also the European Medicines Agency to approve these compounds for treatment of patients with haematological tumors (46, 50). These are still to be accepted for treatment of solid cancers; despite this, many are the studies to show the cytotoxic effect of these drugs (50, 111). It is known that sensitivity to 5-azacytidine depends of a high expression of DNMT3B (112). Hence, the hypothesis is that these agents can be useful in treatment of TGCT patients, mainly of one of the most aggressive NS tumors – embryonal carcinoma, which overexpresses these enzymes (50). Indeed, there are already some studies reporting use of DNMTis in TGCTs.

It is proposed that DNMTis have an anti-neoplasic effect in TGCT cell lines and can restore cisplatin sensitivity by demethylation of tumor suppressor genes, decrease of DNMTs (mainly DNMT1 and DNMT3B), decrease of pluripotency genes, induction of p53 targets (and consequently apoptosis), and increase of ATM and pH2AX that is associated with DNA damage (62, 104, 112-115) – Figure 8. However, despite all these mechanistic clues and studies showing efficacy, clinical studies/trials have been overall disappointing, with no significant efficacy (116, 117). New compounds have been synthesized as a

DNMTis. MLo-1302 is a compound derived from flavanones and was designed and developed to inhibit DNMTs (118). In fact, results in our group demonstrated that MLo-1302 inhibit tumor growth in renal cell carcinoma cell lines. Similarly, it decreases DNA methylation at global levels, as well as loci specific levels, essentially acting in DNMT3A (*Marques-Magalhães et al.*, *under submission*).

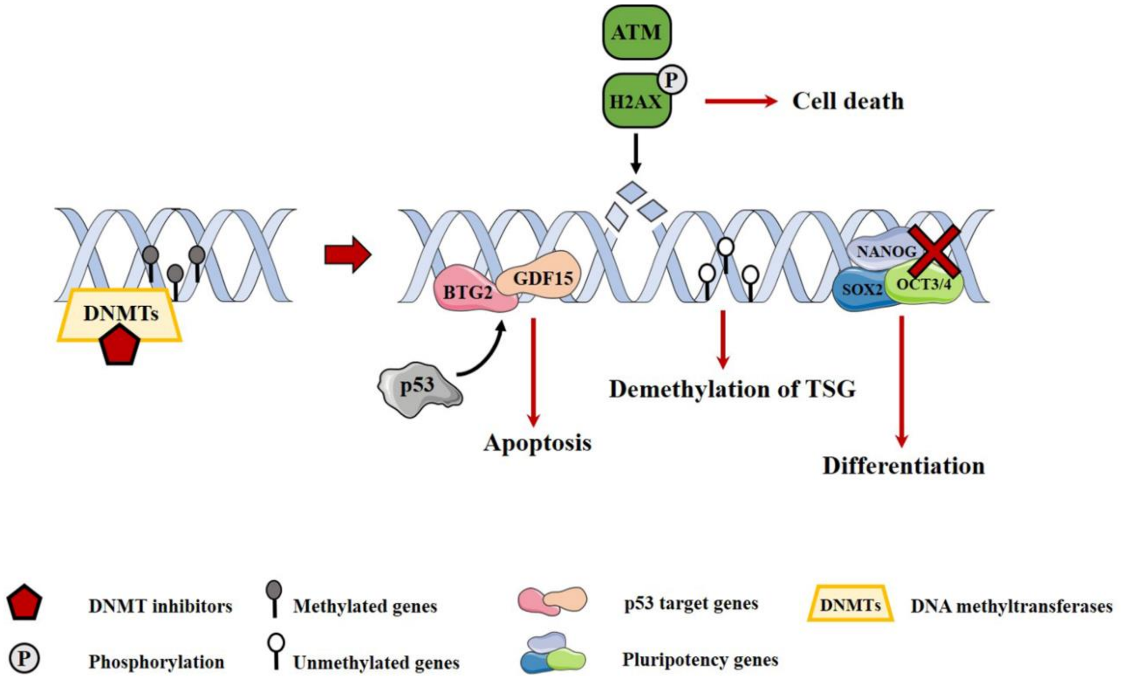

ATM
H2AX
P
Cell death
DNMTs
BTG2
GDF15
p53
Apoptosis
Demethylation of TSG
NANOG
SOX2
OCT3/4
Differentiation
DNMT inhibitors
Methylated genes
p53 target genes
DNMTs
DNA methyltransferases
Phosphorylation
Unmethylated genes
Pluripotency genes

Figure 8: Main molecular mechanisms associated with DNMT inhibitors. DNMT inhibitors can lead to induction of p53 targets, resulting in apoptosis. On the other hand, there is an increase of ATM and pH2AX, associated with DNA damage, conducting to cell death. Beside this, there is a re-expression of tumor suppressor genes by demethylation process. Oppositely, pluripotency genes are downregulated, leading to differentiation of cells. These processes can decrease cisplatin resistance. DNMTs – DNA methyltransferases TSG – tumor suppressor genes. In*: Cardoso et al.* 2020 (59).

AIMS

Although TGCTs are considered a model of curable cancer due to the efficacy of cisplatin, a proportion of patients develop resistance to cisplatin. Importantly, there are no therapeutic options for these patients, often culminating in death. On the other hand, it has been reported that epigenetic alterations can confer resistance to cisplatin in these tumors. Knowing that epigenetics is a hallmark of the TGCTs, and its alterations can be reversible, “epidrugs” can be useful for TGCT treatment. In fact, many studies demonstrate efficacy of these agents in the treatment of cancer, including TGCTs. Regarding DAC, it is described that it can restore cisplatin sensitivity, leading to demethylation of TSG, differentiation, and cell death in TGCT cell lines. However, clinical data so far has not been satisfactory. So, new compounds should be synthesized and tested for TGCT treatment. Recently, several natural compounds have been re-investigated for their epigenetic targeting properties and, hence, anti-cancer properties. In this work we tested a compound derived from flavanones (MLo-1302), described to have ability to inhibit DNMTs. The compound has not yet been tested in TGCT cell lines, despite showing anti-neoplastic effects in other tumor models.

Thus, the main goal of this Dissertation is to evaluate the therapeutic use of MLo-1302 as an anti-cancer agent in TGCT cell lines and to compare the effect of this compound with the effect of approved demethylating agent DAC and differentiating agent all-trans-retinoic acid (ATRA).

Specifically, we aim to:

1. Evaluate the influence of MLo-1302 on cell viability, DNMTs expression, methylation pattern and cell differentiation of TGCT cell lines, comparing with the effects of ATRA and DAC.
2. Explore in more detail the effects of MLo-1302 on TGCT cell lines, namely cytotoxic properties, and downstream deregulated pathways.

MATERIALS AND METHODS

1. Cell culture

Cell lines representative of TGCTs were provided by Prof Looijenga, cultured in the Dulbecco's modified Eagle's culture medium (DMEM 1640; Biochrom, Cambridge, UK) supplemented with 10% of Fetal Bovine Serum (Biochrom), 1% of penicillin/streptomycin (GRISP, Portugal); and maintained at 37ºC in a humified atmosphere containing 5% of CO_2 (the key cell lines' characteristics are summarized in Table 5).

Cells were maintained in low passages and were negative for Mycoplasma spp. (Clontech Laboratories; Mountain View, CA, USA; twice a month test).

Table 5: Non-seminoma cell lines features.

Cell line	Origin	*TP53* status
NCCIT	Anterior mediastinal mixed germ cell tumor	Mutated
NTERA-2	Malignant pluripotent embryonal carcinoma from primary of testis	Wild-type
2102EP	Primary human testicular teratocarcinoma	Wild-type

2. ATRA treatment

ATRA was provided from STEMCELL^{TM} Technologies, France. It was diluted in dimethyl sulfoxide (DMSO) in order to obtain a stock solution of 10mM.

For treatment, $4x10^4$ cells were seeded in $25cm^3$ culture flasks in complete DMEM and then treated with 10µM of ATRA for 10 days with drug renewal every 2 days. After that, pellets were collected and the DNA and protein were extracted to perform quantitative methylation-specific PCR (qMSP) and western blot (Figure 9), respectively. For each condition, at least three independent replicates were performed.

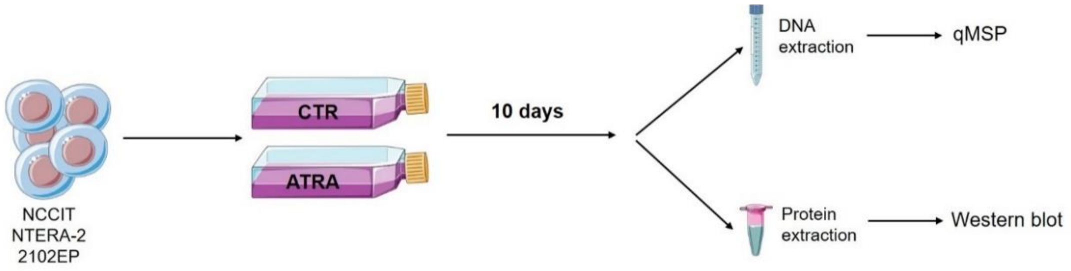


Figure 9: ATRA treatment.

3. Cell viability assay for DAC and MLo-1302

DAC was purchased from Sigma-Aldrich, Germany, and MLo-1302 was synthesized and provided by Dr. Paola Arimondo's Group (ETaC: Pharmacochemistry - Cancer Epigenetic Regulation Unit group, Centre Pierre Fabre Laboratories - Research &

Development, Toulouse, France). Both were dissolved in DMSO to a 10mM stock solution.

Cell viability was assessed at 24h, 48h and 72h after DAC and ML0-1302 treatment through, Resazurin (7-Hydroxy3H-phenoxazin-3-one 10-oxide) method (Canvax Biotech, Córdoba, Spain). Briefly, cells were plated on 96 well plate at density of 8000, 4000 and 6000 cells/well for NCCIT, NTERA-2 and 2102EP cell lines, respectively. After DAC and MLo-1302 treatment with doses reported in Table 6, the culture medium was removed, and cells were incubated during 3h at 37ºC with 1:10 Resazurin solution in culture medium. The solution was then removed, and spectrophotometric measurement was done at 560nm (reference wavelength (λ_{ref}): 600 nm) in a microplate reader (Fluostar Omega, BMG Labtech, Germany). Wells with the Resazurin solution were used as blank to correct the OD values. ODs obtained for each time point were all normalized for the 0 hours-time point. At each time-point, the compounds and controls were freshly added to the wells and the procedure was repeated the next day. All experiments were performed with biological triplicates, each with experimental triplicates. IC_{50} values were estimated from dose-response curves (Table 6) on GraphPad Prism 6 (GraphPad Software, CA, USA).

Table 6: Range of concentrations of DAC and MLo-1302 compounds used to calculate IC_{50}.

DAC	MLo-1302
0.01µM, 0.025µM, 0.05µM, 0.1µM, 0.25µM, 0.5µM, 1µM, 2µM, 4µM, 5µM	0.5µM, 1µM, 2µM, 5µM, 10µM

4. DAC and MLo-1302 treatment

After IC_{50} values calculation, two concentrations of DAC (0.01µM and 1µM) and three of MLo-1302 (0.25µM, 0.5µM, and 1µM) were used to treat TGCT cell lines. Cells were plated in 25cm^3 culture flasks in complete DMEM medium at an optimal density, to obtain about 90% of confluence at ending timepoint. Cells were exposed to different doses of DAC and MLo-1302 for 72h with drug renewal every 24h. After that, RNA, DNA and protein were extracted to perform RT^2 profiler PCR array (for MLo-1302 treatment), qMSP, dotblot, and western blot; culture medium was retrieved to perform the LDH cytotoxicity assay (for MLo-1302) – Figure10. For each condition, at least three independent replicates were performed.

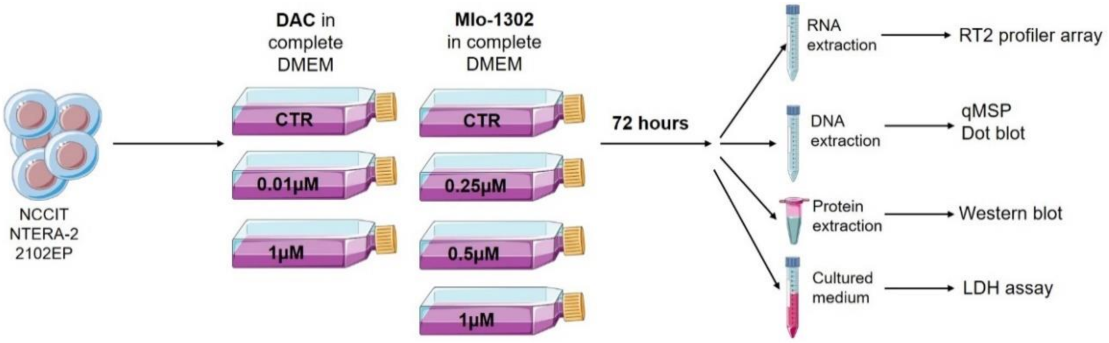

DAC in complete DMEM
Mlo-1302 in complete DMEM
NCCIT
NTERA-2
2102EP
CTR
0.01µM
1µM
CTR
0.25µM
0.5µM
1µM
72 hours
RNA extraction
DNA extraction
Protein extraction
Cultured medium
RT2 profiler array
qMSP
Dot blot
Western blot
LDH assay

Figure 10: DAC and MLo-1302 treatment.

5. Western blot

Total protein was extracted from TGCT cell lines, in biological triplicates, using the RIPA lysis buffer (Santa Cruz Biotechnology Inc., USA) supplemented with protein inhibitor cocktail. After 15min on ice, samples were centrifuged at 13,000rpm for 30min at 4ºC and the supernatant was collected. Protein was quantified using a Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific, USA), according to manufacturer's instructions, which allows to obtain the protein concentration with reference to a bovine serum albumin (BSA) standard curve. Then, 20µg of proteins were separated on 8% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For immunodetection, the resolved proteins were transferred into nitrocellulose membranes (Bio-Rad, Germany) using a Trans-Blot Turbo Transfer system (Bio-Rad, USA) for 16min in a 25mM Tris-base/ glycine transfer buffer. The membranes were then blocked with 5% of milk diluted in tris-buffered saline – tween 20% (TBST pH=7,6) for 1h at room temperature. Subsequently, the membranes were incubated with primary antibodies at 4ºC overnight with gentle shaking, except beta-actin (ACTB) that was incubated for 1h at room temperature. The antibodies used and its incubation conditions are described in Table 7. After that, the membranes were incubated with a secondary horseradish peroxidase (HRP) conjugated antibody (Cell Signaling Technology, Netherlands) – 1:5000 – for 1h at room temperature. The bands of target proteins were detected using enhanced chemiluminescence (Clarity^{TM} western ECL substrate, Bio-Rad, USA). The gray value of each band was measured by ImageJ software and normalized by ACTB.

6. Sodium bisulfite treatment and quantitative methylation-specific PCR

RASSF1A promotor gene methylation levels were assessed in cells treated with ATRA, DAC and ML0-1302 by qMSP method. Firstly, DNA was extracted using the phenol-chloroform, eluted in sterile bi-distilled water, and stored at -20ºC.

Genomic DNA was initially quantified using the NanoDrop Lite Spectrophotometer (Nanodrop Technologies, USA). A total of 500ng were bisulfite-treated with the EZ DNA Methylation-Gold^{TM} Kit (Zymo Research, CA, USA), according to manufacturers' recommendations. At the end, the modified DNA was eluted in 30µL of sterile distilled water and stored at -80ºC until to use.

Table 7: Antibodies and respective incubation conditions used for western blot.

Antibody	Company / clone	Dilution	Secondary antibody
DNMT1	Cell signaling / D63A6	1:500	Anti-rabbit
DNMT3A	Cell signaling / D23G1	1:250	Anti-rabbit
DNMT3B	Cell signaling / D7070	1:1000	Anti-rabbit
NANOG	Abcam / ab109250	1:1000	Anti-rabbit
OCT3/4	Cell signaling / C52G3	1:1000	Anti-rabbit
SOX2	Cell Marque / SP76	1:250	Anti-rabbit
PAX6	Invitrogen / 13B10-1A10	1:1000	Anti-mouse
Cleaved CASPASE 8	Cell signaling / D384	1:250	Anti-mouse
ACTB	Sigma-Aldrich / A1978	1:10 000	Anti-mouse
Abbreviations: ACTB – beta-actin			

The qMSP was carried out in 96-well plates (Applied Biosystems, USA), with 1µL of DNA, 5µL of Xpert Fast SYBER Mastermix Blue supplemented with ROX, 0.3 µL of specific primers (Table 8), and 3.7µL of sterile bi-distilled water in order to obtain a final volume of 10µL in each well. Each sample was carried out in triplicates. Two positive controls (CpGenome Universal Methylated DNA, Millipore, Germamy) and two negative controls (EpiScope Unmethylated HCT116 DKI gDNA, TAKARA, France) were added to validate the technique.

Table 8: Primers sequences and qMSP conditions for each target gene.

Gene	Primer sequence (5'-3')	Volume (µL) F+R*	Annealing Temperature (°C)
RASSF1A	F: AGCGAAGTACGGGTTTAATC	0,3	60
	R: ACACGCTCCAACCGAATA		
ACTB	F: TGGTGATGGAGGAGGTTTAGTAAGT	0,4	60
	R: AACCAATAAAACCTACTCCTCCCTTAA		
Abbreviations: F – forward; R – reverse; *concentration of 10 µM			

All PCR reactions were run on a 7500 Real-Time PCR (Applied Biosystems) with the following conditions: an initial step of polymerase action at 95ºC for 2min followed by 45 cycles at 95ºC for 5 seconds, ending with 20 seconds of denaturation and annealing at a 60ºC (Table 8).

