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Contribution to the *in vitro* evaluation of trisubstituted harmine derivatives effects on the protein synthesis in cancer cell lines

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TABLE OF CONTENTS

ABBREVIATIONS

2-DE: Two-dimensional electrophoresis

35S: Sulfur-35

3D-QSAR: Three dimensional quantitative structure-activity relationship

3-PG: 3-phosphoglycerate

4E-BP: Eukaryotic translation initiation factor 4E-binding protein

5-FU: 5-fluorouracil

ABC: ATP-binding cassette

ABCE1: ATP-binding cassette sub-family E member 1

ABCB1: ATP-binding cassette sub-family B1

Akt: Serine/threonine-protein kinase

AR: Androgen receptor

ASO: Antisense oligonucleotides

asTORi: Active-site mTOR inhibitors

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

AUC: Area Under the Curve

AUG: Adenine-uracil-guanine

BBB: Blood Brain Barrier

BCA: Bicinchoninic acid assay

BCL2: Apoptosis regulator Bcl-2

BRAF: B-Raf proto-oncogene, serine/threonine kinase

BSA: Bovine serum albumin

BTF3: Transcription factor BTF3

CCC: COMPARE correlation coefficient

Cdk: Cyclin-dependent kinases

CFL1: Cofilin-1

CHAPS: 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate

Cip: Cyclin-dependent kinases interacting protein

CK-18: Cytokeratin-18

CKI: Cyclin-dependent kinases inhibitor

CO2: Carbon dioxide

CPMs: Counts per minute

c-RAF: RAF proto-oncogene serine/threonine-protein kinase

CSC: Cancer stem cell

CT: Control

CTLA4: Cytotoxic T lymphocyte-associated antigen 4

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dom34: Protein DOM34

dTMP: Deoxythymidine monophosphate

DTT: Dithiothreitol

dTTP: Deoxythymidine triphosphate

dUMP: Deoxyuridine monophosphate

dUTP: Deoxyuridine triphosphate

DYRK1A: Dual specificity tyrosine-phosphorylation-regulated kinase 1A

Ebp1: ErbB3-binding protein 1

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

eEF1A: Elongation factor 1-alpha 1

eEF2: Elongation factor 2

EGFR: Epidermal growth factor receptor

EIC: Extracted Ion Chromatogram

eIF: Eukaryotic translation initiation factor

eIF1: eukaryotic translation initiation factor 1;

eIF1A: Eukaryotic translation initiation factor 1A

EIF1AX: Eukaryotic translation initiation factor 1A, X-linked gene;

eIF2: Eukaryotic translation initiation factor 2

eIF2α: Eukaryotic translation initiation factor 2 subunit 1

eIF2α: Eukaryotic translation initiation factor 2 subunit 1

eIF2β: Eukaryotic translation initiation factor 2 subunit 2

eIF2γ: Eukaryotic translation initiation factor 2 subunit 3

eIF3: Eukaryotic translation initiation factor 3

eIF3e: Eukaryotic translation initiation factor 3 subunit E

EIF3E: Eukaryotic translation initiation factor 3, subunit E gene

eIF3h: Eukaryotic translation initiation factor 3 subunit H

EIF3H: Eukaryotic translation initiation factor 3, subunit H gene

eIF4A: Eukaryotic initiation factor 4A

eIF4A1: Eukaryotic initiation factor 4A-I

eIF4B: Eukaryotic translation initiation factor 4B

eIF4E: Eukaryotic translation initiation factor 4E

eIF4F: Eukaryotic translation initiation complex 4F

eIF4G: Eukaryotic translation initiation factor 4 gamma

eIF5: Eukaryotic translation initiation factor 5

eIF5: eukaryotic translation initiation factor 5;

eIF5A2: Eukaryotic translation initiation factor 5A-2

eIF5B: Eukaryotic translation initiation factor 5B

eIF6: Eukaryotic translation initiation factor 6

EMT: Epithelial-mesenchymal transition

ER: Endoplasmic reticulum

eRF: Eukaryotic peptide chain release factor

ERK: Extracellular signal-regulated kinase

ESI: Electrospray ionization

EU: 5-ethynyl-uridine

Fas: Tumor Necrosis Factor Receptor Superfamily, Member 6

FBM: Fibroblast Basal Medium

FBS: Foetal bovine serum

FDA: Food and Drug Administration

FDR: False Discovery Rate

G1 phase: Gap 1 phase

G1 phase: Gap 2 phase

Gal1: galectin-1

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GBM: glioblastoma multiform

GCN2: eukaryotic translation initiation factor 2 alpha kinase 4;

GI_{50}: Half maximal (50%) growth inhibitory concentration

GnRH: Gonadotropin-releasing hormone

GTP: Guanosine triphosphate

Hbs1: Elongation factor 1 alpha-like protein

HCA: Hierarchical clustering analysis

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HER2: Human epidermal growth factor receptor 2

hFGF-b: Human fibroblast growth factor basic

HPV: Human papillomavirus

HRI: eukaryotic translation initiation factor 2 alpha kinase 1;

HRP: Horseradish peroxidase enzyme

HSP90: Heat-shock protein 90

HSPA5: heat shock 70 kDa protein 5

HspB1: Heat shock protein beta-1

IC_{50}: Half maximum (50%) inhibitory concentration

IDH: Isocitrate dehydrogenase

IEF: Isoelectric focusing

Ink4: Family of cyclin-dependent kinase inhibitors

IP: Intraperitoneal

IRES: Internal Ribosome Entry Site

IS: Internal Standard

ITAF: IRES transacting factor

IV: Intravenous

Kip: Cyclin-dependent kinase inhibitor protein

LC_{50}: Half maximal (50%) lethal concentration

LC-MS: Liquid chromatography–mass spectrometry

M phase: Mitosis phase

M7G: 7-methylguanylate cap

mAB: Monoclonal antibody

MAO: Monoamine-oxidase

MAO-A: Monoamine-oxidase A

MAPK: Mitogen-activated protein kinase

MDR1: Multi-drug resistance protein 1

MEK: Dual specificity mitogen-activated protein kinase kinase

MET: Mesenchymal-epithelial transition

Met: Methionine

miRNA: MicroRNA

MNK: MAP kinase interacting serine/threonine kinase

mRNA: Messenger RNA

MS/MS: Tandem mass spectrometry

MTD: Maximum tolerated dose

mTOR: Serine/threonine-protein kinase mTOR

mTORC1: Serine/threonine-protein kinase mTOR complex 1

mTORC1: Serine/threonine-protein kinase mTOR complex 2

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MYC: MYC proto-oncogene

Myc: Myc proto-oncogene protein

NAMPT: Nicotinamide phosphoribosyltransferase

NCI: National Cancer Institute

NF-κB: Nuclear factor kappa-B

PABP: Polyadenylate-binding protein

PBS: Phosphate buffered saline

PC: Principal component

PCA: Principal component analysis

PDCD4: Programmed cell death protein 4

PDGFR: Platelet-derived growth factor receptor

PDK1: 3-phosphoinositide dependent protein kinase 1

PERK: Eukaryotic translation initiation factor 2 alpha kinase 3

PGAM1: Phosphoglycerate mutase 1

pH: Potential of hydrogen

pI: Isoelectric point

PI: Propidium iodide

PI3K: Phosphatidylinositol 3-kinase

PIC: Preinitiation complex

PKR: eukaryotic translation initiation factor 2 alpha kinase 2;

PLK1: Polo-like kinase 1

PM_{10}: particulate matter of 10 µm

$PM_{2.5}$: particulate matter of 2.5 µm

PMSF: Phenylmethylsulfonyl fluoride

Pol I: RNA polymerase I

PPARγ1: Peroxisome proliferator-activated receptor γ1

pRb: Retinoblastoma-associated protein

PTEN: Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase

PVDF: Polyvinylidene difluoride

RAPTOR: Regulatory-associated protein of mTOR

Ras: Family of small GTP-binding proteins

RBP: RNA-binding protein

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

RNase: Ribonuclease

RPMI: Roswell Park Memorial Institute

rRNA: Ribosomal RNA

RSK: Ribosomal S6 kinase protein family

S phase: Synthesis phase

S.E.M.: Standard error of the mean

S6K: Ribosomal protein S6 kinase beta

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIMCA: Soft independent modelling by class analogy

siRNA: Small interfering RNA

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

TC: Ternary complex

TCA: Trichloroacetic acid

TEMED: Tetramethylethylenediamine

TGF-β: Transforming growth factor beta

TIC: Total Ion Current

TNF-α: Tumor necrosis factor

TP53: tumour protein p53

TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain protein

TRAIL: TNF-related apoptosis-inducing ligand

tRNA: Transfer RNA

tRNAi: Initiator transfer RNA

UTR: Untranslated region

v/v: Volume/volume

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

w/v: Weight/volume

WHO: World Health Organisation

SUMMARY

Cancers represent one of the main causes of death worldwide. Together with surgery and radiotherapy, chemotherapy constitutes a main therapeutic tool in cancer treatment. However, combat remains challenging because of the intrinsic and/ or acquired resistance mechanisms displayed by cancers to these agents. In order to maintain their continuous growth, multiplication and dissemination, cancer cells display a number of biological hallmarks. Growing evidence of the remarkable association of the protein synthesis process with the onset and progression of cancer has led to extensive revision and research on the role of translation in this disease as well as its potential in therapy. Initiation of translation is especially dysregulated in cancer. Thus, strategies targeting different translation steps, ranging from upstream inhibitors - like mTOR inhibitors - to direct inhibition of specific translation initiation factors, represent potential and selective recent alternatives to conventional chemotherapies. In this work, we have investigated the antiproliferative effects of the previously synthetized harmine derivative CM16, with a particular emphasis on its effects on the protein synthesis of cancer cells. We confirmed CM16 cytostatic effects and showed its selectivity towards cancerous cells. The correlation of the growth inhibition profile of CM16 in the NCI 60-cell-line with those of other protein synthesis inhibitors led us to investigate such potential inhibition *in vitro*. CM16 induced inhibition of protein synthesis and it seems to specifically affect the initiation phase of translation, as it affected the organization of ribosome and polysomes. Phosphorylation on the initiation factor 2α (eIF2α) could be partly responsible for the inhibitory effect observed, as evidenced in this work. Also, the transcriptomic comparison of cell models displaying different levels of sensitivity to CM16 suggested that *EIF1AX*, *EIF3E* and *EIF3H* could drive, at least partly, their sensitivity to this compound. Proteomic study of glioma cells treated or not with CM16 was then conducted. Although the proteins of the genes mentioned above were not identified by this technic, we evidenced tiny but significant changes in Hs683 glioma cell proteomic profile through LC-MS shotgun approach. Thanks to 2-DE gel comparison, proteins differentially expressed in these conditions were identified, such as HspB1, Ebp1, BTF3, galectin, cofilin, dUTPase, PGAM1 and CK-18. These might be involved in the antiproliferative and protein synthesis inhibitory activities of CM16, particularly when considering their roles in cancer cell biology, bringing additional insights to the elucidation of the mechanism of action of this harmine derivative in cancer cells.

RÉSUMÉ

Les cancers figurent parmi les principales causes de mortalité dans le monde. Avec la chirurgie et la radiothérapie, la chimiothérapie reste une des principales manières de lutter contre le cancer. Néanmoins, en raison des mécanismes de résistance intrinsèques et / ou acquis à ces agents, le traitement du cancer reste difficile. Pour assurer leur prolifération, leur dissémination et le développement de la maladie, les cellules cancéreuses présentent certaines caractéristiques biologiques. La mise en évidence de liens remarquables entre la synthèse protéique et l'apparition et la progression du cancer a conduit à une révision et à une recherche plus approfondie de la dérégulation de la traduction au sein des cellules cancéreuses ainsi que de son potentiel en tant que cible thérapeutique. La phase d'initiation de la traduction est particulièrement dérégulée dans le cancer. Ainsi, les stratégies ciblant différentes étapes de la traduction, depuis l'inhibition des voies de signalisation en amont du processus - comme les inhibiteurs de mTOR - à l'inhibition directe des facteurs spécifiques d'initiation de la traduction, représentent de potentielles alternatives sélectives aux chimiothérapies actuelles. Dans le cadre de ce travail, nous avons étudié les effets antiprolifératifs du composé CM16, un dérivé de l'harmine préalablement synthétisé, et, en particulier, ses effets sur la synthèse des protéines des cellules cancéreuses. Nous avons confirmé les effets cytostatiques du composé CM16 et avons montré sa sélectivité vis-à-vis des cellules cancéreuses. Le profil de réponse des 60 lignées cellulaires cancéreuses du panel du NCI s'est avéré corréler avec ceux d'autres inhibiteurs connus de la synthèse protéique, ce qui nous a conduits à investiguer *in vitro* cette potentielle inhibition. Le CM16 inhibe la synthèse protéique et semble affecter spécifiquement la phase d'initiation de la traduction étant donné que nous avons observé une désorganisation des ribosomes et polysomes. L'induction de la phosphorylation du facteur d'initiation 2α (eIF2α) pourrait en partie être responsable de l'effet inhibiteur de la synthèse protéique. La comparaison transcriptomique des modèles du NCI présentant des degrés divers de sensibilité au CM16 suggère que *EIF1AX, EIF3E* et *EIF3H* puissent, au moins en partie, être impliquées dans la sensibilité des cellules cancéreuses au composé CM16. Nous avons ensuite réalisé une étude du profil protéomique des cellules de gliomes traitées ou non par le CM16. Bien que les cibles identifiées ci-dessus n'ont pu être identifiées par cette technique, de légères mais significatives différences dans le protéome des cellules de gliomes traitées avec le CM16 ont été mises en évidence par LC-MS shotgun. Grâce à étude comparative de gels en deux dimensions, des protéines

différentiellement exprimées dans ces conditions ont été identifiées, telles que HspB1, Ebp1, BTF3, galectine 1, cofiline, dUTPase, PGAM1 et CK-18. Celles-ci pourraient être impliquées dans les effets antiprolifératifs et inhibiteurs sur la synthèse protéique induits par le CM16, notamment suite à leurs rôles dans la biologie tumorale, contribuant ainsi à l'élucidation du mécanisme d'action de ce dérivé harmine dans les cellules cancéreuses.

INTRODUCTION

1 Cancer

1.1 Epidemiology

Cancer remains one of the leading causes of death worldwide, being responsible for 15% of all deaths worldwide, killing approximately 8 million people while 14 million new cases were diagnosed in 2012 according to the latest World Cancer Report [1,2]. Rates are estimated to keep escalating and expected to reach 20 million new cases per year by 2025. The type of cancer with higher mortality worldwide in both men and women is lung cancer (19%). Among men, lung (17%) and prostate (15%) cancer are the most incident ones, while in women, breast (25%) cancer has the highest incidence compared to other types of cancers [2]. Mortality rates in the different regions of the world are very heterogeneous and would be related not only to lifestyle choices, environmental aspects and other non-modifiable risk factors, but also to factors such as early diagnosis and access to treatment [2]. While overall in lower income countries the major mortality causes are related to infectious diseases, the more developed regions and those in development seem to face high cancer incidence and mortality [3]. Although the high-income countries have higher cancer incidence, it is noteworthy that 60% of the cancer cases occur in Asia, Central and South America and Africa, where mortality rates account for 70% of all cancer cases (**Figure 1**) [2]. This shows a shifting framework from the developed to the developing regions of the world. This fact, among other reasons, might be due to a change in lifestyle in the developing regions of the world and better access to early diagnosis and treatment in the high-income countries [1,2].

Prevention programs are considered essential to fight cancer in a more effective way, however it requires long-term efforts and results are likely to be seen only after many years [2]. Together with preventive measures, efforts to improve care, early diagnosis, access to and availability of treatment as well as the research and development of anticancer therapies offer possibilities to contribute to combat this growing burden in public health worldwide.

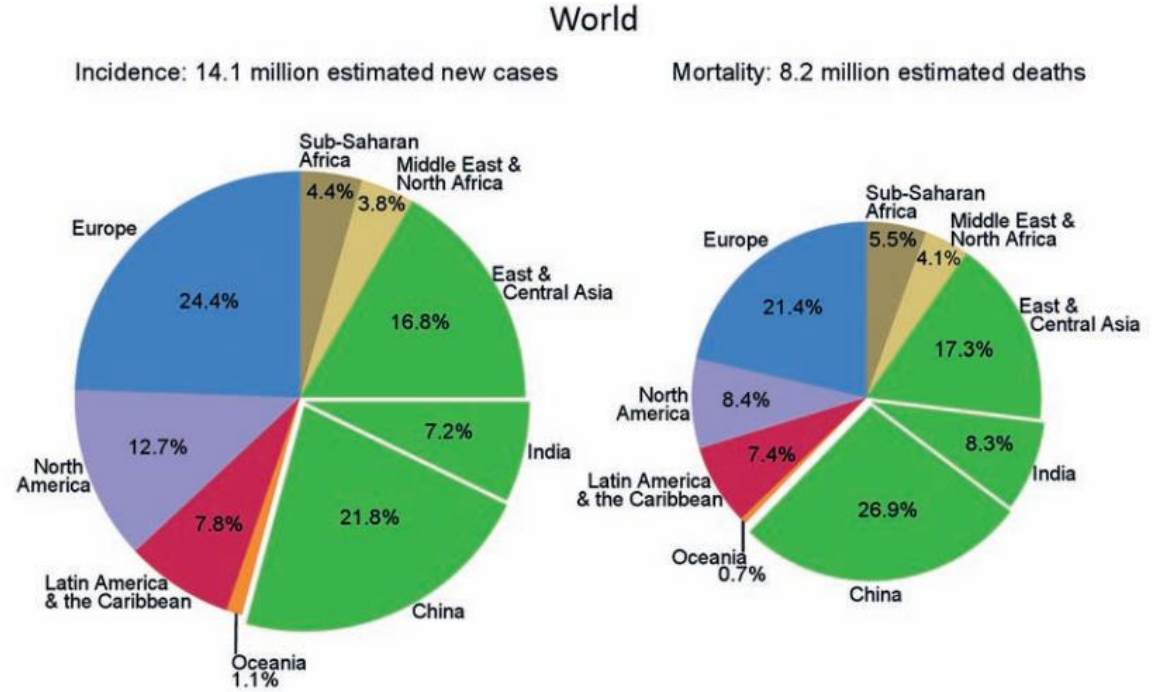


Figure 1. Estimated world cancer incidence and mortality by major world regions in 2012 [2].

1.2 What is cancer? A biological definition

In a general way, cancer can be defined as the unrestrained growth and proliferation of transformed cells, i.e., normal healthy cells that have undergone mutations, and that acquired invasive characteristics, being able to spread to other tissues and organs. The latest feature is called metastasis and it characterizes what we call malignant tumours, being responsible for the majority of cancer deaths [4,5].

There are more than 100 distinct types of cancers originating from most of the tissues and organs of the body [6]. Oncogenesis or carcinogenesis, i.e., the processes through which normal cells are transformed into cancer cells and how this is initiated and formed, is a field of extensive and costly research. Also, the understanding of the roles of the cells present in the tumour microenvironment gained a lot of interest in these past 20 years with the same ultimate goal: propose therapeutic approaches that are able to eradicate cancer cells without significant toxicity for the patient.

1.3 Etiology: risk factors

Distinct risk factors for different types of cancers have been identified to date and they arise from environmental, lifestyle and behaviour exposure. To mention some, tobacco smoking has been extensively associated to lung cancer as well as other cancers, excessive exposure to

sun ultraviolet radiation to skin cancer and 100% of the cervical cancers are attributable to infection by the high risk human papillomavirus (HPV) types [2].

Carcinogens are factors able to potentially cause cancer development. Those include chemicals, physical and biological agents. The real ability of these agents to cause cancer will depend on several factors, such as intensity of exposure, duration as well as genetic background. The majority of cancers is associated with environmental carcinogens which include: tobacco smoking, alcohol consumption, exposure to radiation, pollution, – particularly small particulate matter (PM_{10}, $PM_{2.5}$) – viral and bacterial infections, drugs such as cyclosporine and food contaminant mycotoxins, such as aflatoxins present in peanuts [2]. Tobacco use is the leading cause of cancer morbidity and mortality worldwide, being responsible for approximately 22% of cancer deaths, which includes lung cancer for tobacco smokers and oral cancer for smokeless tobacco users [2,7]. Another important risk factor especially for liver cancer, is alcohol consumption, which caused more than 330,000 deaths in 2010 [2]. In addition to that, there are also occupational carcinogens, such as asbestos, heavy metals, diesel engine emissions and silica [2]. All of the above mentioned are recognized as carcinogens and potential risk factors for different types of cancers that, linked with a lifestyle of junk food consumption leading to obesity, lack of physical activities and stress increases the risk and sets the stage for DNA damage and cancer progression. It is noteworthy that these risks vary a lot depending on geographical and socio-economic settings, age, sex as well as predisposing factors like chronic inflammation and genetic background [2]. The genetic aspects involved in carcinogenesis as well as the metabolic and signalling pathways involved in tumour onset and progression are described in more details in section 2.

1.4 Cancer therapy

Recently, a lot of emphasis has been given to targeted cancer therapy in this era of huge omics data and promising discoveries in the biology of cancer. However, this has not been the case until the late 1960's, when surgery and radiotherapy were the dominating anticancer therapies. Only after data showing that very radical surgeries trying to cure local cancers were not succeeding in avoiding metastases or recurrences, as well as new data suggesting that the addition of chemotherapy could potentially heal patients with advanced cancers, the way was paved for chemotherapy to become part of the standard clinical practice aiming at maximal anticancer effect and minimal toxicity [8].

Since then, a lot has advanced in this field and these strategies have increased patients survival and quality of life. The current treatment of cancer is tackled by a multidisciplinary team that will select different approaches depending on a variety of factors, among which the type of cancer and stage [9]. Guidelines for each specific type of cancer are available for the clinical practice, but the general approaches remain surgery, radiotherapy and chemotherapy, which can be used separately or in combination. Surgery and radiotherapy are used to control locally the disease while chemotherapy and immunotherapy will be needed to prevent or treat spread diseases, i.e. metastases. **Surgery** is still the first choice in the treatment of solid tumours, given that they are a distinct mass that can be excised with the aim to avoid its progression [9]. The problem lies on the development of metastases in other sites of the body encountered after surgery, which then needs further treatment with radio and/or chemotherapy. **Radiotherapy** makes use of ionizing radiation to cause cancer cells death mostly by directly damaging their DNA and also indirectly by interacting with cellular water and producing free radicals that can interact with the DNA. It relies on the fact that rapidly proliferating cells are more sensitive to the radiation and the use of focused beams to target specific sites increases selectivity to tumour cells [10]. Despite being a successful strategy and part of the standard treatment of many cancers, radiotherapy can also cause damage to the normal cells surrounding the tumour [11]. **Chemotherapy** is also a central strategy in combating cancers and most of the drugs rely on targeting characteristics of cells that undergo uncontrolled proliferation, which unfortunately also affects normal rapid proliferating cells, being responsible for the deleterious side effects. **Immunotherapy** has been developed as the new complement to cancer therapy in the past years. Given the roles that the immune system plays in tumorigenesis, immunotherapy holds a great potential by enhancing the anti-tumour immunity, for example through activating tumour-specific immune responses and stimulating effector cells of the immune system [12,13]. Therefore, research is focusing on promising emerging therapies, interfering with specific molecular, metabolic, epigenetic and immunological targets in cancer cells that would be less harmful to normal cells [14] but also on pharmaceutical devices that offer selectivity and modulation of pharmacokinetic properties.

1.5 Overview on brain cancers

This section is dedicated to brain tumours, specifically gliomas, because the present work encompasses the study of the mechanism of action of a new harmine derivative with good predictive permeability of the blood brain barrier.

While the incidence of primary brain tumours accounts 1.4% of all cancers only (17th most common cancer type), it has a high fatality rate of 60% (12th most deadly cancer), as illustrated in **Figure 2-A** and **B** by the fact that colour intensity remains nearly the same for both incidence and mortality [2,15]. Its poor prognosis seems to relate to their resistance to treatment and challenges in the delivery of therapy across the blood brain barrier.

The brain is also a main site for metastases of primary tumours, the main ones being lung, breast and melanoma cancers [16]. They represent the most common malignancies of the Central Nervous System (CNS) and thus represent a major concern [15].

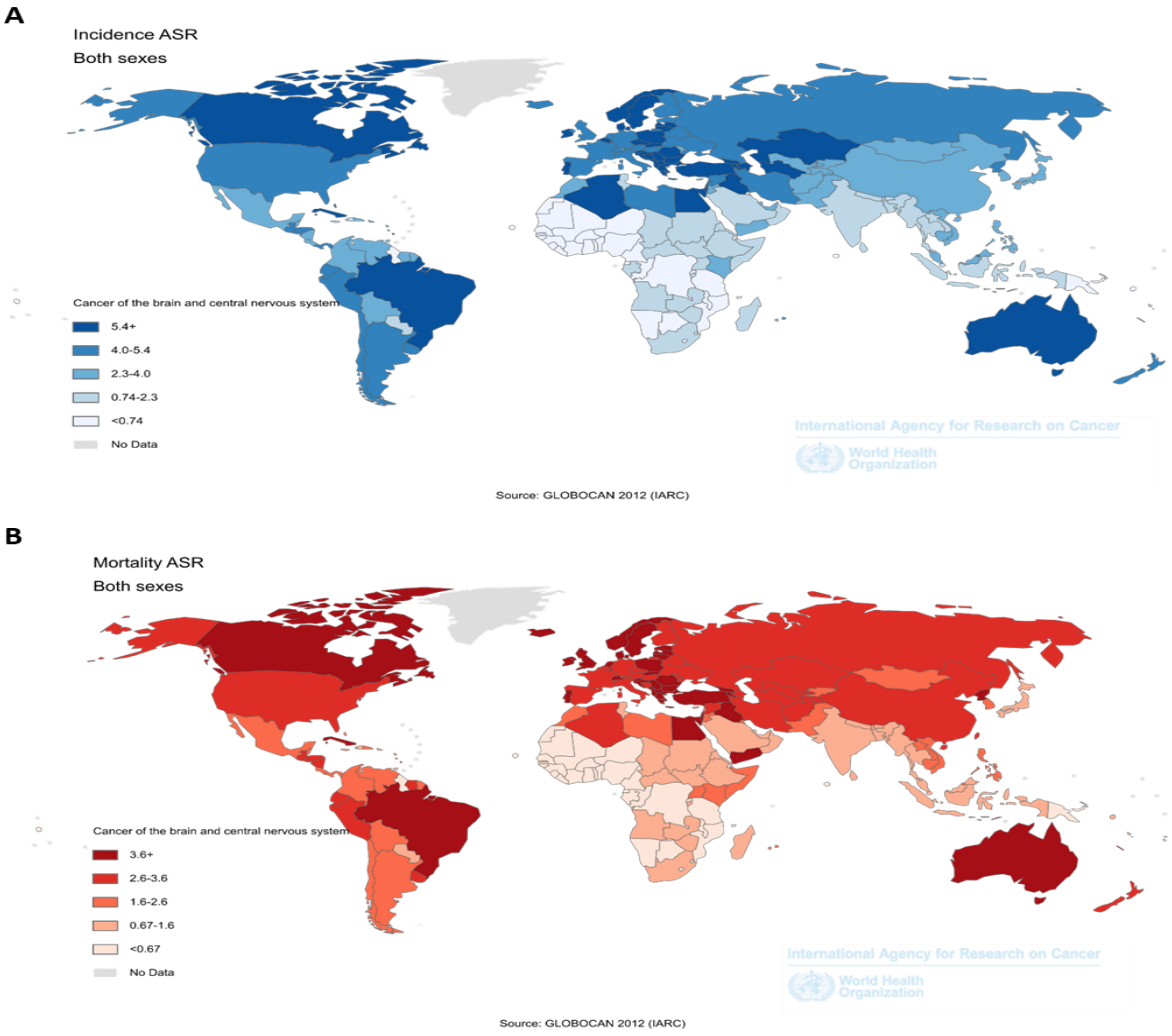


Figure 2. World distribution of estimated age-standardised incidence (A) and mortality (B) rates per 100,000 people for cancer of the brain and central nervous system [17].

Previously, the World Health Organisation (WHO) classification of CNS tumours was majorly based on histological parameters, but very recently molecular aspects were also revised and introduced, drawing a restructured classification [18]. This classification has a detailed histological and molecular separation of the different types of tumours, which encompasses seventeen major classes and several sub-types (Appendix 1). It also includes a categorization of brain tumours in grades I – IV, which are indicative of prognosis and survival. A more detailed attention will be given here to glial tumours and particularly to glioblastoma (grade IV WHO). Other types of gliomas include astrocytomas, oligodendrogliomas, ependymomas and oligoastrocytomas. Glioblastomas are not only the most aggressive, but also the most common (60-70%) type of central nervous system tumour [15]. This later type is characterized by high brain infiltration, necrotic areas and are highly vascularized, all of which being important features for the diagnosis of glioblastomas [19]. With a very poor prognosis, the mean survival of glioblastomas patients is of 15 months only, with a 5-year relative survival of approximately 5% [20,21].

The vast majority (90%) of glioblastomas are primary glioma, meaning that it is originated from normal brain glial cells that become tumorigenic, while the secondary glioblastomas accounts for 10% and arise of lower-grade brain tumours (**Figure 3**). They also differ in prognosis and mean age of the affected people: while primary glioblastomas progress in a time-spam of months and affects more often people in the average age of 62 years, the secondary ones develop in a range of years, affecting people with a mean age of 45 years and having a better prognosis [2,22,23]. At the molecular level, primary glioblastoma development is linked to *PTEN* mutation, *EGFR* amplification and loss of chromosome 10, while secondary glioblastomas present generally chromosomes 1p19q deletion, as well as mutation in *TP53* and *IDH1* [22] and these features are exemplified in **Figure 3**.

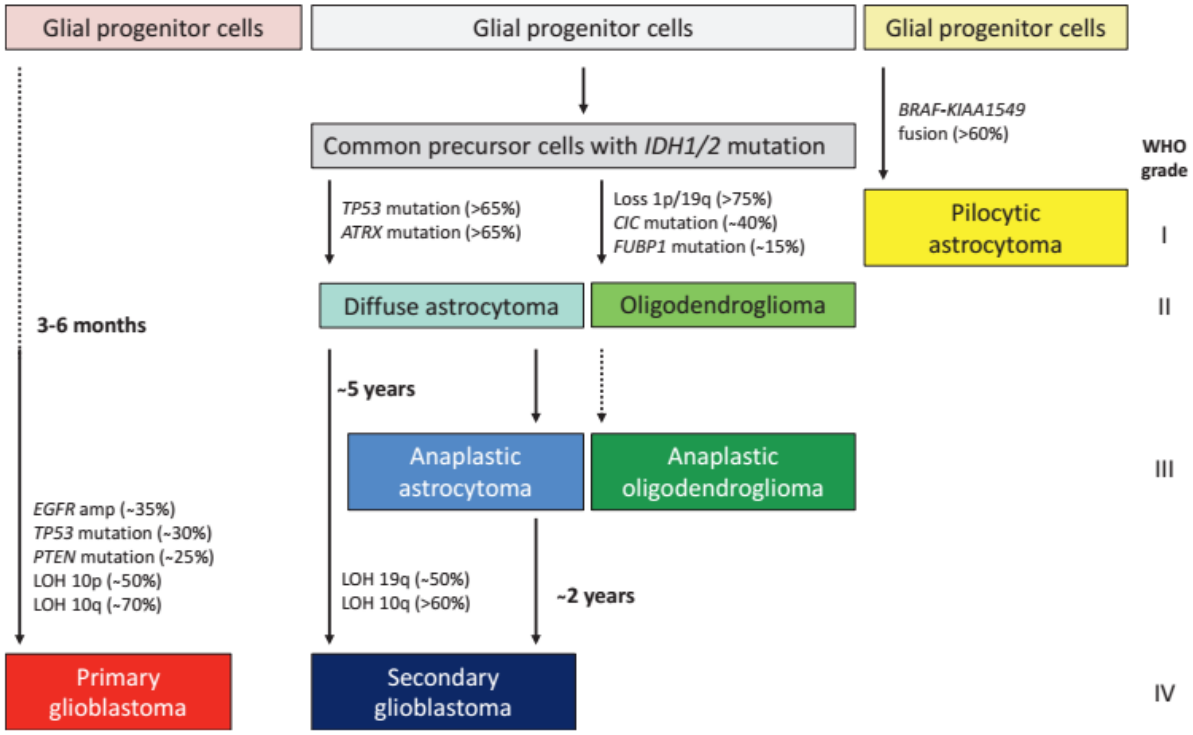


Figure 3. Genetic pathways leading to primary and secondary gliomas, development time and WHO grading [2]. TP53: tumor protein p53; IDH: Isocitrate dehydrogenase; LOH: Loss of heterozygosity; ATRX: ATRX, chromatin remodeler; CIC: capicua transcriptional repressor; FUBP 1: far upstream element binding protein 1; BRAF: B-Raf proto-oncogene, serine/threonine kinase.

The standard treatment for glioblastomas encompasses surgery followed by radiotherapy and administration of temozolomide, an alkylating agent, as the chemotherapeutic agent of choice. This protocol was established following the trial that showed better survival with this regimen [24]. Despite those treatments, the recurrence attributed to chemotherapy and radiation resistance as well as marked migration of the tumour cells into the distant brain parenchyma is the rule and accounts for the poor prognosis. For this reason, efforts from different fields are being sought in an attempt of improving therapy. Approaches using immunotherapy, molecular targeted therapy and gene therapy, among others, have been under investigation [25]. In addition to that, the blood brain barrier (BBB) limits the access of several therapeutic agents, thus finding agents that are able to cross this barrier without being toxic to the brain is an additional challenge to be circumvented [26].

2 The unique features of cancer

Cancer cells display several characteristics that enable their continuous growth, multiplication and dissemination. This neoplastic transformation is a complex process in which features need to be acquired by the cells in order to become malignant and, importantly, not only the tumour cells are involved in this scenario but also the recruited normal cells in the tumour microenvironment contribute to the acquisition and development of such cancerous characteristics, emphasizing the heterogeneity of a tumour [27]. Below are summarized these unique features that enable cancer to arise, develop, progress and disseminate.

2.1 Cancer genome

In recent years, world-wide efforts like the International Cancer Genome Consortium [28], have been made in order to track, characterize and understand the involvement of a plethora of genetic alterations that ultimately cause the phenotypical changes observed in cancers [6,29].

Cancer cells, like the normal cells of our bodies, are, in a very first instance, clonal descendants of the fertilized egg from which we all come from, the one carrying a diploid genome. Throughout one's life, the DNA sequence of the cell genome undergoes alterations, which makes it distinct from its progenitor egg. These changes are called somatic mutations as opposed to the parents-offspring inherited mutations, called germline mutations [6,29]. In a cancer cell genome, several types of DNA sequence alterations, like insertion or deletion of DNA segments, as well as acquisition of new DNA sequences (from viruses, for example) are part of what we call somatic mutations. These mutations in cancer genome can be acquired in different ways and are accumulated throughout a person's life. Mutagens constantly damage the DNA of normal cells, which is mostly repaired. Some of these mutations, however, are not repaired and are further replicated into the daughter cells. Other somatic mutations are linked to lifestyle and environmental mutagenic exposures, an example being the link between tobacco smoke carcinogens and lung cancer. Although these somatic mutations will be responsible for the arousal of cancer, not all of them participate in the development of cancer. The mutations known as **'driver' mutations** are the ones directly implicated in oncogenesis as they offer growth advantages to the cancer cells and have been selected at some point in the microenvironment where the cancer originated from, whereas the **'passenger' mutations** do not contribute to cancer development, they have been carried

down in the clonal expansion of the cancer genome cells but do not affect it functionally. One important type of 'driver' mutation is the one seen in recurrent cancers, the resistance mutations that, as the name indicates, confers resistance to therapy [6]. **Figure 4** schematically represents the concepts here introduced.

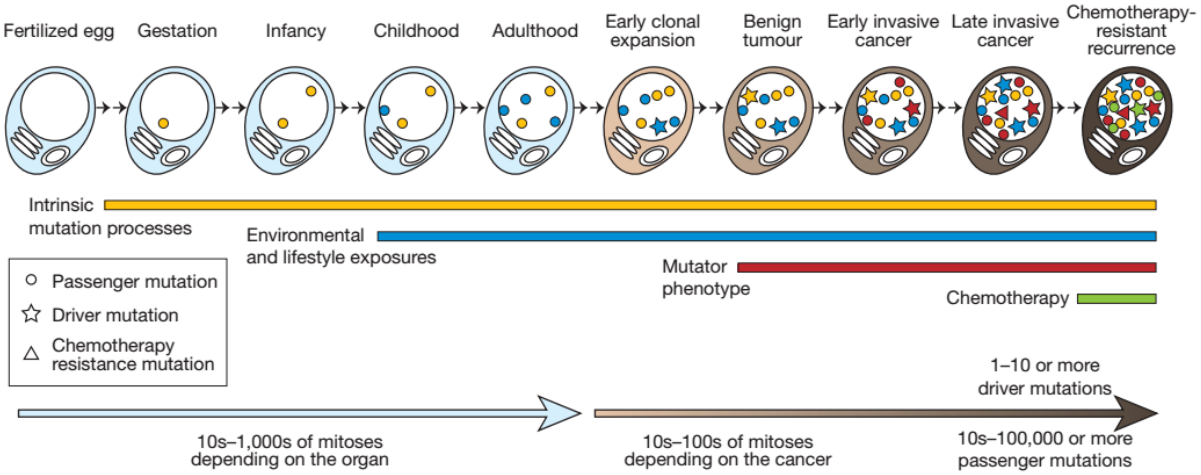


Figure 4. Somatic mutations acquired by a cancer cell throughout life and the processes contributing to it. Adapted from [6].

Mutations in the cancer cell genome can provoke effects in opposite directions: some will inactivate tumour suppressor genes that protect cells from cancerous proliferation, while other mutations will end up promoting cell growth and proliferation through the activation of growth-promoting oncogenes. In either direction, the final effect will be a mediation of changes in the downstream cellular pathways that might result in cancer. In addition to mutations, there are epigenetic alterations that alter chromatin structure and gene expression, as well as defects that affect the DNA maintenance machinery. Together they set the frame for **genome instability** in cancer cells, enabling them to acquire neoplastic features. This and the other unique cancer features, the so-called *cancer hallmarks*, are depicted in the sessions below and illustrated in **Figure 5**.

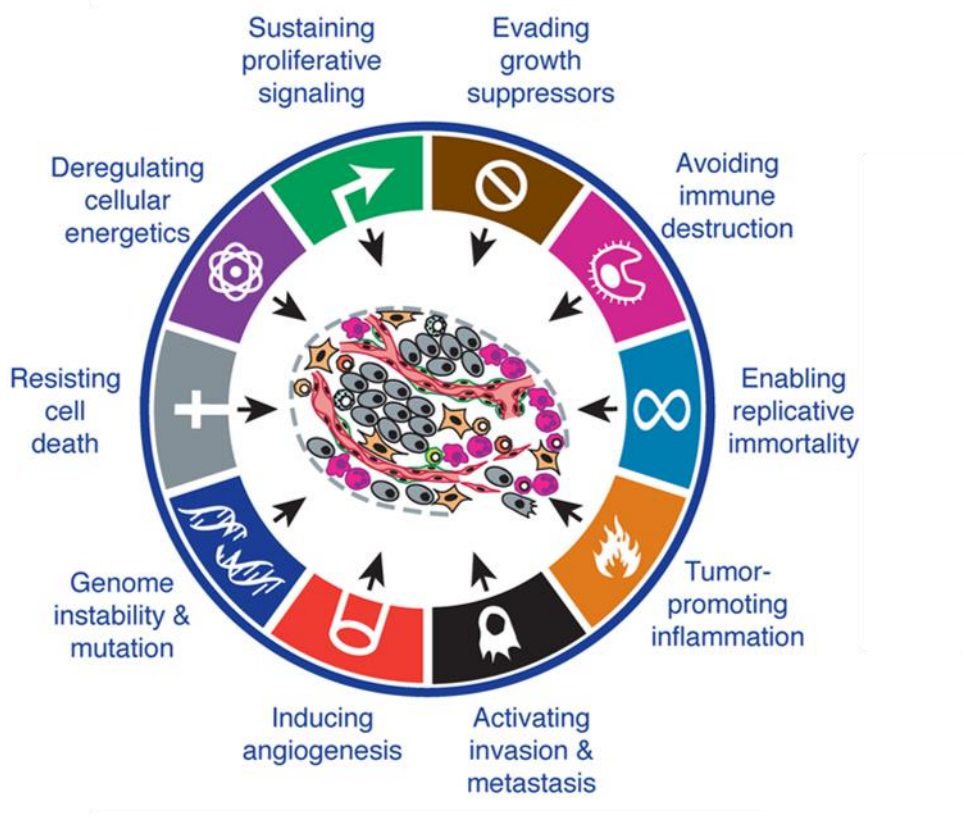


Figure 5. The hallmarks of cancer. Adapted from [27].

2.2 Cancer hallmarks

The equilibrium between cell proliferation and cell death keeps the homeostasis in a healthy system. Its disruption either by a loss of control of the cell cycle or by affecting cell death mechanisms might lead to tumour development. During the cell cycle (**Figure 6**), the DNA is replicated in the synthesis (S) phase and chromosomes are equally passed onto two daughter cells in the mitosis (M) phase. The other phases of the cycle are called gap (G). In the first gap period (G_1) cells grow and the DNA is prepared to enter the S phase while during the second gap (G_2) cells are preparing for mitosis. The cell cycle is tightly regulated and regular checks are made in between every phase transition so that the cell may progress into the next phase or stop in case damage is detected. These checkpoints are sensors of DNA damage and loss of checkpoints may contribute to genomic instability and uncontrolled cell proliferation. Cyclin-dependent kinases (Cdk) and its activators, the cyclins, are the main regulatory complexes present in the different phases of the cell cycle being responsive to mitogenic signalling [30]. Inhibitors of Cdk mediate cell cycle arrest under antiproliferative signals and the deregulation of this system is also implicated in tumorigenesis. Other important regulators of the cell cycle are the tumour suppressors retinoblastoma-associated protein (pRb) and cellular tumour

antigen p53 protein [30], whose activities are clarified in the paragraph below. The ability of the cancer cells to constantly stimulate and remain in the cell cycle is clearly a hallmark of cancer cells achieved notably through **sustained proliferative signalling.** This feature is due to various deregulations in the growth promoting signals making cancer cells autonomous for triggering proliferative signalling, enter and progress into the cell cycle [27]. These alterations may concern the growth factors themselves, their receptors or their downstream signalling. Cancer cells can, for example, independently produce growth factors and secrete them. An additional mechanism of deregulation is through stimulation of the tumour-associated stroma cells which will in turn supply them with growth factors [31]. The growth factor receptors can be overexpressed, as observed for the epidermal growth factor receptor (EGFR) in head and neck cancers [32] or present structural alterations associated with constitutive activity. Similar alterations can also be found at the intracellular signalling cascade level, for example with mutations in oncogenes like *RAS* [27,32].

The disequilibrium of cancer cells in favour of cell growth also relates to their capability to **evade growth suppressors**. The so-called tumour suppressor genes are the ones mainly responsible for encoding proteins such as the retinoblastoma-associated (pRb) and the p53 proteins, which are very important in regulating cell proliferation and death and therefore, defects in these supressing pathways might result in uncontrolled cell proliferation [27]. Both exert complementary roles in the regulation of cell proliferation in response to certain stimuli: whereas pRb integrates signals mainly from extracellular sources and is a negative regulator of the cell cycle progression [33], p53 will sense mainly intracellular stress or abnormalities and cause cells to suicide by apoptosis or to stop further progression into the cell cycle, until repair is made [30].

