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Cátia Filipa Sousa Marques

Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação da Professora Doutora Luísa Barreira e coorientação do Professor Doutor Américo Lemos

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DECLARAÇÃO DE AUTORIA DE TRABALHO

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Cátia Marques

(Cátia Filipa Sousa Marques)

Dedico este trabalho aos meus pais, Helena e Rui,

e à minha irmã, Inês.

"The best way to predict the future is to create it."

Abraham Lincoln

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ABSTRACT

Cancer is defined as a group of more than 100 diseases responsible for uncontrolled cellular growth, local tissue invasion and distant metastases. In 2012, the World Health Organization expected a rise by about 70% of the number of new cases of cancer in the next two decades.

With the exponential development of cancer and the low survival ratio for leukaemia and lymphoma, it is urgent to develop new drugs able to fight cancer cells and improve anticancer treatments. The study of bis-indolyl methane (BIM) compounds may constitute a valuable contribution to tackle this problem.

BIM, also known as 3,3-di-indolylmethane, is a natural metabolite of indole-3-carbinol that occurs in *Brassica* genus plants (cruciferous plants); and with described biological activities such as antibacterial, antifungal, analgesic, anti-inflammatory, anthelmintic, cardiovascular and anticancer.

The main objectives of this work were to study the cytotoxic activity of BIMs with oxime and hydrazone derivatives, to relate their chemical structure with the cytotoxic activity and to compare BIMs with actual therapeutics. The cytotoxic assays were done in THP-1 (leukaemia), EL-4 and U937 (lymphoma) and S17 (bone marrow) cell lines; and revealed that CG-141 was the most active compound towards EL-4, THP-1 and U937 while AL-522B was the most selective compound towards S17. When compared with hydrazone derivatives, oxime derivatives had a stronger biological activity in the studied cell lines.

Relating the studied compounds with the compound 9a, it was possible to conclude that 9a continues to be the most biological active compound towards EL-4, THP-1 and U937 and the most selective towards S17.

None of the studied compounds presented significant hemolytic activity in a hemolytic assay, which corroborated the theory of a nuclear action mechanism. BIMs and vinca alkaloids have the same pharmacophores, which suggests that they might have a similar mechanism of action.

Keywords: bis-indolyl methane, cytotoxic activity, oxime and hydrazone derivatives, cancer, leukaemia, lymphoma.

RESUMO ALARGADO

O cancro é definido com um grupo de mais de 100 patologias responsáveis pelo crescimento celular descontrolado, invasão de tecidos e metástases. Classificado de acordo com o tipo de células que o constituem, este pode ser designado por carcinoma, sarcoma, leucemia, linfoma, mieloma múltiplo, melanoma ou outros tipos de cancro.

Sendo a segunda causa de morte na Europa e nos Estados Unidos da América, a Organização Mundial de Saúde esperava, em 2012, um aumento de cerca de 70% de novos casos de cancro nas duas décadas seguintes.

Com mecanismos bastante complexos, a carcinogénese é classificada em três fases, sendo elas: iniciação – exposição das células normais a substâncias carcinogénicas ou a alterações genéticas; promoção – quando as células sujeitas a agentes cancerígenos e fatores de crescimento ambientais se tornam cancerígenas; e progressão – quando há o aumento de proliferação celular, resultando num tumor ou desenvolvimento de metástases. Além destas três fases, existem também um total de dez características que são adquiridas durante o desenvolvimento de um tumor, compreendendo capacidades biológicas fundamentais das células cancerígenas: i) sinal proliferativo permanente; ii) fuga aos supressores de crescimento; iii) resistência à morte celular; iv) capacidade de imortalidade replicativa; v) indução da angiogénese; vi) ativação da invasão e metastização das células cancerígenas; vii) reprogramação da energia celular; viii) evasão às defesas imunológicas; ix) instabilidade do genoma; e x) inflamação promotora de tumores.

Dentro das doenças oncológicas incluem-se as leucemias e os linfomas. Existem quatro tipos principais de leucemias: leucemia mieloide aguda, leucemia mieloide crónica, leucemia linfoblástica aguda e leucemia linfocítica crónica; e dois grandes grupos de linfomas, Hodgkin e Não-Hodgkin, que incluem um total de mais de 50 subtipos.

Existem diferentes modalidades de combate ao cancro entre as quais se inclui a quimioterapia. Esta utiliza fármacos classificados como:

i) Agentes alquilantes que podem atuar por DNA *cross-link* (ex.: mecloretamina, carmustina, busulfano), por metilação do DNA (ex.: procarbazina) e por ligação cruzada intra e inter cadeias de DNA (ex.: cisplatina, carboplatina e oxaliplatina);

ii) Antimetabolitos que atuam por competição, inibindo a biossíntese de nucleótidos (ex.: antifolatos como o metotrexato, fluoropirimidinas como o 5-fluoruracilo, análogos da desoxicitidina como a citarabina e análogos das purinas como a 6-mercaptopurina);

iii) Produtos naturais: alcaloides da vinca (ex.: vimblastina, vincristina e vinorelbina) que inibem a mitose por polimerização da tubulina; taxanos (ex.: paclitaxel) que também inibem a mitose; epipodofilotoxinas (ex.: etoposido que inibe a topoisomerase II); e camptotecinas (ex.: topotecano e irinotecano) que inibem a topoisomerase I;

iv) Antibióticos, incluindo antraciclinas (ex.: doxorrubicina) que inibem a topoisomerase II, intercalam o DNA e libertam radicais livres de oxigénio, mitomicina e bleomicina que atuam por um mecanismo de alquilação e de libertação de radicais, respetivamente;

v) Fármacos mistos que incluem o imatinib, o dasatinib, o nilotinib, inibidores dos recetores dos fatores de crescimento e asparaginase.

Com o desenvolvimento exponencial do cancro e a baixa taxa de sobrevivência a leucemias e linfomas, há uma grande necessidade de desenvolvimento de novos fármacos capazes de combater células cancerígenas e melhorar os tratamentos oncológicos, uma vez que estes apresentam bastantes efeitos adversos (náuseas, vómitos, diarreia, fadiga, mielossupressão, neutropenia, trombocitopenia, hepatotoxicidade, alopecia, mucosite, entre outros). O estudo de bis(indolil)metanos (BIMs) poderá constituir uma vantagem face a este grande grupo de doenças, em especial, leucemia e linfomas.

BIM, também conhecido como 3,3-diindol metano, é um metabolito natural do indole-3-carbinol, que está presente em plantas do género *Brassica* (plantas crucíferas como brócolos, couves de bruxelas, couve-flor, couve e repolho) e tem descritas atividades biológicas como atividade antibacteriana, antifúngica, analgésica, anti-inflamatória, anti-helmíntica, cardiovascular e anticancerígena.

Durante o processo de desenvolvimento de um fármaco existem diferentes etapas envolvidas: descoberta, *design* e desenvolvimento. A etapa de *design* de um fármaco consiste na identificação de relação estrutura-atividade deste, assim como do seu farmacóforo, de modo a melhorar as propriedades farmacodinâmicas e farmacocinéticas. Contudo, devido à utilização de recursos limitados e às limitações impostas à experimentação animal ao longo dos últimos anos, esta componente do desenvolvimento tem-se tornado cara e longa. Assim, e como forma de colmatar estas dificuldades, surgiram os modelos computacionais de relação estrutura-atividade e estudos *in silico*.

Os principais objetivos deste trabalho consistiram no estudo da atividade citotóxica dos BIMs com derivações de oximas e hidrazonas, na relação da estrutura química com a respetiva atividade citotóxica e na comparação dos BIMs com os fármacos atualmente usadas em terapêutica.

Desta forma, começou por se determinar a atividade citotóxica dos compostos em estudo (derivados de oximas: CG-99-1, CG-109, CG-141 e CG-142; derivados de hidrazonas: AL-516, AL-522B, CG-92, CG-105, CG-147, CG-151 e CG-153) e do composto etoposido nas linhas celulares de EL-4 (linfoblastos de murino), THP-1 (leucemia aguda monocítica humana), U937 (linfoblastos de pulmão humanos) e S17 (estroma de medula óssea de murino), mediante ensaio colorimétrico com MTT, seguindo-se o cálculo da respetiva seletividade. Foi também determinada a atividade hemolítica dos compostos em estudo. Os resultados revelaram que o composto CG-141 foi o que apresentou melhor atividade biológica: EL-4 (IC_{50}=3,01-3,47 µM), THP-1 (IC_{50}=5,79-6,94 µM) e U937 (IC_{50}=9,79-10,3 µM). Já o composto AL-522B apresentou melhores valores de seletividade face à linha celular S17: 3,10 para EL-4, 2,49 para THP-1 e 2,55 para U937. Quando comparados com os derivados de hidrazonas, os BIM derivados de oximas revelaram maior atividade biológica nas linhas celulares estudadas.

Relativamente ao composto 9a, analisado num estudo prévio, este continua a apresentar melhor atividade biológica e seletividade nas linhas celulares estudadas, destacando assim a importância do hidrogénio na posição R_2 do BIM. Quando compara a atividade citotóxica do composto 9a com o etoposido, verificaram-se valores de IC_{50} semelhantes, traduzindo-se numa atividade citotóxica bastante similar entre ambos.

Nenhum dos compostos estudados apresentou atividade hemolítica significativa, corroborando desta forma a teoria de um mecanismo de ação a nível nuclear.

Ao comparar a estrutura dos BIMs com os fármacos atualmente utilizados em terapêutica, foi possível concluir que os BIMs e os alcaloides da vinca apresentam o mesmo farmacóforo – dois índoles -, o que pode sugerir um mecanismo de ação semelhante.

Em conclusão, novas estratégias de abordagem química e ensaios biológicos devem ser feitos de modo a complementar os dados obtidos e a melhorar moléculas com potencial de utilização citotóxica. A utilização de substituintes alquil e aril juntamente com oximas e hidrazonas sugerem a criação de compostos promissores no tratamento de células tumorais não aderentes. Grupos heteroarilo, fosforilo, carbonilo e carboxilo poderão ser abordagens interessantes na medida em que também fazem parte da estrutura de fármacos atualmente utilizados. Relativamente aos ensaios biológicos, são necessárias melhorias nos ensaios realizados assim como a realização de ensaios complementares, com o objetivo de permitir esclarecer o mecanismo de ação dos compostos em causa.

Palavras-chave: bis(indolil)metano, atividade citotóxica, derivados de oxima e hidrazona, cancro, leucemia, linfoma.

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ABBREVIATIONS

ABC- DLBCL	Activated B-cell-like Diffuse large B cell lymphoma
ABL	Abelson murine leukaemia
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
Bak	BCL2-antagonist/killer Protein Gene
Bax	BCL2 Associated X Protein Gene
BC	Before Christ
Bcl	B-cell lymphoma gene
BCR	Breakpoint cluster region
BIM	Bis-indolyl methane
BRCA	Breast cancer, early onset
CAFs	Cancer-associated fibroblasts
CLL	Chronic lymphocytic leukaemia
CLL/SLL	Chronic lymphocytic leukaemia / Small lymphocytic lymphoma
CML	Chronic myeloid leukaemia
CSF-1	Colony-stimulating factor 1
DCs	Dendritic Cells
DLBCL	Diffuse large B cell lymphoma
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Endothelial growth factor
EL-4	Murine lymphoblasts cell line

FL	Follicular lymphoma
FLT3	FMS-Like Tyrosine Kinase 3
GCB DLBCL	Germinal centre B cell-like Diffuse large B cell lymphoma
GLUT1	Glucose Transporter 1
HL	Hodgkin Lymphoma
IC_{50}	Half-maximal inhibitory concentration
IDH	Isocitrate Dehydrogenase
IL	Interleukin
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
MZL	Marginal zone lymphoma
NF-κB	Nuclear factor kappa B
NHL	Non-Hodgkin Lymphoma
NIK	NF-κB inducing kinase
NK	Natural killer cells
OXPHOS	Oxidative phosphorylation
p53	Tumour-suppressor protein p53
PBS	Phosphate-buffered saline
PI3-K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog protein coding gene
Rb	Retinoblastoma protein
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute 1640 medium
S17	Murine bone marrow stromal cell line
SAR	Structure-Activity Relationship
STAT	Signal transducers and activators of transcription pathway
TAM	Tumour-associated macrophages
TCA Cycle	Tricarboxylic acid cycle, or Krebs cycle

TH1	T helper 1 cells
THP-1	Human acute monocytic leukaemia cell line
TME	Tumour Microenvironment
TNF	Tumour necrosis factor
U937	Human lymphoblast lung cell line
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

I. INTRODUCTION

1.1 Cancer

The oldest evidences suggesting cancer date back to ancient Egypt being referred in ancient manuscripts from about 1600 BC. However, the word cancer comes from the Greek word *karkinos* and was firstly used by Hippocrates, a physician who lived between 460-370 BC, to described carcinoma tumours (1).

Cancer is defined as a group of more than 100 diseases responsible for uncontrolled cellular growth, local tissue invasion and distant metastases. If cancer cells remain encapsulated or localized we are towards a benign cancer, while cells that invade and destroy surrounding tissues characterize malign cancers (2).

According to the type of cells that form a cancer, it has different classifications:

- Carcinoma: is the most common type of cancer and is formed by epithelial cells. Carcinomas can be classified in (3):
    - Adenocarcinoma: cancer from epithelial cells that produce fluids or mucus (e.g.: breast, colon and prostate cancer) (3).
    - Basal cell carcinoma: cancer that begins in the lower or basal layer of the epidermis (e.g.: skin cancer) (3).
    - Squamous cell carcinoma: cancer in squamous cells (e.g.: stomach, intestines, lungs, bladder and kidney cancer) (3).
    - Transitional cell carcinoma: cancer in transitional epithelium or urothelium (e.g.: some bladder, ureters and kidney cancer) (3).
- Sarcoma: cancer formed in bone (osteosarcoma) and soft tissues as muscle, fat, blood and/or lymph vessels and fibrous tissue (tendons and ligaments) (3).
- Leukaemia: not-solid tumour that begins in blood-forming tissue of the bone marrow (3). This type of carcinoma will be explored in Section 1.1.2.
- Lymphoma: cancer that begins in lymphocytes (T or B cells) (3). This type of carcinoma will be explored in Section 1.1.2.

- Multiple Myeloma: cancer formed in plasma cells causing myeloma cells, which forms tumours in bones (3).

- Melanoma: formed when melanocytes are affected causing, mostly, skin cancer (3).

- Other types of tumours: brain and spinal cord tumours, germs cell tumours, neuroendocrine tumours and carcinoid tumours (3).

The risk factors that are in the origin of cancer can be different, depending on the type of cancer. The use of tobacco is the riskiest factor, followed by obesity and pathogens (Fig. I-1) (5).

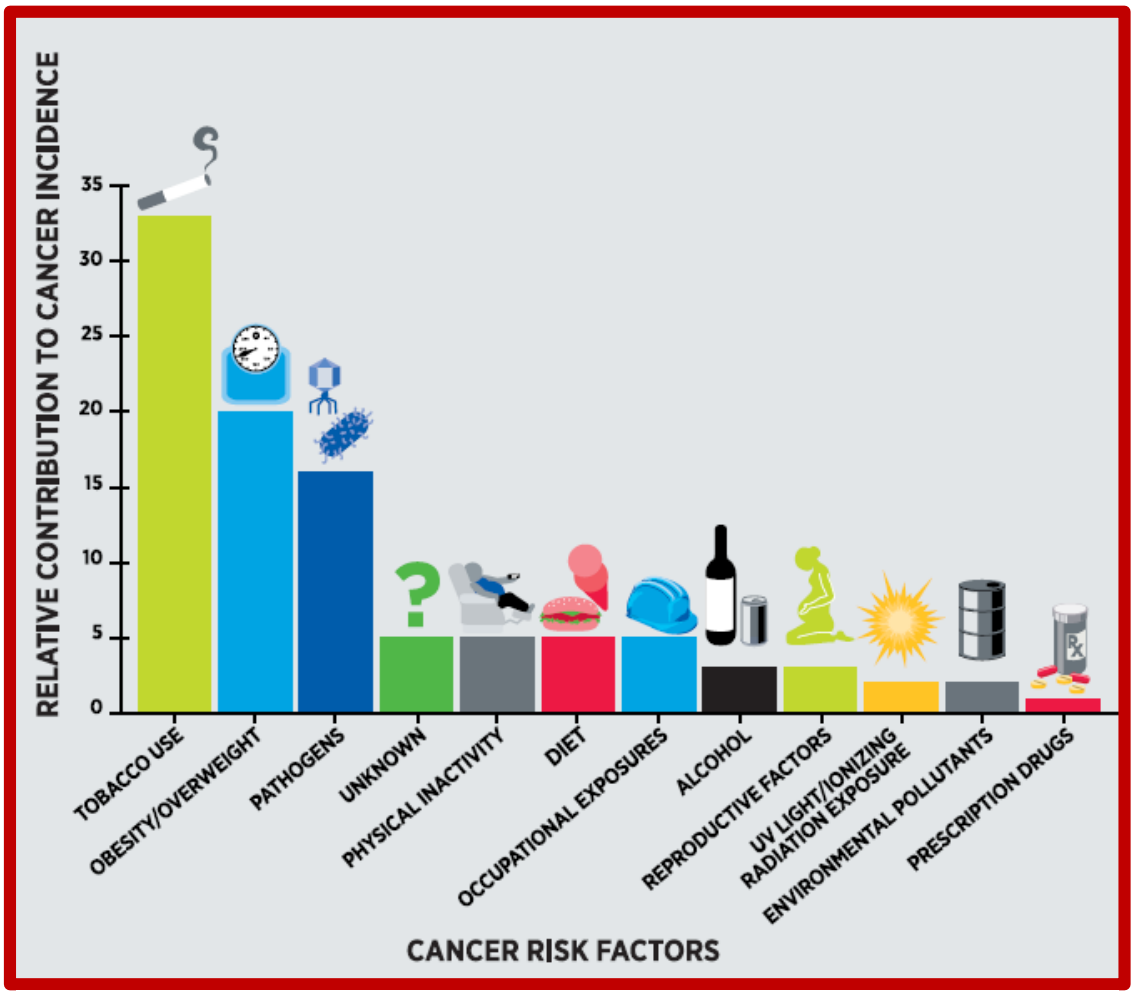


Figure I-1. Cancer risk factors that increase individual risk for developing cancer *(Adapted from* (5)*).*
Despite not all risk cancer factors have the same impact on cancer, some of them are recognized as higher risk: tobacco use, obesity, infections by some pathogens, lack of physical activity and wrong diet habits are among the most common cancer causes.

According to GLOBOCAN 2012, 14.1 million new cases of cancer and 8.2 million deaths were reported in 2012 worldwide and the World Health Organization (WHO) expected, in 2012, a rise by about 70% of the number of new cases of cancer in the next two decades (6,7). Actually, cancer is the second cause of death in Europe and in USA, after cardiovascular diseases, with lung, breast, colorectal, prostate, stomach and liver cancer as the most common in the World (Fig. I-1) (5,8,9).

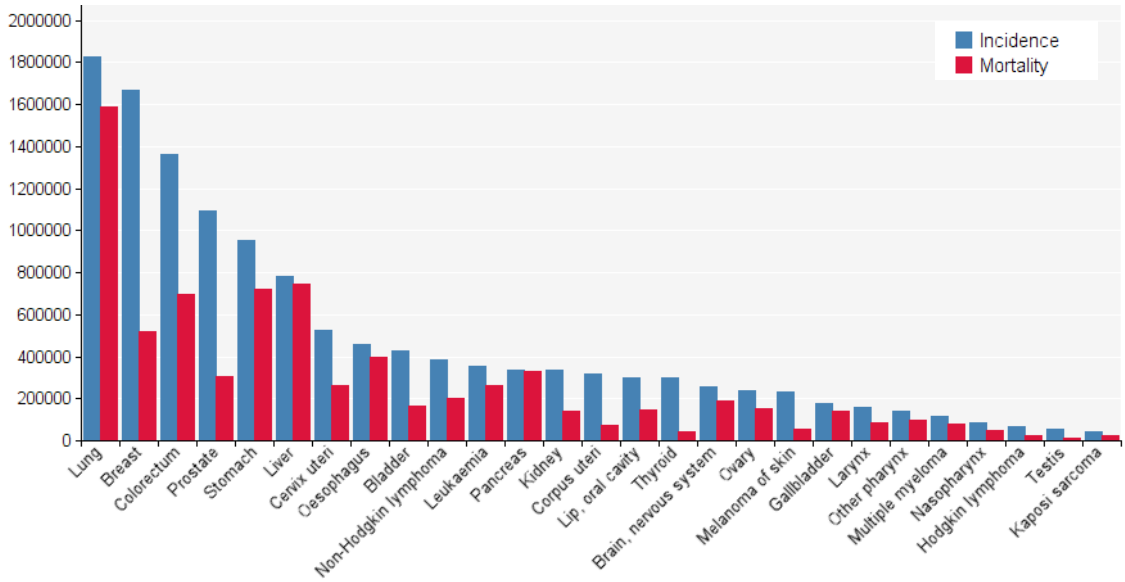


Figure I-2. Estimated number of cancer incidence and mortality in the World (6).
Lung, Breast, Colorectal, Prostate, Stomach and Liver Cancer are the most common.

1.1.1 Carcinogenesis mechanisms

The cell cycle is a complex biological process, which is controlled by a series of signalling pathways in order to regulate cell grow, DNA replication and division. To ensure minimum faults in this process, it includes mechanisms to avoid cellular errors (correction mechanisms or suicide mechanism – apoptosis), in order to prevent uncontrolled cell proliferation (10). When cells are submitted to modifications in genetic programmes (influencing cell proliferation and lifespan) and the relationship with neighbouring cells and the capacity to escape the immune system are affected, cells change their behaviour and their metabolism, turning into cancer cells. Cancer cells are

able to proliferate at a faster rate and invade distant tissues to form metastases, perturbing physiological functions (11).

Basing on the capacity of normal cells to turn into cancer cells, carcinogenesis is classified in three phases: initiation, promotion and progression. The initiation phase is characterized by the exposition of normal cells to carcinogenic substances (e.g.: tobacco) or to genetic changes which are not repaired by the organism, resulting in irreversible cellular mutations. When cells reach the promotion phase, they are subjected to carcinogens and/or environmental growth factors, being prone to become cancerous (conversion or transformation). In the last phase, progression, there is an increase of cell proliferation, which results in tumour invasion into local tissues and development of metastasis (2).

In the initiation phase, the genes affected by genetic changes can be classified into three types: proto-oncogenes, tumour suppressor genes and DNA repair genes (table I-1 presents examples of these genes and the genetic alteration that they can be subjected). Proto-oncogenes (or oncogenes) result from normal genes that were altered, being more active than normal (there is a gain of function). Thus, oncogenes allow faster cell growth and cell proliferation. Like oncogenes, tumour suppressor genes also control cellular growth and division, being responsible for the inhibition of cell proliferation. When mutated, these genes lose this function, which results in uncontrolled cell division. DNA repair genes work as DNA repairers, correcting mutations (3).

