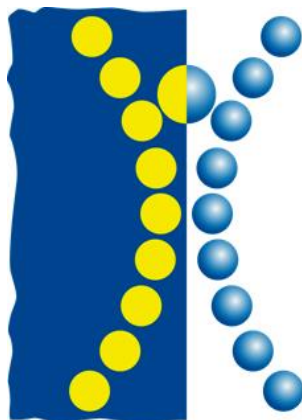


Coimbra Health School - ESTeSCoimbra



Master of Pharmacy

Specialization in Applied Pharmacotherapy

Dissertation

*A Novel Integration Approach: Perturbation Studies *in vitro* Using PDK1 Inhibitors in Glioblastoma Multiforme*

Luís Carlos Fernandes de Oliveira

Coimbra, MARCH 2014



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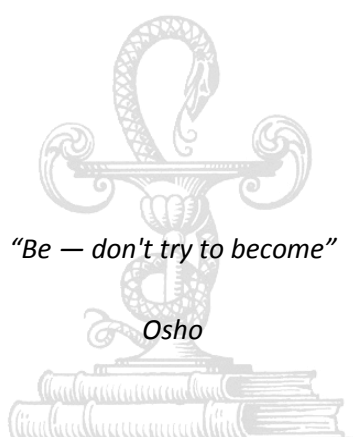
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Coimbra, March 2014



“Be — don't try to become”

Osho

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Resumo

Os objetivos a atingir em 2020 no que respeita ao processo de investigação e desenvolvimento de medicamentos estão claramente focados na redução em termos temporais na investigação pré-clínica e clínica e na diminuição da taxa de atrito entre as novas moléculas. De forma a atingir estes objetivos, um novo conceito tem sido desenvolvido e aplicado a este complexo e moroso processo, este é a Farmacologia Quantitativa e de Sistemas. Além disso, esta abordagem inovadora pode ser crucial para o tratamento de determinados tipos de tumores cerebrais letais – Glioblastoma Multiforme (GBM) – que permanecem um desafio terapêutico, e por tanto, uma doença com um destino fatal para os doentes. Por estas razões, esta dissertação de mestrado apresenta uma especial relevância, tendo por objetivos avaliar o potencial impacto e importância biológica da variação de parâmetros farmacológicos, para além da potência, no contexto da resposta celular ao fármaco, pela avaliação da perturbação induzida em células do GBM por inibidores do PDK1 e pela realização de uma caracterização multiparamétrica dose-resposta destas novas moléculas.

A presente dissertação assume em Portugal a vanguarda na área da Farmacologia Quantitativa e de Sistemas aplicada ao processo de investigação e desenvolvimento de medicamentos. Em última estância, esta dissertação poderá contribuir para uma melhor previsão dos fármacos durante este processo, significando assim possíveis vantagens para os utentes, indústrias farmacêuticas, institutos de investigação, governo e institutos superiores.

Palavras-chave

PDK1 | Glioblastoma Multiforme | Farmacologia Quantitativa e de Sistemas | Investigação e Desenvolvimento de Medicamentos

Abstract

The target objectives for 2020 regarding the drug discovery & development process are clearly focused on the reduction of the years of both preclinical and clinical research and decrease drug attrition rate. With a view to achieve this goal, a novel concept can be applied to this complex and time-consuming process – the Quantitative and Systems Pharmacology. In addition, this innovative approach can be critical for the treatment of a lethal type of primary brain tumour – Glioblastoma Multiforme (GBM) – which remains therapeutically challenging, therefore a disease with a clear dramatic fatal destination for patients. For these reasons, the present MSc thesis project has a special relevance by aiming to evaluate the potential impact and biological importance of variation in pharmacological parameters other than potency, in the context of cellular drug response, by measure the induced perturbations in GBM cells by PDK1 inhibitors and performing a multiparametric characterization dose-response of these drugs.

This project assume in Portugal the vanguard on the area of Systems Pharmacology applied to drug discovery & development process. Ultimately, it will hopefully contribute to a better prediction of specific drugs, which means possible advantages for patients, pharmaceutical companies, research institutes, governments and academia.

Keywords

PDK1 | Glioblastoma Multiforme | Quantitative & Systems Pharmacology | Drug Discovery & Development

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Abbreviations

%CV – Coefficient of variation

AEDs – Antiepileptic drugs

AKT or PKB – Protein kinase B

ALERTS – Number of structural alerts

ALOGP – Octanol-water partition coefficient

AROM – Aromatic rings

AUC – Area under the dose-response curve

C₀ – Untreated control well

cAMP – Cyclic adenosine monophosphate

CBTRUS – Central Brain Tumour Registry of the United States of America

CDKN2A – Cyclin-dependent kinase inhibitor 2A

ceRNAs – Competitive endogenous RNAs

cGMP – Cyclic guanosine monophosphate

CMap – Connectivity Map

CNS – Central Nervous System

CNS MPO – Central Nervous System Multiparameter Optimisation

CO₂ – Carbon dioxide

C-terminal – Carboxyl-terminal

DMEM – Dulbecco's modified eagle's medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

DTA – Doubling time analysis

DTT – Dithiothreitol

E_0 or E_{max} - Maximum Effect

EC_{50} - Concentration at half-maximal effect

EGFR – Epidermal growth factor receptor

E_{inf} - Minimum Effect

EMA – European Medicines Agency

FBS – Fetal bovine serum

FDA – Food and Drug Administration

FI – Functional interaction

FOXO – Forkhead box transcription factors

GBM – Glioblastoma Multiforme

GI_{50} - Concentration needed for growth inhibition by 50%

GICs – Glioma-initiating cells

GSCs – Glioma stem cells

HBA – Hydrogen-bond acceptors

HBD – Hydrogen-bond donors

HDAC – Histone deacetylase

HEPES – 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HER1 – Epidermal growth factor receptor type1

HM – Hydrophobic motif

HS – Hill Slope

IC₅₀ – Half-maximum inhibitory concentration

IDH1 – Isocitrate dehydrogenase 1

lncRNAs – Long non-coding RNAs

LOA – Likelihood of approval

Log – Logarithmic

LOH – Loss of heterozygosity

MAPK – Mitogen-activated protein kinase

MeOH – Methanol

MF – Molecular formula

MgCl₂ – Magnesium chloride

MGMT – O-6-methylguanine-DNA methyltransferase

miRNA – Micro-ribonucleic acid

MoA – Mechanism of action

mTOR – Mammalian target of rapamycin

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

MVP – Microvascular proliferation

MW – Molecular weight

NaCl – Sodium chloride

ncRNAs – Non-coding RNAs

NF1 – Neurofibromin 1

NSCs – Neural stem cells

N-terminal – Amino-terminal

OPCs – Oligodendrocyte precursor cells

PAK1 – p21-activated kinase-1

PBS – Phosphate Buffered Saline

PC – Permeability coefficient

PDGF – Platelet-derived growth factor

PDGFRA – Platelet-derived growth factor receptor, alpha polypeptide

PDK1 – 3-phosphoinositide-dependent protein kinase-1

PDT – Population doubling time

PH – Pleckstrin homology

PI3K – Phosphatidylinositol-3-kinase

PIF – 3-phosphoinositide-dependent protein kinase-1 interacting fragment

PK – Pharmacokinetics

PKA – Protein kinase A

PKC – Protein kinase C

PKG – Cyclic guanosine monophosphate-dependent protein kinase

PKN1 – Serine/threonine-protein kinase N1

PKN2 – Serine/threonine-protein kinase N2

PMSF – Phenylmethanesulfonyl fluoride solution

PRIM – Patient Rule Induction Method

PRKACA – Cyclic adenosine monophosphate-dependent protein kinase

PRKCD – Protein kinase C delta type

PRKCZ – Protein kinase C zeta type

PS – Permeability-surface area product

PSA – Surface area

PTEN – Phosphatase and tensin homolog

QED – Quantitative estimate of drug-likeness

QSP – Quantitative and Systems Pharmacology

R&D – Research and development

R² – Coefficient of determination

Raf – Raf murine sarcoma viral oncogene homolog

RAS – Rat Sarcoma

ROTB – Rotatable bonds

RPS6KA1 – p90 ribosomal protein S6 kinase alpha-1

RPS6KA2 – p90 ribosomal protein S6 kinase alpha-2

RPS6KA3 – p90 ribosomal protein S6 kinase alpha-3

RPS6KB1 – p70 ribosomal protein S6 kinase beta-1

SB – Sleeping Beauty

SD – Standard deviation

SEM – Standard error of the mean

SGK1 – Serum/glucocorticoid-inducible kinase 1

SGK2 – Serum/glucocorticoid-inducible kinase 2

SGK3 – Serum/glucocorticoid-inducible kinase 3



SRB – Sulforhodamine B

TGI – Total growth inhibition

TMZ – Temozolomide

TP53 – Tumour protein p53

WHO – World Health Organisation

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Introduction

Drug Discovery & Development

Nowadays and regarding the actual world economic conjuncture, on the science community the inefficiencies of the drug discovery and development process are a fashionable subject (Antman *et al.*, 2012; Csermely *et al.*, 2013). As we look back, we can see that this process has faced multiple paradigm shifts since its beginning, which dates back to the early days of human civilization (Chandra, 2009; Ji *et al.*, 2009). Back there, drugs were much more than science, they were also associated with religious and spiritual healing. Those medicines were derived mainly from natural products, such as plants and supplemented by animal materials and minerals. Its discovery was probably done by combination of trial and error experimentation, observing the human and animal reactions. Just in the late 1800s, the drug discovery and development started to follow scientific techniques. This approach increased after the World War I, with the born of the modern pharmaceutical industry, rising and establishing the scientific principles of this complex process (Ji *et al.*, 2009; Ng, 2008). In spite of this huge step on the modern pharmaceutical industry, the science behind the research and development (R&D) process has been changing, and the early process entirely focused on serendipity or systematic screening (Figure 1A) has been replaced by other process models, such as the ligand-centric approach (Figure 1B), and lately the target-centric approach (Figure 1C) (Chandra, 2009).