The relative methylation levels were determined as the ratio between the *RASSF1A* mean quantity and ACTB was used as housekeeping gene, using ΔΔCt method.

7. Dotblot

DNA was extracted and quantified as mentioned before. 1000ng of DNA were diluted in TE buffer. After that, DNA was denaturated in 0.1M of NaOH at 95ºC during 10min and single chains were stabilized in 1M of ammonium acetate on ice. DNA was pipetted into nitrocellulose membranes and these membranes were left to dry for 30min at 37ºC. Membranes were exposed to UV light for 1min to produce crosslinking between DNA and membranes. Subsequently, membranes were blocked in 5% milk diluted in TBST and incubated with primary antibody for 5mC (Millipore, 1:1000) at 4ºC overnight. In the next day, membranes were incubated with anti-mouse HRP conjugated antibody (1:5000, Cell Signaling Technology) for 1h at room temperature. Similar to western blot, the blots were detected using enhanced chemiluminescence. Sybergreen I nucleic acid stain (Molecular Probes, 57567, Invitrogen, USA) was used as loading control. Dotblot analysis was performed by ImageJ software.

8. Immunofluorescence

Immunofluorescence (IF) was performed to assess the presence of 5mC in MLo-1302 treated cells. For this, cells were plated in dark 96-well plates, at density of 4000, 2000 and 3000 cells/well for NCCIT, NTERA-2 and 2102EP cell lines, respectively. After MLo-1302 treatment for 72h, cells were fixed 15min in 4% paraformaldehyde, following cell permeabilization with 0.5% Triton X, 0.5% Tween20 for 30min. Before blocking with 5% BSA 1h at room temperature, cells were treated with 4M HCL during 20min following trypsin at 37ºC for 2 min. After trypsin activity inactivation with complete cultured medium, cells were incubated with primary antibody for 5mC (Millipore, 1:100) overnight at 4ºC. In the next day, cells were incubated with secondary antibody anti-mouse Alexa Fluor 594 (Molecular Probes, Invitrogen), for 1h at room temperature. Subsequently, cells were stained with 4’,6’-diamidino-2-phenylindole (DAPI,1:5 dilution, AR1176, BOSTER Biological Technologies, China). Pictures were obtained in a fluorescence microscope Olympus IX51 with a digital camera Olympus XM10 using CellSens software (Olympus, Japan) (400x magnification).

9. Lactate dehydrogenase release assay

The cytotoxic effect of MLo-1302 compound was evaluated LDH activity in supernatant cell culture medium by NADH kinetics function. Briefly, TGCT cells were plated at desired density on 96 well plates and allowed to adhere overnight at 37ºC, 5% CO_2. Then, TGCT cells were treated with MLo-1302 as mentioned before (Figure 10). The supernatants were collected every 24h during 72h of MLo-1302 treatment and stored at -20ºC. For LDH quantification, samples were incubated with 0.21mM β-NADH in 0.05M phosphate buffer at 30ºC for 5min, followed by 22.72mM sodium pyruvate. The kinetics of NADH disappearance was followed at 340nm during 3min at 30ºC. The levels of LDH released were normalized to positive control and to the number of live cells. LDH levels in MLo-1302 conditions were normalized for control condition.

10. RT^2 profiler array

400ng of cDNA were synthetized using Transcriptor High Fidelity cDNA Synthesis Kit (Qiagen, Germany), according to manufacturer’s instructions. The RT^2 Profiler PCR Array System Kit (Qiagen) included 96 genes corresponding to cancer research molecular pathways and adequate controls in quadruplicates (Annex 1). The expression levels were determined by reverse transcription polymerase chain reaction (RT-PCR) in a LightCycler 48 (Roche Diagnostics) and ACTB, β2M, GAPDH, HPRT1 and RPLP0 were used as endogenous controls. The RT^2 profiler PCR array analysis was performed in Qiagen specific platform. The data analysis in web portal calculates fold change using ΔΔCT method. Genes with a logarithm fold change above 1.5 or below -1.5 were considered. Additionally, DNA genomic contamination (GDC), as well as first strand synthesis (RTC) and real-time PCR efficiency (PPC) were monitored in Qiagen platform for RT^2 profiler PCR array analysis. The lower limit detection was set at CT≥35.

11. Cell death assay

The effect on apoptosis was measured by APOPercentage™ kit (Biocolor, United Kingdom). Briefly, cells were seeded in 24 well plate at density of $4x10^4$, $3.5x10^4$ and $3x10^4$ cell/well for NCCIT, NTERA-2 and 2102EP cell lines, respectively, and treated with 0.5µM and 1µM MLo-1302 as mentioned before (Figure 10). After that, cells were incubated with 300µL/well of APOPercentage dye solution at ratio 1:20 respectively, during 20min at 37ºC. Then, cells were washed with phosphate-buffer saline (PBS) and detached from well plate with TrypleTM Express (GBICO, Invitrogen, USA) during 10min at 37ºC. Finally, APOPercentage Dye Release reagent was added, and plate were vigorously agitated during 15min, following colorimetric measurement at 550nm with λ_{ref}=620nm (Fluostar Omega). The H_20_2 was used as a positive control. The OD obtained for apoptosis assay was normalized for the OD obtained by viability assay at the same time point. At least three independent experiments were performed.

12. Cell proliferation assay

The effect on cell proliferation was assessed by Cell Proliferation ELISA 5-bromo-2'-deoxyuridine (BrdU) assay (Roche). Cells were plated into 96-well plates in complete DMEM medium at 8000, 4000 and 6000 cells/well for NCCIT, NTERA-2 and 2102EP cells, respectively, and incubated overnight. Then, cells were treated for 72h with 0.5µM and 1µM, according to Figure 10. Before timepoint ending TGCT cells were incubated with 20µM BrdU labelling solution for 12h. After removing medium, cells were fixed for 30min at room temperature with FixDenat solution following Anti-BrdU-POD antibody (dilution 1:100) incubation for 90min at room temperature. Cells were washed 3 times with 1X PBS and then incubated for 5-10min with substrate solution until colour development. Then, the reaction was stopped with 1M H_2SO_4 and the product was quantified in a microplate reader (Fluostar Omega, BMG Labtech, Germany) by measuring absorbance at 450nm (λ_{ref}= 690nm). The OD values obtained for 72h was normalized for the 0h time point.

13. Statistical analysis

Differences between two groups were assessed by non-parametric Mann-Whitney U test. For comparison between three or more groups, non-parametric Kruskal-Wallis test was performed. For this, we used GraphPad Prism6.

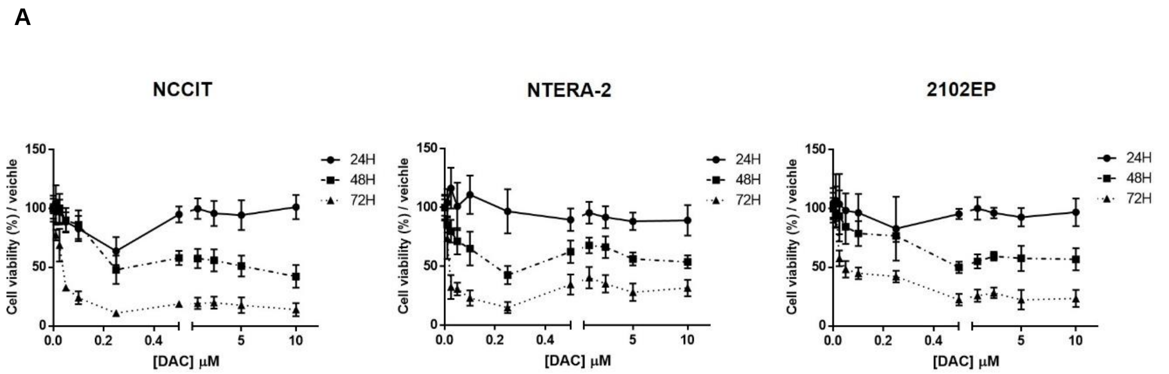
$p<0,05$ was considered to indicate a statistically significant difference. Significance is show *vs* the respective control and depicted as follows: $*/^{\#}p<0.05$, $**/^{\#\#}p<0.01$, $***/^{\#\#\#}p<0.001$, $****/^{\#\#\#\#}p<0.0001$, and $^{ns}p>0.05$ (non-significant).

RESULTS

1. Cell viability

Cell viability and IC_{50} was determined for the tested TGCT cells for each drug at 24, 48 and 72h treatment exposure.

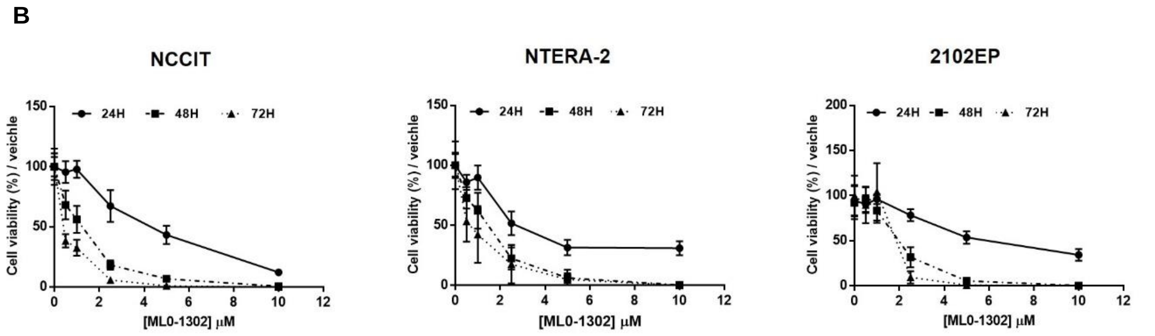
Regarding DAC, cell viability (in all cell lines) decreased with low nanomolar doses until around 10-30% viability after 72h of treatment. No additional effect on viability was observed with concentrations above 0.25µM. A time dependent response was shown, once for all concentrations cell viability was significantly lower at 72h, compared to 48h and 24h. Moreover, IC_{50} decreased significantly over time, being the IC_{50} of 0.035µM for NCCIT, 0.009µM for NTERA-2, and 0.12µM for 2102EP at 72h of exposure (Figure 11A).



	24h	48h	72h
IC_{50}	>10µM	2.97µM	0.035µM

	24h	48h	72h
IC_{50}	>10µM	>10µM	0.009µM

	24h	48h	72h
IC_{50}	>10µM	7.15µM	0.12µM



	24h	48h	72h
IC_{50}	3.92µM	1.11µM	0.42µM

	24h	48h	72h
IC_{50}	3.24µM	1.02µM	0.57µM

	24h	48h	72h
IC_{50}	6.25µM	2.08µM	2.17µM

Figure 11: NCCIT, NTERA-2, and 2102EP cell viability after exposure to drugs, at 24, 48 and 72h, and respective IC50. **(A)** DAC treatment. **(B)** MLo-1302 treatment.

NCCIT, NTERA-2, and 2102EP cell viability and their respective IC_{50} were assessed at 24, 48 and 72 hours after MLo-1302 treatment (Figure 11B). Overall, cell viability decreased with increasing doses of the compound, for all time points. Also, cell viability was found to be dependent on exposure time, being the lowest after 72h of exposure. This is illustrated by the lower IC_{50} values at 72h of drug exposure, namely an IC_{50} of 0.42µM for NCCIT, 0.57µM for NTERA-2, and 2.17µM for 2102EP.

2. Differentiation

To evaluate the effect of studied drugs in differentiation, the pluripotency-related markers (NANOG, OCT3/4 and SOX2) expression was assessed by western blot. For ATRA, PAX6, a (neuronal) differentiation-related marker was also evaluated. It was used 10µM of ATRA, 0.01µM and 1µM doses of DAC, and 0.25µM and 0.5µM of MLo-1302.

All pluripotency-related markers were expressed across baseline cell lines. However, NCCIT showed the highest expression of pluripotency markers, mainly OCT3/4 and SOX2. This latter was significantly lower in 2102EP cell line, with significant differences between NCCIT and 2102EP cell lines (Figure 12A).

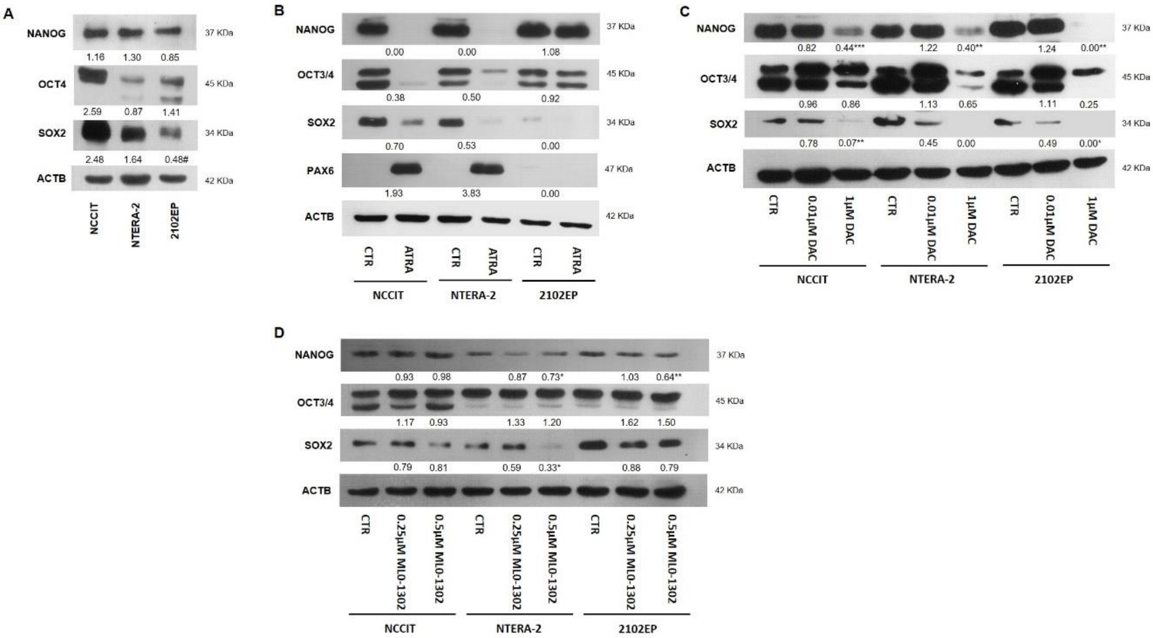

A
NANOG
37 KDa
1.16
1.30
0.85
OCT4
45 KDa
2.59
0.87
1.41
SOX2
34 KDa
2.48
1.64
0.48#
ACTB
42 KDa
NCCIT
NTERA-2
2102EP
B
NANOG
37 KDa
0.00
0.00
1.08
OCT3/4
45 KDa
0.38
0.50
0.92
SOX2
34 KDa
0.70
0.53
0.00
PAX6
47 KDa
1.93
3.83
0.00
ACTB
42 KDa
CTR
ATRA
CTR
ATRA
CTR
ATRA
NCCIT
NTERA-2
2102EP
C
NANOG
37 KDa
0.82
0.44***
1.22
0.40**
1.24
0.00**
OCT3/4
45 KDa
0.96
0.86
1.13
0.65
1.11
0.25
SOX2
34 KDa
0.78
0.07**
0.45
0.00
0.49
0.00*
ACTB
42 KDa
CTR
0.01µM DAC
1µM DAC
CTR
0.01µM DAC
1µM DAC
CTR
0.01µM DAC
1µM DAC
NCCIT
NTERA-2
2102EP
D
NANOG
37 KDa
0.93
0.98
0.87
0.73*
1.03
0.64**
OCT3/4
45 KDa
1.17
0.93
1.33
1.20
1.62
1.50
SOX2
34 KDa
0.79
0.81
0.59
0.33*
0.88
0.79
ACTB
42 KDa
CTR
0.25µM ML0-1302
0.5µM ML0-1302
CTR
0.25µM ML0-1302
0.5µM ML0-1302
CTR
0.25µM ML0-1302
0.5µM ML0-1302
NCCIT
NTERA-2
2102EP

Figure 12: Differentiation pattern in TGCT cell lines at **(A)** basal levels; **(B)** after ATRA treatment; **(C)** after DAC treatment; **(D)** after MLo-1302 treatment. $^{\#}$NCCIT vs 2102EP; *Treated *vs* CTR.

After ATRA treatment, NCCIT and NTERA-2 cell lines displayed a significantly decrease in all pluripotency-related markers, while PAX6 expression levels were increased. Conversely, in 2102EP cell line, no significant differences were observed for NANOG and OCT3/4, and SOX2 and PAX6 expression levels were very minor/absent (Figure 12B).

Regarding DAC treatment, a remarkable decrease of all pluripotency-related markers after treatment with 1µM of DAC was apparent, although a minor effect was observed at 0.01µM (Figure 12C).

Furthermore, MLo-1302 at 0.5µM also impacted in cells' differentiation. Indeed, NANOG protein levels were significantly decreased in all cell lines, whereas SOX2 expression only significantly decreased in NTERA-2. No significant differences were found between treated cells and control at lower dose of 0.25µM MLo-1302 (Figure 12D).

3. DNMTs expression

DNMTs expression was assessed by western blot technique for 0.01µM and 1µM concentrations of DAC and 0.25µM and 0.5µM of MLo-1302. For ATRA, 10µM was used.

Firstly, we characterized cell lines at baseline levels (Figure 13A). NCCIT cell line presented the highest expression of all DNMTs, especially the DNMT3B. DNMT3A was the least expressed in all studied cell lines.