Table I-1. Example of oncogenes and tumour suppressor genes, as well as the kind of genetic alteration that they can be subjected *(adapted from* (12)*).*

	Protein	Genetic Alterations	Evading Apoptosis	Self-Sufficiency in Growth Status	Insensitivity to Anti-Growth Signals	Tissue Invasion & Metastasis	Sustained Angiogenesis
Akt Signalling Pathway							
🔵	Akt	Point mutation, amplification, increased expression	✓				
🔵	Bax	Point mutation	✓				
🟠	PI3k	Point mutation	✓				
🟠	Bcl2	Translocation	✓				
🟠	PTEN	Point mutation, deletion	✓				

Table I-1 (Continuation). Example of oncogenes and tumour suppressor genes, as well as the kind of genetic alteration that they can be subjected (adapted from (12)).

Cell Cycle Control: G1/S Checkpoint							
●	Abl	Translocation		✓			
●	p57	Point mutation		✓			
●	Rb	Point mutation		✓			
Cell Cycle Control: G2/M DNA Damage Checkpoint							
●	ATM/ATR	Point mutation			✓		
●	BRCA1	Point mutation		✓	✓		
●	p53	Point mutation, deletion	✓		✓		
Death Receptor Signaling Pathway							
●	Fas	Point mutation	✓				
Notch Signaling Pathway							
●	Notch	Translocation	✓				
Ras							
●	Ras	Point mutation		✓			
●	Integrin	Deletion				✓	
TGF-β Signalling Pathway							
●	Myc	Point mutation, amplification		✓			
●	TGFβR	Point mutation			✓		
Wnt/β-Catenin Signalling							
●	β-catenin	Point mutation		✓			
●	Wnt1	Increased expression		✓			
●	APC	Point mutation		✓			
●	α-catenin	Point mutation				✓	
●	E-cadherin	Point mutation		✓	✓	✓	
●	Wnt5A	Point mutation		✓			
Other/Transcription Factors							
●	BCL6	Translocation, point mutation					

● Oncogenes ● Tumour Suppressor ✓ Protein Function

Besides the classification in three phases, there are six hallmarks of cancer, which are acquired during tumour development and comprise six biological capabilities fundamentals to cancerous cells (Fig. I-3): i) sustaining proliferative signalling; ii) evading growth suppressors; iii) resisting cell death; iv) enabling replicative immortality; v) inducing angiogenesis; and vi) activating invasion and metastasis (13,14).

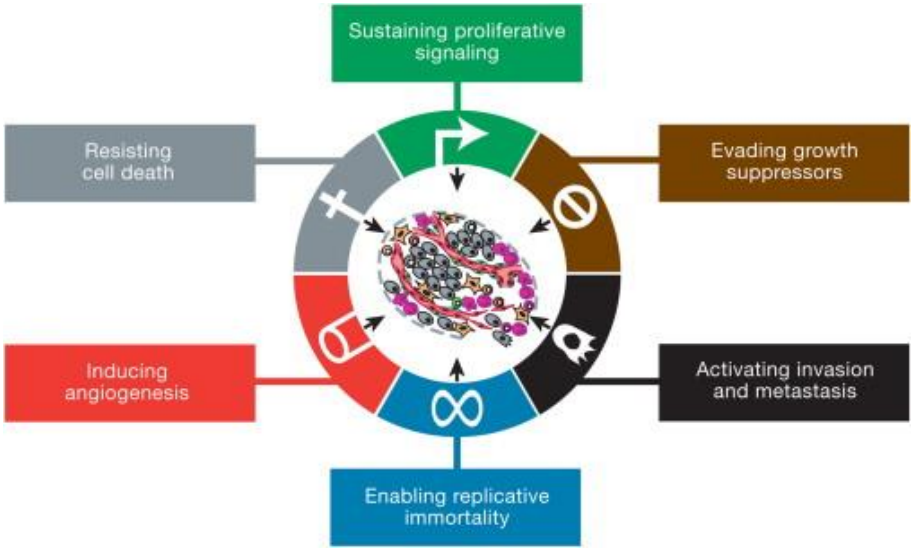


Figure I-3. The six hallmarks of cancer (13,14)
The six hallmarks of cancer represent the six basic mechanisms that a cell has to acquire to become cancerous.

Recent studies described two additional hallmarks (Fig. I-4): vii) deregulating cellular energetics and viii) avoiding immune destruction; and two enabling characteristics: ix) genome instability and mutation and x) tumour-promoting inflammation (13).

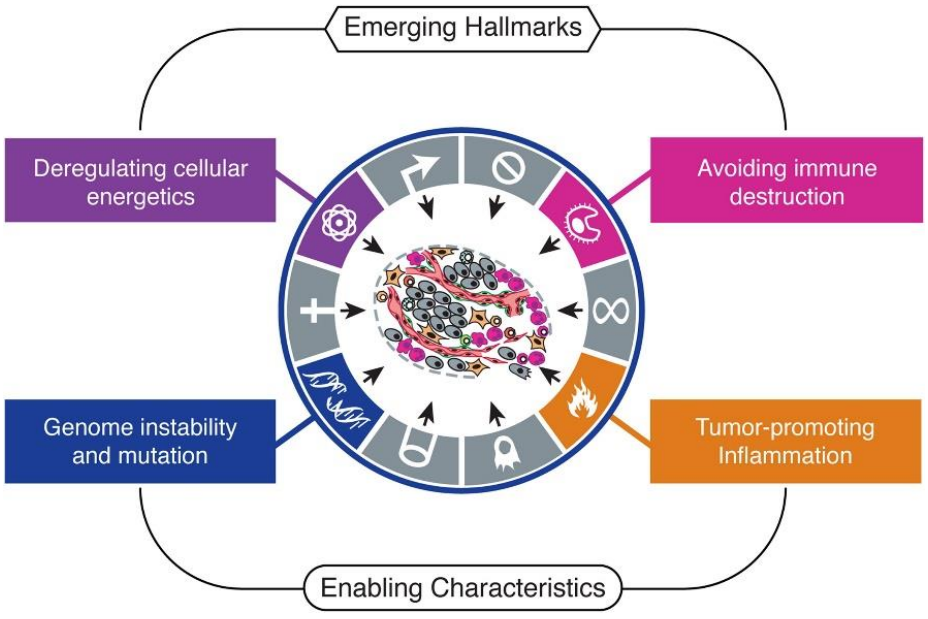


Figure I-4. Additional hallmarks of cancer. (13)
Emerging hallmarks include the capacity to modify or reprogram cellular mechanism, supporting neoplastic proliferation and evade immunological destruction (T and B lymphocytes, macrophages and natural killer cells). Enabling characteristics refer to genome instability and consequently cells mutability as well as inflammation by innate immune cells.

1.1.1.1 Sustaining Proliferation Signalling

While normal cells are able to control all the proliferative signals, namely production and release of growth-promoting signals, in cancer cells this regulation is compromised (13).

Due to this non-regulation, cancer cells have developed some capabilities to sustain proliferative signalling:

- Increased growth factor production (13);
- Stimulated normal cells in the microenvironment to provide cancer cells with growth factors (13);
- Increased number of receptors on the cancer cell surface (13);
- Structurally altered receptors to facilitate cancer cell Signalling; (13)
- Activated proteins in the downstream signalling pathway (13);
- Disrupted negative feedback mechanisms that attenuate proliferative signalling (e.g.: Ras oncoprotein which is modify by oncogenic mutations affecting *ras* gene and, consequently, Ras GTPase activity (Fig. I-5)). PTEN phosphatase counteracts PI3-kinase and can also be mutated; the loss of function by mutations in PTEN amplifies PI3K signalling and promotes tumorigenesis. Another example is mTOR kinase, which functions as coordinator of cell growth and metabolism; mTOR activation results in the inhibition of PI3-K signalling. When mTOR is pharmacologically inhibited in cancer cells, PI3-K activity increases and cellular proliferation is enhanced (13).

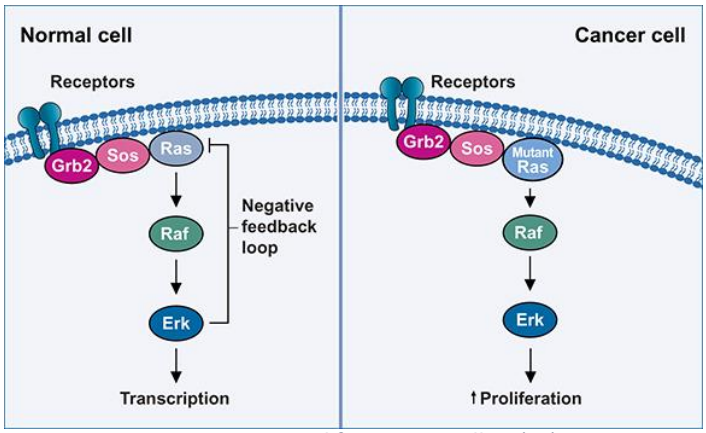


Figure I-5. Sustaining proliferative signalling (15).
Disruption of negative feedback example: When Ras oncoprotein is mutated, Ras GTPase activity is conditioned and signalling proliferative pathway affected, enhancer the proliferation.

1.1.1.2 Evading Growth Suppressors

Normal tissues control the production and release of growth suppressors by two mechanisms: induction of the G_0 phase and induction of a post-mitotic state, usually related with differentiation of the cell (14). However, cancer cells have the ability to circumvent the regulated cell proliferation programme (13).

In cancer cells, the two tumour suppressors most commonly dysregulated are the retinoblastoma protein (Rb) and p53 (13). Rb is responsible for inhibiting cell passage through the restriction point in the G1 cell-cycle phase; in cancer cells, Rb is mutated, moving this restricting point and, consequently, allowing cell proliferation (14,16). p53 activation occurs towards DNA damage or oncogene activation, resulting in G_1 cell cycle arrest and apoptosis; if this activation does not occur, cellular proliferation continues, despite any damage or error (17).

1.1.1.3 Resisting Cell Death

Apoptosis is a highly conserved mechanism responsible for maintaining cell number and tissue homeostasis. This mechanism is controlled by a complex regulatory network containing pro-apoptotic (Bax and Bak), which enhances the expression and is in charge of the apoptotic cascade and anti-apoptotic regulatory molecules that regulate anti-apoptotic factors (e.g.: NF-κB, AKT, Bcl-2 and IAP family of proteins) (13,18,19). This mechanism is delicately regulated and balanced by physiological features, characterized by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (19).

Apoptosis occurs through two major pathways: the intrinsic and extrinsic pathways. The intrinsic pathway (or the mitochondrial pathway) is activated by intrinsic signals including DNA damage induced by radiation or chemicals, growth factor deprivation or oxidative stress, resulting in the formation of a complex – apoptosome – composed of procaspase-9, apoptotic protease activating factor and cytochrome c. The release of cytochrome c is regulated by Bcl-2 family members (e.g.: Bax, Bak, Bcl-2 and Bcl-X_L) through mitochondrial membrane permeabilization. The extrinsic pathway (or

death receptor pathway) is initiated by apoptotic stimuli mediated by cell surface death receptors such as Fas ligand, TNF-related apoptosis inducing ligand (TRAIL) and TNF-α (18).

In cancer situations, cancer cells can develop resistance to apoptosis by two different ways: up-regulation or over-activation of anti-apoptotic proteins or down-regulation of pro-apoptotic proteins (19). One example is the influence of p53 in apoptosis: in normal cells, p53 induces apoptosis by up-regulating the expression of the Noxa and Puma BH-3-only proteins, in response to substantial levels of DNA breaks and chromosome abnormalities (Fig. I-6). However, in cancer cells, the loss of p53 tumour suppressor function eliminates this critical damage sensor from the apoptosis-inducing circuitry (13).

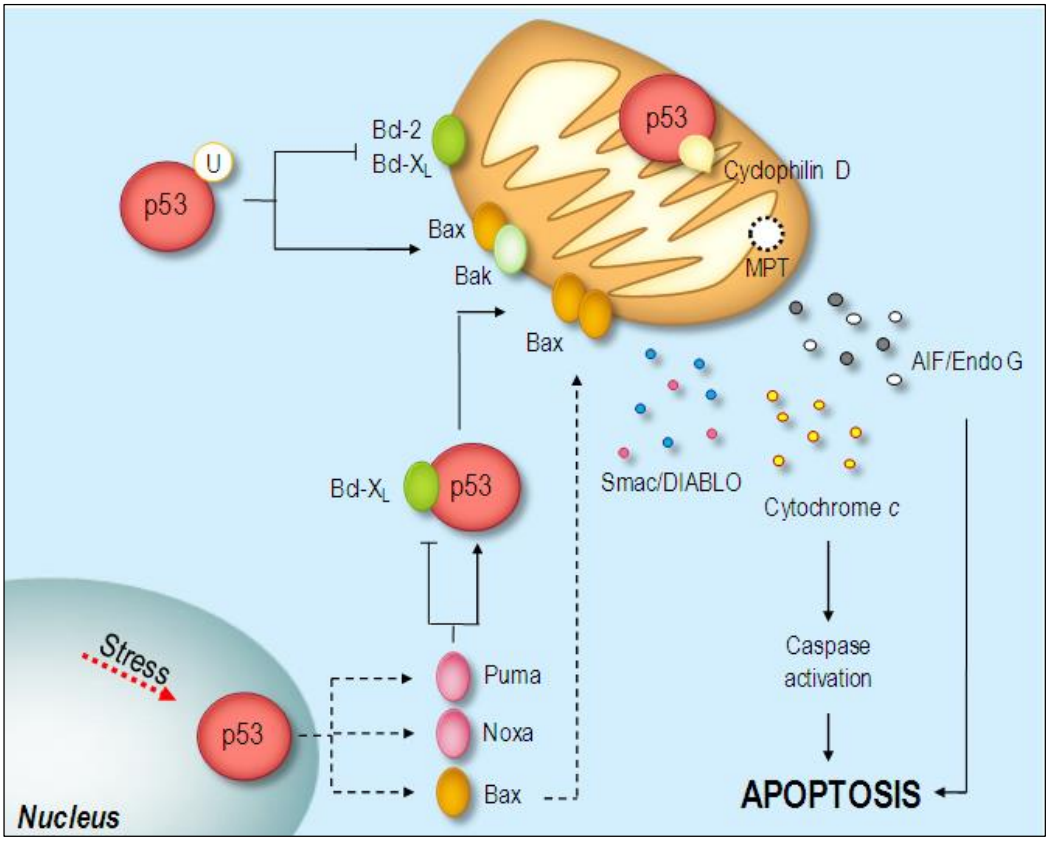


Figure I-6. p53 apoptotic pathway (20).
In the cytosolic p53 apoptotic pathway, nuclear p53 induces Puma expression, which releases inactive cytosolic p53 in the cytoplasm through binding to Bcl-X_L, inducing Bax oligomerization and mitochondrial translocation. In the mitochondria, p53 induces Bax and Bak oligomerization, antagonizes the Bcl-2 and Bcl-Xl antiapoptotic effect and forms a complex with cyclophylin D in the mitochondrial inner membrane. These changes result in marked disruption of mitochondrial membrane and subsequent release of soluble and insoluble apoptogenic factors.

Besides apoptosis, there are other mechanisms that regulate cell death such as cell-to-cell contacts, the recognition and destruction of the cancer cells by the immune system, autophagy, phagocytosis and necroptosis (18). The role of autophagy and necroptosis, however, is not consensual since some articles refer advantages to cancer cells through these mechanisms; autophagy is apparently cytoprotective for cancer cells and necrosis can activate tumour promoters and recruit tumour promoting inflammatory cells (13).

1.1.1.4 Enabling Replication Immortality

Normal cells have a limited number of successive cell growth and division cycles, which are controlled by senescence (irreversible non-proliferative but viable state of cell) and crisis (process of cell death). This limited number of division cycles is regulated by telomeres: telomeres are composed of hexanucleotide repeats, which get shortened progressively in non-immortalized cells, losing the ability to protect the end of chromosomal DNA from end-to-end fusions. In non-immortal cells, telomerase – a DNA polymerase that adds telomere repeat segments to the end of telomeric DNA – is almost absent. However, when telomerase is active, cells develop resistance to induction of senescence and crisis/apoptosis, resulting in continuous cell proliferation and cancer development (Fig. I-7) (13).

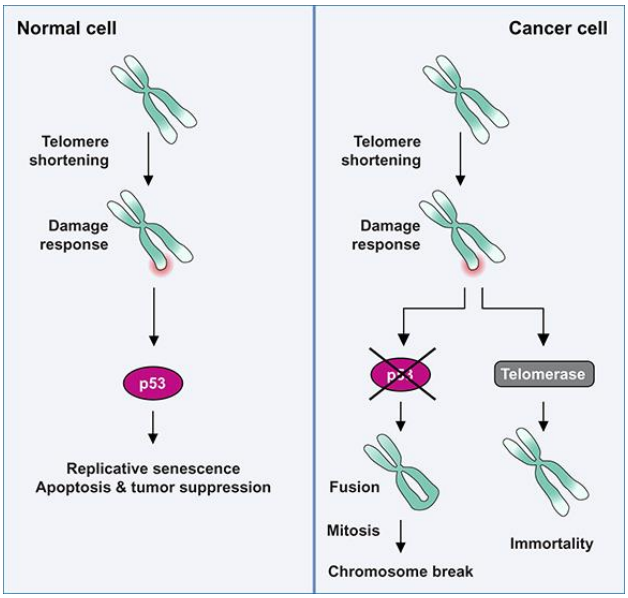


Figure I-7. Enabling replicative immortality (15).
In normal cells, shorten telomeres activate replicative senescence while in cancel cells, telomerase is active, resulting in telomere length maintenance and cell immortality.

1.1.1.5 Inducing Angiogenesis

All cells require nutrients and oxygen supply as well as metabolic wastes and carbon dioxide removal; requirements provided by the normal vasculature of cells. In cancer situations, the higher number of cells requires an amplified vasculature of cancer cells through a process named angiogenesis (13).

Angiogenesis is the sprouting of new blood vessels from already established vessels; it is a complex multistep process that follows stage and tissue specific regulations (21). This process (angiogenesis switch) occurs in an early state of cancer development and is regulated by signalling proteins that bind to stimulatory or inhibitory cell-surface receptors displayed by vascular endothelial cells (e.g. vascular endothelial growth factor (VEGF), as inducer; and thrombospodin-1 (TSP-1), as inhibitor) (13).

Besides these receptors, pericytes and bone marrow derived cells are also an important part of angiogenesis. Pericytes are supporting cells that, in a normal situation, provide mechanisms and physiologic support to endothelial cells; in a cancer situation, pericytes maintain a functional tumour neovasculature. Bone marrow derived cells such as macrophages, neutrophils, mast cells and myeloid progenitors, infiltrate premalignant lesions, aiding the progression of tumours and sustaining angiogenesis (13).

1.1.1.6 Activating Invasion and Metastasis

The word metastasis refers to a multistage process during which cancer cells spread from the primary tumour to discontiguous organs (22). There are some prerequisites to allow such process: unlimited proliferation of cells, evasion of cell-intrinsic and environmental constraints, attraction of blood supply, and capacity to detach and move away from the original location (23).

The metastasis process consists of interrelated and sequential steps including: i) transformation of initial cells and progressive and slow neoplastic growth; ii) vascularization for tumour and synthesis and secretion of angiogenic factors; iii) local

invasion of the host stroma by tumour cells; iv) detachment and embolization of tumour cell aggregates; v) circulation of these emboli within the vascular, hematologic and lymphatic systems; vi) survival of tumour cells in circulation and arrest in capillaries; vii) extravasation of the tumour cells resulting in metastasis; viii) proliferation of tumour cells in a new organ; ix) establishment of vascularization and defences against host immune responses; x) re-initiation of metastasis process, developing metastases from metastases (Fig. I-8) (22).

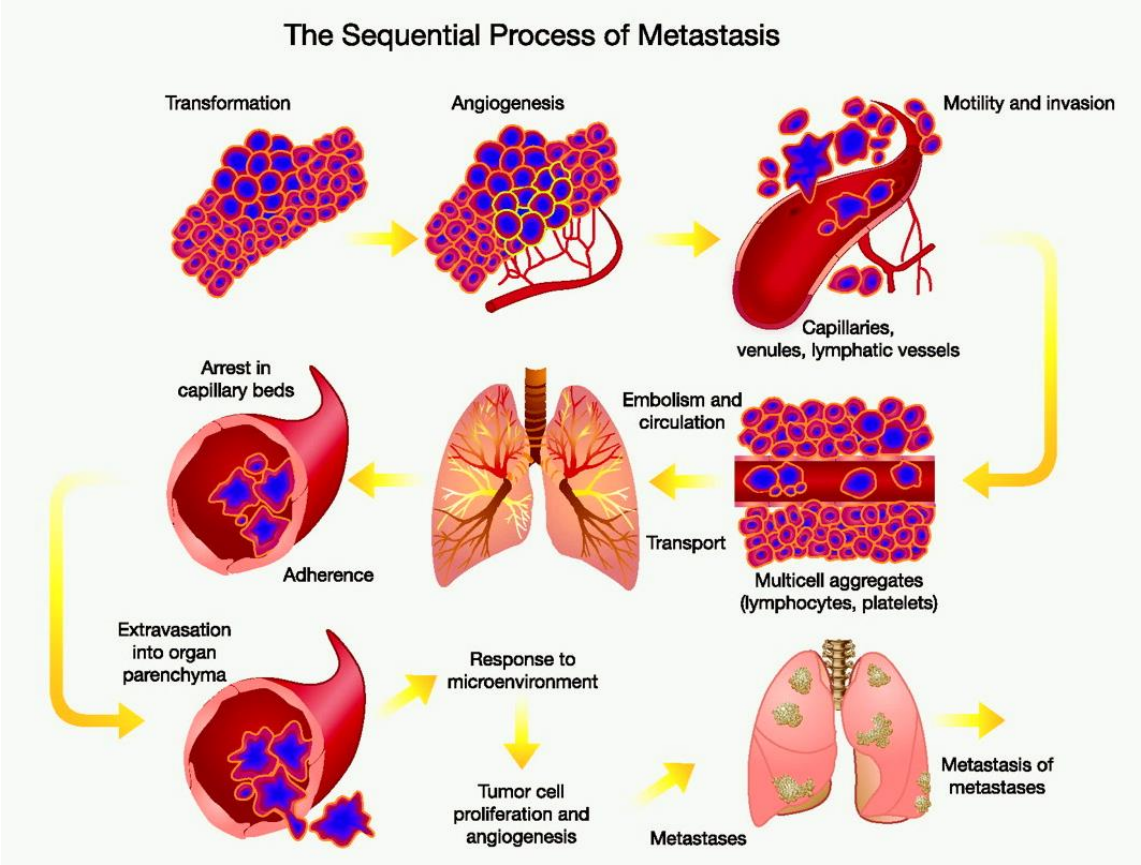


Figure I-8. The sequential process of metastasis (22).