In the last decades, within critical discoveries in science and technology, powerful tools to identify and optimize potential drug candidates were developed, however, the R&D productivity remains one of the biggest challenges (Brown *at al.*, 2003; Abou-Gharbia *et al.*, 2014). Analysing the concept of R&D productivity can be a complex task, but it can be simplified by considering the following two dimensions: efficiency and effectiveness. When we talk about efficiency of R&D, it means the ability to translate inputs – ideas, investment and effort – into defined outputs, such as milestones that represent resolved uncertainties (Swinney *et al.*, 2011). This parameter has been highly discussed by financial markets that highlight fundamental inefficiencies in the current business model. And so, there is an urge for the industry to reduce costs and increase the efficiency of R&D (Williams, 2011). On the other hand, effectiveness of the process can be considered as the ability to produce outputs with certain intended and desired qualities (Swinney *et al.*, 2011). In

order to achieve the required qualities of outputs, the European Medicines Agency (EMA), the Food and Drug Administration, (FDA), and other regulatory entities have assumed that it is critical the development of the regulatory science through this process, and so, increase the effectiveness of R&D (Gispén-de Wied *et al.*, 2013).

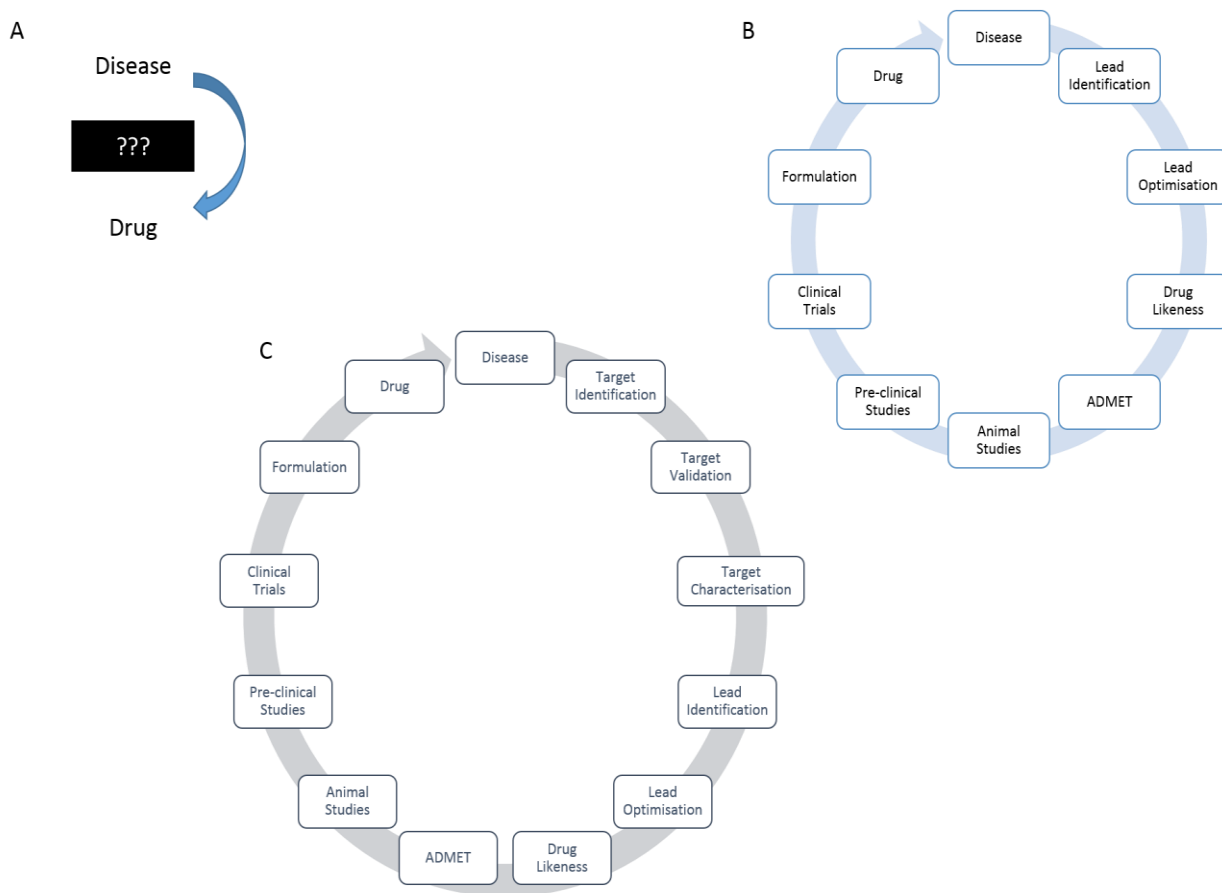


Figure 1 - Scientific background shifts for the drug discovery process. A) In the early days, the process started from the disease to drug treated as a ‘black box’ - serendipity or systematic screening. **B)** The ligand-centric approach with focus on medicinal chemistry. **C)** The subsequent shift to target focus called target-centric approach. Figure adapted from Chandra, 2009.

Challenges

In spite of the innovative developments during the last decades and the huge investments made, the R&D process is still largely inefficient, thus raising expressions such as “valley of death” & “curse of attrition” in the science community to express the drug failures during this process (Abou-Gharbia *et al.*, 2014; Brown *et al.*, 2003). Table 1 comprises a relatively high percentage of failure (29.9 %) for projects in the preliminary stage of discovery than projects achieving the later stages of the process. This proportion decreases to around 14 % in phase I and II of clinical trials. As well, around 10 % of the projects that are in phase 3 clinical trials fail to be launched in the market (Barrenho *et al.*, 2013). Even for the drugs that pass these “valleys”, the understanding of their mechanism is lacking, and this fact is exacerbated by the gap on the respective representative disease models. Therefore, both therapeutic and side effect are poorly predictable (Zhao *et al.*, 2012; Hoeng *et al.*, 2013).

Table 1 – Descriptive statistics across the years between 1980 – 2012 for failures and successes in R&D, duration of the projects, competition, alliances and market size proxies.

Variables	Discovery			Phase 1			Phase 2			Phase 3		
	Total	Failures	Successes	Total	Failures	Successes	Total	Failures	Successes	Total	Failures	Successes
Duration	3.48	5.82	3.66	2.48	4.78	2.22	2.70	5.52	3.08	2.24	5.59	2.70
Competition in the final product market – new drugs	6.06	13.61	8.04	7.21	18.28	10.18	6.69	17.72	10.37	7.59	21.91	7.65
Competition in the final product market – old drugs	0.88	2.00	1.21	0.95	2.22	1.28	1.02	1.86	1.34	1.07	2.07	1.00
Competition within the R&D process –potential competitors	11.02	22.79	14.21	13.08	30.67	17.98	12.37	30.60	18.37	13.70	39.42	13.78
Population, total	1.7E+09	2.0E+09	1.8E+09	1.7E+09	1.9E+09	1.8E+09	1.7E+09	1.9E+09	1.8E+09	1.9E+09	2.3E+09	1.8E+09
GDP pc (constant 2000 US\$)	29058.08	31988.49	30052.19	29882.39	32233.52	30570.54	30734.38	32398.69	31483.51	30503.2	32320.02	30037.68
Intensity of alliances	1.65	1.43	1.69	2.05	1.43	2.08	2.24	1.65	2.46	2.87	2.46	1.78
Type of alliances - big firm participant	0.59	0.56	0.54	0.66	0.64	0.66	0.68	0.67	0.73	0.76	0.69	0.53
Type of alliances - Academia participant	0.12	0.13	0.11	0.09	0.06	0.08	0.08	0.08	0.08	0.08	0.07	0.12
Number of observations	54600			11109			9644			5789		
Number of drug projects	10952	3277		3257	444		2446	361		1597	148	
Proportion of failed projects	29.90%			13.60%			14.80%			9%		
Number of therapeutic classes	78											
Years covered	1980-2012											

Reference: Barrenho *et al.*, 2013

The high drug attrition rates – percentage of new molecules that start in one of the clinical evaluation phases and that fail to succeed on that phase (Moreira, 2014) – are the biggest challenge for R&D model, and understanding it is a key to better understand pharmaceutical industry performance, the magnitude of the long-term investments involved in R&D, and improving investment activity in the future (Barrenho *et al.*, 2013; Csermely *et al.*, 2013). In order to analyse the success of drugs during this process, there is reported two different types of metrics of success: ‘Phase Success’ and ‘Likelihood of Approval’ (LOA). The first metric, ‘Phase Success’, is calculated as the number of drugs that moved from one phase to the next phase divided by the sum of the number of drugs that progressed to the next phase and the number of drugs that were suspended. The n value associated with the Phase Success represents the number of drugs that have advanced plus the number of drugs that have been suspended, labelled as transitions. The LOA denotes the probability of reaching approval from the current phase, being expressed in percentage. It is calculated as the product of each Phase Success probability leading to approval. The n value associated with LOA is the sum of the n values for each Phase Success included in the LOA calculation (Hay *et al.*, 2014).

Still on the side of the business model of R&D, additional challenges are related with key patents expiration, the increase of generic competition and the increasingly constrained healthcare budgets (Williams, 2011). These business concerns are strictly related with the scientific side of the question, since payers will look increasingly to only reimburse medicines that show a clear improvement in patient outcomes relative to existing therapies in defined patient populations, and so, there are some factors that might be critical to analyse and improve in this context:

- Poor target quality, known as “druggability-trap” (Csermely *et al.*, 2013);
- Chemical leads are often discovered to have unwanted side-effects and/or be toxic at later development phases (Csermely *et al.*, 2013);
- Underestimation of the complexity of cells, organisms and human disease (Csermely *et al.*, 2013);
- Lack of mechanistic understanding of how drugs interactions at the molecular level manifest themselves as alterations in tissue/organ-level function (Zhao *et al.*, 2012);
- The knowledge gained to assess other related drugs, or to develop new drugs is not used (Baird, 2012);

- Cellular and tissue/organ-level systems are treated as black boxes (Zhao *et al.*, 2012);
- Patient-to-patient variability in drug response (Fallahi-Sichani *et al.*, 2013);
- Inability to predict adverse events when the drug is brought to market and used by the population at large (Zhao *et al.*, 2012);
- The more limited phenotypic screening in recent years (Swinney *et al.*, 2011);
- Data unreliable (Csermely *et al.*, 2013).