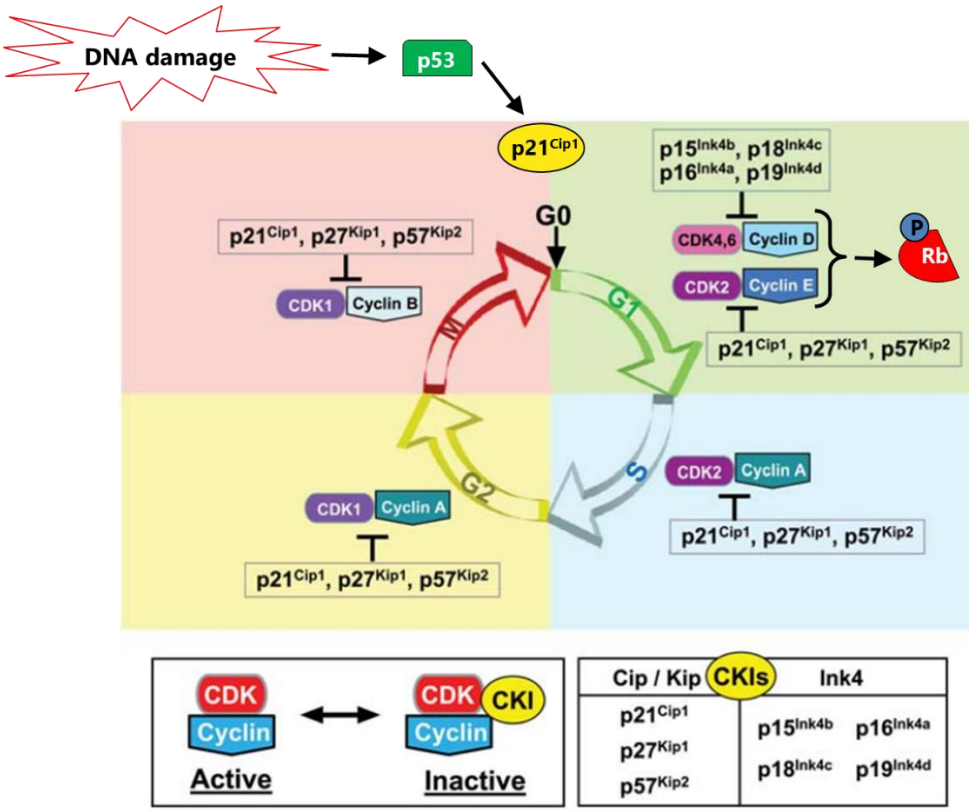


Figure 6. Cell cycle phases and regulation. Activated CDK/cyclin complexes allow progression of the cell cycle, while CKIs block it. Cip/Kip and Ink4 are CKIs families. p53 senses DNA damage and induces cell growth arrest by activating CKI p21. In tumorigenesis, pRb (G1-S regulator) is inactivated through phosphorylation by cell cycle kinase complexes CDK4,6-cyclin D and CDK-2-cyclin E inducing gene transcription of regulators of cell growth and those encoding for DNA synthesis, resulting in cell cycle progression. Adapted from [34].

Resisting cell death is also an ability that cancer cells have acquired in order to progress into tumours. Cell death occurs under physiological stress or DNA damage in order to keep the homeostasis. Cell death should thus be triggered in the case of tumorigenesis [27].

There are different types of deaths triggered by the cells as a means to keep the balance between death and survival. The most known and studied one is **apoptosis**, a type of programmed cell death, important in embryonic development, immune-system function and in keeping the homeostasis. If, for example, mutations in a cell are not repaired it should undergo apoptosis [35]. In the context of tumorigenesis, apoptosis can be triggered in response to diverse physiologic stresses such as increased oncoprotein signalling (e.g., via Myc protein) and DNA damage, (e.g., via *TP53*) [27]. The apoptotic process is highly regulated and encompass a diversity of signalling proteins, which can initiate the death cascade through an extrinsic – death receptor mediated – or through an intrinsic program, mitochondrial pathways, both leading to completion by effector protease caspases, culminating in cell disassembly into apoptotic bodies that will undergo phagocytosis without inflammatory

damage [36]. Anoikis, a type of apoptosis induced by detachment from the extracellular matrix or by inappropriate cell adhesion, also plays an important role as it helps avoiding formation of metastases in distant organs [37].

Autophagy is a homeostasis mechanism triggered in stress conditions for recycling cellular components including damaged proteins and other cell organelles [36,38]. Autophagy is triggered especially in the lack of nutrients [36]. In this process, the components are enveloped by autophagosomes that further fuse with lysosomes, where degradation occurs. These organelles and proteins will break down and go through a recycling process, becoming thus available for biosynthesis and energy metabolism. In providing these resources, autophagy can mediate the survival of cancer cells. However, on the contrary, autophagy may also act by eliminating damaged organelles, toxics and oncogenic proteins, playing a tumour suppressor role [38]. Even, sustained autophagy has been shown to lead to cell death explaining that it can be considered as the type II programmed cell death [39].

A third main type of cell death is **necrosis**, a process triggered in response to important cell damage or stresses, being also a type of death that can occur on a regulated manner (for example, necroptosis). It is characterized by cellular swelling and rupture of plasma membrane, features not observed in apoptosis and autophagy [40,41]. Although necrosis might be seen as protective against cancer development, the release of cellular components during necrosis triggers pro-inflammatory signals. These recruited inflammatory cells have been suggested to participate in angiogenesis, cell proliferation and invasiveness [27]. The role of the immune system in tumorigenesis is further explained in section 2.3.

Another cancer hallmark required for sustained proliferation is **enabling replicative immortality**. Normal cells which have undergone a certain number of growth and division cycles enter in replicative senescence or crisis due to their telomeric shortening over the cell division. Senescence is the process in which cells enter a state of division arrest, but keeping the plasma membrane integrity [27]. Cancer cells, in order to circumvent this physiological barrier to immortality, maintain their telomeres length via the upregulated expression of telomerase, an enzyme that adds telomere repeat segments to the ends of telomeric DNA and that is nearly absent in nonimmortalized cells [27].

A natural adaptation of the cancer cells that walk hand-in-hand with the uncontrolled growth and proliferation is the metabolic adjustment necessary to it and thus **deregulating cellular**

energetics is another cancer hallmark [27]. As a much known matter of fact, cancer cells can switch into a glycolytic state, reprogramming the way they produce energy in order to strive through situations of hypoxia present in many tumour environments. Not only that, but somehow surprisingly, cancer cells also turn to glycolysis and lactate production even in the presence of oxygen, an observation first made by Otto Warburg and thus known as the 'Warburg effect' [42–44]. The problem with the glycolysis metabolism chosen by the cancer cells lies in its poorer efficiency if compared to the amount of ATP produced by mitochondrial oxidative phosphorylation, but cancer cells manage to circumvent this inefficiency partially by upregulating nutrient transporters through the activation of the PI3K/Akt/mTOR pathway. Activated oncogenes such as *RAS* and *MYC* also play roles in the deviation to glycolysis observed in cancer cells. The glycolytic switch of cancer cells has broader consequences as it affects other biosynthetic pathways, involved in the production of nucleosides and amino acids which are required for the formation of new cells, warranting them an advantage in the constant proliferation process [44].

2.3 Tumour microenvironment

As mentioned earlier, tumour is also constituted of normal cells (**Figure 7**) that were reprogrammed in order to help tumour proliferation and dissemination.

To meet their needs for nutrients and oxygen, tumours develop a supporting vasculature thanks to the "**angiogenic switch**". Endothelial cells of the cancer stroma are activated by growth factors such as VEGFs from a quiescent form into active blood vessel builders. In order to stimulate angiogenesis, which physiologically is only activated transiently in the adult (e.g. in the placenta during pregnancy and in wound healing process) [45], cancer cells may induce oncogene signalling like Ras, which will upregulate angiogenic factors [46]. Bone marrow derived myeloid cells, like macrophages (tumour associated macrophages - TAM), can also contribute to tumour angiogenesis through the production of pro-angiogenic growth factors, like VEGF. [47].

Immune cells are present in basically every tumour. The immune system plays an important role as endogenous defence against neoplasia in its different stages. The fact that tumours develop and progress implies that cancer cells have to **evade such immune control** [27,48]. For that purpose, cancer cells may, for example, secrete TGF-β and paralyze the infiltrating cytotoxic T lymphocytes and the natural killer cells that display cytotoxic activities towards

them [48,49]. In-depth investigations on the roles that the immune system plays in cancer have been shedding light on how immune targeted therapy could combat cancer [13]. Paradoxically, the recruitment of immune cells in the tumour microenvironment, particularly innate immune cells like macrophages, has major implications in several tumorigenic processes. These inflammatory cells release signalling molecules that will participate in angiogenesis, cell survival, proliferation and dissemination, endowing them with a **tumour-promoting inflammatory characteristic**. [27,47,48].

Cancer stem cells (CSCs) are also present in the tumour microenvironment and contribute to tumour heterogeneity, aggressiveness and disease recurrence [27,50]. Indeed CSC are so called because of the similar features they share with stem cells: the self-renewal potential and the capacities to differentiate and migrate. This sub-population in a tumour has implications for therapy as they seem to contribute, at least partially, to the resistance to chemotherapy as well as recurrence of some cancers. Among the mechanisms through which CSCs might cause chemoresistance are the expression of ABC transporters, such as MDR1 and ABCG2, and the aberrant activity of pro-survival BCL-2 family members in CSCs may be a mechanism of resistance to chemotherapy-mediated apoptosis [51].

The origin of these cells and its implications in cancer biology have yet to be better established, but research to combat specifically those CSCs has gained increased interest this last decade [52].

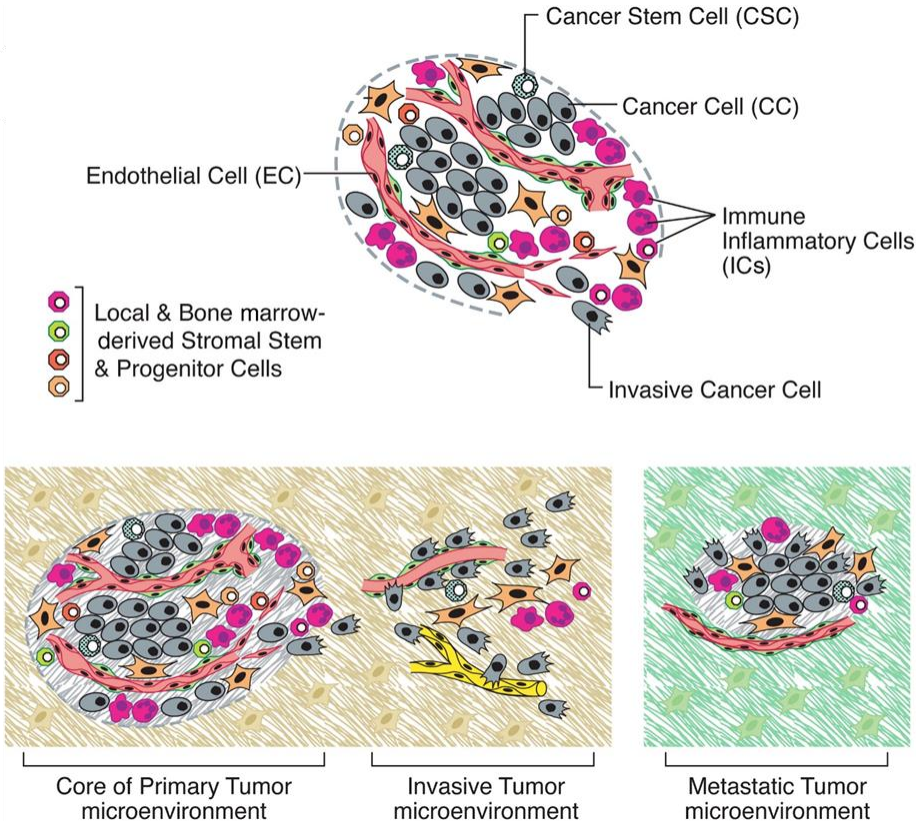


Figure 7. Cells of the tumour microenvironment. Adapted from [27].

2.4 Metastases

Malignancy of cancer cells is characterized by their ability to disseminate from their primary location to other sites and to colonize them, a process known as **metastases**, which is responsible for the largest mortality caused by cancer [53]. To actively invade and spread, cancer cells change shape and pattern of attachment to other cells and to the extracellular matrix (ECM). Loss of expression and downregulation of the important cell-to-cell adhesion molecule and tumour suppressor E-cadherin in carcinomas was reported as associated with the ability of those cells to invade and metastasize [27,54]. Tumour cells can undergo the so called *epithelial-mesenchymal transition* (EMT) [55]. A set of transcriptional regulators like Snail/Slug are involved in the EMT, regulating the process: alterations in morphology, followed by loss of cell-to-cell receptors (E-Cadherin mentioned above) as well as integrins and secretion of metalloproteinases, the last ones being able to cleave cell-surface proteins and degrade components of the ECM (**Figure 8**) [27,54]. It is noteworthy that this is not a process that would happen in all the cancer cells. In a secondary site, these cells might undergo the opposite transition: the *mesenchymal-epithelial transition* (MET) in order to form a secondary tumour in the new site [55]. Although better described and understood in the recent years,

EMT is not the only way for cancer cells to invade other tissues and they can, instead, penetrate as a group of cells moving together, a process called *collective invasion* [56].

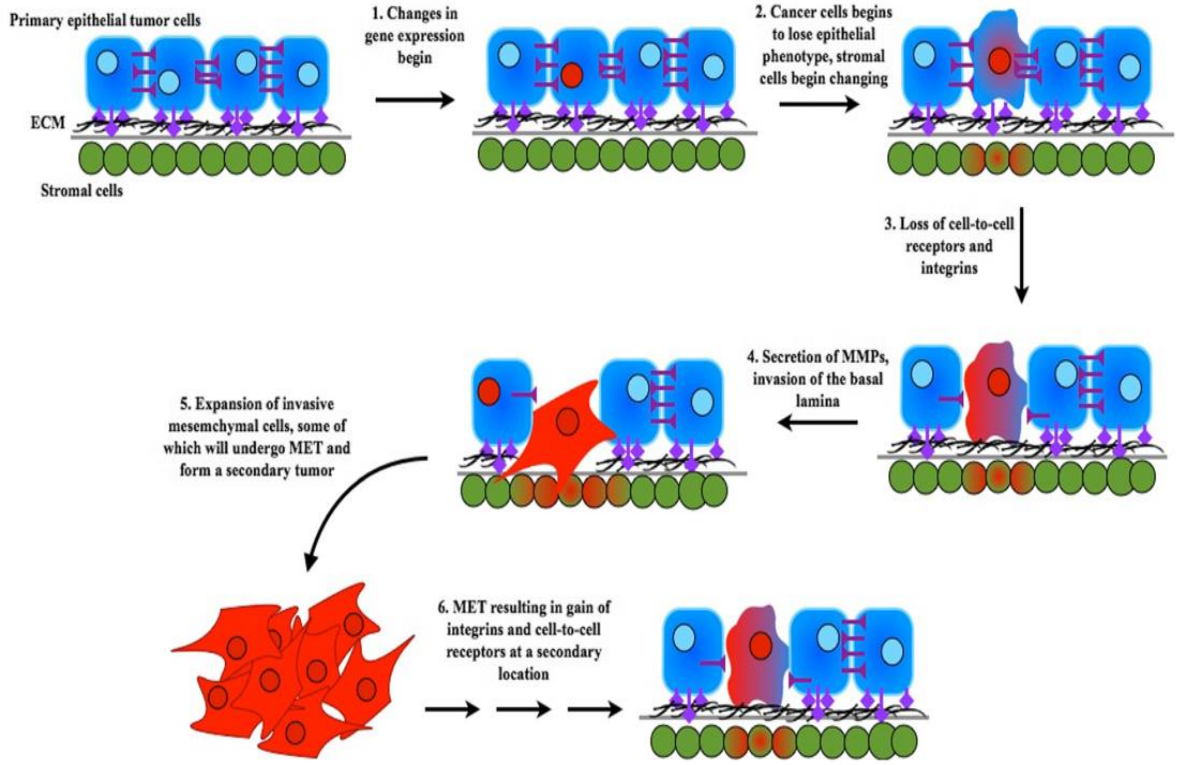


Figure 8. Cancer cells undergoing epithelial-mesenchymal-transition (EMT) as a proposition for metastases. The gradual EMT process in which cancer cells undergo alterations in morphology, followed by loss of cell-to-cell receptors and integrins and secretion of metalloproteinases, the last ones being able to cleave cell-surface proteins and degrade components of the ECM. The MET process might follow for cells to attach to new location. MMPs: Matrix metalloproteinases. Adapted from [55].

3 An introduction to protein synthesis and cancer

3.1 General considerations

The continuous proliferative signalling as well as the genome instability observed in the tumour and its environment provoke substantial effects on the protein synthesis of these cells, which has proven to be subverted in order to warrant the uncontrolled proliferation characteristic of cancer cells. As we evaluated in this work the effects of a potential anticancer drug on the protein synthesis of cancer cells that represents an emerging and promising strategy in anticancer therapy [57], we detail below the protein synthesis process in eukaryotes, its roles in cancer and the strategies to inhibit it as part of an anticancer therapy strategy.

Protein synthesis plays a pivotal role in the regulation of gene expression, especially affecting the homeostasis, controlling cell proliferation, growth and development as it will define the proteome of a cell. In addition, protein synthesis also plays a very important role in the cellular metabolism, since it consumes considerable energy of a cell, especially proliferating cells [58]. Protein synthesis rate is generally proportional to the translational efficiency of the mRNA, which is highly regulated and thus plays an important role in the modulation of gene expression [58]. The regulation of the mechanisms involved in the synthesis of proteins is thus very essential and deregulation of this process in cancer contributes to the disease.

3.2 Transcription

Transcription is the process by which the RNA molecules are produced from a DNA template. The coding RNA carrying the information that will be translated into a protein is called mRNA (messenger RNA) but other non-coding RNAs with different cellular functions are also produced such as: the rRNA (ribosomal RNA), tRNA (transfer RNA), miRNA (microRNA) and siRNA (small interfering RNA). Transcription occurs with the assistance of RNA polymerases. In the case of mRNA production, this process will be carried out by the RNA polymerase II, which, with the help of transcription factors, will bind to a start site and unwind the DNA double helix. The enzyme then moves along the DNA, unwinding new segments and adding nucleotides to the RNA strand according to its complementary nucleotide sequence, growing in the 5'-to-3' direction. In eukaryotes, this transcript will be further processed by **i)** the addition of a cap to the 5'end and **ii)** a poly (A) tail to the 3' end of the mRNA and **iii)** by splicing to remove non-coding introns. The newly formed and mature mRNA transcript will be transported from the nucleus to the cytoplasm in order to meet the ribosomes and the translational machinery present mostly in the endoplasmic reticulum [59].

3.3 Translation

In general terms protein synthesis is a process which binds together amino acids based on the sequence of a mRNA template, doing so essentially through the ribosomes [58]. The main actors that compose or are involved in the translational process in eukaryotes are the mRNAs, the ribosomes subunits 40S and 60S, the tRNAs (transfer RNA) and the so-called factors, including initiation factors (eIF) and elongation factors (eEF), which assist and regulate translation.

In eukaryotic cells, protein synthesis occurs in four stages: initiation, elongation, termination and ribosome recycling (**Figure 9**). Among them, initiation is believed to be pivotal in the regulation of translation [60]. In the initiation phase all the machinery for translation will come together through a set of reactions needed to place the mRNA in the recruited 40S ribosome containing the start codon AUG and to further bind the large ribosome subunit 60S, forming the final 80S complex and enabling protein synthesis to progress to the elongation phase [60].

At the other end of the protein life-cycle, is - among other processes including autophagy - the ubiquitin proteasome pathway contributing to the protein degradation. The degradation of proteins is important to keep the normal cell metabolism. Proteins tagged with ubiquitin will be recognized by the 26S proteasome, a large multi-enzyme complex that mediates protein degradation into peptides and amino acids [61]. This process should not be confounded with the recycling process that the ribosomes and the translation machinery undergo after delivering a protein[62]. Although the proteasome pathway is not involved in the translation process it plays roles in cancer and as such, proteasome inhibitors, like bortezomib and carfilzomib, have been developed and are in use as anticancer therapy for multiple myeloma [63].

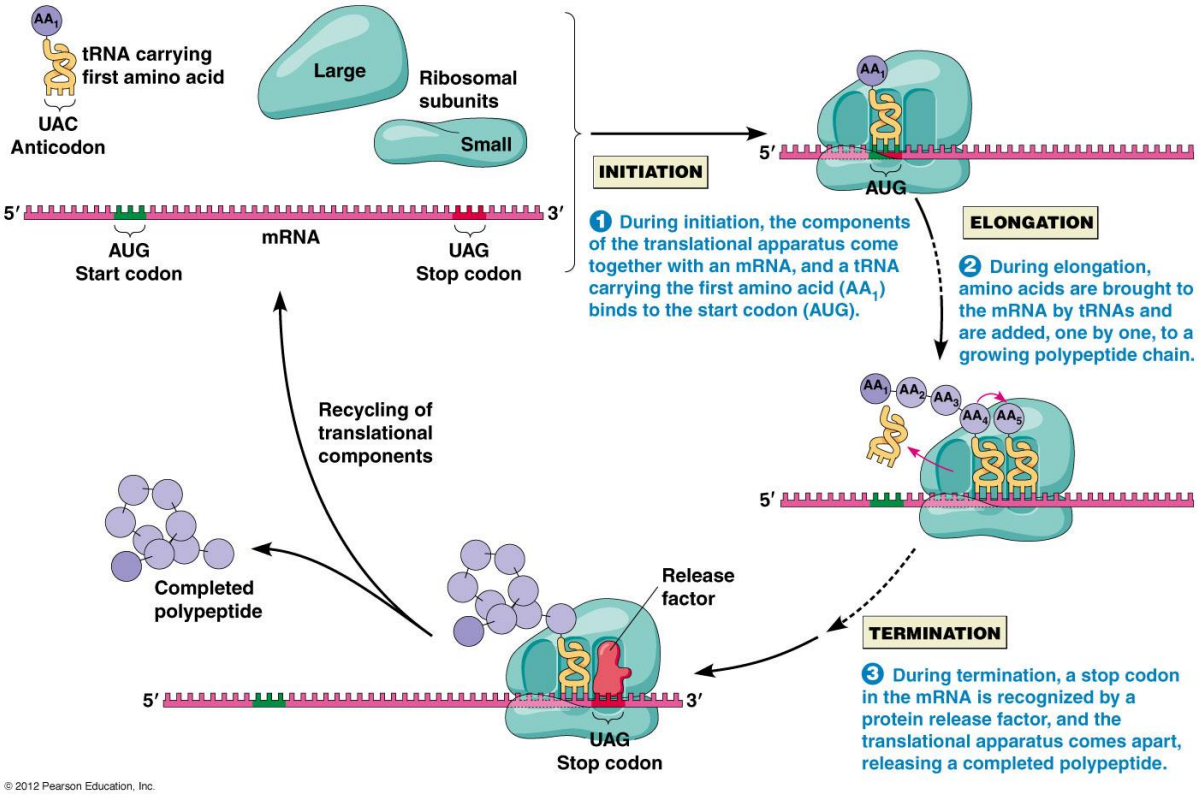


Figure 9. The different steps of protein synthesis. Adapted from [64].

3.3.1 Translation initiation

There are two ways through which mRNAs can recruit the 40S ribosome subunit and initiate translation: the so called *cap-dependent initiation* that requires a scanning process, which is the most predominant process in the cell. The other way in which the mRNA bypasses this scanning process and directly recruits the ribosome to start translation is called *cap-independent initiation* [60,65].

3.3.1.1 ***CAP-dependent translation***

In order to place the peptidyl (P) decoding site of the ribosome with the AUG start codon of the mRNA, this last one needs to be identified through a scanning mechanism, which is depicted in details in **Figure 10**. Initially, the ribosome subunits need to be separated for translation to start. Therefore, the 80S complex is dissociated by initiation factors (eIFs) 1, 1A and 3. These last ones, together with eIF5, will then assist in the assembly of the preinitiation complex (PIC) 43S. This complex is composed of the 40S ribosome, the aforementioned eIFs and the *ternary complex* (TC), which consists of the initiator methionine-tRNA (Met-tRNAi) and eIF2-GTP. Meanwhile, the mRNA undergoes a circularization process by the poly(A)-binding protein (PABP) that binds to the 3' poly(A) tail of the mRNA and the eIF4F (eIF4G-eIF4E-eIF4A) at the 5' cap simultaneously, activating thereby the mRNA. The 43S complex is then recruited to the 7-methyl-guanosine cap at the 5' end of the mRNA transcript, a reaction facilitated by the interaction of eIF3/eIF5 with eIF4G/eIF4B. Once this system (48S complex) is in place, the scanning will start and inspection of the triplets goes on until it finds the AUG codon complementary sequence to the anticodon Met-tRNAi. Scanning is arrested thanks to the anchorage of the Met-tRNAi to the PIC mediated by eIF2-GTP. Then, the GTP bound to eIF2 undergoes hydrolysis, being released (eIF2-GDP) together with other eIFs, a reaction catalysed by eIF5B-GTP that leads to the final step of initiation, i.e. the joining of the 60S subunit to form the active 80S complex [60].

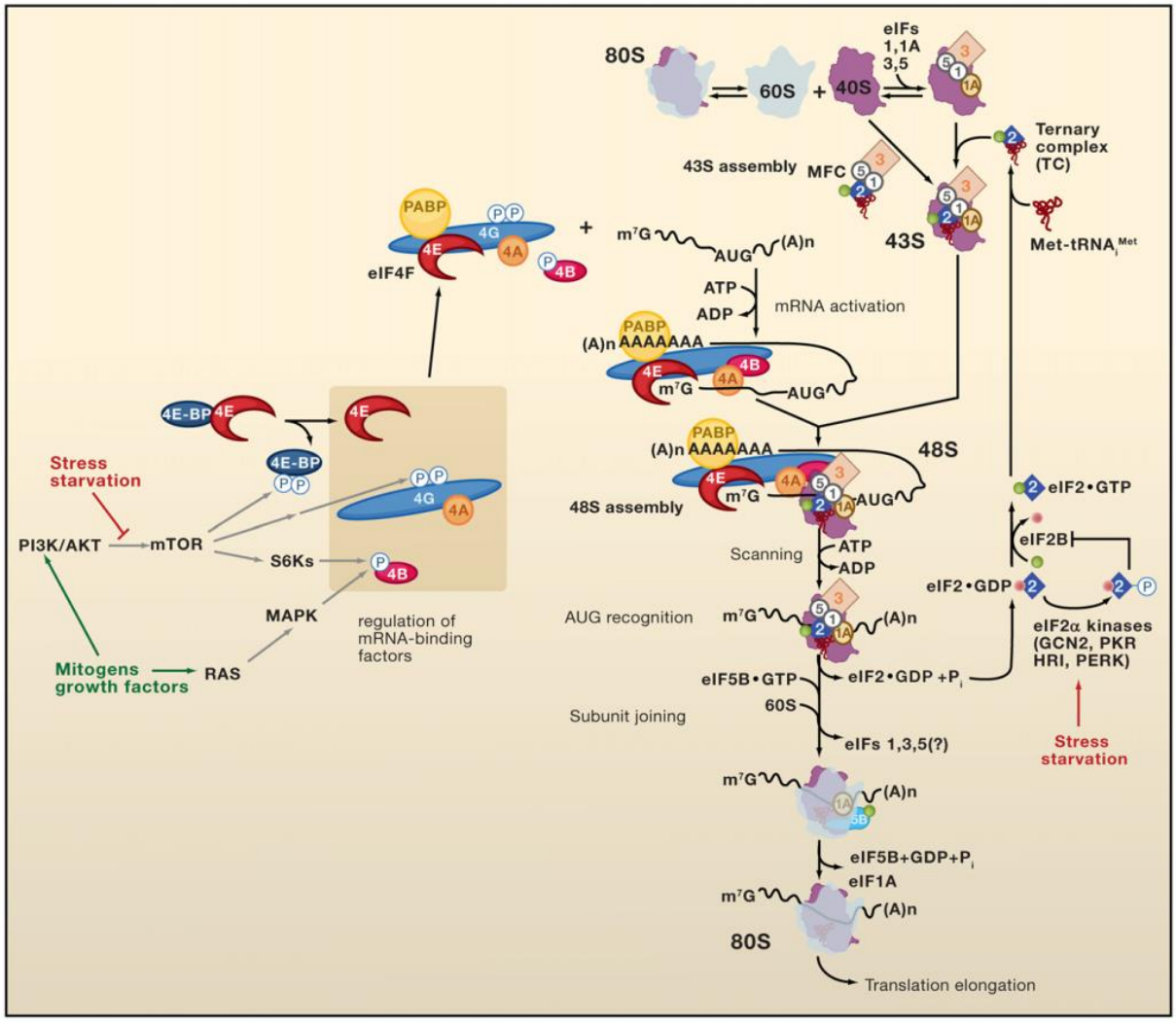


Figure 10. Cap-dependent translation initiation steps and regulation. 80S ribosome are dissociated by eIFs 1, 1A and 3. Together with eIF5 and the ternary complex (eIF2-GTP-Met-tRNAi), these factors will bring together the preinitiation complex (PIC) 43S (40S ribosome-ternary complex-eIFs). mRNA is activated and circularized by binding of eIF4F (eIF4E-eIF4G-eIF4A) to the cap and PABP to the poly(A) tail. The 43S PIC binds near the mRNA cap (facilitated by eIF3/eIF5 interactions with eIF4G/eIF4B), forming the 48S complex. 43S PIC then scans for the AUG codon in an ATP-dependent reaction (partial hydrolysis of the eIF2-bound GTP in the ternary complex to eIF2-GDP-Pi). The 60S subunit is joined, releasing other factors, which is catalysed by eIF5B-GTP, and forming the 80S complex. Different initiation factors (eIFs) are implicated in all these steps, as detailed in the figure. The ternary complex formation is reduced by eIF2α phosphorylation, caused by eIF2α kinases under stress or starvation conditions. The activation of mTOR via PI3K/Akt or RAS/MAPK signalling affects translation. Assembly of eIF4F is blocked by 4E-BP binding to eIF4E and mTOR phosphorylation dissociates 4E-BP from eIF4E. mTOR can also promote eIF4G and eIF4B phosphorylation directly or via S6Ks. Adapted from [60].

Two main nodes involved in the regulation of translation initiation include thus the eIF2 and eIF4F complex proteins. Certain conditions of stress and starvation will lead to phosphorylation of eIF2α by the kinases GCN2, PKR, PERK or HRI, which results in decreased formation of the TC and thus decreased translation [60]. Mitogens and growth factors might also activate mTOR, via PI3K/Akt or Ras/MAPK signalling. Phosphorylation of mTOR will cause dissociation of 4E-BP from eIF4E that is made free for the assembly of eIF4F complex and

consequently promotes translation. In addition, mTOR also phosphorylates eIF4G and eIF4B either directly or through S6 kinases [60].

3.3.1.2 *CAP-independent translation*

Alternatively to the cap-dependent mechanism, a small amount of mRNAs, notably under certain stress conditions when the cap-dependent translation is decreased (e.g., hypoxia, nutrient limitation and apoptosis) use *Internal Ribosome Entry Site* or IRES to recruit the 40S ribosome to the initiation region, circumventing the scanning process. This is, therefore, a more direct process but it still needs the presence and assistance of eIFs. For IRES highly structured sequence elements within the 5' untranslated region (UTR) in the mRNA are stimulated by IRES transacting factors (ITAFs) that stabilize their active conformations [60,66,67].

3.3.2 Translation elongation, termination and recycling

Following initiation, the 80S complex is ready to start the process of peptide chain elongation by accepting the tRNA with the appropriate anticodon for the second codon that waits in the A-site of the ribosome (**Figure 11**). Directing the tRNA to the A site is assisted by the elongation factor eEF1A-GTP, which will be released once the codon recognition is made by the tRNA. Then a peptide bond is rapidly formed between the methionine and the second amino acid and a translocation of tRNAs to the E and P sites takes place, which is promoted by eEF2-GTP, the ribosome now being ready for the next elongation cycle until it reaches a stop codon. Upon recognition of a stop codon the release factors (eRF) eRF1 and eRF3 bind to the A site of the ribosome, GTP is hydrolysed and eRF3 is released, leaving the new synthetized protein ready for further processing [68]. The translational machinery, however, still needs to be dissociated once at this point the 80S complex is still bound to the mRNA, tRNA and eRF1 all in need to be recycled to be used again in translation [68]. This recycling processes is assisted by three proteins, ABCE1 (*Homo sapiens*), Dom34 and Hbs1 (*Saccharomyces cerevisiae*), which bind to the A-site of the ribosome complex and promote subunit dissociation. The eRF1 and eRF3 also cooperate by triggering slower rates of ribosomal dissociation. Following recycling, tRNA and mRNA are likely dissociated from the subunits [68].

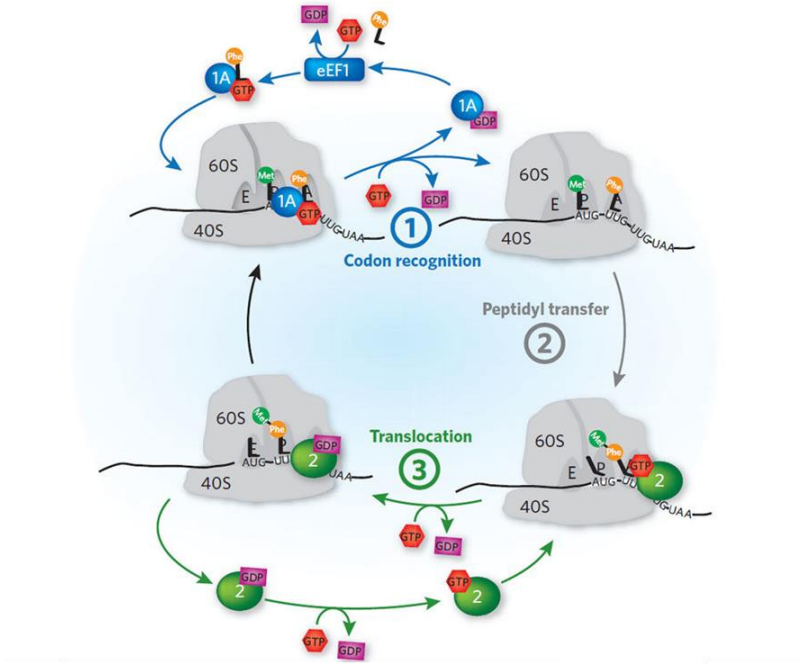


Figure 11. The elongation phase of translation. In the A-site of the ribosome eEF1A-GTP delivers the aminoacyl-tRNA that will be released after codon recognition. Peptide bond is catalysed and the nascent polypeptide transferred to the A-site t-RNA. eEF2 assists in translocating the peptidyl-tRNA into the P-site and deacylated tRNA into the E-site, freeing the A-site. Adapted from [69].

3.4 Protein synthesis and cancer

In recent years the growing evidence of the remarkable association of the protein synthesis process with the onset and progression of cancer has led to extensive revision and research on the role of translation in this disease as well as its potential in therapy [57,66,70,71].

It is clear that reprogramming the cellular metabolism of cancer cells involves protein synthesis to meet their increased production and turn-over needs, as well as to change their proteome to adapt and this will ultimately affect the different phases of tumorigenesis [72,73]. It was previously thought that transcriptional regulation was mainly responsible for specific gene expression, but it is now known that posttranscriptional (translational) control also plays different roles in the control of cellular metabolism, growth, migration, adhesion, cell cycle and tumorigenesis [72,74].

3.4.1 Regulation of translation in cancer

As initiation seems to be the most controlled step in translation, the association of its factors (eIFs) with cancer progression has already been observed at the gene level, such as genes encoding for eIF3h, eIF4G, eIF4E, eIF5A2 that are amplified and result in increased expression [72]. Deregulation of translation initiation proteins in cancer also includes alteration of their

activity via modifications of their phosphorylation status such as eIF4E and 4E-BP1 that have been associated with cancer, often displaying also altered expression [72,74–76]. **Table 1** details the specific deregulation of the initiation factors observed in different cancer types as well as their biological significance.

Table 1. Translation initiation factors and regulators deregulated in human cancers. Adapted from [57]. The references for each information can be found in the Supplementary Information of the cited article.

Factor	Dysregulation	Clinical correlates in cancers
eIF4E	Overexpression	• Decreased survival in breast, head and neck, liver, prostate, bladder and stomach cancers • Correlates with disease progression and aggressive subtypes in many cancers, and with resistance to chemotherapy
eIF4E	Phosphorylation	• Elevated in early stages of development of breast, colon, gastric and lung cancers • Increased in prostate cancer and correlates with androgen independence • Poor-prognosis marker in non-small-cell lung cancer
4E-BP1	Overexpression	• Inversely correlates with tumour grade • Correlates with better survival in lung and prostate cancers • Correlates with absence of lymph node and distant metastases in gastric cancer
4E-BP1	Loss	Possibly responsible for loss of translational control in 50% of pancreatic tumours
4E-BP1	Phosphorylation	Correlates with tumour grade and poor prognosis in breast, lung, ovarian and prostate cancers
eIF4G	Increased expression	• Amplification correlates with aggressive stages in lung cancer • Overexpressed in inflammatory breast cancer and cervical cancer • Correlates with poor prognosis in nasopharyngeal carcinoma
eIF4A	Increased expression	Overexpressed in lung and cervical cancer; lowered expression after radiation predicts better survival in cervical cancer
PDCD4	Decreased expression	• Associated with poor prognosis in breast, lung, colon and ovarian cancers and gliomas • Inversely correlated with advanced tumour stage in renal cell carcinoma
eIF2α	Increased expression	Correlates with aggressive lymphoma subtypes
eIF5A	Increased expression and hypusination	Correlates with poor prognosis in early-onset colorectal cancer. Overexpression of eIF5A2 correlates with local invasion in non-small-cell lung cancer and hepatocellular carcinoma
eIF6	Altered expression and function	• Regulates ribosome biogenesis and 40S–60S joining. Promotes transformation and lymphomagenesis • Elevated in colorectal cancer, head and neck carcinomas and ovarian serous carcinoma; low expression correlates with reduced disease-free survival in ovarian serous carcinoma; mediates lymphomagenesis in Shwachman–Diamond syndrome
eIF3a	Increased expression	Associated with breast, cervical, oesophagal, lung and stomach cancers
eIF3b	Increased expression	Associated with bladder, breast and prostate cancers
eIF3c	Increased expression	Associated with meningioma and testicular seminoma
eIF3h	Increased expression	Associated with breast, colon, liver and prostate cancers
eIF3i	Increased expression	Associated with breast, head and neck, and liver cancers, as well as melanoma and neuroblastoma
eIF3m	Increased expression	Associated with colon cancer
eIF3e	Decreased expression	Associated with breast, lung and prostate cancers
eIF3f	Decreased expression	Associated with breast, colon, small intestine, ovarian, pancreatic and vulval cancers and melanoma

Additionally, several oncogenic pathways like Ras-MAPK and PI3K-Akt-mTOR promote cellular transformation in cancer through modulation of the translational regulation and they do so by acting on the activity and expression of specific translational components (**Figure 12**) [77–80]. The **PI3K-Akt-mTOR** pathway, which is activated in many cancers, can activate **mTORC1** that phosphorylates ribosomal protein S6 kinase 1/2 (S6K1/2) and the eIF4E binding proteins (4E-BPs). Their phosphorylation releases eIF4E, allowing eIF4E-eIF4G association and eIF4F complex assembly allowing translation to proceed[72,74,76]. The other consequence of active S6K is a stimulation of eIF4A activity, another factor that is part of the eIF4 complex. The Programmed cell death protein 4 (PDCD4) binds to eIF4A, inhibiting its binding to eIF4G. The activated S6 kinase phosphorylates PDCD4 and liberates eIF4A to be assembled to eIF4F, which will further recruit the ribosome subunits [81]. The eIF4E can also be modulated by the **Ras pathway**, once activated Ras pathway causes the phosphorylation of the **MAP kinases Mnk1/2** which bind to eIF4G and phosphorylate eIF4E. Thus, eIF4E plays an important role in response to stimuli like growth factor and oncogenic signalling [73,81]. Accordingly, its overexpression and altered phosphorylation levels have been observed in several tumours (**Table 1**) [72,76]. Another important translational control that results in increased protein synthesis is the overexpression of **eIF2α** in cancers, an essential factor of the ternary complex to bring the tRNA to the 40S ribosome and a negative regulator of translation. Under normal conditions, the eIF2α-GDP is recycled to eIF2α-GTP - its active state - by eIF2B. When phosphorylated p-eIF2α inhibits eIF2B and consequently the recycling of eIF2α for initiation and further protein synthesis [66,72,74]. The joining of the 60S ribosome with the preinitiation complex is assisted by **eIF6**, which is a rate-limiting step, important in cell growth and transformation and thus its deregulation has clear implications in cancer [72,75]. Under stimulation, mTORC1 associates with **eIF3**, releasing S6K and thus promoting the assembly of the 40S ribosome with mRNA, activating protein synthesis. The overexpression of several eIF3 subunits has been observed in different types of cancers [66,82] (**Table 1**). Another regulatory component affecting translation directly is the transcription factor **Myc**, often deregulated in cancers. Myc increases transcription of multiple components of translation, including eIF4E mRNA, promoting protein synthesis [72,74]. At the elongation step, translation can be regulated by S6K inhibition of eukaryotic translation elongation factor 2 (eEF2) kinase (eEF2K). S6K phosphorylates eEF2K and liberates eEF2, allowing the translocation step of elongation to

continue [83]. **Figure 12** shows the major pathways modulating translation initiation in cancer and their cooperation to different steps of tumorigenesis.

Transformed cells also increase the use of cap-independent translation. Due to the fact that certain oncogenes and growth factors present in cancer cells such as c-Myc and VEGF carry **IRES** sites, these cells might take advantage of those in order to promote protein synthesis, being thus able to survive and proliferate [66,74].