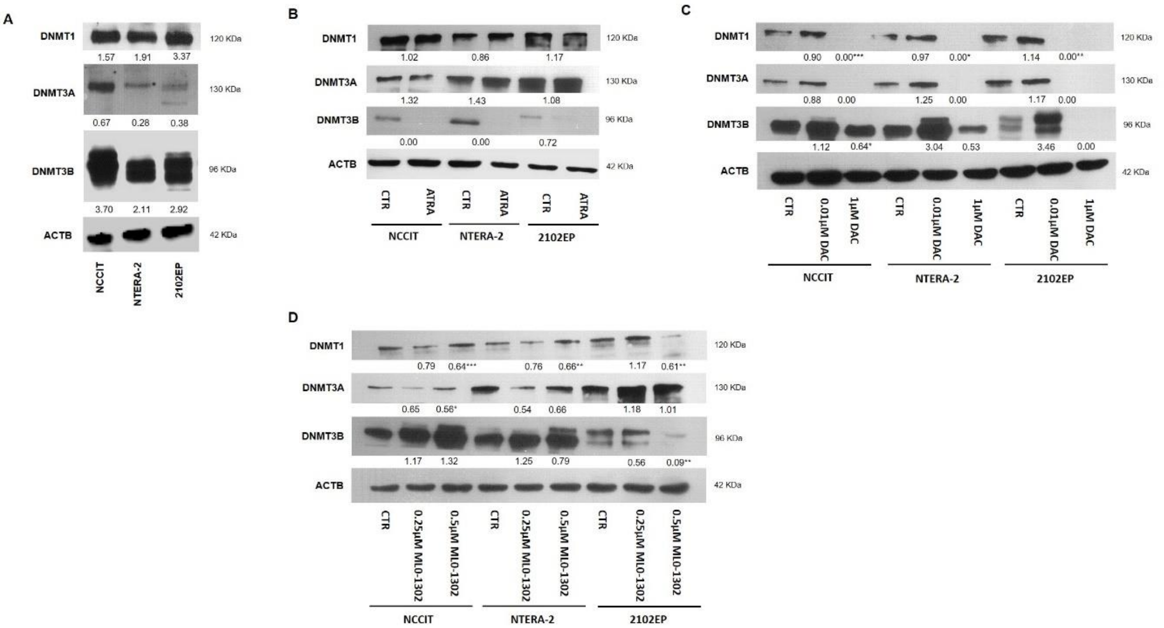

A
DNMT1
1.57
1.91
3.37
120 KDa
DNMT3A
130 KDa
0.67
0.28
0.38
DNMT3B
96 KDa
3.70
2.11
2.92
ACTB
42 KDa
NCCIT
NTERA-2
2102EP
B
DNMT1
120 KDa
1.02
0.86
1.17
DNMT3A
130 KDa
1.32
1.43
1.08
DNMT3B
96 KDa
0.00
0.00
0.72
ACTB
42 KDa
CTR
ATRA
CTR
ATRA
CTR
ATRA
NCCIT
NTERA-2
2102EP
C
DNMT1
120 KDa
0.90
0.00***
0.97
0.00*
1.14
0.00**
DNMT3A
130 KDa
0.88
0.00
1.25
0.00
1.17
0.00
DNMT3B
96 KDa
1.12
0.64*
3.04
0.53
3.46
0.00
ACTB
42 KDa
CTR
0.01µM DAC
1µM DAC
CTR
0.01µM DAC
1µM DAC
CTR
0.01µM DAC
1µM DAC
NCCIT
NTERA-2
2102EP
D
DNMT1
120 KDa
0.79
0.64***
0.76
0.66**
1.17
0.61**
DNMT3A
130 KDa
0.65
0.56*
0.54
0.66
1.18
1.01
DNMT3B
96 KDa
1.17
1.32
1.25
0.79
0.56
0.09**
ACTB
42 KDa
CTR
0.25µM ML0-1302
0.5µM ML0-1302
CTR
0.25µM ML0-1302
0.5µM ML0-1302
CTR
0.25µM ML0-1302
0.5µM ML0-1302
NCCIT
NTERA-2
2102EP

Figure 13: DNMTs expression in TGCT cell lines **(A)** at basal levels; **(B)** after ATRA treatment; **(C)** after DAC treatment; **(D)** after MLo-1302 treatment. *Treated *vs* CTR.

No significant differences were apparent between control and ATRA for DNMT1 protein levels, despite the slight increase found in 2102EP cell line. The same pattern was seen for DNMT3A expression, where no significant differences were displayed, although a tendency for increased expression was apparent. On the contrary, decreased DNMT3B protein levels were shown by all cell lines (Figure 13B).

Concerning DAC, DNMTs expression changes were achieved. Indeed, 1µM dosage led to significant DNMT1, DNMT3A and DNMT3B protein decrease in all three cell lines, while 0.01µM DAC did not impact in DNMTs expression (Figure 13C).

Lower concentrations of MLo-1302 (0.25µM) showed a minor effect in DNMTs expression. However, treatment with 0.5µM of this compound significatively decreased DNMT1 expression in all cell lines and, additionally, of DNMT3A in NCCIT and 2102EP and of DNMT3B in 2102EP cells. Both DNMT3A and DNMT3B proteins were decreased in NTERA-2, although not significantly (Figure 13D).

4. Methylation global (5mC) and loci specific (*RASSF1A* promoter) status

Previous studies by our group showed that *RASSF1A* (among others) gene promoter was hypermethylated in TGCTs, mainly in NSs (66). Based on this, methylation status of this gene promoter was evaluated by qMSP at baseline and after exposure to the different compounds. Additionally, 5mC levels were quantified after DAC and MLo-1302 treatments, using dotblot and IF (the latter only for the MLo-1302). Treatments with 0.01µM and 1µM DAC, and 0.25µM, 0.5µM, and 1µM MLo-1302 were performed.

At baseline (Figure 14A), NCCIT and NTERA-2 present similar *RASSF1A* methylation levels, whereas 2102EP displayed the lowest levels.

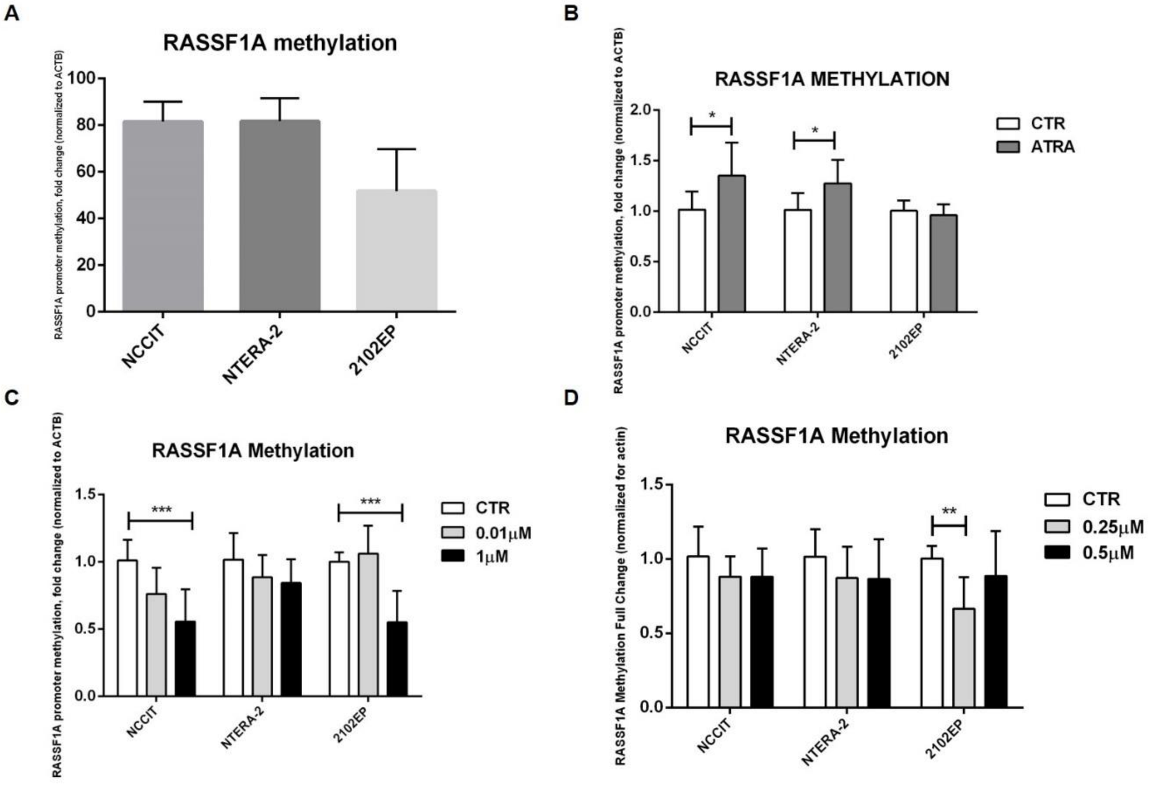


Figure 14: Distribution of RASSF1A promoter's methylation levels: **(A)** Basal levels. **(B)** ATRA treatment. **(C)** DAC treatment. **(D)** MLo-1302 treatment. *Treated *vs* CTR.

After ATRA treatment, *RASSF1A* methylation levels increased significantly in NCCIT and NTERA cell lines, while no differences were observed in 2102EP cell line.

Regarding DAC treatment, 1µM significantly decreased *RASSF1A* methylation levels, particularly in NCCIT and 2102EP cell lines (Figure 14B), although no effect was observed with 0.01µM. Nonetheless, no alterations were apparent for 5mC levels at both DAC concentrations (Figure 15A).

Concerning MLo-1302 compound, only a significant effect was found for 2102EP treated with 0.25 µM (Figure 14D). Conversely, significant 5mC levels decrease were observed in NCCIT cell line treated with 0.5µM (Figure 15B). Moreover, 5mC levels were significantly reduced in NTERA-2 and 2102EP cell lines treated with 1µM MLo-1302 (Figure 15C).

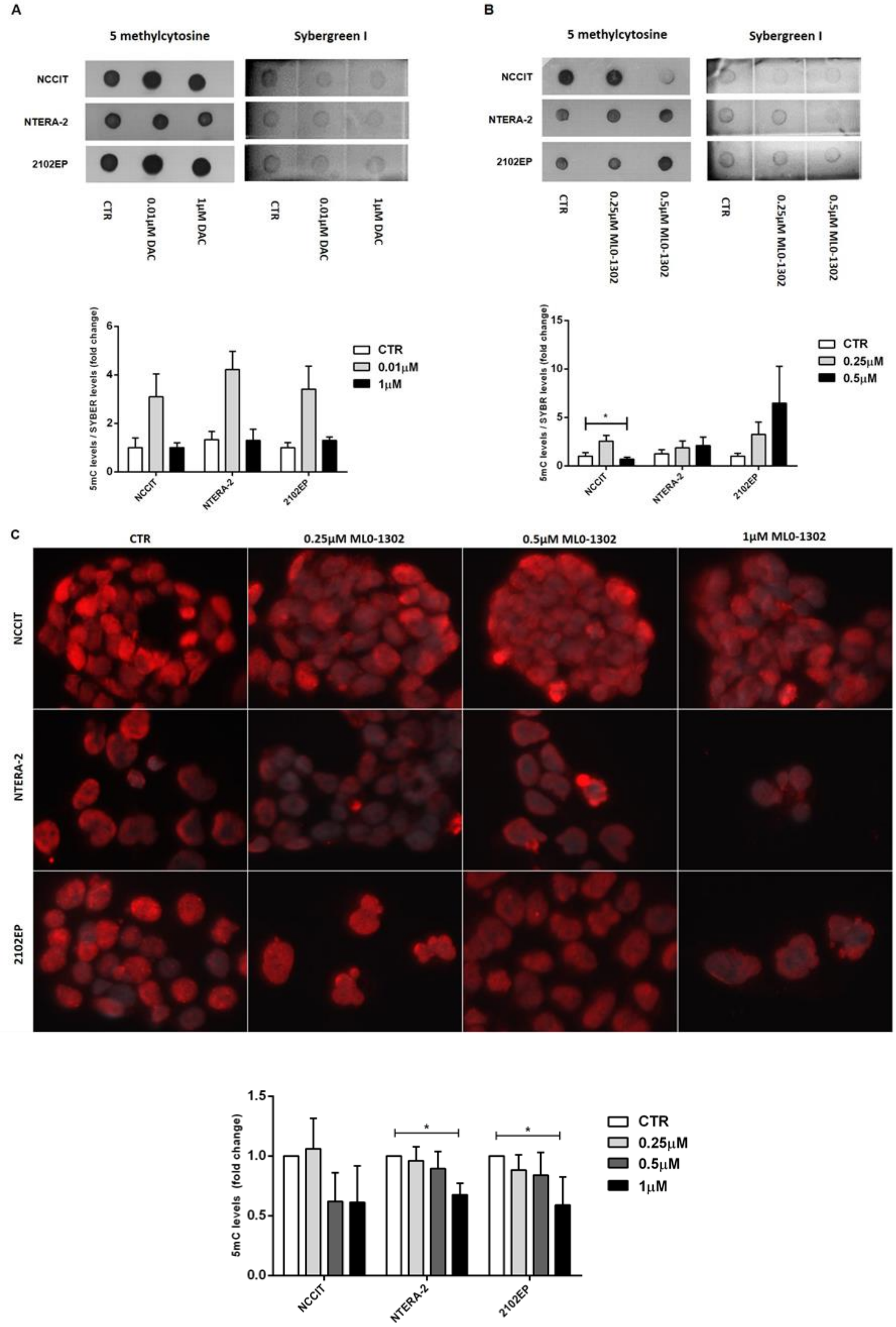

A
5 methylcytosine
Sybergreen I
NCCIT
NTERA-2
2102EP
CTR
0.01µM DAC
1µM DAC
5mC levels / SYBER levels (fold change)
CTR
0.01µM
1µM
B
5 methylcytosine
Sybergreen I
CTR
0.25µM ML0-1302
0.5µM ML0-1302
5mC levels / SYBR levels (fold change)
CTR
0.25µM
0.5µM
*
C
CTR
0.25µM ML0-1302
0.5µM ML0-1302
1µM ML0-1302
NCCIT
NTERA-2
2102EP
5mC levels (fold change)
CTR
0.25µM
0.5µM
1µM
*
*

Figure 15: 5mC levels after DAC and MLo-1302 treatments. **(A)** Dot blot for DAC. **(B)** Dot blot for MLo-1302. **(C)** IF for MLo-1302. *Treated *vs* CTR.

Overall, although MLo-1302 modulated DNMTs expression and, more importantly, methylation profile, the results were not consistent in all cell lines and conditions. Given the impact on cell viability, we hypothesize that other off-target effects or non-methylation-related effects may be elicited upon treatment with this compound and set out to explore downstream pathways that could be affected.

5. LDH assay

LDH assay was performed to assess the possible MLo-1302's cytotoxic effect. For this, cells were treated with 0.25µM, 0.5µM, and 1µM MLo-1302.

NCCIT and NTERA-2 cell lines showed increased LDH percentage with increasing concentrations of MLo-1302, achieving significance in NTERA-2 treated with MLo-1302 1µM. Hence, MLo-1302 showed a cytotoxic effect in these two cell lines, particularly at 1µM. Conversely, no apparent cytotoxic effect was observed in 2102EP (Figure 16).

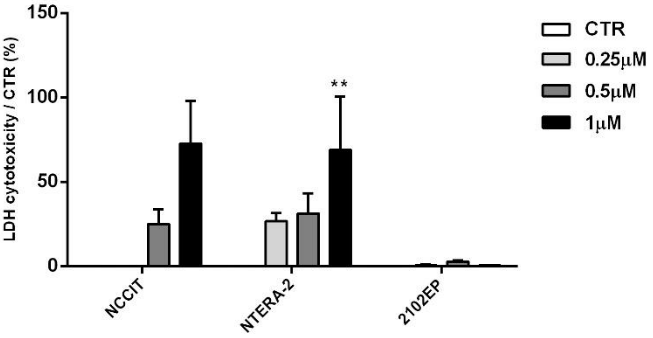


Figure 16: LDH cytotoxicity after MLo-1302 treatment.

6. RT^2 profiler array

To better dissect other possible mechanisms of action of this compound RT^2 profiler array was performed in NCCIT cell line treated with 0.5µM of MLo-1302.

Herein, carnitine palmitoyltransferase 2 (CPT2), keratin 14 (KRT14), protein phosphatase 1 regulatory subunit 15A (PPP1R15A), snail homolog 3 (SNAI3), and placental growth factor (PGF) genes were found to be upregulated, while, baculoviral IAD repeat containing 3/Inhibitor of apoptosis protein 1 (BIRC3), DNA damage inducible transcript 3 (DDIT3), kinase insert domain receptor (KDR)/Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), and nucleolar protein 3/apoptosis repressor with CARD domain (NOL3) genes were downregulated, the latter one with a statistically significant decrease between the treated and control group (Figure 17 and Table 9).