Oncologic Drug Discovery & Development Challenges

In oncology, the R&D seems to be even more complex as the success rate of drug candidates is abysmally lower when compared with the other areas of healthcare. On that field, the success rates for drugs entering clinical development range from 3 to 8 % and less than 5% will be on the pharmacy shelf (Williams, 2011; Bhattacharjee, 2012).

According to the analysis of discontinued drugs in oncology reports shown elsewhere (Williams, 2011; Williams, 2013a; Williams, 2013b), from 2006 to 2012 there is an upwards trend in the number of development terminations over the years, with no clear pattern, in 2010/11, to either the type of molecules terminated, with both small molecules and biological represented, or the biological axes targeted. However, the failure trend in 2012 benefited the biological therapeutic approaches (Table 2). Particularly noteworthy was the increase in Phase I terminations (Table 3) and the increase in discontinuous for strategic, including financial, reasons (Table 4). And so, the analysis of drugs discontinued from the global oncology pipeline in 2012 reflects similar trends to those reported for 2011 cohort.

Table 2 – Evolution trend on the type of molecules terminated from 2011 to 2012.

2011	2012
20 Small molecules	19 Small molecules
13 Antibodies	8 Antibodies
2 DNA-based therapeutics	1 Antisense oligonucleotide
2 Non-antibody proteins	1 Cyclic Peptide

Reference: Williams, 2013a; Williams, 2013b

Table 3 – Discontinued drugs by phase in 2006, 2007, 2008, 2010, 2011 and 2012.

Year	Phase I	Phase II	Phase III	Total I-III
2006	8	5	2	15
2007	9	10	4	23
2008	12	5	6	23
2010	11	12	5	28
2011	23	9	5	37
2012	13	9	7	29

Reference: Williams, 2013b

Table 4 – Reasons for discontinued development of oncology drugs candidates in 2006, 2007, 2008, 2010, 2011 and 2012.

Year	Reasons						Total
	Unspecified	Strategic	Efficacy	Toxicity	PK	Accrual	
2006	3	2	8	2	0	0	15
2007	10	2	9	1	1	0	23
2008	8	4	3	6	0	2	23
2010	8	9	6	4	1	0	28
2011	8	20	5	1	3	0	37
2012	10	7	11	0	1	0	29

PK – Pharmacokinetics. Reference: Williams, 2013b

In addition to the challenges shown above, the lack of resources, wrong incentives, aggressive pricing strategies or adverse regulatory environments, no consideration of tumour microenvironment, cross-talk and negative feedback loops, development of resistance, exposure time, drug delivery or the choice of preclinical models must be considered. Much remains to be done in terms of increasing the efficiency of drug development, although new and more effective medicines are being approved for the treatment of cancer. Some of these drugs have made improvements in overall survival for all patients with a given condition, whereas others have only show benefit in smaller groups of patients with known molecular aberrations (Moreno *et al.*, 2013).

Innovation on the Drug Discovery & Development Process

Dramatic changes to the scientific and business environments have made it impossible for pharmaceutical and biotech companies to continue operating as they have over the past 30 years (Csermely *et al.*, 2013). Also, there is currently impossible to predict precisely the effects of a particular candidate drug in humans, making initially promising drugs lack efficacy or to have unsupportable levels of toxicity-typically at a late stage of a clinical trials, costing years of effort and up to \$1 billion (Leo, 2011).

To overcome these limitations, efforts and innovative approaches have been developed. One of them, which has been already reported as reaping benefit in terms of new, effective therapies seems to be the collaboration among academics, industry, regulators, government and not-for-profit organisations (Williams, 2013b). This has led to creation of large open-access databases of chemicals and associated bioactivity data, providing fantastic opportunities for computational research to contribute to a systemic understanding of drug-perturbed molecular and physiological processes, hopefully, leading to more rational drug discovery (Iskar *et al.*, 2012). The integration of computational systems approach for drug target discovery with high-throughput screening technologies – covering gene expression, proteomics, metabolomics or cellular phenotypes – will contribute in a wide extent to elucidate a drug's general mechanism of action (MoA) on a biological system, and so, shortcut many aspects by generating hypotheses early on the process. Just by curiosity, the large-scale profile-based comparison of drug treatments were pioneered by the Connectivity Map (CMap) project (Iskar *et al.*, 2012). Other integrations approaches have been developed, which is the case of the idea of 'reversing disease profiles', in

which diseases are characterized by disruptions of processes and networks at the cellular level, integrating profiles of altered gene expression, metabolite concentrations, and other. Also the incorporation of drug-drug similarity measures, side-effect profiles, properties of the drugs' targets, disease-disease similarity, toxicity phenotypes, compound's mechanism-based toxicity, characterize drug-induced organ and organism failures will be in the future part of the routine of the novel drug discovery and development process (Figure 2) and integrating modeling by computational systems approach is expected in a long run to be critical on this process (Figure 3) (Chandra, 2009; Iskar *et al.*, 2012; PwC, 2011).

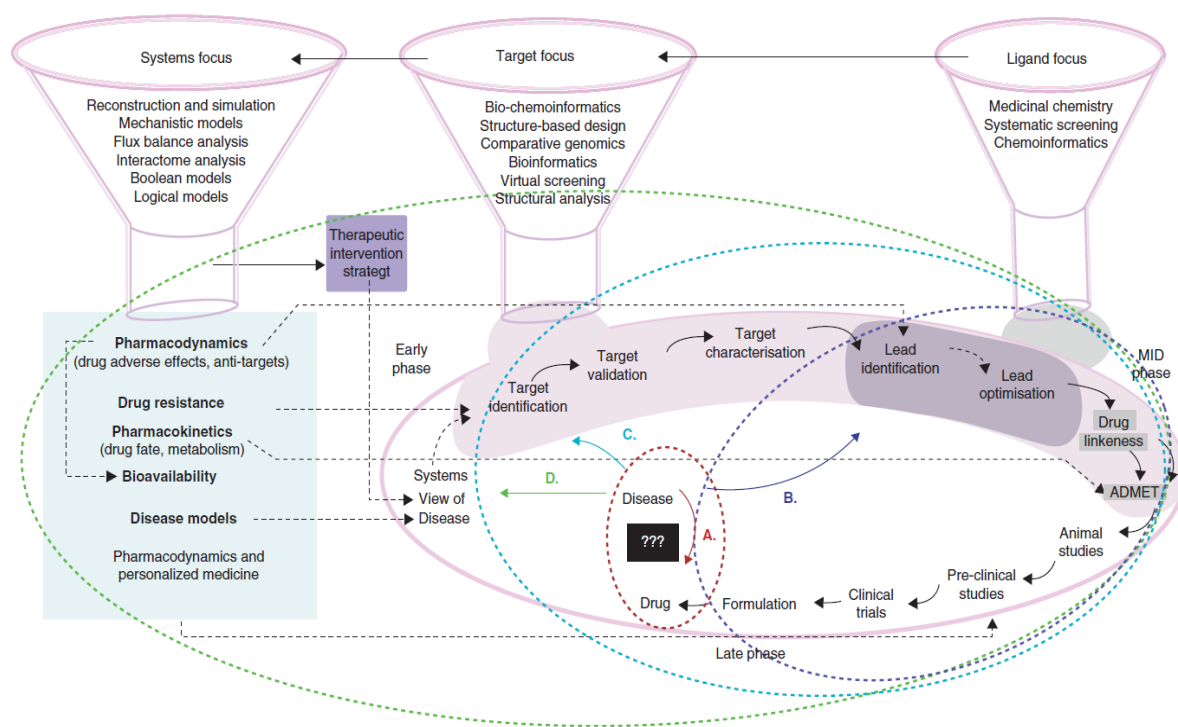


Figure 2 – Drug discovery and development models. Evolution of the drug discovery processes has resulted in changing the discovery pipeline. **A)** ‘black-box’ model (red). **B)** Ligand-centric approach (Blue). **C)** Target-centric approach (cyan). **D)** Newly emerging shift to systems level focus (green). Various steps of the pipeline(s) as well the broad techniques that are required and the issues that they can address are also indicated in the figure. *Reference:* Chandra, 2009

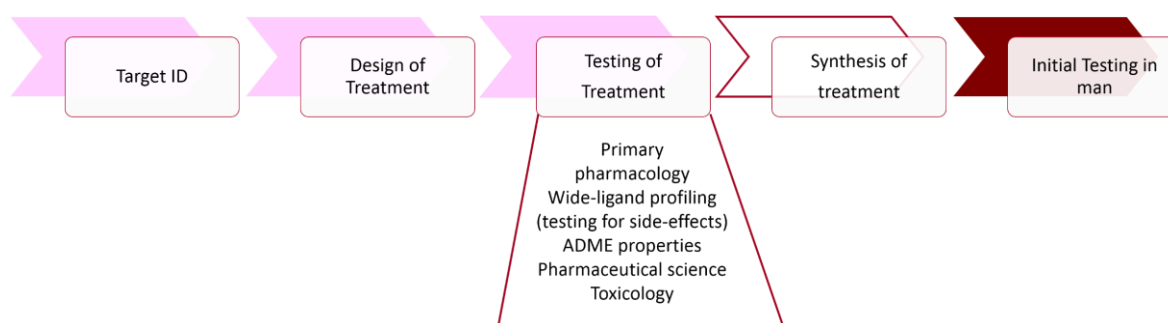


Figure 3 – What the drug discovery process might look like with a fully developed computational systems approach – “Virtual Man”. Reference: PwC, 2011

Nowadays, so far, some different metric approaches have been well established with the aim to improve the survival of development candidates and increase the speed at which these candidates are identified (Wager *et al.*, 2010). These approaches are called ‘Target Product Profiles’, which are typically defined based on the existing body of knowledge and experience of development of orally dosed drugs, focusing the analysis of simple drug-like properties for known drugs, such as molecular weight (MW), lipophilicity ($\log P$ or $\log D$), polar surface area (PSA), counts of hydrogen-bond acceptors (HBA), hydrogen-bond donors (HBD), aromatic rings (AROM) and rotatable bonds (ROTB). Some existing profiles based on different criteria are defined below (Yusof *et al.*, 2014; Ritchie *et al.*, 2014; Wager *et al.*, 2010):

- Lipinski’s Rule of Five;
- 3/75 concept introduced by Pfizer;
- Patient Rule Induction Method (PRIM);
- Central Nervous System Multiparameter Optimisation (CNS MPO) Algorithm;
- Drug-likeness Score.