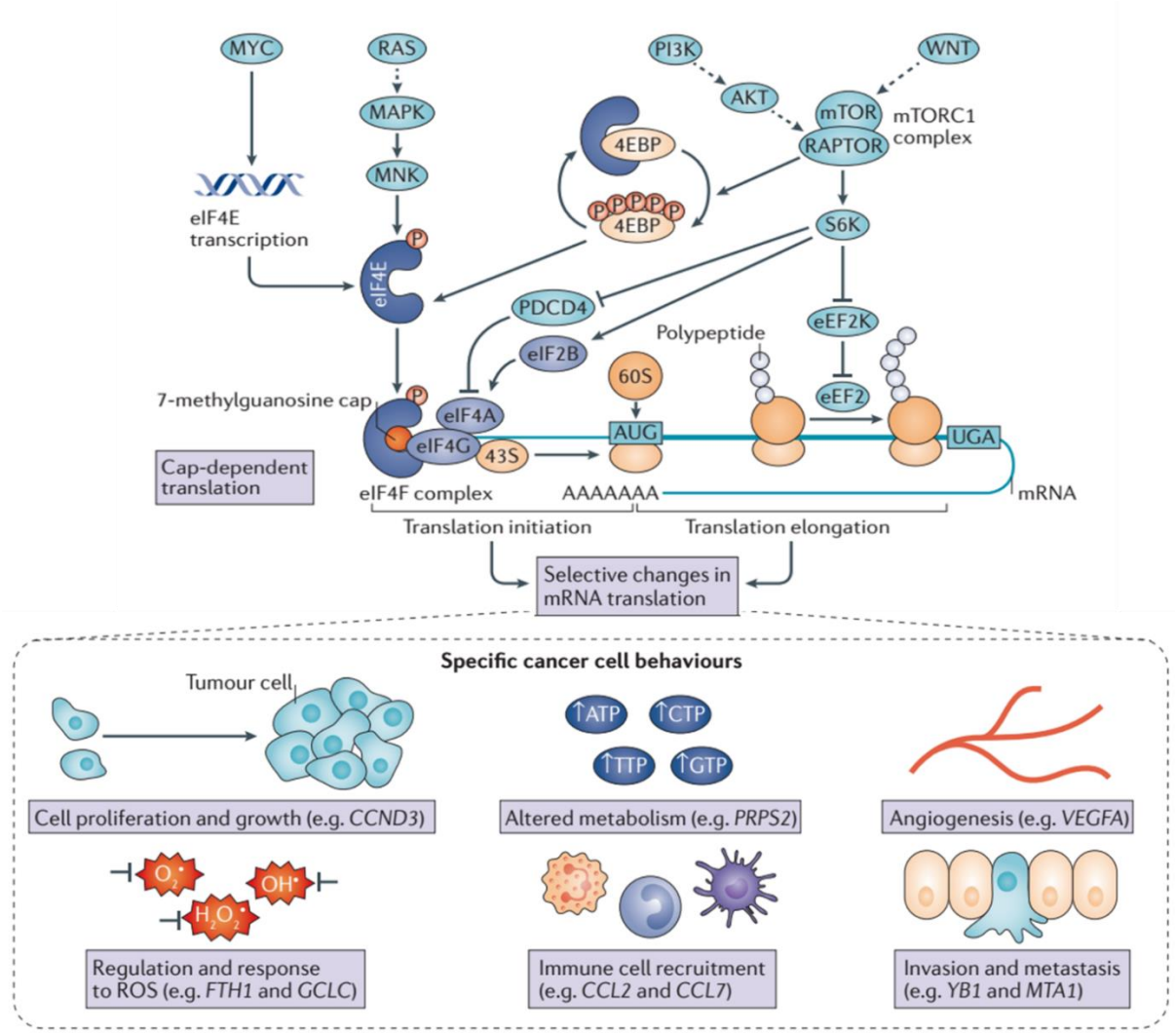


Figure 12. Main signalling pathways affecting translation and their resulting influences on tumorigenesis. These stimuli regulate translation and cause global changes in protein synthesis as well as specific changes in the translation of specific mRNAs. CCL: C-C motif chemokine ligand; CCND3: cyclin D3; FTH1: ferritin heavy polypeptide 1; GCLC: glutamate–cysteine ligase catalytic subunit; MTA1: metastasis-associated 1; PRPS2: phosphoribosyl pyrophosphate synthetase 2; RAPTOR: regulatory associated protein of mTORC1; ROS: reactive oxygen species; VEGFA: vascular endothelial growth factor A; YB1: Y-box binding protein 1. Adapted from [83].

Therefore, the elevated activity of the translation machinery components and regulators in cancer cells makes them potential targets for selective therapeutic development [57,66,71], a field of research that is increasingly growing. Additionally, the involvement of translation in

cancer resistance to some therapies has been observed. For example, the eIF4F complex has been associated with resistance to anti-BRAF and anti-MEK therapies in *BRAF* mutant tumours [84] while phosphorylation of eIF4E, which is associated with malignant progression, has been implicated in resistance to DNA-damaging agents [85]. These findings also support that targeting translational control is an interesting approach for selectively eliminating cancer cells [83] and will be further explored in the next section.

3.5 Protein synthesis inhibitors

Protein synthesis inhibitors are certainly not a novelty in the prokaryotic universe as antibiotics (e.g. macrolide and tetracycline) are used as drugs acting on different components of the bacteria translation machinery, but no inhibitors of eukaryotic translation had been explored for medical use before the last two decades [71].

Nonetheless, some protein synthesis inhibitors in eukaryotes are of valuable use in research, but their toxicity does not allow them to be used for therapeutic purposes. Puromycin, for example, is a natural nucleoside antibiotic that is structurally analogue to the adenosine in the 3' of the tRNA. Therefore it enters the A site of the ribosome and it stops the elongation of the nascent polypeptide chain, inhibiting protein synthesis [59]. Cycloheximide is another important protein synthesis inhibitor used as research reactive; it inhibits translation by binding the ribosome and inhibiting eEF2 translocation, thus halting elongation [86].

3.5.1 Targeting cancer with protein synthesis inhibitors

Several efforts were already conducted in the aim of targeting cancer cells protein synthesis, ranging from upstream inhibitors - like PI3K and mTOR inhibitors - to direct inhibition of specific factors [57,66,71,87,88]. It is noteworthy that strategies to target the elongation phase of translation were investigated earlier but are generally considered to have limited therapeutic value due to their non-specific effect in blocking global protein synthesis [71]. Contrarily, because the regulation of translation occurs mainly at the initiation step and has been shown to be deregulated in cancer, it is believed that targeting the initiation phase would offer more selectivity to cancer cells. Therefore, below we will consider the nodes of translation initiation known to participate in tumorigenesis that are already under investigation. **Figure 13** summarizes the different inhibitors and their mode of action that are briefly detailed further.

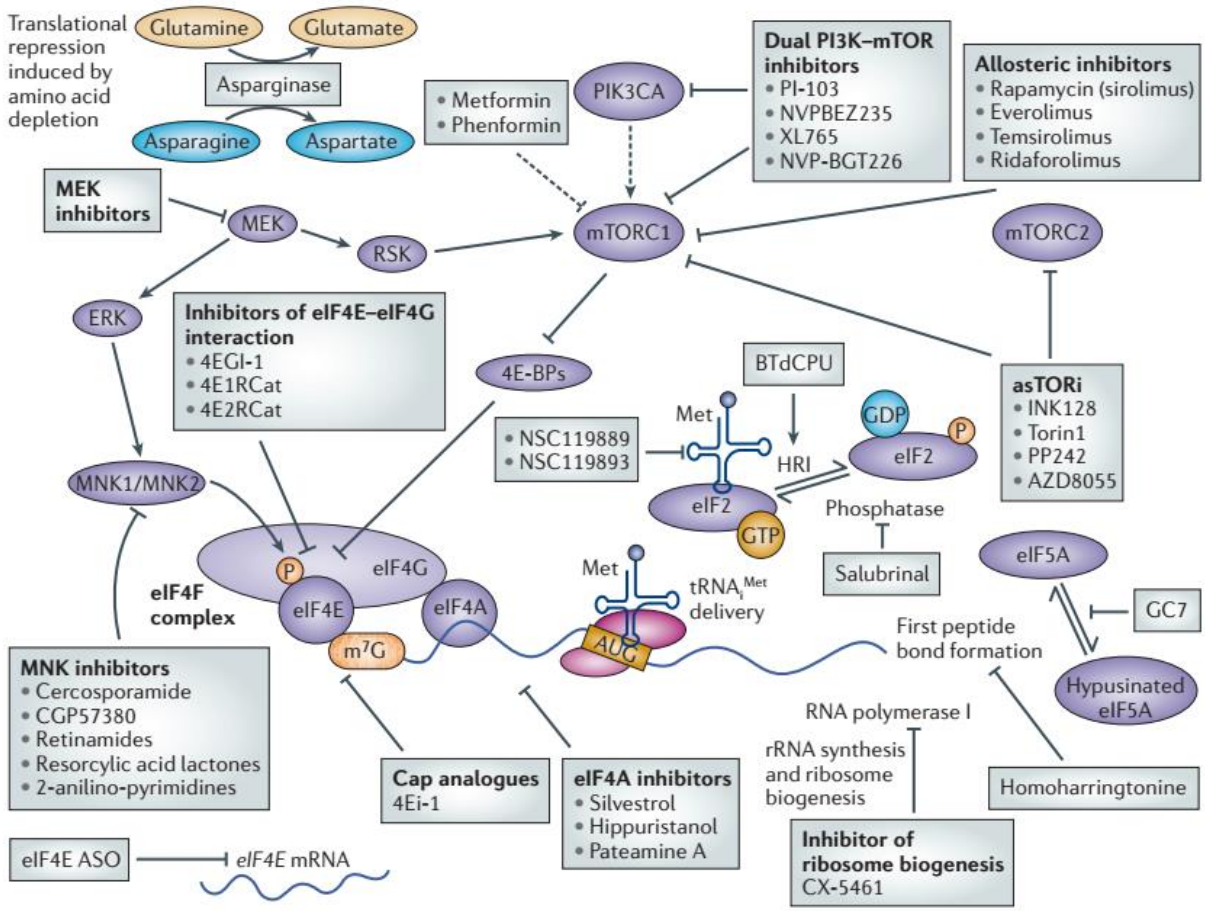


Figure 13. Drugs in use and compounds under investigation targeting the translation machinery in cancer. Adapted from [57].

3.5.1.1 *Miscellaneous drugs in clinical use affecting protein synthesis*

There are a few agents used in clinic that affect protein synthesis. Homoharringtonine is a natural product that prevents the formation of the first peptide bond and it has been approved for the treatment of chronic myeloid leukaemia [57,89]. Another natural product, asparaginase is an enzyme from bacteria that catalyses hydrolysis of L-asparagine and, in a small amount, L-glutamine. Depletion in these amino acids will cause perturbation in the amino acids pool, activate GCN2 and thus increase eIF2α phosphorylation and inactivation of mTORC1 [90]. This enzyme is currently used in the treatment of acute lymphoblastic leukaemia and paediatric acute myeloid leukaemia [91,92].

3.5.1.2 *The eIF4F complex*

The implications of deregulation of the components of the eIF4F complex in cancer are the most known to date and thus strategies to target this complex have also been the aim of more

intensive studies. Two distinct classes can be envisaged here: one targeting signalling pathways upstream, which regulate translation and other cellular processes, and one directly targeting the eIF4F complex [57,71].

3.5.1.2.1 *Upstream inhibitors: mTOR and MNK inhibitors*

The prototype inhibitor of mTOR is rapamycin (to which mTOR even owe its alternative name to: ***m****echanistic* ***T****arget* ***O****f* ***R****apamycin*) also known as sirolimus, a naturally occurring agent produced by the bacteria *Streptomyces hygroscopicus*. It binds to mTOR inducing conformational changes that impair its interaction with the regulatory-associated protein of mTOR (RAPTOR), impairing the formation of mTORC1 complex [93] – a major regulator of eIF4F assembly (Figure 13). Rapamycin analogues, i.e. the rapalogues (e.g., everolimus and temsirolimus), were developed in order to improve the pharmacodynamics properties of rapamycin. Although all of them are either in use in clinics or in clinical trials for the treatment of cancer, their efficacy is weaker than expected, probably because of partial inhibition of 4E-BPs as well as induction of PI3K-Akt signalling pathway via negative feedback loops [94,95]. Overcoming mTOR inhibitors' resistance has been attempted with dual-inhibition of both mTOR and PI3K, a strategy that is being tested in clinical trials but the possible increased toxicity is higher due to targeting of general nodes that have numerous downstream effects [95]. Still other resistance mechanisms, including activation of MAPK pathway and the switch of the cancer cells to cap-independent translation constitute a challenge in targeting mTOR to inhibit protein synthesis. Synthetic inhibitors of mTOR, called asTORi (Active-site mTOR inhibitors) have also been designed to potently inhibit both mTORC1 and mTORC2 [96], because this last one is able to activate Akt. The asTORi appeared superior to rapalogues in preclinical models [97], but it also seems to face resistance [57].

MNK inhibitors act downstream of the MEK-ERK pathway that controls eIF4E phosphorylation. Small-molecule inhibitors such as cercosporamide and CGP57380 were described as such and causing consequent decrease of malignant cells growth in culture [98,99]. However they also have off-target effects [98,99] and therefore other MNK inhibitors have been developed but associated resistance mechanisms still need to be assessed [57]. Naturally occurring rocaglamides also inhibit proliferation of several human cancer cell lines via MNK signalling–dependent protein synthesis inhibition [100].

3.5.1.2.2 *Direct eIF4F complex inhibitors*

The structures of the inhibitors of the eIF4F complex and eIF2 are shown in **Figure 14**.

eIF4E cap-interaction inhibitors

The strategy to inhibit the cap-interaction lies in that it is mainly the cap-binding activity and not only the overexpression of eIF4E that cooperates to the acquisition of transformation in cells [57]. Although cap analogues work *in vitro* for functional studies, they have poor stability *in vivo* and thus delivery strategies with virus-like particle were developed [101]. Alternatively, the pro-drug 4Ei-1 was developed and able to inhibit cap-dependent translation and EMT in zebrafish [102] as well sensitize lung cancer cells to gemcitabine treatment [103]. Ribavirin, a physical mimic of the cap structure, despite its controversial effects as an eIF4E cap-inhibitor [86,104] has shown benefits in clinical trials on patients with acute myeloid leukaemia alone [105], or in combination with cytarabine [106].

eIF4E-eIF4G interaction inhibitors

After eIF4E binding, eIF4G binds to the mRNA and this is thought to stabilize the association eIF4E-5'-mRNA-cap [57]. Three compounds were identified from a high-throughput screening as inhibitors of the eIF4E-eIF4G interaction and showed promising results in preclinical tests: 4EGI-1, 4E1RCat and 4E2RCat. The compound 4EGI-1 induced apoptosis in several cancer cell lines and inhibited melanoma and breast cancer xenograft growth [107–109].

eIF4E inhibitors

Antisense oligonucleotides (ASOs) are short single-stranded RNA or DNA molecules complementary to a specific sequence in the mRNA of a specific target gene [110]. The use of ASOs to target the synthesis of eIF4E is a successful strategy in preclinical models. Here, the ASO designed to target eIF4E RNA formed a RNA-RNA duplex that inhibits the synthesis of the corresponding protein product [111,112]. It reduced tumours in breast and prostate models *in vivo* with low toxicity, decreased pro-survival and pro-growth proteins encoded by eIF4E and reduced angiogenesis [113]. Clinical trials with eIF4E ASO combined with other chemotherapeutic agents clinically used are ongoing. Results of one study show that the treatment of colorectal cancer with irinotecan combined with the eIF4E ASO ISIS1883750 was successful in approximately half of the patients [114].

eIF4A inhibitors

eIF4A has a helicase activity, unwinding secondary structure of the 5' UTR of poorly translated mRNAs, facilitating their attachment to the 40S ribosome. This activity is dependent on the interaction of eIF4A with other components of the eIF4F complex which will stimulate ATP hydrolysis and eIF4A binding to mRNA [66,72]. In addition, the incorporation of eIF4A to the eIF4F complex is controlled by the PDCD4 protein: association of eIF4A with eIF4G through degradation of PDCD4 allows translation to occur. Three eIF4A inhibitors from natural sources (**Figure 17**, section 4.4) have been investigated for their potential anticancer effects:

- hippuristanol is a steroid that prevents eIF4A interaction with RNA and blocks its helicase activity [115];
- pateamine A forms a complex between eIF4A and eIF4B, reducing eIF4A-eIF4G association, depleting it from the eIF4F complex [116].
- silvestrol, like pateamine A, induces dimerization and forces non-sequence specific interaction between eIF4A and RNA, causing depletion of eIF4A from the eIF4F complex [117].

These compounds inhibit eIF4A, reducing ribosomal recruitment and therefore translation [57,66]. The three compounds and synthetic derivatives have shown efficacy in different preclinical models [118,119], but silvestrol seems to have best potency *in vivo* [117], although resistance mediated by overexpression of ATP-binding cassette sub-family B1 (ABCB1) impedes the development of silvestrol as an anticancer agent [120].

The silvestrol-related family, rocaglamides, in addition to the aforementioned MNK-activity, also inhibit protein synthesis directly by preventing incorporation of eIF4A in the eIF4F complex. In addition to the *in vitro* antiproliferative and *in* vivo antitumor effects, these compounds displayed no or very little toxicity to non-malignant cells [100].

3.5.1.3 ***Inhibitors of the Ternary Complex: eIF2***

As explained earlier, the phosphorylation of eIF2α will ultimately lead to translation inhibition via eIF2B sequestration. eIF2B catalyses the GDP-GTP exchange to form the eIF2-GTP, the active form. The phosphorylation of eIF2α is depending on the following kinases: PERK, HRI, PKR and GCN2 under certain stress conditions. Activating those kinases is thus a strategy to inhibit translation. Examples are the HRI kinase activators N,N'-diarylureas and related compounds, the most potent being BTdCPU. They showed good activity *in vitro* and *in vivo* [121,122]. Another possible strategy is to inhibit dephosphorylation of eIF2α which was

attempted with the phosphatase inhibitor salubrinal [123]. A combination of this inhibitor with the proteasome inhibitor bortezomib showed promising results for multiple myeloma cells *in vitro* [124].

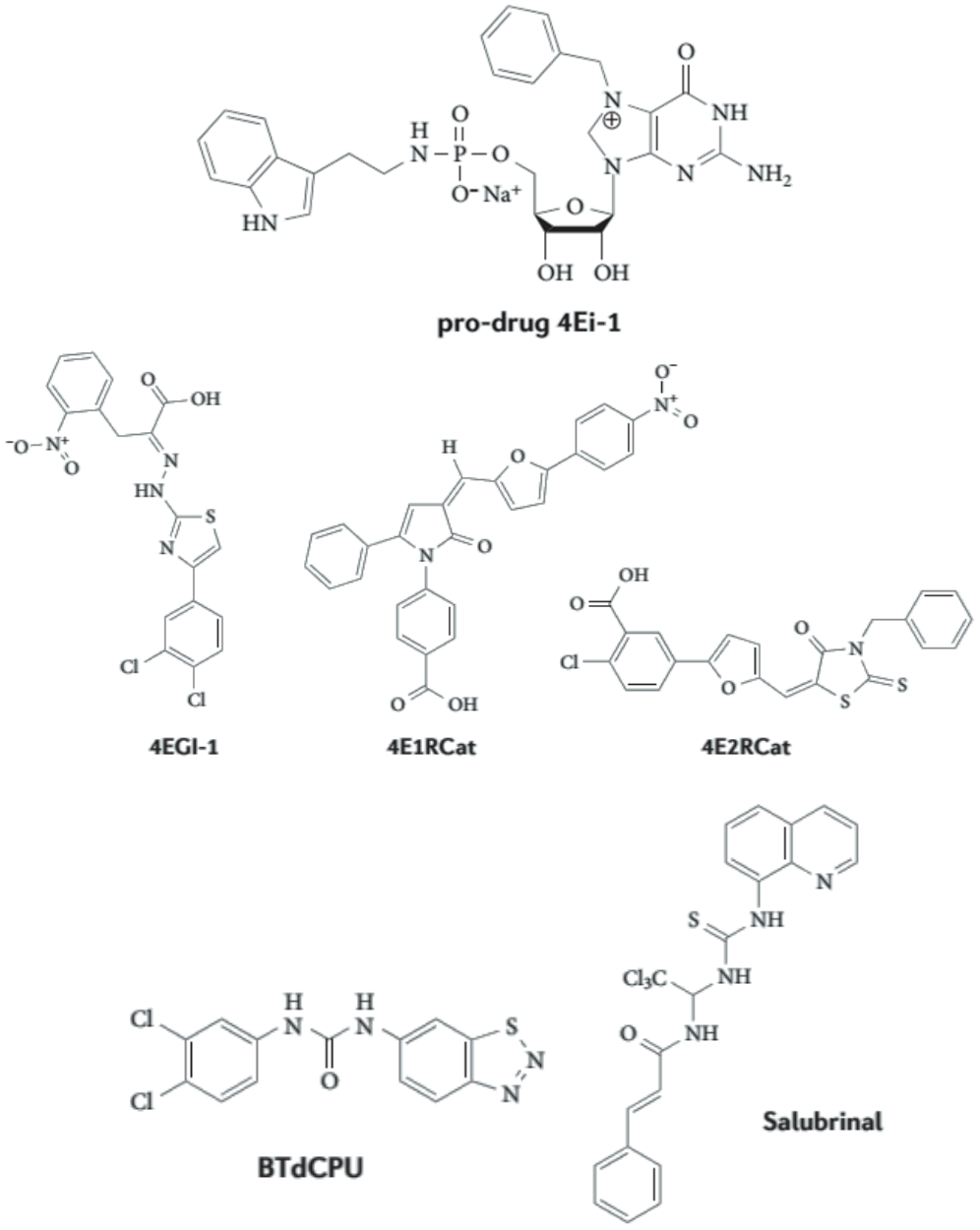


Figure 14. Structures of translation inhibitors acting directly on the eIF4F complex and on eIF2α phosphorylation. The structures of the eIF4A inhibitors are presented in **Figure 17**, section 4.4.

3.5.1.4 ***Other inhibitors***

There are other inhibitors of protein synthesis that are not necessarily classified above but deserve consideration. GC7 (N1-guanyl-1,7-diaminoheptane) is also a protein synthesis inhibitor because it prevents the formation of the first peptide bond, doing so through

inhibition of the hypusination of eIF5A [125]. Hypusination is a posttranslational modification in which an amino-butyl residue is transferred to lysine and, interestingly, eIF5A is the only known protein to have the amino acid hypusine [126]. Inhibition of eIF5A hypusination led to apoptotic cell death and tumour cell growth impairment in a mouse model of melanoma [125]. A nicotinamide phosphoribosyltransferase (NAMPT) inhibitor FK866 also blocked translation in leukemia cells through two initiation mechanisms: phosphorylation of eIF2 and inhibition of mTOR/4E-BP1 signalling [127]. The small molecule CX-5461 induced inhibition of ribosomal RNA (rRNA) synthesis and accumulation of free ribosomal proteins through inhibition of the RNA polymerase I (Pol I), since transcription of rRNA by Pol I is often increased in cancer [128].

As we can see, the inhibition of the different proteins involved in translation and their signalling pathways have a great potential as anticancer therapy. This is due to the prominent roles that have been and keep being unveiled for protein synthesis in cancer, confirming it as an emerging and promising approach in therapy.

4 Anticancer drugs and natural products

4.1 A brief history of anticancer drugs

Chemotherapy was recognized and combined to surgery and radiotherapy for cancer therapy only in the 1960's, when it became clear that metastases needed a systemic approach rather than the localized one offered by the other two methods [8]. In fact, research to use chemical entities to treat cancer had begun in the 1940's during World War II, when investigators observed that those men exposed to the mustard gas had the bone marrow and lymph nodes severely affected. In the following decades several drugs, such as methotrexate, chlorambucil and 5-fluorouracil were discovered and used in the treatment of different types of cancers [8]. In addition to that, some of the most promising agents from natural products that are still in clinical use were discovered: the plant alkaloids from *Vinca rosea* (vincristine and vinblastine) [8], camptothecin, from the Chinese tree *Camptotheca acuminata* and taxol from the Pacific yew tree *Taxus brevifolia* [129]. In the 1970's chemotherapy combinations were established allowing long-term remission of certain cancers by targeting different mechanisms and being adjuvant after surgery and/or radiotherapy. Later, there were a few promising discoveries, like cisplatin and other platinum compounds, but perhaps the greatest change was the shift

in the landscape of the random drug screening to a more targeted one. These agents aiming at less cytotoxic side effects and overcoming resistance to current chemotherapy are targeting one or more aspects of the unique features of cancer and acting at specific cellular molecules such as growth factors, signalling molecules, cell-cycle proteins, modulators of apoptosis and molecules that promote angiogenesis and metastases [130]. They include not only small-molecules, but also biological compounds such as vaccines or antibodies.

4.2 Anticancer drugs used in chemotherapy

Below is a simple recapitulation on how the most clinically used drugs employed in chemotherapy affect cancer cells. A table with the chemotherapeutic drugs per class and their indication can be found in the Appendix 2.

Antimetabolites

Due to the evident difference in metabolism between cancer and normal cells, the first ones are more susceptible to antimetabolites. This class of agents interferes mainly by inhibiting DNA synthesis, thus in the S phase of the cell cycle by mimicking essential DNA and RNA elements such as purine and pyrimidine or compounds needed to synthetize these last two. Some examples of drugs clinically used include the **folate analogue** methotrexate, **pyrimidine antagonists** 5-fluoracil and cytarabine and **purine analogues** 6-mercaptopurine and 6-tioguanine [131].

Alkylating and intercalating agents

These agents cause cytotoxicity to the cancer cells by interaction with the DNA basically because of their electrophilic characteristic: through alkylating reaction they bind covalently to the nucleophilic centres of DNA, the most frequent binding site being the N-7 position of guanine. There are several classes of alkylating agents and the most known categories are: **nitrogen mustards, nitrosureas and platinum analogues** [131,132].

Topoisomerase inhibitors

DNA topoisomerases I and II are nuclear enzymes that allow the DNA to be tightly packed. Topoisomerase I binds to double-stranded DNA, cleaves one of the strands and forms an enzyme-DNA covalent bond, allowing the unbroken strand to pass through it and release the torsional stress of the DNA double helix. The topoisomerase I inhibitors, such as **camptothecin**, interact with the enzyme-DNA complex thus blocking synthesis and

transcription of DNA. Although in a different way, topoisomerase II also acts on the cleavage and release on torsional stress and thus topoisomerase II inhibitors, such as the semisynthetic **podophyllotoxin etoposide**, block the religation of the DNA strand after cleavage performed by these enzymes [133].

Antimicrotubule agents

Tubulins compose the microtubules, which are components of the mitotic spindle responsible for the segregation of chromosomes into the daughter cells during mitosis. The vinca alkaloids like **vincristine** and **vinblastine**, bind to the tubulins preventing their assembly into microtubules, while taxanes, like **paclitaxel**, prevent the depolymerisation of the microtubules, the resulting effect being anyway the inhibition of cell division [134].

Antitumor antibiotics

Various antibiotics are actually used as anti-tumour agents. While they belong to this class, they display different mechanisms of action that cause cell cytotoxicity: they may cause DNA intercalation, alkylation and cross-linking and also act at the level of the topoisomerases. **Anthracyclines**, for example, intercalate the DNA and their most known agents used in chemotherapy are doxorubicin and daunorubicin. Other examples of antibiotics clinically used are mitomycin and bleomycin [131].

Agents acting on growth factor signalling

As signal transduction pathways that control cell survival, proliferation and migration are deregulated in cancer cells, targeting some specific factors of them that are mutated or overexpressed led to the approval and use of new drugs. Those inhibitors are small chemical agents or antibodies [135]. The most famous example of targets are members of the EGFR family tyrosine kinase receptors that are deregulated and/ or activated and are implicated in many aspects of the cancer cells malignancy [136]. Some of the tyrosine kinase inhibitors (small molecules or antibodies) currently in use include Imatinib (Gleevec, against EGFR), Sorafenib (Nexavar, against VEGFR, c-RAF, PDGFR) and Lapatinib (Tykerb, against EGFR, HER2) [137].

Hormonal agents

These agents are usually employed in the treatment of hormonally responsive cancers, like breast, prostate and endometrial cancers [138]. Selective estrogen receptor modulators, like

Tamoxifen for example, inhibit estrogen stimulation of breast cancer cells, by blocking translocation and nuclear binding of the estrogen receptor. The inhibition of estrogen synthesis by aromatase inhibitors is another strategy used in breast cancer therapy. As for prostate cancer, the depletion of testosterone is targeted with gonadotropin-releasing hormone (GnRH) agents and antiandrogens [138].

Other agents

Given the role that angiogenesis play in tumour growth, invasion and metastasis, antiangiogenic agents are an important strategy in cancer therapy. Angiogenesis inhibitors target Angiogenesis inhibitors target signalling pathways, endothelial cells and/or proangiogenic factors like VEGF, and belong to different classes [139], like the aforementioned tyrosine kinase inhibitors and monoclonal antibodies.

The ubiquitin proteasome system, implicated in protein degradation, is also a target in cancer therapy. Proteasome inhibitors, like Bortezomib and Carfilzomib were approved for use in multiple myeloma [63].

A new area of development relates to antibody-conjugated-drugs aiming to couple antibody against an overexpressed target to a cytotoxic drug. Trastuzumab-emtansine has been marketed for HER2+ breast cancers recently [140].

4.3 Resistance to chemotherapy

Chemotherapy and molecularly targeted therapies are thus currently mainstays in the treatment of cancers. However, their effectiveness is hindered by various resistance mechanisms developed by the tumour cells [141]. Tumour resistance to drugs can be classified as intrinsic, meaning that they are present prior to the treatment, or acquired, which is developed during treatment and induced by drugs [142]. Diverse mechanisms enable cancer cells to resist the anticancer drugs and include alterations of the drug target or its metabolism, increase in the rate of drug efflux, adaptation through activation of signalling pathways that enable cell survival or disable cell death, epigenetic changes. Also, the tumour microenvironment and cancer stem cells are believed to be involved in resistance mechanisms [141].

Drug efflux is a major cause of resistance to chemotherapy and cell membrane transporter proteins, especially the ATP-binding cassette (ABC) transporter family, has been implicated in

this type of resistance (**Figure 15** for more details). These transporters cause the efflux of several compounds, including chemotherapeutics such as taxanes and antimetabolites, which will result in less intracellular drug concentration. MDR1 (multi-drug resistance protein 1, P-glycoprotein) is an example associated with treatment failure in many cancers, due to its overexpression in many tumours or to an increase in expression followed by administration of chemotherapy [141,142].

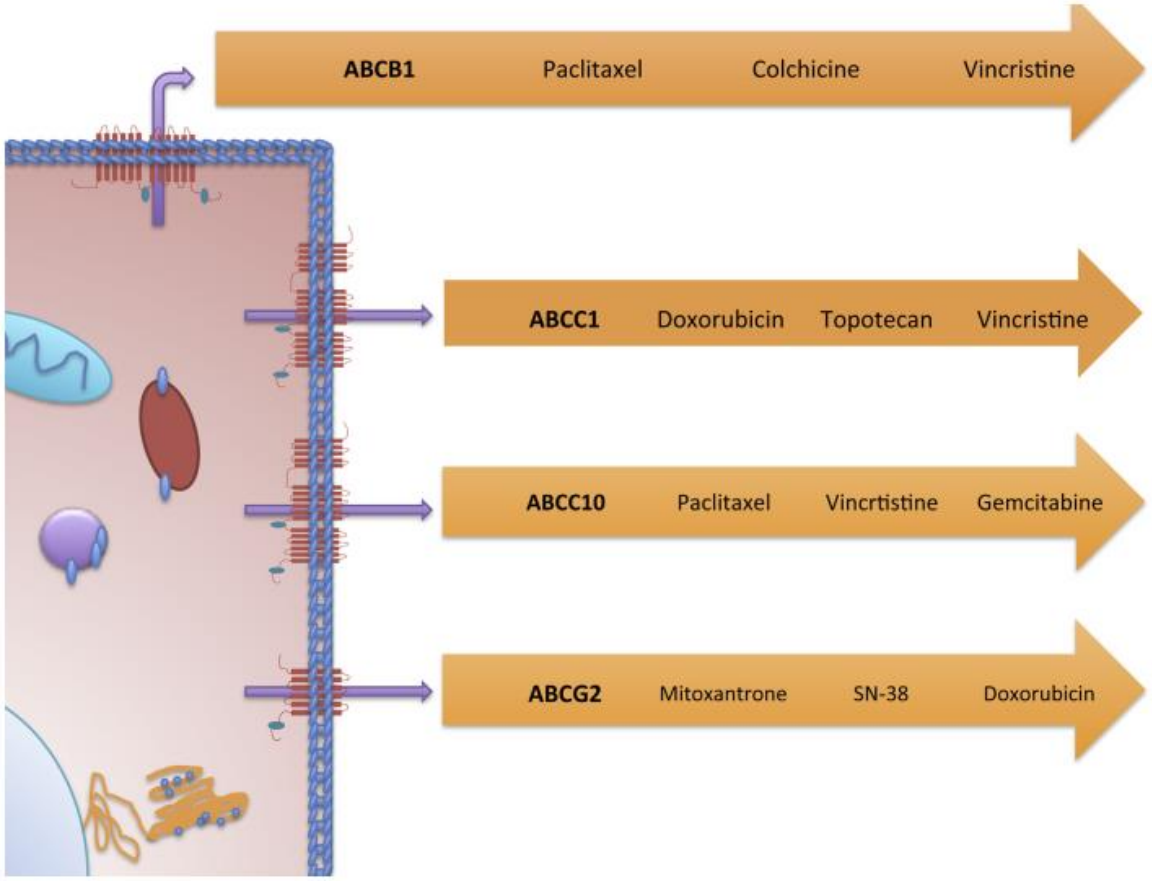


Figure 15. MDR-ABC transporters and examples of substrates anticancer drugs. ABCB1 (MDR1/P-glycoprotein), ABCC1 (MRP1), ABCC10 (MRP7) and ABCG2 (MXR), located on the cell surface, are the major mediators of efflux of anticancer drugs from the cells. The transporters use energy from the hydrolysis of ATP to ADP to transport their substrates across the membrane against a concentration gradient. ABCB1 is an apical membrane transporter found in the kidney, placenta, liver, adrenal glands, intestine and blood–brain barrier cells, protecting against xenobiotics and cellular toxicants. ABCC1 is present on the basolateral surface of the epithelial membrane and has a similar resistance profile with ABCB1, although it does not confer resistance to taxanes. ABCC10 is located to the basolateral cell surface and also confers resistance to several anticancer drugs. The ABCG2 is a half transporter, active upon dimerization with itself or other transporters. It is mainly present in the plasma membrane and its substrates include tyrosine kinase inhibitors, camptothecin-derived topoisomerase I inhibitors and methotrexate. Adapted from [143].

The fact that many chemotherapeutic drugs induce DNA damage implies that the cancer cell will attempt to respond either by **repairing the DNA damage** or by causing cell death. DNA damage causes cell cycle arrest, but the deregulation of cell cycle checkpoints, for example

through inactivation of the tumour suppressor p53, allows genomically instable cancer cells to repair it and/or continue proliferating. Therefore, the inhibition of DNA damage repair is a strategy to overcome resistance when combined with DNA damaging agents [144].

Alterations in drug targets due to mutations or increased expression levels also hinders the effects of anticancer drugs that specifically inhibit those targets. One example is the androgen receptor (AR) in prostate cancer, which is amplified following acquired resistance to therapy using an AR antagonist and testosterone-lowering drugs [141].

The **deregulation of cell death** is another mechanism of resistance to the drugs targeting cancer cells. The activation of mutations of oncogenes as well as the inactivation of tumour suppressor genes that characterize tumour cells, as explained above, plays a role *per se* in intrinsic resistance [142]. In addition, the anti-apoptotic proteins, like Bcl-2, have a role in resisting to chemotherapy: combined approaches between chemotherapy and inhibitory strategies of BCL-2 members were investigated to overcome resistance [141]. Although autophagy cell death is induced by many chemotherapeutic and targeted therapies, its ability to promote cell survival might also play a role in the resistance to therapy [141].

As seen earlier, sustaining proliferative signalling is one of the cancer hallmarks and **activation of pro-survival signalling** has been linked to chemoresistance. The activation of EGFRs, for example, has been reported as a resistance mechanism to some chemotherapies. Thus, therapies targeting EGFR were able to sensitize different tumour types to chemotherapeutics like 5-FU, irinotecan and paclitaxel [141]. Other types of resistance to chemotherapy and targeted therapies have been associated with **cancer cells undergoing EMT**, the presence of **cancer stem cells**, which are endowed with several of the aforementioned resistance mechanisms, and also with the **tumour microenvironment**, that offers protection for cancer cells and facilitate their apoptosis evasion [141].

The understanding of drug resistance offers challenges and opportunities on how to overcome it by applying rational drug combinations assisted by the use of predictive biomarkers for different types of cancers and populations. It also becomes clear that the search for more drugs with different mechanisms of action compared to the available ones to tackle resistance to chemotherapy is essential.

4.4 The role of natural products in anticancer drugs research

Natural products are source of a variety of small secondary metabolite molecules with broad structural diversity. Plants, marine and microbial organisms compose the vast source of natural products available to be explored in the research and development of new drugs [145].

Natural products have always been part of the humanity as source not only of nutrients but also traditionally used in preparations in ritual and folk medicine practices [146]. Maybe intuitively, one would think that with the advent of technology and other major developments, the drug discovery path would turn towards the synthetic path to achieve its goals. Recent years have indeed seen a shift to organic-synthesis due to the easy access to small molecules that may produce drug candidates as opposed to the long-term period usually needed to develop projects with natural products [147]. It is also important to highlight the increase on the research and development of new macromolecular drugs, such as antibodies. They are interesting therapeutic agents, especially because of their high selectivity and specificity [148]. The use of monoclonal antibodies (mABs) to treat cancer has been established in the past 20 years. The killing of the cancer cells using mABs may occur through direct mechanisms, (like receptor blockade), immune-mediated cell killing mechanisms (like antibody-dependent cellular cytotoxicity) as well as specific effects of the antibody on the tumour vasculature and/or stroma [149]. Several mABs are in use for both haematological malignancies and solid tumours, e.g. Trastuzumab (anti-HER2) and Bevacizumab (anti-VEGF).

Nature remains a major source of hundreds of thousands of molecules associated with chemical and biological diversity still unexplored. Based on the impressive success recorded in recent history of natural products eventually becoming medicines and, particularly in the oncological domain, it makes it a compelling pursue for continued exploration [146,147,150]. The successful use of drugs derived from natural products in cancer therapy, such as podophyllotoxin, paclitaxel, camptothecin and the vinca alkaloids, intensified the research and development of new compounds prevenient from natural products as potential chemotherapeutic agents [151]. **Figure 16** assessing small molecules approved for anticancer therapy from the 1940s to 2014 shows that out of the 207 (removing the vaccines and biologicals, which are high-molecular weight), 77% are either natural or natural-derived products [150].

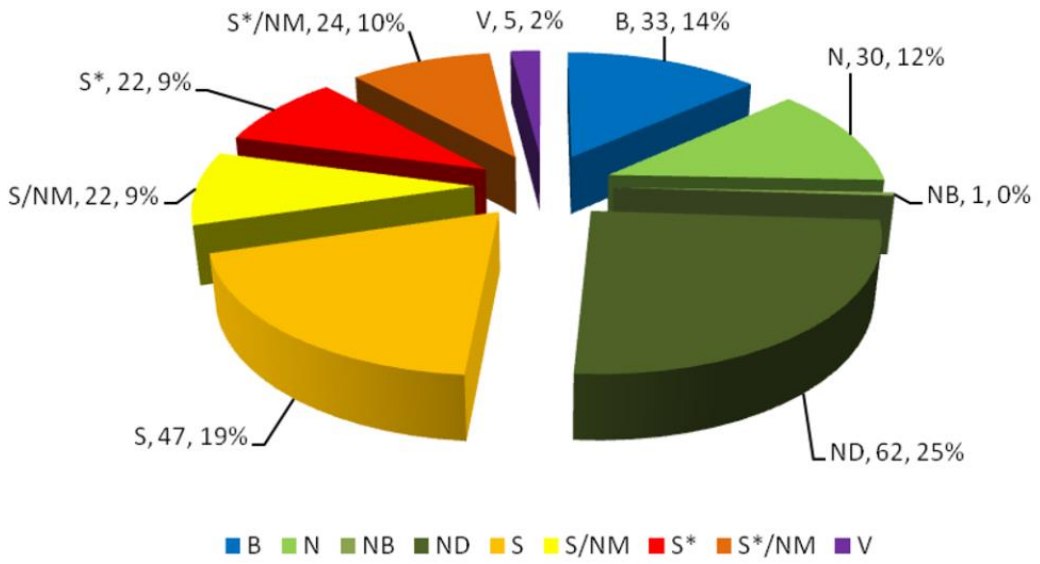


Figure 16. Source of all anticancer drugs from 1940's to 2014 (n = 246). "B" Biologicals (usually a large peptide or protein either isolated from an organism/cell line or produced by biotechnological means), ''N'' Natural product (unmodified in structure, though might be semi- or totally synthetic), ''NB'' Natural product ''Botanical drug'', ''ND'' Derived from a natural product and is usually a semi-synthetic modification, ''S'' Totally synthetic drug, "S/NM" Synthetic but a competitive inhibitor of an enzyme or receptor (natural product mimic), ''S*'' Made by total synthesis, but the pharmacophore is/was from a natural product, ''S*/NM'' Natural product pharmacophore that is a competitive inhibitor, "V" Vaccines. Adapted from [150].

Not only the much known and clinically used chemotherapeutic agents aforementioned, but also new ones continue to be studied, included in clinical trials and approved. This is the case of homoharringtonine, a cephalotaxine alkaloid from *Cephalotaxus fortunei* (family *Taxaceae*) that inhibits protein synthesis by preventing formation of the first peptide bond and that was approved for the treatment of chronic myeloid leukaemia by the FDA (*Food and Drug Administration*) in 2012 in the USA [150]. Homoharringtonine inhibits translation by acting on the initial elongation step of protein synthesis. It interacts with the ribosomal A-site, causing inhibition of protein synthesis by competing with the amino acid side chains of incoming tRNAs for binding to the A-site of the ribosome [152]. The research on new translation inhibitors for anticancer therapy continues to show diverse examples of natural products as source of potential and successful candidates exemplified with the eIF4A inhibitors. Silvestrol was extracted from fruits and twigs of *Aglaia silvestris*, Pateamine A was isolated from the marine sponge *Mycale* sp. and Hippuristanol is a natural compound from *Isis hippuris*, a branching coral present in the Western Pacific Ocean. By inhibiting the elongation factor 4A, these compounds reduce ribosomal recruitment and consequently translation [57,66]. Hippuristanol prevents eIF4A interaction with RNA and blocks its activity [115], while pateamine A and silvestrol deplete eIF4A from the eIF4F complex preventing 40S loading onto

the mRNA, halting translation [116,117] (for detailed mechanism see section 3.5.1.2.2). Unlike homoharringtonine, these compound are still in pre-clinical development [118,119]. Among the three silvestrol has shown better activity *in vivo* [117], but its resistance mediated by tumours overexpressing of P-glycoprotein remains an obstacle to the development of this compound as an anticancer agent [120]. The structure of the aforementioned translation inhibitors are shown in **Figure 17**. These are promising agents and examples that encourages us to investigate new protein synthesis inhibitors from natural sources.