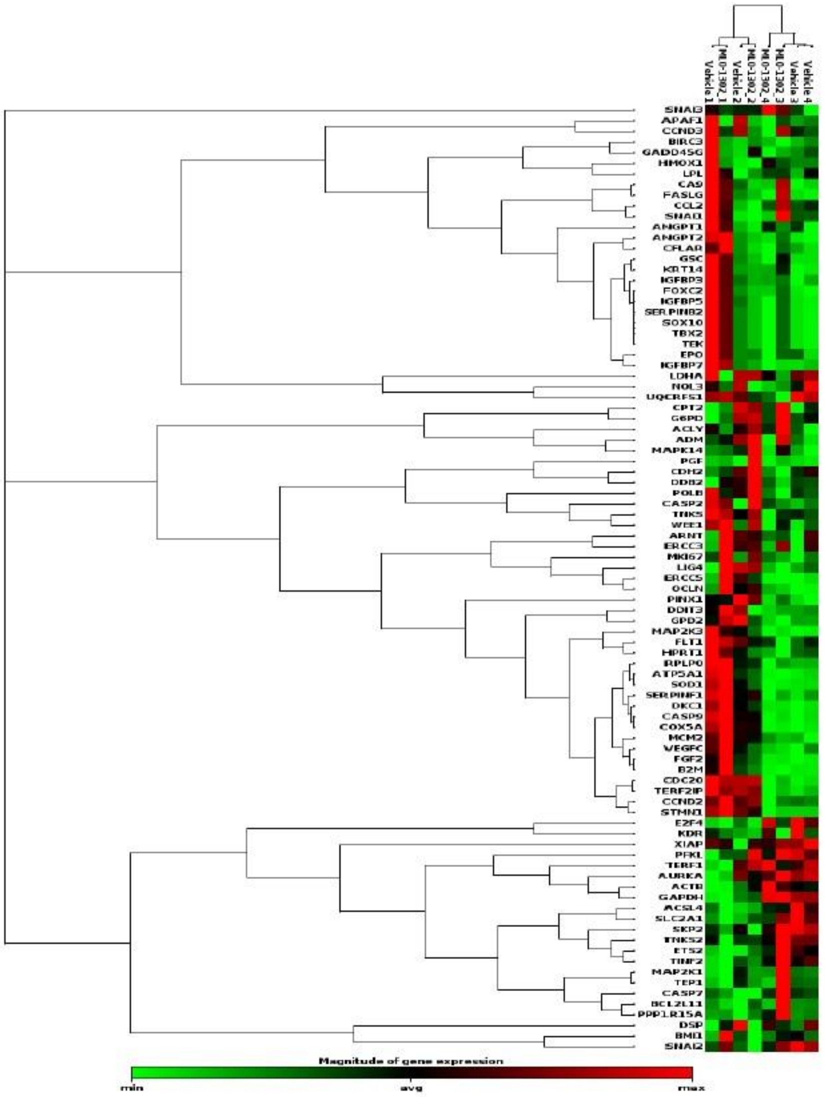


Figure 17: Clustergram representative of genes that are down and upregulated with 0.5µM MLo-1302 treatment.

Table 9: Main function of genes that are deregulated with MLo-1302 treatment.

Gene	Main Functions	Fold regulation
CPT2	Transport of fatty acids in the mitochondria	1.64
KRT14	Cell cytoskeleton constituent	1.52
PPP1R15A	Stress-induced DNA damage Growth arrest Apoptosis	2.39
SNAI3	Mesodermal formation during embryogenesixs	1.73
PGF	Angiogenesis Trophoblast growth and differentiation	2.13
BIRC3	Apoptosis inhibition	-1.57
DDIT3	Apoptosis and cell cycle arrest promotion, induced by ER stress	-2.13
VEGFR2	Promotes angiogenesis	-1.56
NOL3	Anti-apoptotic protein that downregulates CASP8	-1.86**

Abbreviations: ER - endoplasmic reticulum; CASP8 – caspase 8; **treated vs control; Red: upregulated genes; Green: downregulated genes.

Globally, the most deregulated pathways were related to apoptosis and cell cycle arrest. According with these results, and since NOL3 negatively regulates caspase 8 (CASP8), CASP8 expression was evaluated as well as, cell apoptosis and proliferation.

6.1. Caspase 8

To evaluate the effect of MLo-1302 in cleaved CASP8 expression 0.5µM and 1µM doses was used in all cell lines. Herein, increased cleaved CASP8 protein expression was found in treated NCCIT and NTERA-2 cell lines with MLo-1302 0.5µM concentration, with no significant changes in protein expression for 2102EP (Figure 18).

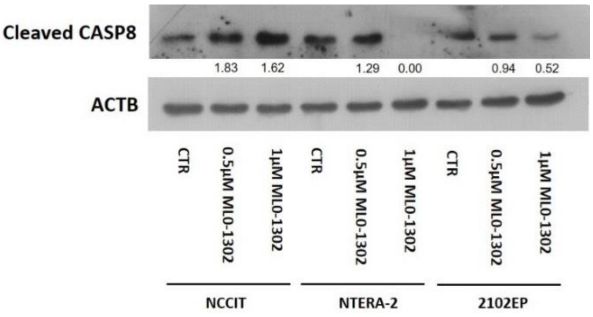


Figure 18: Cleaved caspase 8 expression with TGCT cell lines were treated with 0.5µM and 1µM of MLo-1302. CASP8 – Caspase 8.

6.2. Apoptosis

Apoptosis was evaluated for all studied cell lines treated with 0.5µM and 1µM. MLo-1302 exposure with the 1µM concentration at 72h significantly increased apoptosis levels in treated NCCIT and NTERA-2 cell lines, whereas no significant effect was apparent in 2102EP (Figure 19).

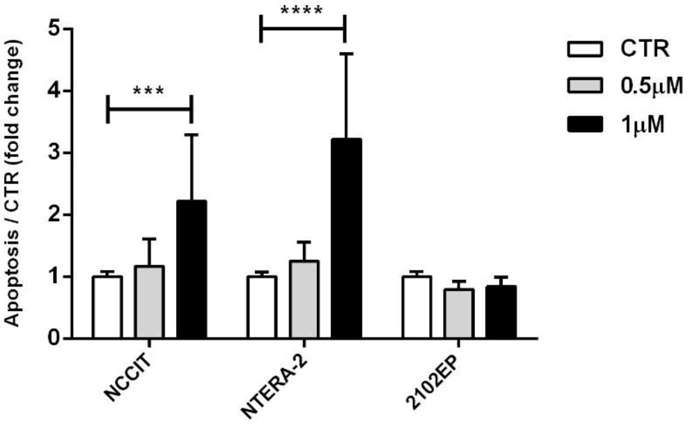


Figure 19: Effect in apoptosis when TGCT cell lines was exposed to MLo-1302 compound. *Treated *vs* CTR.

6.3. Proliferation

Herein, 1µM MLo-1302 significantly decreased proliferation in all cell lines, while 0.5µM only significantly affected NTERA-2 cell line (Figure 20).

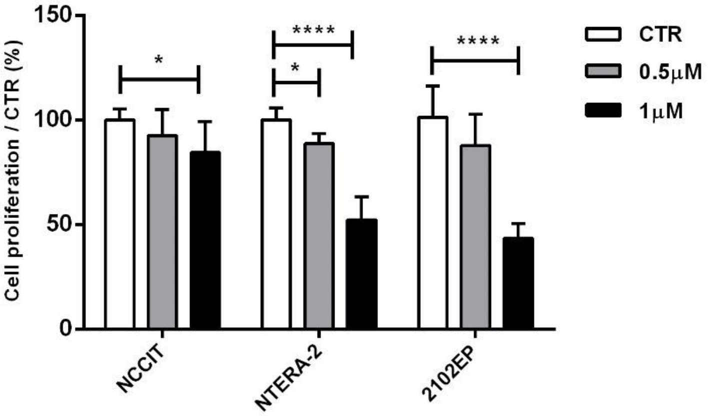


Figure 20: MLo-1302’s effect in cell lines’ proliferation. *Treated *vs* CTR.

DISCUSSION

Over the last years, epigenetic therapies have gained special attention as novel cancer therapies and have been increasingly studied, with the ultimate goal of enhancing specificity and selectivity, while decreasing side effects. Importantly, it is known that “epidrugs” exhibit less toxicity than conventional chemotherapy (46). To date, only a limited number of trials have included TGCT patients, which should deserve more attention, especially those acquiring cisplatin resistance for which no curative treatments are available. Although in pre-clinical studies these drugs appear to have an anti-cancer effect, no significant efficacy was demonstrated in clinical trials (59). Thus, the challenge is to synthesize and test new compounds for TGCT treatment, possibly natural compounds that have been demonstrated to be anti-cancer agents associated with low toxicity (46). Therefore, the main goal of this Dissertation is to evaluate the effect of a new compound, MLo-1302 (demonstrated in other tumor models to have anti-tumor properties) in comparison with ATRA and DAC, previously demonstrated to act both as differentiation and demethylating agents. MLo-1302 is derived from flavanones and was designed and synthetized to inhibit DNMTs (118). ATRA is the all-trans-retinoic acid, already approved for the treatment of acute promyelocytic leukemia, being involved in several anti-neoplastic processes, acts by inducing differentiation of these neoplastic cells (119). DAC is a well-known demethylating agent, with anti-cancer proprieties, already approved for treatment of haematological tumors (46, 50).

We started to evaluate the effect of MLo-1302 in cell viability, comparing with DAC. We observed that DAC reduced tumor cell viability, even with low doses since viability decreased until 10-30% with nanomolar concentrations. However, from 0.25µM, we no longer found an effect in viability, concluding that this concentration was the limiting dose to kill cells. According with these results, our hypothesis was that the cells that survive can efficiently differentiate and fail to be eliminated by DAC. Indeed, there is a pluripotency markers’ decrease associated with DAC treatment, more obvious with 1µM than with 0.01µM. Importantly, the effect on viability was dependent of time, with a IC_{50} at 72h of approximately 0.01µM (lethal minimum dose). *Albany et al.* (120) reported similar results, with an IC_{50} of 0.01µM in NTERA-2 cell line. In the same line, in TGCTs, DAC IC_{50} was described to be lower than in other solid tumors (121, 122).

Regarding MLo-1302, decreased cell viability associated with increased doses and time of exposure was observed. IC_{50} was lower at 72h, being approximately of 0.5µM for NCCIT and NTERA-2. 2102EP was the least responsive drug (IC^{50}~2µM). This is in line with previous findings, as 2102EP was less sensitive to both DAC and ATRA (see above). Moreover, in our hands, renal cell carcinoma cell lines, displayed IC_{50} values at much higher ranges than those obtained with TGCT lines (1.5 to 3µM) (*Marques-Magalhães et* *al., under submission*), which seems to be in line with the higher sensitivity of these tumor cells to demethylating agents in general (like observed for DAC).

Since DAC (and demethylating agents in general) has been shown to also induce cell differentiation (59, 110, 113), MLo-1302 effect in differentiation was evaluated and compared with DAC and with the classical potent differentiating agent ATRA. Overall, all TGCT cell lines presented high protein expression of pluripotency markers, as described by *Josephson et al.* (123), *van der Zwan et al.* (124), and *Perrett et al.* (125), but with more prevalence in NCCIT, possibly due to its p53-mutated state. Indeed, some studies (126, 127) reported that p53 alterations lead to pluripotency markers' overexpression. The mechanism can be associated with miRNAs, since it has already been described that the presence of p53 activates expression of miR-34a and miR-145, which repress stem factors such as OCT3/4 and SOX2 (126). The exposure of these cell lines to ATRA was associated with decrease pluripotency markers' expression, while PAX6 – (neuronal) differentiation-related marker drug-specific for ATRA – was increased. However, this was not visible for 2102EP, because this cell line did not show the capacity to differentiate with retinoic acid (123). As mentioned before, DAC decreased pluripotency markers expression, mainly when cells were treated with 1µM. Comparing with ATRA and DAC, MLo-1302 showed a partial effect in differentiation, as there was only NANOG decreased expression in all lines, and of SOX2 in NTERA-2.

Because MLo-1302 compound was designed to inhibit DNMTs, the effect of this new compound in DNMTs expression was also evaluated. In fact, previous results in our group in renal cell carcinoma cell lines demonstrated a significant DNMT3A expression reduction in all cell lines and an additional downregulation of DNMT1, mainly in Caki-2 cell line (Marques-Magalhães *et al*., *under submission*). In TCGT cells, a significant DNMT1 decrease was found in all cell lines, whereas DNMT3A and 3B expression was only reduced in some cell lines, when treated with 0.5µM. Moreover, 0.25µM MLo-1302 did not significantly affect DNMTs expression. Hence MLo-1302 effect is not as evident as DAC, where a significant decrease/absence of expression of DNMTs expression (mainly with 1µM) was found as previously described (112, 113). Some studies (128, 129) suggest that azacitadine/DAC may form links with DNA, preventing the interaction of DNMTs (mainly DNMT1) with DNA, with this enzyme being then targeted for degradation by the proteasome.

Importantly, DNMTs are differently expressed in TGCTs (50). Indeed, in our hands, NCCIT presented highest expression of all DNMTs, specially DNMT3B. Immunohistochemical evaluation in primary tumors showed that DNMTs expression was greater in more differentiated tumors (130) which is in accordance with our results, since ATRA induced DNMT1 and DNMT3A expression, although not reaching statistical significance (except for DNMT1 in NTERA cell line), and this was actually accompanied by increased methylation of *RASSF1A* gene promoter. Conversely, DNMT3B expression was decreased with ATRA, which is in accordance with its role in pluripotency regulation (112, 131).

Because MLo-1302 had a partial effect in DNMTs expression, cells lines methylation profile was tested. MLo-1302 associated with reduced *RASSF1A* promoter methylation levels, although not significantly, while 1µM DAC treatment significantly decreased *RASSF1A* promoter methylation levels, further supporting previous results (112, 120). When assessing the global 5mC levels, a significant decrease was only achieved in NCCIT cell line when treated with 0.5µM MLo-1302 and in NTERA-2 and 2102EP cell lines when treated with 1µM MLo-1302, although an apparent concordant progressive decrease of 5mC was displayed by all cell lines. Surprisingly, DAC did not produce a significant effect in global methylation. This results are in the same line as those obtained by *Juttermann et al.* (132), that showed the anti-neoplastic effect of DAC is more dependent on DNMTs expression levels than on actual genome demethylation.

Overall, MLo-1302 seems to have a partial effect on DNMTs expression and methylation profile, depending on cell type. Therefore, suggesting that other mechanisms might also be activated upon cells treatment with this compound. Indeed, the compound was cytotoxicity in NCCIT and NTERA-2 cell lines, but not in 2102EP (the cell line with less effect on viability as well).

Furthermore, we found that genes associated with apoptosis and cell cycle arrest (essentially) were deregulated, despite only NOL3 showed a significant decrease. This gene is a known CASP8 inhibitor, which, in turn, activates CASP3, leading to apoptosis (133). Herein, NOL3 was downregulated, while cleaved CASP8 was upregulated, leading subsequently to apoptosis. Indeed, a slight increase of cleaved CASP8, mainly in NCCIT cell line and significant increased apoptosis was observed in NCCIT and NTERA-2 cell lines treated with 1µM MLo-1302. In the same line, proliferation was significantly decreased in 2102EP cell line. Henceforth, our results indicate that the compound induces apoptosis and cytotoxicity/cell death in NCCIT and NTERA-2 cell lines, while in 2102EP the effect was mainly in cell cycle.

Moreover, other apoptosis-related genes were also deregulated, although without statistical significance. Namely, BIRC3, an inhibitor of CASP3, was downregulated (134). Contrarily, CPT2 allows the entry of fatty acids into the mitochondria which, when oxidized, increases the reactive oxygen species (ROS), leading to an apoptosis increasing (135) – and this was upregulated. Furthermore, PPP1R15A is responsible for inducing cell cycle arrest, which is in the same line the proliferation results (136).

Globally, that gathered data suggests that MLo-1302 not only acts as a demethylating and differentiating agent, but also acts in pathways associated with apoptosis and proliferation – Figure 21.

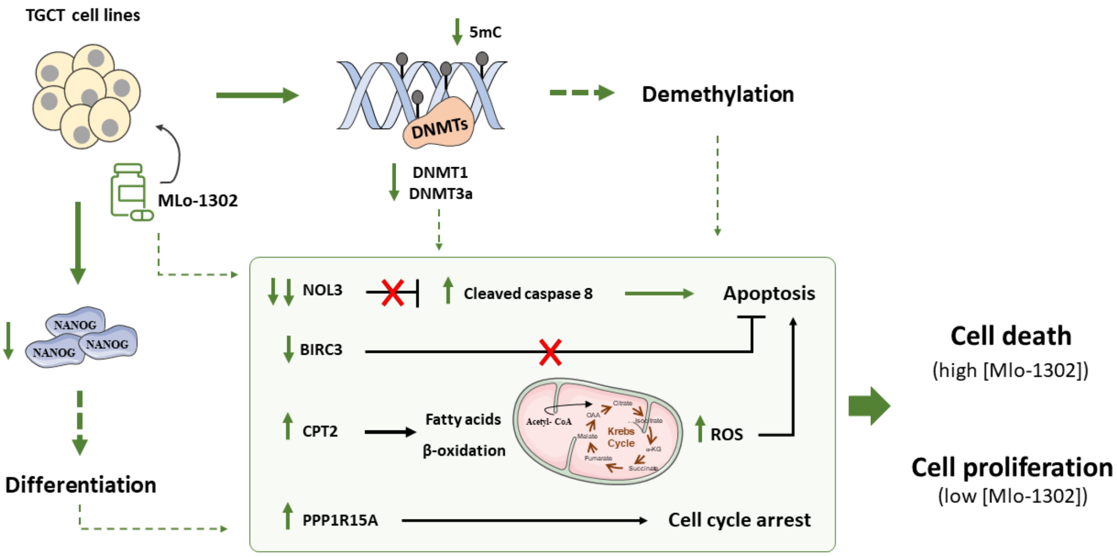

TGCT cell lines
MLo-1302
5mC
DNMTs
DNMT1
DNMT3a
Demethylation
NANOG
NANOG
NANOG
Differentiation
NOL3
Cleaved caspase 8
Apoptosis
BIRC3
CPT2
Fatty acids
β-oxidation
Acetyl- CoA
OAA
Citrate
Isocitrate
α-KG
Succinate
Fumarate
Malate
Krebs Cycle
ROS
PPP1R15A
Cell cycle arrest
Cell death
(high [Mlo-1302])
Cell proliferation
(low [Mlo-1302])

Figure 21: Possible anti-neoplastic mechanisms of MLo-1302 in TGCT cell lines. MLo-1302 influences DNA methylation, with a reduction of DNMT1 and DNMT3A expression and, consequently demethylation of DNA. On the other hand, MLo-1302 decreases NANOG expression, leading to differentiation of cells. This can be indirectly associated with cell death. However, MLo-1302 also decreases anti-apoptotic genes (NOL3 and BIRC3) and increases pro-apoptotic genes, like CPT2, that consequently increase apoptosis. Still, this compound leads an increase of PPP1R15A, that is associated with cell cycle arrest. TGCT – testicular germ cell tumor; DNMTs – DNA methyltransferases; ROS – reactive oxygen species.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this Master Dissertation, we were able to demonstrate that newly synthesized flavanone-derived compound, MLo-1302, attenuated malignant phenotype in TGCT cell lines. Specifically, it has a partial effect on differentiation, decreasing pluripotency-related markers, and on demethylation, with a slight reduction of DNMTs expression and DNA methylation at both global and loci specific levels. Additionally, MLo-1302 has an important impact in cell death and proliferation, increasing apoptosis mediated by caspases. Hence, MLo-1302 might be a promising anti-neoplastic DNMTi for TGCT therapy, but more studies are required to confirm its efficacy, safety, and reliability.