Some of the cell alterations in metastasis situation include:

- Decrease of cell-to-cell adhesion capacity through E-cadherin (high values of E-cadherin induce an antagonist of invasion metastasis due to high cell to cell adhesion) and N-cadherin (associated with cell migrations; high values correspond to invasive carcinomas);

- Epithelial-mesenchymal transition – ability of transformed epithelial cells to invade tissues, to resist apoptosis and to disseminate to other tissues;

- Contribution of stroma cells - tumour-associated macrophages (TAM) supply epidermal growth factor (EGF) while cancer cells stimulate macrophages with colony-stimulating factor 1 (CSF-1) (Fig. I-9) (13).

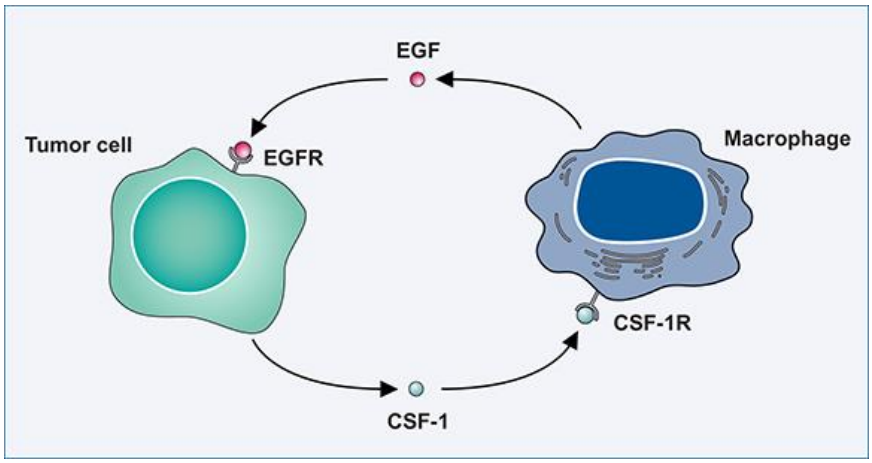


Figure I-9. Activating invasion and metastasis (15).
Process focus on stroma cells: macrophages stimuli epidermal growth factor (EGF) receptor of tumours cells while cancer cell stimuli macrophage with colony-stimulating factor 1 (CSF-1).

1.1.1.7 Deregulating Cellular Energetics

Cellular metabolism is essential to cells since it provides energy through the supply of adenosine triphosphate. This energy is used in functions such as DNA replication, transcription, translation, protein transport, assembly of multi-molecule complex and organelles, cell mobility and enzymatic reactions (24).

When cells suffer metabolic transformation, there are some alterations that contribute to oncogenes or tumour suppressors origin, namely: increase of glycoses consumption, decrease of mitochondrial respiration and increased production of reactive oxygen species (ROS). Based on these alterations, when normal cells turn into cancer cells, they can present different phenotypes (25):

1) Apoptosis resistance (25);
2) Increased anabolic activity, supporting uncontrolled growth and proliferation (25);
3) Increase ROS production, activating metastatic proteases, tumour-promoting inflammation, gene instability and DNA mutagenesis (25);

4) Mitochondrial oxidative phosphorylation (OXPHOS) decrease, increasing aerobic glycolysis and decrease of extracellular pH (25).

In normal situations, when cells are under aerobic conditions, they process glucose to pyruvate, via glycolysis in the cytosol, and then to carbon dioxide in the mitochondria. Under anaerobic conditions, glucose is preferentially processed via glycolysis and little pyruvate is used by mitochondria. Cancer cells process glucose almost always via aerobic glycolysis, even under hypoxic conditions (The Warburg effect). Thus, energy production in cancer cells is adjusted by reprogramming glucose metabolism, up-regulating glucose transporters (Glucose Transporter 1 – GLUT1) and depend on alternate metabolic pathways (13).

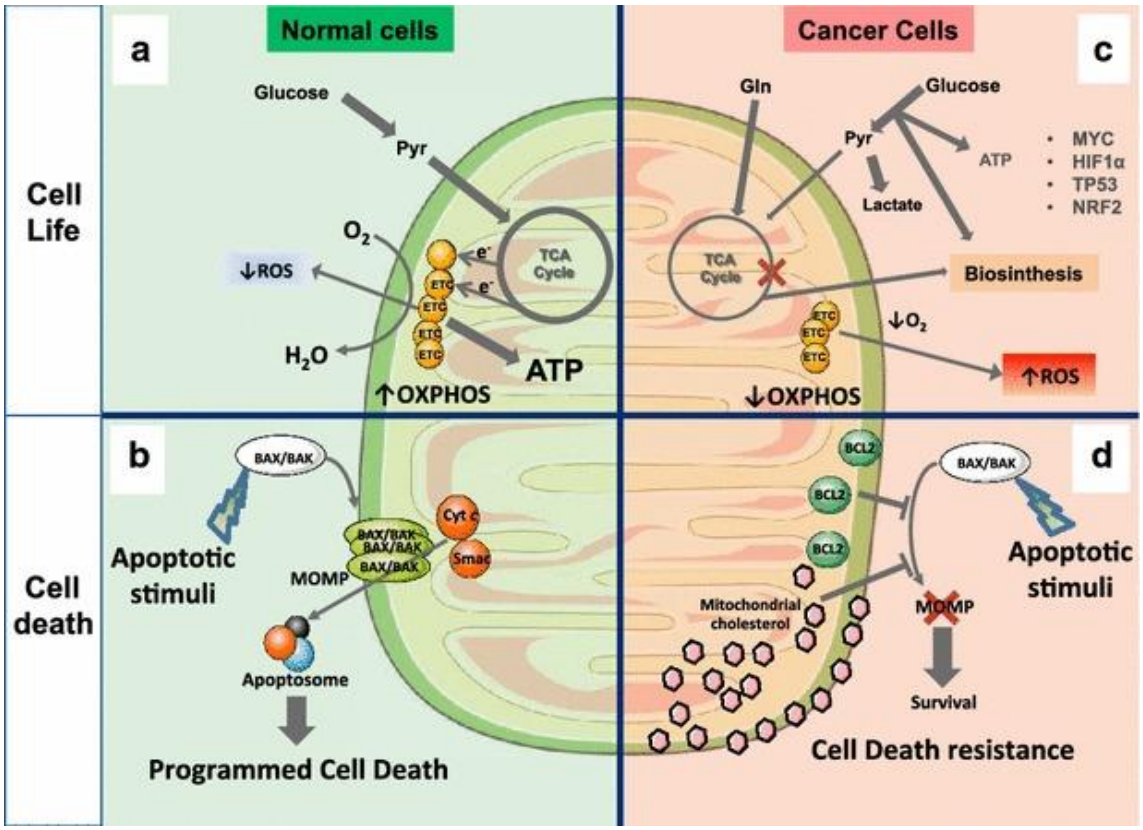


Figure I-10. Summary of altered mitochondrial functions in cancer cell life and death (25).
a) Normal non-transformed cells; b) Normal cells sensitive to apoptotic stimuli; c) Cancer cells with altered metabolism (gain-of-function of oncogenes and loss-of-function of tumour suppressor genes); d) Cancer cells with cancer death resistance.

Figure I-10 describes the different situations that can affect cellular metabolism, in cancer cells life and death:

a) In normal situations, and aerobic conditions, cells process glucose through the glycolysis pathway: glucose is metabolized to pyruvate, entering the mitochondrial TCA Cycle (tricarboxylic acid cycle, or Krebs cycle) and producing reduced equivalent and adenosine triphosphate (ATP), through OXPHOS (25).

b) In normal cells sensitive to apoptosis stimuli, Bax/Bak are activated and MOMP (mitochondrial outer membrane permeabilization) releases cytochrome c into the cytosol, stimulating the formation of apoptosome and apoptotic cell death (25).

c) In cancer cells, glucose is degraded by glycolysis and the resulting pyruvate is reduced to lactate to generate ATP. These cells have the ability to reprogram glucose metabolism and upregulate glucose transporters (GLUT1), having a low OXPHOS activity and increased mitochondrial ROS production (25).

Altered genes such as MYC, HIFα (Hypoxia-inducible factor) and p53 are examples of altered metabolism by the Warburg effect. MYC is an oncogene activated in cell progression, apoptosis and cellular transformation. MYC induces transcription of metabolic enzymes required for anabolism and fast cell growth, regulates the conversion of glucose to pyruvate through glycolysis genes and glucose transporters, and blocks the entry of pyruvate into the TCA cycle. That is, MYC is responsible for the metabolic adaptation of tumour cells. p53, when lost, increases the glycolytic flux to promote anabolism and redox balance while H1Fα promotes the conversion of glucose to pyruvate and lactate by up-regulating GLUT-1 and other transporters, decreasing the conversion of pyruvate to Acetyl-CoA, which compromises OXPHOS (25,26).

d) Cancer cells resistant to cell death present anti-apoptosis proteins or inactivated pro-apoptotic proteins that counteract Bax/Bak and MOMP (described in b)). In addition, mitochondrial cholesterol loading shields mitochondrial membrane and impairs Bax/Bak and MOMP (25).

The major problem of the described metabolic alterations is probably the production of reactive oxygen species. ROS promote tumour growth, angiogenesis and metastasis. Due to their higher reactivity, ROS induce DNA damage, transforming normal

cells into cancer cells. However, there is no consensus about the use or not of anti-oxidants in anticancer therapies. If, on one hand, there are some evidences of the reduction of cancer risk development and slow cancer progression due to the use of anti-oxidants, on another hand, there are evidences of cancer cells adaptation to these compounds, developing strategies of proliferation (25).

1.1.1.8 Avoiding Immune Destruction

The immune system plays an important role in preventing the formation and in fighting neoplastic cells. In fact, there are some evidences that the deficient development of $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ T_h1 helper T cells or natural killer (NK) cells increases tumour incidence (13).

The cancer immune-editing process (a process of the immune system that impedes tumour growth and progression) is classified in three phases named the "three Es": elimination, equilibrium and escape. The elimination (or protection) phase refers to the recognition and elimination of cancer cells by the immune system, returning tissues to normal conditions. The equilibrium phase (or persistence phase) is poorly understood; in this phase, the immune system controls tumour growth without eliminating the transformed cells. In the third phase, the escape phase (or progression), tumours are no longer susceptible to the immune system, being able to use in their advantage enhancing tumour cell growth (27,28).

Figure I-11 illustrates the immune-editing process. In the first phase, elimination phase, the immune system (innate and adaptive) proceeds to the recognition of transformed cells. Natural killer cells, $CD4^+$ and $CD8^+$ T cells recognize tumour cells, allowing the immune system to kill them and through the production of chemokines and other cytokines that facilitate the process. If tumour cells are not killed in the first phase, the immune system prevents their development. The escape phase consists on tumour cells, which evade the immune pressure of the equilibrium phase, tumour cell variants (tumour cells that suffer immune selection pressure, being resistant to the immune

effector cells) and non-immunogenic transformed cells. This phase is clinically detectable and allows the progression of tumours (28,29).

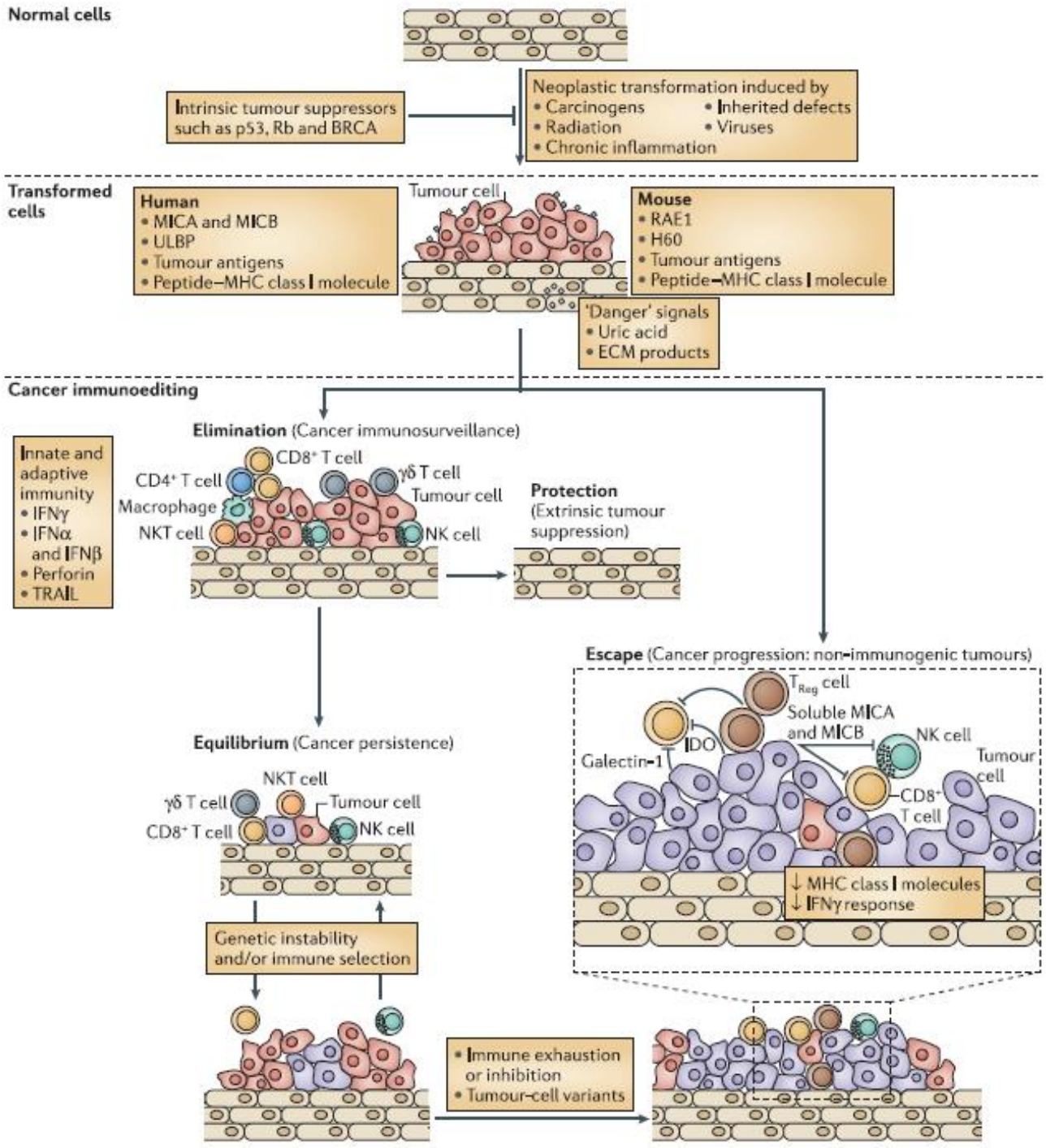


Figure I-11. Cancer immune-editing (28).

BRCA (Breast cancer, early onset); ECM (extracellular matrix); IDO (indoleamine 2,3-dioxygenase); IFN (interferon); MIC (MHC-class- I-polypeptide-related sequence); NK (natural killer); NKT (natural killer T); p53 (tumour-suppressor protein p53); RAE1 (retinoic acid early transcript 1); Rb (retinoblastoma protein); TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand); T_{Reg} ($CD4^+CD25^+$ regulatory T); ULBP (cytomegalovirus UL16-binding protein).

1.1.1.9 Genome Instability and Mutation

Genome instability plays an important role in cancer development since it can manifest during all the cancer process. Additionally, mutated genotypes represent a selective advantage since it allows the growth and proliferation of cancer cells in normal tissues (13,30).

The most relevant pathways that affect genomic instability include telomere damage, centrosome amplification, epigenetic modifications and DNA damage. Telomeres are located in the end of a chromosome and function as protective caps used by the cells to control cellular division, guiding the cell to cellular death. Centromeres are the primary microtubules used in mitoses to allow an equal division of the genetic material. Epigenetic modifications refer to heritable changes of the primary DNA sequence without changing itself while DNA damage can be cause by diverse pathways, as already mentioned (30).

DNA maintenance machinery, also named caretakers, has a relevant work in this hallmark and complements the work of the cellular constituents described in the previous paragraph. These caretaker genes are responsible for the detection and repair of damage DNA, direct repairing of damage DNA and inactivation or interception of mutagenic molecules before DNA damages (13).

Based on the mechanisms that influence genomic instability, it is possible to define some targets: prevention of DNA damage; improvement of DNA repair; signal of DNA repair weakness; impairment of centrosome clustering; inhibition of telomerase activity (30).

1.1.1.10 Tumour-Promoting Inflammation

Towards cancer development, there are some conditions that could be modified and contribute to cancer proliferation. The set of all these modifications - Tumour Microenvironment (TME) - consists in the interactions between malignant and non-malignant cells, sustaining the growth, invasion and metastasis processes (31,32).

Tumour Microenvironment contains different kinds of cells, including malignant cells, cell of the immune system, tumour vasculature and lymphatics, fibroblasts, pericytes and adipocytes, which are responsible for the complex communication between these cells through cytokines, chemokines, growth factors and inflammatory and matrix remodelling enzymes (Fig. I-12) (31). These include:

- T Lymphocytes - cytotoxic $CD8^+$ memory T cells, able to kill tumour cell; $CD4^+$ T helper 1 (TH1) cells, responsible for the production of interleukin-2 (IL-2) and interferon gamma (IFN-γ); TH2 cells, that produce IL-4, IL-5 and IL-3), supporting B cell responses; TH17 cells promote tumour growth through tissue antibacterial inflammation mediated by IL-17A, IL-17F, IL-21 and IL-22; immunosuppressive T regulatory cells (Treg) are immunosuppressed by $CD4^+$, inhibiting the recognition and clearance of tumour cells by the immune system (31).

- B Lymphocytes - more frequently found in draining lymph nodes and lymphoid structures (31).

- Natural killer and natural killer T cells - both cells are not found in contact with cancer cells and NK do not exert tumour-killing function (31).

- Tumour-associated macrophages - these cells exhibit a pro-tumorigenic activity, being the major contributor to tumour angiogenesis. TAMs tend to accumulate themselves near cells in hypoxia and/or necrosis, probably due to hypoxia-induced chemo-attraction (31).

- Myeloid-derived suppressor cells - increased in cancer situations, these cells inhibit immune cells (31).

- Dendritic Cells (DCs) - antigen processing and presentation function. It is thought that the activity of DCs in the TME is defective, in other words, cannot perform the stimulation of the immune to tumour-associated antigen (31).

- Tumour-associated neutrophils – their contribution is controversial. In spite of existing some evidences that neutrophils are involved in mechanisms such as tumour growth promotion and immune suppression, neutrophils are also involved in tumour cells destruction and inhibition of TGF-β (31).

- Cancer-associated fibroblasts (CAFs) - myofibroblasts that secret growth and pro-inflammatory factors as well as induce vascular permeability and angiogenesis (31,32).

- Adipocytes - these cells collaborate in the recruitment of tumour cells and provide fatty acids required for cellular growth (31).

- Vascular endothelial cells - in association with pericytes, these cells contribute to the neovascularization of tumours (31).

- Pericytes - cells that provide structural support to blood vessels (31).

- Lymphatic endothelial cells - cells with an important role in malign cells dissemination (31).

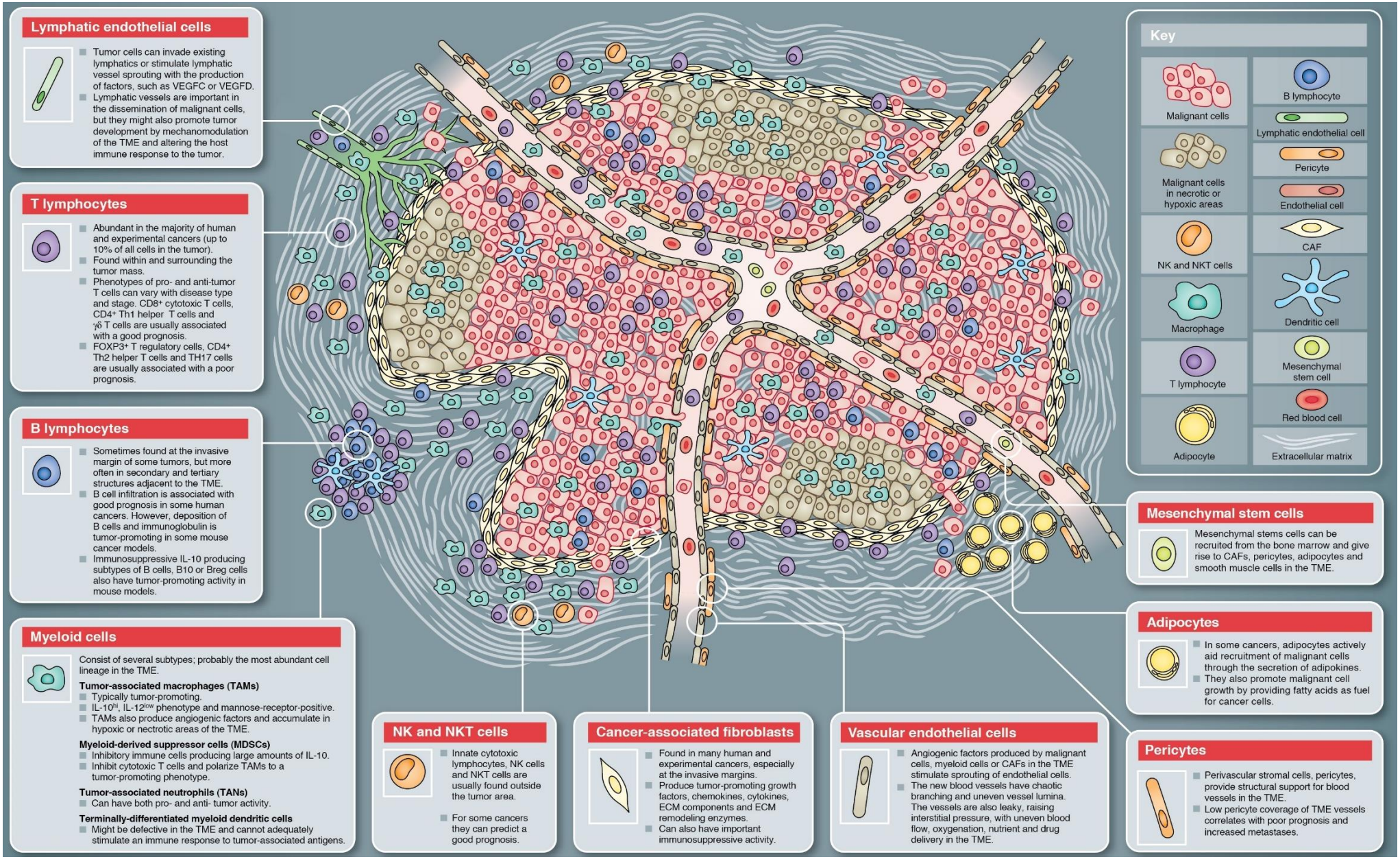


Figure I-12. The Tumour Microenvironment (31).

TME constists on the presence of different cells and their products, which contribute to cancer development through the supply of proliferative conditions. This figure illustrats these cells and their influence.