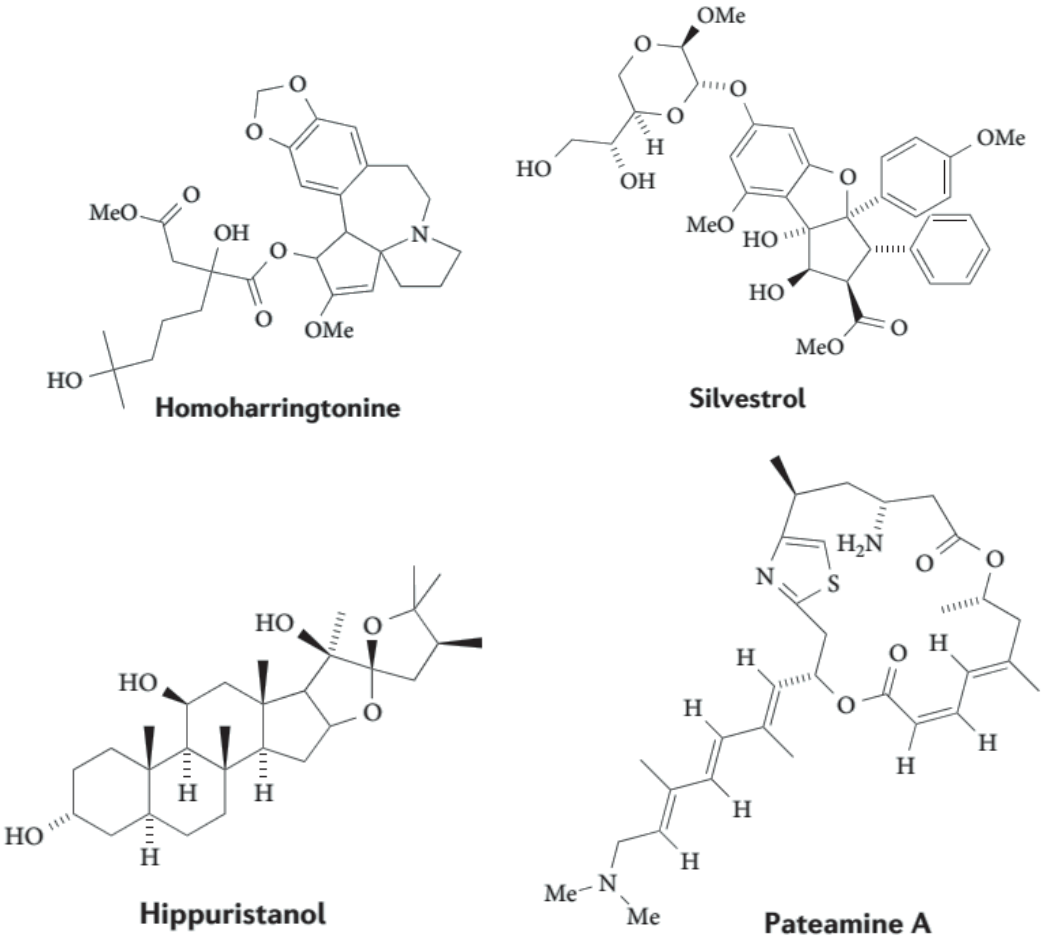


Figure 17. Structures of translation inhibitors originated from natural products.

As part of the research projects of our group, we investigate the potential of new natural products and derivatives as anticancer agents. In that context, we have investigated β-carbolines, from which a lead compound was identified and investigated *in vitro.* We therefore present below a brief report on this class of compound as well as introduce previous research conducted in our group with this chemical family and derivatives.

5 β-Carbolines

5.1 Origin and chemical structure

β-carbolines are indole alkaloids of natural origin. These alkaloids are heterocyclic amines, biosynthesized from a combination of cyclic five and six carbon structures that contain an amine group. These compounds are made up of planar tricyclic ring (**Figure 18**) derived from the amino acid L-tryptophan (which contains an indole ring system) [153]. This class of compounds was firstly isolated from the seeds of *Peganum harmala* (Syrian Rue, family *Zygophyllaceae*), a plant used in traditional medicinal practices in the Middle East, North Africa and Asia [154]. Preparations form mainly the seeds were used in folk medicine as a hypotensive (cardiovascular system), analgesic, antiparkinson, in psychiatric conditions, halucinogenic (nervous system), antispasmodic and antidiarrheal (gastrointestinal), antidiabetic, antileishmanial, among many other uses [155]. The main components in the seed extract of this plant are the β-carbolines, also known as harmala alkaloids: harmine (**Figure 16**), harman and harmaline. β-carbolines are also found in a variety of sources other than plants, such as marine organisms, fungi, algae, food and beverages as well as endogenously in mammalian tissues, organs and biological fluids [153,154]. A ritual hallucinogen drink in the Amazon regions prepared from *Banisteriopsis caap*i (family *Malpighiaceae*), called *ayahuasca*, contains harmine and N,N-dimethyltryptamine (DMT), the latter being a potent hallucinogen that is inactive orally due to degradation by the monoamine-oxidase (MOA). Harmine, however, is a potent reversible inhibitor of the monoamine-oxidase A (MAO-A) – as evidenced in kinetic studies using human enzymes [156] – and thus able to render DMT active, potentiating the hallucinogen effects of the hallucinogen drink [157].

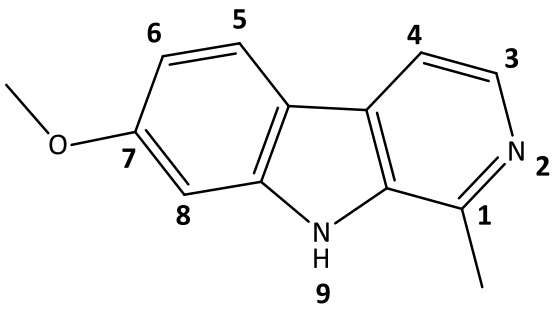


Figure 18. Structure of the β-carboline harmine.

5.2 Pharmacological activities

5.2.1 Diverse pharmacological activities of harmine

In the central nervous system, other than the antidepressant effect through inhibition of MAO-A, which also degrades serotonin and other neurotransmitters [158], harmine inhibits phosphorylation (competitive inhibitor of the ATP pocket) of the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) and thereby its activation, as verified by kinase assay (DYRK1A inhibition; IC_{50} = 33 nM) and cellular assay (DYRK1A phosphorylation on its substrate, the splicing factor 3B1; IC_{50} = 48 nM) [159]. This protein is implicated in neurodegenerative disorders such as Alzheimer's disease, Down syndrome and Parkinson's disease, having a potential for therapeutic use in diseases where DYRK1A is elevated [159].

Additionally, harmine has an effect on inflammation by i) decreasing the pro-inflammatory cytokine TNF-α, limiting cartilage degradation promoted by this cytokine in chondrocytes [160], ii) decreasing other pro-inflammatory mediators that play a role in chronic inflammation, like nitric oxide and interleukin 6, [161], and iii) inhibiting the pro-inflammatory myeloperoxidase [162] and the signalling pathway NF-κB [163]. Therefore, harmine could be of potential interest in the treatment of degenerative joint diseases, such as osteoarthritis [160].

This ability of harmine to decrease cytokines was also associated with an anti-diabetic (diabetes type 2) effect *in vivo*, once inflammation of the adipose tissues plays a role in the resistance to insulin. PPARγ1 is a regulator of insulin resistance in adipose tissues, its transcriptional activity is required for maintenance of insulin sensitivity and lipid metabolism. Harmine seems to act as a cell type specific regulator of PPARγ1 expression [164]. The administration of harmine to mice (30 mg/Kg, for 6 weeks), mimicked the effects of PPARγ1 ligands on PPARγ1-dependent gene expression in adipose tissue and improved glucose tolerance in diabetic mice [164]. The anti-diabetic effect of harmine in diabetes was also attributed to stimulation of the pancreatic insulin-producing beta cells (rat cells), deficient in diabetes. However the mechanism of how harmine stimulates these cells remains unclear [165]. The antimalarial effect of harmine was attributed to its binding to the ATP domain of the heat-shock protein 90 (HSP90) in the *Plasmodium falciparum* [166]. Not only that, but also a synergistic effect was observed with the antimalarial chloroquine against the *Plasmodium berghei* [167].

5.2.2 *In vitro* and *in vivo* effects of harmine and derivatives in cancer cells

Harmine has been shown to hold both antiproliferative effects in several cancer cell lines and antitumor effects in different *in vivo* mice models as detailed in **Table 2**. The majority of the studies show the effects of harmine in cancer cells and in tumours but the underlying mechanisms were not always investigated. In spite of it, some studies associate these effects to harmine inhibition of DYRK1A and ability to intercalate the DNA [168–171]. The intercalation of harmine to the DNA (calf-thymus DNA) has been demonstrated in non-cellular assays [171,172] and has been associated with its antiproliferative and antitumor activity (see **Table 2** for details) [171]. Caspase 9 is a critical component of the intrinsic apoptotic pathway, activated by apoptotic stimuli that can lead to cell death. Its activity is inhibited by phosphorylation and DYRK1A has been shown to phosphorylate caspase 9 at its inhibitory site threonine 125 [168,173]. The inhibitory effect of harmine on DYRK1A phosphorylation of caspase 9 was evidenced in a cellular assay (1μM, 30 min) [168] and associated with increased apoptosis in head and neck squamous cell carcinoma [170]. In addition to that, DYRK1A prevents the degradation of EGFR, leading to recycling of the receptor (**Figure 19**). The inhibition of DYRK1A promoted by harmine led to EGFR degradation and resulted in reduced tumour growth in a glioblastoma mouse model [169].

Table 2. Harmine effects in cancer cells *in vitro* and antitumor *in vivo*.

Effects	Models evaluated	Concentration/ dose/ treatment time	Reference
DYRK1A inhibition of caspase 9 phosphorylation	*In vitro*: U2.C9– C287A cells expressing caspase 9 (derivative of U-2 OS human bone osteosarcome cell line)	1 µM – 30 min	[168]
DYRK1A inhibition causing EGFR degradation, decreasing self-renewal capacity *in vitro* and tumorigenic capacity *in vivo*	Primary human glioblastoma cells and glioblastoma mouse model	20 µM – 3 days 15mg/kg/day – 5 times per week (70 days)	[169]
Induced intrisic and extrinsic pathways of apoptosis, caused cell cycle arrest at G1; inhibited production of pro-inflammatory cytokines (TNF-a, IL-1b, IL-6 and GM-CSF)	*In vitro* – B16F10 mice melanoma cells	2 µg/mL – 3 days	[174]
Induces cell cycle arrest (G2/M) and apoptosis of endothelial cells (HUVECs) by increasing activation of p53. Blocks angiogenesis *in vitro* and *in vivo* and supresses tumor growth (no DNA damage in HUVECs)	*In vitro*: Endothelial cells (HUVECs) *In vivo*: xenograft lung tumor (A549) in mice	50 µM – 48 h 30 mg/kg for 21 days	[175]
Inhibition of telomerase activity inducing senescence by overexpressing p53	*In vitro*: human breast cancer cells (MCF-7)	20 µM – 96 h	[176]
Cyclins inhibition by binding to the ATP-Mg2+ pocket of CDKs (Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25) Antiproliferative effect; inhibition of DNA replication	CDKs inhibition: non-cellular kinases assay *In vitro* antiproliferative assay: human cancer cell lines - HeLa (cervical cancer), MCF-7 (breast cancer), and SW480 (colon cancer) DNA replication: SW480 cell line	CDKs: 17 to 33 µM Antiproliferative : 8 – 22 µM DNA replication: 23 µM	[177]
Antiproliferative effect, induces apoptosis	*In vitro*: HepG2 (human hepatocellular carcinoma cell line)	10 µg/mL – 48 h	[178]
Induces apoptosis, inhibition of migration and invasion of cancer cells mediated by down-regulation of COX-2; inhibition of tumor growth	In vitro: human gastric cancer cell lines (BGC-823 and SGC-7901) *In vivo*: gastric cancer model (BGC-823) in mice 60 mg/Kg - 5 days/week for 15 days	3-4 µg/mL – 72h	[179]
Induces apoptosis, inhibition of migration and invasion in thyroid cancer cells; antitumor effect	*In vitro*: TPC-1 (human thyroid cancer cell line) *In vivo*: thyroid cancer model in mice	5 µg/mL – 48 h 40 mg/Kg – 15 days	[180]
DNA intercalation, inhibition of topoisomerase I and antiproliferative effects	Non-cellular assays: DNA melting temperature assay (calf-thymus DNA), DNA relaxation reaction with topoisomerase I assay *In vitro*: human cancer cell lines - liver carcinoma (HepG2) and gastric carcinoma (SGC-7)	Topo I inhibition: 150 µM – 30 min IC50: 46-74 µM – 48 h	[171]

Inhibition of angiogenesis *in vitro* (decreased endothelial cells migration) and *in vivo* (inhibition of microvessel formation, reduction of VEGF levels)	***In vitro*: endothelial cells (HUVECs) *In vivo*: B16F10 mice melanoma cells inducing angiogenesis**	**33 µg/mL 10 mg/Kg for 5 days**	[181]
DYRK1A inhibition, resulting in apoptosis in head and neck cancer cell lines (*in vitro*) and antitumor effect	*In vitro*: various head and neck squamous cell carcinoma (HNSCC) cell lines *In vivo*: HNSCC tumors in mice	Harmine concentration for apoptosis induction *in vitro* not indicated 15 mg/Kg evry 3 days for 21 days	[170]

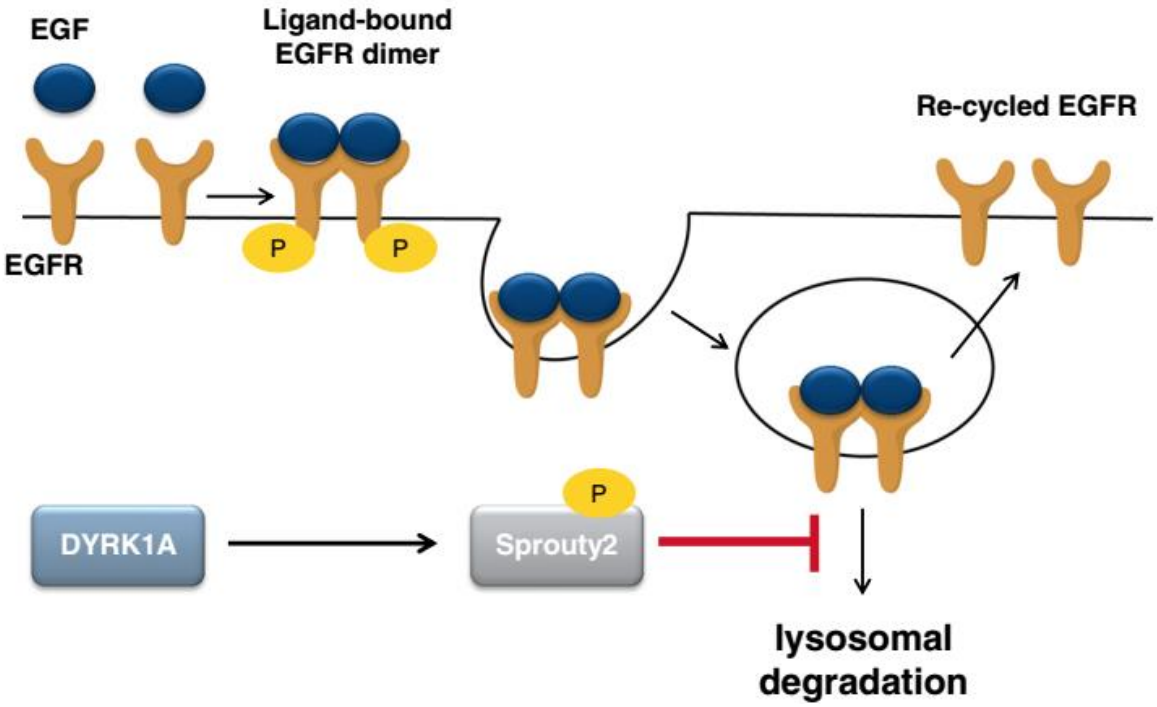


Figure 19. EGFR stabilisation mediated by DYRK1A. The kinase phosphorylates Sprouty2 (modulator of receptor tyrosine kinases acting in a growth factor- and cell-specific manner), which will inhibit EGFR lysosomal degradation after endocytosis, leading to the recycling of EGFR to the cell surface. This will cause increased EGFR-dependent proliferation and self-renewal of the cell. Adapted from [182].

Despite the various potential pharmacological activities of harmine, its neurotoxicity observed *in vivo* causing tremble, twitch and jumping in mice limits its potential for therapeutic uses [183,184]. Nonetheless, given the interesting effects of harmine in cancer cells *in vitro* and tumors *in vivo*, it raised the interest and led to the use of harmine as a starting point for the development of derivatives that would offer less toxicity while still keeping its pharmacological activities.

Therefore, **harmine derivatives** were synthetized and investigated by several groups [183,185–194]. Complexing harmine with palladium had a cytotoxic effect on leukemia mouse cell lines in similar concentration range than cisplatin and 5-FU [185]. The effect of introducing a single substituent in the harmine skeleton did not improve markedly the antiproliferative activity compared to harmine [183]. However, JKA97, a molecule substituted in position 1 has shown to provoke apoptotic effects and cell cycle arrest effects in breast and colon cell lines, as well as inhibition of tumour growth *in vivo* [186,187]. Compounds with substitution in position 9 also showed antiproliferative effects and inhibition of angiogenesis [188,189]. Nevertheless, an i*n vivo* study with compounds containing a single substitution in this position showed similar neurotoxicity compared to harmine [183].

While the effect of single substitution was not very promising, adding multiple substitutions seemed to have shown a superior antiproliferative activity when compared to harmine in multiple cancer cell lines [190,191]. Especially compounds trisubstituted in positions 2, 7 and 9 showed good activity, exemplified by a series of compounds inducing cell cycle arrest and apoptosis, through inhibition of the polo-like kinase 1 (PLK1) that participates in the cell cycle progression [192]. *In vivo* studies showed that the trisubstituted derivatives are also superior in the inhibition of the tumour growth in a sarcoma model and a lung model [193]. Finally, it is noteworthy that these and other derivatives had less systemic toxicity than harmine [193,194]. Another recent study showed the potential of a disubstituted compound (positions 1 and 9) with pro-apoptotic effects *in vitro* and tumour growth inhibition *in vivo* [188]. **Table 3** details the information here presented.

Table 3. Harmine derivatives with effects in cancer cells *in vitro* and antitumor *in vivo*.

Compound	Structure (substituent position in harmine – Fig. 16)	Information	IC_{50} (µM)	Reference
Harmine-Pd complex	2 – trans-[Pd(DMSO)2Cl2]	Cytotoxic effect on leukemia mouse cell lines	0.4	[185]
Compounds 1-4 bearing a 3,4,5-trimethoxyphenyl substituent at position-1	1	Weak antiproliferative effects	36 to < 200 In different cancer cell lines of different origins	[191]
Compounds monosubstituted at position 9 vs dissubstituted at positions 3 and 9	3 and 9	Compounds dissubstituted at positions 3 and 9 had better antiproliferative and antitumor activities; Compounds monosubstituted at position 3 were not better than harmine	The most potent compound: 8C – from 11 to 116 µM depending on the cell line – tested in different cancer cell lines of different origins	[183]
JKA97 benzylidene harmine 1-styryl-9H-pyrido-[3,4-b]-indole	1	Induces apoptosis (mitochondrial pathway) in colon cancer cells; Induces apoptosis and cell cycle arrest (G1 phase) in breast cancer cell lines; inhibits tumour growth *in vivo* (colorectal cancer and breast cancer models)	10 (colon cancer cell line) 7, 10 and 19 (breast cancer cell lines)	[186,187]
Screen with 17 new derivatives (N2-benzylated)	1 and 9	Tested in several cancer cell lines; 3c most potent: induces apoptosis (via inhibition of Akt phosphorylation) and promoted production of ROS; inhibited tumour growth *in vivo* (colorectal cancer model)	> 10 for R9 (N2-benzylated) substituted in several cancer cell lines; The most potent compound: 3c: 0.5 to 5.0 in several cancer cell lines	[188]
B-9-3 (1, 4-bis (1-methyl-9H-pyrido [3, 4-b] indol-9-yl) butane)	9	Induces apoptosis and inhibits angiogenesis (inhibits migration of endothelial cells by blocking VEGFR2); inhibition of tumour growth *in vivo* (mice lung cancer model)	6 µg/mL in endothelial cells (*in vitro*) and 20 mg/Kg (*in vivo*)	[189]
N2-benzylated β-carbolinium bromates	1, 2, 7 and 9	Antiproliferative effect in several cancer cell lines	> 10 in several cancer cell lines of different origins	[190]

Screen with 60 derivatives	1, 2, 7 and 9	Antiproliferative effect in several cancer cell lines	Most active: N2-benzylated β-carbolinium bromides: > 10 in several cancer cell lines of different origins	[191]
β-carboline derivatives: DH281, DH285 and DH287	2, 7 and 9	Induced cell cycle arrest and apoptosis (cervical cancer cell line), through inhibition of the polo-like kinase 1 (PLK1) that participates in the cell cycle progression	0.5 to 10 in several cancer cell lines	[192]
Several derivatives (N2-alkylated quaternary β-carbolines) screened	2, 7 and 9	Antiproliferative and antitumor activities (mice lung cancer and sarcoma); lower acute toxicity	Best compounds trisubstituted > 5 in several cancer cell lines – inferior to cisplatin	[193]
2DG-Har-01 and MET-Har-02	2, 7 and 9	Induction of apoptotic cell death (MET-Har-02 – 7.5 µM in liver carcinoma cells); antitumor effect of both compounds (mice sarcoma model); lower acute toxicity and neurotoxicity than harmine	Better than harmine (~ 15 to 60) several cancer cell lines	[194]

AIMS

Based on the interest and previous investigations with the harmine derivatives as explained in the last section of the introduction, the main aim of this work was to elucidate, at least partly, the mechanism of action of the potential anticancer harmine derivative CM16.

The specific aims can be summed up as follows:

- To evaluate the antiproliferative effects of the three best novel harmine derivatives in different cancer cell lines (initiated at NAMEDIC – University of Namur);
- To investigate the potential of the lead compound CM16 to inhibit protein synthesis of cancer cells *in vitro*;
- To identify potential targets of CM16 in cancer cells;
- To evaluate how this compound affects the proteome of cancer cells.

In order to meet these aims this work will be divided in two parts. In the first one we will evaluate the general antiproliferative effects of the harmine derivatives and the mechanism of protein synthesis inhibition of the lead compound CM16. The second part of this work will be dedicated to the evaluation of the proteome of glioma cells affected by treatment with CM16. We have selected a glioma model to conduct this study because of the theoretical BBB penetration properties of CM16 making brain tumours as first cancer type candidate for future perspectives.

PART I: DECIPHERING THE ANTIPROLIFERATIVE EFFECTS OF HARMINE DERIVATIVES: *IN VITRO* EVALUATION OF CELLULAR AND BIOCHEMICAL EFFECTS WITH AN EMPHASIS ON PROTEIN SYNTHESIS

1 INTRODUCTION AND AIMS

The search for new harmine derivatives with anticancer properties: the beginning

Harmine derivatives were synthetized in the context of a first project aiming at discovering new potential MAO inhibitors carried out at NAMEDIC – University of Namur. MAO inhibitors are clinically used in the treatment of psychiatric and neurological disorders, such as depression. The side effects of these inhibitors, however, limit their clinical use [195]. Given the fact that harmine is a potent MAO-A inhibitor, harmine was used as a starting point to synthetize new derivatives (NAMEDIC – University of Namur) [196]. It was observed that an increase in the number of substituents markedly decreased the MAO inhibition in comparison to harmine. Then, the new trisubstituted harmine derivatives were preliminarily tested in cancer cell lines to evaluate their antiproliferative potential and, interestingly, they displayed effects with lower concentrations than harmine (e.g. compound 5a, results detailed in the section below). Due to this fact and to the aforementioned evidence from the literature showing that harmine and derivatives have showed activity *in vitro* and *in vivo* in different cancer models, the group at NAMEDIC (University of Namur) synthetized new trisubstituted harmine derivatives to investigate their potential as anticancer agents. The biological activities of these compounds were evaluated in collaboration with our lab prior to the present work.

Novel harmine derivatives: CV and CM series

A first series of derivatives was synthetized and the compounds trisubstituted in positions 2, 7 and 9 tested in cancer cell lines *in* vitro. This first generated series revealed three best candidates (5a, 5k and 5o, **Figure 20**) inhibiting proliferation with activity superior than harmine (IC_{50} 0.3-3.0 µM versus 28 µM, see **Table 4**) when screened in three glioma (U373, Hs683 and T98G) and two oesophageal cancer (OE21 and OE33) cell lines as carcinoma models. Importantly, while harmine seems to exert its effects in cancer cells *in vitro* through

DNA intercalation and DYRK1A inhibition (reviewed in Introduction, section 5.2.2), the evaluation of DNA intercalation with 5a, 5o and CM16 turned out to be negative (**Figure 22**, results from thesis of C. Meinguet, NAMEDIC – University of Namur). Similarly, 5a and 5k did not affect DYRK1A activity as opposed to harmine. Those kind of tri-substitutions on harmine skeleton thus appeared to impair its main mechanisms of action leading to different mode of action. By contrast, those compounds appeared rather to be possible protein synthesis inhibitors after having been screened on the NCI 60 cell line panel and compared to the large NCI compound database [197].

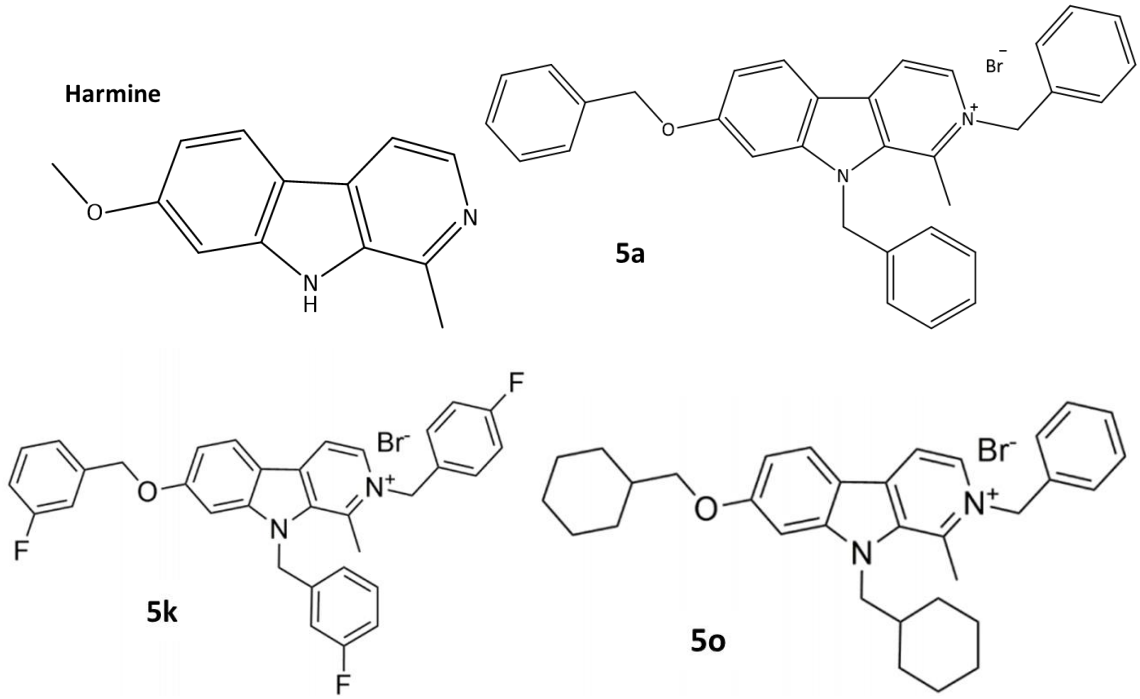


Figure 20. Structure of harmine and the previously investigated harmine derivatives of the CV series 5a, 5k and 5o.

Considering the requirements in the development of a new drug, however, the solubility of these compounds at physiological pH was a major drawback (**Table 4**). After a guided synthesis to improve solubility while keeping the antiproliferative activity, a novel series of harmine derivatives was yielded: the CM series, with the three best candidates (CM11, CM14 and CM16) shown in **Figure 21**. This new study was carried out by Céline Meinguet during her doctoral thesis (NAMEDIC – University of Namur). The optimization of the molecules encompassed a 3D-QSAR (three dimensional quantitative structure-activity relationship)

study, followed by the synthesis of a second series of derivatives (CM series) and characterization of their solubility at the physiological pH and calculation of lipophilicity.

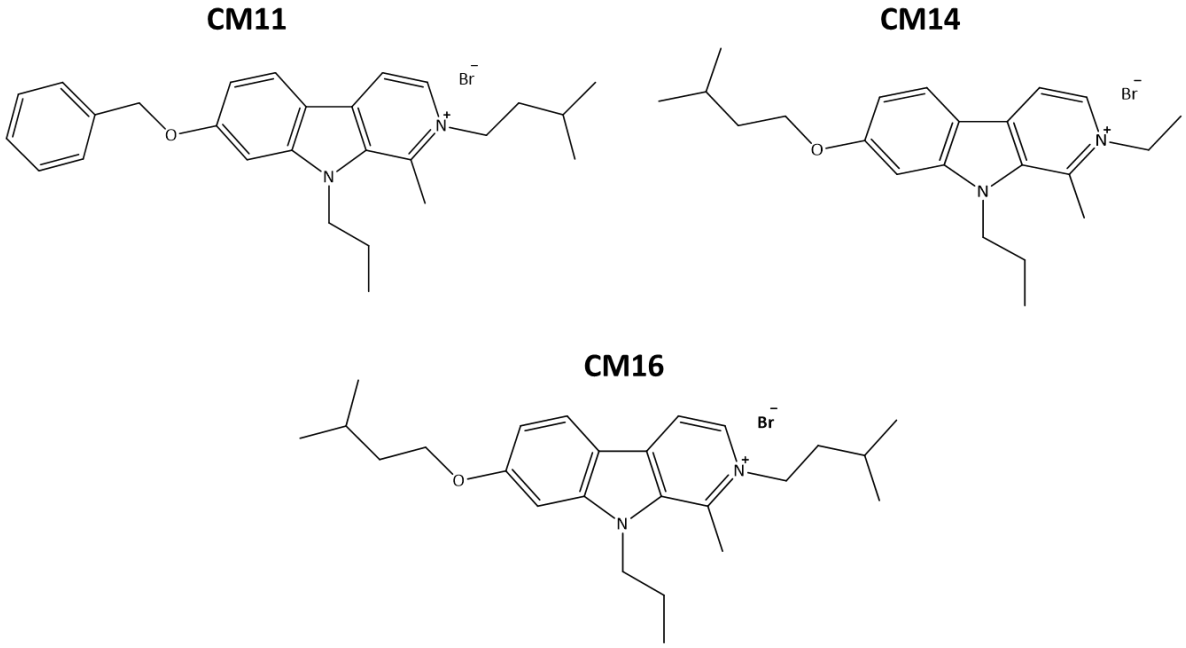


Figure 21. Structure of the harmine derivatives of the CM series: CM11, CM14 and CM16.

The results were encouraging, with an overall increase in solubility and decrease in lipophilicity for all the new synthetized compounds keeping the good antiproliferative activity, superior to harmine (**Table 4**). The best compound concerning the ability to inhibit cancer cells proliferation was CM16 (**Figure 21**) with an average IC_{50} lower than 0.3 µM when tested in five different cell lines (U373, Hs683, T98G, SK-MEL-28 and A549) of different origins (glioma, melanoma and lung cancer) and a solubility of 189 µg/mL in phosphate buffer (pH 7.4) [198]. In addition to that, a predictive model (software Discover Studio) showed that CM16 has a high blood-brain-barrier (BBB) penetration potential, an important characteristic for the development of a potential drug targeting brain cancers [198].

Table 4. Summary of main results previously obtained for harmine and derivatives.*

Compound	Harmine	5a	5k	5o	CM16	CM11	CM14
IC_{50}[a] ± SD[b] (µM)	28±3	0.7±0.2	2.7± 0.6	0.3±0.05	<0.3	1.0±0.7	0.6±0.4
Solubility ± SD (pH 7.4; 25 °C (µg/mL)	NT[c]	73±4	2±1	<2	189±13	164±6	186±5
Calculated lipophilicity (cLogP)	NT	5.3±1.51	5.4±1.5	6.7±1.5	3.32±1.51	2.74±1.51	2.95±1.51
DNA intercalation	Yes	No	NT	No	No	NT	NT
DYRK1A inhibition	Yes	No	No	NT	NT	NT	NT

*** Results obtained by C. Meinguet for her thesis presented in 2015 (NAMEDIC – University of Namur).**

[a]IC_{50} determined in different cell lines, as described in the text; [b]SD: Standard Deviation; [c]NT: not tested.

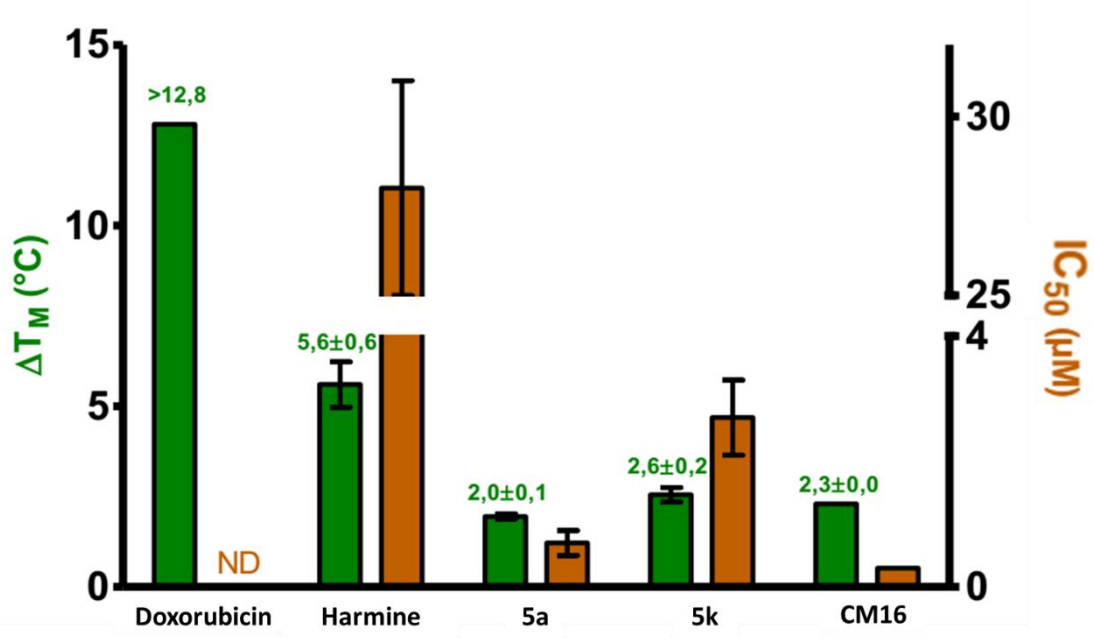


Figure 22. Correlation between the different DNA (calf thymus) melting temperatures, ΔT_M ($\Delta T_M = T_{M\,(DNA + compound)} - T_{M\,(DNA)}$), measured in the presence of the evaluated compounds and the mean IC_{50} obtained in five human cancer cell lines. Ratio DNA:compound was 1:4 for doxorubin and 1:2 for the other compounds tested. The results presented here were collected from the doctoral thesis of C. Meinguet (NAMEDIC – University of Namur, presented in 2015).

Given the interesting drug-like characteristics of the new series of synthetized harmine derivatives (CM series) [198] and the indication of a potential protein synthesis inhibition effect for the previous series (CV series – 5a and 5k) [197], this study was dedicated to confirm

and elucidate the mechanism of action of these molecules as potential protein synthesis inhibitors in cancer cells *in vitro,* notably by investigating on which phase of translation they could exert their antiproliferative effects and eventually identifying specific potential targets. Importantly, CM16 was also shown to not intercalate into the DNA (**Figure 22**). The majority of the results obtained from this first study are published in the European Journal of Pharmacology (vol. 805, pg. 25-35, 2017, DOI: 10.1016/j.ejphar.2017.03.034) (Appendix 3).

2 MATERIAL AND METHODS

2.1 Compounds

The new harmine derivatives used in this study were synthetized in and provided by the NAMEDIC laboratory of the University of Namur under the supervision of Profs. Johan Wouters and Bernard Masereel in the context of a fruitful collaborative FNRS and Télévie project (FNRS 3.4525.11; Télévie 7.4547.11F). Their synthesis are described in [198]. The structures of the β-carbolines CM11 (MW=481 g/mol), CM14 (MW=438 g/mol) and CM16 (MW=461 g/mol), as well as harmine (MW=212.25 g/mol) itself are shown in **Figure 20** and **Figure 21**. The structure and the purity of each harmine derivatives synthesized at NAMEDIC (University of Namur) were confirmed by analytical methods. Indeed, the values of experimental elemental microanalyses (C, H, N) were within 0.4% of the theoretical values (Thermo-Finnigan-Flash EA 1112 series), and 1H-NMR spectra were in accordance with the proposed structures (Jeol JNM ECX 400MHz. The data are in the thesis of Dr. C. Meinguet (University of Namur) [199].

2.2 Cell lines, media and cell culture reagents

The following human cancer cell lines were used in this study:

- oligodendroglioma Hs683 (ATCC code HTB-138),
- melanoma SK-MEL-28 (ATCC code HTB-72) and
- breast adenocarcinoma MDA-MB-231 (ATCC HTB26).

The non-cancerous and non-transformed human dermal fibroblasts NHDF (Normal Human Dermal Fribroblast - Lonza CC-2509) and lung fibroblasts NHLF (Normal Human Ling Fibroblast - Lonza CC-2512) were also selected for the evaluation of the selectivity and possible toxic effects of the β-carbolines *in vitro*.

Cells were cultivated in 25 or 75 cm^2 culture flasks (Sarstedt, Berchem, Belgium) at 37 °C in a humidified atmosphere of 5% CO_2 in specific cell culture media. RPMI culture medium supplemented with 10% fetal bovine serum (FBS – Thermo Fisher Scientific, Aalst, Belgium), 200U penicillin-streptomycin, 0.1 mg/ml gentamicin and 4 mM L-glutamine was used to cultivate Hs683, SK-MEL-28 and MDA-MB-231 cells, while fibroblast medium FBM (Lonza, Verviers, Belgium) supplemented with 2% fetal bovine serum, 0.1% insulin, 0.1% hFGF-b and

0.1% gentamicin/amphotericin B (GA-1000) was used for NHDF and NHLF cell lines. RPMI medium and supplements were purchased from Westburg (Leusden, The Netherlands). Some of the experiments described below with MDA-MB-231 and NHDF cell lines were performed by our collaborators in Canada (Jennifer Chu and Jerry Pelletier, McGill University, Canada). In their lab they cultivated MDA-MB-231 cells in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine at 37 °C and 5% CO_2.

2.3 Cell growth inhibition evaluation

Determination of cell growth inhibition was performed by means of the MTT colorimetric assay. Briefly, cells were seeded in 96 well plates (Sarstedt, Berchem, Belgium) at concentrations ranging from 10,000-50,000 cells/ml (depending on the cell type) 24 h prior to treatment to ensure attachment and then treated with harmine, CM11, CM14 or CM16 at concentrations ranging from 0.01 to 100 µM or left untreated for 72 h. Cell viability was estimated by means of the mitochondrial reduction of a yellow MTT salt - (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - Sigma Aldrich, Overijse, Belgium) into the purple product formazan solubilized in DMSO as previously described [200]. For this purpose, following the treatment period, supernatants were replaced by a MTT solution at 0.5 mg/ml and after 4 hours incubation at 37 °C, plates were centrifuged at 290x *g* for 6 min (Eppendorf, Rotselaar, Belgium), the MTT solution removed and DMSO added to each well. The amount of living cells is proportional to the intensity of the purple colour, making relative quantification possible through measurement of the optical density by spectrophotometry. Measurements were made in a plate reader Biorad 680XR (Biorad, Nazareth, Belgium) at 570 nm (reference wavelength 630 nm).

2.4 Evaluation of cytostatic effects through videomicroscopy

Computer-assisted phase contrast microscopy was performed as previously described [201]. Cell lines Hs683, SK-MEL-28, MDA-MB-231 and NHDF were seeded in 25 cm^2 culture flasks and left untreated or treated with harmine, CM11, CM14 or CM16 at cytostatic concentrations. Culture flasks were placed under the microscope (Leica, Diegem, Belgium) in a 37 °C atmosphere in an in-house built system (**Figure 23**) and pictures of one field were taken every four minutes during a 72 h period and further compiled into a short movie [201]. Quantitatively, global growth ratio was determined based on cell counting on pictures

corresponding to 24 h, 48 h and 72 h in comparison to 0 h. Cell counting was performed with MatLab software (The Mathworks, Massachusetts, United States).

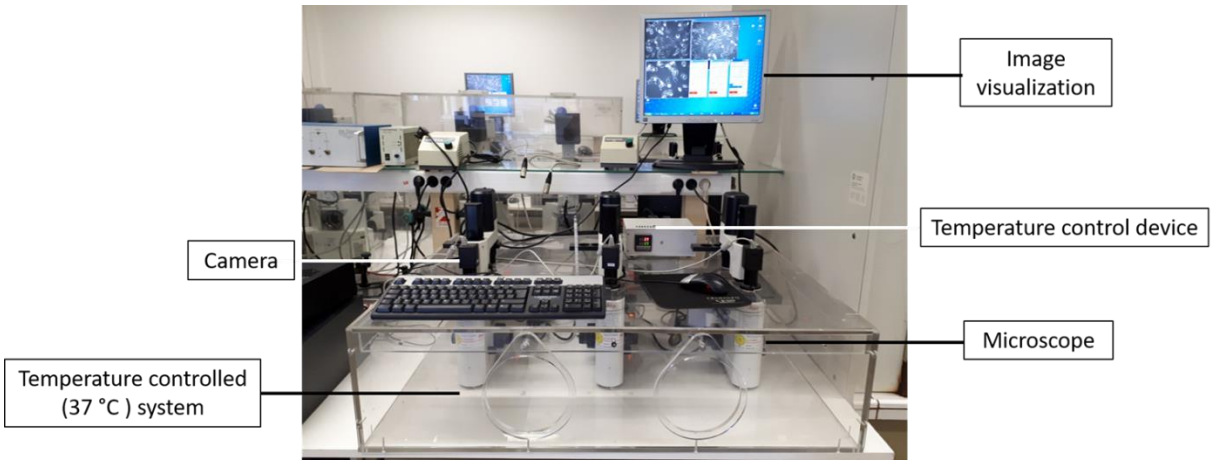


Figure 23. Videomicroscopy system at our laboratory (Laboratoire de Cancérologie et de Toxicologie Experimentale, Faculté de Pharmacie - ULB).

2.5 Cell cycle evaluation

Cell cycle analysis was performed by the measurements of DNA content stained with propidium iodide (PI) with flow cytometer. Hs683 and SK-MEL-28 cells were seeded in cell culture flasks and left untreated or treated with CM11, CM 14 or CM16 at their cytostatic concentrations for 24 h, 48 h and 72 h. After their detachment by trypsin, the cells were centrifuged, washed in PBS, centrifuged again (Hermle Z400K, Wehingen, Germany) (10 min, 1500x g, 4 °C), and the pellets were resuspended in ice-cold ethanol 70% for fixation and permeabilization overnight. Staining with a 0.08 mg/ml propidium iodide (Sigma Aldrich, Overijse) solution (0.08 mg/ml PI; 0.2 mg/ml RNase in PBS) was conducted at 37°C for 30 min after a PBS wash. Samples were kept on ice till analysis by a Cell Lab Quanta flow cytometer (Beckman Coulter, Analis, Suarlée, Belgium). 10,000 events per sample were analysed and each experimental condition was evaluated in four replicates.

2.6 National Cancer Institute (NCI) screening

The harmine derivative CM16 was sent to the NCI and evaluated in their screening panel of 60 cancer cell lines. The detailed information on this screening is presented in the Results and Discussion section.

2.7 Transcriptomic analysis of the National Cancer Institute data

We have conducted a transcriptomic comparison of cell lines displaying various levels of sensitivity to CM16 among the NCI 60 cell line panel. The transcriptomic analysis was carried out using information available from the NCI *CellMiner* database, which provided us with a score on each investigated gene in the cell line considered. The Z score is determined for each probe/cell line pair by subtracting its intensity from the probe mean across the 60 cell lines and followed by division by the standard deviation of the probe across the whole panel of the 60 cell lines. The average transcript Z score, i.e. combined gene expression for a determined gene, is calculated as the mean across all probes and probe sets that passed quality control criteria. Comparison of each gene between the least and most sensitive groups of cell lines was performed by T-test comparison of the Z score with the Statistica Software.