In fact, this compound showed an effect in DNA methylation, but only DNMTs expression was addressed and TETs have also been implicated in this process. Thus, these proteins should also be investigated. Furthermore, the lack of expression alterations is a very limited approach to test the effect of the compound in DNMTs function. Hence, it is important to study DNMTs/TETs activity. Additionally, as epigenetic mechanisms do interact, it will be important to study other epigenetic mechanisms (not only DNA methylation), such as HDACs deregulation and alterations in histone acetylation.

Finally, other phenotype effects, such as migration and invasion might be studied, since some genes that were deregulated in the array have been implicated in these processes (for example, VEGFR2).

Moreover, to better ascertain the safety and putative toxicity of this compound, *in vivo* studies should also be performed.

Overall, and taking in to account the increasing knowledge of epigenetic regulation mechanisms, there are many opportunities for targeted treatment with epigenetic-base strategies in TGCTs. Indeed, more studies have been developed, including combined therapies (not only with epigenetic agents, but also with these drugs and other therapies, like immunotherapies), natural compounds, proteolysis targeting chimeras (PROTACs), histone mark (ubiquitination), non-coding RNAs, and epitranscriptomics (Figure 22).

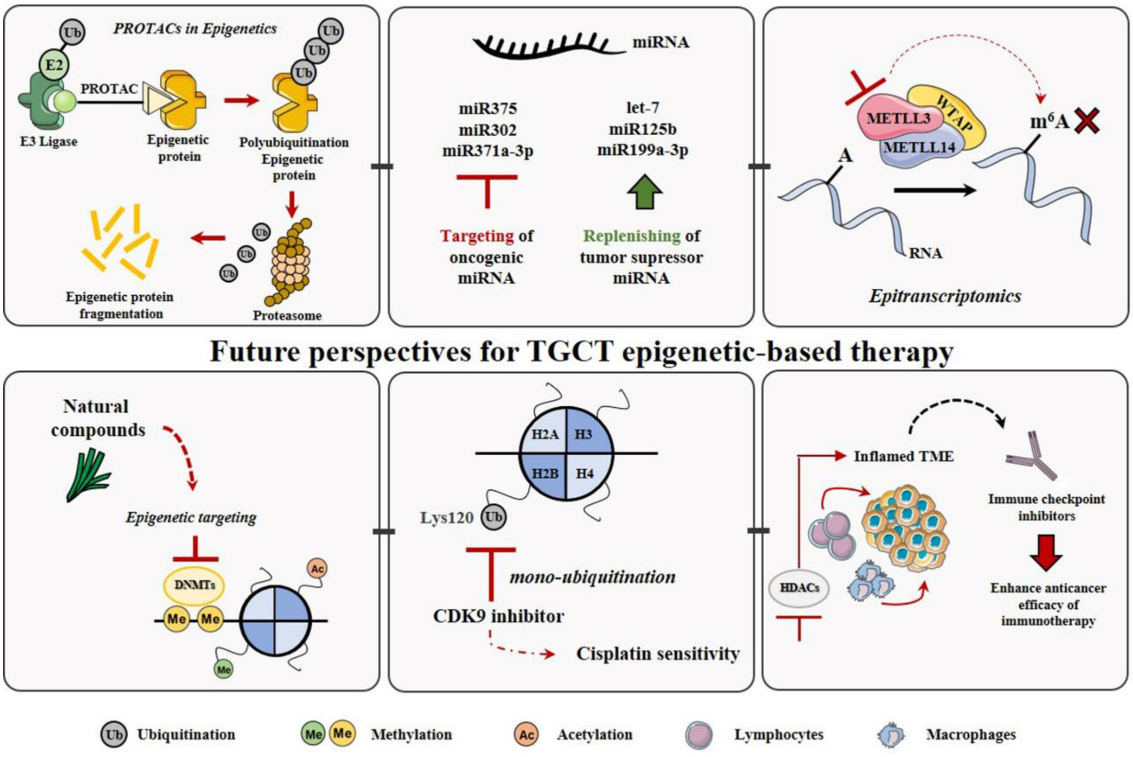

PROTACs in Epigenetics
Ub
E2
PROTAC
E3 Ligase
Epigenetic protein
Polyubiquitination Epigenetic protein
Proteasome
Epigenetic protein fragmentation
miRNA
miR375
miR302
miR371a-3p
Targeting of oncogenic miRNA
let-7
miR125b
miR199a-3p
Replenishing of tumor supressor miRNA
METLL3
WTAP
METLL14
A
m6A
RNA
Epitranscriptomics
Future perspectives for TGCT epigenetic-based therapy
Natural compounds
Epigenetic targeting
DNMTs
Me
Ac
H2A
H3
H2B
H4
Lys120
mono-ubiquitination
CDK9 inhibitor
Cisplatin sensitivity
Inflamed TME
HDACs
Immune checkpoint inhibitors
Enhance anticancer efficacy of immunotherapy
Ubiquitination
Methylation
Acetylation
Lymphocytes
Macrophages

Figure 22: Overview of future directions in epigenetic-based therapies for testicular germ cell tumour. In: *Cardoso et al*., 2020 (59).

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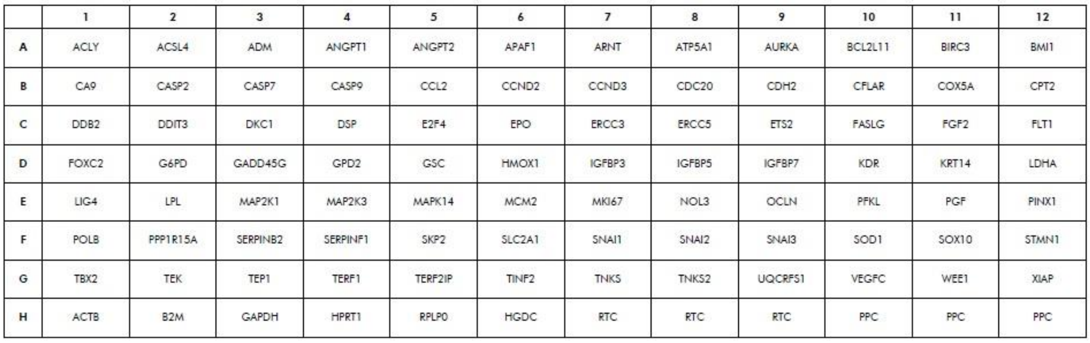
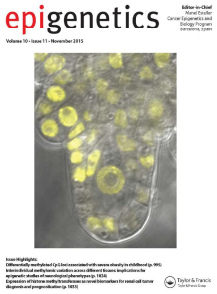
ANNEXS

Annex 1: Layout of RT2 profiler array plates



	1	2	3	4	5	6	7	8	9	10	11	12
A	ACLY	ACSL4	ADM	ANGPT1	ANGPT2	APAF1	ARNT	ATP5A1	AURKA	BCL2L11	BIRC3	BMI1
B	CA9	CASP2	CASP7	CASP9	CCL2	CCND2	CCND3	CDC20	CDH2	CFLAR	COX5A	CPT2
C	DDB2	DDIT3	DKC1	DSP	E2F4	EPO	ERCC3	ERCC5	ETS2	FASLG	FGF2	FLT1
D	FOXC2	G6PD	GADD45G	GPD2	GSC	HMOX1	IGFBP3	IGFBP5	IGFBP7	KDR	KRT14	LDHA
E	LIG4	LPL	MAP2K1	MAP2K3	MAPK14	MCM2	MKI67	NOL3	OCLN	PFKL	PGF	PINX1
F	POLB	PPP1R15A	SERPINB2	SERPINF1	SKP2	SLC2A1	SNAI1	SNAI2	SNAI3	SOD1	SOX10	STMN1
G	TBX2	TEK	TEP1	TERF1	TERF2IP	TINF2	TNKS	TNKS2	UQCRFS1	VEGFC	WEE1	XIAP
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC



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Ana Rita Cardoso , João Lobo , Vera Miranda-Gonçalves , Rui Henrique & Carmen Jerónimo

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REVIEW

Epigenetic alterations as therapeutic targets in Testicular Germ Cell Tumours : current and future application of ‘epidrugs’

Ana Rita Cardoso [a,b], João Lobo [a,c,d], Vera Miranda-Gonçalves [a,d], Rui Henrique [a,c,d], and Carmen Jerónimo [a,d]

[a]Cancer Biology and Epigenetics Group, IPO Porto Research Center (GEBC CI-IPOP), Portuguese Oncology Institute of Porto (IPO Porto) & Porto Comprehensive Cancer Center (P.CCC), 4200-072, Porto, Portugal; [b]Master in Oncology, Institute of Biomedical Sciences Abel Salazar, University of Porto (ICBAS-UP), 4050-513, Porto, Portugal; [c]Department of Pathology, Portuguese Oncology Institute of Porto (IPOP), 4200-072, Porto, Portugal; [d]Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar, University of Porto (ICBAS-UP), 4050-513, Porto, Portugal

ABSTRACT

Testicular germ cell tumours (TGCTs) are heterogeneous neoplasms mostly affecting young-adult men. Despite high survival rates, some patients with disseminated disease acquire cisplatin resistance, entailing the need for less toxic therapies. Epigenetic alterations constitute an important feature of TGCTs, which are also implicated in resistance mechanism(s). These alterations might be used as potential targets to design epigenetic drugs. To date, several compounds have been explored and evaluated regarding therapeutic efficacy, making use of pre-clinical studies with *in vitro* and *in vivo* models, and some have already been explored in clinical trials. This review summarizes the several epigenetic mechanisms at play in these neoplasms, the current challenges in the field of TGCTs and critically reviews available data on ‘epidrugs’ in those tumours.

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Introduction

The testis is composed by different types of cells, and each cell type may give rise a neoplasm, justifying the wide variety of tumours within this organ. However, around 95% of testicular cancers are derived from germ cells, which fail to complete their normal differentiation, and these are called testicular germ cell tumours (TGCTs) [1,2].

Overall, TGCTs are not prevalent cancers. According to worldwide statistics for 2018 (Globocan) [3], they represent the 21st most incident neoplasm in men worldwide, with 71,105 new cases diagnosed in 2018. Nonetheless, from an Epidemiological perspective, they constitute a real concern, since they are the most incident solid tumour arising specifically in young-adult men (aged until 34 years), a figure only surpassed by the liquid tumours (lymphomas/leukaemias) [3]. Indeed, the ‘genvironmental model’ of understanding this disease fits both with the changing epidemiology of this cancer type and the intricate association with disorders of sex development [1].

Over the years, there has been better and more complete understanding of the biology of these tumours. A developmental perspective of these cancers has led to an integrated classification, universal to all types of germ cell tumours (GCTs) and genders (both testicular, ovarian and extragonadal), based on the biology of the cell of origin; this is a mostly due to its epigenetic status [4]. Among the seven subclasses of GCTs, type II GCTs of the testis are the most frequent and, at the same time, the most clinically challenging, due to malignant behaviour, being the focus of this Review. TGCTs are derived from the precursor lesion germ cell neoplasia *in situ* (GCNIS), which emerges from primordial germ cells/gonocytes that get arrested in differentiation, and are categorized into two major groups: the more homogeneous seminomas (SEs) and the heterogeneous group of non-seminomas (NSs), which includes embryonal carcinoma, yolk-sac tumour, choriocarcinoma and teratoma, as well as tumours composed of mixtures of two or more components, the so-called mixed tumours [1,5]. Clinical behaviour (and hence therapeutic strategy) is distinct and morphology is strikingly different, despite the low

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CONTACT Prof. Dr. Carmen Jerónimo ✉ carmenjeronimo@ipoporto.min-saude.pt; cljeronimo@icbas.up.pt 4200-072, Porto, Portugal

[1]Authors contributed equally

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mutational rate and paucity of genomic aberrations (similar to other pediatric/young-onset tumours), the most frequent being the (almost) universal presence of isochromosome 12p [6]. The contribution of epigenetics is remarkable [7], and dictates phenotypic and clinical variety [5].

Clinical challenges remain in TGCT field, and research efforts should be directed to them. Overall, about 70% of patients are diagnosed with localized disease (approximately 80% of SE and 60% of NS) [1,8,9], and the cure rate is around 95%, making TGCTs a model of curable cancer, even in the case of disseminated disease. Whereas for a substantial proportion of patients (around 75%) orchiectomy alone is curative, for some patients, adjuvant chemotherapy should be given to avoid disease relapse [10]. The major challenge nowadays is to uncover biomarkers (for instance, epigenetic) with high accuracy for determining the subset of patients that will never relapse and may be spared cisplatin-based treatments and its associated long-term side effects [11]. On the other side of the spectrum, patients presenting with (or developing) metastases receive cisplatin-based chemotherapy, the mainstay drug for treatment of GCTs and responsible for the major drop in mortality of these patients since its incorporation around the 1970s [12]. Despite its remarkable efficacy, due to the extreme sensitivity of TGCTs to DNA damage, a subset of patients develop resistance to cisplatin. The major relevance of this event relates to the absence of validated therapeutic options for these patients, which are the ones experiencing morbidity and mortality from disease at present [1,13]. Several studies attempted to elucidate the mechanisms of resistance to cisplatin [14–17], but a specific mechanism is still lacking. Again, there is evidence that epigenetic deregulation may also provide insight on this matter [18–20].

Epigenetics focuses on reversible changes in gene expression, which occur without altering DNA sequences, and is an expanding research field, namely (but not only) in cancer. Three major epigenetic mechanisms are known: DNA methylation, histone post-translational modifications and chromatin remodelling, and regulation by non-coding RNAs [21]. Importantly, and because these alterations are reversible, they can be therapeutically targeted with ‘epidrugs’, some of them already approved for cancer treatment or under investigation in clinical trials [22]. Given the parallelism between development/TGCTs/epigenetics, it is expected that targeting TGCTs’ epigenetic landscape may help reverse the cisplatin-resistant phenotype, which would be of major clinical impact [18].

The main goal of this Review is to briefly summarize the main epigenetic mechanisms implicated in TGCTs, and to highlight and discuss the current status of epigenetic-based therapies for these patients, namely its achievements and also areas where improvement is still lacking. Finally, we intend to provide rationale for researchers to pursue, and move forward within the field of epigenetic targeting in TGCTs, creating new opportunities.

Brief summary of DNA methylation in TGCTs

DNA methylation is the most well studied epigenetic mechanism, and methylation-based biomarkers are expanding as promising non-invasive means of diagnosis and follow-up of cancer patients. As mentioned, TGCTs tumorigenesis is tightly linked to epigenetic phenomena; specifically, the methylation profiles of SE and NS types (and even within NS subtypes) are remarkably distinct (see below) [2,6]. Altogether, these data seem to indicate that DNA methylation could be explored to uncover both TGCT-specific biomarkers and alternative treatment options (‘epidrugs’).

The covalent addition of a methyl group to the 5′ carbon cytosine of DNA (occurring mainly at CpG sites) and is catalysed by a family of enzymes, the DNA methyltransferases (DNMTs). There are three main DNMTs: *DNMT1, DNMT3A* and *DNMT3B*; the former is responsible for the maintenance of parental cell DNA methylation in a replication-dependent manner, whereas the remainder are associated with *de novo* methylation, which occurs during embryogenesis/germ cell development and ensures re-establishment of parental imprinting marks [23,24]. The DNA methylation process is dynamic and may be reversed through DNA demethylation. The so-called ‘active demethylation’ process consists of removal of the methyl group from DNA [25], which involves the action of ten-eleven translocation enzymes (TETs; including *TET1, TET2* and *TET3*), which catalyse oxidation of 5-methylcytosine to 5-hydroximetylcytosine [23,25]. Active demethylation occurs, for instance, in the paternal genome in the zygote, while the maternal genome undergoes 'passive demethylation', due to failure of DNMTs in establishing methylation during replication, creating a CpG methylation dilution effect [26].

Concerning TGCTs, SEs, in general, display global hypomethylation and erasure of imprinting marks, resembling their originating cell – primordial germ cells. Hypomethylation and the initial phenomenon of polyploidization lead to instability and tumour progression. Studies have shown erasure of methylated peaks in SE samples after correcting for methylation provided by infiltrating lymphocytes [2,6]. Conversely, there is locus-specific hypermethylation in NSs [2,27], including several gene promoters (e.g., *MGMT, CALCA, HIN1 (SCGB3A1), RASSF1A, HOXA9, CRIPTO, MCAM, MLH1, S100A2, SSBP2, APC, VGF* and *PGP9.5*) [7,19,28,29]. Data from genome-wide studies also showed distinct methylation patterns among NS subtypes, for instance with non-canonical methylation (CpH methylation) occurring in embryonal carcinoma (and correlating with *DNMT3A/B* expression), a pattern followed by embryonic stem cells, whereas more differentiated components lose such non-CpG methylation and display patterns approximating somatic cancers [6], indicating shifts in methylation that follow tumour differentiation. Importantly, *MLH1, RASSF1A* and *HIC1* hypermethylation was associated with cisplatin resistance [19,30], and *CALCA, MGMT, HOXA-9* and *SCGB3A1* hypermethylation was associated with poor prognosis in TGCT patients [7,31]. Demethylation of specific DNA segments is also typical of TGCTs biology, namely hypomethylation of *LINE1* sequences and *XIST*, the latter related to the phenomenon of X-chromosome inactivation that is patent in TGCTs, which show extra copies of X-chromosome [2,32]. A summary of DNA methylation patterns of TGCTs is illustrated in Figure 1.