1.1.2 Leukaemia and Lymphomas

The WHO refers a total of 14.1 million new cases of cancer and 8.2 million deaths in 2012, as mentioned before. From all the new cancer cases, 2.5% were leukaemia and 3.2% were lymphoma (Hodgkin and Non-Hodgkin). The low survival ratio (compared to the number of deaths / new cases, in 2012) - 25% for leukaemia and 50% for lymphoma - justifies the necessity to develop new drugs and to improve current treatments (6).

1.1.2.1 Leukaemia

Blood consists of a wide variety of cells: erythrocytes, also known as red blood cells, which are responsible for the oxygen transport; platelets that are involved in blood clotting; and leukocytes, or white blood cells, that play an important role in immune response. All these cells have a common characteristic: they originate from the myeloid pathway of haematopoiesis process, having a common progenitor cells, hematopoietic stem cells. Due to external pressures and different cell conditions, when leukocytes stem cells suffer abnormal modifications, they turn into cancer cells – Leukaemia (33,34).

Leukaemia's classification is based on four characters: onset (acute or chronic), affected blood cell type (lymphoblastic/lymphocytic or myeloid/myelogenous), maturity stage and phenotypic expression (34). Thus, there are 4 major types of leukaemia:

- Acute myeloid leukaemia (AML);
- Chronic myeloid leukaemia (CML);
- Acute lymphoblastic leukaemia (ALL);
- Chronic lymphocytic leukaemia (CLL).

Leukaemia symptoms are common to the different types of the disease and include fever, fatigue, weakness, loss of appetite and weight loss, night sweats and frequent infections. Leukaemia incidence depends on the type of leukaemia: ALL is more common in childhood, when CLL affects predominantly older adults; AML is common in children and adult while CML affects adults (35).

1.1.2.2 Lymphoma

According to the WHO classification system, there are more than 50 distinct lymphomas classified in two big types: Hodgkin (HL) and Non-Hodgkin Lymphoma (NHL) (Table I-2) (36).

Table I-2. 2016 WHO classification of mature lymphoid, histiocytic and dendritic neoplasm. (73)
In 2016, WHO revised the previous classification of hematopoietic and lymphoid tumours, published in 2008.

Mature B-cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
Monoclonal B-cell lymphocytosis*
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma
Waldenström macroglobulinemia
Monoclonal gammopathy of undetermined significance (MGUS), IgM*
μ heavy-chain disease
γ heavy-chain disease
α heavy-chain disease
Monoclonal gammopathy of undetermined significance (MGUS), IgG/A*
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases*
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
In situ follicular neoplasia*
Duodenal-type follicular lymphoma*
Pediatric-type follicular lymphoma*
*Large B-cell lymphoma with IRF4 rearrangement**
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
In situ mantle cell neoplasia*
Diffuse large B-cell lymphoma (DLBCL), NOS
Germinal center B-cell type*
Activated B-cell type*
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the central nervous system (CNS)
Primary cutaneous DLBCL, leg type
EBV+ DLBCL, NOS*
*EBV+ mucocutaneous ulcer**
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV8+ DLBCL, NOS*
Burkitt lymphoma
*Burkitt-like lymphoma with 11q aberration**
High-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements*
High-grade B-cell lymphoma, NOS*
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Mature T and NK neoplasms
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK cells
Aggressive NK-cell leukemia
Systemic EBV+ T-cell lymphoma of childhood*
Hydroa vacciniforme–like lymphoproliferative disorder*
Adult T-cell leukemia/lymphoma
Extranodal NK-/T-cell lymphoma, nasal type
Enteropathy-associated T-cell lymphoma
Monomorphic epitheliotropic intestinal T-cell lymphoma*
*Indolent T-cell lymphoproliferative disorder of the GI tract**
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous CD30+ T-cell lymphoproliferative disorders
Lymphomatoid papulosis
Primary cutaneous anaplastic large cell lymphoma
Primary cutaneous γδ T-cell lymphoma
Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma
*Primary cutaneous acral CD8+ T-cell lymphoma**
*Primary cutaneous CD4+ small/medium T-cell lymphoproliferative disorder**
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic T-cell lymphoma
*Follicular T-cell lymphoma**
*Nodal peripheral T-cell lymphoma with TFH phenotype**
Anaplastic large-cell lymphoma, ALK+
Anaplastic large-cell lymphoma, ALK–*
*Breast implant–associated anaplastic large-cell lymphoma**
Hodgkin lymphoma
Nodular lymphocyte predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-depleted classical Hodgkin lymphoma
Posttransplant lymphoproliferative disorders (PTLD)
Plasmacytic hyperplasia PTLD
Infectious mononucleosis PTLD
Florid follicular hyperplasia PTLD*
Polymorphic PTLD
Monomorphic PTLD (B- and T-/NK-cell types)
Classical Hodgkin lymphoma PTLD
Histiocytic and dendritic cell neoplasms
Histiocytic sarcoma
Langerhans cell histiocytosis
Langerhans cell sarcoma
Indeterminate dendritic cell tumor
Interdigitating dendritic cell sarcoma
Follicular dendritic cell sarcoma
Fibroblastic reticular cell tumor
Disseminated juvenile xanthogranuloma

Provisional entities are listed in italics.
* Changes from 2008 classification.

The etiology of Lymphomas remains unknown but there are some factors that seem to influence this disease's development; the Epstein-Barr virus, autoimmune conditions and human immunodeficiency virus (HIV) are frequently present in patients who suffer Lymphoma disease. Certain industrial products, such as pesticides and hair dyes prior to 1980 appeared also to be related with some Lymphoma cases (36).

Hodgkin Lymphoma

Hodgkin lymphoma consists in a malignant transformation of a germinal or post-germinal centre B cell, presenting a small number of Reed-Sternberg cells (large and multi-nucleated tumour cells of HL that derived from pre-apoptotic germinal centre B cells, losing their B cell identity) (36,37).

During the last years, the cellular origin of Hodgkin Lymphomas has been clarified. Due to the silencing of regulators of B cell differentiation (ABF-1, ID2 and NOTCH1, for example) or the disruption of B-cell specific gene expression (BCL6, NOTCH1 inhibition, for example), Reed-Sternberg cells, the tumour cells of HL, seem to result from a defect in the cytokinesis of cells. Some mechanisms responsible for Hodgkin-Reed-Sternberg cells include NF-κB activity encouraged by TNF/death-receptor family members, constitutive IκB kinase (IKK) complex and NF-κB inducing kinase (NIK). The disruption of JAK/STAT Signalling (Janus kinase/signal transducers and activators of transcription pathway) and deregulation of the AP-1/CREB complex are other drivers to HL pathology (38).

The clinical manifestations expressed include symptomatic lymphadenopathy, mediastinal mass and systemic symptoms (fever, weight lost, sweats) (39).

Non-Hodgkin Lymphoma

Non-Hodgkin Lymphoma (NHL) is characterised by the presence of a group of malignant cells derived from B cells (most common), T cells, Natural killer cells and their precursors. Non-Hodgkin Lymphoma differs from Hodgkin Lymphoma since Reed-Sternberg cells are not present (40).

The most common NHL are Diffuse large B cell lymphoma (DLBCL), Mantel cell lymphoma, Burkitt lymphoma (BL), Chronic lymphocytic leukaemia / Small lymphocytic lymphoma (CLL/SLL), Follicular lymphoma (FL) and Marginal zone lymphoma (MZL). The classification in these subtypes is based on the cell of origin, namely morphologic, immunophenotypic and genetic features of the malignant cells, as illustrated in Fig. I-22 (41).

The clinical manifestations of NHL depend if we are in the presence of aggressive or indolent lymphomas. Aggressive lymphomas (DLBCL and BL, for example) are characterised by systemic B symptoms (fever, night sweats, weigh loss), an increase of lactate dehydrogenase and uric acid concentration as well as a rapidly growing mass. Indolent lymphomas (CLL/SLL, FL and MZL) cause a slow growing lymphadenopathy, splenomegaly, hepatomegaly and cytopenias (40).

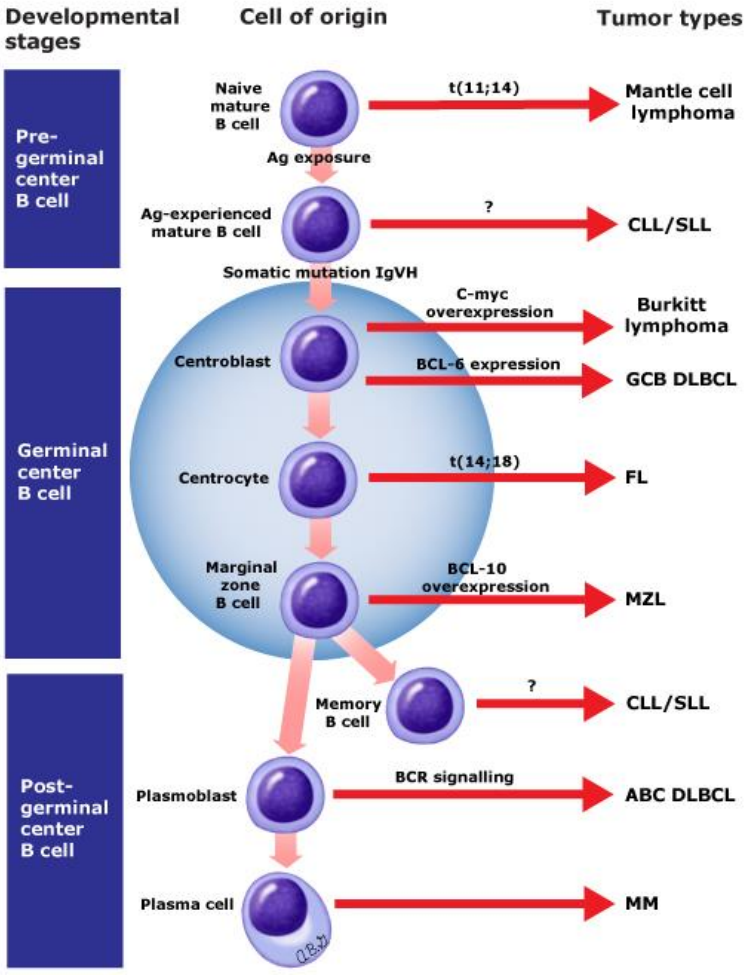


Figure I-13. Schematic Non-Hodgkin Lymphoma cells development and respective mechanisms (41)***.***
Ag: antigen; CLL/SLL: Chronic lymphocytic leukaemia / Small lymphocytic lymphoma; GCB DLBCL: germinal centre B cell diffuse large B cell lymphoma; FL: follicular lymphoma; MZL: marginal zone lymphoma; ABC DLBCL: activated B cell diffuse large B cell lymphoma; MM: multiple myeloma.

1.1.3 Actual therapies: mechanisms and adverse effects

There are different modalities for cancer treatment: surgery, radiation, chemotherapy and biologic therapy (2). This work only focuses on chemotherapy treatment.

Cancer chemotherapeutic drugs can be classified as alkylating agents, antimetabolites, natural products cancer chemotherapy drugs, antitumor antibiotics or miscellaneous anticancer drugs (42).

Alkylating agents (Fig. I-14) exercise their anticancer activity through the transfer of alkyl groups to different constituents of the cell, causing the cross-linking of DNA strands. There are three characteristic mechanisms such as DNA cross-links with consequently inhibition of DNA synthesis and functions (e.g.: mechlorethamine (a), carmustine (b) (nitrosourea), busulfan (c)); DNA methylation with DNA synthesis and function inhibition (e.g.: procarbazine (d)) and DNA intra and inter-strand cross link in nucleous and cytoplasmic proteins (nitrosoureas: cisplatin (e), carboplatin (f) and oxaliplatin (g)) (42).

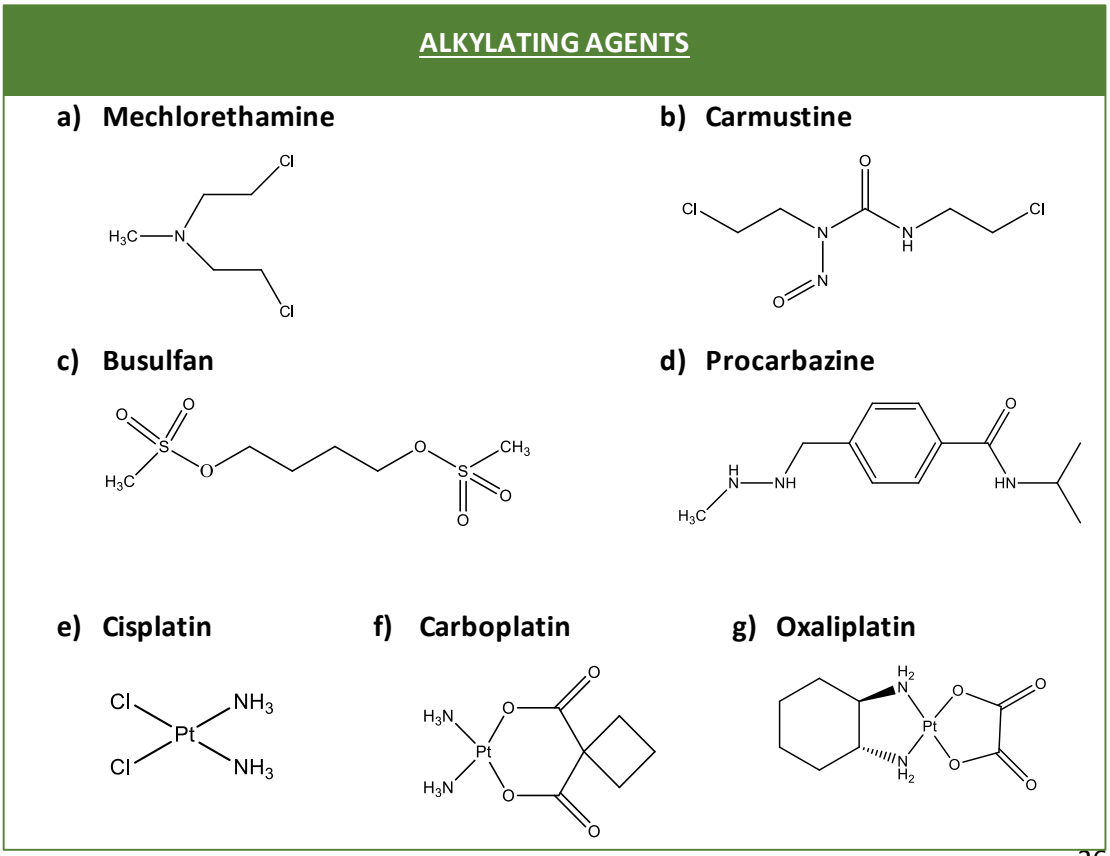


Figure I-14. Chemical structure of alkylating agents (44).

Antimetabolite drugs (Fig. I-15) are compounds with structural similarities to metabolites competing with the same enzymes to inhibit nucleotide biosynthesis and cell death (43). This class of drugs includes the antifolates like methotrexate (h), fluoropyrimidines like 5-fluorouracil (i), deoxycytidine analogues like cytarabine (j) and purine antagonists like 6-mercaptopurine (k) (42).

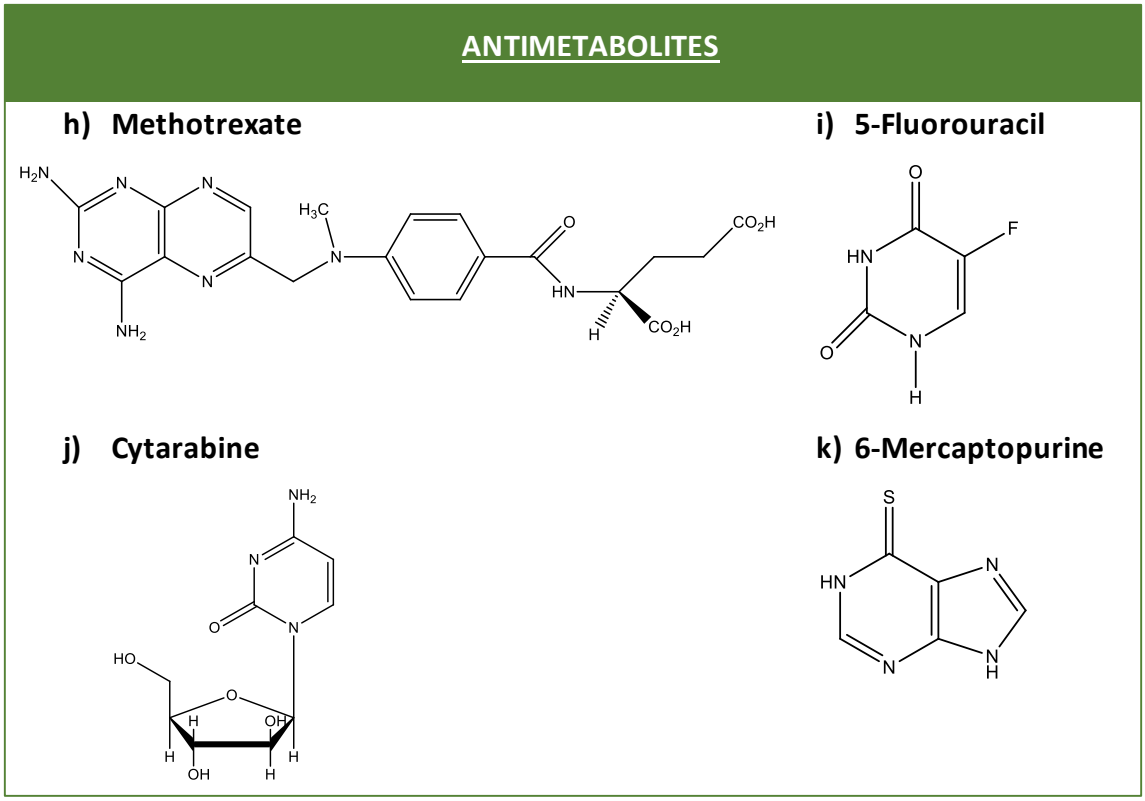


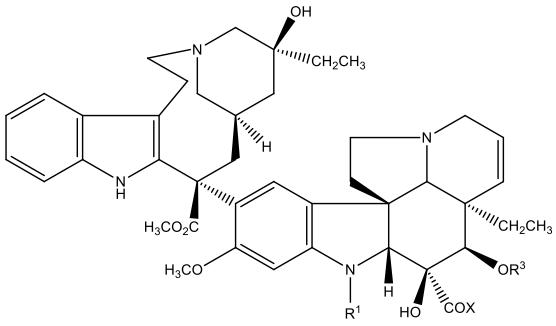
Figure I-15. Chemical structures of antimetabolites (44).

Natural products cancer chemotherapy drugs (Fig. I-16) are divided in vinca alkaloids, taxanes and related drugs, epipodophyllotoxins and camptothecins. Vinca alkaloids (vinblastine (l), vincristine (m) and vinorelbine(n)) inhibit mitosis through tubulin polymerization. Taxanes and related drugs like paclitaxel (o) also act in mitosis inhibition. Etoposide (p), an epipodophyllotoxin, inhibits the DNA enzyme topoisomerase II while camptothecins like topotecan (q) and irinotecan (r) inhibit topoisomerase I (42).

NATURAL PRODUCTS

l) Vinblastine

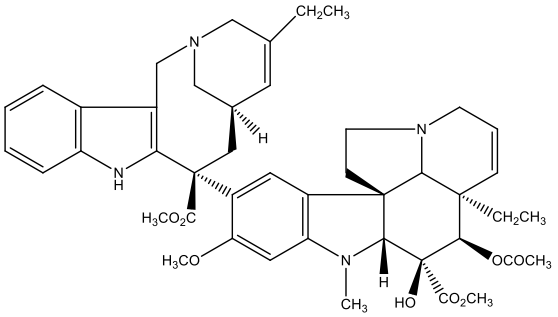
m) Vincristine



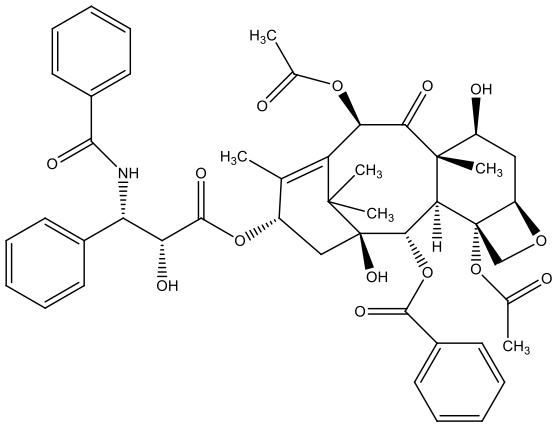
Vinblastine $R^1=CH_3$, $X=OCH_3$, $R^3=COCH_3$

Vincristine $R^1=CHO$, $R^2=OCH_3$, $R^3=COCH_3$

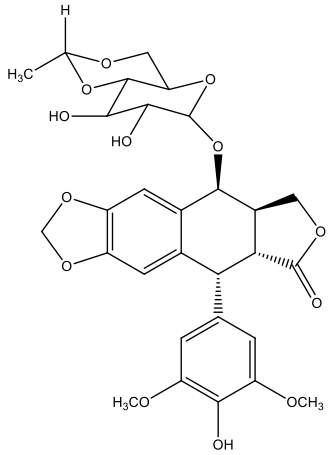
n) Vinorelbine



o) Paclitaxel

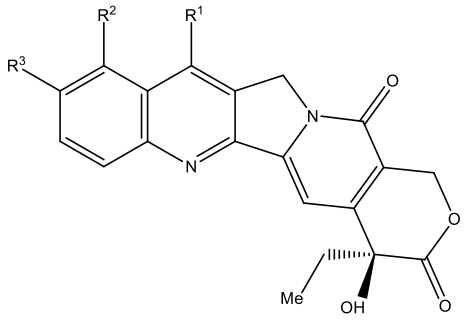


p) Etoposide



q) Topotecan

r) Irinotecan



Camptothecin $R^1=R^2=R^3=H$

Topotecan $R^1=H$, $R^2=CHNMe$, $R^3=OH$

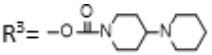
Irinotecan $R^1=Et$, $R^2=H$, $R^3=$ 

Figure I-16. Chemical structures of natural products used in chemotherapy (44).

Antitumor antibiotics (Fig. I-17) include anthracyclines (doxorubicin (s), for example), which inhibit topoisomerase II, intercalate into DNA and release oxygen free radicals that cause DNA breaks. Mitomycin (t) and bleomycin (u) are also antitumor antibiotics, which act by an alkylating mechanism and act as oxygen free radicals' releaser, respectively (42).