For this project, drug-likeness score assumes a special importance regarding its previous application to the compounds provided by the Department of Pharmacy of the University of Pisa (Italy). Therefore, this profile aims to identify virtual or real molecules that fall into what is considered to be a drug-like chemical space, based on the calculated physicochemical properties of marketed oral drugs (quantitative estimate of drug-likeness, QED) and published human data, high-scoring and low-scoring drugs, from 0 (all properties are completely undesirable) to 1 (all properties

are ideal). This calculation uses eight important properties, namely: MW, octanol-water partition coefficient (ALOGP), HBD, HBA, PSA, ROTB, AROM and number of structural alerts (ALERTS) (Ritchie *et al.*, 2014; Yusof *et al.*, 2014). This approach seems to be attractive as it integrates several important molecular properties and provides a continuous scale against which structures can be compared, where compounds can still achieve reasonable scores even if one or two properties are unfavourable because the others are close to ideal. Also, it solves the issues arising from the use of hard cut-off thresholds for physicochemical properties (Ritchie *et al.*, 2014).

Recently, the Harvard Medical School aiming to transform the drug discovery process launched an 'Initiative in Systems Pharmacology', which is a comprehensive strategy convening biologist, chemists, pharmacologists, physicists, computer scientist and clinicians to explore together how drugs work in complex systems, and so, transforming therapeutics. There are two broad goals of this initiative: significantly increase the knowledge of human disease mechanisms, the nature of heterogeneity of disease expression in different individuals, and how therapeutics act in human system; the other – based in this knowledge, trying to provide more effective translation of ideas to patients, by improving the quality of drug candidates as they enter clinical testing and regulatory approval process, aiming to increase the number of efficacious diagnostics and therapies reaching patients (Leo, 2011).

Quantitative & Systems Pharmacology

What is it?

As defined by the Quantitative and Systems Pharmacology (QSP) White Paper (Sorger *et al.*, 2011), QSP is an emerging discipline, which focus on identifying and validating drug targets, understanding existing therapeutics and discovering new ones. Its goal is to understand, in a precise, predictive manner, how drugs modulate cellular networks in space and time, moreover, how they impact human pathophysiology.

The aim of this discipline is to develop formal mathematical and computational models that incorporate data at several temporal and spatial scales (Sorger *et al.*, 2011; Iskar *et al.*, 2012). The focus of these models will be on the interactions among multiple elements – biomolecules, cells, tissues and others – promoting the understanding and prediction of safety/efficacy profile (Agoram *et al.*, 2011). Creation of multi-scale models that ultimately span knowledge of molecules, cells, tissues and patients will be particularly critical for preclinical and clinical research teams evaluating target selection and testing therapeutic proof of concept (Geerts *et al.*, 2013; Sorger *et al.*, 2011).

QSP draws on several existing disciplines, including classic pharmacology, chemical biology, biochemistry and structural biology, molecular genetics and genomics, pathology, applied mathematics, and medicine, and has an intrinsic and extensive experimental component that incorporates approaches from tissue and organ physiology, pharmacology and cell biology as well as bioinformatics and “-omics” approaches, for example genomics, proteomics and metabolomics analysis (Sorger *et al.*, 2011; Zhao *et al.*, 2012). QSP will accelerate drug discovery and development by helping to identify and validate targets (and druggable) networks, uncover drug-response biomarkers, design better drugs and drug combinations, select appropriate doses and dosage regimens and identify those patients most likely to respond to new therapeutic agents and combinations. It will therefore become a core discipline of translational medicine, which aims to convert the understanding of biological mechanisms into effective ways of treating and preventing diseases (Cucurull-Sanchez *et al.*, 2012; Baird, 2012; Sorger *et al.*, 2011).

Parameterising perturbagen dose-response relationships

In order to assess the prediction of the drug response, and so, better characterize the drug and the whole system, other metrics than variation in potency (half-maximum inhibitory concentration – IC_{50}) must be systematically measurement and integrated with other scientific technologies (Fallahi-Sichani *et al.*, 2013).

The focus to date on potency ignores the potential impact and biological importance of variation in other parameters, such as the (Fallahi-Sichani *et al.*, 2013; Heiser *et al.*, 2011):

- Steepness of the dose-response curve, called Hill Slope (HS);
- Maximum Effect (E_0 or E_{max});
- Minimum Effect (E_{inf});
- Area under the dose-response curve (AUC);
- Concentration needed for growth inhibition by 50% (GI_{50});
- Total Growth Inhibition (TGI);
- Concentration at half-maximal effect (EC_{50}).

During the development of a *novel* compound, reducing IC_{50} is certainly an important goal, but when the aim is to understand variability in patient responses to an existing drug, it is likely to be more informative to focus on E_0 and HS. Furthermore, different dose-response parameters encode distinct information (Fallahi-Sichani *et al.*, 2013):

- E_0 , IC_{50} , AUC are parameters for simplicity;
- EC_{50} , IC_{50} are classic measures of drug potency;
- E_0 , E_{inf} are measures of drug efficacy;
- AUC combines potency and efficacy of a drug into a single parameter.

Some of the parameters mentioned above are reported to vary systemically with cell line and others with drug. For example, for cell-cycle inhibitors, E_0 often but not always correlate with cell proliferation rate. In the case of drugs targeting protein kinase B/phosphatidylinositol-3-kinase/mammalian target of rapamycin (Akt/PI3K/mTOR) pathway, dose-response curves are reported to be unusually shallow. Also, HS and E_0 shown to be frequently uncorrelated with each other or with GI_{50} , but the parameters varied in a consistent way with drug class. On that line, it

was shown so far that HS was particularly high for drugs such as proteasome and histone deacetylase (HDAC) inhibitors, whereas inhibitors of the Akt/PI3K/mTOR pathway had low and variable HS, special drugs as PP242, temsirolimus, everolimus and rapamycin. In many cases, classical pharmacology has no ready explanation for this phenomenon of variation in dose-response parameters. Association with drug class or target is confounded by polypharmacology, which almost certainly affects the shape of dose-response curves at high drug concentrations, particularly with phenotypic measures of response (Fallahi-Sichani *et al.*, 2013).

Systems Pharmacology: Glioblastoma Multiforme

Epidemiology

Glioblastoma Multiforme (GBM) is the most common intrinsic primary brain tumour in adults, and represents over 80% of diffuse gliomas (Toda, 2013). The age-adjusted rate for glioblastoma is 3.19/100 000, according to the last Central Brain Tumour Registry of the United States of America (CBTRUS) statistical report. It is evident that glioblastoma increases with age and incidence is 75-84 years old, being more common in white males. When it comes to children, GBM accounts approximating 3 % of all primary brain and Central Nervous System (CNS) tumours. The 5 year survival rate is approximately 12 % in children and <5% in adults (Olar *et al.*, 2014).

Definitions & Histopathological Diagnosis

According to the World Health Organisation (WHO) Classification of Tumours of the CNS (4th edition, 2007), GBM is by far the most aggressive diffuse glioma of the astrocytic lineage. Others malignant diffuse gliomas are additionally comprised of oligodendroglioma, and mixed oligoastrocytic neoplasms based solely on morphology and are further subdivided by tumour grade based on additional histologic features present in the tumour (Dunn *et al.*, 2012). GBM corresponds to grade IV and it is most common in the cerebral hemispheres, but it may involve any neuroanatomical level or structure (Olar *et al.*, 2014).

Histologically, glioblastoma is composed of pleomorphic cells, mitotic activity, intravascular microthrombi, necrosis with or without cellular pseudopalisading and/or microvascular proliferation (MVP) (Figure 4). Either one of the latter two features are the *sine qua non* criteria for diagnosis. This aggressive tumour encloses under its umbrella multiple distinct patterns (small cell, giant cell, gliosarcoma, etc.) (Olar *et al.*, 2014).

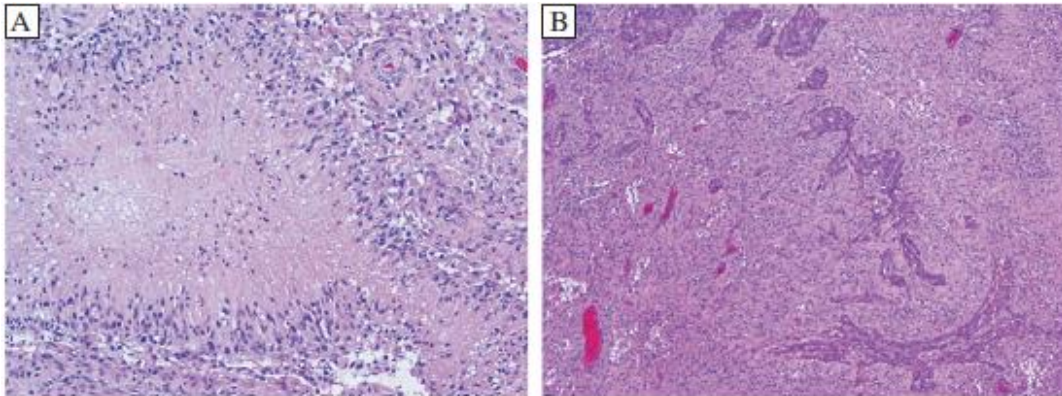


Figure 4 – Histopathology of glioblastoma. Proliferating atypical cells with mitotic activity, necrosis with pseudopalisading (left) and microvascular proliferation (right). *Reference: Olar et al., 2014*

The WHO classification is based on subjective criteria and is imperfect in predicting patient outcome. Tumours may appear virtually identical by histology, yet still have very different outcomes. Beyond their histological criteria, progress in molecular techniques has allowed the identification of a number of markers and genetic profiles that can characterize gliomas (Hofer *et al.*, 2014).