Overall, deregulation and specific expression patterns of DNMTs and/or TETs may be expected in TGCTs, considering the shifting methylation profile across subtypes. Several studies (both *in vitro, in vivo* and using patient samples) have showed this, with NSs displaying higher DNMTs expression levels and lower/variable TETs expression levels [25,33–35]. This should be taken into account when treating patients with specific inhibitors (see below).

Overview of 'epidrugs' over time: DNMT inhibitors in TGCTs

In the last decades, some DNMT inhibitors (DNMTis) have shown therapeutic efficacy, effectively contributing to tumour cell death [23]. Overall, DNMTis block DNMTs action, leading to global hypomethylation and, consequently, to re-expression of genes (especially tumour suppressor genes), reversing or attenuating the malignant phenotype. According to the mode of action, DNMTis can be divided into two main groups: nucleoside and non-nucleoside analogues; the former incorporate directly into DNA, during S phase of cell cycle, disrupting replication; the latter bind to the catalytic site of DNMTs, preventing their activity [23].

5-azacytidine and 5-aza-2′deoxytidine are two nucleoside analogues approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of patients with haematological cancers [23,25]. Although not yet approved for treatment of solid cancers, many studies have demonstrated the cytotoxic effect of those drugs in a pre-clinical setting [25,36]. It is acknowledged that sensitivity to 5-azacytidine depends on high *DNMT3B* expression [37]. Thus, it has been hypothesized that these agents might be useful for treatment of TGCT patients, mostly for one of the most aggressive NS – embryonal carcinoma – which overexpresses that enzyme [25].

Indeed, some studies have explored DNMTis in TGCTs (Table 1). The first study dates already from 1994, when *Juttermann et al* [38] exposed embryonic stem cells to 5-aza-2′-deoxycytidine, observing demethylation and re-expression of tumour suppressor genes, culminating in apoptosis, direct or indirectly mediated by this drug. Subsequently, *Lind et al* [28] demonstrated that TGCT cell lines cultured with 5-aza-2′-deoxycytidine reversed the aberrant epigenetic gene silencing pattern, specifically the *SCGB3A1* gene promoter [7].

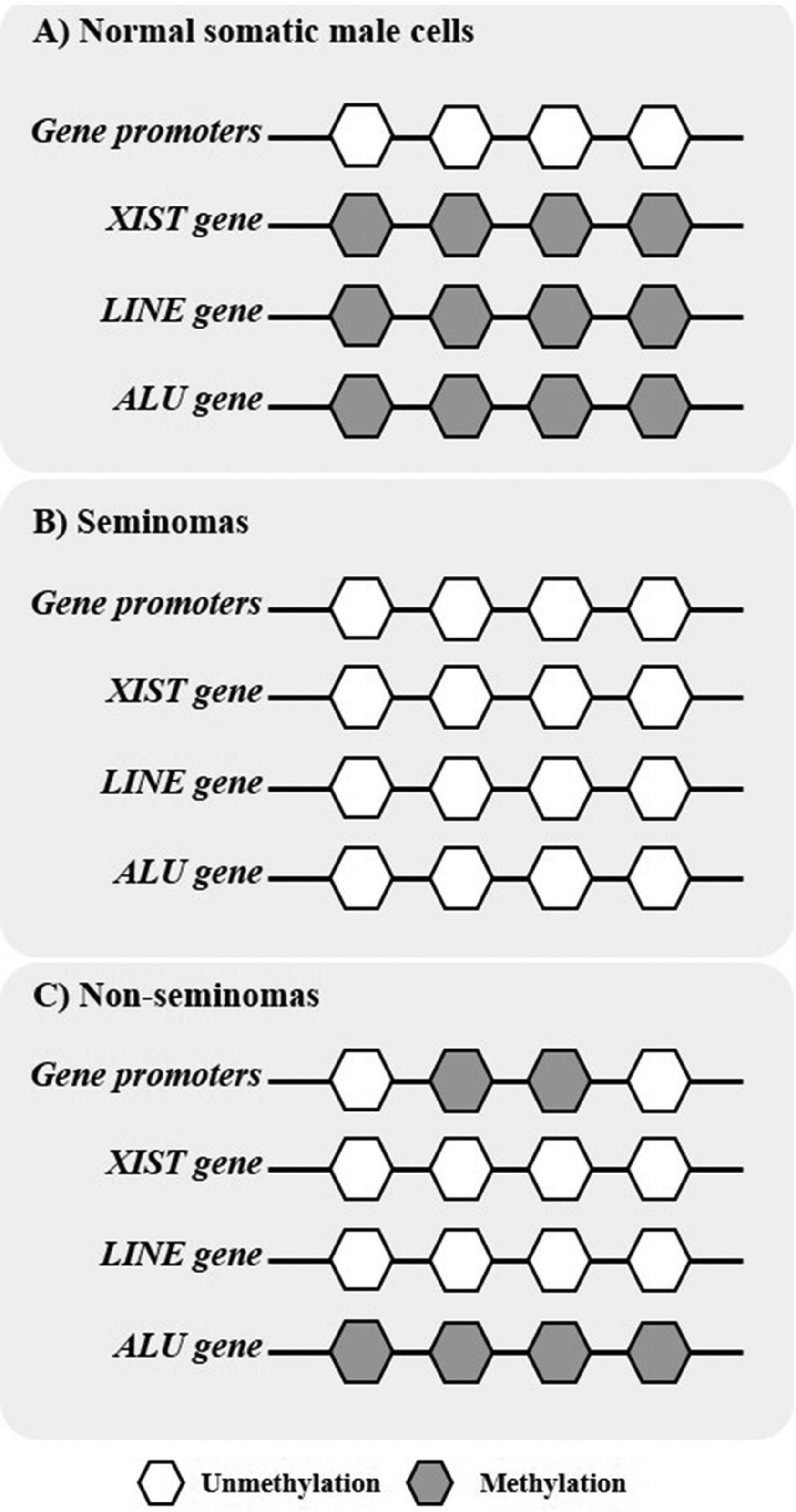
A) Normal somatic male cells
Gene promoters
XIST gene
LINE gene
ALU gene
B) Seminomas
Gene promoters
XIST gene
LINE gene
ALU gene
C) Non-seminomas
Gene promoters
XIST gene
LINE gene
ALU gene
Unmethylation
Methylation

Figure 1. Methylation pattern of (a) normal somatic cells, (b) seminomas and (c) non-seminomas. In normal somatic cells, gene promoters are unmethylated in general, while repetitive sequences, like *LINE1* and *ALU* are densely methylated. The *XIST* promoter (a long non-coding RNA involved in X-chromosome inactivation) is methylated in normal somatic male cells (due to XY chromosome constitution) but demethylated overall in TGCTs, both seminomas and non-seminomas (due to the initial step of polyploidization and universal gain of X chromosome). Seminomas show an overall unmethylated profile. Non-seminomas present locus-specific methylation of gene promoters and methylated *ALU* sequence.

SEs: Seminomas; XIST: X–inactive specific transcript; LINE1: Long interspersed nuclear element 1.

Table 1. Studies with epidrugs in testicular germ cell tumours.

Drug	Category	Phase of study	Cell lines/Patients	Dosage	Assays/Endpoints	Main Results	Notes	Ref.
5-aza-2'deoxycytadine	DNMT inhibitor	Pre-clinical	TERA1, TERA2 and NCCIT	10 µM	MSP and bisulphite sequencing (methylation)	Can reverse aberrant DNA methylation, mainly in *SCGB3A* promoter		[29]
5-aza-2'deoxycytidine	DNMT inhibitor	Pre-clinical	NT2/D1, 833 K and their cisplatin-resistant clones, TERA-1, 577 M and 2102EP	10 nM	Cell-TiterGlo (viability)	Pre-treatment with low doses of this compound restores cisplatin cytotoxic response by inducing p53 and/or re-expressing genes (*MGMT, RASSF1A*, and *HOXA-9*)	The effect of 5-aza-2 ´deoxytidine depends on *DNMT3B* expression, but cisplatin sensitivity not	[40]
5-aza-2'deoxycytadine	DNMT inhibitor	Pre-clinical	2102EP, TERA1, NTERA2 and H7	10 nM	Flow cytometry (apoptosis) qRT-PCR (transcript levels) Western Blot (protein levels)	There is an induction of apoptosis and differentiation	First study that shows that *DNMT3B* can act as oncogene	[42]
5-azacytidine	DNMT inhibitor	Pre-clinical	TCam-2	10 µM	qRT-PCR (transcript levels) MTT (viability) MSP and bisulphite sequencing (methylation)	Restores sensitivity to cisplatin, causing changes in methylation pattern and downregulation of pluripotency genes (*NANOG* and *OCT3/4*)		[28]
5-aza	DNMT inhibitor	Pre-clinical	NT2/D1 and its cisplatin-resistant clone	10 nM	Cleavage of PARP1 and Western Blot (apoptosis) MSP and bisulphite sequencing (methylation)	Low doses of 5-aza lead to DNA damage and apoptosis by activation of p53 targets, global hypomethylation and downregulation of pluripotency genes	First study that shows that induction of p53 is not associated with increased p53 mRNA, but with p53 stability	[43]
Guadecitadine (SGI-110)	DNMT inhibitor	Pre-clinical	NT2/D1 and its cisplatin-resistant clone	5 nM	Cell-TiterGlo (viability)	Exposure to low concentration results in a decrease of tumour cells growth, induces p53 target genes, re-expresses *RASSF1A* and *SOX15* and, consequently, restores cisplatin sensitivity	Using *in vivo* (mouse) models, combination of SGI-110 with cisplatin causes complete tumour regression	[44]
5-aza	DNMT inhibitor	Pre-clinical	2102EP and NCCIT and their cisplatin-resistant clones	10–20 nM	Trypan Blue (viability) Cleavage of Caspase 3 and PARP1 Western Blot Flow cytometry (apoptosis)	Anti-tumour activity as a single agent at low concentration, but more effectively when combined with cisplatin	Demonstrated that the effect of 5-aza is independent of *TP53* mutational status	[17]
5-aza alone or in combination with TSA	DNMT inhibitor and HDAC inhibitor	Pre-clinical	JEG-3 and primary choriocarcinoma stem-like cells	75 µM (5-aza) 100 nM (TSA)	qRT-PCR (transcript levels) Western Blot (protein levels)	5-aza as a single agent leads to decreased *DNMT1* and *DNMT3B*. In combination with TSA it also reduces the expression of pluripotency genes (*NANOG, OCT3/4, SOX2*, and *ABCG2*)	This study introduces a natural compound (curcumol) for treatment of choriocarcinoma cells with satisfactory results	[36]

(*Continued*)

Since platinum-based therapies are extremely effective in treating these patients (and not likely to be replaced), studies rapidly focused on the ability of epidrugs to restore sensitivity to cisplatin, which is a major clinical issue in current times. Pre-treatment with DNMTis was indeed reported to rescue sensitivity to chemotherapy with cisplatin in TGCT cell lines. *Beyrouthy* and colleagues [37] demonstrated that 5-aza-2′deoxycitidine not only affected cisplatin-resistant embryonal carcinoma cells, but also cells pre-treated with cisplatin. They also demonstrated that this effect might be caused by induction of p53 target genes, like *GDF15* and *BTG2*, or by re-expression of other genes (*MGMT, RASSF1A* and *HOXA-9*) through demethylation of respective promoters. Moreover, low doses of 5-aza-2′deoxycitidine increased activated *ATM* and phosphorylated *H2AX* levels, inducing DNA damage. This study also reports that the effect of this compound depends on *DNMT3B* expression, as previously mentioned. Indeed, *Wongtrakoongate* and co-workers [39] demonstrated that *DNMT3B* behaves as an oncogene, and that 5-aza-2′deoxycitidine treatment of TGCT cell lines causes a reduction of DNMT3B, which associates with increased apoptosis.

In 2010, *Wermann et al* [27], treated SE-like cell line (TCam-2) with 5-azacytidine and showed that the increased sensitivity to cisplatin was not only caused by changes in methylation pattern, but also through downregulation of pluripotency genes (*NANOG, OCT3/4*). *Biswal* and co-workers [40] reported similar results with 5-aza treatment in NS cell lines (NT2/D1 and NT2/D1-R1), and demonstrated that p53 induction was not associated with increased p53 transcript levels, but rather with p53 stability.

Second generation demethylating agents were also explored; in 2016, *Albany et al* [41] demonstrated that low concentrations of guadecitadine (SGI-110) had similar effects to 5-aza in TGCT cell lines. Combination therapies started to be documented as the most promising. Using *in vivo* mouse models, authors observed that 0.5 mg/kg of guadecitabine as single agent resulted only in incomplete tumour inhibition, whereas combination with cisplatin resulted in complete tumour regression at 48 hours, without significant toxicity. Furthermore, they observed an early and extensive activation of p53 pathway and induction of immune tumour cell recognition components, like HLA class I and cancer testis antigens. This deserves further investigation, especially due to the potential for combination with immunotherapies (see below).

Very recently, in 2018, the findings of *Oing et al* [18] reinforced the idea that the anti-tumour effect is more prominent when DNMTis are used in combination with cisplatin. Authors demonstrated that the effect of this ‘epidrug’ is independent of *TP53*-mutational status in TGCT cell lines. Combination of several ‘epidrugs’ was also investigated to explore potential synergistic mechanisms. *Peng et al* [35] combined DNMTi 5-azacytidine with histone deacetylase inhibitor (HDACi) trichostatin A (TSA), as well as the natural compound curcumol (an active component of *Curcuma zedoria*, used in traditional Chinese Medicine for treatment of gynaecological tumours). Choriocarcinoma cell lines (JEC-3 and primary choriocarcinoma stem-like cells), an aggressive tumour type, were treated with 5-azacytidine alone, TSA alone, 5-azacytidine in combination with TSA and curcumol. With 5-azacytidine treatment as single agent there was a decreased *DNMT1* and *DNMT3B* expression, whereas the combination with TSA resulted in a decrease of pluripotency genes, such as *NANOG, OCT4, SOX2* and *ABCG2*. Curcumol’s effects were studied in cell lines and *in vivo* models, with cell lines losing the stem-like phenotype and treated mice surviving longer than the control group. The regulatory mechanism of this compound, however, is not well understood at present. This study shows that natural compounds may have potential as anticancer therapy, eventually through modification of the epigenetic background of tumour cells. This supports research on this kind of compounds, and should also trigger investigation on repurposing drugs [42]. A summary of the mechanisms of action of DNMTis in TGCTs is illustrated in Figure 2.

According to these results in pre-clinical studies, DNMTis have also been tested in patients. However, clinical data so far has not been satisfactory, and these drugs have not moved forward to

Table 1. (Continued).

Drug	Category	Phase of study	Cell lines/Patients	Dosage	Assays/Endpoints	Main Results	Notes	Ref.
5-azacytidine	DNMT inhibitor	Clinical trial, phase II	17 patients with advanced germ cell tumours	150 mg/m^2/day	Response to treatment Disease-free survival	All patients progressed; 16/17 died		[45]
5-azacytidine	DNMT inhibitor	Clinical trial, phase II	4 patients with testicular cancer	150–225 mg/m^2	Response to treatment	Two of four patients presented partial responses		[46]
Hydralazine in combination with valproate	DNMT inhibitor and HDAC inhibitor	Clinical trial, phase II	One patient with non-seminoma	83–182 mg (hydralazine) 700 mg (valproate)	Response to treatment Disease-free survival Overall survival	Patient with stable disease		[47]
TSA	HDAC inhibitor	Pre-clinical	P19	10–100 ng/mL	TUNEL and flow cytometry (apoptosis)	TSA inhibits cell progression but alone does not induce differentiation (only in combination with retinoic acid)		[61]
CBB	KDM inhibitor	Pre-clinical	F9, NCCIT, NTERA2, HELA, 293, NIH3TS	5,27–11,16 µM	Spectrometry	CBB blocks demethylation activity of *LSD1* on mono- and- di-methylated H3K4, inducing differentiation	To mimic *in vivo* models, authors treated mouse F9 embryonic stem cells and obtained similar results in *in vitro* analysis	[62]
CBB3001	KDM inhibitor	Pre-clinical	F9	21,25 µM	Spectrometry	CBB3001 inhibits specifically *LSD1*, reducing cell growth and downregulates *SOX2* and *OCT3/4*		[63]
TSA and vorinostat as a single agent	HDAC inhibitor	Pre-clinical	GH	Various concentrations	qRT-PCR (transcript levels) Cleavage of PARP-1 and caspase 3 and Western Blot (apoptosis)	Both drugs restore GTAp63 and induce apoptosis. Combination with cisplatin causes complete cell death	First study that reports the induction of GTAp63 as relevant for treatment	[66]
Romidepsin	HDAC inhibitor	Pre-clinical	TCam-2, 2102EP-R, NCCIT-R, NT2/D1-R, JAR and JEG-3	1–10 nM	XTT (viability) Cleavage of PARP-1 and Western Blot and flow cytometry (apoptosis) qRT-PCR (transcript levels)	Romidepsin is highly toxic at low concentration, inducing stress, apoptosis and cell cycle arrest	Used *in vivo* models (mouse). Romidepsin kills SurePath cells by inducing apoptosis	[67]
JQ1	Bromodomain inhibitor	Pre-clinical	NCCIT, NT2/D1, 2102EP and their cisplatin-resistant clones, TCam-2, FS1 and MPAF	100–500 nM	XTT (viability) Western Blot (protein levels) Flow cytometry (apoptosis) qRT-PCR (transcript levels)	JQ1 not only increases G1 arrest and apoptosis, but also differentiation, and inhibits angiogenesis	First study that used bromodomain inhibitor in TGCTs	[58]

(*Continued*)

Table 1. (Continued).