2.8 Analysis of CM16 effects on transcription

RNA neosynthesis was evaluated through incorporation of a nucleoside analogue, 5-ethynyl-uridine (EU), using the Click iT®-RNA HCS (Invitrogen, Life Technologies, Merelbeke, Belgium) according to the manufacturer's instruction. In this assay, the alkyne-containing nucleosides that have been incorporated in nascent RNA inside the cell react with a fluorescent dye containing an azide moiety (**Figure 24**). Quantity of neosynthesized RNA is thus proportional to the fluorescent level measured. Briefly, Hs683 or SK-MEL-28 cells were seeded and after attachment they were either left untreated (negative control) or treated with 0.5, 1.0 or 5.0 µM of CM16 for 3, 6 and 24 h or with 25 µM of the positive control actinomycin (Life Technologies, Paisley, UK) for an hour. Cells were then incubated with 4 mM of the alkyne analogue 5-ethynyl uridine for two hours and further fixed with 3.7% formaldehyde solution in PBS. Afterwards cells were incubated with the fluorophore Alexa Fluor 488 azide for 30 minutes in the absence of light and fluorescence readings (ex/em: 495/520 nm) were carried out on a microplate reader (SynergyMX Biotek, Winooski, USA). Normalization according to cell number was carried out with DNA staining (HCS NuclearMask®) and reading was performed in the same equipment (ex/em: 350/460 nm).

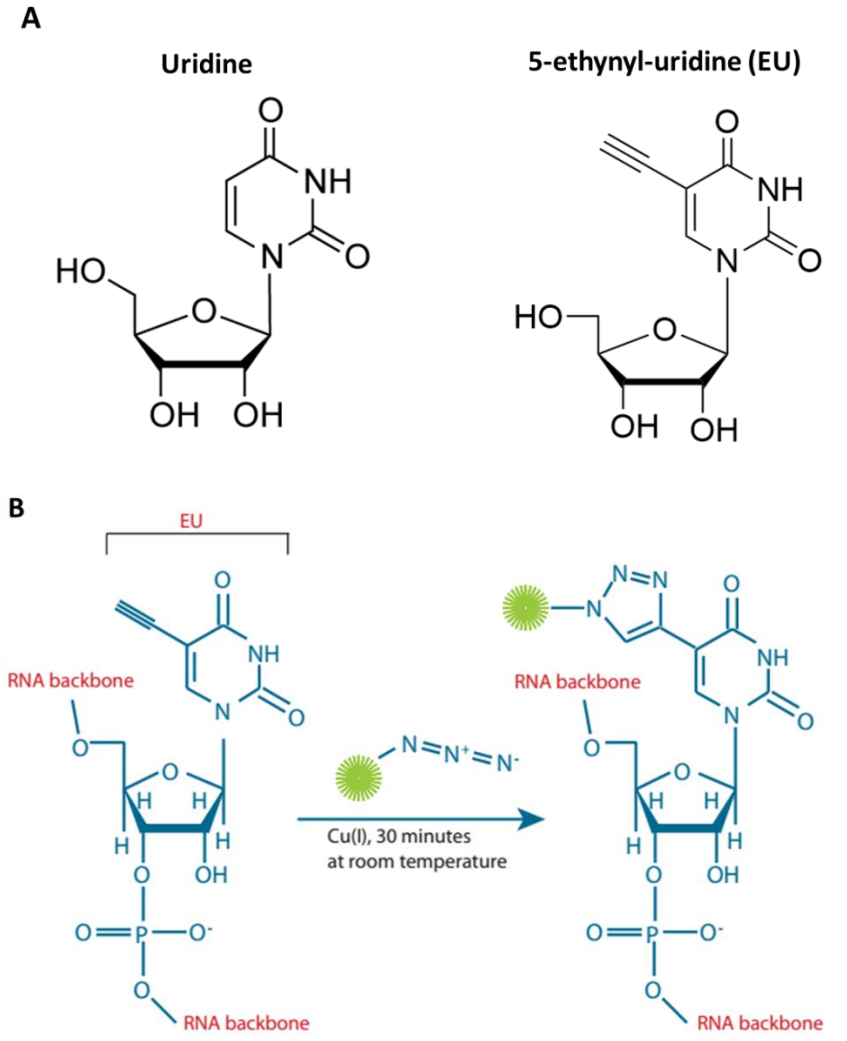


Figure 24. (A): Uridine and 5-ethynyl-uridine (EU) and (B) the reaction between EU and azide modified dye. Adapted from the Invitrogen protocol for Click-iT® RNA HCS assays.

2.9 Protein synthesis evaluation

2.9.1 Fluorescent method

To evaluate the effects of CM16 on neosynthetized proteins of cancer cells, the Click-iT® AHA alexa fluor 488 kit (Invitrogen, Life Technologies, Merelbeke, Belgium) was used. Similarly to the analogue nucleoside incorporation assay described before, the L-azidohomoalanine methionine analogue is incorporated in newly synthesized proteins and reacts with an alkyne coupled to alexa 488 fluorescent dye (ex/em: 495/520nm) allowing measurement and relative quantification. Hs683 and SK-MEL-28 cells were seeded in 96 wells plates and left untreated or treated with 0.5 and 5.0 µM of CM16 or with the positive control cycloheximide (Santa Cruz Biotech., Heildelberg, Germany) 0.1 mM for 1 h. The treatment was followed by the addition of L-azidohomoalanine (1/1000) for four hours. After fixation with formaldehyde, the

neosynthesized proteins were stained with Alexa Fluor 488 according to the manufacturer's recommendations. Normalization according to cell number was carried out as described in the user manual with Hoescht counterstaining. The fluorescent signal was measured in a microplate reader (SynergyMX Biotek, Winooski, USA: ex/em: 495/520 nm for Alexa Fluor 488 and 350/460 nm for Hoescht).

2.9.2 ^{35}S Methionine incorporation

These experiments have been conducted by Jennifer Chu in the lab and under the supervision of Prof J. Pelletier (McGill University, Canada). The general procedure they used is provided here below. MDA-MB-231 or NHDF cells were seeded at a density of 50,000 cells per well in a 12 well cell culture plate one day prior to the labelling experiment. Cells were then incubated with CM16 at concentrations ranging from 1 to 15 µM or left untreated in methionine/cysteine-free DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-Glutamine for a total of 1 hour and 20 minutes. During the last 20 minutes, [^{35}S]-methionine/cysteine (150-200 µCi/ml) (Perkin Elmer, Waltham, MA) was added to the cells. At the end of the incubation, cells were washed twice with ice-cold PBS and incorporation of the labelled amino acid was terminated through the addition of RIPA buffer. Newly synthesized radiolabelled proteins were precipitated on 3 MM Whatman™ paper (pre-blocked with 0.1% L-methionine) using trichloroacetic acid (TCA) and washed twice with 5% TCA, followed by two washes of ethanol. Samples were then dried and quantitated using scintillation counting. CPMs (counts per minute) were normalized to total protein, which was determined using the Detergent Compatible (DC) Assay (Bio-Rad).

2.10 Fluorescence assays

2.10.1 Cell penetration and distribution analysis

The fluorescence properties of CM16 allowed us to qualitatively analyse the CM16 cell penetration and distribution. These properties were studied by Céline Meinguet who synthesized the compound during her PhD (NAMEDIC – University of Namur). At pH 7.4, the maximum excitation is observed at 330 nm and the emission peak at 439 nm (**Figure 25**).

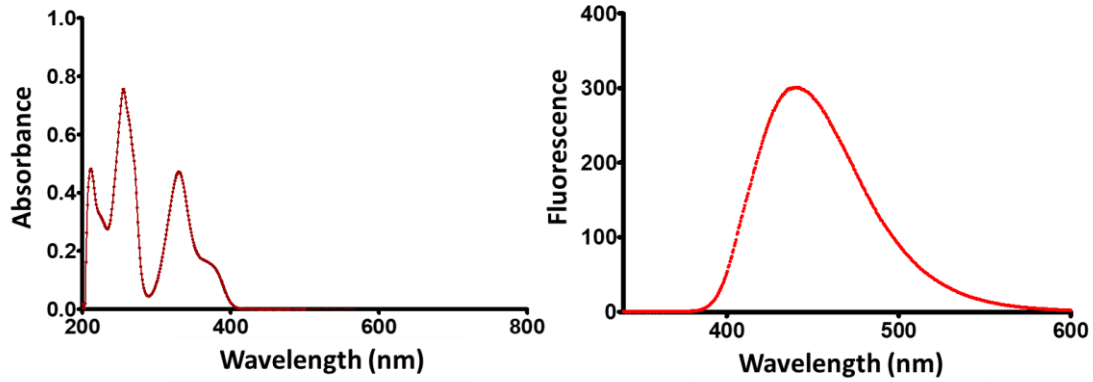


Figure 25. Absorption and fluorescence emission spectra of CM16 4 µM in phosphate buffer (pH 7.4), performed at NAMEDIC – University of Namur.

Cancer cells (Hs683, SK-MEL-28, MDA-MB-231 and NHDF) were seeded on glass coverslips in 6 wells plates and, after attachment, treated with 5 µM CM16 or left untreated. For imaging of the living cells, coverslips were rapidly rinsed with PBS and transferred to a slide. Images were captured with the Imager M2 fluorescence microscope (Carl Zeiss, Zaventem, Belgium) coupled with the AxioCam ICm1 and AxioImager software (Carl Zeiss, Zaventem, Belgium). Hs683 and NHDF cells were treated for periods ranging from 5 min to 3 h, SK-MEL-28 for 3 and 6 h and MDA-MB-231 for 3 h.

2.10.2 Endoplasmic reticulum staining

Endoplasmic reticulum (ER) staining in Hs683, SK-MEL-28 and MDA-MB-231 cells was performed with glibenclamide ER-tracker red dye (Molecular Probes - Life Technologies, Merelbeke, Belgium) and analysed by fluorescence microscopy. Glibenclamide will bind to receptors of ATP-sensitive K^+ channels, which are abundant in the ER. Cells were seeded on coverslips and after attachment they were either left untreated or treated with 5.0 µM CM16 for 3 h. After the treatment period, cells were incubated with 1 µM of the dye solution in PBS for 30 min at 37 °C. The staining solution was then replaced with cell culture medium and sample-containing coverslips were transferred to microscope slides. The imaging of living cells was performed similarly to description in the prior section (2.9.1) with fluorescence microscope (Carl Zeiss, Zaventem, Belgium).

2.10.3 Microfilament staining

Microfilament staining of Hs683 and SK-MEL-28 cells treated with 0.5 and 5.0 µM of CM16 for 1, 3 and 24 h was performed. Briefly, cells were seeded on glass coverslips in 6 well plates and

after attachment treated with CM 16 at 0.5 or 5.0 μM for 1, 3 and 24 h or left untreated. Cells were then washed with cold PBS, fixed with 3.5% formaldehyde for 15 min at 4 °C, permeabilized with a lysis buffer (2 mM $MgCl_2$, 0.2 mM DTT, 10% glycerol, 0.01% triton X100) and labelled with phallacidin conjugated with Alexa Fluor 488 fluorochrome in 0.1% BSA (bovine serum albumin) in PBS (w/v) (Molecular Probes; Invitrogen, Merelbeke, Belgium). After being transferred to slides, coverslips containing cells were analysed under fluorescence microscope (Imager M2 coupled with the AxioCam ICm1 and AxioImager software - Carl Zeiss, Zaventem, Belgium).

2.11 Investigation of the translation initiation: ribosome and polysome organization study

This study was conducted through the separation of the ribosomes and polysomes by ultracentrifugation in a sucrose gradient as described in Cencic et al. [202]. This method allows the analysis of the organization of ribosomes and polysomes (mRNA bound to ribosomes) as it occurs in the initiation phase of translation. The sucrose density gradient that separates the ribosomes and polysomes according to their density will be divided in fractions whose absorbance at 254 nm (specific for RNA) generate a profile. Briefly, Hs683, SK-MEL-28, NHDF, NHLF cells were seeded in cell culture flasks and left untreated (negative control) or treated either with the positive control puromycin (184 μM) for 1 h or 0.5 and 5.0 μM of CM16 for 3 h. Cells were then scraped and collected in a PBS buffer containing 100 μg/ml cycloheximide and centrifuged (400x g, 4 °C, 10 min). Pellets were resuspended in a hypotonic lysis buffer (5 mM Tris pH 7,5; 2,5 mM $MgCl_2$; 1,5 mM KCl), supplemented with cycloheximide 100 μg/mL, DTT 2 mM, 5 μL RNasin Ribonuclease Inhibitor (Promega, Leiden, Netherlands), 10 μL of 10% Triton X-100 and 10 μL of 10% sodium deoxycholate. Samples were centrifuged (16,100x g, 2 min, 4 °C) and supernatant loaded onto a 10 – 50% sucrose gradient and centrifuged for 2 hours at 39,000 rpm (156,213x g) at 4 °C (SW 60 Ti rotor, Beckman, Ramsey, USA). The obtained gradients were then collected in fractions through a constant pump flow and their absorbance measurement carried out at 254 nm in a microplate reader (SynergyMX Biotek, Winooski, USA).

The experiment with the MDA-MB-231 cells was performed once by our collaborators in Canada (J. Pelletier, McGill University). Our original protocol was set up from their own previously. The only differences concern the equipment. Therefore their samples were

centrifuged for 2 hours and 15 minutes at 217,290x g and the gradients were then fractionated while reading UV_{254} absorbance using the Foxy® R1 fraction collector (Teledyne, ISCO).

2.12 Western blot analyses

Treated and untreated Hs683 and SK-MEL-28 cells were scraped and lysed in an ice cold buffer (1% triton-X, 10% glycerol, 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 10 µg/ml aproptinin, 1 µg/ml pepstatin A, 10 mM sodium pyrophosphate, 0.2 mM Na_3VO_4). Samples were centrifuged (16,100x g, 15 min, 4 °C) and supernatant collected. Protein concentration was measured by the bicinchoninic acid assay (BCA) (Thermo Scientific, Leuven, Belgium) prior to gel loading. Samples were denatured for 5 min at 95 °C and 15 to 25 µg proteins were loaded for electrophoresis in polyacrylamide gel (10 or 12%). Proteins were then transferred to PVDF membrane (PerkinElmer, Zaventem, Belgium), blocked with 5% BSA (MP Biomedicals, Illkirch, France) or milk powder (Nestlé, Brussels, Belgium) for 1 h. The primary antibodies used were as follows: eIF2α: 1/400, phospho-eIF2α: 1/400 (Cell Signaling, Leiden, Netherlands), eIF4E: 1/500, eEF2: 1/1000, α-Tubulin: 1/8000, GAPDH: 1/10000 (Abcam, Cambridge, UK), eIF4A1: 1/400 (Origene, Rockville, USA), eEF1A: 1/1000 (Millipore, Darmstadt, Germany). Secondary antibodies coupled with horseradish peroxidase enzyme (HRP) to allow detection by chemiluminescence were purchased from Pierce (Thermo Fisher Scientific, Aalst, Belgium) and used at 1/200 to 1/8000 dilutions for the anti-rabbit IgG and 1/10000 for the anti-mouse IgG respectively. Chemiluminescence was monitored with the Pierce SuperSignal system (Thermo Scientific, Leuven, Belgium) and detected in the ChemiDoc® XRS+ imaging system (BioRad, Nazareth, Belgium).

The western blots with MDA-MB-231 cells were performed by Jennifer Chu in Canada (Prof J. Pelletier, McGill University). Here are their experimental conditions: cells were treated with 10 µM CM16 for 80 mins and lysates were collected by washing the cells with ice cold PBS followed lysis using a buffer composed of 20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton-X100, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM PMSF, 4 mg/ml aprotinin, and 2 mg/ml pepstatin A. Samples were resolved on a 10% NuPAGE gel and transferred to a PVDF membrane (BioRad, Ontario, Canada) for immunoblotting. The following antibodies were used in this study: eEF2, pan eIF2α, phospho-eIF2α (Cell Signaling, Ontario, Canada).

2.13 PERK activity

PERK activity was evaluated by the Life Technologies screening service (Lantha Screen, Madison, USA). The *in vitro* assay used is based on Time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) between a terbium-labelled antibody and the phosphorylated product of the active kinase: TR-FRET increases proportionally to their binding and thereby to the quantity of the phosphorylated product. CM16 compound at concentrations ranging from 0.001 to 100 µM or the control solutions were mixed with the kinase (PERK), its substrate (GFP-eIF2α) in presence of ATP in the wells. After 60 min of reaction at room temperature, the detection mix containing the terbium-labelled antibody (Tb-anti-p-eIF2α) was added and left to equilibrate for one h prior to fluorescence reading.

3 RESULTS AND DISCUSSION

3.1 Antiproliferative effects of harmine and derivatives

3.1.1 Growth inhibition and selectivity to cancer cells

Together with harmine, the novel derivatives were assayed for their potential to inhibit cell growth *in vitro* in three cancer cell lines, i.e., glioma Hs683 cells, melanoma SK-MEL-28 cells and breast carcinoma MDA-MB-231 and two non-cancerous fibroblasts strains, the dermal NHDF and the lung NHLF cells. **Figure 26 A-D** shows the concentration-response curves of each compound on cancerous and non-cancerous cell lines (for the concentration-response curves of each compound on the five cell lines separately see Appendix 4). **Table 5** contains the values of the concentration needed to inhibit 50% of the cell growth after 72 h of treatment (IC_{50}) as compared to the non-treated control. It is noteworthy that the CM compounds are able to inhibit the cell growth of cancerous cells (<1 µM) with a much lower concentration than harmine (~25 µM). Those data allowed us to calculate the selectivity index, i.e. the ratio between the mean IC_{50} concentration on non-cancerous cell lines and the mean IC_{50} on cancer cell models. According to **Table 5**, while these compounds display all a certain level of selectivity towards cancerous cells, the three derivatives are more selective towards cancer cells than harmine, with CM16 displaying the highest selectivity index of 13. The selectivity of this compound towards cancer cell lines was also confirmed by our collaborators (NAMEDIC – University of Namur) when comparing A549 lung cancer model to non-cancerous human fibroblasts (BJ cells) and human endothelial cells (HUVEC cells) [199]. Due to i) its potent activity *in vitro* with a mean IC_{50} of 0.3 µM on cancer cells, ii) its high *in vitro* selectivity index of 13 and iii) its solubility at the physiological pH, CM16 was chosen for the in depth investigation of its potential as an anticancer agent. CM11 and CM14 derivatives were also used in some experiments to verify that results were similar for those analogues and that conclusions are not specific for CM16.

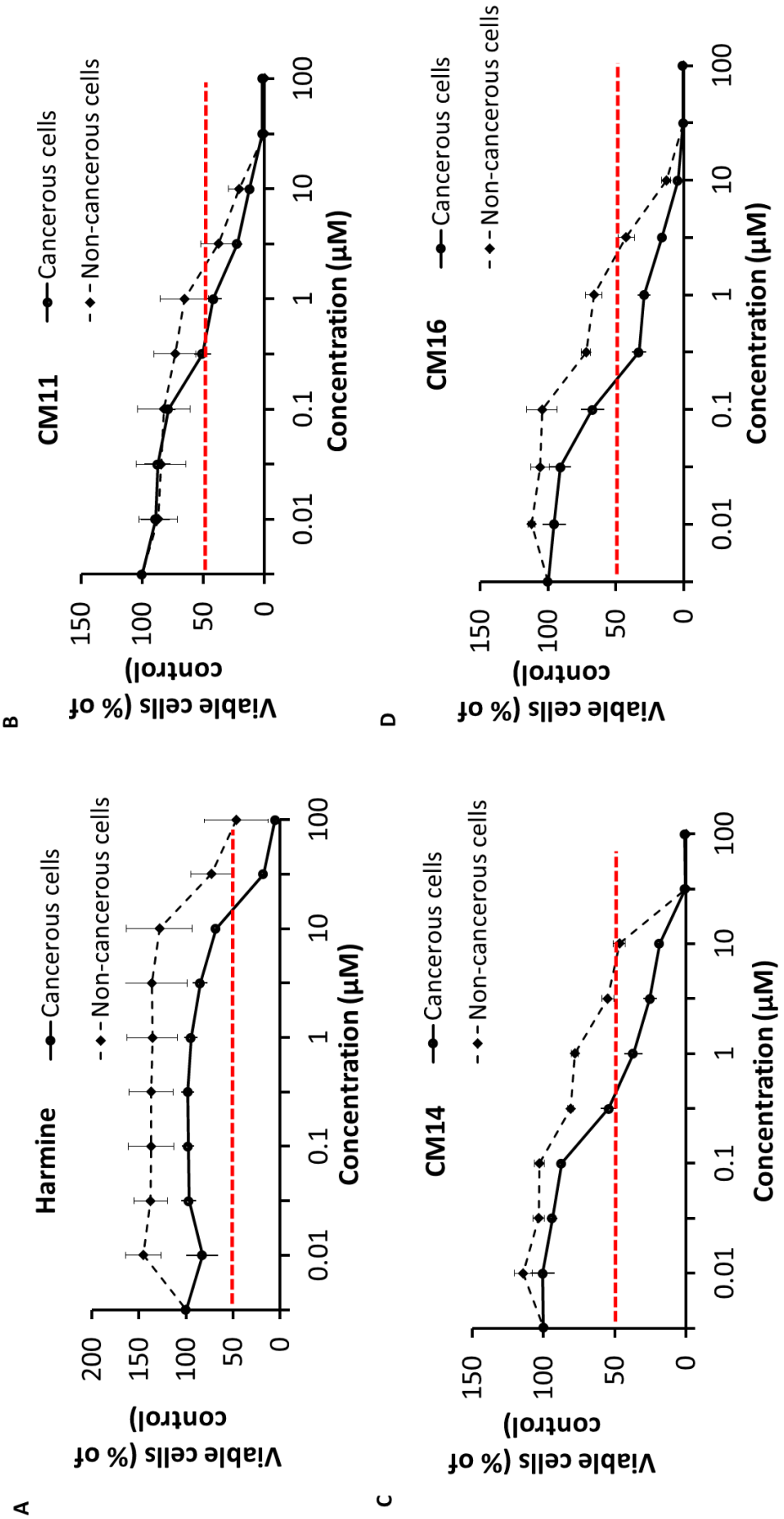


Figure 26. Cell growth inhibition in cancer cells (solid line) versus non-cancerous cells (dashed line) treated with harmine (A), CM11 (B), CM14 (C) and CM16 (D) for 72 h. Cancerous cell lines: Hs683, SKMEL-28 and MDA-MB-231. Non-cancerous cell lines: NHLF and NHDF non-transformed fibroblasts. The red dashed line in the 50% cell viability mark aids the visualization of the concentration of each compound needed to inhibit 50% of cell proliferation for each cell line (IC_{50}). Data are expressed as the mean of viable cells relative to control (100%) ± S.E.M. of the six replicates of one representative experiment.

Table 5. Concentration of harmine and derivatives needed to inhibit 50% of the cell growth (IC_{50}) after 72 h of treatment and selectivity index. Means of these concentrations are also separately presented for cancerous versus non-cancerous cell lines. Selectivity index is based on the mean IC_{50} in cancerous versus non-cancerous cell lines. The selectivity towards cancerous cells is the ratio between the non-cancerous and cancerous cell lines.

Compounds	**IC_{50} (µM) *In Vitro* Growth Inhibitory Concentrations**							**Selectivity Index**
	Human Cancerous Cell Lines				**Human Non-Cancerous Cell Lines**			
	HS683 (oligodendroglioma)	SKMEL-28 (melanoma)	MDA-MB-231 (breast carcinoma)	**Mean ± SEM**	NHDF (Dermal Fibroblast)	NHLF (Lung Fibroblast)	**Mean ± SEM**	
Harmine	25.6	20.7	26.0	**24.1 ± 1.7**	>100.0	51.5	**75.8 ± 24.2**	**3**
CM11	1.5	0.6	0.3	**0.8 ± 0.3**	5.4	0.8	**3.1 ± 2.3**	**4**
CM14	0.8	0.8	0.9	**0.8 ± 0.03**	10.5	5.7	**8.1 ± 2.4**	**10**
CM16	0.2	0.4	0.3	**0.3 ± 0.1**	2.4	5.2	**3.8 ± 1.4**	**13**

3.1.2 Cytostatic effects of harmine and derivatives

According to the cytostatic effects of the previous CV series [197] we aimed first to confirm similar activity of the new selected harmine derivatives in glioma Hs683, melanoma SK-MEL-28 and breast cancer MDA-MD-231 cell lines by means of videomicroscopy. **Figure 27** shows that treatment with the cytostatic concentrations of these compounds (harmine, CM11, CM14 and CM16) reduced the proliferation of the Hs683 glioma cell line as compared to the non-treated control. However, we still observe few cell proliferation in the treated conditions. The same was observed with respect to the other cell lines (Appendix 5).

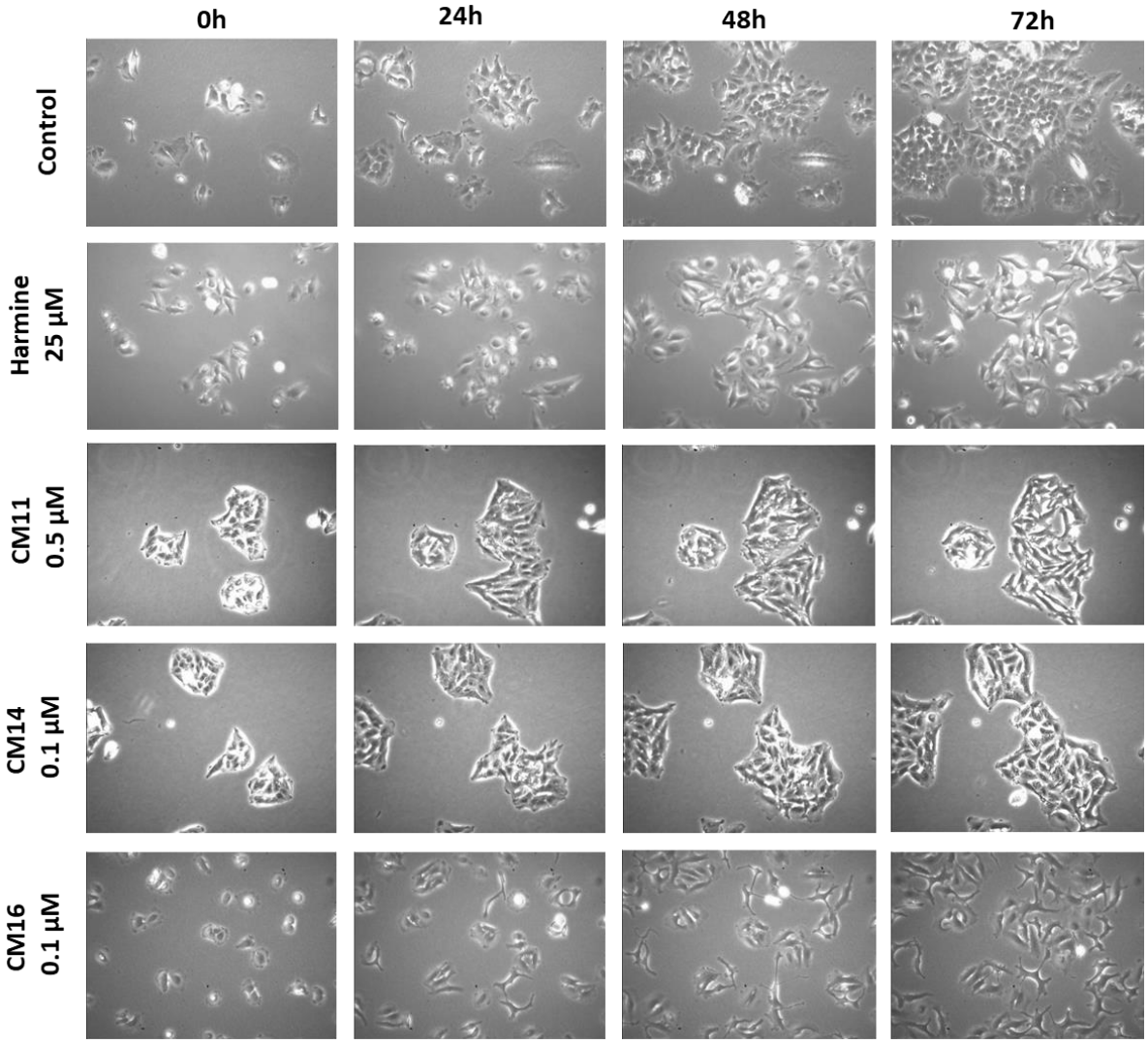


Figure 27. Videomicroscopy of the induced *in vitro* effects of harmine and derivatives CM11, CM14 and CM16 in the Hs683 glioma cell line. Figures are representative of one experiment performed in three replicates. Experiments were performed once with harmine and at least twice with the CM derivatives, each in triplicates.

For CM16, the cytostatic effect at its IC_{50} concentration is illustrated morphologically and quantitatively in the three cancer cells lines and in the non-cancerous fibroblast NHDF in **Figure 28 A-E**. We observe in **Figure 28 E** that treating the different cell lines with their respective IC_{50} concentration causes, as expected, a decrease of 50% (1=100%) of their growth within 48 h (Hs683, SK-MEL-28 and NHDF) to 72 h (MDA-MB-231) of treatment. However, note that NHDF IC_{50} concentration was 5 µM while the cancerous models were treated with 0.1 µM for Hs683 and 0.5 µM for SK-MEL-28 and MDA-MB-231 respectively, confirming high selectivity. Furthermore, the inhibition of some cell proliferation compared to the control caused by CM16 was also observed in the non-cancerous fibroblast NHDF in its 5 µM IC_{50} (**Figure 28 D**).

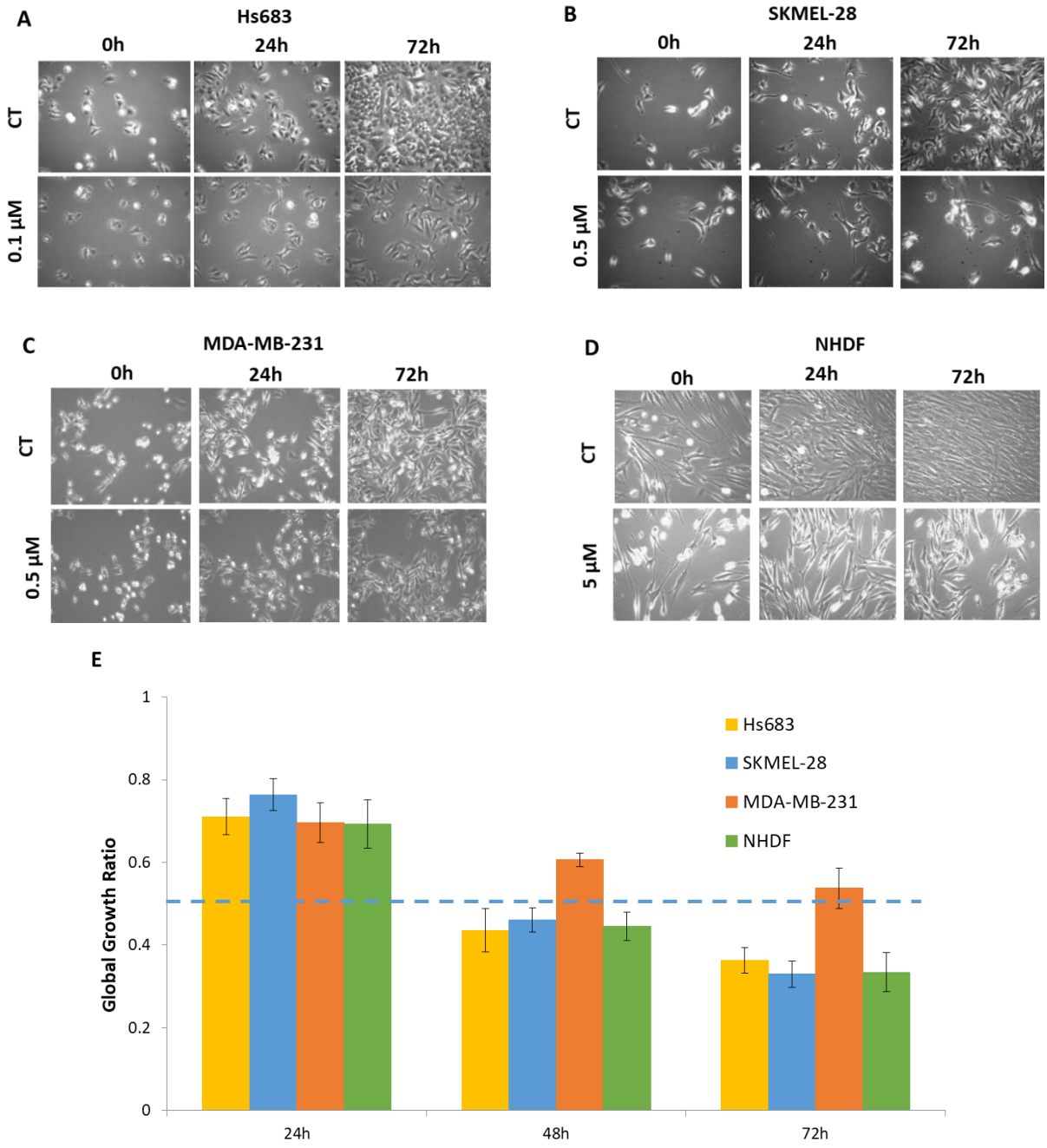


Figure 28. A-D: Videomicroscopy of CM16-induced *in vitro* effects in Hs683 (A), SK-MEL-28 (B), MDA-MB-231 (C) and NHDF (D) cell lines. Figures are representative of one experiment performed in three replicates. Experiments were performed at least twice in triplicates. E: Global growth ratio in HS683, SK-MEL-28, MDA-MB-231 and NHDF cells after 24 h, 48 h and 72 h treatments with CM16. The global growth ratio corresponds to the amount of treated cells present in a determined time divided by the amount of cells present at that same time in the non-treated cells. Results are expressed as the mean growth ratio between treated cells relative to control (1) ± S.E.M. of three replicates of one representative experiment.

3.1.3 Evaluation of harmine derivatives effects on the cell cycle

Given the cytostatic effect of these compounds, we further investigated the effects of CM11, CM14 and CM16 derivatives on the cell cycle of Hs683 and SK-MEL-28 cell lines by propidium

iodide staining but did not observe any significant effect (**Figure 29** – for cell cycle profiles of glioma cells treated with CM16 see Appendix 8). Thus we did not evaluate their effects in the MDA-MB-231 breast cancer cell line. The data here obtained suggest that the CM compounds-induced proliferation inhibition is not related to a specific cell cycle phase or arrest. Consistently, CM16 and its previously studied analogue 5a [197] did not interact with DNA when assayed *in vitro* (see **Figure 22**), as opposed to harmine and related compounds that display DNA intercalating and groove binding properties [172,203]. Interestingly, 5a also did not inhibit DYRK1A, which has been associated to the pro-apoptotic effect of harmine and other derivatives [168,169], again pointing to a different mechanism of inhibition of proliferation in cancer cells for these derivatives.

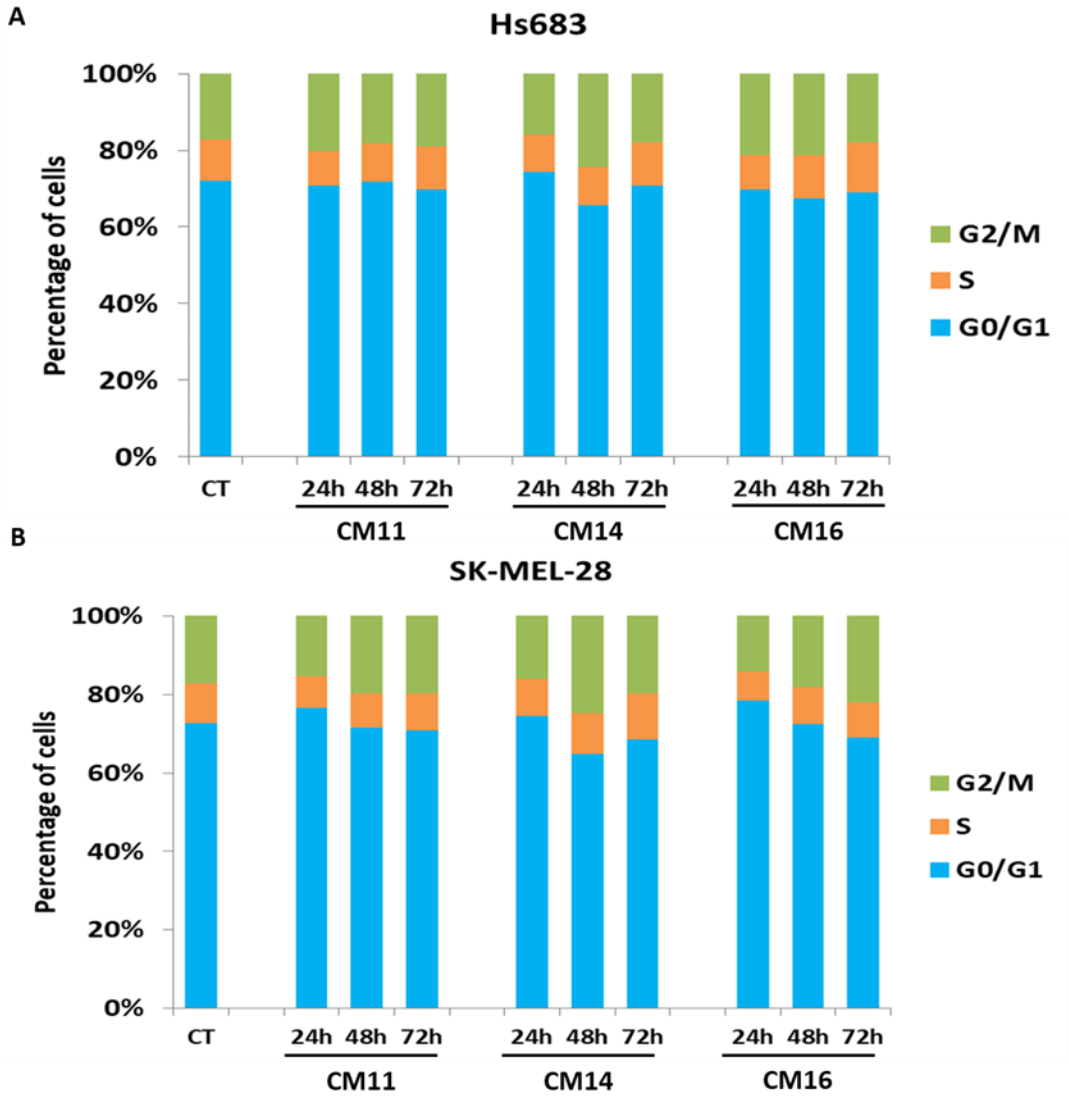


Figure 29. Cell cycle effects of of CM11, CM14 and CM16 on (A) Hs683 at 0.5 µM (CM11) and 0.1 µM (CM14 and CM16); and (B) SKMEL-28 at 1.0 µM (CM11 and CM14) and 0.5 µM (CM16). Data are expressed as the mean percentage of cells in each phase of the cell cycle of four replicates. The experiment was performed once in quadruplicate.

3.1.4 60-cancer-cell-line screening of CM16

As the lead compound chosen for investigation, CM16 was selected and sent to the National Cancer Institute (NCI, Bethesda, USA) in order to confirm the *in vitro* activity in cancer cell lines. The Development Therapeutics Program (DTP) of the NCI performs an *in vitro* screen of novel compounds on 60 human cancer cell lines representing nine different cancer types: leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate, and kidney [204].

The evaluation of CM16 in the 60 cancer cell line panel shows that the mean 50% growth inhibitory concentration (GI_{50}) obtained by the NCI is ~0.2 µM and varies from <0.01 to 4 µM **Figure 30 A**. Of note, 80% of the cell lines display GI_{50} close to the mean value, between 0.1 and 0.5 µM (**Figure 30 A**), which is similar to our mean IC_{50} (i.e. GI_{50}) of 0.3 µM (**Figure 26 D** and **Table 5**). The mean 50% lethal concentration (LC_{50}) in the NCI panel is 9 µM and ranges from 0.5 to >100 µM (**Figure 30 B**). Interestingly, the response profiles of the 60 cell lines based on the GI_{50} values differ from those obtained with the LC_{50} counterparts. For example, for the leukemia sub-panel, these differential sensitivities are opposite to each other. These observations suggest that CM16 exerts cytostatic activity at lower concentrations (close to GI_{50}) through a mechanism different from that associated with its cytotoxic action at higher concentrations (close to LC_{50}).

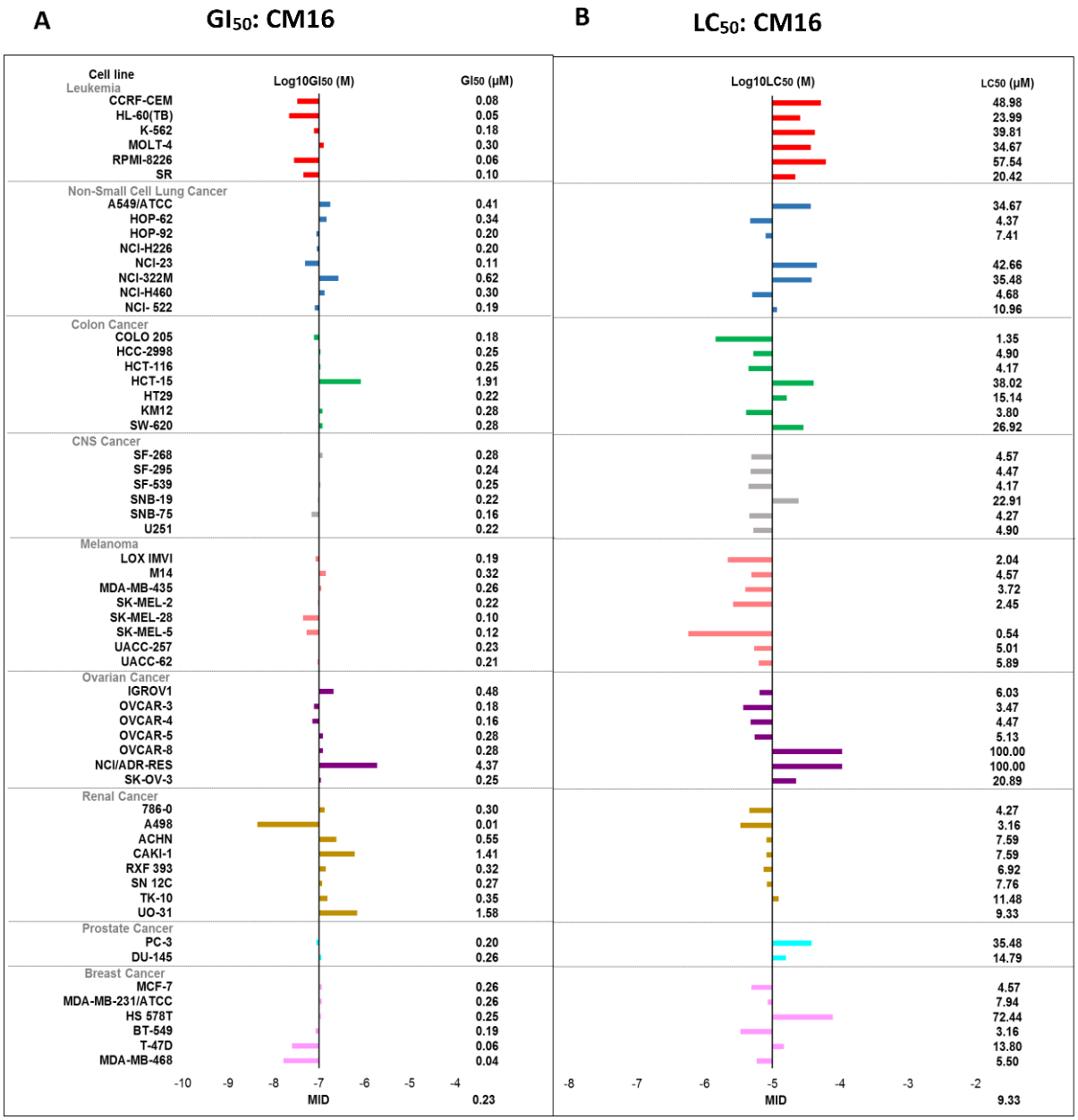


Figure 30. *In vitro* evaluation of the cell growth inhibition and lethal concentrations of CM16 in the NCI 60-cell line panel. (Adapted presentation - shown with the permission of the NCI.) (A): Global growth inhibition [GI_{50}] of each cell line after 48 h of culture with CM16. "-7" represents the mean GI_{50} of the 60 cell lines, i.e. 0.2 µM. Log10 differences are represented by the bars. (B): Lethal concentration [LC_{50}] of CM16 for each cell line compared to the mean LC_{50} ["-5"]. The scale of the bars is in log10 as for A and B.