Glioblastoma may be divided into two identical morphological subtypes, based on the presence or absence of a precursor lesion. Primary glioblastoma is the most common type (~90%); it arises *de novo*, without evidence of a precursor lesion, and is common in older adults (> 50 years). Secondary glioblastoma represents progression from a pre-existent, lower-grade astrocytoma (WHO grades II or III) (Dunn *et al.*, 2012). The time to progression from diffuse astrocytoma (WHO grade II) to glioblastoma is longer (~5 years) than the time to progression from anaplastic astrocytoma (WHO grade III), which is about 2 years. However, this is not a straight process (Olar *et al.*, 2014).

Cellular and Molecular Biology Heterogeneity

Although morphologically identical, different glioblastoma tumours has been shown different clinical behaviours, and so, different clinical outcomes (Olar *et al.*, 2014). Also, so far, it is well recognized that GBM shows high complex heterogeneous cellular, molecular, microenvironmental characteristics, which might partially explain the variety of clinical responses (Olar *et al.*, 2014; Hofer *et al.*, 2014; Dunn *et al.*, 2012). Therefore, it is highly important to identify biomarkers able to predict individual prognosis and to develop specifically directed therapies (Denysenko *et al.*, 2014).

Recently, there is increasing evidence that tumour bulk mass contain subpopulations of cells, such as glioma-initiating cells (GICs) and glioma stem cells (GSCs), which renders the so-called ‘*novel strategies*’ an old fashioned procedure (Persano *et al.*, 2011; Sampetean *et al.*, 2013). When it comes to the glioma cells of origin – their cellular origin that give rise to the initial tumour – continues to be a source of debate (Table 5) (Sampetean *et al.*, 2013; Dunn *et al.*, 2012). There is reported that neural stem cells (NSCs) or oligodendrocyte precursor cells (OPCs) might be putative glioma cells of origin (Figure 5) (Sampetean *et al.*, 2013; Clarke *et al.*, 2013).

Table 5 – Similarities and differences among GSCs, glioma cells of origin, and GICs.

According to cancer stem cell definition		
<i>Conceptual</i>		
Term	Glioma cell of origin	Glioma stem cell (GSC)
Definition	Cell that gives rise to the tumor = cell undergoing initial transformation event	Cell that sustains the tumor = cell with self-renewal ability and ability to give rise to heterogeneous offspring
Characteristics	Tumorigenic ability: ability to initiate primary tumor Preference for location in brain	Tumorigenic ability: ability to initiate recurrent tumor Preference for location in tumor: stem cell niche
<i>Experimental</i>		
Term	Glioma-initiating cell (GIC)	Glioma stem cell (GSC)
Requirement	(May be induced from normal cells in vitro)	Are or have been part of a tumor (in vivo or ex vivo)
Assessment	Capacity to form a tumor in a mouse model	Sphere formation ability Multilineage differentiation potential Capacity to recapitulate initial tumor pathology
<i>Clinical importance</i>		
Identification important in	Prevention Early diagnosis	Curative treatment Prevention of recurrence

Reference: Sampetean *et al.*, 2013

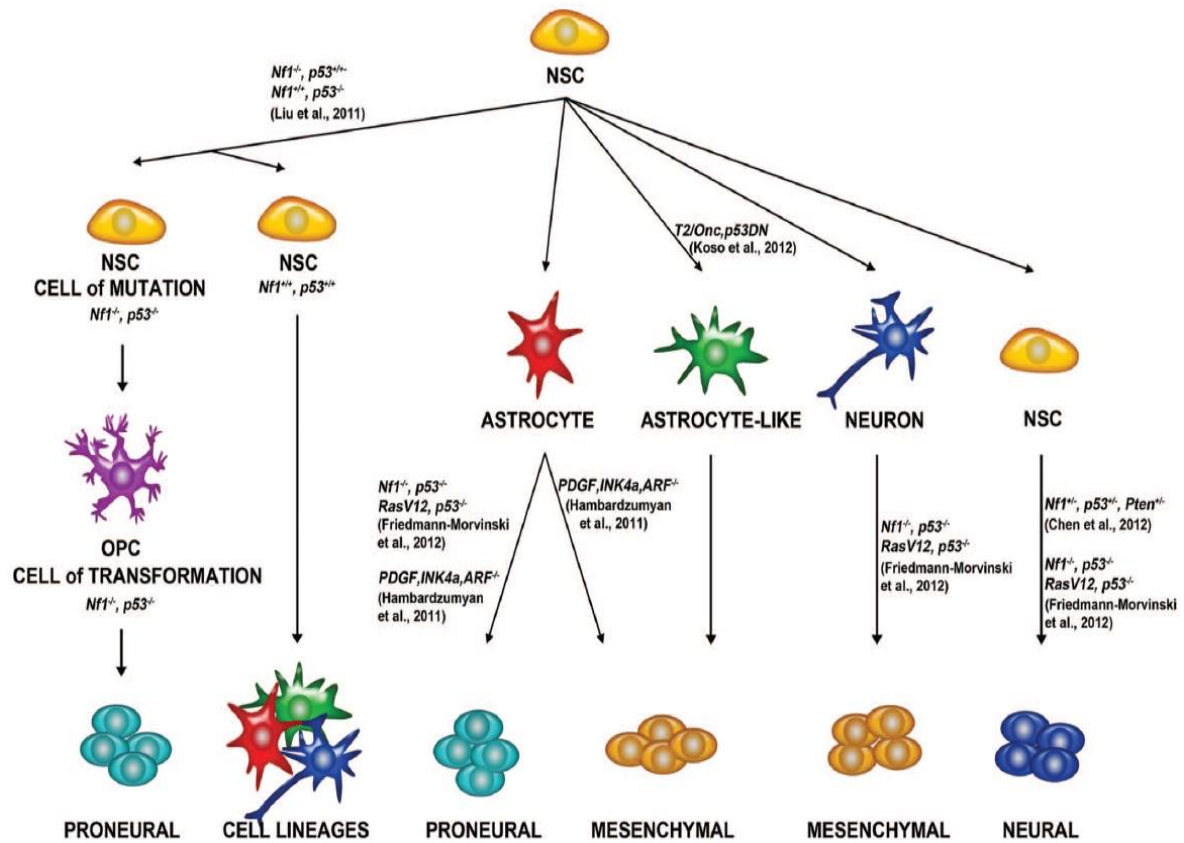


Figure 5 – Possible cells of origin of glioma. Studies in mouse models have shown that various cell types can give rise to glioma. Neural stem cells (NSCs) give rise to other Neural Stem Cells, Astrocytes, Astrocyte-like cells, and neurons. It is demonstrated that NSCs give rise to OPCs, which can give rise to glioma (Liu et al., 2011). Others shown that astrocytes and neurons can give rise to glioma (Marumoto, Singer, Ellisman, & Verma, 2013). Also, astrocytes can give rise to glioma after platelet-derived growth factor (*PDGF*) overexpression and *Ink4a*, and *ARF* deletion as said somewhere (Hambardzumyan, Cheng, Haeno, Holland, & Michor, 2011). As well, overexpression of a mutagenic *Sleeping Beauty* (*SB*) transposon (*T2/Onc2*) along with a dominant negative p53 in astrocyte like cells can give rise to glioma (Koso et al., 2012). Additionally, it is reported that NSCs could give rise to glioma after *Nf1*, *p53*, and *Pten* deletion (Chen, McKay, & Parada, 2012). Neural stem cells can give to proneural, mesenchymal, and neural cell lineages. *Reference:* Clarke *et al.*, 2013

At the moment, there is absent unequivocal biomarkers to well characterize this newly discovered type of cells – GICs – making it as a remaining working hypothesis to be adjusted and validated through further research. Hopefully, a better characterization of GICs, and through them the glioma cells of origin, will increase the understanding of the initial steps of gliomagenesis and could lead to early detection and even prevention of this intractable disease (Sampetean *et al.*, 2013).

The cellular microenvironment also plays a critical role in the regulation of cells in GBM. The heterogeneous cell populations do not only catch external signals from the environment but also respond to the latter in order to take advantage of it (Schonberg *et al.*, 2013). Therefore, there is reported and well accepted the following tumour-associated parenchyma cells interact with GBM cells, controlling the course of the pathology (Goffart *et al.*, 2013):

- Involvement of Microglia;
- Involvement of the Immune System;
- Involvement of Neural Progenitors;
- Involvement of the Vascular Niche.

For instance, GSCs often reside and thrive in perivascular and hypoxic (region of irregular blood flow that experience fluctuating abnormal hypoxic oxygen tension levels, ranging from less than 1-5%) niches (Figure 6), which are anatomical and functional locations.

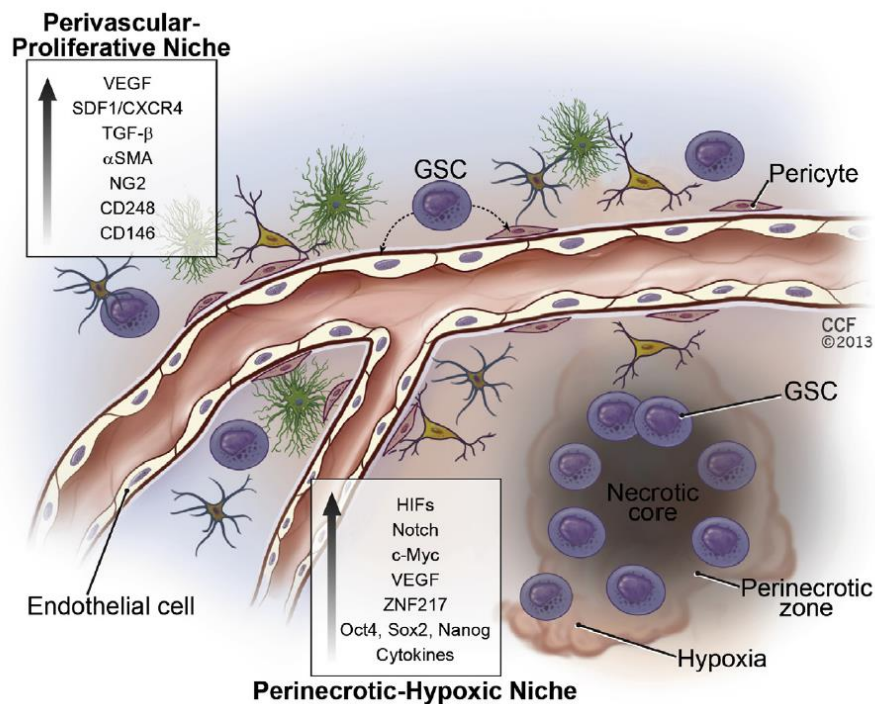


Figure 6 – GSCs microenvironment. GSCs often reside and thrive in perivascular and hypoxic niches where growth factors and cytokines promote their maintenance. In turn, GSCs up-regulate the expression, activation and secretion of a number of niche-dependent signaling molecules and transcription factors involved in pathways such as angiogenesis, proliferation, migration, hypoxia response, etc. GSCs interact with other tumour cells and have the potential to differentiate into other cell types including endothelial cells and pericytes. *Reference:* Schonberg *et al.*, 2013