Drug	Category	Phase of study	Cell lines/Patients	Dosage	Assays/Endpoints	Main Results	Notes	Ref.
Animacroxam	HDAC inhibitor and cellular cytoskeletal dynamics inhibitor	Pre-clinical	2102EP and resistant clone, NCCIT	0,1–3,2 µM	ELISA iCELLigence AC-DEVD-AMC Flow cytometry	Animacroxam has an anti-proliferative effect and inhibits cell migration	Authors used CAM as *in vivo* model, observing reduction of tumour growth	[69]

DNMT: DNA methyltransferase; MSP: Methylation-specific PCR; qRT-PCR: Real-Time Quantitative Polymerase Chain Reaction; TSA: trichostatin A; HDAC: Histone deacetylase; KDM: Histone demethylases; ELISA: Enzyme-Linked Immunosorbent Assay; CAM: Chick Chorioallantoic Membrane

the clinic. In 1993, *Roth* and colleagues [43] enrolled 17 patients with advanced GCTs undergoing four weeks of cisplatin therapy. Subsequently, patients were treated with 5-azacytidine, 150 mg/m^2/day, for three weeks (with one patient refusing therapy beyond the first course). All patients progressed after the therapy and 16/17 died, 15 from progressive disease and one from sepsis. The only surviving patient was reported as disease-free at 38.5 months. Furthermore, patients experienced toxicity: nine patients with granulocytopenia and three with anaemia and thrombocytopenia. *Quagliana* and colleagues [44] treated 214 patients with solid tumours, including four with testicular cancer cases (with no further specifications), with the same drug. First, they received 225 mg/m^2, but owing to associated toxicities, it was decreased to 175 mg/m^2 and then, 150 mg/m^2. Remarkably two of those four patients disclosed partial response. However, drug toxicity limited prolonged use. Overall, these studies constitute a mismatch to pre-clinical findings, in which demethylating drugs were quite effective. The clinical setting, namely giving the drug in monotherapy to patients with advanced GCTs, may not have been the most appropriate setting. Combination strategies with cisplatin or other drugs should be better explored (discussed below).

Combinations of several ‘epidrugs’ were also tested. A study combined hydralazine, a drug used for treating hypertension and repurposed for its action as weak non-nucleoside DNMTi (demonstrated by us and others [45]), and magnesium valproate, a histone deacetylase inhibitor (HDACi) [46]. In that study, only one patient with testicular cancer was included and although no disease regression was achieved, no progression was observed, either. Nevertheless, in general, there was increased sensitivity to chemotherapy, with stable clinical response, with a 5.6 month progression-free survival and overall survival of 5.7 months. We consider that further studies are needed and preferentially should test other ‘epidrugs’, such as zebularine and procainamide, already evaluated in other urological cancers [23]. Moreover, some studies suggested TET inhibitors’ therapeutic effect for treatment of TGCTs [47].

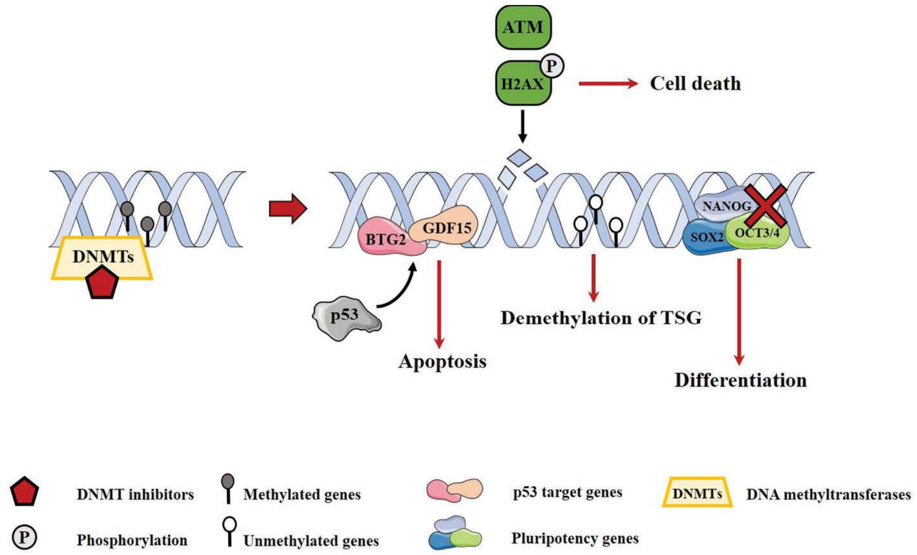

Figure 2. Main molecular mechanisms associated with DNMT inhibitors. DNMT inhibitors can induce p53 targets, leading to apoptosis. On the other hand, they lead to increase of ATM and pH2AX, associated with DNA damage, conducting to cell death. Besides this, there is re-expression of tumour suppressor genes by demethylation process. Oppositely, pluripotency genes are downregulated, leading to differentiation of cells.

DNMTs: DNA methyltransferases TSG: tumour suppressor genes.

Brief summary of histone post-translational modifications in TGCTs

Histones are proteins that provide structural support to chromatin. Importantly, histones possess a flexible N-terminal tail, which can undergo post-translational modifications, such as methylation, acetylation (the most well studied) and others. These mechanisms alter chromatin pattern with implications on the accessibility of transcription factors to DNA and, consequently, altering gene expression without changing the genetic code [25]. All these modifications are mediated by histone-modifying enzymes. Acetylation is controlled by the balanced activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add/remove acetyl groups on lysine residues of histones [25,48]. In general, acetylation of histones is associated with chromatin unfolding and gene transcription [48], whereas HDACs are seen as transcription co-repressors [25]. Histone methylation (transfer of methyl groups to histone lysine or arginine residues) is mediated by histone methyltransferases (KMTs), and its removal is catalysed by histone demethylases (KDMs). These can associate with transcriptional activation or repression, depending on the modified amino acid and its position (Figure 3). In addition, a heterogeneous family of chromatin remodelling complexes (ChRCs) can alter the nucleosome structure, again affecting DNA accessibility and gene expression; therapeutic targeting of these complexes is less well developed compared to histone (de)acetylation, and could be alternative therapies to explore, as discussed below [25,49].

In TGCTs, differential expression of HATs and HDACs between SEs and NSs has been reported. An *in silico* analysis of The Cancer Genome Atlas (TCGA) dataset disclosed that SEs present higher HATs expression levels, whereas NSs display HDACs overexpression [25]. Moreover, HDACs isoforms' immunoexpression profile significantly differs among TGCTs histological subtypes. Whereas HDAC2 and 3 were shown to be highly expressed in all types of TGCTs, HDAC1 displayed low levels. Choriocarcinomas, an aggressive tumour subtype, presented high expression of all HDACs isoforms [50].

Studies on histone methylation are more conflicting. *Hinz* and colleagues [51] did not observe significant differences in activating or repressive modifications between SEs and NSs. However [25], in an *in silico* analysis we showed that SEs depict higher expression levels of enzymes primordially implicated in establishment of activations marks, like *KDM4D, KDM3A, KMT2B/C/D and*

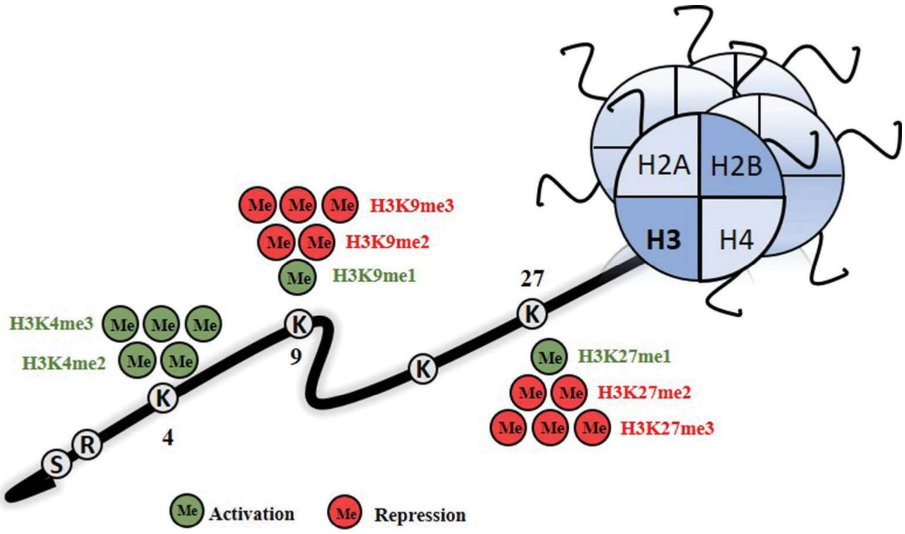

Figure 3. Repression and activation marks associated with methylation of histones. H3K4me2/3, H3K9me1 and H3K27me1 are activation marks, while H3K9me2/3 and H3K27me2/3 are repression marks.
ME: methylation; H3: histone 3.

SETD1A, whereas overexpression of enzymes that catalyse addition of repressive marks, like *EHMT2* and *EZH2*, was found in NSs. *Almstrup* and co-workers [52] reported that SEs disclose high levels of selective repressive modifications. *Lambrot et al* [53], and other authors [54,55], observed that *SMYD3*, an H3K4 methyltransferase, was upregulated in NS-like NT2 and NCCIT cell lines.

Altered ChRCs dynamics have seldom been addressed in TGCTs, with *Jostes et al* [56] recently reporting that *BRD2, BRD3* and *BRD4* are overexpressed in TGCTs.

Overview of 'epidrugs' over time: histone-modifying enzymes and bromodomain inhibitors in TGCTs

Histone-modifying enzyme inhibitors, such as HDACis, KMT inhibitors (KMTis), KDM inhibitors (KDMis) and bromodomain inhibitors, have been synthetized and explored in several tumour types. Until now, FDA has approved four HDACis for treatment of haematological malignancies: vorinostat (SAHA), romidepsin (FK288), panobinostat (LBH589) and belinostat (PXD101) [57,58], although many more compounds are under testing in clinical trials and for several malignancies. An advantage of these inhibitors (for instance, HDACis) is its specificity, with some agents being pan-inhibitors (i.e., inhibiting all classes of HDACs, from I to IV), such as vorinostat, and others being more specific (such as entinostat, a class I HDACi) [58]. Moreover, some drugs are amenable to be repurposed for targeting HDACs and treat cancer, such as valproic acid (a class I and IIa HDACi), which is routinely used for treating epilepsy [57]. For the time being, no histone-modifying enzyme inhibitors have been approved for treatment of TGCT patients, yet, but they have been a subject of scientific interest, and some studies are already exploring these agents (Table 1).

The first study using histone-modifying enzyme inhibitors for cancer treatment dates from 1997 when *Minucci et al* [59] exposed P19 cell lines to TSA in combination with retinoic acid (which induces differentiation). Authors found TSA to synergistically promote differentiation when combined with retinoic acid, activating the transcription of retinoic acid responsive promoters [35]. Interestingly, *Wang* and collaborators [60] synthesized a KDM-inhibitor (CBB), which specifically blocked *LSD1* activity, but not other demethylases, such as *LSD2* and *JARID1A*. Again, the compound could induce the expression of genes responsible for differentiation in TGCT cell lines. Indeed, high *LSD1* levels associated with overexpression of *OCT3/4* and *SOX2*, two well-known pluripotency genes. In the same line, *Hoang* and co-workers [61] synthesized another drug targeting *LSD1*

(CBB3001) and verified that F9 cell lines developed growth arrest and downregulation of *SOX2* and *OCT3/4*.

Subsequently, in 2011, GTAp63 was shown to be downregulated during the development of TGCTs [62]. GTAp63 is a transcriptionally active isoform of p63, localized in the endogenous retrovirus type 9 (ERV9)-LTR which is able to induce apoptosis upon genotoxic stress [63]. In TGCTs, this process can be restored by HDACs' inhibition. TGCT cell lines firstly treated with TSA and SAHA endured cell death when exposed to cisplatin, mediated by induction of GTAp63 transcription (demonstrating a synergistic effect between HDACis and cisplatin) [64].

More recently, pre-clinical studies have focused on more newly approved HDAC inhibitors, namely romidepsin. *Nettersheim* and co-workers [65] used this drug, already shown to induce apoptosis in TCam-2 cells [66], for treating cisplatin-resistant TGCT cells lines with distinct p53 mutational status, and demonstrated a reduction of cell viability at low nanomolar concentrations, supported by tumour growth inhibition in an *in vivo* mouse model. The proposed mechanism of action comprises a chromatin remodeller, *ARID1A*, whose inhibition consequently triggers apoptosis and induces cell cycle arrest. Mechanistically, high *DUSP1* levels in response to romidepsin blocked the *MAPK/ERK* cascade, leading to decreased *pRBS5* levels and, subsequently, to cell cycle arrest and apoptosis. Importantly, authors also tested the effects in non-GCT cell lines (fibroblasts and Sertoli cell lines), in which apoptosis was not triggered, suggesting that toxicity in other tissues is likely to be low. Since class I HDACs seem to be the particularly relevant in TGCT, future studies using class I-specific HDACi would be of interest. Moreover, combination of the latter with immunotherapies could be particularly effective, following the immunomodulatory supportive data on a phase I/II clinical trial on renal cell carcinoma [67]. In this line, follow-up on studies, including one on lung cancer which used newly synthetized inhibitors that overcame drug resistance, could be relevant for TGCT treatment, particularly given the inhibitory interaction with the pluripotency factor SOX2, fitting well with the TGCT model [68].

Recently, the same group mentioned above [56] reported promising effects of the bromodomain inhibitor JQ1. JQ1 is a small molecule that inhibits the binding pocket of bromodomains, mainly *BRD4*, which interferes with the histone code. TGCT cells treated with JQ1 endured increased apoptosis and growth arrest, along with differentiation. Importantly, in mice, JQ1 treatment associated with reduction of tumour size and blood vessel density, suggesting an anti-angiogenic effect; we hypothesize that combination with anti-angiogenic agents, such as VEGF inhibitor sunitinib, should be explored, considering the previously reported effects of this drug in TGCTs, including cisplatin-resistant tumours [69]. Moreover, this might also allow for a dose reduction, avoiding the reported apoptosis observed in a Sertoli cell line (but not in fibroblasts), which suggests toxicity for testis microenvironment.

Drugs that have dual effects on cells could also be of use. *Steinemann et al* [70] tested a single drug (animacroxam) that targets two distinct molecules: HDACs and cellular cytoskeletal dynamics. As an HDACi, animacroxam induced apoptosis and G0-G1 phase arrest in TGCT cell lines, along with induction of cytoskeletal fibres' stress. The latter reduced cell migration by 96%, suggesting that it might reduce the propensity for metastization. Drugs like animacroxam or combinations between different agents/treatments with similar properties have shown promising results and should be further explored.

Non-coding RNAs in TGCTs: are these possible therapeutic targets?

In the past few years, non-coding RNAs (ncRNAs), mainly microRNAs (miRNAs), have gained special attention, as they have been acknowledged as key gene expression regulators [71]. Specifically, miRNAs are very attractive, since they can be easily detected in biofluids in a cost-effective manner, allowing for patient diagnosis and monitoring. They modulate gene expression post-transcriptionally by targeting specific messenger RNA (mRNA) molecules, functioning as oncomiRNAs or as tumour supressor miRNAs [72].

In TGCTs, the remarkable clinical impact of a set of embryonic miRNAs, especially miR-371a-3p, as a disease biomarker must be acknowledged. Whereas in several malignancies issues like tissue/

cell specificity have been of concern in liquid biopsy studies, TGCTs (and GCTs in general) are fortunate since a cluster of miRNAs (miR-371-3 and miR-367-3p, in particular) are involved in regulation of embryonic development. Hence high levels are detectable across TGCT subtypes (except mature teratoma), but low or completely absent in healthy males or carriers of other conditions, as shown in several clinical settings and in large retrospective and prospective multicentric studies [73,74] (for a review see [75]). Based on its performance, miR-371a-3p is deemed to be available for clinical use within the near future.

However, if miR-371a-3p (and miRNAs, in general), are currently revolutionizing the field owing to their biomarker capabilities, authors also foresee the potential of these molecules to be used as therapeutic vehicles [76]. Because miRNAs can target many mRNA segments simultaneously, they might affect the transcript levels of several players and target several pathways; because the mechanisms of cisplatin resistance are most likely multifactorial, miRNA modulation may more efficiently target the cisplatin resistant phenotype. This field of research is growing, with novel ways of conceiving synthetic miRNAs and, especially, of delivering them selectively to tumour cells under active development (like via nanoparticles) [77]. Given the set of embryonic miRNAs regulating TGCTs biology, there is a rationale for pursuing miRNAs as an additional form of epigenetic-based therapy. Indeed, miRNA-based therapies are real, considering the phase II study with delivery of an anti-miR-122 (miravirsen) to hepatitis C patients, demonstrating prolonged reduction of viral RNA levels [78]. On the other hand, instead of using anti-miRs, replenishing the downregulated miRNAs might also be envisioned and has been already attempted, with delivery of miR-16 to patients with lung cancer and pleural malignant mesothelioma [79]. Murray *et al* report an example of this: a mimic of let-7 (which is downregulated in TGCTs) delivered to TGCT cells was able to activate anti-tumour mRNA targets and reduce tumour growth [80]. Indeed, miRNA-based therapies are already being pursued by several groups (and taken over by specific companies), using both the miRNA inhibition or miRNA restoration strategies, with published works in both pre-clinical and clinical settings, including solid cancers (renal cell carcinoma, melanoma, hepatocellular carcinoma), as summarized in [81].