These results also confirmed that CM16 does not display specificity towards one defined cancer type in the NCI panel and thus corroborates our decision to use cancer derived cell lines of distinct origins, i.e., glioma (Hs683), melanoma (SK-MEL-28) and breast carcinoma (MDA-MB-231) for our study in order to avoid cell-type specific and context-dependent factors.

3.2 Investigation of CM16 as a protein synthesis inhibitor of cancer cells

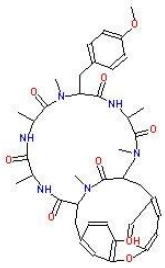
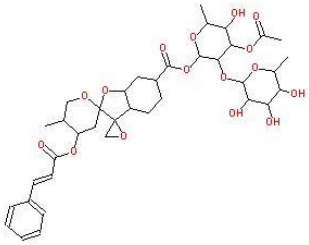
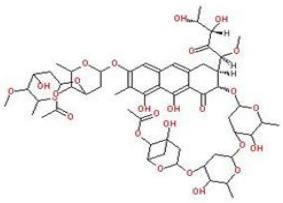
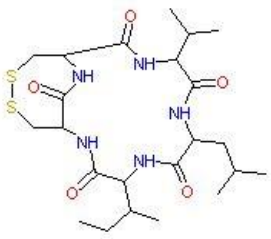
3.2.1 NCI data analysis suggests CM16 as a protein synthesis inhibitor

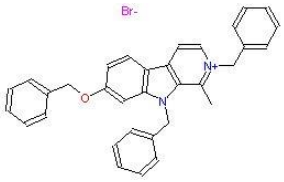
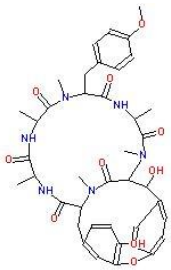
The NCI COMPARE analysis (Paull et al., 1989) is a tool which allows the comparison of the outcome data from the 60 cell line screen (growth inhibitory – GI_{50} – and lethal concentration – LC_{50} – profiles) of a compound with the profile of the previously screened compounds of the database. Pattern recognition algorithms (COMPARE) ranks the previously tested compounds present in the large NCI library based on the similarity of responses profile in relation to the novel compound, which is expressed as Pearson correlation coefficient [205]. The growth inhibitory profile (GI_{50}) of CM16 was thus compared to the NCI compound library using the COMPARE tool. The correlations represent the similarity of the growth inhibitory profile in the cancer cell line panel between two compounds, therefore the higher the correlation coefficient, the higher the similarity between the profiles of the compounds.

This comparative approach has been used by different groups in order to help identify possible mechanism of action of new compounds. Several compounds have been screened using this strategy, which helped pointing out for a variety of possible mechanism of actions for different molecules, such as inhibitors of the tubulin polymerization [206,207], cyclin-dependent kinases inhibitors [208], topoisomerase inhibitors [207] and also proteins synthesis inhibitors [209]. Our group itself has already employed this approach in the study with the harmine derivatives of the CV series, which identified the harmine derivative 5a as a potential protein synthesis inhibitor [197].

In the outcome of the current analysis, among 11 compounds displaying the COMPARE correlation coefficient (CCC) with CM16 above 0.7, mechanistic information was available for 8 (Pubmed or the SCOPUS database as of July, 2017), of which 7 have been described as potential protein synthesis inhibitors and are presented in **Table 6**, including importantly the harmine derivative 5a of the first derivative series (CCC of 0.71) that was previously also shown to correlate with protein synthesis inhibitors [197]. This last information further supports the hypothesis that the new mechanism of action of these trisubstituted derivatives relates to the inhibition of the protein synthesis of cancer cells.

Table 6. Correlations of CM16 with protein synthesis inhibitors in the NCI 60-Cell-Line Screen using the COMPARE Algorithm.

Compound	Correlation CM16 (NSC 779185)	Information	Chemical structure	References
NSC 656902	0.744	Quassinoid tested by NCI among other quassionoids that were protein synthesis inhibitors		[210]
DEOXYBOUVARDIN – NSC 259969	0.724	1. Protein synthesis inhibitor 2. Apoptotic effect (phosphorylation inhibition Akt and PDK1) 3. Angiogenesis inhibition (ERK1/2 dowregulation)		1. [209,211] 2. [212] 3. [213]
PHYLLANTHOSIDE,S3'-DESACETYL – NSC 342443	0.723	Protein synthesis inhibitor – elongation phase		[209,214]
CHROMOMYCIN A3 – NSC 58514	0.722	1. Protein synthesis inhibitor – transcription inhibition 2. NF-κB inhibitor + effect on estrogen receptor 3. Effect on TRAIL and Wnt signaling pathways		1. [209,215,216] 2. [217] 3. [218]
MALFORMIN A – NSC 324646	0.713	1. Protein synthesis inhibitor 2. Effect on cell cycle 3. Fibrinolytic activity 4. Activity against cancerous cell lines 5. Cytotoxic effect on cancerous cell lines 6. Anti-Tobacco mosaic virus		1. [219] 2. [220] 3. [221] 4. [222–224] 5. [225] 6. [226]

5a – NSC 760180	0.713	Protein synthesis inhibitor		[197]
BOUVARDIN – NSC 259968	0.707	Protein synthesis inhibitor - elongation phase		[227–230]

Additionally, one of the four compounds with a high similarity profile to 5a is also present among the ones identified as highly similar to CM16, which is compound NSC342443 (phyllanthoside). This compound was identified as a protein synthesis inhibitor in a screen attempting to identify new potential eukaryotic protein synthesis inhibitors through the COMPARE analysis, a study made by Chan and colleagues [209]. In this study, the natural product phyllanthoside was evaluated biologically for the first time as protein synthesis inhibitor and indeed was confirmed as such, being an inhibitor of the elongation phase of translation *in vitro*. More recently, in a study aiming at bringing new insight in the mechanism of action of eukaryotic ribosome inhibitors, phyllanthoside and other protein synthesis inhibitors were complexed with the 80S ribosome of yeast and their x-ray crystal structure revealed, shedding light on its binding sites on the ribosomes, where it acts at the mRNA-tRNA translocation level at the elongation phase [214].

Among the other compounds on **Table 6**, bouvardin and deoxybouvardin are structure-related protein synthesis inhibitors. Bouvardin has been recently investigated in head and neck cancer and glioma cells *in vitro*, confirming its inhibition of translation elongation and suggesting its potential in combination with radiation as demonstrated in an *in vivo* model [230]. Chromomycin A3 is an antitumor antibiotic that inhibits protein synthesis by binding to the DNA and therefore blocking transcription [209,215,216]. It has been used for testicular carcinoma but is no longer in use because of its cytotoxicity and immunosuppressive characteristics [215,216]. Malformin A has shown its potential against cancer cell lines in different studies as shown in **Table 6**. Although its role as protein synthesis inhibitor is not clear, there is a suggestion that its inhibition of protein synthesis prevents the pro-inflammatory interleukin 1 (IL-1) to induce endothelial changes [219].

Taken together this data generated a hypothesis for the harmine derivative CM16 acting as a protein synthesis inhibitor and encouraged its further evaluation. Noteworthy however, those results do not exclude possible similar effects on normal cells.

3.2.2 CM16 effects on transcription

As the first step for a cell to synthesize a protein, the RNA transcription under treatment with CM16 was evaluated. For this evaluation, we have preliminarily investigated if CM16 was affecting transcription in the Hs683 and SK-MEL-28 cells lines, treated for periods of 3, 6 and 24 h with 0.5, 1.0 and 5.0 µM of CM16. However, no alteration of the incorporation of the

nucleotide analogue EU into the newly-transcribed RNA was observed in these cells lines up to 24 h and in the presence of up to 5.0 µM CM16, as opposed to the positive control actinomycin (**Figure 31 A-B**). Because no effect could be observed, we did not perform the assay with MDA-MB-231 cells as we aimed to decipher a common mechanism of action on these three sensitive models to CM16.

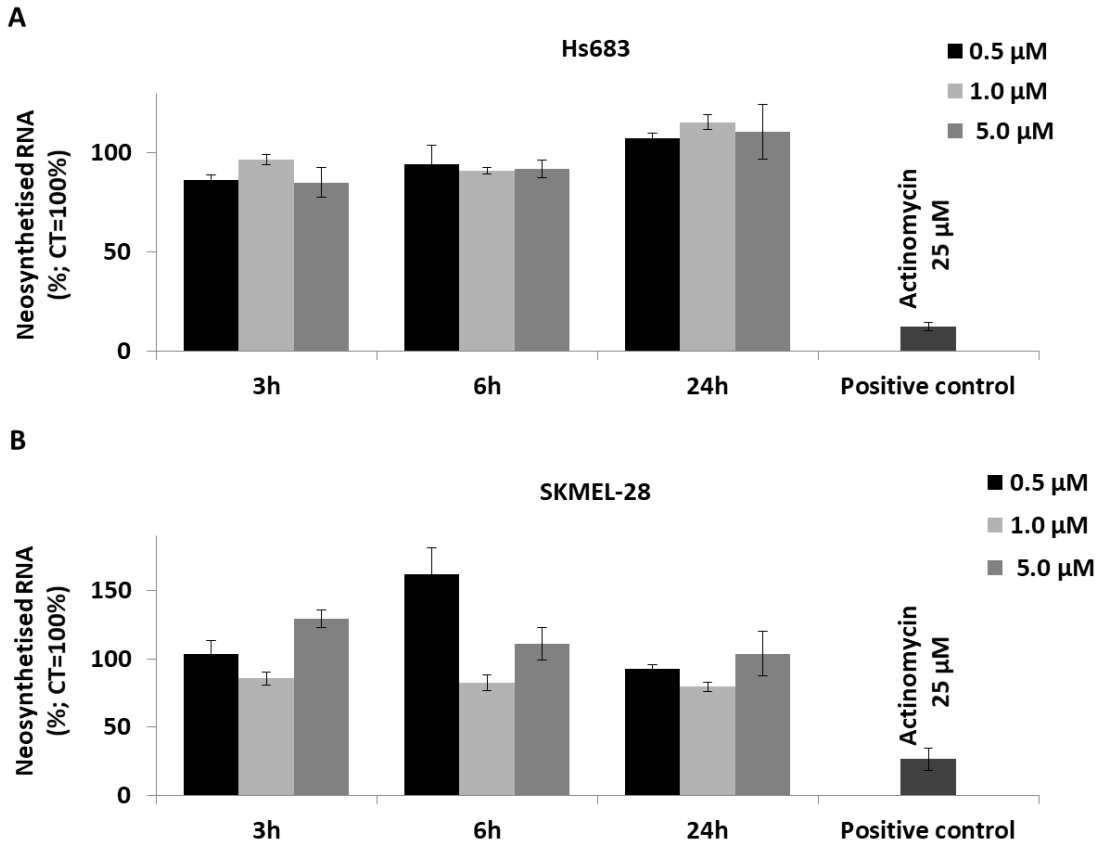


Figure 31. Effects of CM16 on newly synthesized RNA in (A): Hs683 and (B): SK-MEL-28 cell lines. Results are expressed as the mean neosynthezised RNA amounts normalized to the control (100%) ± SEM of six replicates. The experiment was performed once in sextuplicate.

3.2.3 CM16 inhibition of protein synthesis *in vitro*

Having tested CM16 effects on RNA transcription we moved on to investigate the effects of this compound on newly translated proteins in transformed Hs683, SK-MEL-28 and MDAMB-231 and in NHDF non-transformed cell lines (**Figure 32 A-D**). This study was actually conducted together with the other CM derivatives, i.e. CM11 and 14 as well as the first series (CV series – 5a, 5k and 5o) for practical issues and to allow better comparison. We thus selected two concentrations close to their respective mean IC_{50} i.e. 0.5 µM and 5.0 µM (see **Table 4**).

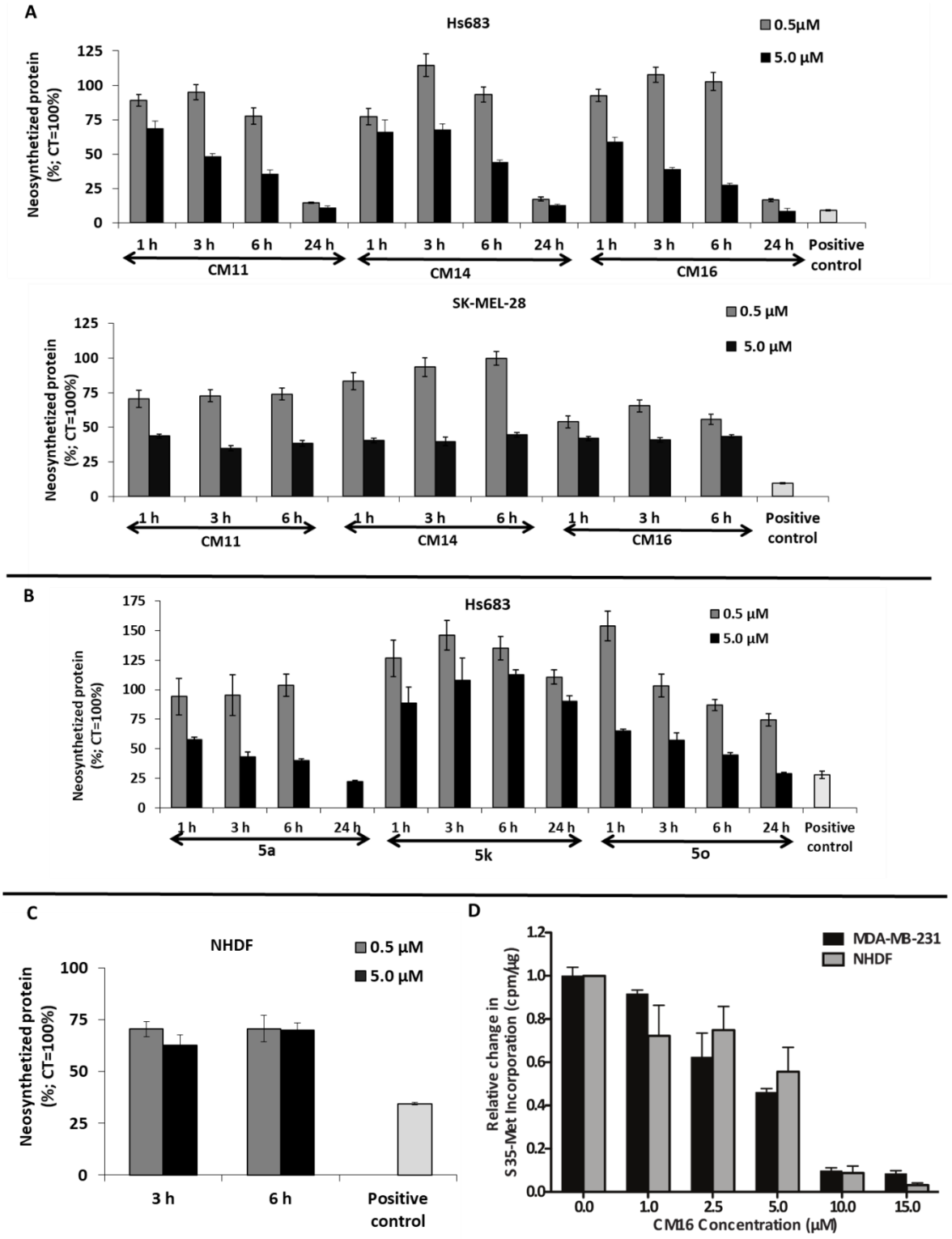


Figure 32. Analysis of the newly synthesized proteins under treatment with 0.5 and 5.0 µM of the harmine derivatives from the CV and CM series. (A): CM series (CM11, CM14 CM16) in Hs683 and SK-MEL-28 cell lines. (B): CV series (5a, 5k and 5o) in Hs683 cell line, previously performed by V. Mathieu. (C): CM16 in non-cancerous NHDF. Positive control: cycloheximide 0.1 mM for 1 h. No results of 24 h of treatment in SK-MEL-28 and NHDF cell lines. Data are expressed as the mean neosynthezised protein amounts normalized to the control (100%) ± S.E.M. of the six replicates of one representative experiment. Two independent experiments were performed, each in sextuplicate. (D): Dose-dependent evaluation of ^{35}S-methionine labelling in MDA-MB-231 and NHDF cells after 80 min of treatment. CPMs were normalized to total protein. Experimental results represent three biological replicates, each performed in technical duplicate.

We observed a concentration and time-dependent decrease in neosynthesized protein in the Hs683 cell line with all the compounds (**Figure 32 A-B**). However, the first series of derivatives (5a, 5k and 5o) appeared slightly less potent in inhibiting methionine analogue incorporation than the optimized CM series comparing the results obtained in the Hs683 cell line (no evaluation was performed with the first series in SK-MEL-28 cell line). These results are consistent with their higher antiproliferative concentrations as determined by means of the MTT assay (**Table 4**). Regarding CM16 in particular, the neosynthesized protein level was decreased by 50% at 5 µM after only a 1 h treatment in SK-MEL-28 cells and 3 h in HS683 cells. At 0.5 µM, the treatment period had to be increased from 6 to 24 h to obtain a similar decrease (**Figure 32 A**). Similarly, our collaborators in Canada observed a concentration-dependent decrease in incorporated ^{35}S methionine in MDA-MB-231 breast cancer cells leading to nearly a complete inhibition of protein synthesis after only 80 min of treatment with CM16 with the concentration of 10 µM (**Figure 32 D**). However, evaluation of newly synthesized proteins in NHDF normal fibroblast cells showed no difference with the breast cancer cell model (**Figure 32 C-D**). Importantly, we verified by videomicroscopy that the higher concentrations, i.e. 5 and 10 µM, did not induce major cytotoxic effects in all cell line models, at least in the time frame of the present analysis of protein synthesis (**Figure 33**), being aware that higher concentrations still could trigger also other intracellular pathways. Cytotoxic effects were reached only at later time points.

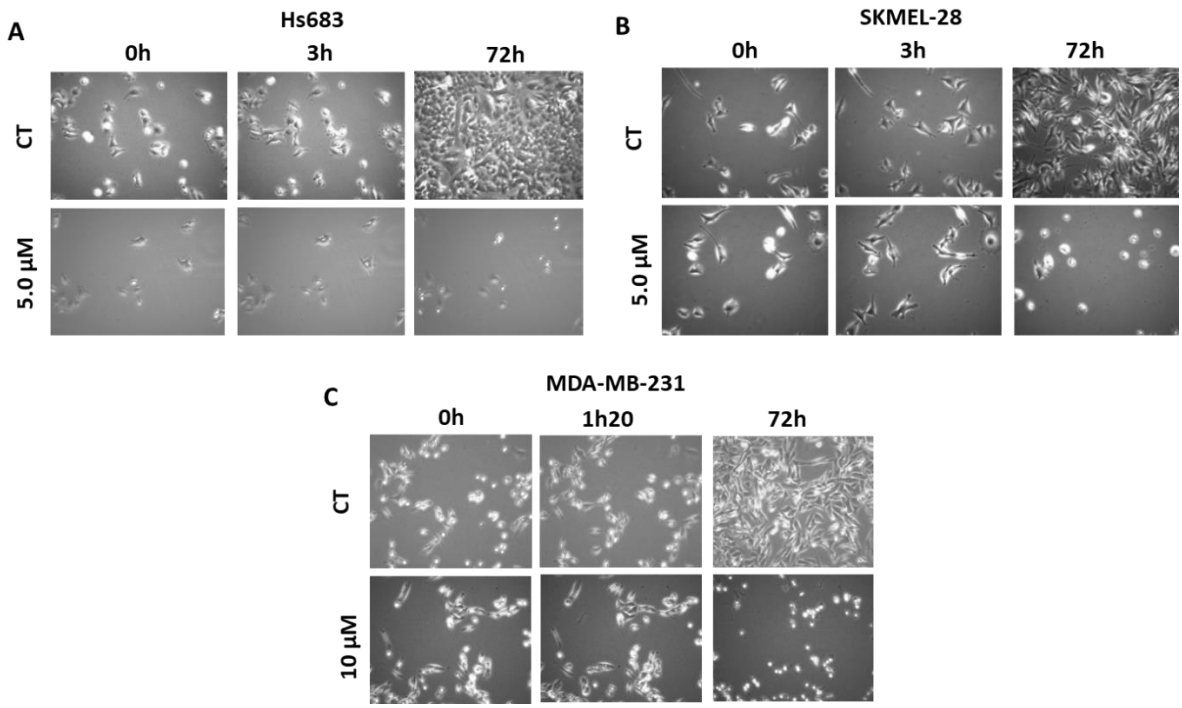


Figure 33. A-C: Videomicroscopy of CM16-induced *in vitro* effects in Hs683 (A), SK-MEL-28 (B) and MDA-MB-231 (C) cell lines after 3 h and 72 h treatments with 10 times their IC_{50} (Hs683 and SK-MEL-28) and 10 µM for MDA-MB-231. Figures are representative of one experiment performed in three replicates. Experiments were performed at least twice in triplicates.

Considering that protein synthesis inhibition was observed as a common feature induced by the different tri-substituted derivatives and in different cell type models while no effect on transcription could be observed, at least with respect to CM16, we concluded that protein synthesis inhibition indeed seems to contribute to the antiproliferative effects of these compounds.

3.2.4 CM16 cellular penetration and distribution

Once we verified the general inhibition of protein synthesis caused by CM16, we profited from the fluorescent properties of this compound (ex/em: 330/439 nm) to study its cellular penetration and distribution by fluorescence microscopy. CM16 appeared to penetrate as early as 5 min after initiating treatment in the perinuclear region of the glioma Hs683 cells, while the nucleus itself remained unstained even after 6 h of treatment (**Figure 34 A**). Similar penetration characteristics were observed for SK-MEL-28, MDA-MB-231 and NHDF cells in the periods of treatment evaluated, as exemplified in **Figure 34 B** with respect to 5 µM treatment for 3h. However, no obvious differences were revealed in cell penetration of CM16 between non-transformed fibroblasts (**Figure 34 B**, right panel) and cancer cells that could explain, even

partly, the selectivity we observed above in the evaluation of cancer cells growth inhibition (**Table 5**).

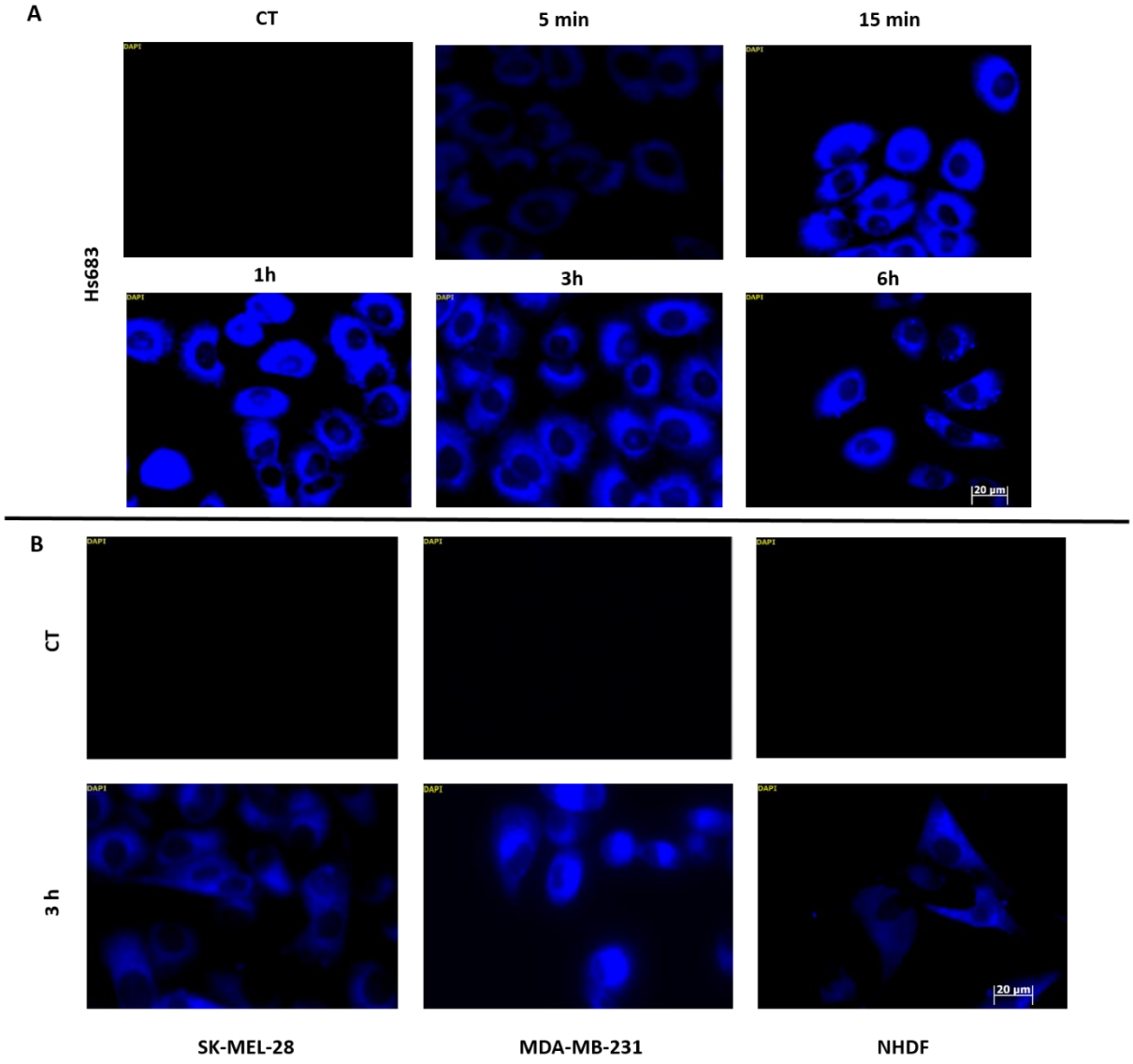


Figure 34. (A): CM16 fluorescence properties allow its visualization in blue colour (filter ex/em: 359-371/397 nm) in Hs683 cells over time after 5.0 µM treatment. (B): Visualization of CM16 in the other cell lines after 3 h treatment with 5.0 µM of CM16. Cancerous cell lines: SK-MEL-28 and MDA-MB-231 and non-cancerous cell line: NHDF. Exposure times for blue filter (ex/em) 359–371/397 nm: 176 ms (Hs683), 145 ms (SK-MEL-28), 80 ms (MDA-MB-231) and 53 ms (NHDF). Two experiments in duplicate were conducted with the cancerous cell lines and one with the non-cancerous fibroblast. Three images per condition were taken with a 40x objective.

In addition, we have also compared CM16 distribution to the endoplasmic reticulum (ER) using an ER dye in live cells. As showed in the last column of **Figure 35 A-C** for Hs683, SK-MEL-28 and MDA-MB-231, respectively, it appears that the CM16 distribution in the perinuclear region seems to be associated to the endoplasmic reticulum (for a better visualization of the

staining of the perinuclear region see Appendix 9), a major site for mRNA translation [231]. These results appeared consistent with the protein synthesis inhibition of CM16 shown earlier.

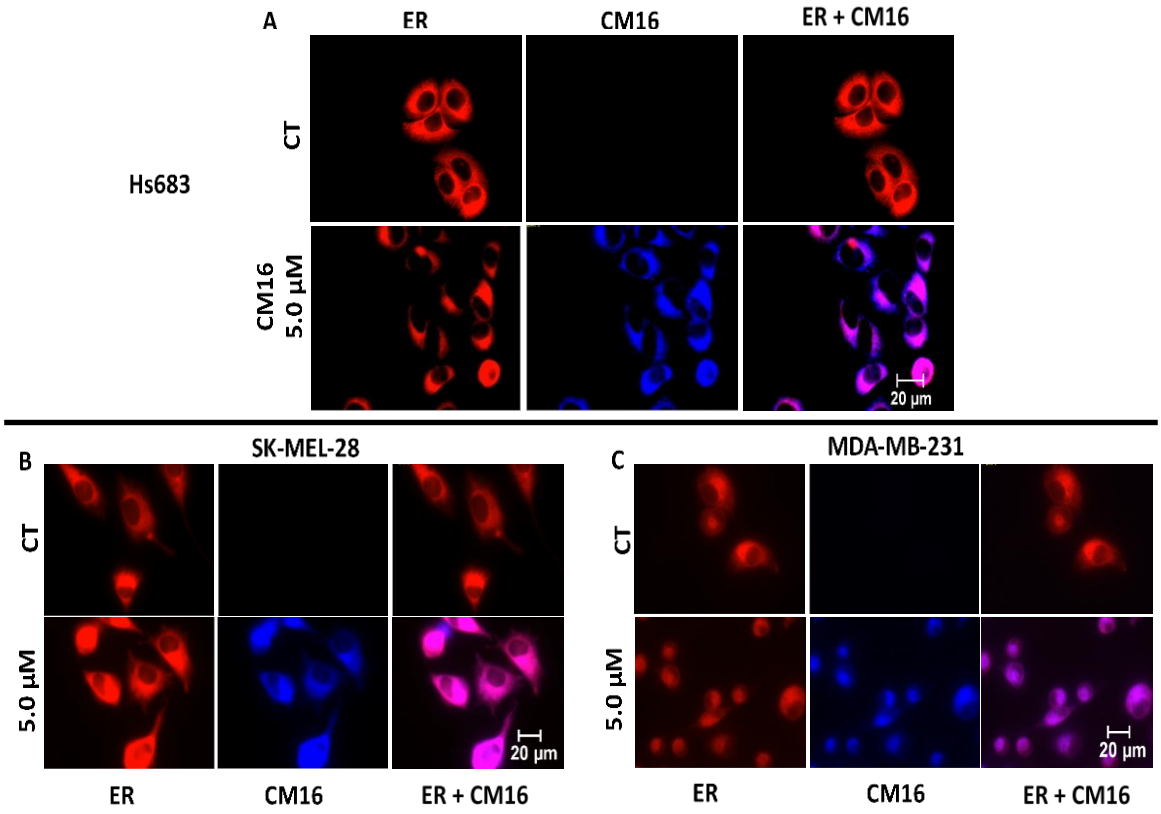


Figure 35. CM16 parallel distribution to the endoplasmic reticulum fluorescent probe (ER-tracker) after a 3 h treatment in (A) Hs683, (B): SK-MEL-28 and (C): MDA-MB-231 cell lines. Exposure times for blue filter (ex/em) 359-371/397 nm: 40 ms (SK-MEL-28) and 80 ms (Hs683 and MDA-MB-231); and for red filter (ex/em) 540-580/593-668 nm: 283 ms (SK-MEL-28) and 850 ms (Hs683 and MDA-MB-231). All pictures were taken with a 40x objective. Illustrations are representative of one experiment performed in two replicates. The experiments were conducted twice in duplicate and five images per condition were taken.

3.2.5 CM16 affects ribosomal organization in cancer cells

To investigate the effects of CM16 further on translation, we evaluated the ribosomal assembly into 80S functional subunit and polysome organization by means of sucrose gradients. CM16 induced the accumulation of 80S ribosomes in MDA-MB-231 treated cells, while polysomes decreased after 80 min of treatment at 10 µM (**Figure 36 A**) (the concentration that completely inhibited ^{35}S methionine incorporation in 80 min in MDA-MB-231 – **Figure 32 D**). We confirmed that ribosomal organization in Hs683 and SK-MEL-28 cells was affected as early as after a 3 h treatment with lower concentrations, i.e. 0.5 and 5.0 µM CM16 (**Figure 36 B-C**). Again, we observed an accumulation of the fractions corresponding to the free 80S ribosome when compared to puromycin-induced effects (**Figure 36 B**), a known

protein synthesis inhibitor used as positive control [65,209]. Because of these results, we hypothesized that CM16 affects translation initiation of cancer cells while those effects were observed at higher concentrations only in the non-transformed fibroblasts (**Figure 36 D-E**).

Note that the experiment on MDA-MD-231 cells was conducted by our collaborators in Canada while we performed the assays on the other cell models. Difference in their equipment allowing automated collection of the fractions could explain, at least partly, the shift of the profile in comparison to ours obtained manually. The difficulty in observing polysomes in our profiles was not surprising according to the literature in the field [232].

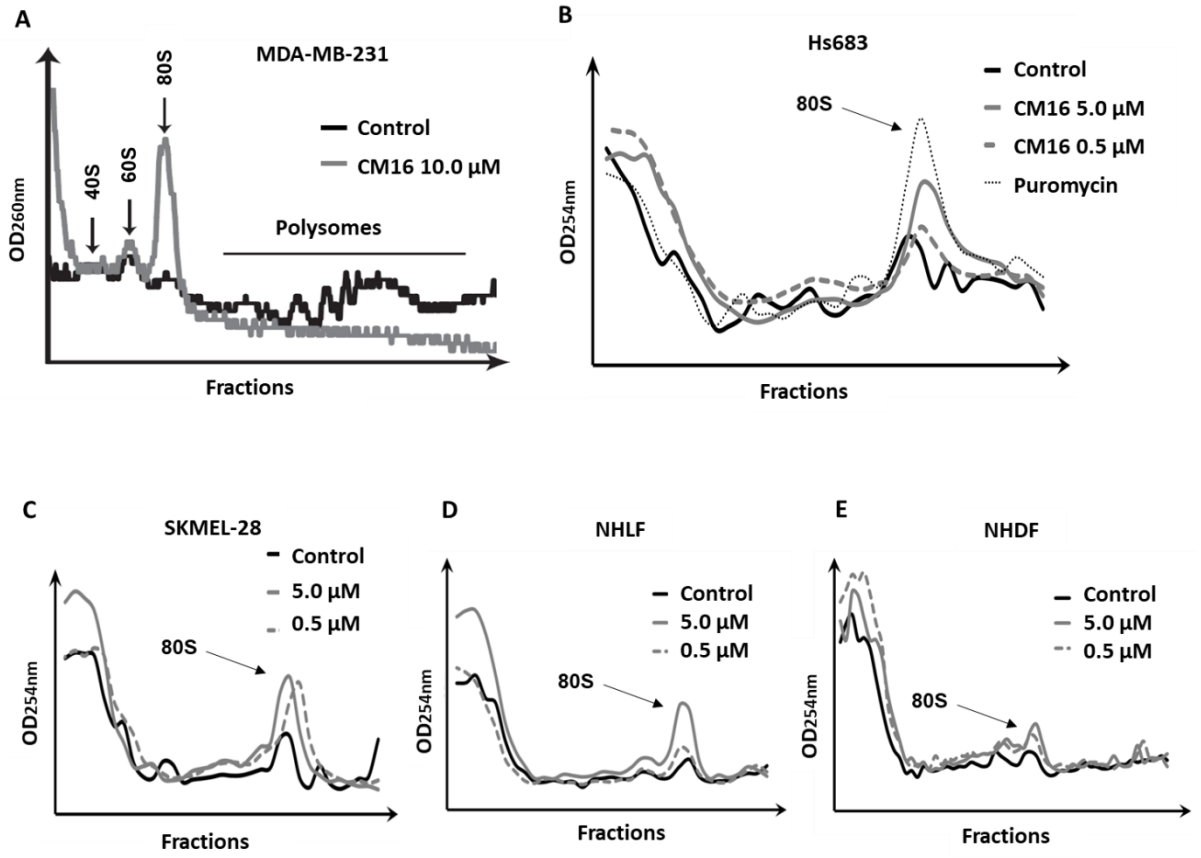


Figure 36. Effect of CM16 on ribosomal units and polysome organization after treatment. (A): MDA-MB-231 cells incubated for 80 min with CM16 at 10 µM in comparison to the control. (B): Cancerous cell lines Hs683 and (C): SK-MEL-28 and non-cancerous cell lines (D): NHDF and (E): NHLF incubated with CM16 at 0.5 µM or 5.0 µM for 3 h (grey lines) in comparison to the non-treated control (solid black line). Puromycin was used as positive control (1 h, 184 µM) to visualize the 80S peak containing fractions. A minimum of three independent experiments were carried out with the Hs683 and SK-MEL-28 cancer cell lines and one experiment in the non-cancerous non-transformed fibroblasts NHLF and NHDF. Each profile is representative of one experiment.

3.2.6 CM16 effects on the actin microfilaments

Organization of actin cytoskeleton has been associated with protein synthesis, since the elongation factor 1A (eEF1A) interacts with actin fibres and participates in polysome

organization [233]. Therefore evaluation of CM16 effects on the actin cytoskeleton of Hs683 and SK-MEL-28 was performed by fluorescent staining of actin microfilament in the absence or presence of 0.5 and 5.0 µM of CM16 over a 24 h period. As no modification was noticed (**Figure 37,** conventional, non-confocal microscopy) in two cancer models, we did not perform the assay on MDA-MB-231.

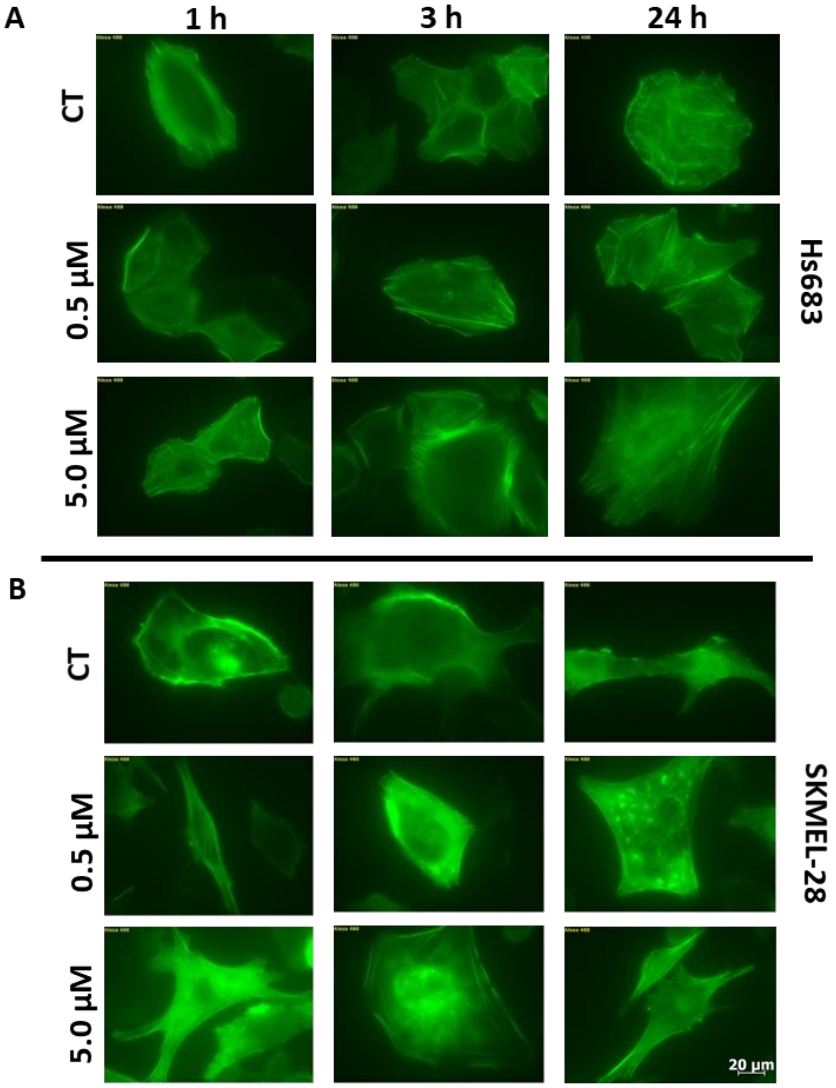


Figure 37. Fibrillar actin staining in (A) Hs683 and (B) SK-MEL-28 treated with 0.5 and 5.0 µM of CM16 for periods of 1 h, 3 h and 24 h. The experiment was conducted once in triplicate and ten images per condition were taken.

3.2.7 Evaluation of CM16 effects on translation initiation and elongation factors

Once we had confirmed CM16 ability to interfere with the ribosome and polysome assembly of cancer cells, we have investigated the possible effect of this compound on specific actors of the different phases of translation. Because translation termination is not known to play an important role in cancer [66], further investigation was focused on the effects of CM16 on

translation initiation and elongation steps in cellular assays. Translation initiation is dependent on the formation of eIF4F complex (eIF4G, eIF4A and eIF4E), which recruits the 40S ribosomal unit, and the ternary complex (met-tRNA-eIF2-GTP), necessary for initiation. Targeting initiation proteins instead of elongation ones would offer greater selectivity in inhibiting the growth of cancer cells as elongation inhibitors seem to have a narrow therapeutic window due to the inhibition of global protein synthesis of non-transformed cells [71,87]. Moreover, several initiation proteins are dysregulated in cancer cells as compared to normal cells. We thus first investigated the total expression levels of crucial initiation and elongation factors, i.e. eIF4A1, eIF2, eIF4E, eEF1A and eEF2 but they remained unchanged in Hs683 and SK-MEL-28 cells, at least following 24 h of treatment with CM16, even at concentrations 10 times higher than the IC_{50} in Hs683 and SK-MEL-28 cells (**Figure 38 A-B**). Thus we did not perform this evaluation in MDA-MB-231 cells. The fact that the expression of these factors remained unchanged under treatment with CM16 is not entirely surprising because fine tuning control of translation is usually achieved or influenced by the activation status (phosphorylation) of these proteins or the regulators that interact with them [234–236]. As the harmine derivative 5a was shown to decrease eIF2α expression and phosphorylation levels as part of the confirmation of its potential as a protein synthesis inhibitor in Hs683 and U373 glioma cell lines [197], we continued with the investigation of eIF2α phosphorylation. Indeed, the change in the phosphorylation status of this factor controls the formation of the ternary complex and binding of $tRNA^{met}$ to the ribosome [237]. If this step is compromised, protein synthesis is inhibited [238]. Moreover, eIF2 phosphorylation has been closely linked to tumorigenesis [238–240]. Similarly to the observations in the glioma cell lines treated with the compound 5a, eIF2α phosphorylation was induced in MDA-MB-231 cells when treated with 2.5 or 10 µM of CM16 for 80 min, an effect also found in NHDF cells (**Figure 38 C**; assays performed by Jennifer Chu in J. Pelletier team, McGill University, Canada). In contrast, CM16 did not affect the total expression level of the protein eIF2, as 5a did. A family of four kinases – eukaryotic translation initiation factor 2 alpha kinase 1 (HRI), eukaryotic translation initiation factor 2 alpha kinase 2 (PKR), eukaryotic translation initiation factor 2 alpha kinase 4 (GCN2) and PERK – regulates eIF2α phosphorylation [238,241]. The effect observed with CM16 is unlikely due to direct effects of CM16 on PERK kinase, as evaluated *in vitro* in a kinase activity assay (**Figure 38 D**). However, it remains possible that CM16 acts, at least partly, through ER stress-

mediated activation of PERK in cellular assay [238] and/or via the other kinases mentioned above.