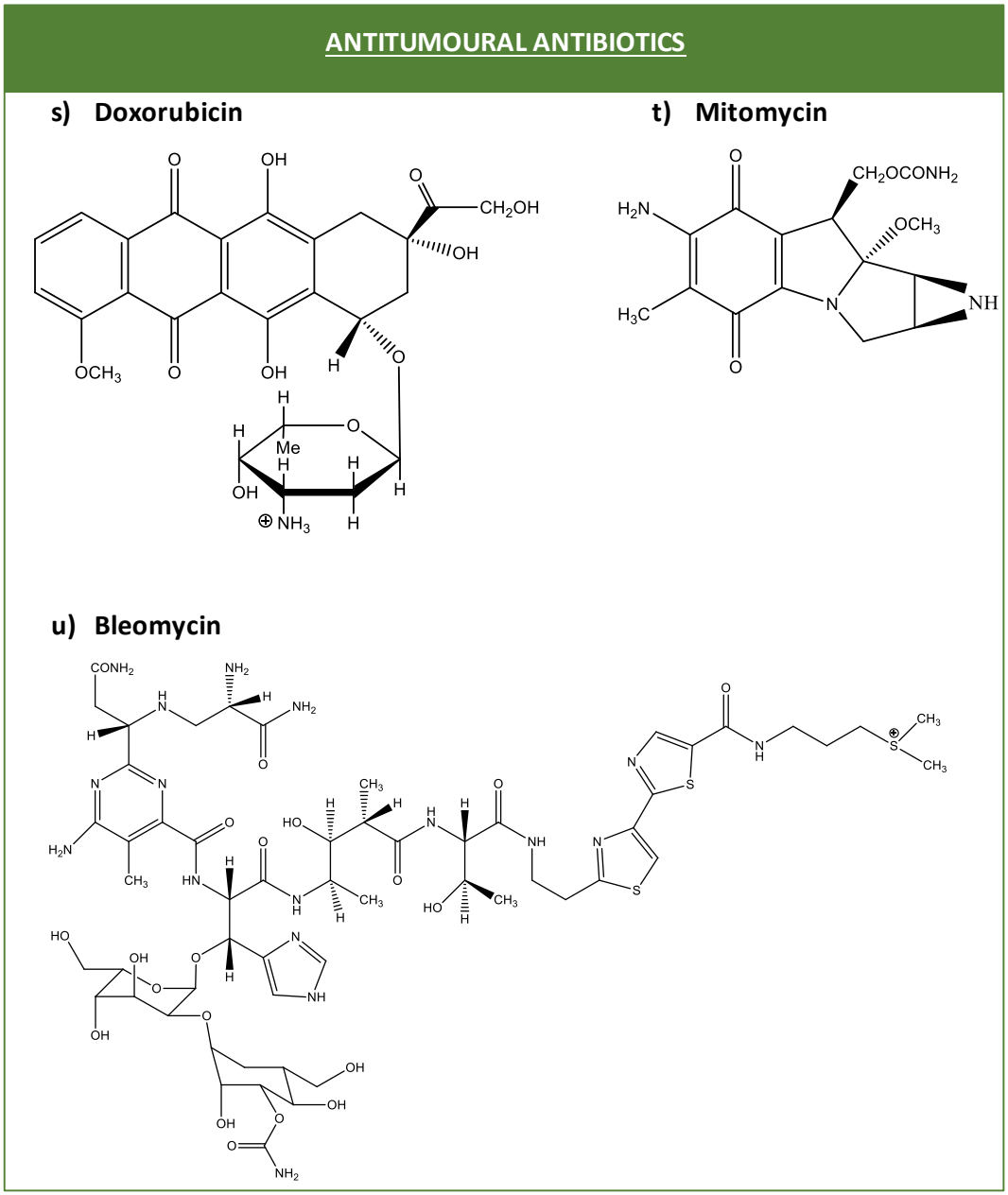


Figure I-17. Chemical structures of antitumoural antibiotics (44).

The last drug classification, miscellaneous anticancer drugs, includes various types of drugs: imatinib (v), dasatinib (w) and nilotinib (x) (inhibitors of tyrosine kinase domains of the BCR-ABL oncoprotein, platelet-derived growth factor receptors, stem cell factor and c-kit), growth factor receptor inhibitors (inhibitors of epidermal growth factor receptor signalling and vascular endothelial growth factor which lead to angiogenesis, invasion and metastasis inhibition) and asparaginase (exogenous source of L-asparagine amidohydrolase) (42).

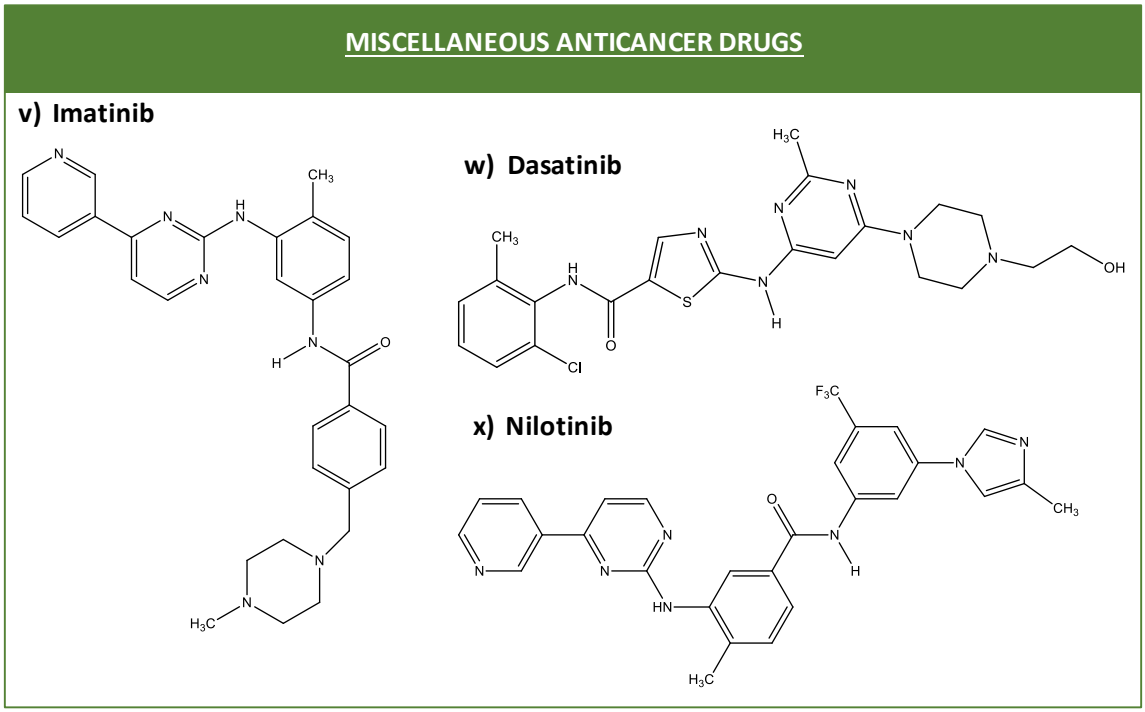


Figure I-18. Chemical structures of miscellaneous anticancer drugs (44).

Despite the existence of different drug mechanisms, all of them present side effects, the most common being: nausea, vomiting, diarrhoea, fatigue, myelosuppression, neutropenia, thrombocytopenia, hepatotoxicity, alopecia, mucositis, among others (45).

In addition, tumour cells have the ability to adapt to adverse conditions provided by anticancer drugs through resistance drug mechanisms such as: i) mutations in the target; ii) reactivation of the targeted pathway; iii) hyperactivation of the alternative pathway; iv) cross-talk with the microenvironment; v) clonal evaluation (46).

1.1.4 New Approaches to Therapy

The existence of side effects and the development of tumour resistance, associated with the necessity of more specific anticancer drugs amply justify the necessity for new drugs able to improve the efficacy and reduce the toxicity of therapeutics.

Table I.3 presents some of the new agents that are being developed in the acute myeloid leukaemia field.

Table I-3. New agents for the treatment of Acute Myeloid Leukaemia *(adapted from*(47,48)*).*

Agent	Mechanism of action
CPX-351	Liposomal formulation of cytarabine + daunorubicin in 5:1 molar ratio
Vosaroxin	Novel topoisomerase II inhibitor
Guadecitabine	Hypomethylating agent resistant to deamination
SGN-CD33A	Antibody-drug conjugate against CD33 with stable linker
Volasertib	Novel PLK1 (polo-like kinase 1) inhibitor
Quizartinib	FLT3 inhibitor
Crenolanib	FLT3 inhibitor with activity against TKD (tyrosine kinase domain) -resistance mutation
ASP-2215	FLT3 inhibitor with activity against TKD-resistance mutation
AG-221	IDH2 inhibitor
AG-120	IDH1 inhibitor
EPZ-5676	DOT1L inhibitor
ABT-199	BCL2 inhibitor
OTX-015	BET (bromodomain) inhibitor
Pracinostat	HDAC (histone deacetylase) inhibitor
Sorafenib	Tyrosine kinase inhibitor (TKI)
Midostaurin	FLT3 TKI
STAT inhibitors	STAT3 tyrosine phosphorylation
Clofarabine	Purine nucleoside analog

1.2 Bis-indolyl Methanes

Bis-indolyl methane (BIM), also known as 3,3-di-indolylmethane (DIM), is a natural metabolite of indole-3-carbinol that occurs in *Brassica* genus plants (cruciferous plants): broccoli, brussels sprouts, cauliflower, kale and cabbage (49–51).

BIM have different biological activities described, such as antibacterial, antifungal, analgesic, anti-inflammatory, anthelmintic, cardiovascular and, the most important to our study, anticancer activity (52). Besides these, BIM is also described as a colourimetric and ratiometric chemosensor for Cu^{2+} (53).

Exploring the anticancer activity, it is possible to find studies about BIM's activity in breast, cervical, ovarian, colon, hepatoma and prostate cancer (54–59).

1.2.1 Structure of Bis-indolyl Methanes

Bis-indolyl methane is composed by two indole molecules (pharmacophores), which are responsible for the anticancer activity of some drugs, attached to a methane group.

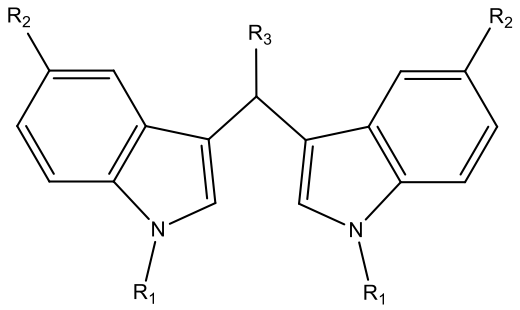


Figure I-19. Bis-indolyl methane structure.

The indole group is characteristic of anticancer groups as vinca alkaloides (vincristine, vinblastine, vinorelbine, vindesine) (60).

1.2.2 Tumour anti-progression activity of Bis-indolyl Methanes

As mentioned before, there are different biological activities described to BIMs, namely anticancer activity, and different mechanisms of action associated:

- Breast Cancer: Some articles refer BIMs as inhibitors of proliferation (estrogen-dependent and non-estrogen dependent cells), inducing apoptosis by decreasing Bcl-2 levels and increasing Bax and Bax/Bcl2 ratio levels; inductors of G_1 cell cycle and p21 gene expression; up-regulators of interferon γ expression; inhibitors of Akt, NF-κB and mTOR (51,57,61).
- Cervical Cancer: MAPK (Mitogen-activated protein kinases) and PI3K pathways are modified by BIMs interference (62).
- Ovarian Cancer: BIMs inhibit the signal transducer and activator of transcription pathway (STAT) through the anti-IL6 activity. Thus, cancer cell proliferation processes (adhesion, migration and invasion) are inhibited too (55).
- Colon Cancer: treatments with BIM showed a significant reduction of c-Myc, **β**-catenin and cyclin D1 protein levels, which are important oncogenes, by inactivation of **β**-catenin and targeting the Wnt/ **β**-catenin signalling pathway, as well as induction of G_1 phase (63). Another study concluded that class I histone deacetylases are selectively induced by BIM, increasing p21 and 27 expressions and causing DNA damage and consequently apoptosis (64).
- Hepatoma Cancer: BIMs present an inhibitor activity against topoisomerase IIα and topoisomerase I and Ii**β** (partial inhibition), which block DNA synthesis and mitosis processes (58). Phosphorylation of FAK and Estrogen signalling pathway mechanisms contribute to inhibition of cell proliferation and induction of tumorigenesis, respectively (65).
- Prostate Cancer: PI3K and Akt seems to be inhibited by BIMs (61). NF-κB, VEGF, Androgen receptor signal, uPA/uPAR (Urokinase receptor), MAPK (Mitogen-activated protein kinases) pathway and p75 (prostate epithelial cells tumour suppressor) are also subject to BIM influence (66).

In summary, BIMs lead to cellular apoptosis and anti-proliferation mechanisms through interferences in Bcl2, Bax, Akt, NF-κB pathway, mTOR STAT, Wnt/ β-catenin signalling pathway, VEGF, among other processes. BIM is also involved in reactive oxygen species production and cellular endoplasmatic reticulum stress, which cause DNA damage and consequently apoptosis (65).

1.3 Structure-activity relationship

During the drug development process, many steps are involved: drug discovery, drug design and drug development. Drug discovery includes the choice of the study disease as well as the drug target and the lead compound; drug design consists of the identification of structure-activity relationship (SAR) and the pharmacophore and on the improvement of pharmacodynamic and pharmacokinetic properties. Drug development includes pre-clinical and clinical trials (44).

Due to limited resources and limitations in experimental animal use, the investigation of new chemical compounds became too costly and time consuming (67). Thus, the development of structure-activity relationship and *in silico* studies, both computer models, became crucial to drug development.

Structure-activity relationship intents to obtain a statistical model of the compound activity considering its physicochemical properties and biological activities. SAR takes into account parameters as: ligands and their binding sites, inhibition constants, rate constants, lipophilicity, polarizability, electronic and steric properties; and allows relating chemical structure to qualitative biological activity, avoiding the experimental part of non-active compounds (67,68).

In silico studies are used to discover and optimize novel molecules with affinity to a target, to test ADMET properties (adsorption, distribution, metabolism, excretion and toxicity) and to characterize physicochemical properties. These studies include databases, quantitative SAR, pharmacophores, molecular modelling approaches and analysis tools (69,70).

1.3.1 Bis-indolyl Methanes in Cancer

The article *Novel approach to bis(indolyl)methane: De novo synthesis of 1-hidroxyminomethyl derivatives with anticancer properties* studied the influence of this compound in leukaemia and lymphoma cells. In this study, it was possible to conclude that (E)-1-((4-bromophenyl)-1-hydroxyiminomethyl)bis(1H-indol-3-yl)methane (henceforth designed as 9a), was worth pursuing due to its lower half maximal inhibitory concentration (IC_{50}) and higher selectivity. In this context, different compounds were chemical synthetized and their biological activity determined in order to continue the work already done in this area (71).

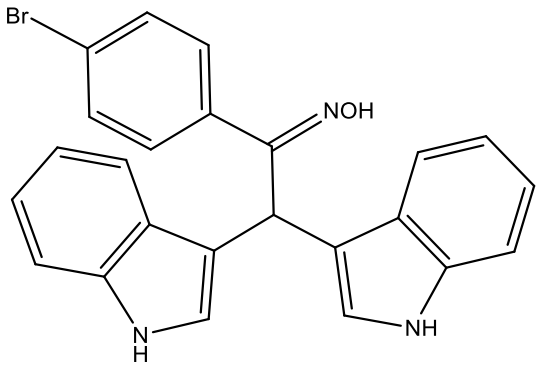


Figure I-20. Chemical structure of 9a ((E)-1-((4-bromophenyl)-1-hydroxyiminomethyl)bis(1H-indol-3-yl)methane).

1.4 Aim of the thesis

The number of cancer patients continues to increase, demanding for new anticancer therapeutics development.

The use of BIMs is already being investigated for some cancers as breast, cervical, ovarian, colon, hepatoma and prostate cancer. The major goal of this work was to study BIMs cytotoxic activity in lymphoma and leukaemia cells.

The specific objectives of this work were:

1) To study the cytotoxic activity of two classes of compounds of new bis-indolyl compounds (oximes and hydrazones) in four different cell lines - S17 (murine bone marrow), THP1 (human acute monocytic leukaemia), U937 (human leukaemic

monocytic lymphoma) and EL4 (murine T-lymphoma) – using the MTT colorimetric assay;

2) To relate the compounds' chemical structure with the cytotoxic activity;

3) To compare the effects of the studied compounds with drugs that are currently being used to treat cancer.

II. MATERIALS AND METHODS

2.1 Chemicals, culture media and supplements

Dimethyl Sulfoxide (DMSO) was purchased from Merck while Sigma-Aldrich (Germany) supplied 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RMPI) 1640 medium, foetal bovine serum, trypsin, L-glutamine and penicillin/streptomycin were provided by Lonza (Belgium). Additional reagents and solvents were obtained from VWR International (Belgium).

2.2 Study compounds

The studied compounds in this project were provided by Professor Américo Lemos and his PhD student Carla Grosso. All the compounds, except otherwise indicated, were unknown compounds and obtained by chemical synthesis via two consecutive Hetero Diels-Alder reactions of nitroso- or azo-alkenes with the particular indole. The derivatives of bis-indolyl methane: CG-99.1, CG-109, CG-141 and CG-142 were oximes and AL-516, AL-522B, CG-92, CG-105, CG-147, CG-151 and CG-153 were hydrazones. All the compounds tested are listed below in Table II-1 and II-2 and illustrated in Annex 7.1.

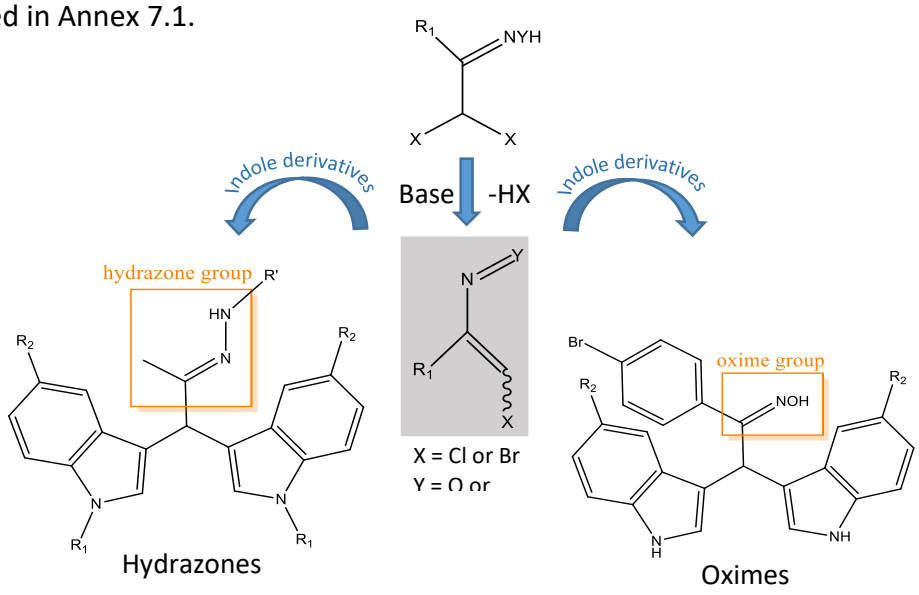
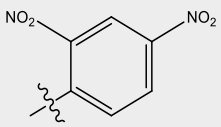
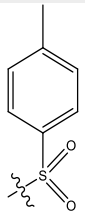
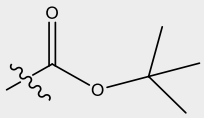
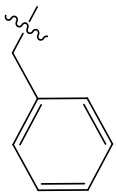
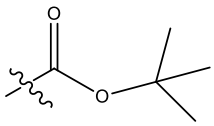
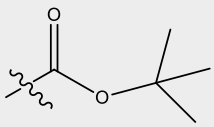
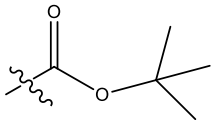


Figure II-1. One-pot synthesis of Bis-indolyl methane.
AL-516, AL-522B, CG-92, CG-105, CG-147, CG-151 and CG-153 compounds have hydrazone groups while CG-99.1, CG-109, CG-141 and CG-142 compounds have oxime groups.

Table II-1. Derivatives of bis-indolyl methane with oxime group, as illustrated in Figure II-1.
[1] Compounds analysed in previous experiments.

Compound	R2
CG-99-1 [1]	Br
CG-109	OMe
CG-141	Me
CG-142	OH

Table II-2. ***Derivatives of bis-indolyl methane with hydrazone group, as illustrated in Figure II-1.***
[1] Compounds analysed in previous experiments.

Compound	R1	R2	R'
AL-516	H	H	
AL-522B	H	H	
CG-92 [1]	CH3	H	
CG-105		H	
CG-147	H	H	
CG-153	H	OH	

2.3 Cell culture

This study used four types of cells: three cancerous cell lines (THP-1 - human acute monocytic leukaemia; EL-4 - murine lymphoblast; and U937 - human lymphoblast lung); and a non-tumoural cell line (S17 - murine bone marrow stromal). All the cell lines belonged to the Marbiotech Group Lab from CCMAR.

S17 and EL-4 cell lines were cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. THP-1 and U937 cell lines were maintained in RPMI medium with the same supplementation of DMEM: 10% foetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. All cell lines were cultured at 37°C and 5% carbon dioxide (CO_2) atmosphere.

2.4 Tumour anti-progression activity

2.4.1 Cytotoxicity assay

Cytotoxicity assay was based on the MTT colourimetric assay, described in 2.4.1.1.

This assay was performed in 96-well microplates at a density of $5x10^3$ cells/well (adherent cell lines: S17) or $1x10^4$ cells/well (suspension cell lines: THP-1, U937 and EL-4).

After incubation to allow the adherence of S17 cells, the studied compounds were applied (dissolved in DMSO) and incubated for 72 hours. The first test concentration of each compound was 100µM and, if active (<50% cell viability), lower concentrations were tested and the half maximal inhibitory concentration (IC_{50}) was determined. Two hours before the end of the incubation time, 20 µL of MTT (5 mg/mL in PBS - Phosphate-buffered saline) were added and the plates returned to the incubator. After the 72 hours, the supernatant was removed and 150 µL of DMSO was added to each well. The absorbance of the produced formazan cristals was measure at 590nm (Biotek Synergy 4).

Control cells were treated with DMSO at the highest concentration used in test wells (0,5%) and etoposide, a currently used cytotoxic anticancer drug, was used as a positive control.

The tested compound AL-516 had the particularity of being coloured, whereby an additional step was necessary and a colour control containing medium and the compound at the same concentration as in the assay was tested.

The absorbance of AL-516 test samples was calculated as the difference between the absorbance of sample and colour control. Cell viability was determined as a ratio of absorbance of test sample and DMSO control (negative control) media. Results were expressed in terms of cell viability (%) and half maximal inhibitory concentration (IC_{50}, µM).

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ test\ sample}{Absorbance\ of\ DMSO\ control\ media} \times 100$$

$$\%\ Cell\ viability\ (AL-516)\ (\%) = \frac{Absorbance\ of\ test\ sample\ -Absorbance\ of\ colour\ control}{Absorbance\ of\ DMSO\ control\ media} \times 100$$

2.4.1.1 MTT colourimetric assay

MTT colourimetric assay belongs to Tetrazolium Reduction Assays and consist in a coloured product that can be detected with a spectrophotometric plate reader (72).