The morphological heterogeneity that prompted the original description of high-grade glioma as glioblastoma multiforme has also extended to the molecular level and these molecular alterations are extremely complex (Dunn *et al.*, 2012). Based on genomic profiling of hundreds of human samples, four subtypes of GBM have been proposed and these subgroups might develop from different cells of origin (Clarke *et al.*, 2013):

- Proneural GBMs – altered expression of platelet-derived growth factor receptor, alpha polypeptide (*PDGFRA*); isocitrate dehydrogenase 1 (*IDH1*); tumour protein p53 (*TP53*) mutation; loss of heterozygosity (LOH) along with phosphatase and tensin homolog (*PTEN*) mutation; and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) loss;
- Mesenchymal GBMs – deletion of neurofibromin 1 (*NF1*), mutation of *TP53* and *PTEN*, and loss of *CDKN2A*;
- Classical GBMs – typified by epidermal growth factor receptor (*EGFR*) amplification and lack of *PTEN*, and *CDKN2A*;
- Neural GBMs – show a strong expression of neuron markers and genes associated with neuron projection and axon and synaptic transmissions.

Epigenetic alterations has been increasingly debated and studied in GBM. Some regulator agents on that matter are *micro-ribonucleic acid* – miRNA – approximately 21-nucleotides in length, single stranded endogenous non-coding RNAs (ncRNAs) that post-transcriptionally modulate gene expression and by that regulate a wide array of biological processes including differentiation, cell cycle, cell proliferation, apoptosis and angiogenesis; *competitive endogenous RNAs* – ceRNAs – control the amount of each miRNA species; *long non-coding RNAs* – lncRNAs – control global gene repression. Also, epigenetic enzymes, which are controlled by these RNAs, might play a critical role as GBM drivers (Clarke *et al.*, 2013).

Network Analysis

A network is defined as a series of entities connected to one another on the basis of a defined criterion (Zhao *et al.*, 2012). The entities are named network nodes or vertices or network elements, which represent different types of objects such as genes, proteins, drugs, and disease.

On these networks there are edges, which are often called interactions, connections, or links. In terms of local topology, there can be described hubs, motifs and graphlets. A hub is a node having a much higher number of neighbours than average. Usually, hubs are attractive as drug targets. In the case of network motifs, they are circuits of 3 to 6 nodes in directed networks that are highly overrepresented as compared to randomized networks. Lastly, graphlets are similar to motifs but are defined as undirected networks (Csermely *et al.*, 2013).

Recently, there was created a functional interaction (FI) network to study GBM by developing a human-curated map of the molecular pathways involved in GBM (Figure 7) (Wu *et al.*, 2010).

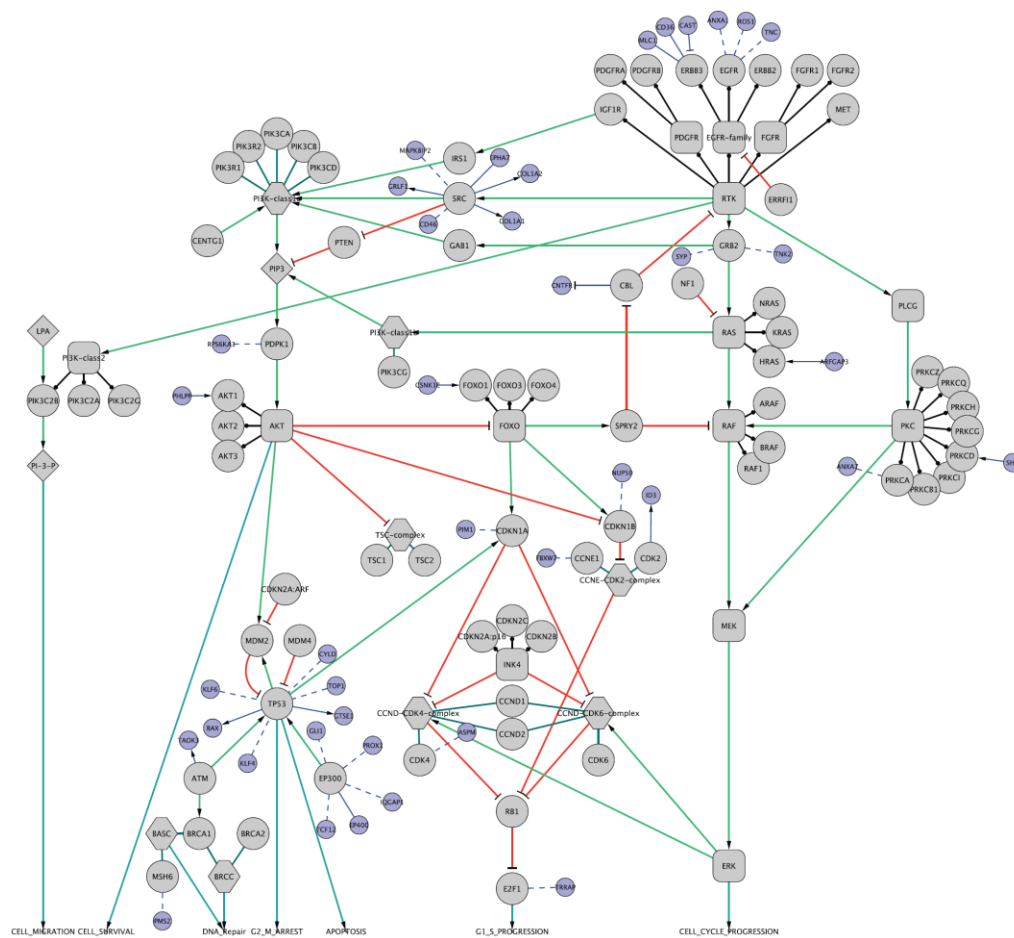


Figure 7 – Overlay of predicted functional interactions onto a human curated GBM pathway. Many genes can interact with multiple pathway genes. In this diagram, only genes interacting with one pathway gene are shown to minimize diagram clutter. Newly added genes are colored in light blue, while original genes are colored in grey. Newly added FIs are in blue, while original interactions are in other colors. FIs extracted from pathways are shown as solid lines, while those predicted based on Naïve Bayes classifier (NBC) are shown as dashed lines. Extracted FIs involved in activation, expression regulation, or catalysis are shown with an arrowhead on the end of the end of the line, while FIs involved in inhibition are shown with a ‘T’ bar. *Reference: Wu et al., 2010*

PDK1 Structure & Pathway

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) is a serine/threonine kinase, discovered over a decade ago. Its structure consists of an N-terminal kinase domain (amino acids 71-359) and a carboxyl-terminal (C-terminal) pleckstrin homology (PH) domain (amino acids 459-550), which binds PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Figure 8) (Nesi, 2011a).

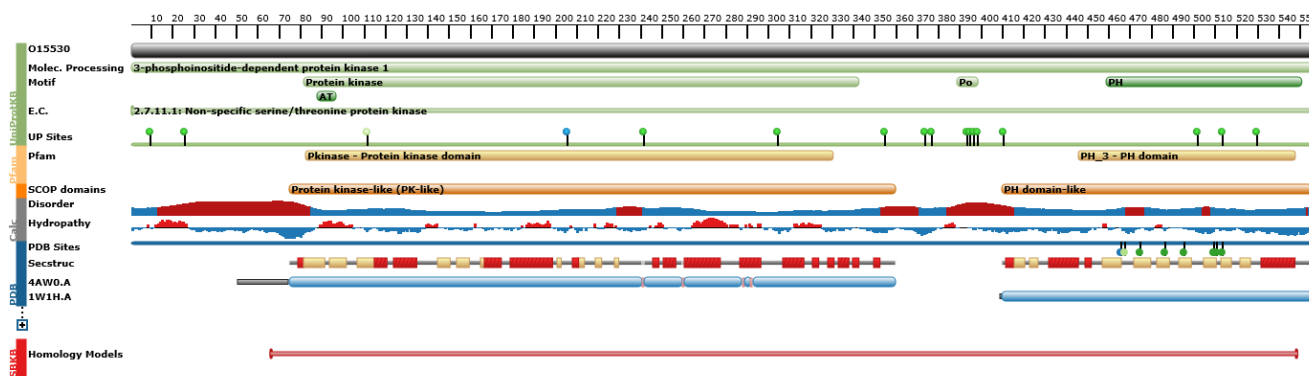


Figure 8 – PDK1 feature view of Protein Data Bank (PDB) entries mapped to a UniProtKB sequence. Data in green originates from UniProtKB. Data in yellow originates from Pfam, by interacting with the HMMER3 web site. **Data in grey** has been calculated using BioJava. Protein disorder predictions are based on JRONN, a Java implementation of RONN: red – potentially disorderd region; blue – probably ordered region. Hydropathy has been calculated using a sliding window of 15 residues and summing up scores from standard hydrophobicity table: red – hydrophobic; blue – hydrophilic. **Data in blue** originates from PDB. Secstruc: Secondary structure projected from representative PDB entries onto the UniProt sequence. **Data in red** indicates combined ranges of Homology Models from SBKB and the Protein Models Portal. *Reference:* From Protein Data Bank (Rutgers, 2014)

Identification of the PH domain as a specialized lipid-binding module has been an important clue in understanding the mechanism by which membrane-bound lipids convey signals to the cytoplasm. Deletion of the PH domain prevents PDK1 recruitment to the plasma membrane and affects the activation and membrane localization of Akt. Binding of PDK1 to PtdIns(3,4,5)P3 induces a major conformational change that is likely required for the activation of substrates. However, PtdIns(3,4,5)P3 binding to the PH domain of PDK1 does not affect the activity of PDK1 directly (Nesi, 2011a).