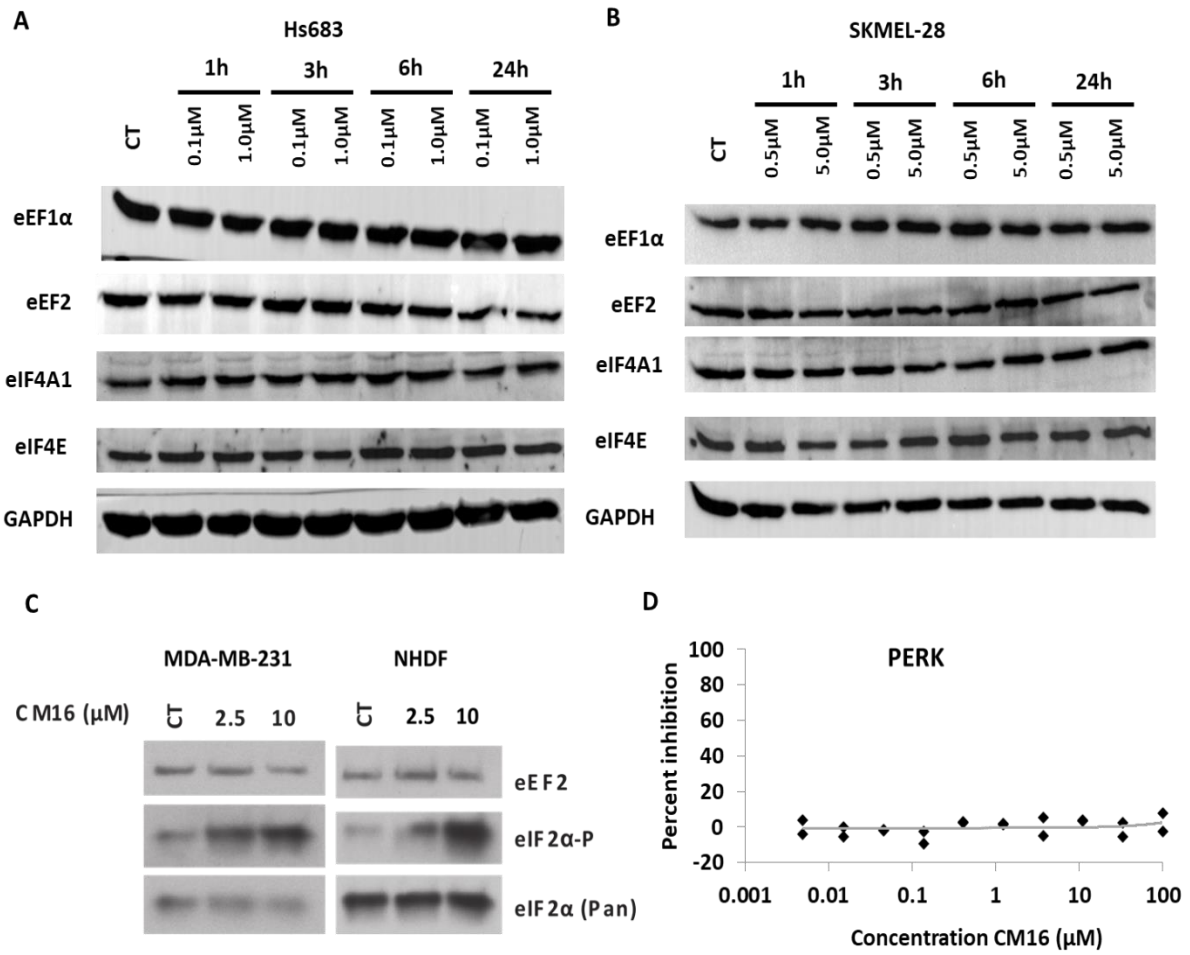


Figure 38. Effects of CM16 on elongation and initiation factor expression and PERK activity. Immunoblotting of different factors after treatment with CM16. (A): Hs683 cell line. (B): SKMEL-28 cell line. Representative western blot of three independent experiments. (C): Effects of CM16 treatment (80 min) on the expression and phosphorylation status of eIF2α in MDA-MB-231 cancerous and NHDF non-cancerous cells by western blot. Representative western blot of two experiments (D): PERK kinase activity *in vitro* in the presence or absence of CM16.

We also tried to assess the induction of eIF2α phosphorylation in the two other cell lines used throughout this study, Hs683 glioma and SK-MEL-28 melanoma cell lines, however we have encountered difficulties, including very intense additional bands with different molecular weight on the blots. Despite our efforts to optimize the conditions (blocking, washes, concentration of antibody), we did not succeed to get improved and specific results. We then tried to use an immunoprecipitation step prior to the separation of proteins in SDS-PAGE, but again no satisfactory results could be obtained even with a positive control (Appendix 6).

3.2.8 NCI transcriptomics analysis

To carry on with the specific investigation on how the harmine derivative CM16 is indeed inhibiting protein synthesis, we have decided to take benefit from the NCI data. To further evaluate whether the expression level of eIF2α and its partners eIF2β and eIF2γ could drive, at least partly, the sensitivity of cells to CM16 we utilized the NCI *in vitro* evaluation of CM16 anti-cancer effects and their cell line transcriptomic characterization. Although the GI_{50} of CM16 in the majority of the NCI cell line panel are close to the average of 0.2 µM (**Figure 30 A**) without large variation, four cell lines appeared poorly sensitive to CM16 with GI_{50} > 1µM (least sensitive, LS) while five other appeared highly sensitive, i.e. those with a GI_{50} < 0.1 µM (most sensitive, MS). The cell lines and their origin are described in the legend of **Figure 39**. We thus compared the transcriptomic expression levels of the genes encoding eIF2α (*EIF2S1*) and its partners eIF2β (*EIF2S2*) and eIF2γ (*EIF2S3*) between these two groups of cell lines displaying two orders of magnitude difference of sensitivity to CM16 but no statistical significance could be observed (**Figure 39 A**). To help in orienting us to other possible targets and mechanism(s) of action of CM16, we extended the comparison of the highly versus poorly sensitive cell lines of the NCI to a more extensive list of 57 components, actors and regulators of translation (**Table 7**). This list contains the main components of the cap-dependent translation machinery, as well as kinases and important proteins involved in the protein synthesis pathways linked to tumorigenesis. Following the colour code in the table, the initiation factors are presented first, followed by the elongation factors, the various kinases involved in the regulation of translation and finally other proteins with diverse roles on the translation pathways that have been linked to tumorigenesis (as detailed in the introduction section of this manuscript). Interestingly, among these 57 targets of the translation machinery selected, only three initiation factors were found to be significantly differentially expressed between the two groups: *EIF1AX, EIF3E* and *EIF3H* (**Figure 39 B**), while neither elongation nor signalling pathways investigated were different (data not shown). The expression level of these initiation factors could thus drive, at least partly, the sensitivity of a cell line to CM16 and further support the effects of this compound on the initiation phase of translation.

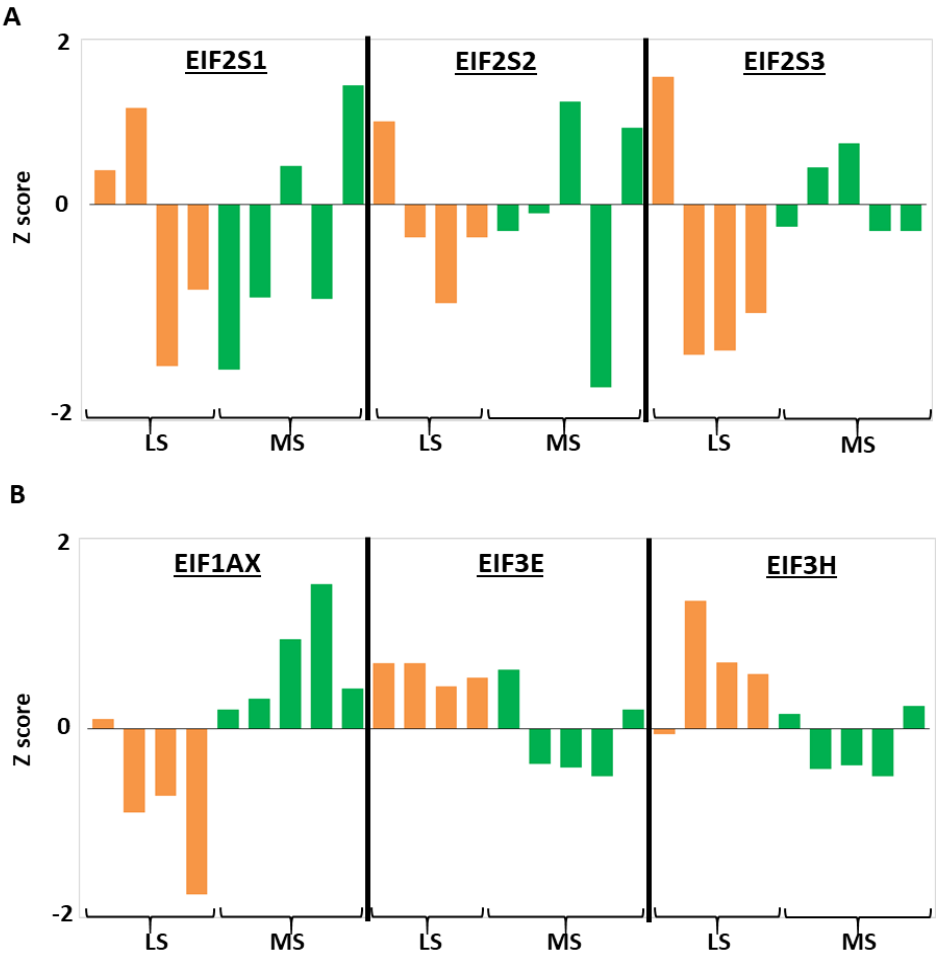


Figure 39. Comparison of the transcriptomic expression levels of main translation initiation genes in the most (MS, green) versus least sensitive (LS, orange) cell lines to CM16 effects among the NCI-cell-line-panel screening. The four least sensitive cell lines (i.e. those with a GI50 > 1 µM) are colon HCT-15 [-5.72], ovarian NCI/ADR-RES [-5.36] and renal cancer cell lines Caki-1 [-5.85] and UO-31 [-5.80]. The five most sensitive cell lines (i.e. with a GI50 < 0.1 µM) are renal A498 [-8.00], three leukemia cell lines HL60 [-7.30], CCRF-CEM [-7.12], RPMI-8226 [-7.19] and a breast cancer cell line T47D [-7.23]. (A): Transcriptomic comparison of eIF2α, β and γ subunits between the most and least sensitive cell lines to the CM16 growth inhibitory effects identified in the NCI cell line panel. (B): Transcriptomic comparison of the targets with significantly different expression levels between the cell lines most and least sensitive to CM16 growth inhibitory effects identified in the NCI cell line panel by means of t-test comparison. Increased expression levels as compared to the 60 cell line mean appear above 0 while decreased expression levels appear below 0. Results are expressed as Z scores as provided by the NCI database. Z scores are determined for each probe/cell line pair by the subtraction from its intensity of the probe mean (across the 60 cell lines), and division by the standard deviation of the probe (across the 60 cell lines). The z score average was then calculated as the mean across all probes and probe sets that passed quality control criteria.

eIF1A, the protein encoded by *EIF1AX*, is important in the formation of the preinitiation complex, composed of the 40S subunit, eukaryotic translation initiation factor 1 (eIF1), eukaryotic translation initiation factor 5 (eIF5), eIF3 and the ternary complex [57,75], and together with eIF1, is required for mRNA scanning and binding at the initiation codon [75]. Mutations in the *EIF1AX* gene have been associated with tumour development and

progression in thyroid cancer [242,243] uveal melanomas [244,245] and possibly ovarian tumour carcinogenesis [246]. Knowledge of its functional roles in cancer biology is still currently limited [75] and warrant further investigations. Similarly, eIF3e and eIF3h have been both reported to be dysregulated in cancer [57]. They are part of the largest initiation complex, i.e. the eIF3 complex, which is composed of 13 subunits acting together in the initiation process. Their main roles include recruitment of the mRNA to the 40S ribosomal unit and stabilizing the ternary complex [57,75]. At the protein level, eIF3e affects proliferation and survival of glioblastoma cells [247], is involved in colon tumour progression [248] and breast tumour formation, [249] progression [250] and metastasis [251]. High levels of eIF3h maintain the malignancy of several cancer cell lines *in vitro* [252] and have been indeed observed in breast, prostate and hepatocellular carcinomas [253,254]. The *EIF3H* gene was also found amplified in breast and prostate cancers, together with MYC proto-oncogene [74]. Whether the possible targeting of these initiation factors could participate to the relative selectivity of CM16 against cancerous over non-cancerous cells remains to be investigated.

Indeed the CM16 selectivity towards cancer cells that was observed in the *in vitro* evaluation of growth inhibition (**Figure 26** and **Table 5**) and modestly in the polysome organisation analysis (**Figure 36**) cannot be explained by difference in the intracellular penetration of CM16 as shown in **Figure 34 B** or by a specific induction of eIF2-α phosphorylation (**Figure 38 C**).

Table 7. List of genes analyzed for the transcript intensity from the NCI cell line panel.

Protein	Protein code (UniProt)	Gene (HGNC Symbol)	Gene code (Entrez Gene)
Eukaryotic translation initiation factor 2 subunit 1	P05198	EIF2S1	1965
Eukaryotic translation initiation factor 2 subunit 2	P20042	EIF2S2	8894
Eukaryotic translation initiation factor 2 subunit 3	P41091	EIF2S3	1968
Translation initiation factor eIF-2B subunit alpha	Q14232	EIF2B1	1967
Translation initiation factor eIF-2B subunit beta	P49770	EIF2B2	8892
Translation initiation factor eIF-2B subunit gamma	Q9NR50	EIF2B3	8891
Translation initiation factor eIF-2B subunit delta	Q9UI10	EIF2B4	8890
Translation initiation factor eIF-2B subunit epsilon	Q13144	EIF2B5	8893
Eukaryotic translation initiation factor 4E	P06730	EIF4E	1977
Eukaryotic translation initiation factor 4E-binding protein 1	Q13541	EIF4EBP1	1978
Eukaryotic translation initiation factor 4 gamma 1	Q04637	EIF4G1	1981
Eukaryotic translation initiation factor 4 gamma 2	P78344	EIF4G2	1982
Eukaryotic translation initiation factor 4 gamma 3	O43432	EIF4G3	8672
Eukaryotic initiation factor 4A-I	P60842	EIF4A1	1973
Eukaryotic initiation factor 4A-II	Q14240	EIF4A2	1974
Eukaryotic initiation factor 4A-III	P38919	EIF4A3	9775
Eukaryotic translation initiation factor 5A-1	P63241	EIF5A	1984
Eukaryotic translation initiation factor 5A-2	Q9GZV4	EIF5A2	56648
Eukaryotic translation initiation factor 5B	O60841	EIF5B	9669
Eukaryotic translation initiation factor 6	P56537	EIF6	3692
Eukaryotic translation initiation factor 1	P41567	EIF1	10209
Eukaryotic translation initiation factor 1A, X-chromosomal	P47813	EIF1AX	1964
Eukaryotic translation initiation factor 1A, Y-chromosomal	O14602	EIF1AY	9086
Probable RNA-binding protein EIF1AD	Q8N9N8	EIF1AD	84285
Eukaryotic translation initiation factor 3 subunit A	Q14152	EIF3A	8661
Eukaryotic translation initiation factor 3 subunit B	P55884	EIF3B	8662
Eukaryotic translation initiation factor 3 subunit H	O15372	EIF3H	8667
Eukaryotic translation initiation factor 3 subunit I	Q13347	EIF3I	8668
Eukaryotic translation initiation factor 3 subunit M	Q7L2H7	EIF3M	10480
Eukaryotic translation initiation factor 3 subunit E	P60228	EIF3E	3646
Eukaryotic translation initiation factor 3 subunit F	O00303	EIF3F	8665
Elongation factor 1-alpha 1	P68104	EEF1A1	1915
Elongation factor 2	P13639	EEF2	1938
Eukaryotic translation initiation factor 2-alpha kinase 3	Q9NZJ5	EIF2AK3	9451
Eukaryotic translation initiation factor 2-alpha kinase 4	Q9P2K8	EIF2AK4	440275
Interferon-induced, double-stranded RNA-activated protein kinase	P19525	EIF2AK2	5610
Eukaryotic translation initiation factor 2-alpha kinase 1	Q9BQI3	EIF2AK1	27102
MAP kinase-interacting serine/threonine-protein kinase 1	Q9BUB5	MKNK1	8569
MAP kinase-interacting serine/threonine-protein kinase 2	Q9HBH9	MKNK2	2872
Serine/threonine-protein kinase mTOR	P42345	MTOR	2475
RAC-alpha serine/threonine-protein kinase	P31749	AKT1	207
RAC-beta serine/threonine-protein kinase	P31751	AKT2	208
RAC-gamma serine/threonine-protein kinase	Q9Y243	AKT3	10000
Ribosomal protein S6 kinase beta-1	P23443	RPS6KB1	6198

Ribosomal protein S6 kinase beta-2	**Q9UBS0**	**RPS6KB2**	**6199**
3-phosphoinositide-dependent protein kinase 1	O15530	PDPK1	5170
Mitogen-activated protein kinase 1	P28482	MAPK1	5594
Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	P42336	PIK3CA	5290
Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN	P60484	PTEN	5728
Myc proto-oncogene protein	P01106	MYC	4609
Programmed cell death protein 4	Q53EL6	PDCD4	27250
Hamartin	Q92574	TSC1	7248
Tuberin	P49815	TSC2	7249
Cellular tumor antigen p53	P04637	TP53	7157
Retinoblastoma-associated protein	P06400	RB1	5925
Vascular endothelial growth factor A	P15692	VEGFA	7742
78 kDa glucose-regulated protein	P11021	HSPA5	3309

3.3 Toxicity, antitumor evaluation and formulation of CM16

Due to the results obtained preliminarily with CM16, our collaborators (NAMEDIC – University of Namur) have evaluated its effects *in vivo*. Firstly, a toxicity evaluation was carried out with CM16, after having verified its stability in reconstituted human plasma up to minimum 8 hours. The maximum tolerated dose (MTD) was determined as 1 mg/Kg, with a unique intraperitoneal (IP) administration of the CM16 suspension (**Figure 40**).

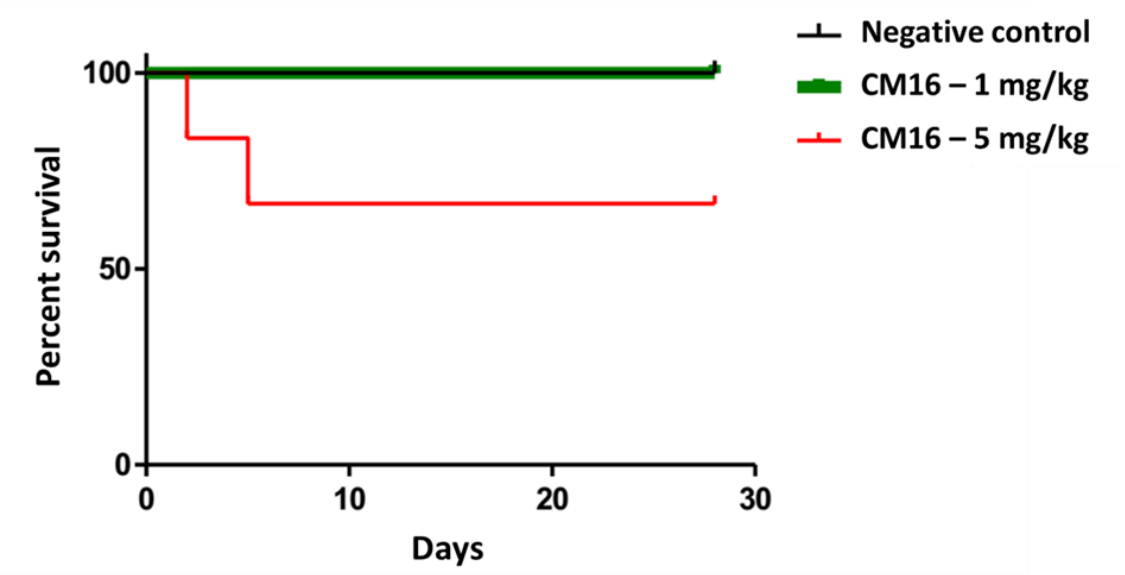


Figure 40. Percent survival obtained during 28 days after unique intraperitoneal administration of the excipient (99.9% NaCl 0.9% w/v and 0.01% Tween-80) (Negative control) or CM16 at 1 mg/kg or 5 mg/kg. Each group contained six animals (mice).

Secondly, the anticancer effect of this dose was then tested in a mouse lung melanoma pseudometastases model (six weeks C57/BL6 mice). This model is obtained by intravenous injection of mouse melanoma B16F10 cells into the tail vein leading to development of pseudometastases into the lung [255]. The survival of the treated group did not differ from the non-treated (**Figure 41**). Other than the possibility that CM16 is not active in the tested model, the lack of activity could be explained by the low absorption of the compound in the blood stream following the IP administration, preventing it to reach the effective therapeutic dose, which was confirmed by a pharmacokinetic study [199]. In an attempt to circumvent this difficulty, a complexation with cyclodextrin was successfully performed to render CM16 soluble [256] enough to allow intravenous (IV) administration, which, we believe would render a better anticancer activity of this compound. However this remains to be verified in a study

in vivo. The results presented in this section were collected from the doctoral thesis of C. Meinguet (NAMEDIC – University of Namur, presented in 2015) [199].

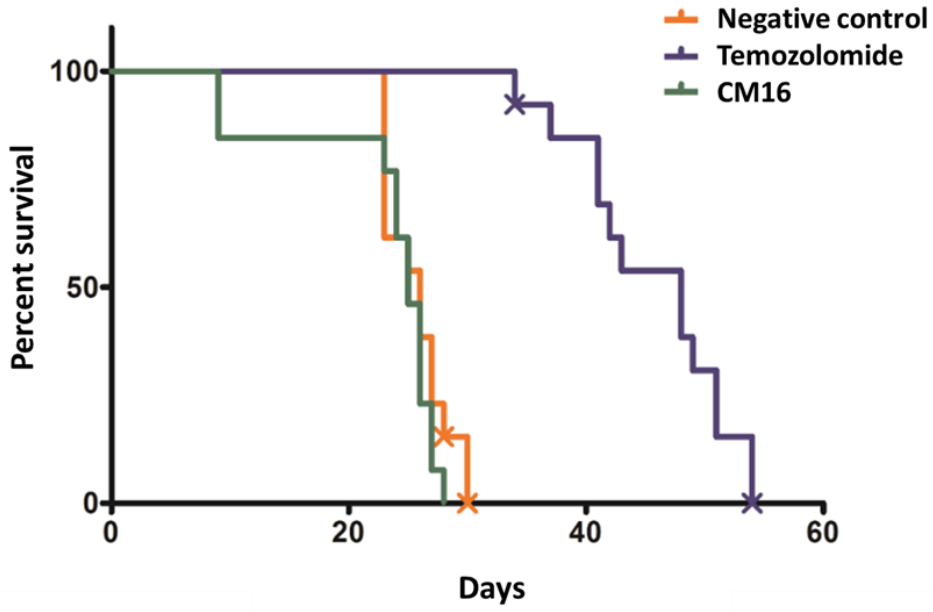


Figure 41. Determination of survival percent for the negative control group (13 animals) treated via intraperitoneal administration of the excipient three times per week during one week (99.9% NaCl 0.9% w/v and 0.01% Tween-80), the group treated via oral administration with temozolomide (80 mg/kg) three times per week during three weeks (13 animals) and the group treated with CM16 (1 mg/kg) via intraperitoneal administration once a week during three weeks (13 animals), five days after injection of 250,000 B16F10 cells (murine melanoma) per mouse. Statistical analysis (*p* value) were performed with a Log-Rank test. Not significant ($p>0.05$), highly significant ($p<0.0001$). The cross designates the animal euthanasia.

4 CONCLUSIONS

The systematic optimization of this series of β-carbolines included Q-SAR studies amelioration of physicochemical properties, i.e., increase of solubility, decrease of lipophilicity and a molecular weight lower than 500 g/mol. This optimization of the first series allowed to keep the antiproliferative effects in cancer cells and led to the selection of a hit compound, CM16.

In this part of the work we showed the antiproliferative and cytostatic effects of CM16 as well as its lack of interaction with the DNA through fluorescent staining and cell cycle evaluation. The analysis of the NCI 60-cancer-cell line panel evidenced no selectivity to a specific type of cancer, which led us to investigate its mechanism of action in three distinct cancer models from different origins, i.e., glioma, melanoma and breast cancer.

The COMPARE analysis of CM16 with the vast NCI compounds library revealed correlation of this harmine derivative with other protein synthesis inhibitors, including the previously synthetized 5a compound, also identified earlier as a protein synthesis inhibitor. Firstly, we verified that CM16 indeed inhibits protein synthesis. More precisely, we showed that CM16 might be acting on the initiation phase of translation, affecting the ribosomal organization. The further investigation of possible proteins targeted by CM16 showed that the phosphorylation on the initiation factor 2α (eIF2α) could be partly responsible for the inhibitory effect observed. In addition to that, the transcriptomic analysis of the NCI data of the main proteins involved in or controlling translation suggested that *EIF1AX, EIF3E* and *EIF3H* could drive, at least partly, the sensitivity of cancer models to CM16. To provide further insights in the antiproliferative effects of CM16, we conducted a proteomic comparison of cells after treatment with CM16.

GENERAL DISCUSSION

The search for new anticancer drugs coming from natural sources has been very promising over the years [150,321] with many successful examples, such as paclitaxel, camptothecin and the vinca alkaloids. Harmine, a natural β-carboline, displays activity against cancer cell lines *in vitro* and antitumor effects *in vivo*. This compound seems to act mainly through DNA intercalation and inhibition of the anti-apoptotic protein DYRK1A [168,172]. However, the neurotoxicity of this compound is important, causing tremble, twitch and jumping in mice [183,184]. Beta-carbolines are known for their modulation of neurotransmitters and binding to central nervous system receptors, such as MAO, and this could be associated to the neurotoxicity observed for harmine [184].

In order to look for compounds that could potentially be less toxic while keeping activity against cancer cells, harmine derivatives with substituents in different positions were synthetized by several research groups [183,185–194], as reviewed in the introduction of this work (section 5.2.2). These new derivatives di- or trisubstituted were less toxic than harmine and displayed similar or superior antiproliferative and antitumor effects [193,194,322]. In the context of this work, new trisubstituted harmine derivatives were synthetized in the NAMEDIC (University of Namur). This work was carried out previously in two main phases: the first one led to a chemical series displaying higher antiproliferative activity than harmine but appeared not to be intercalating into the DNA nor inhibiting DYRK1A anymore [197,199]. Their analysis by the NCI (COMPARE GI_{50} profile analysis) suggested those to inhibit protein synthesis [197]. The second phase of chemical development aimed at improving the physicochemical properties of these trisubstituted derivatives according to drugability criteria. This second phase led to the CM series to which the main compound of interest of this work, i.e CM16 belongs.

Again the evaluation of CM16 by the NCI (COMPARE GI_{50} profile analysis) on their panel containing 60 different cell lines of nine distinct types of cancers (leukemia, non-small cell lung cancer, colon cancer, central nervous system cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer) suggested it as a potential protein synthesis inhibitor. Although targeting protein synthesis of prokaryotes for therapy is routinely used with several antibiotics [323], the interest in targeting the eukaryotic machinery for human therapy arose from the new discoveries of its fine-tuning and regulation. In particular, malignant cells require

higher levels of protein synthesis to maintain oncogenic programs [74] and in recent years it has been extensively shown that several distinct dysregulations occur at the translational level in cancer cells [72,73,75]. It is true that there are long-known protein synthesis inhibitors used for research purposes, like cycloheximide and puromycin. However, their toxicity impedes those to be clinically used. Therefore, research with compounds targeting specific deregulated proteins and signalling pathways of protein translation in cancer cells have been carried out. Our work is thus in line with the search and investigation of compounds from natural sources to combat cancer through translation inhibition.

Although our work is restricted to the investigation of the effects of the lead compound CM16 in cancer cell lines, we have herein deepened the understanding of how this compound is causing antiproliferative effects. Importantly, other harmine derivatives with antiproliferative and antitumor activities have been described in the literature but no study has shown compounds acting on the translation of cancer cells before those series, at least to our best knowledge. Indeed, the harmine derivatives that were tested in cancer cell lines *in* vitro (reviewed in the Introduction) seem mainly to induce cell cycle arrest and/or apoptosis. Those effects could relate to DNA intercalating properties or DYRK1A inhibition like harmine. On the contrary, the harmine derivative CM16 did not induce cell cycle arrest in the cancer cell lines studied (glioma and melanoma, see part I). In this work, we observed that the harmine derivative CM16 had similar cancer cells inhibitory profile to other protein synthesis inhibitors tested by the NCI. Among those, other than the related compound 5a from the first series, are the translation inhibitors phyllanthoside and bouvardin. Phyllanthoside acts at the mRNA-tRNA translocation level at the elongation phase [214], while bouvardin inhibits elongation by blocking the dissociation of the elongation factor 2 (eEF2) from the ribosome [230]. Bouvardin has been recently investigated in head and neck cancer and glioma cells *in vitro* and its potential in combination with radiation was suggested, according to a study in an *in vivo* model [230]. As for phyllanthoside, it has been studied in the 1980's by the NCI as a potential anticancer agent (*in vitro* and *in vivo* studies) but it showed to be toxic in dogs [324]. Perhaps due to that, no other studies were carried out with this compound in order to develop it further.

Other strategies are more advanced in their development as potential anticancer drugs. The eIF4A inhibitors hippuristanol, pateamine A and silvestrol that are from natural sources (i.e. the marine coral *Isis hippuris*, the marine sponge *Mycale* sp. and fruits and twigs of *Aglaia*

silvestris, respectively) are by example in preclinical development while homoharringtonine from *Cephalotaxus fortunei* that prevents the formation of the first peptide bond is already on the market in the USA for the treatment of chronic myeloid leukaemia [57,89]. The eIF4E antisense oligonucleotide (ASO ISIS1883750) was recently clinically tested (PhaseI/II) in combination with irinotecan for the treatment of irinotecan-refractory colorectal cancer. The results showed that the treatment stabilized the disease (halted the progression) in 47% of the treated patients, although without objective clinical responses.

While we were not able to determine specific targets involved in the mode of action of CM16 in cancer cells *in vitro* yet, with this work, we are able to suggest that CM16 affects the initiation of translation according to the effects observed in the sucrose gradient assays for ribosomal and polysomal organisation evaluation. We also showed that CM16 induced eIF2α phosphorylation, an effect which could be partially responsible for the protein synthesis inhibition observed. Four kinases regulate eIF2α phosphorylation: HRI, PKR, GCN2 and PERK [238,241], with the three last ones being implicated in tumorigenesis (the role of HRI is unclear). These kinases are activated in response to stress stimuli, such as amino acid deficiency and accumulation of misfolded protein on the ER [238]. Although CM16 did not seem to act on PERK when tested in a non-cellular assay, it remains possible that CM16 acts, at least partly, through ER stress-mediated activation of PERK in cellular assay. Thus, CM16 effects on these three kinases (PERK, PKR and GCN2) should be investigated in cellular models.

Additionally, we highlighted the *EIF1AX*, *EIF3E* and *EIF3H* genes as possible modulators of the sensitivity of the cancer cells to CM16. Mutations in the *EIF1AX* gene have been associated with tumour development and progression in thyroid cancer [242,243], uveal melanomas [244,245] and possibly ovarian tumour carcinogenesis [246]. The *EIF3H* gene was also found amplified in breast and prostate cancers [74]. The proteins expressed by the genes *EIF3E* and *EIF3H*, i.e. eIF3e and eIF3h have also been associated to cancer [247]. Therefore, these genes could contribute, at least partly, to CM16 activity but also to its selectivity towards cancer cells in comparison to non-cancerous models (see below the discussion about selectivity). Interestingly, none of those initiation-related proteins have been described as biomarkers or targets in anti-cancer drug therapy to date, at least to our best knowledge.

Finally, we have employed proteomic approaches to investigate how CM16 was affecting early the proteome of glioma cells to try to contribute further in understanding the mode of action of CM16 and possibly highlight other molecular targets of this harmine derivative. Notably, a

recent work employed a mass spectrometry based strategy and identified brusatol as protein synthesis inhibitor [325]. This second study highlighted that CM16 affects expression of proteins involved in cancer progression and related to translation, as 6 out of 8 proteins identified are poly (A) RNA binding proteins and play different roles in cancer. HspB1 is involved in proliferation, migration and invasion of cancer cells and is related to poor prognosis in cancer [287], and it has been associated to translation inhibition by binding to eIF4G [288]. dUTPase is involved in the nucleotides metabolism and inhibitors of this enzyme sensitizes cancer cells to the chemotherapeutic agent 5-FU [294]. The transcriptor factor BTF3 is involved in apoptosis and cell cycle regulation [295] and is overexpressed in several cancers [295,298–301]. Ebp1 promotes cell survival and proliferation and it affects translation by inhibiting phosphorylation of eIF2α [317–319], while cofilin-1 and galectin-1 are involved in cancer cells migration and invasion [310,313].

This work provide thus several arguments that trisubstituted harmine derivatives exert potent antiproliferative effects *in vitro* against cancer cell lines (< 1 µM) through a different mode of action than harmine itself. These chemical substitutions led to a switch in activity from DNA intercalating activity to translation inhibition, probably more particularly at the initiation level. All arguments found to date in this work point indeed to proteins involved in initiation factors and/ complexes but we cannot rule out that CM16 acts via other kind of modifications in the regulation of protein synthesis. Indeed, not only specific targets in the translation machinery but also inhibitors of upstream signalling pathways affecting translation have been shown to inhibit protein synthesis and are under investigation for anticancer therapy [57,66,71]. Those include inhibitors of mTOR, that controls eIF4F assembly, and of MNK, that controls eIF4E phosphorylation. However, these signalling pathway mediators regulate other cellular processes, thus being less specific. Finally, important discoveries have unveiled deregulation of the ribosome biogenesis in cancer that can also be explored as targets [83,326]. Due to oncogenic signalling and tumour suppressor loss, transcription of rRNA by Pol I (RNA polymerase I) is often increased in cancer cells leading to increase in ribosome biogenesis to meet their needs. The small molecule CX-5461 that inhibits Pol I and results in the inhibition of ribosomal RNA (rRNA) synthesis without genotoxicity is undergoing Phase I/II clinical trial for the treatment of solid tumours [129,333 and clinicaltrials.gov].

Further defining if CM16 could be an interesting strategy among all those described above depends on its mode of action and targets that still remain to be identified but also to other issues including selectivity and toxicity.

We have included in our *in vitro* MTT assay several non-cancerous cell lines, i.e. dermal and lung fibroblasts and previously in the NAMEDIC endothelial cell lines and fibroblasts and we observed that the harmine derivative CM16 is at least 10 times more selective to the cancer cells tested than to these normal cell models. We have qualitatively compared the intracellular penetration of CM16 by means of fluorescent microscopy but we did not observed any difference between cancerous and non-cancerous cell model penetration that would have possibly contributed to this selectivity. Additionally the effects on protein synthesis were also observed in NHDF fibroblasts. While a suitable *in vitro* selectivity index would be >100 times [327], it is important to interpret these data with caution and to note that *in vitro* selectivity can only be considered as indicative in such preliminary phase. Indeed, jumping from *in vitro* to *in vivo* remains challenging and using fibroblast or endothelial cells as non-cancerous is clearly restrictive when considering that *in vivo* administration could lead to distribution in all organs, or not, being each more or less sensitive to the effects of the compound of interest. Thus pharmacokinetic studies are mandatory for any further development, keeping in mind that selectivity/ decrease in toxicity can be achieved or improved *in vivo* via pharmaceutical development as exemplified by paclitaxel or doxorubicin last formulation developments [328,329]. The difference in rate of proliferation between cancer and normal cells as well as in protein synthesis demand and turn over should also be taken into consideration and could be part to the difference of sensitivity we observed. Our transcriptomic comparison of the cancer cell models from the NCI with high versus low sensitivity was focused only on genes related to translation. We would have liked to perform a full transcriptomic comparison but were, however, not able to do so because of practical issues (these data were retrieved in the accessible format for our collaborator Pr A. Kornienko). However this very preliminary analysis pointed initiation genes *EIF1AX, EIF3E* and *EIF3H* described above that have been recently pointed to be deregulated in cancers. Further evaluation of their possible involvement in cell sensitivity to CM16 would need i) validation at the proteomic level, ii) with over- or down-expression assays and comparison between cancer models and non-cancerous models. The fact that we did not performed the proteomic validation together with the other investigated initiation and elongation factors is due to the fact that the results of the transcriptomic

analysis were obtained much later. Finally, we also have to consider that due to the hostile cancer environment, not only cap-dependent translation is altered, but also IRES-dependent translation is increased in cancers. Therefore we cannot rule out the possibility of CM16 acting on this system, which needs to be further assessed. This scenario is indeed different in normal cells, where approximately only 10% of mRNA translation is initiated via IRES-dependent translation [66]. Thus, it remains a possibility that CM16 could be targeting IRES-dependent translation components, which could make it more selective to cancer cells.

Further evaluation of CM16 toxicity *in vivo* should also be addressed before any further development. CM16 has favourable drug-like characteristics, such as moderate solubility (189 µg/mL), molecular weight under 500 g/mol and logP lower than five. In addition to that, this compound has the ability to penetrate the BBB according to a theoretical model. Considering that harmine has been shown to be neurotoxic with induction of tremble, jumping and twitch symptoms in mice, particular attention to neurotoxicity should be considered. While neurotoxicity of harmine is well established, the mechanisms of this later effect have not been clearly evidenced. Nevertheless, the inhibitory effect of harmine on MOA appears as a hypothesis thereof [156]. Importantly, the CM16 parental compound 5a does not inhibit the MAO anymore in comparison to harmine (results obtained in the NAMEDIC) but whether it is still the case with respect to CM16 remains to be validated.

One main strength of this work relies on its multidisciplinary character: several laboratories and/ or institution have obtained similar and cross-validating results of i) the antiproliferative concentration of CM16 confirmed by two academic labs (ULB, current thesis and NAMEDIC, University of Namur) and the NCI and ii) the protein synthesis inhibition suggested by high correlation from NCI COMPARE data further validated by two academic labs (ULB, current thesis and Pr J.P. Pelletier, McGill University). Our attempts to decipher the intracellular mechanisms of CM16 pointed initiation factors and protein translation regulators in i) our lab by the proteomic approach, ii) J.P. Pelletier lab on eIF2α as well as iii) with the NCI transcriptomic comparison. Those results have been observed in cancer cell models from different origins including breast, brain and melanoma cancer cell lines but also in normal dermal fibroblasts, allowing to us to hypothesize that these effects are not cell type/ context specific.

However our work still lack of numerous validations and no specific target could be identified yet. This could be partly due to lack of time and workflow that is not always fitting with the

scientific plans and progression of the results considering the numerous collaborations involved. Of course financial, technical and biological factors are also taken into account day by day so that we are aware that our work could have been improved. For example, there were clear limitations concerning the approaches here used to study the proteome of glioma cells treated with CM16. The first and most evident one is the low number of proteins identified in the gel-free approach (shotgun proteomics). This could be due to instrumental and method limitations, since the resolution of the LC and the low frequency of acquisition by MS, as well as a consequence the acquisition time, did not allow us to detect higher numbers of peptides. Furthermore, no sample preparation (like desalting and fractionation) was performed.

Our aim being the analyses of major changes in the proteome of glioma cells treated with CM16, the label-free shotgun was our first method of choice. However, changes are minor due to the treatment conditions (early time points) and thus difficult to be evidenced with this approach. Despite the low number of proteins identified, we have attempted to identify the changes caused by treatment with CM16 through label-free quantification approach. Firstly, with the available software we have attempted to calculate the AUC of extracted ion chromatograms of identified peptides based on a list, which revealed to be a very manual process prone to errors if pursued to quantify all the proteins found in the samples. Yet, this method of quantification proved useful later in the study when comparing specific proteins that were identified in the gel-based approach with the gel-free approach. There are other label-free methods and free software that have been reported and used in the literature [277,330–332] and perhaps could be further explored in order to try and highlight possible differences between cells treated and non-treated in this study. Other than the MS-based analysis, which relies on the relationship between the MS signal and protein abundance [272], other methods are also available. The spectral count of a protein, for example, relies on the fact that peptides of more abundant proteins present in the sample will be more selected for fragmentation and therefore produce a higher number of MS/MS spectra [273]. Still, the task of comparing protein abundance from information collected at the peptide level is recognizably very challenging. This is due to the complexity of the data structure, exemplified by dependence on ionization efficiency, abundancy of peptides, instrument performance as well as other aspects such as proteins that share same peptides and factors that affect peptide measurability [271]. In our case, perhaps the way that we have tried to identify peptides from

the TIC chromatograms profiles of glioma cells treated or not with CM16, in which so many intensities and thus information is contained, was not the best method and could be the reason why finally after identifying a set of proteins we were not able to see a difference between treated or not when analysing.

For future possible development and application, although CM16 seemed to affect different cancer cell lines with a similar concentration, as evidenced by the NCI results (approximately 80% of the cell lines had GI_{50} close to the mean) and our own results both suggesting that CM16 effects did not seem to depend on the type of cancer cell line tested, we observed when selecting the most sensitive and the most resistant ones to CM16 to perform the transcriptomic evaluation, that three out of five of the most sensitive cell lines are leukemia cells. Thus, this observation could also help in the decision of targeting a specific type of cancer for future *in vivo* evaluations with CM16. We, however, selected to target glioma because this compound is theoretically able to cross the BBB, a feature that was known before getting the NCI results and conducting the transcriptomic comparison. The cancer type(s) to be chosen for further possible development could thus need to be questioned again, notably when more toxicological and pharmacokinetic data will be obtained.

GENERAL CONCLUSIONS AND PERSPECTIVES

In the framework of investigating potential anticancer activity of compounds coming from natural sources, this manuscript describes the effects of new trisubstituted harmine derivatives in cancer cells *in vitro*. Previously, our collaborators (NAMEDIC – University of Namur) synthetized a series of novel β-carbolines, using the natural compound harmine as point of depart for searching new monoamine-oxidase (MAO) inhibitors. However, because harmine and derivatives were previously shown to exert potential anticancer effects *in vitro*, the evaluation of the effects of those new trisubstituted derivatives against cancer cell lines was also conducted and revealed a higher *in vitro* activity than harmine while losing its MAO inhibition, DNA intercalating and DYRK1A inhibition activity. The synthesis of a second analogue series of derivatives was rationally designed with 3D-QSAR, evaluation of physicochemical characteristics like solubility and logP followed by *in vitro* and *in vivo* tests of the antiproliferative and antitumor properties of the best candidate, i.e. CM16.