MTT is positively charged and is able to penetrate viable cells. Viable cells have an active metabolism that allows the transformation of MTT into a purple coloured formazan product (Fig. II-3). The mechanisms of this reaction are still not well understood but they likely involve reactions with NADH or a similar reducing compound. The product of this reaction is insoluble and tends to accumulate inside cells and to deposit near their surface and in the culture medium. When DMSO is added, the formazan crystals solubilize and the solution acquire the purple colour than is measured in a plate absorbance reader (72).

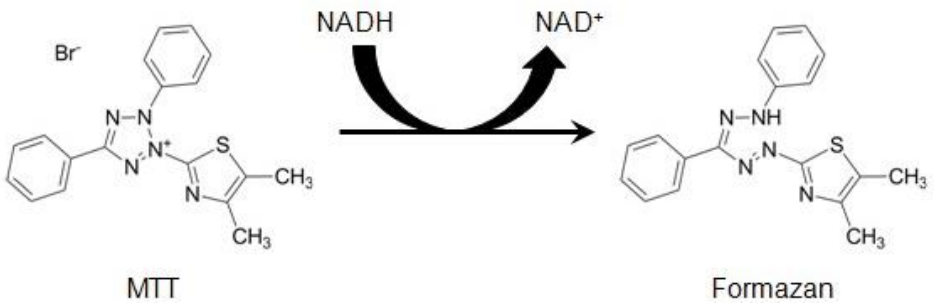


Figure II-2. Structure of MTT and the product of reaction, formazan (72).

2.4.2 Hemolytic activity

The first step of hemolytic activity determination was to remove the plasma and white cells from a type O blood sample (1000g, 5 minutes). After that, the appropriate controls and the compounds were prepared: each Eppendorf tube had 4µL of red blood cells and 196µL of Milli-Q water (positive control), PBS (negative control) or the tested compound. The different Eppendorf tubes were incubated during 1 hour at 37°C, and then centrifuged (1000g, 5 minutes). 100µL of each Eppendorf tube were add to a well (3 replicates each) and the absorbance was measured (Biotek Synergy 4).

The percentage of hemolysis was determined by the ratio between the absorbance of the sample and positive control (media).

$$Hemolysis\ (\%) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ positive\ control\ (media)} \times 100$$

2.5 Statistical analysis

The results of cytotoxicity assays where expressed in 95% confidence intervals and best-fit value of half-maximal inhibitory concentration, using at least six replicates, and were calculated using a variable slope model. Hemolytic assay results were compared using a one-way analysis of variance (ANOVA) and were expressed in mean ± SEM.

All the data was analysed in GraphPad Prism 6 program, version 6.01.

III. RESULTS AND DISCUSSION

3.1 Tumour anti-progression activity

3.1.1 Cytotoxicity assay and selectivity of compounds

This assay intended to study the cytotoxic activity of bis-indolyl methane derivatives in four cell lines, representative of three pathologic and one non pathologic cell lines: THP-1 (leukaemia), EL-4 and U937 (lymphoma) and S17 (bone marrow).

The objective was to find active compounds with cytotoxic activity towards cancerous cells (EL-4, THP-1 and U937) but not to non-cancerous cells (S17), in order to decrease the side effects of these compounds to healthy cells.

In a first step, all the compounds were study at 100µM. If active, the studies proceeded to the IC_{50} determination. CG-105 was the only compound not active in U937. All the others were active and allowed the elaboration of an IC_{50} curve (Annex 7.2). Results are summarized in table III-1.

Table III-1. ***IC_{50} (μM) (Best-fit value and 95% Confidence Interval) of etoposide and BIM derivatives with oxime and hydrazone groups in suspended and non-suspended cell lines (EL-4, THP-1, U937 and S17).***

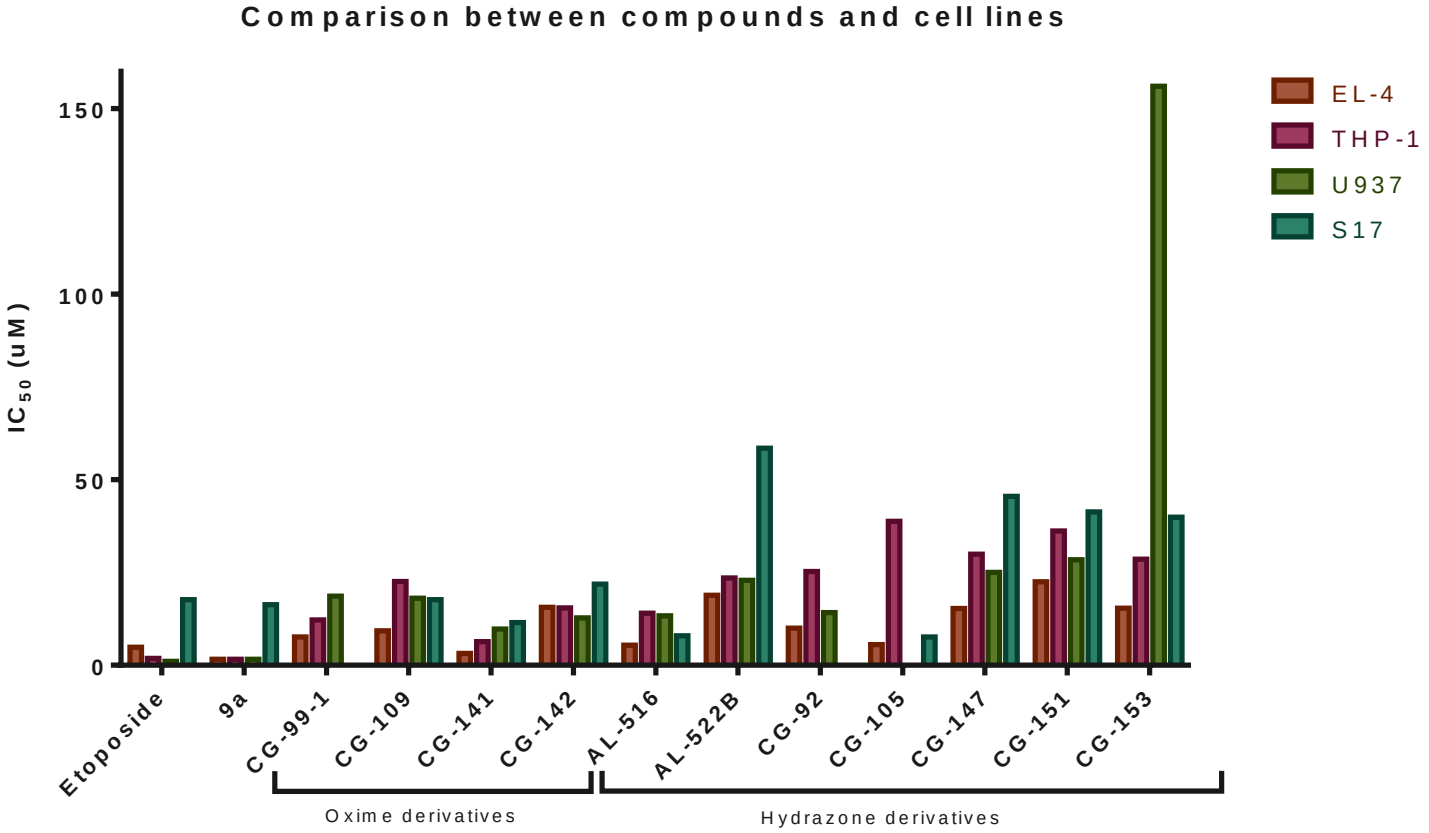
		IC_{50} (μM)							
		EL-4		THP-1		U937		S17	
	Compound	IC50	CI (95%)	IC50	CI (95%)	IC50	CI (95%)	IC50	CI (95%)
	Etoposide [1]	4,90 ± 0,47		1,82 ± 0,06		1,10 ± 0,04		17,65 ± 0,31	
BIMs derivate with oxime group	CG-99-1 [1]	7,65 ± 1,07		12,2 ± 1,0		18,6 ± 1,1			
	CG-109	9,30	(8,77 - 9,85)	22,6	(21,0 - 24,3)	18,1	(16,2 - 20,1)	17,7	(14,9 - 21,1)
	CG-141	3,23	(3,01 - 3,47)	6,34	(5,79 - 6,94)	9,79	(9,36 - 10,3)	11,5	(11,2 - 11,9)
	CG-142	15,6	(13,7 - 17,9)	15,5	(14,2 - 16,9)	12,8	(12,0 - 13,7)	21,9	(16,7 - 28,7)
BIMs derivate with hydrazone group	AL-516	5,45	(5,20 - 5,73)	14,0	(12,2 - 16,1)	13,3	(12,0 - 14,8)	7,92	(6,88 - 9,11)
	AL-522B	18,9	(16,4 - 21,7)	23,5	(20,2 - 27,2)	22,9	(20,3 - 25,89)	58,5	(48,9 - 70,0)
	CG-92 [1]	9,99 ± 1,05		25,3 ± 1,1		14,2 ± 1,0			
	CG-105	5,56	(5,12 - 6,05)	38,8	(31,3 - 48,2)	--		7,65	(3,95 - 14,8)
	CG-147	15,3	(13,7 - 17,1)	29,9	(24,9 - 35,8)	25,0	(23,7 - 26,4)	45,5	(42,6 - 48,6)
	CG-151	22,5	(19,6 - 25,9)	36,2	(32,5 - 40,4)	28,4	(21,3 - 37,9)	41,3	(36,9 - 46,2)
	CG-153	15,4	(12,9 - 18,4)	28,6	(24,4 - 33,5)	156	(4,83 - 5105)	39,9	(33,4 - 47,6)

[1] Compounds analysed in previous experiments. Results are expressed as IC_{50}, 95% Confidence Interval or mean ± SEM;
-- Not active compounds at 100uM.

Results of IC_{50} showed that, in a general way, CG-141 was the most active compound and EL-4 was the most sensitive cell line, that means, the cell line which require a lower drug concentration to kill 50% or more of their population. Furthermore, when compared with the hydrazone derivatives, oxime derivatives had a stronger biological activity in these cell lines (graphic III-1).

As referred in section 1.3.3, the study of oximes continued from a previous investigation. In that studied, 9a structure presented an IC_{50} of 1.65±0.02, 1.62±0.08, 1.64±0.08 and 16.3±0.08 µM for EL-4, THP-1, U937 and S17, respectively (71).

When compound 9a is compared with the oxime derivatives, it was possible to conclude that the hydrogen in the R_2 BIM position increases the biological activity in EL-4, THP-1 and U937.



***Graphic III-1. Comparison between compounds and cell lines**. Representation of table III.1 in order to help its interpretation. CG-99-1, CG-109, CG-141 and CG-142 include an oxime group in their structure. AL-516, AL-522B, CG-92, CG-105, CG-147, CG-151 and CG-153 consist in structures with a hydrazone group.*

CG-147 was the simplest model of a hydrazone derivative. CG-151 and CG-153 had different R_2 group whereby CG-153 (R_2=OH) presented higher biological activity in EL-4 and THP-1. Comparing CG-153 with CG-147, the biological activity in EL-4 and THP-1 was similar and CG-147 was more active in U937.

The R_1 activity influenced by CG-92 and CG-105 was cell dependent. CG-92 was more active in THP-1 and CG-105 was in EL-4. Comparing CG-147 with CG-92 and CG-105, it was possible to affirm that in EL-4, R_1=CH_2-phenil was more active biologically and in THP-1 and U937, R_1=CH_3 was a better choice.

AL-516 and AL-522B differed in R_3. This difference allowed to conclude that AL-516 was more active in all the cancer cells, however, not selective (as explained below). Between AL-516 and CG-147, AL-516 showed lower IC_{50}.

Comparing 9a, the oxime with the best biological activity, with AL-516, the hydrazone with better results, 9a had better biological activity results: 1,65 µM versus 5,45 µM (EL-4), 1,62 µM versus 14,00 µM (THP-1) and 1,64 µM versus 13,3 µM (U937).

Other comparative analysis consisted in 9a and etoposide (positive control), which presented similar IC_{50} values and, consequently, similar cytotoxic activity.

Based in the cytotoxic assay best-fit value, the selectivity of compounds was calculated. This value was obtained by the ratio between IC_{50} of the compounds towards S17 cell line and the IC_{50} of the same compound towards each tumoural cell line.

$$Selectivity = \frac{IC_{50}(S17, compound\ x)}{IC_{50}(tumoural\ cell\ line, compound\ x)}$$

Table III-2. ***Selectivity results.*** *This table shows the selective value of studied compounds for the different tested cell lines (EL-4, THP-1 and U937), comparatively to S17. Green numbers signalize the higher selectivity values.*

Selectivity			
Compounds / Cell lines	**EL-4**	**THP-1**	**U937**
Etoposide	**3,60**	**9,70**	**16,05**
AL-516	1,45	0,57	0,59
AL-522B	**3,10**	**2,49**	**2,55**
CG-105	1,37	0,20	---
CG-109	1,91	0,79	0,98
CG-141	**3,57**	**1,82**	1,18
CG-142	1,40	1,41	1,71
CG-147	2,97	1,52	**1,82**
CG-151	1,83	1,14	1,46
CG-153	2,59	1,40	0,25

Comparing the selectivity values of BIM derivatives, compounds AL-522B and CG-141 were the most selective between tumoural and non-tumoural cells. AL-522B was the most selective compound, considering the three cell lines, with higher value in EL-4.

Compound 9a remains the most selective towards S17, presenting selectivity values of the same order to the three different cell lines: 9,86 (EL-4), 10,04 (THP-1) and 9,92 (U937) (71).

When compared with etoposide, compound 9a was more selective in U937 but less in EL-4.

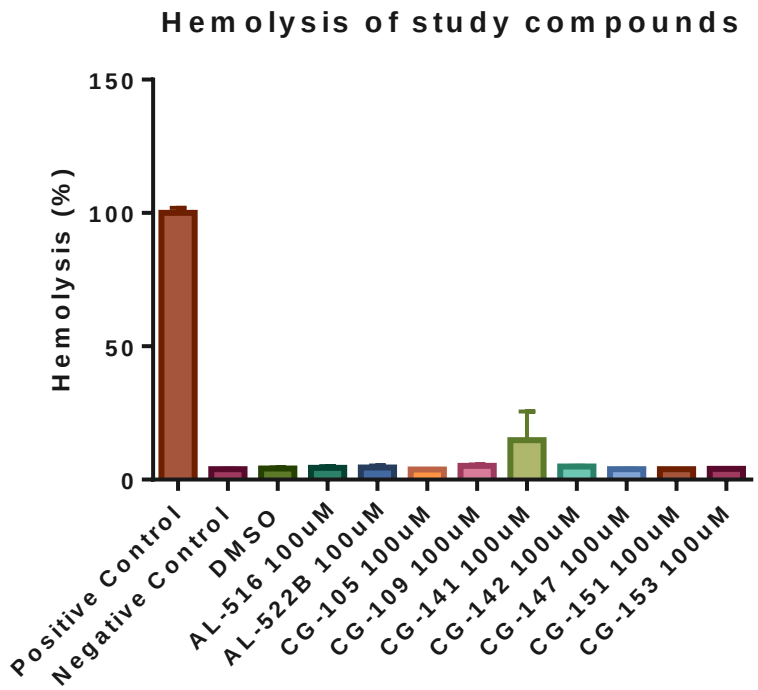
3.1.2 Hemolytic activity

Hemolysis consists on the damage to red blood cells, releasing hemoglobin that can be measured determining the hemolysis percentage.

The assay studied compounds as well as DMSO (solvent of compounds – negative control), milli-Q water (positive control) and PBS (negative control).

CG-141 was the compound with higher hemolysis percentage (14,8%). All the other compounds presented very low hemolytic activities; between 3,9% and 5,2%.

The hemolytic assay allowed concluding that the study compounds do not have significant activity against red blood cells. These results corroborate some data referred in the introduction, which mention BIM activity in nuclear mechanisms. Red blood cells are anucleated cells which justify the lack of activity.



Graphic III-3. Hemolysis percentage graph of tested compounds. *Results are expressed as mean ± SEM, 95% Confidence.*

Table III-3. Hemolytic activity results of tested compounds, expressed as mean ± SEM, 95% Confidence.

	Positive control	Negative control	DMSO	AL-516, 100µM	AL-522B, 100µM	CG-105, 100µM	CG-109, 100µM	CG-141, 100µM	CG-142, 100µM	CG-147, 100µM	CG-151, 100µM	CG-153, 100µM
Mean	100	3,90	4,2	4,48	4,55	3,78	5,19	14,8	4,88	3,93	3,88	4,02
SEM	1,98	0,17	0,39	0,54	0,82	0,06	7,41	10,7	0,29	0,10	0,07	0,17

3.2 BIMs versus actual therapeutics

The third proposed objective was the structural comparison of the studied compounds with drugs that are currently being used to treat cancer. Comparing BIMs with the anticancer drugs presented in section 1.1.3, the two indoles present in BIMs (pharmacophore) are also present in the structures of vinca alkaloids (Fig. III-1). Vinca alkaloids present anticancer activity through mitosis inhibition due to tubulin polymerization, and such activity is attributed to the presence of the indole structures. It is thus possible that the compounds studied in this work act upon cancer cells through a similar mechanism of action. Further studies are, however, needed to confirm this hypothesis.

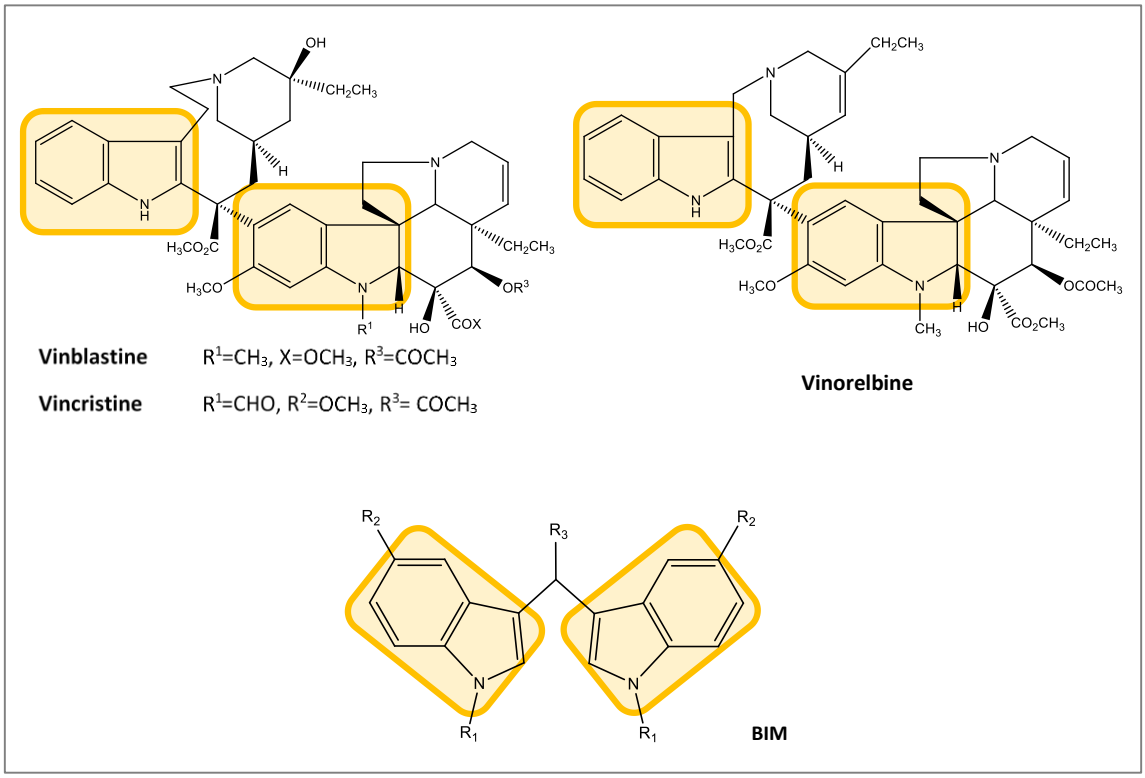


Figure III-1. Comparison between BIM and actual therapeutic chemical structures.

3.3 Experimental Limitations

During the experimental component, there were some difficulties, which are important to refer:

- the use of cell lines coming from different organisms can justify the different response to the tested compounds (EL-4 and S17 are murine cells while THP-1 and U937 where human);
- the use of suspension cell lines (EL-4, THP-1 and U937) and adherent cell lines (S17) could have influenced the cellular response to the compounds and hence affect the evaluation of the testes compounds selectivity;
- the use of small volumes during the cell culture and during the compounds preparation could have resulted in measurement errors;
- the existence of suspended cells and non-suspended cells contributed to additional steps during the assays and consequently loss of cells, affecting the final result;
- in the hemolysis assay, the blood viscosity conditioned the drop's volume used in the assay as well as the removal of the supernatant.

IV. CONCLUSIONS AND FUTURE PERSPECTIVES

The existence of side effects, development of resistance by tumour cells and the necessity of more specific anticancer mechanisms are the main reasons to investigate new drugs able to improve the efficacy and reduce the toxicity of therapeutics. Actually, BIMs have described biological activities such as antibacterial, antifungal, analgesic, anti-inflammatory, anthelmintic, cardiovascular and anticancer activity (52).

In the course of this work, the main objectives were to study the cytotoxic activity of BIMs derivatives, to relate their chemical structure with the cytotoxic activity and to compare BIMs with actual therapeutics. The results revealed that CG-141 was the most active compounds towards EL-4 (IC_{50}=3,01-3,47 µM), THP-1 (IC_{50}=5,79-6,94 µM) and U937 (IC_{50}=9,79-10,3 µM) while AL-522B was the most selective compound towards S17 (EL-4: 3,10; THP-1: 2,49; U937:2,55). When compared with hydrazone derivatives, oxime derivatives had a stronger biological activity in the studied cell lines.

As result of the comparison between the studied compounds with the compound 9a, it was possible to conclude that 9a continues to be the most biological active compound towards EL-4 (IC_{50}=1.65±0.02µM), THP-1 (IC_{50}=1.62±0.08µM) and U937 (IC_{50}=1.64±0.08µM) and the most selective towards S17 (9,86; 10,04; and 9,92, respectively). This comparison allowed to conclude that a hydrogen in the R_2 BIM position increased the biological activity results.

The similar IC_{50} between the etoposide and 9a compound suggest that 9a has potential to be a therapeutic drug, due to the cytotoxic activity demonstrated. BIMs and vinca alkaloids have the same pharmacophores, which suggest possible resemblance to the action mechanism, namely in tubulin polymerization.

None of the studied compounds presented significant hemolytic activity in hemolytic assay, which corroborated the theory of a nuclear action mechanism.

As future perspectives, new structural approaches and biological studies can be considered. Structurally speaking, the studies undertaken shown that BIMs bearing oxime and hydrazone moieties with alkyl and aryl substituents in R_3 BIM position may be promising compound for treatment of non-adherent tumour cells. Further studies of BIMs involving heteroaryl, phosphoryl, carbonyl and carboxyl motifs at the oxime and/or

hydrazone moiety would be very interesting, since these groups are also present in anticancer drugs used in therapeutic.