PDK1 belongs to the protein kinase superfamily, AGC protein kinase family, PDPK1 subfamily, acting as a master upstream protein kinase, phosphorylating and activating a subgroup of the AGC family of protein kinases implicated in the control of cell growth, proliferation, survival and metabolism regulation (Nesi, 2011a; Sephton *et al.*, 2009). The AGC group is named after three of its substituents: cyclic adenosine monophosphate (cAMP)-dependent (protein kinase A, PKA), cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and protein kinase C (PKC). Members of this group preferentially phosphorylate serine/threonine residues close to arginine and lysine, the basic residues. Like all members of this family, the catalytic core of PDK1 possess (Figure 9) an amino-terminal (N-terminal) lobe that consists mainly of a β -sheet and a predominantly α -helical C-terminal lobe, whereas, unlike other AGC kinases, PDK1 possesses an HM pocket in the small lobe of its catalytic motif. The α C-helix (residues 124-136), located in the small lobe of the kinase domain, is a key regulatory domain because it links a substrate interacting site termed the hydrophobic motif (HM) pocket with Ser-241 in the activation loop. The HM pocket in the kinase domain of PDK1 has been termed the PIF pocket after the first discovery that the C terminus of PKC-related kinase-2, which contains an HM motif, interacts with the kinase domain of PDK1. Then, PDK1 interacting fragment (PIF) pocket act as a docking site, which enables the kinase to interact with some of its physiological substrates (Biondi *et al.*, 2002; Feldman *et al.*, 2005).

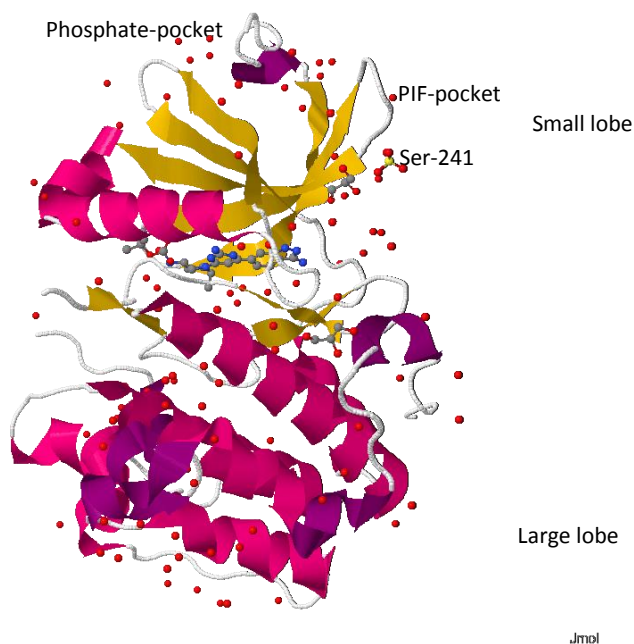


Figure 9 – Overview of the PDK1 structure. Generated by Jmol Java Console.

Protein phosphorylation plays a key regulatory role in numerous facets of protein behaviour including localization, activity and intermolecular interactions and it is mediated by kinases and phosphatases. PDK1 show to be a constitutive active kinase that can use distinct mechanisms to phosphorylate different substrates within cells. Its targets include (Swiss-prot, 2013; Sephton *et al.*, 2009):

- Protein kinase B (PKB/AKT1, PKB/AKT2, PKB/AKT3);
- p70 ribosomal protein S6 kinase beta-1 (RPS6KB1);
- p90 ribosomal protein S6 kinase alpha-1 (RPS6KA1), p90 ribosomal protein S6 kinase alpha-2 (RPS6KA2) and p90 ribosomal protein S6 kinase alpha-3 (RPS6KA3);
- cAMP-dependent protein kinase (PRKACA);
- Protein kinase C delta type (PRKCD) and Protein kinase C zeta type (PRKCZ);
- Serum/glucocorticoid-inducible kinase 1 (SGK1), serum/glucocorticoid-inducible kinase 2 (SGK2) and serum/glucocorticoid-inducible kinase 3 (SGK3);
- p21-activated kinase-1 (PAK1);
- Serine/threonine-protein kinase N1 (PKN1) and Serine/threonine-protein kinase N2 (PKN2).

In glioblastoma multiforme, PDK1 is directly or indirectly part of critical molecular pathways which regulate several growth factors and oncogenes (Krakstad *et al.*, 2010; Mao *et al.*, 2012):

- Phosphatidylinositol-3-kinase (PI3K)/PDK1/Akt pathway: one of the most critical and best characterized pathways in gliomas, being important in cell survival, migration and proliferation;
- PI3K-mTOR crosstalk pathway: critical for proliferation and angiogenesis;
- PTEN-Akt-mTOR signaling pathway: regulates normal cellular functions that can be crucial in tumorigenesis, including cellular proliferation, apoptosis, cell invasion, and mobility.

Additionally as shown above, cytokines such as interleukin-6 (IL-6) have been reported to influence the PI3K/AKT signaling pathway (Guyen-Maiorov *et al.*, 2014).

Treatment Options

In spite of the increased understanding of the complex processes underlying GBM development, this disease still has a dramatic prognosis that has not improved during the last two decades, therefore, a pathology with a clear fatal outcome for patients (Denysenko *et al.*, 2014).

Standard treatment upon initial diagnosis of GBM consists of maximal surgical resection, radiotherapy, and concomitant and adjuvant chemotherapy with temozolomide (TMZ) (Fialho *et al.*, 2012). In the case of patients older than 70 years less aggressive therapy is sometimes employed, through radiation or sole administration of temozolomide. For these patients, recent evidence suggests that temozolomide or hypofractional radiotherapy is associated with prolonged survival than treatment with standard fractioned radiotherapy. For patients over 60 years old, in turn, temozolomide therapy has been shown to lead to a longer survival than treatment with standard radiotherapy (Malmström *et al.*, 2012).

Average time to recurrence after standard therapy is presently 6.9 months for GBM patients. For recurrent tumours, surgery is appropriate in selected patients, and various radiotherapeutic, chemotherapeutic, biologic, or experimental therapies are also employed (Stupp *et al.*, 2005; Chamberlain *et al.*, n.d.).

The radiation therapy for GBM patients involves focal, fractioned external beam therapy while current drugs are alkylating agents such as temozolomide, often in combination with inhibitors of growth factors that promote tumour growth such as the inhibitor (erlotinib) of epidermal growth factor receptor (EGFR) (Fialho *et al.*, 2012). Dose response relationships for GBM demonstrate that a radiation dose of less than 4500cGy results in a median survival of 13 weeks compared with a median survival of 42 weeks with a dose of 6000 cGy. This is usually administered in a therapeutic scheme of 5 days per week in doses of 1.8-2.0 Gy (Bruce *et al.*, 2014b).

The optimal chemotherapeutic regimen for GBM is not yet defined, however, there are some therapeutic options, even not being specific to treat GBM (Bruce *et al.*, 2014a; Bruce *et al.*, 2014b; Mao *et al.*, 2012):

- Temozolomide (Temodar): an oral alkylating and chemotherapeutic agent, first used to treat primary brain tumours in 1993. It alkylates or methylates DNA, causing cancer cells to die. O-6-methylguanine-DNA methyltransferase (MGMT) or O-6-alkylguanine-DNA alkyltransferase can diminish the effect of TMZ by repairing the DNA damage. Also, TMZ has been combined with other inhibitors, in many phase I and II clinical trials, such as EGFR inhibitor, erlotinib, and radiotherapy and shown improvement on patient survival;
- Carmustine (BiCNU): Alkylates and cross-links DNA strands, inhibiting cell proliferation;
- Cisplatin (Platinol): DNA damaging agent (DNA crosslinks and denaturation of double helix), inhibits protein synthesis and, thus, cell proliferation.
- Erlotinib (Tarceva): a human epidermal growth factor receptor type1/epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor. EGFRs are overexpressed in multiple GBM cases and play a significant role in regulating other intracellular significant pathways that contribute to GBM pathogenesis, including mTOR/PI3K/Akt and Rat Sarcoma/mitogen-activated protein kinase (RAS/MAPK).
- Gefitinib (Iressa): An aniliniquinazoline, which mechanism is not fully understood, but seems to inhibit tyrosine kinase intracellular phosphorylation associated with transmembrane cell surface receptors.

Certain conditions related with this type of tumour require medical treatment. For seizures, the patient usually is started on levetiracetam (Keppra), phenytoin (Dilantin), or carbamazepine (Tegretol). Levetiracetam is often used because it lacks the effects on the P450 system seen with phenytoin and carbamazepine, which can interfere with antineoplastic therapy. Vasogenic cerebral edema is typically managed with corticosteroids (for example, dexamethasone), usually in combination with some form of antiulcer agent (for instance, famotidine, ranitidine). The American Academy of Neurology's practice parameters state that prophylactic antiepileptic drugs (AEDs) should not be administered routinely to patients with newly diagnosed brain tumours (standard) and should be discontinued in the first postoperative week in patients who have not experienced a seizure (Glantz *et al.*, 2000).

Therapeutic Challenges

In glioblastoma multiforme drug discovery, one of the biggest challenges is defining the cellular origin of GBM since it is difficult to develop a successful GBM treatment without first uncovering the responsible cell or cells types to eliminate, having in mind the complex heterogeneity of this specific cancer type (Clarke *et al.*, 2013). Nevertheless, the tumour location in a region where it is beyond the reach of local control, and rapid, aggressive tumour relapse are further challenges related to tumour aspects to overcome. Other factors that frustrate therapeutic interventions are the physiologic barriers (blood brain barrier, blood-cerebrospinal fluid barrier and blood-tumour barrier), mechanisms of transport (for example, efflux) and physicochemical factors (for instance, log octanol-water partition coefficient ($\log P_{o/w}$), permeability-surface area product (PS) or permeability coefficient (PC)) (Misra *et al.*, 2003). As well as, identifying epigenetic targets for GBM treatment and subsequently develop therapeutic strategies against GBM are further steps to overcome.