Although there was an indication that compounds from the first series were possibly translation inhibitors of cancer cells, the mechanism of action of CM16 as a potential translation inhibitor remained to be investigated. In this thesis we deepened the understanding of the effects of CM16 and thereby contributed to elucidating its mechanism of action in cancer cells as a potential translation inhibitor. The COMPARE analysis of the NCI revealed correlation with other protein synthesis inhibitors, among which the previously studied harmine derivative 5a. Then, we verified that CM16 inhibits the protein synthesis of cancer cells and it appears to possibly affect the initiation phase of translation. CM16 intracellular distribution seems to be parallel to the endoplasmic reticulum but additional experiments should be conducted to confirm co-localization. CM16 was also shown to phosphorylate the initiation factor 2α (eIF2α) in breast cancer cells, which might be, at least partially, responsible for their inhibition of translation. Again validation in other cellular models are mandatory to validate the role of eIF2α in the mode of action of CM16. The transcriptomic analysis made on the basis of the NCI data set suggested that *EIF1AX*, *EIF3E* and *EIF3H* could drive, at least partly, the sensitivity of cancer models to CM16, further supporting translation initiation as one of the processes affected by this compound. While the proteomic investigation of glioma cells under CM16 treatment still needs to be optimized, our preliminary results revealed that CM16 affects it significantly as early as 15 h after a treatment with 1 µM of CM16. Gel-based approach enabled us to identify eight proteins differentially

expressed in the glioma cells under treatment with CM16. These proteins are all linked to cancer biology, several of which being directly involved in protein synthesis regulation and process. Therefore they also might participate in the antiproliferative effect of CM16 in glioma cells.

Translation inhibition in cancer is emerging as a new and promising alternative to the existing therapies due to the specific alterations of the translation machinery in cancer cells. In this work we have observed translation inhibition of cancer cells by a harmine derivative – CM16 – that has good physicochemical characteristics to be further developed as a drug. Moreover, no previous study highlighted harmine derivatives as possible protein synthesis inhibitors.

In terms of perspectives, it would be interesting to investigate the translation inhibition of CM16 in some of the cancer cell lines that were most resistant and most sensitive to CM16 with the aim to evidence whether the genes differentially expressed in these cell lines and their products could indeed drive sensitivity to CM16's treatment. Deepening the possible roles of eIF2α, *EIF1AX, EIF3H, EIF3E,* transcription factor BTF3, Ebp1 and HspB1 in the antiproliferative effects of CM16 should be carried out and made in both cancerous and non-cancerous cell lines models to contribute in understanding the selectivity of CM16 against cancer cell models. We could first perform western blotting to study the effects of CM16 on these proteins. Then, validation of these results and even another proteomic study in another cancer cell model can be envisaged in order to evaluate if the effects we observed are not cell specific. In a similar way, the upregulated proteins found in our proteomics study, i.e. galectin 1 and Ebp1 could be envisaged as response or resistance mechanism to CM16 treatment.

Moreover, to evaluate if CM16 also inhibits the cap-independent translation, IRES, since transformed cells also enhance their use of this type of translation [66], is a perspective of this work. This could be achieved by using the IRES element of the cricket paralysis virus (CrPV), a reporter construct containing luciferase to be detected in a rabbit reticulocyte lysate [107] in the presence of CM16.

Regarding shotgun proteomics, it remains as a perspective to perhaps apply other methods of label-free quantification for a better exploitation of the data. Using labelled and targeted proteomics approaches can also be envisaged to quantify or to investigate specific targets, for example those suggested in our transcriptomic comparison, i.e. EIF1AX, EIF3E and EIF3H.

Studying those as possible biomarkers of cancer cells sensitivity to CM16 should also be envisaged.

Although the GI_{50} of CM16 in 80% of the 60 cell lines was close to the mean value (between 0.1 and 0.5 µM), the study with these most and least sensitive cell lines could also help in the decision of targeting a specific type of cancer for future *in vivo* evaluations with CM16. Because CM16 showed very high blood brain barrier penetration in a theoretical model [198], we have considered glioma as a promising area for future assay and development. However, pharmacokinetic study would be required to evaluate the distribution and elimination of CM16 following its intravenous (IV) administration. Very importantly, the toxicity of this compound should be investigated *in* vivo. According to outcomes of those studies, we could envisage preparing CM16 pharmaceutics for *in vivo* efficacy study in cancer bearing mice. With respect to drug development against glioma, we would first assay a syngeneic orthotopic glioma mouse model such as GL261 [333] treated intravenously and chronically with CM16 as it is an antiproliferative compound *in vitro*.

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APPENDICES

APPENDIX 1: THE WORLD HEALTH ORGANIZATION CLASSIFICATION OF THE TUMOURS OF THE CENTRAL NERVOUS SYSTEM IN 2016.

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	9400/3
Gemistocytic astrocytoma, IDH-mutant	9411/3
Diffuse astrocytoma, IDH-wildtype	*9400/3*
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH-mutant	9401/3
Anaplastic astrocytoma, IDH-wildtype	*9401/3*
Anaplastic astrocytoma, NOS	9401/3
Glioblastoma, IDH-wildtype	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epithelioid glioblastoma	*9440/3*
Glioblastoma, IDH-mutant	9445/3*
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M–mutant	9385/3*
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	9451/3
Anaplastic oligodendroglioma, NOS	*9451/3*
Oligoastrocytoma, NOS	*9382/3*
Anaplastic oligoastrocytoma, NOS	*9382/3*
Other astrocytic tumours	
Pilocytic astrocytoma	9421/1
Pilomyxoid astrocytoma	9425/3
Subependymal giant cell astrocytoma	9384/1
Pleomorphic xanthoastrocytoma	9424/3
Anaplastic pleomorphic xanthoastrocytoma	9424/3
Ependymal tumours	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Papillary ependymoma	9393/3
Clear cell ependymoma	9391/3
Tanycytic ependymoma	9391/3
Ependymoma, *RELA* fusion–positive	9396/3*
Anaplastic ependymoma	9392/3
Other gliomas	
Chordoid glioma of the third ventricle	9444/1
Angiocentric glioma	9431/1
Astroblastoma	9430/3
Choroid plexus tumours	
Choroid plexus papilloma	9390/0
Atypical choroid plexus papilloma	9390/1
Choroid plexus carcinoma	9390/3

Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	9413/0
Gangliocytoma	9492/0
Ganglioglioma	9505/1
Anaplastic ganglioglioma	9505/3
Dysplastic cerebellar gangliocytoma (Lhermitte–Duclos disease)	9493/0
Desmoplastic infantile astrocytoma and ganglioglioma	9412/1
Papillary glioneuronal tumour	9509/1
Rosette-forming glioneuronal tumour	9509/1
Diffuse leptomeningeal glioneuronal tumour	
Central neurocytoma	9506/1
Extraventricular neurocytoma	9506/1
Cerebellar liponeurocytoma	9506/1
Paraganglioma	8693/1
Tumours of the pineal region	
Pineocytoma	9361/1
Pineal parenchymal tumour of intermediate differentiation	9362/3
Pineoblastoma	9362/3
Papillary tumour of the pineal region	9395/3
Embryonal tumours	
Medulloblastomas, genetically defined	
Medulloblastoma, WNT-activated	9475/3*
Medulloblastoma, SHH-activated and *TP53*-mutant	9476/3*
Medulloblastoma, SHH-activated and *TP53*-wildtype	9471/3
Medulloblastoma, non-WNT/non-SHH	9477/3*
Medulloblastoma, group 3	
Medulloblastoma, group 4	
Medulloblastomas, histologically defined	
Medulloblastoma, classic	9470/3
Medulloblastoma, desmoplastic/nodular	9471/3
Medulloblastoma with extensive nodularity	9471/3
Medulloblastoma, large cell / anaplastic	9474/3
Medulloblastoma, NOS	9470/3
Embryonal tumour with multilayered rosettes, C19MC-altered	9478/3*
Embryonal tumour with multilayered rosettes, NOS	*9478/3*
Medulloepithelioma	9501/3
CNS neuroblastoma	9500/3
CNS ganglioneuroblastoma	9490/3
CNS embryonal tumour, NOS	9473/3
Atypical teratoid/rhabdoid tumour	9508/3
CNS embryonal tumour with rhabdoid features	*9508/3*
Tumours of the cranial and paraspinal nerves	
Schwannoma	9560/0
Cellular schwannoma	9560/0
Plexiform schwannoma	9560/0

Appendix 1 continued

Melanotic schwannoma	9560/1
Neurofibroma	9540/0
Atypical neurofibroma	9540/0
Plexiform neurofibroma	9550/0
Perineurioma	9571/0
Hybrid nerve sheath tumours	
Malignant peripheral nerve sheath tumour	9540/3
Epithelioid MPNST	9540/3
MPNST with perineurial differentiation	9540/3
Meningiomas	
Meningioma	9530/0
Meningothelial meningioma	9531/0
Fibrous meningioma	9532/0
Transitional meningioma	9537/0
Psammomatous meningioma	9533/0
Angiomatous meningioma	9534/0
Microcystic meningioma	9530/0
Secretory meningioma	9530/0
Lymphoplasmacyte-rich meningioma	9530/0
Metaplastic meningioma	9530/0
Chordoid meningioma	9538/1
Clear cell meningioma	9538/1
Atypical meningioma	9539/1
Papillary meningioma	9538/3
Rhabdoid meningioma	9538/3
Anaplastic (malignant) meningioma	9530/3
Mesenchymal, non-meningothelial tumours	
Solitary fibrous tumour / haemangiopericytoma**	
Grade 1	8815/0
Grade 2	8815/1
Grade 3	8815/3
Haemangioblastoma	9161/1
Haemangioma	9120/0
Epithelioid haemangioendothelioma	9133/3
Angiosarcoma	9120/3
Kaposi sarcoma	9140/3
Ewing sarcoma / PNET	9364/3
Lipoma	8850/0
Angiolipoma	8861/0
Hibernoma	8880/0
Liposarcoma	8850/3
Desmoid-type fibromatosis	8821/1
Myofibroblastoma	8825/0
Inflammatory myofibroblastic tumour	8825/1
Benign fibrous histiocytoma	8830/0
Fibrosarcoma	8810/3
Undifferentiated pleomorphic sarcoma / malignant fibrous histiocytoma	8802/3
Leiomyoma	8890/0
Leiomyosarcoma	8890/3
Rhabdomyoma	8900/0
Rhabdomyosarcoma	8900/3
Chondroma	9220/0
Chondrosarcoma	9220/3
Osteoma	9180/0
Osteochondroma	9210/0
Osteosarcoma	9180/3
Melanocytic tumours	
Meningeal melanocytosis	8728/0
Meningeal melanocytoma	8728/1
Meningeal melanoma	8720/3
Meningeal melanomatosis	8728/3
Lymphomas	
Diffuse large B-cell lymphoma of the CNS	9680/3
Immunodeficiency-associated CNS lymphomas	
AIDS-related diffuse large B-cell lymphoma	
EBV-positive diffuse large B-cell lymphoma, NOS	
Lymphomatoid granulomatosis	9766/1
Intravascular large B-cell lymphoma	9712/3
Low-grade B-cell lymphomas of the CNS	
T-cell and NK/T-cell lymphomas of the CNS	
Anaplastic large cell lymphoma, ALK-positive	9714/3
Anaplastic large cell lymphoma, ALK-negative	9702/3
MALT lymphoma of the dura	9699/3
Histiocytic tumours	
Langerhans cell histiocytosis	9751/3
Erdheim–Chester disease	9750/1
Rosai–Dorfman disease	
Juvenile xanthogranuloma	
Histiocytic sarcoma	9755/3
Germ cell tumours	
Germinoma	9064/3
Embryonal carcinoma	9070/3
Yolk sac tumour	9071/3
Choriocarcinoma	9100/3
Teratoma	9080/1
Mature teratoma	9080/0
Immature teratoma	9080/3
Teratoma with malignant transformation	9084/3
Mixed germ cell tumour	9085/3
Tumours of the sellar region	
Craniopharyngioma	9350/1
Adamantinomatous craniopharyngioma	9351/1
Papillary craniopharyngioma	9352/1
Granular cell tumour of the sellar region	9582/0
Pituicytoma	9432/1
Spindle cell oncocytoma	8290/0
Metastatic tumours	

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) (742A). Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification, taking into account changes in our understanding of these lesions.
*These new codes were approved by the IARC/WHO Committee for ICD-O. *Italics*: Provisional tumour entities. **Grading according to the 2013 *WHO Classification of Tumours of Soft Tissue and Bone*.

APPENDIX 2: TABLE CONTAINING CHEMOTHERAPEUTIC DRUGS PER CLASS AND THEIR INDICATION ACCORDING TO THE RÉPERTOIRE COMMENTÉ DES MÉDICAMENTS OF THE CENTRE BELGE D'INFORMATION PHARMACOTHÉRAPEUTIQUE, BELGIUM, 2015 AND 2017.

	Class	Drug	Indications
Antimetabolites	Folate analogues	- methotrexate Emthexate® Ledertrexate® Metoject®	Various malignancies
	Pyrimidine analogues	- cytarabine Cytosar® Depocyte® - fluorouracil Fluracedyl® Fluroblastine® - gemcitabine Gemtamycine®Sandoz - azacytidine Vidaza® - decitabine Dacogen® - capécitabine Xeloda®	Leukemia, colorectal and gastric carcinoma, breast cancer, Hodgkin's disease, head and neck cancers, lung, pancreatic, ovarian, vesicle and breast carcinomas
	Purine analogues	- cladribine Leustatin® Litak® - clofarabine Evoltra® - fludarabine Fludara® - mercaptopurine Puri-nethol® - nelarabine Atriance® - tioguanine Lanvis®	Leukemia
	Others	- pemetrexed Alimta® - hydroxycarbamide Hydrea® - raltitrexed Tomudex®	Leukemia, lung mesothelioma and carcinoma, colorectal carcinoma

Alkylating and intercalating agents	**Nitrogen mustards**	**- melphalan Alkeran® - busulfan Busilvex® - cyclophosphamide Endoxan® - ifosfamide Holoxan® - chlorambucil Leukeran®**	**Several solid tumours and haematological malignancies**
	Nitrosoureas	- fotemustine Muphoran®	Metastatic malignant melanoma and glioma
	Platinum analogues	- carboplatin Carbosin® Carboplatinum® - cisplatin Cisplatine®Teva - oxaliplatin Eloxatin® Oxiplatin®Hospira	Head and neck cancers, lung, ovarian, gastric and colorectal carcinomas
	Other alkylating agents	- bendamustine Levact® - dacarbazine Dacarbazine®Medac - estramustine Estracyt® - temozolomide Temodal®	Chronic lymphocytic leukemia, non-Hodgkin's lymphoma, multiple myeloma, metastatic melanoma, sarcomas, Hodgkin's disease, glioblastoma, astrocytoma, metastatic prostate carcinoma
Topoisomerase inhibitors	Topoisomerase I inhibitors	- irinotecan Campto® Irinosin® - topotecan Hycamtin®	Metastatic colorectal cancer, ovarian, cervical and lung carcinomas
	Topoisomerase II inhibitors	- etoposide Celltop® Eposin® Vepesid®	Solid tumours and haematological malignancies
Microtubule inhibitors	Vinca alkaloids	- vinblastine Vinblastine® Teva - vincristine Vincrisin® - vindesine Eldisine® - vinorelbine Navelbine®	Hodgkin's disease, solid tumours and acute leukemia
	Taxanes	- docetaxel Taxotere® - paclitaxel Paclitaxin® - cabazitaxel Jevtana®	Metastatic prostate and breast carcinomas, lung, gastric, ovarian carcinomas and Kaposi sarcoma

Antitumor antibiotics	Anthracyclines	- doxorubicin Adriblastina® Caelyx® Myocet® - daunorubicin Cerubidine® - epirubicin Farmorubicine® - idarubicine Zavedos® - mitoxantrone Xantrosin®	Solid tumours, haematological malignancies, leukemia
	Bleomycin	- bleomycin Bleomycine®Sanofi	Solid tumours, haematological malignancies
	Others	- mitomycin Mitomycin-C®	Solid tumours

Kinase inhibitors		- imatinib Glivex® - gefitinib Iressa® - sorafenib Nexavar® - dasatinib Sprycel® - sunitinib Sutent® - erlotinib Tarceva® - nilotinib Tasigna® - lapatinib Tyverb® - pazopanib Votrient® - bosutinib Bosulif® - vandetanib Caprelsa® - afatinib Giotrif® - ponatinib Iclusig® - ibrutinib Imbruvica® - axitinib Inlyta® - ruxolitinib Jakavi® - regorafenib Stivarga® - dabrafenib Tafinlar® - osimertinib Tagrisso® - nintedanib Vargatef® - pazopanib Votrient® - crizotinib Xalkori® - vemurafenib Zelboraf® - idelalisib Zydelig® - ceritinib Zykadia®	Lung, pancreatic, hepatocellular carcinomas, kidney cancer, chronic myeloid leukemia, metastatic melanoma, breast cancer

Monoclonal antibodies		- bevacizumab Avastin® - cetuximab Erbitux® - trastuzumab Herceptin® - trastuzumab emtasine Kadcyla® - rituximab Mabthera® - aldesleukin Proleukin® - catumaxomab Removab® - panitumumab Vectibix® - ibritumomab Zevalin® - brentuximab Adcetris® - obinutuzumab Gazyvaro® - pembrolizumab Keytruda® - nivolumab Opdivo® - pertuzumab Perjeta® - ipilimumab Yervoy® - aflibercept Zaltrap®	Metastatic renal adenocarcinoma, colorectal, breast and gastric carcinomas, lymphomas, head and neck cancer, melanoma, soft tissue sarcoma

APPENDIX 3: FIRST PAGE OF ARTICLE PUBLISHED CONTAINING PART OF THE RESULTS PRESENTED IN PART I OF THIS MANUSCRIPT.

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Molecular and cellular pharmacology

A harmine-derived beta-carboline displays anti-cancer effects *in vitro* by targeting protein synthesis

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ABSTRACT

Growing evidence indicates that protein synthesis is deregulated in cancer onset and progression and targeting this process might be a selective way to combat cancers. While harmine is known to inhibit DYRK1A and intercalate into the DNA, tri-substitution was shown previously to modify its activity profile in favor of protein synthesis inhibition. In this study, we thus evaluated the optimized derivative CM16 *in vitro* anti-cancer effects unfolding its protein synthesis inhibition activity. Indeed, the growth inhibitory profile of CM16 in the NCI 60-cancer-cell-line-panel correlated with those of other compounds described as protein synthesis inhibitors. Accordingly, CM16 decreased in a time- and concentration-dependent manner the translation of neosynthesized proteins *in vitro* while it did not affect mRNA transcription. CM16 rapidly penetrated into the cell in the perinuclear region of the endoplasmic reticulum where it appears to target translation initiation as highlighted by ribosomal disorganization. More precisely, we found that the mRNA expression levels of the initiation factors EIF1AX, EIF3E and EIF3H differ when comparing resistant or sensitive cell models to CM16. Additionally, CM16 induced eIF2α phosphorylation. Those effects could explain, at least partly, the CM16 cytostatic anti-cancer effects observed *in vitro* while neither cell cycle arrest nor DNA intercalation could be demonstrated. Therefore, targeting protein synthesis initiation with CM16 could represent a new promising alternative to current cancer therapies due to the specific alterations of the translation machinery in cancer cells as recently evidenced with respect to EIF1AX and eIF3 complex, the potential targets identified in this present study.

1. Introduction

Among various processes that enable the continuous growth, multiplication and dissemination of malignant cells (Hanahan and Weinberg, 2011), protein synthesis plays an important role in the onset and progression of cancer. Growing evidence indicates that targeting mRNA translation as a cancer therapy has the potential of selectively eradicating cancerous cells (Bhat et al., 2015; Nasr and Pelletier, 2012; Spilka et al., 2013). In eukaryotic cells, mRNA translation occurs in four stages: initiation, elongation, termination and ribosome recycling. Among these, initiation is believed to be pivotal in the regulation of translation (Sonenberg and Hinnebusch, 2009), and is often altered in cancer through dysregulation of expression and/or phosphorylation status of translation initiation proteins, including eIF2α, eukaryotic translation initiation factor 3 (eIF3) and members of the eukaryotic translation initiation factor 4F complex (eIF4F) (Bhat et al., 2015; Blagden and Willis, 2011; Silvera et al., 2010). Translational control contributes to maintain several oncogenic programs (Silvera et al., 2010) and is reciprocally affected by oncogenic signaling pathways, which include MAPK and PI3K-AKT-mTOR (Bader et al., 2005; Topisirovic and Sonenberg, 2011). In response to energy and nutrient demand, mTOR is activated by the PI3K signaling cascade and

Abbreviations: DYRK1A, dual specificity tyrosine phosphorylation regulated kinase 1A; EIF1AX, eukaryotic translation initiation factor 1A, X-linked; EIF3E, eukaryotic translation initiation factor 3 subunit E; EIF3H, eukaryotic translation initiation factor 3 subunit H; eIF2α, eukaryotic translation initiation factor 2 subunit 1

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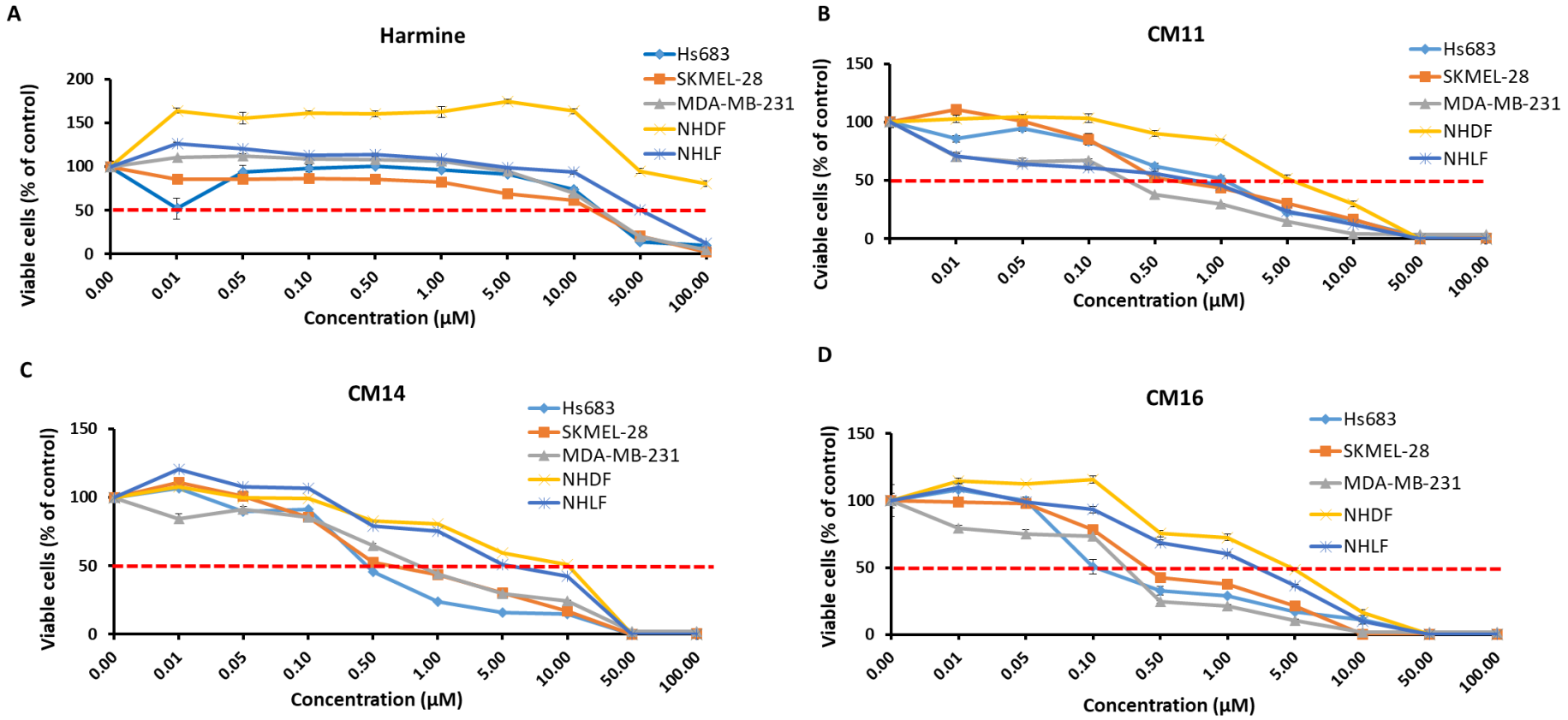
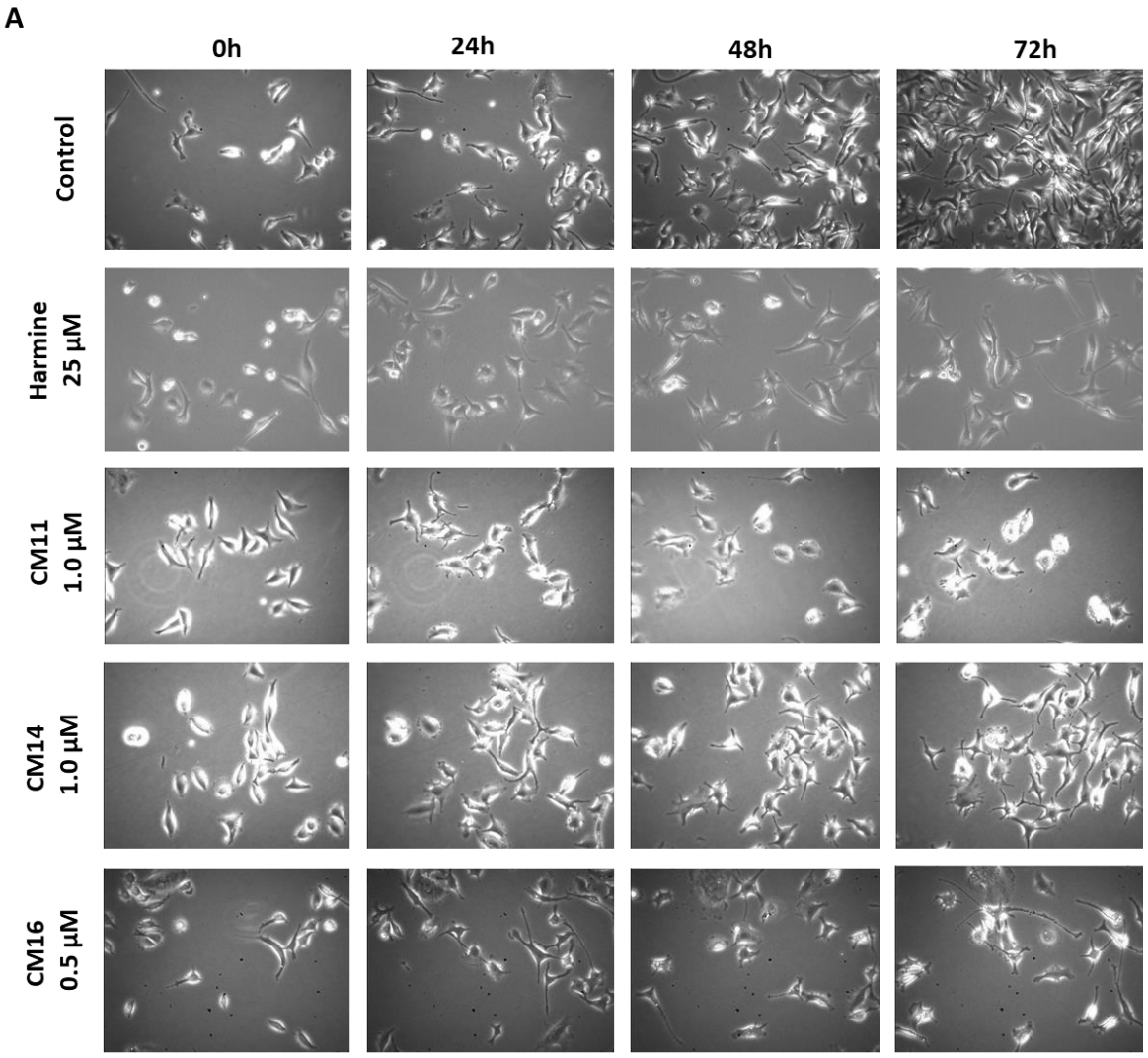
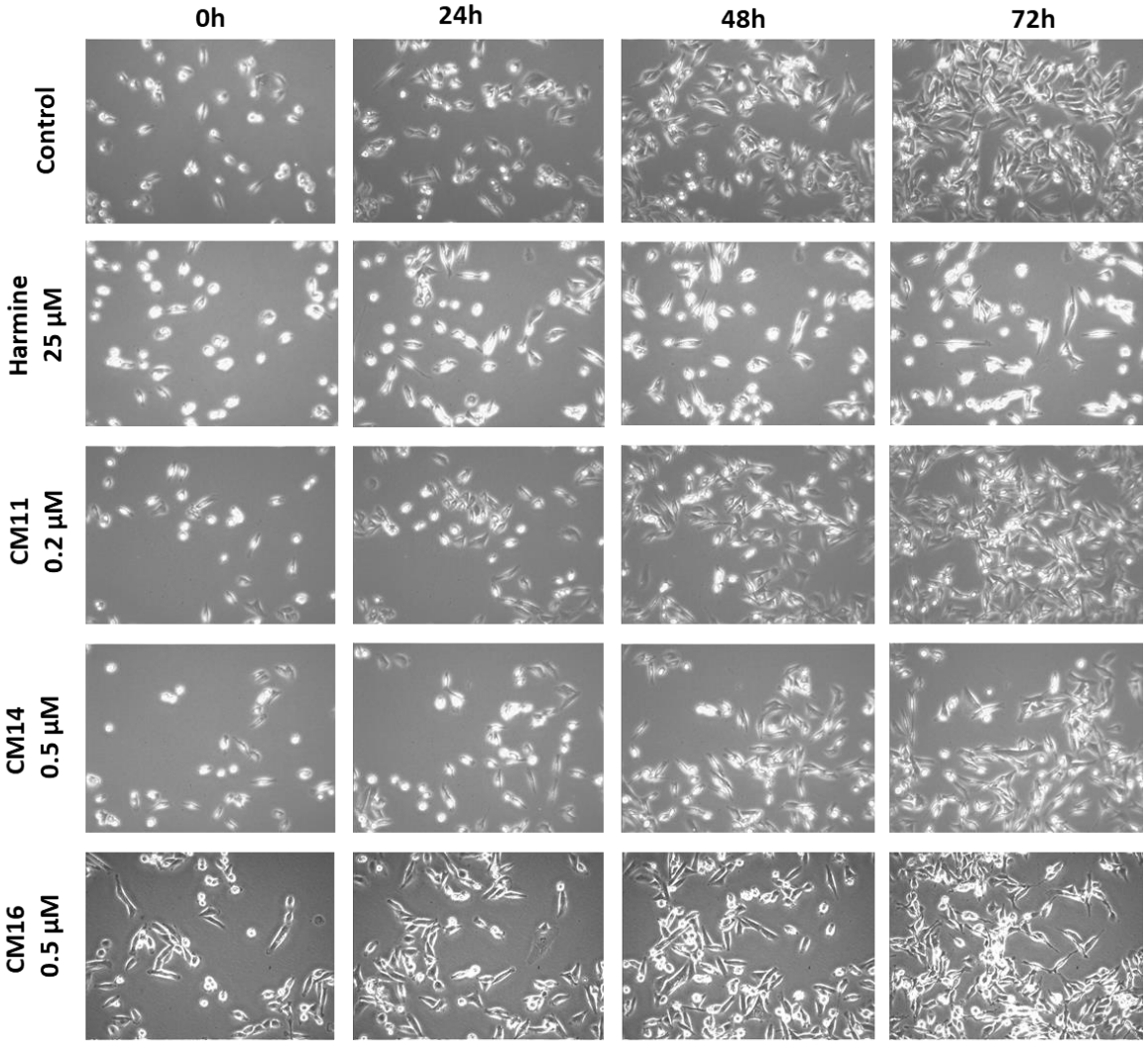
APPENDIX 4: CELL GROWTH INHIBITION IN CANCER CELLS VERSUS NON-CANCEROUS CELLS TREATED WITH HARMINE (A), CM11 (B), CM14 (C) AND CM16 (D) FOR 72 H AS COMPARED TO THE NON-TREATED CONTROL. THE RED DASHED LINE IN THE 50% CELL VIABILITY MARK AIDS THE VISUALIZATION OF THE CONCENTRATION OF EACH COMPOUND NEEDED TO INHIBIT 50 % OF CELL PROLIFERATION FOR EACH CELL LINE (IC_{50}). DATA ARE EXPRESSED AS THE MEAN OF VIABLE CELLS RELATIVE TO CONTROL (100%) ± S.E.M. OF THE SIX REPLICATES OF ONE REPRESENTATIVE EXPERIMENT. TWO EXPERIMENTS WERE PERFORMED IN NON-CANCEROUS CELL LINES (NHDF AND NHLF) AND THREE IN CANCEROUS CELL LINES (HS683 AND SK-MEL-28).



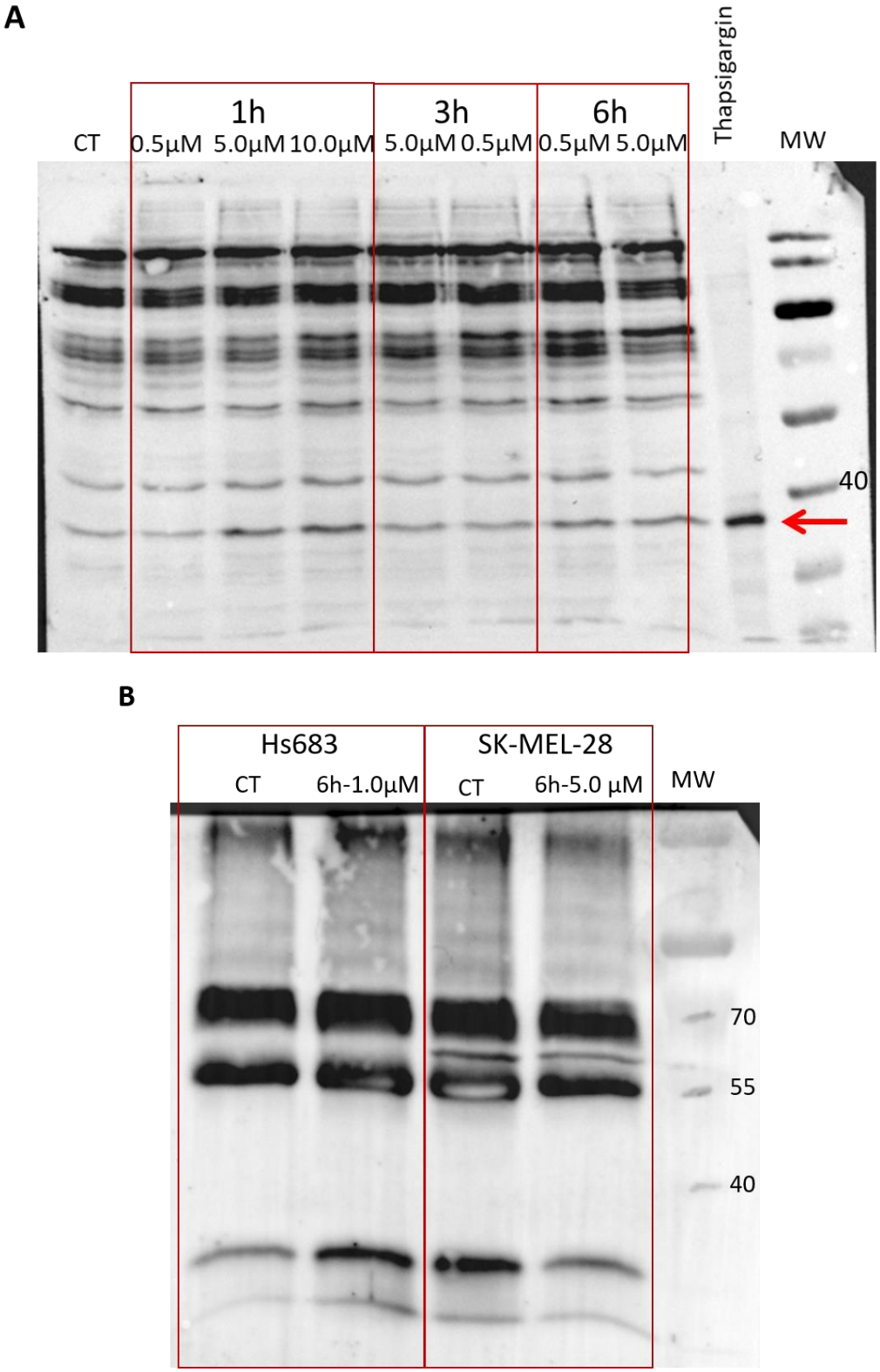
APPENDIX 5: CM11, CM14 AND CM16 VIDEOMICROSCOPY IN SK-MEL-28 AND MDA-MB-231. VIDEOMICROSCOPY OF THE INDUCED IN VITRO *EFFECTS OF HARMINE AND DERIVATIVES CM11, CM14 AND CM16 IN THE (A) SK-MEL-28 AND (B) MDA-MB-231 CELL LINES. FIGURES ARE REPRESENTATIVE OF ONE EXPERIMENT PERFORMED IN THREE REPLICATES. EXPERIMENTS WERE PERFORMED ONCE WITH HARMINE, CM11 AND CM14 AND AT LEAST TWICE WITH CM16, EACH IN TRIPLICATES.*



B


0h
24h
48h
72h
Control
Harmine 25 µM
CM11 0.2 µM
CM14 0.5 µM
CM16 0.5 µM

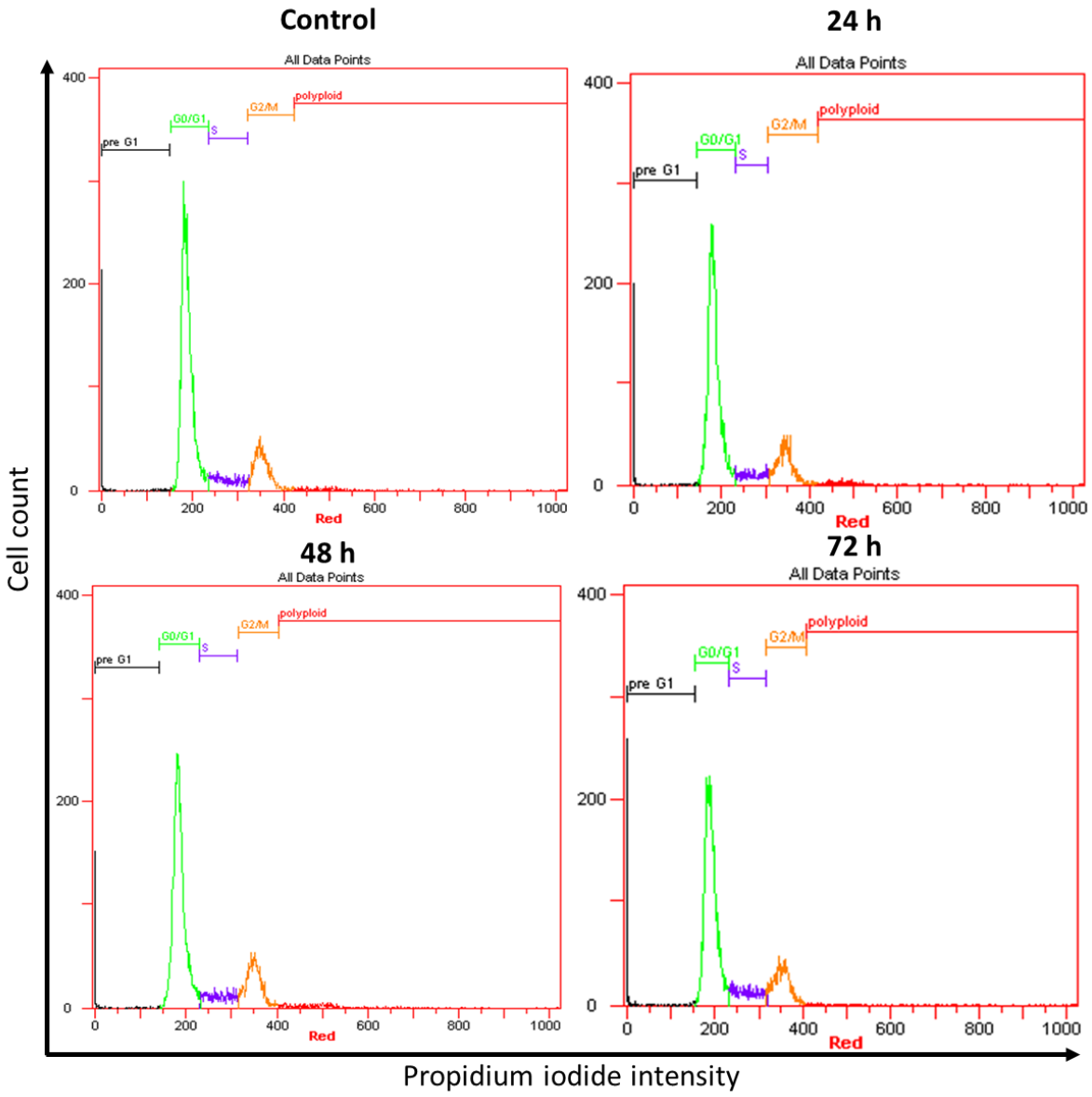
***APPENDIX** 6: EXAMPLE OF IMMUNOBLOTTING OF EIF2-P IN (A) SK-MEL-28 CELL LINE. THE POSITIVE CONTROL THAPSIGARGIN INDUCES EIF2 PHOSPHORYLATION (INDICATED WITH THE RED ARROW). THE MOLECULAR WEIGHT OF EIF2 IS 36 KDA. AT LEAST THREE INDEPENDENT EXPERIMENTS WERE PERFORMED IN BOTH HS683 AND SK-MEL-28 CELL LINES. (B): IMMUNOBLOTTING OF EIF2-P FOLLOWING IMMUNOPRECIPITATION WITH PHOSPHOSERINE. TWO EXPERIMENTS WERE PERFORMED.*



***APPENDIX** 7: NUMBER OF PROTEINS IDENTIFIED FOR EACH SAMPLE IN EACH OF THE FIVE INDEPENDENT EXPERIMENTS (NON-TREATED CONTROL AND TREATED WITH CM16) ON SHOTGUN PROTEOMICS.*

Sample	Amount of proteins identified by auto MS/MS
CT_1	142
CT_2	131
CT_3	145
CT_4	147
CT_5	130
15h_0.1 µM _1	125
15h_0.1 µM _2	116
15h_0.1 µM _3	128
15h_0.1 µM _4	123
15h_0.1 µM _5	120
15h_1.0 µM_1	134
15h_1.0 µM _2	126
15h_1.0 µM _3	118
15h_1.0 µM _4	116
15h_1.0 µM _5	123
24h_0.1 µM _1	128
24h_0.1 µM _2	118
24h_0.1 µM _3	122
24h_0.1 µM _4	111
24h_0.1 µM _5	117
24h_1.0 µM_1	145
24h_1.0 µM _2	114
24h_1.0 µM _3	121
24h_1.0 µM _4	120
24h_1.0 µM _5	117

***APPENDIX** 8: CELL CYCLE PROFILES OF HS683 GLIOMA CELLS TREATED WITH 0.1 µM OF CM16 FOR 24, 48 AND 72 H. PROFILES ARE REPRESENTATIVE OF FOUR REPLICATES OF ONE EXPERIMENT.*



***APPENDIX** 9: (A) CM16 PERINUCLEAR DISTRIBUTION IN GLIOMA CELLS (5.0 µM AFTER 5 MIN TREATMENT) AND (B) ENDOPLASMIC RETICULUM STAINING OF HS683 GLIOMA CELLS WITH FLUORESCENT PROBE ONLY (ER-TRACKER). EXPOSURE TIMES FOR BLUE FILTER (EX/EM) 359-371/397 NM: 80 MS; AND FOR RED FILTER (EX/EM) 540-580/593-668 NM: 850 MS (HS683). PICTURES WERE TAKEN WITH A 40X OBJECTIVE.*