The biological cytotoxicity determination in human suspension cell lines of the compounds, instead of EL-4 and S17 (both murine cell lines), constitutes a possible future assay, since it allows comparisons between tumoural and non-tumoural suspension cell lines of the same organism. Furthermore, the evaluation of proteins such as tubulin, which regulates cellular cycle, and the determination of the antioxidant activity of BIMs, constitute possible assays that could be useful to complement the data presented, explaining BIM anticancer mechanisms.

To conclude, additional strategies of chemical structure and biological assays should be done to complement the data presented here and to improve new molecules with anticancer activity potential.

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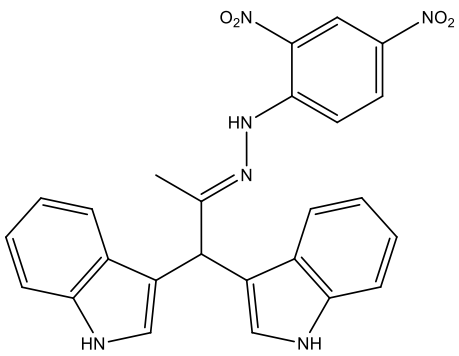
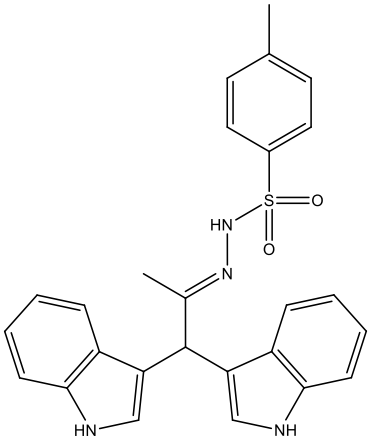
VI. ANNEXES

7.1 Chemical Structures of compounds

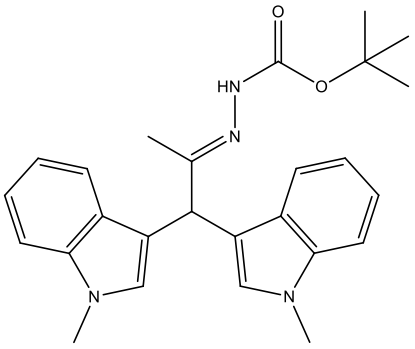
The compounds are illustrated by alphabetic order.



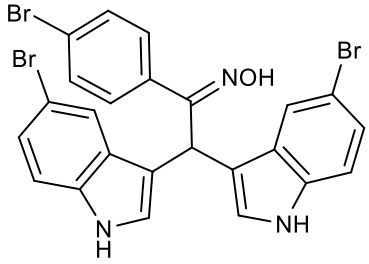
AL-516 chemical structure.



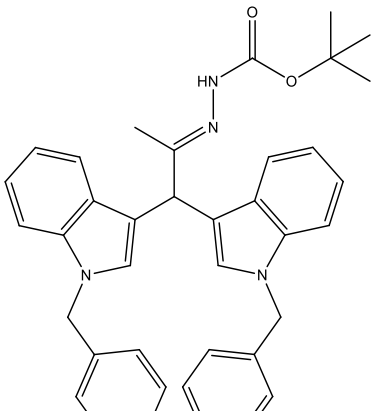
AL-522B chemical structure.



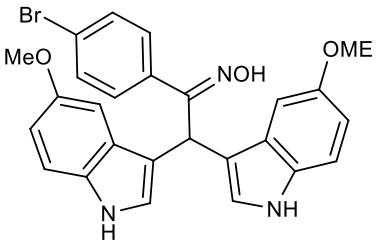
CG-92 chemical structure.



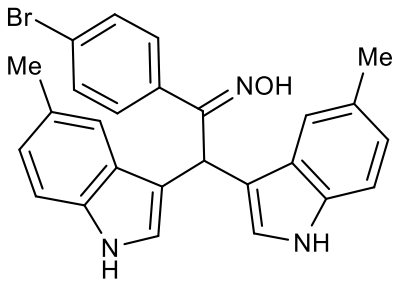
CG-99.1 chemical structure.



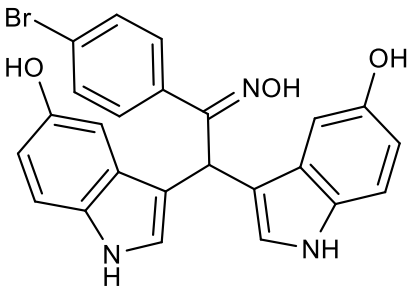
CG-105 chemical structure.



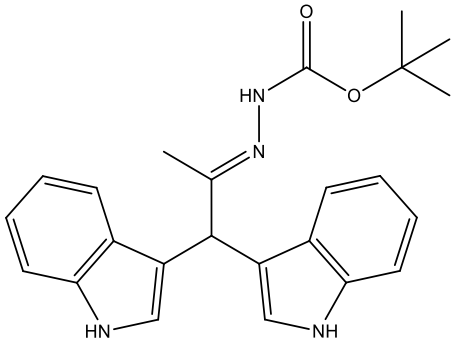
CG-109 chemical structure.



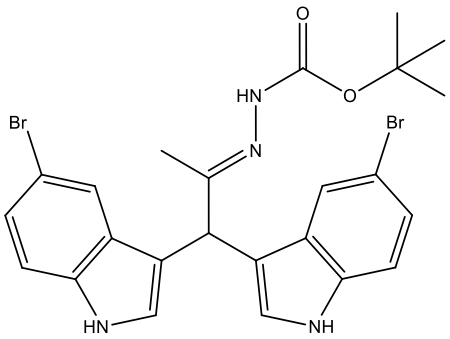
CG-141 chemical structure.



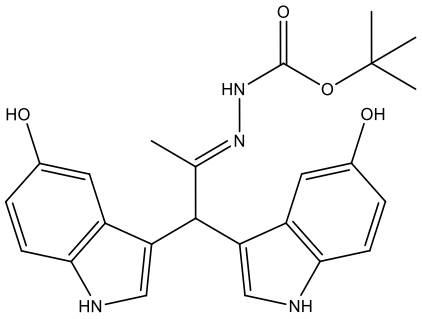
CG-142 chemical structure.



CG-147 chemical structure.



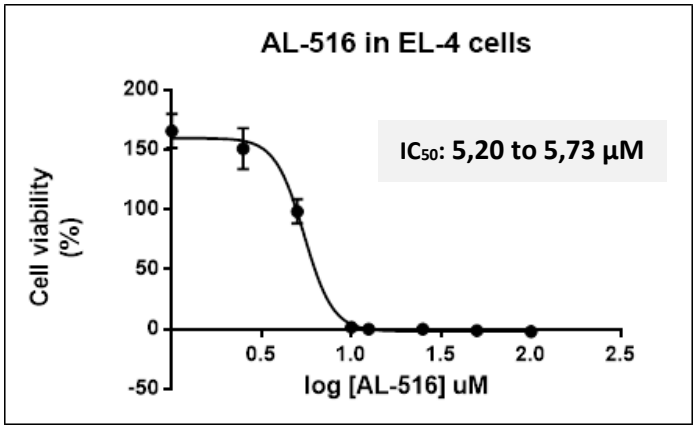
CG-151 chemical structure.



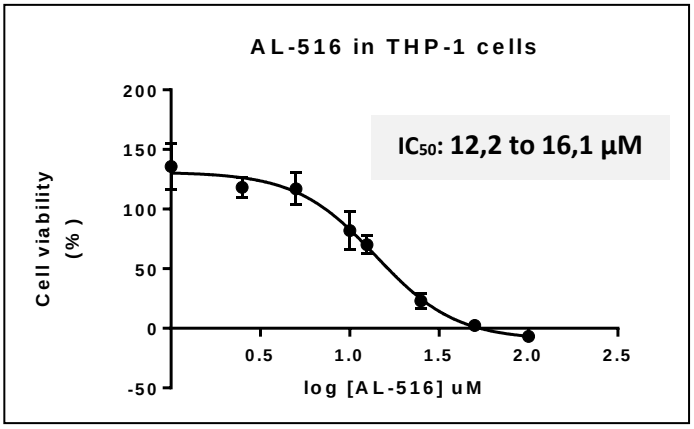
CG-153 chemical structure.

7.2 IC_{50} curves

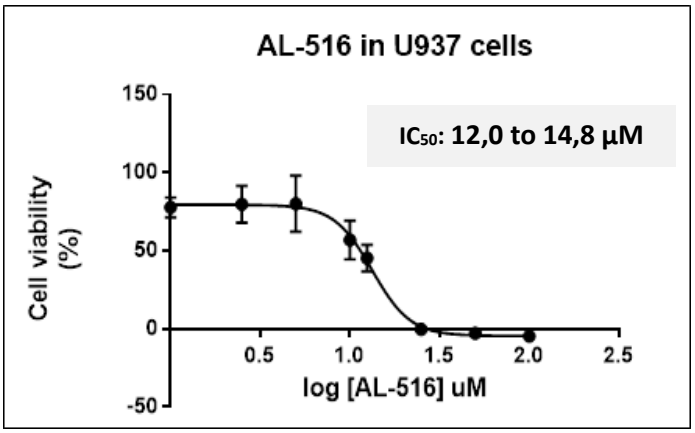
IC_{50} curves with AL-516



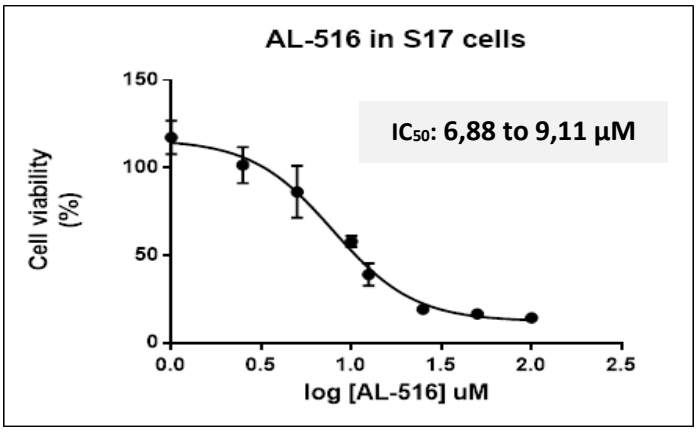
AL-516 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with AL-516 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-516 in EL-4 cell line has a 95% confidence interval of 5,20 to 5,73 µM.*



AL-516 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with AL-516 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-516 in THP-1 cell line has a 95% confidence interval of 12,2 to 16,1 µM.*

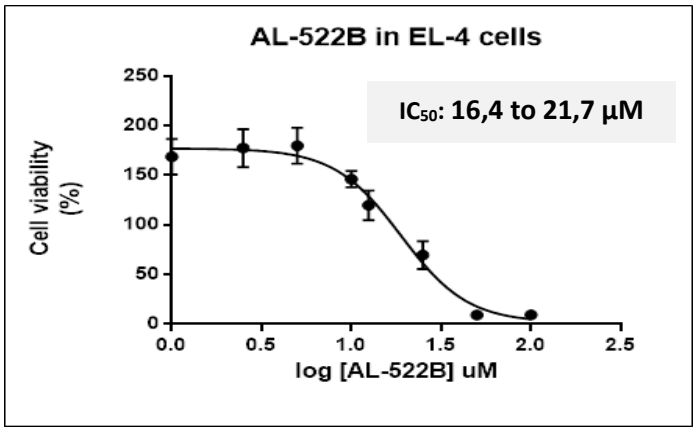


AL-516 IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with AL-516 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-516 in U937 cell line has a 95% confidence interval of 12,0 to 14,8 µM.*

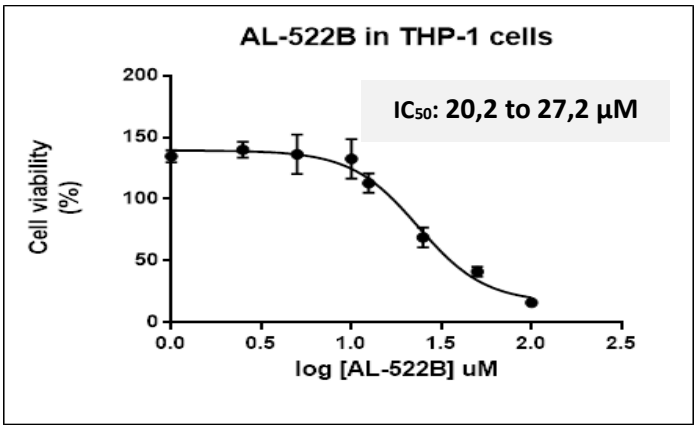


AL-516 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with AL-516 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-516 in S17 cell line has a 95% confidence interval of 6,88 to 9,11 µM.*

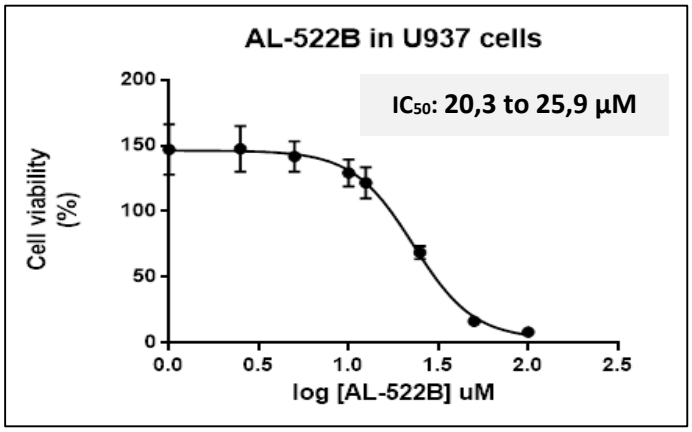
IC_{50} curves with AL-522B



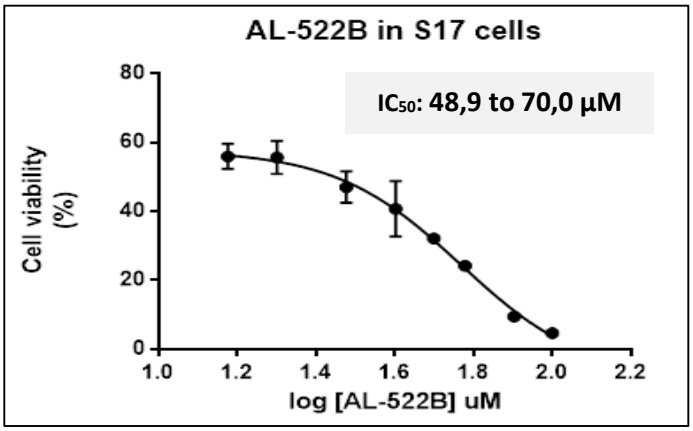
AL-522B IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with AL-522B at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-522B in EL-4 cell line has a 95% confidence interval of 16,4 to 21,7 µM.*



AL-522B IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with AL-522B at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-522B in THP-1 cell line has a 95% confidence interval of 20,2 to 27,2 µM.*

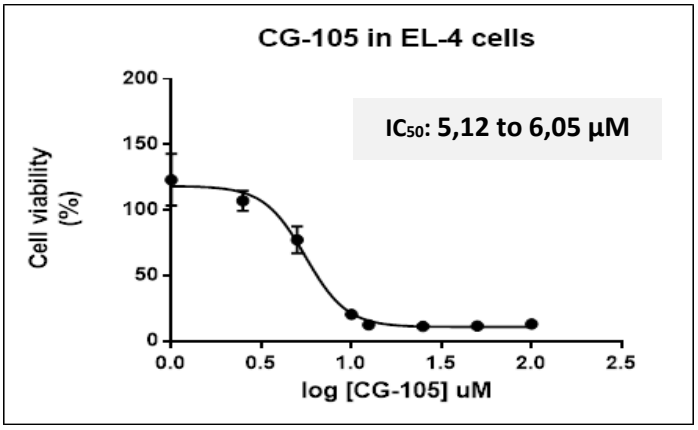


AL-522B IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with AL-522B at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-522B in U937 cell line has a 95% confidence interval of 20,3 to 25,9 µM.*

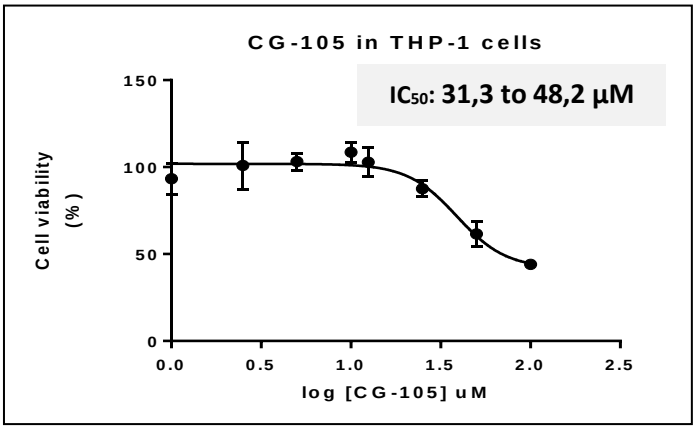


AL-522B IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with AL-522B at 100, 80, 60, 50, 40, 30, 20 and 15 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of AL-522B in S17 cell line has a 95% confidence interval of 20,3 to 25,9 µM.*

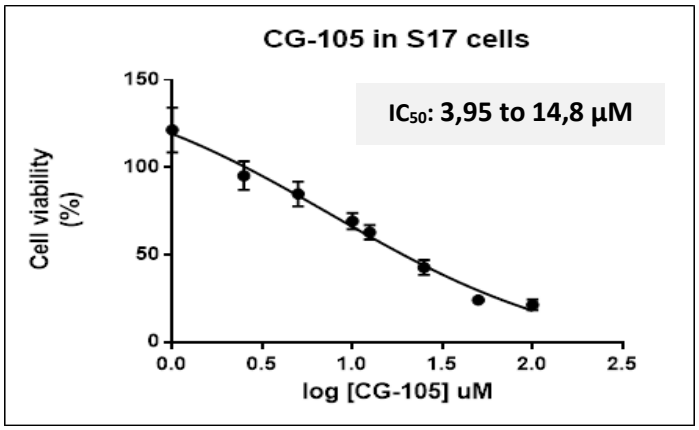
IC_{50} curves with CG-105



CG-105 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with CG-105 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-105 in EL-4 cell line has a 95% confidence interval of 5,12 to 6,05 µM.*

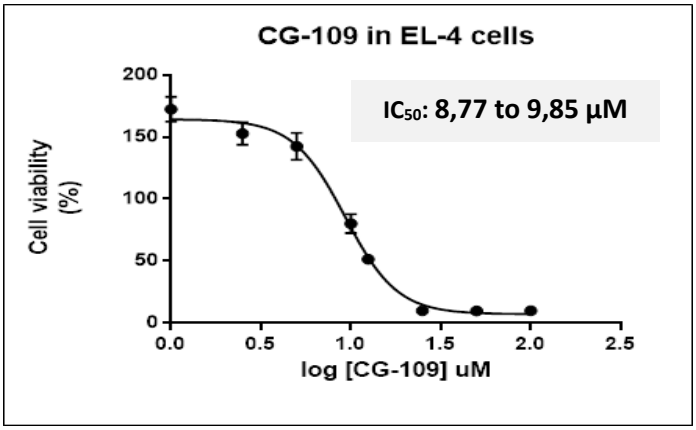


CG-105 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with CG-105 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-105 in THP-1 cell line has a 95% confidence interval of 31,3 to 48,2 µM.*

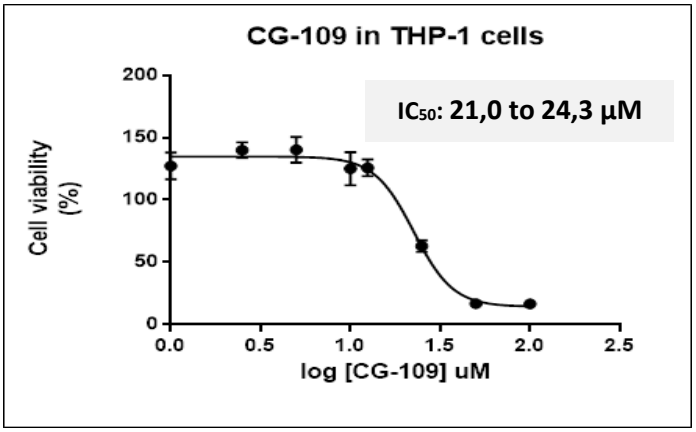


CG-105 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with CG-105 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-105 in S17 cell line has a 95% confidence interval of 3,95 to 14,8 µM.*

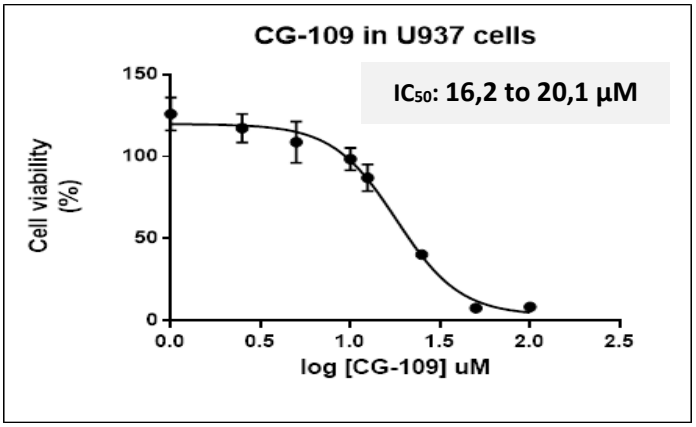
IC_{50} curves with CG-109



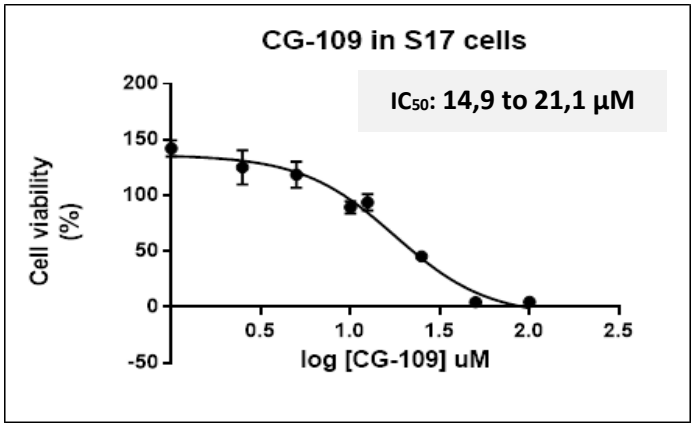
CG-109 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with CG-109 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-109 in EL-4 cell line has a 95% confidence interval of 8,77 to 9,85 µM.*



CG-109 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with CG-109 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-109 in THP-1 cell line has a 95% confidence interval of 21,0 to 24,3 µM.*