Overall, miR-125b, miR-302a, b, c and d, miR-371, miR-372, miR-373 and miR-375 have been implicated in TGCT tumorigenesis. MiR-125b is considered a tumour suppressor miRNA that regulates several mechanisms, like proliferation, apoptosis and, importantly, pluripotency. In TGCTs, low miR-125b levels associated with tumour growth. Importantly, miR-125b also led to pro-tumorigenic macrophage recruitment to the microenvironment [82] and to inhibition of tumour-derived chemokines, contributing to decreased tumour growth [82]. This way, strategies for replenishing miR-125b within these tumours could be have therapeutic effect and potentiate the action of immunotherapies (an expanding field in itself) by recruiting immune cells to the microenvironment and making it more ‘inflamed’.

MiR-375 has been recently considered a promising marker for teratoma (the histology left undetected by miR-371a-3p, and for which clinical attitude may be different) [6], but this has been contradicted in liquid biopsies [73]. Remarkably, miR-375 and miR-302a/b/c/d may disrupt the *TP53* pathway, leading to development of TGCTs and may, thus, constitute valuable therapeutic targets [83]. Of notice, whereas miRNAs of the 371–3 cluster disrupt the p53 pathway (by targeting *LATS2*) [84], miR-885-5p (a p53 activator) was shown to be highly expressed in mature teratomas, which are, by definition, resistant to cisplatin. A miRNA switch (371a-3p to 885–5p) was proposed that might be involved in the process of differentiation, possibly amenable for therapeutic targeting (as seen for the above-mentioned ‘epidrugs’, many of them influencing differentiation) [73]. Also, this might be explored together with other agents aimed at targeting the p53 pathway, namely Nutlin-3, a Mdm2 inhibitor (with Mdm2, in its turn, responsible for targeting p53 for degradation) [16]. Moreover, miR-302 is overexpressed in TGCTs, acting as an oncogene, inducing *SPRY4* expression and, consequently, activating *MAPK/ERK* pathway, which is relevant in TGCTs. Of note, it has been demonstrated that its inhibition results in decreased proliferation of TGCT cell lines [85]. Finally, *Chen* and collaborators [86] also demonstrated that miRNAs could also influence the methylation status of relevant genes (mentioned above in the DNMTi section); mir-199a-3p overexpression restored *APC* and *MGMT* expression in NT2 cells, affecting the methylation status of their promoters, disclosing the crosstalk between epigenetic mechanisms. Overall, therapies with miRNAs still face multiple challenges, related to long-term effects, toxicities and need for better delivery options; however, we do believe that the dependence of these tumours on a more specific cluster of miRNAs constitutes an ideal tumour model for therapeutic targeting using these strategies.

Future perspectives: in which direction are TGCTs epigenetic-based treatments moving?

Over the last years, epigenetic therapies have gained special attention for cancer treatment and have been increasingly studied, with the ultimate goal of enhancing specificity and selectivity, while decreasing the side effects (Figure 4). Moreover, it is acknowledged that 'epidrugs' exhibit less toxicity than conventional chemotherapy [23]. To date, only a limited number of trials have included TGCT patients, which should deserve more attention, especially those acquiring cisplatin resistance for which no curative treatments are available. Although in pre-clinical studies, these drugs used in monotherapy appear to have an anti-cancer effect, no significant efficacy was demonstrated in the clinical trials. This could be due to several reasons, including: epigenetic therapies do not have an immediate effect, since these drugs cause reprogramming of cancer cells, initiating a long-term anti-neoplastic action; the appropriate dosage may not have been achieved; and the clinical context and population characteristics may not have been the most adequate (e.g., refractory and advanced stage disease). To our view, this should not discourage work in the field; on the contrary,

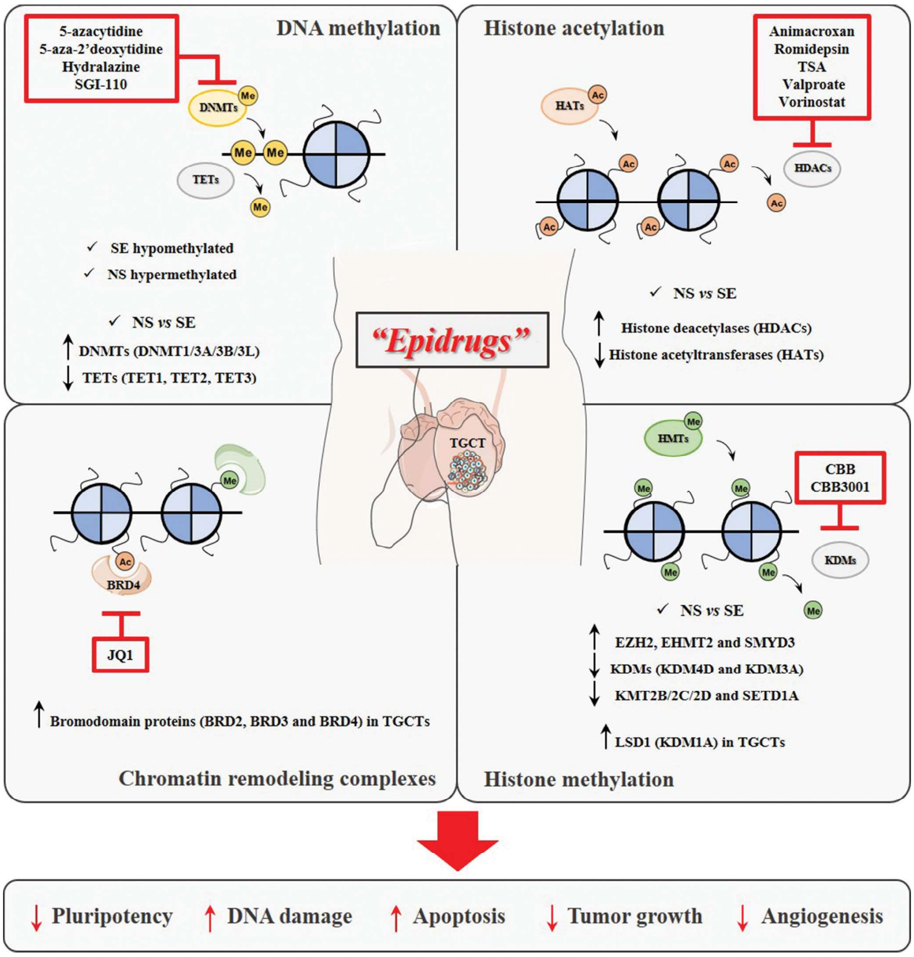
5-azacytidine
5-aza-2'deoxytidine
Hydralazine
SGI-110
DNA methylation
DNMTs
Me
TETs
✓ SE hypomethylated
✓ NS hypermethylated
✓ NS vs SE
↑ DNMTs (DNMT1/3A/3B/3L)
↓ TETs (TET1, TET2, TET3)
Histone acetylation
Animacroxan
Romidepsin
TSA
Valproate
Vorinostat
HATs
Ac
HDACs
✓ NS vs SE
↑ Histone deacetylases (HDACs)
↓ Histone acetyltransferases (HATs)
"Epidrugs"
TGCT
BRD4
JQ1
↑ Bromodomain proteins (BRD2, BRD3 and BRD4) in TGCTs
Chromatin remodeling complexes
HMTs
CBB
CBB3001
KDMs
✓ NS vs SE
↑ EZH2, EHMT2 and SMYD3
↓ KDMs (KDM4D and KDM3A)
↓ KMT2B/2C/2D and SETD1A
↑ LSD1 (KDM1A) in TGCTs
Histone methylation
↓ Pluripotency
↑ DNA damage
↑ Apoptosis
↓ Tumor growth
↓ Angiogenesis

Figure 4. Overview of current challenges in testicular germ cell tumours and the putative role of epidrugs.

it should prompt researchers for tuning their experimental settings, think on combination therapies and find the optimal treatment doses and schemes.

Combination therapies (not only with epigenetic agents, but also with these drugs and other therapies) may be the key issue, by triggering a synergistic effect. Indeed, the combination of different 'epidrugs' was considered very promising [35,46]. Steele *et al* demonstrated that the combination of belinostat with decitabine enhanced the effect of rescuing sensitivity to cisplatin in ovarian cancer cell lines [87], suggesting that the same may be possible for TGCTs. Indeed, belinostat was well tolerated in a phase II trial enrolling women with platinum-resistant ovarian cancer [88]. Dual inhibitors could also be envisioned, such as HDAC1 and LSD1 dual inhibitor Corin, which was shown to reduce tumour growth in melanoma cell lines and in mouse models [89]. On the other hand, the combination between 'epidrugs' and cisplatin, in addition to increase the therapeutic sensitivity to this chemotherapeutic agent, allows for the reduction of cisplatin dose, avoiding intense treatments and reducing the associated toxicities [90]. This should be the way to go, in a field where cisplatin will continue to be the mainstay drug for treating TGCT patients, but also at the cost of building on short-term and long-term side effects, including metabolic syndrome, cardiovascular disease, hearing loss, renal toxicity, secondary malignancies, etc [11].

The immune landscape of tumours is also epigenetically regulated [91]. Combinations of 'epidrugs' with immunotherapies, which have witnessed remarkable progress in several tumours, might be advantageous, as mentioned above. Indeed, the inflammatory infiltrate present in TGCTs is very rich and distinct among subtypes [92,93]. Notwithstanding that recent clinical trials with immunotherapies have not shown impressive results [93,94], combinations with epigenetic-based treatments may be beneficial, as illustrated by the results of combining HDACis with immunotherapies in urological malignancies [95,96]. In other words, epigenetic 'priming' of tumours, facilitating acquisition of a more inflamed tumour microenvironment, may be the required pre-treatment for taking maximal advantage of immune checkpoint inhibitors (and this is currently being explored in multiple clinical trials, summarized in [97]).

Natural compounds are also a source of potential anti-cancer agents. They may influence several biological processes, including epigenetic mechanisms, through which they might exert the antineoplastic properties. Importantly, they are usually associated with low toxicity, as many are included in the diet [23]. In TGCTs, the encouraging results of curcumol upon tumour cells [35], pave the way for more intense research concerning the efficacy for treatment of TGCT patients. Recently, CRISPR-Cas9 technology for epigenetic silencing of aberrantly demethylated epigenetic mediators was indicated as promising therapy [98], but more studies are required to confirm its efficacy, safety and reliability.

Considering the promising results in pre-clinical studies, and the still reduced number of clinical trials and of study participants with TGCT, a more robust approach is needed. Hence, studies with larger patient cohorts and with different previous treatments and comorbidities, are needed to elucidate and validate these results. Importantly, these studies should include different TGCTs types, as well as detailed clinical and pathological characterization and appropriate endpoints. Indeed, it should be recalled that TGCTs are quite heterogeneous neoplasms, each subtype bearing distinct epigenetic alterations that should be considered when designing and selecting 'epidrugs'.

With the increasing understanding of epigenetic regulation mechanisms, we believe that there are still many opportunities for targeted treatment with epigenetic-based strategies. For instance, the expanding field of proteolysis targeting chimeras (PROTACs) could be useful for epigenetics research [99], by inducing degradation of specific proteins (like the androgen receptor, through use of a non-steroidal androgen receptor ligand connected to the Mdm2 ligand Nutlin, which leads to ubiquitination of the receptor and consequent degradation [100]). Remarkably, Mdm2 (which is frequently amplified in TGCTs with cisplatin resistance) is one of the E3 ubiquitin ligases used in this technology. Indeed, ubiquitination is nothing less than a post-translational modification, still seldom explored in TGCTs; our in silico analysis of TCGA database disclosed differential expression of ubiquitin ligases and deubiquitinating enzymes according to TGCT subtype, and some have shown impact on patient survival [25], meaning that these could also be explored in the future. Moreover, Oing *et al* demonstrated that a distinct histone mark, mono-ubiquitination of Lys120 (H2Bub1), was associated with cisplatin resistance, and targeting it with a specific inhibitor like LDC000067 (a CDK9 inhibitor) was shown to increase sensitivity to DNA damage by cisplatin and radiation, meaning that ubiquitination should be another post-translational modification to be explored in TGCTs [101]. Importantly, most PROTACs aimed at targeting epigenetic players also target chromatin remodelling complexes, such as BRD4, the same complex targeted by JQ1, already shown to be effective in vitro and in vivo in TGCTs (see above). It remains to be seen if the higher specificity of this therapy may overcome the toxicity demonstrated by JQ1 [56]. More PROTACs targeting non-BRD4 epigenetic proteins are being explored, such as those involved in other chromatin remodellers (SWI/SNF and SMARC2/4 complexes) and other enzymes such as HDACs (like for SIRT2 [102] and HDAC6 [103]) and PCAF/GCN5 [104].

In another setting, ncRNAs might also be envisioned as therapeutic opportunities, including in chemotherapy resistance, considering their involvement in several pathways frequently triggered upon the resistant phenotype is reached [105]. This was already mentioned for miRNAs, with miR-34a being the first of these therapies introduced in the clinic, as this 'all-around' miRNA targets many tumour-prone pathways, including cyclin-dependent kinases, SIRT1 and SOX2 and is efficiently delivered in liposomal nanoparticles. Currently, long non-coding RNAs (lncRNAs) are also in clinical trials for cancer treatment (summarized in [105]). These have been less explored in TGCTs, and deserve further studies, also exploring novel ways of delivery (lipid nanoparticles, but also carriers and oncolytic adenoviruses). Moreover, one of most expanding fields in recent years has been the niche of RNA modifications, namely methylation of adenosine 6 (m^6A) [106]. There is a current competitive race to effectively drug and target these modifications, which are fundamental for cancer development across all tumour types [71]. We and others have explored the role of these modifications and respective enzymes in TGCTs [107,108], showing that they are related to differentiation. Currently, synthesis of novel small inhibitors of the m^6A writer METTL3 was already achieved and effectively used for treating acute myeloid leukaemia cells [109]; in a fast-progressing field, it is expected that novel inhibitors are uncovered, and

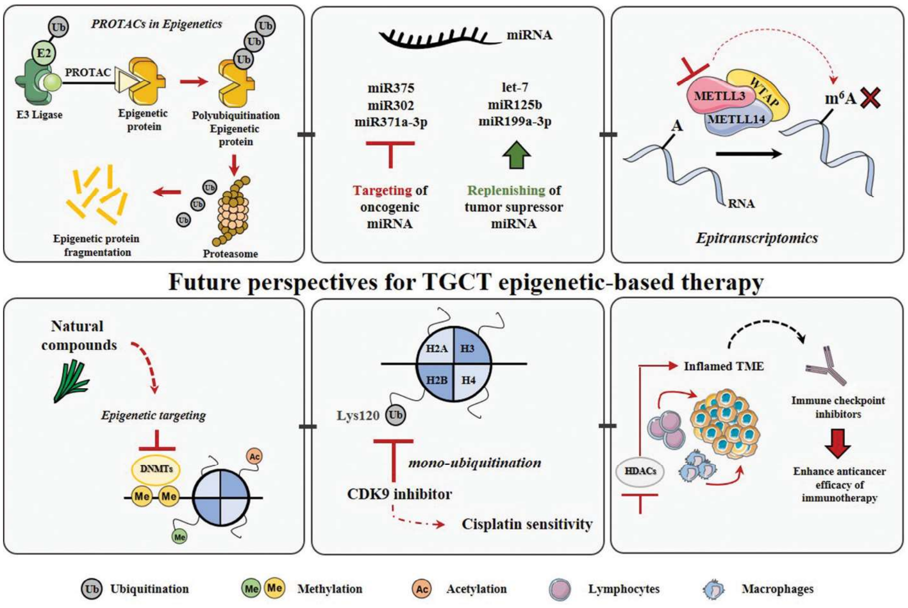
PROTACs in Epigenetics
Ub
E2
PROTAC
E3 Ligase
Epigenetic protein
Polyubiquitination Epigenetic protein
Epigenetic protein fragmentation
Proteasome
miRNA
miR375
miR302
miR371a-3p
let-7
miR125b
miR199a-3p
Targeting of oncogenic miRNA
Replenishing of tumor suppressor miRNA
METTL3
WTAP
METTL14
m⁶A
A
RNA
Epitranscriptomics
Future perspectives for TGCT epigenetic-based therapy
Natural compounds
Epigenetic targeting
DNMTs
Me
Ac
H2A
H3
H2B
H4
Lys120
mono-ubiquitination
CDK9 inhibitor
Cisplatin sensitivity
Inflamed TME
Immune checkpoint inhibitors
Enhance anticancer efficacy of immunotherapy
HDACs
Ubiquitination
Methylation
Acetylation
Lymphocytes
Macrophages

Figure 5. Overview of future directions in epigenetic-based therapies for testicular germ cell tumour patients.

given the high expression of these players in TGCTs, they might be optimally tested in this tumour model (Figure 5).

The spectrum of available tools for modulating and studying epigenetic landscape is developing at high pace and leading to discovery of versatile ways to probe chromatin, including chemical biology tools such as fluorescent ligands, chemical dimerizers, phase separation disruptors, among others. All in all, there are still a lot of opportunities within the 'chemical biology toolkit' for taking advantage of epigenetic features and looking at them as therapeutic opportunities in TGCTs [110].

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Disclosure of interest

The authors declare that they have no conflicts of interest.

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ORCID

Ana Rita Cardoso http://orcid.org/0000-0001-9051-6032
João Lobo http://orcid.org/0000-0001-6829-1391
Vera Miranda-Gonçalves http://orcid.org/0000-0002-4231-5532
Rui Henrique http://orcid.org/0000-0003-3171-4666
Carmen Jerónimo http://orcid.org/0000-0003-4186-5345

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