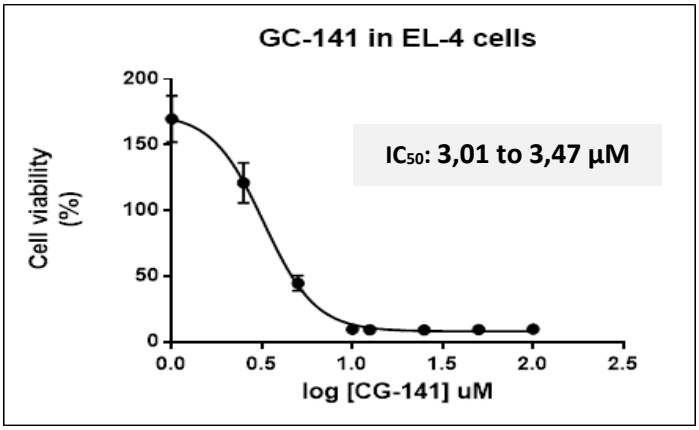


CG-109 IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with CG-109 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-109 in U937 cell line has a 95% confidence interval of 16,2 to 20,1 µM.*

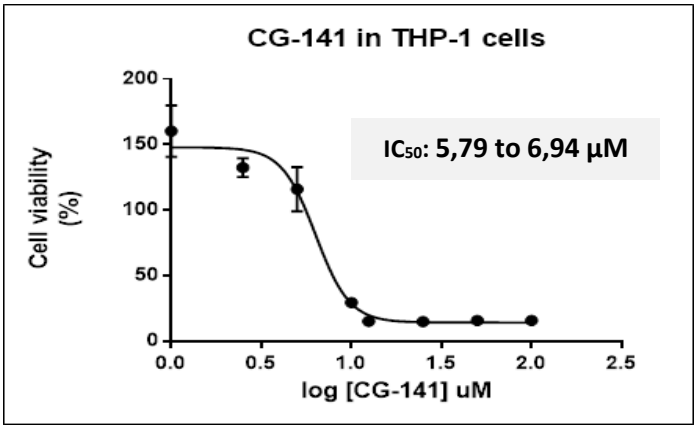


CG-109 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with CG-109 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-109 in S17 cell line has a 95% confidence interval of 14,9 to 21,1 µM.*

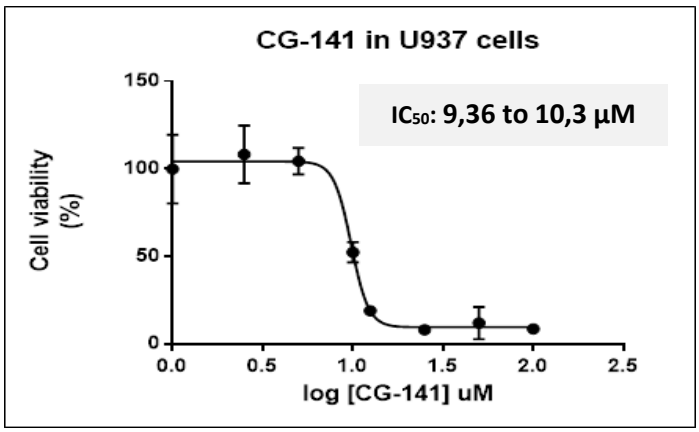
IC_{50} curves with CG-141



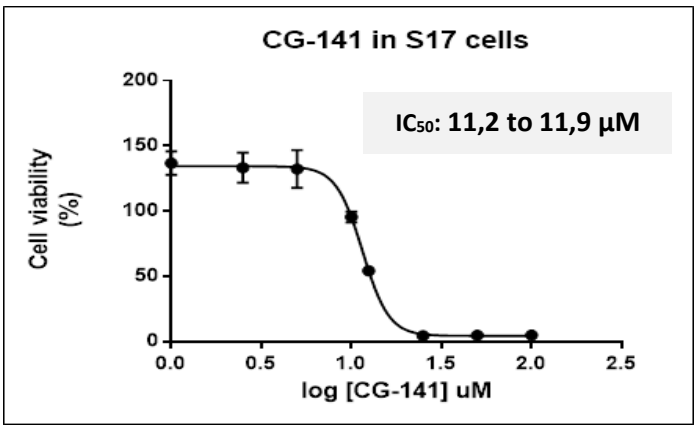
CG-141 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with CG-141 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-141 in EL-4 cell line has a 95% confidence interval of 3,01 to 3,47 µM.*



CG-141 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with CG-141 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-141 in EL-4 cell line has a 95% confidence interval of 5,79 to 6,94 µM.*

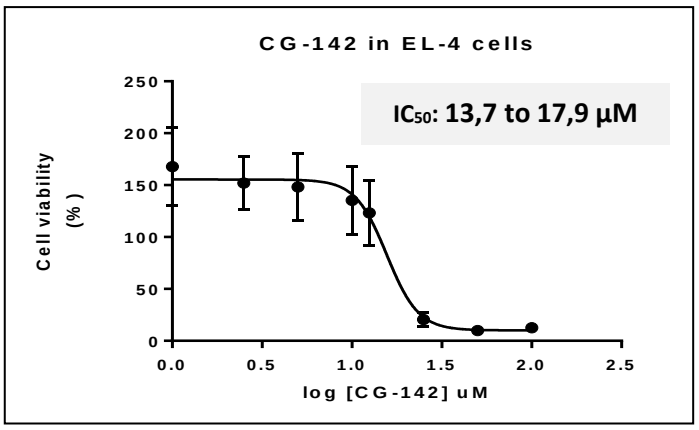


CG-141 IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with CG-141 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-141 in U937 cell line has a 95% confidence interval of 9,36 to 10,3 µM.*

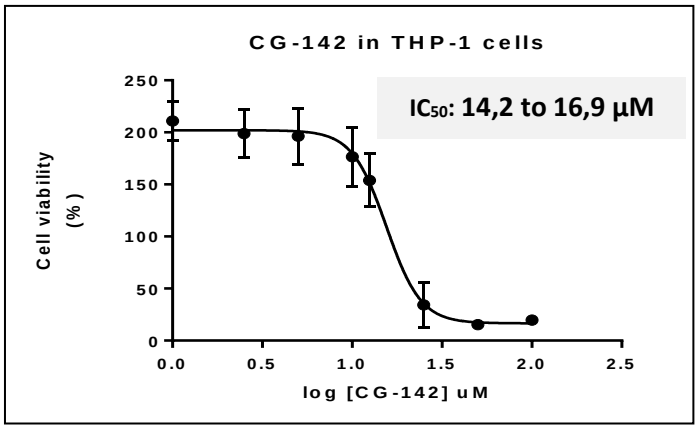


CG-141 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with CG-141 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-141 in S17 cell line has a 95% confidence interval of 11,2 to 11,9 µM.*

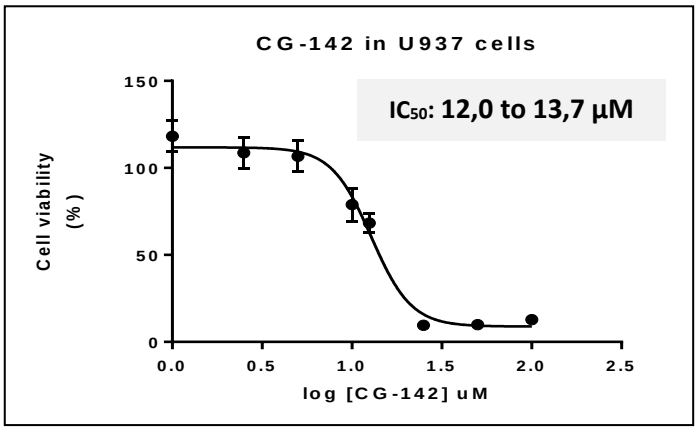
IC_{50} curves with CG-142



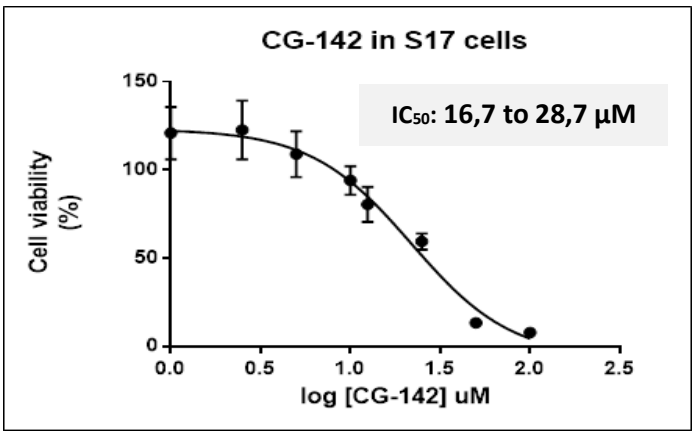
CG-142 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with CG-142 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-142 in EL-4 cell line has a 95% confidence interval of 13,7 to 17,9 µM.*



CG-142 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with CG-142 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-142 in THP-1 cell line has a 95% confidence interval of 14,2 to 16,9 µM.*

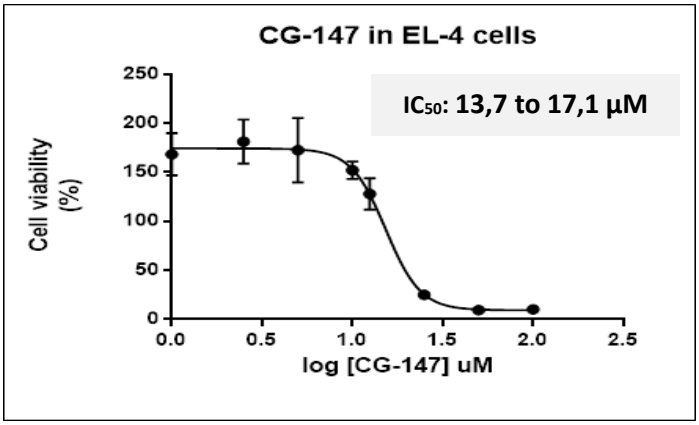


CG-142 IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with CG-142 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-142 in U937 cell line has a 95% confidence interval of 12,0 to 13,7 µM.*

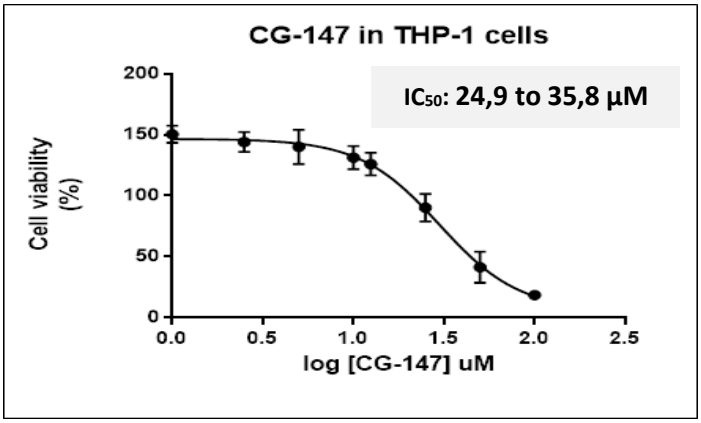


CG-142 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with CG-142 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-142 in S17 cell line has a 95% confidence interval of 16,7 to 28,7 µM.*

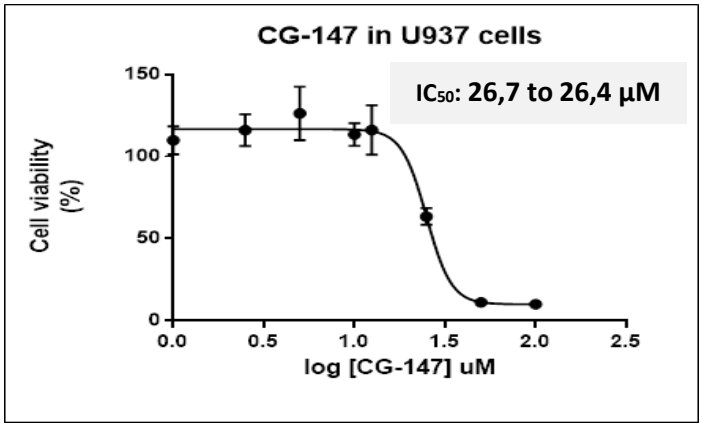
IC_{50} curves with CG-147



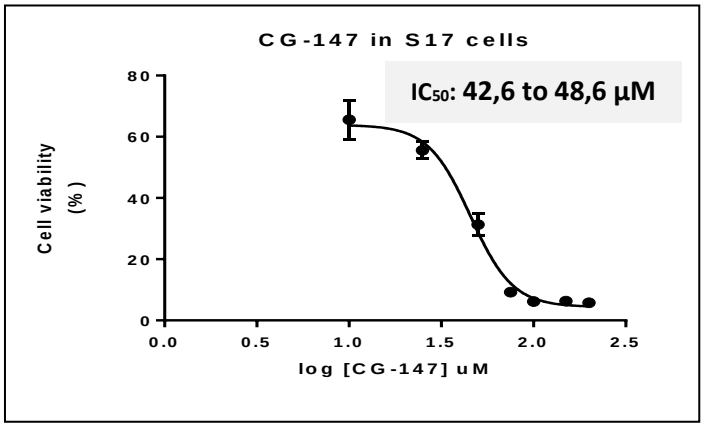
CG-147 IC_{50} curve in EL-4, based on MTT colourimetric assay*. EL-4 was tested with CG-147 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-147 in EL-4 cell line has a 95% confidence interval of 13,7 to 17,1 µM.*



CG-147 IC_{50} curve in THP-1, based on MTT colourimetric assay*. THP-1 was tested with CG-147 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-147 in THP-1 cell line has a 95% confidence interval of 24,9 to 35,8 µM.*

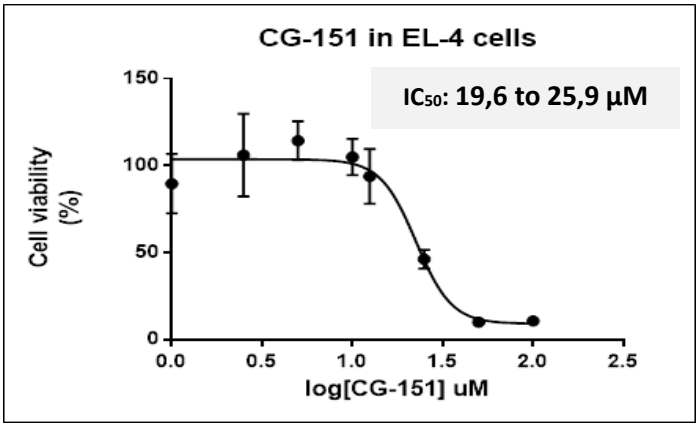


CG-147 IC_{50} curve in U937, based on MTT colourimetric assay*. U937 was tested with CG-147 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-147 in U937 cell line has a 95% confidence interval of 26,7 to 26,4 µM.*

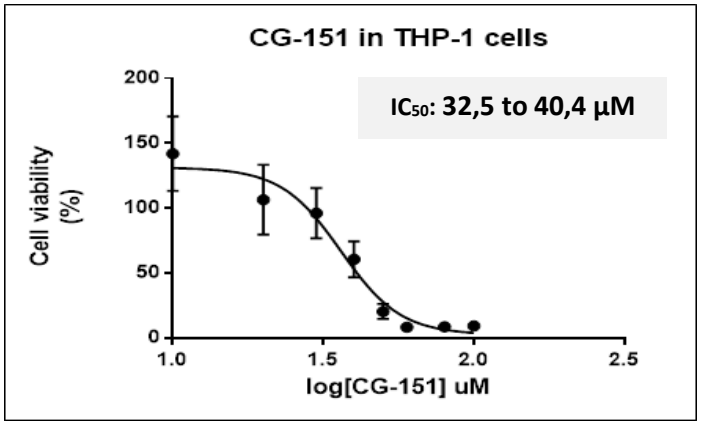


CG-147 IC_{50} curve in S17, based on MTT colourimetric assay*. S17 was tested with CG-147 at 200, 150, 100, 75, 50, 25 and 10 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-147 in S17 cell line has a 95% confidence interval of 42,6 to 48,6 µM.*

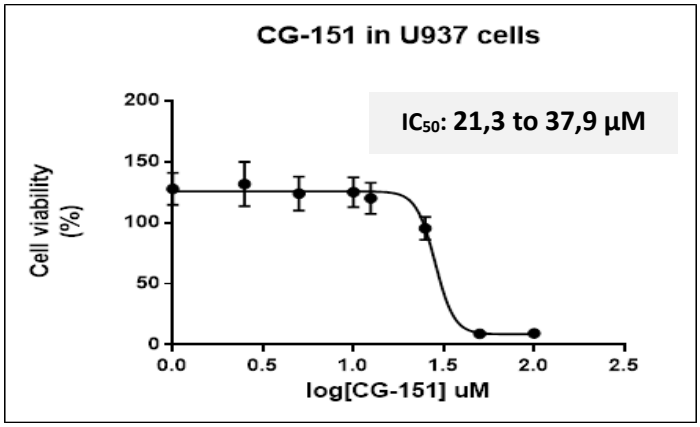
IC_{50} curves with CG-151



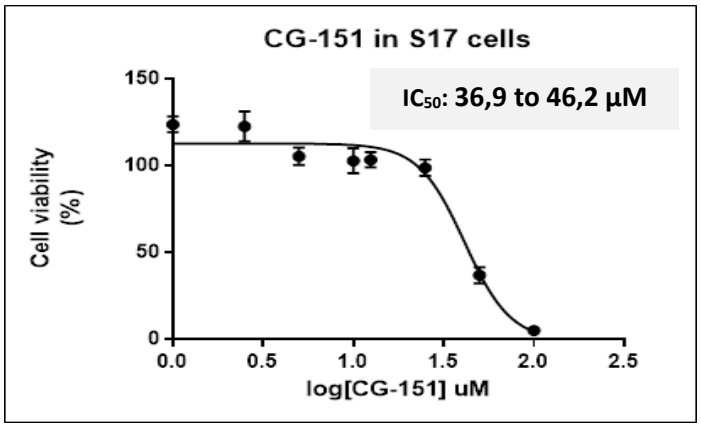
CG-151 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with CG-151 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-151 in EL-4 cell line has a 95% confidence interval of 19,6 to 25,9 µM.*



CG-151 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with CG-151 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-151 in THP-1 cell line has a 95% confidence interval of 32,5 to 40,4 µM.*

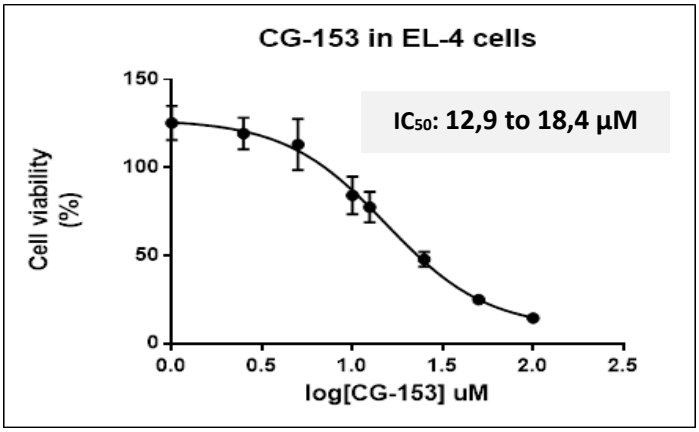


CG-151 IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with CG-151 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-151 in U937 cell line has a 95% confidence interval of 21,3 to 37,9 µM.*

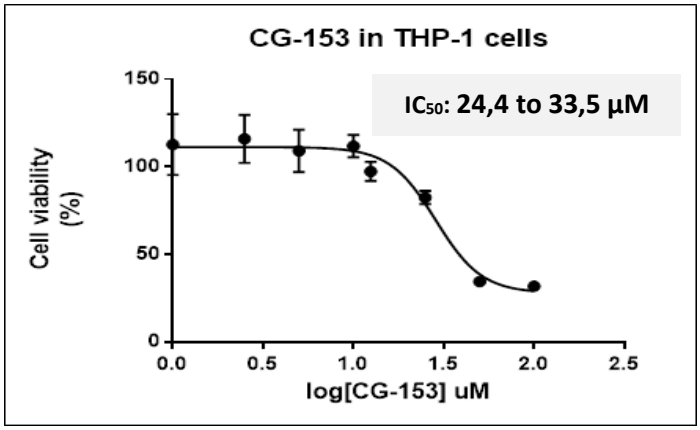


CG-151 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with CG-151 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-151 in S17 cell line has a 95% confidence interval of 36,9 to 46,2 µM.*

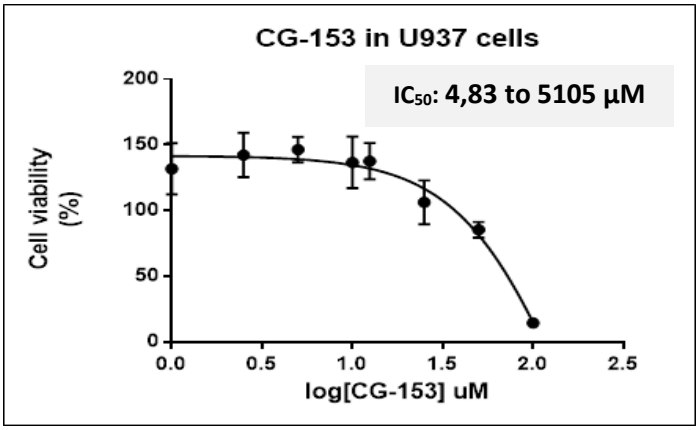
IC_{50} curves with CG-153



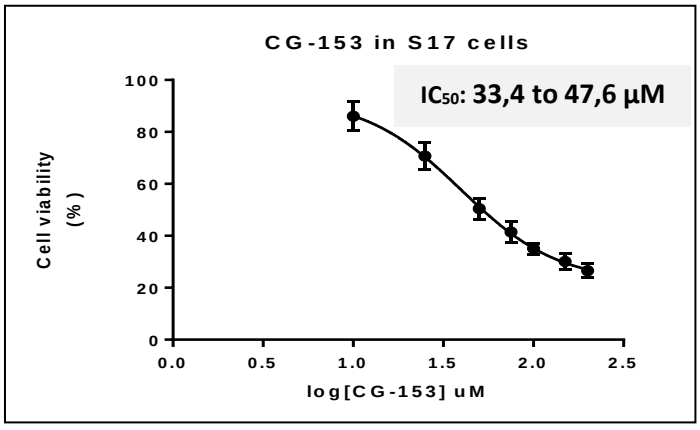
CG-153 IC_{50} curve in EL-4, based on MTT colourimetric assay. *EL-4 was tested with CG-153 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-153 in EL-4 cell line has a 95% confidence interval of 12,9 to 18,4 µM.*



CG-153 IC_{50} curve in THP-1, based on MTT colourimetric assay. *THP-1 was tested with CG-153 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-153 in THP-1 cell line has a 95% confidence interval of 24,4 to 33,5 µM.*



CG-153 IC_{50} curve in U937, based on MTT colourimetric assay. *U937 was tested with CG-153 at 100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-153 in U937 cell line has a 95% confidence interval of 4,83 to 5105 µM.*



CG-153 IC_{50} curve in S17, based on MTT colourimetric assay. *S17 was tested with CG-153 at 200, 150, 100, 75, 50, 25 and 10 µM, prepared in DMSO solution. The half-maximal inhibitory concentration of CG-153 in S17 cell line has a 95% confidence interval of 33,4 to 47,6 µM.*