In a time where the bio – and nanotechnology applied to medicines is growing exponentially, the development and design of *novel* approaches to promote an efficient, effective and safe drug delivery to the specific regions of the brain while planning therapeutic regimen and monitoring the responses to treatment would help to overcome some therapeutic limitations such as neurotoxicity, lack of specificity, poor drug accumulation in tumours and severe side effects (Caruso *et al.*, 2011).

Aims of the Study

This MSc study assume in Portugal the vanguard on the area of Quantitative & Systems Pharmacology applied to glioblastoma multiforme drug discovery & development.

The main goal of the work is evaluate the induced drug perturbation in glioblastoma cancer cells through *in vitro* assays by performing a multiparametric characterization of the PDK1 inhibitors dose-response. Specifically it means:

- Grow the *U-87 MG Human Cell Line* representative of the human glioblastoma multiforme;
- Perform the cellular growth profile;
- Parameterising multiparametric perturbation dose-response relationships for two newly synthesised PDK1 inhibitors (G51 and FC100).

The results obtained and the efforts made, ultimately, might contribute to the development of tools to better predict the drug effects before it reach clinical trials, and so, improve the R&D process and possible clinical outcomes. These would means possible advantages for patients, as well as for pharmaceutical companies, research institutes, governments and academia.

Materials & Methods

In this study, the effects of two PDK1 inhibitors were parameterised in a multiparametric dose-response relationship way on U-87MG Human glioblastoma astrocytoma cell line by fitting pharmacologic experimental methods to dedicated models. The detailed experimental procedures will be described on this section.

Reagents

Table 6 – List of the reagents used along the study.

Reagent	Supplier
Aseptic Technique and Good Cell Culture Practice	
70% Ethanol	Prepared on the laboratory
70% Isopropanol	Prepared on the laboratory
Sodium Hypochlorite	Random
Preparation of Sample Solutions	
Phosphate Buffered Saline (PBS) powder, pH 7.4 in 1L sterile water	Powder from Sigma-Aldrich Chemical S.L., Sintra, Portugal; Solution prepared on the laboratory
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Cell Culture	
Dulbecco's Modified Eagle's Medium – high glucose with 4500 mg/L glucose and L-glutamine, without sodium bicarbonate, powder	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Penicillin-Streptomycin, liquid	Invitrogen, Barcelona, Spain
Fetal Bovine Serum (FBS)	Invitrogen, Barcelona, Spain
Sodium bicarbonate, powder	Sigma-Aldrich Chemical S.L., Sintra, Portugal

Phosphate Buffered Saline (PBS) powder, pH 7.4 in 1L sterile water	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Trypsin-EDTA Solution	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Trypan Blue Solution	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Sulforhodamine B (SRB) Colorimetric Assay	
Phosphate Buffered Saline (PBS) powder, pH 7.4 in 1L sterile water	Powder from Sigma-Aldrich Chemical S.L., Sintra, Portugal; Solution prepared on the laboratory
Methanol (MeOH)	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Sulforhodamine B	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Acetic acid	José M. Vaz Pereira, Lisboa, Portugal
Tris(hydroxymethyl)aminomethane or Tris	National Diagnostic, Hesse Hull, United Kingdom
Cell Lysis and Protein Extraction	
Dithiothreitol (DTT)	Bio-Rad Laboratories, Amadora, Portugal
Glycerol	Sigma-Aldrich Chemical S.L., Sintra, Portugal
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Magnesium chloride (MgCl ₂)	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Sodium chloride (NaCl)	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Phenylmethanesulfonyl fluoride solution (PMSF)	Sigma-Aldrich Chemical S.L., Sintra, Portugal
Pepstatin A	AppliChem, Germany
Leupeptin hemisulfate	AppliChem, Germany

Equipment

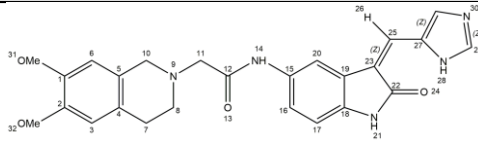
Table 7 – List of the equipment used along the study.

Equipment	Supplier
Aseptic Technique and Good Cell Culture Practice	
Personal protective equipment (sterile gloves, laboratory coat, safety visor, others)	Various
Microbiological Safety Cabinet class II Biowizard (BW)-100	Kojair Tech Oy, Vilppula, Finland
Preparation of Sample Solutions	
Vortex, IKA, MS2 Minishaker	Deutschland, Germany
Falcon 15 mL tubes	Orange Scientific, Braine-l'Alleud, Belgium
Filter	Frilabo, Maia, Portugal
Syringe	Terumo Medical Corporation, Canada
Cell Culture	
Microbiological Safety Cabinet class II Biowizard (BW)-100	Kojair Tech Oy, Vilppula, Finland
MCO-19A Incubator	Sanyo Electric Co. Ltd., Japan
NE1B-14 Water bath	Clifton, England
Olympus CKX41 Microscope with an Olympus DP20-5E Digital	Olympus Optical Co. Ltd., Japan
MPW-350R Centrifuge	MPW Medical Instruments, Poland
Plates, Flasks, Eppendorfs & Falcon tubes	Orange Scientific, Braine-l'Alleud, Belgium
Sulforhodamine B (SRB) Colorimetric Assay	
Plates	Orange Scientific, Braine-l'Alleud, Belgium
Bio-Tek μ Quant MQX200 Spectrophotometer UV-VIS (Gen5 software)	Bio-Tek, United States

Compounds & Stock Solutions

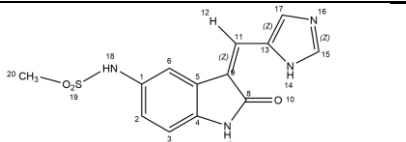
The new PDK1 inhibitors used in this project (Table 8 and 9) – G51 and FC100 – were synthesised and kindly provided upon collaboration with Dr. Simona Rapposelli from the Department of Pharmacy of the University of Pisa, Italy.

Table 8 – Physicochemical properties of G51 compound.

G51						
Purity	clogP	Drug like properties	Melting point	MW	MF	Structure
± 0.4 %	0.8	-4.88	169-171	459.50 g.mol ⁻¹	C ₂₅ H ₂₅ N ₅ O ₄	

MW – Molecular weight; MF – Molecular formula.

Table 9 – Physicochemical properties of FC100 compound.

FC100						
Purity	clogP	Drug like properties	Melting point	MW	MF	Structure
± 0.4 %	-0.25	2.72	205-207	304.32 g.mol ⁻¹	C ₁₃ H ₁₂ N ₄ O ₃ S	

MW – Molecular weight; MF – Molecular formula.

Two different stock solutions of these inhibitor were prepared and it can be visualized on the Figure 10.

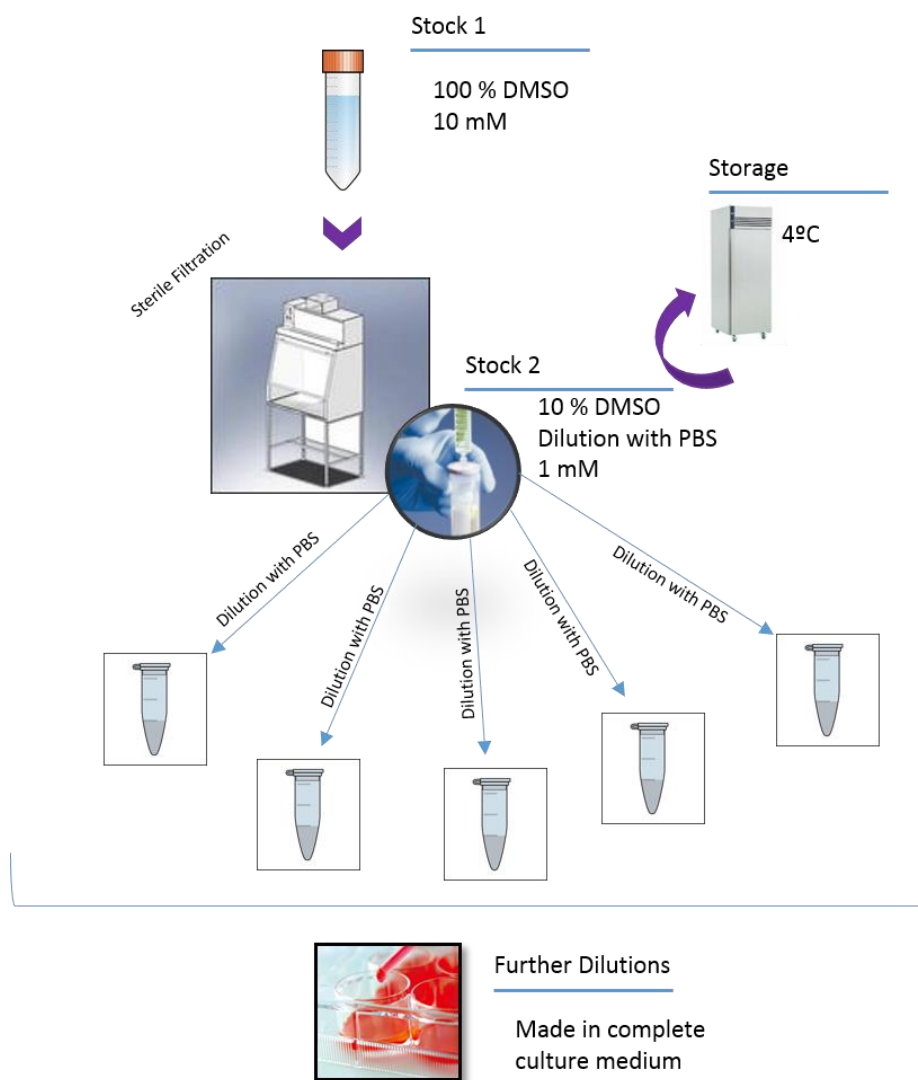


Figure 10 – Schematic representation of compound solutions preparation. Stock solutions 1 of PDK1 inhibitors were made by dissolving each compound in 100% DMSO followed by sterile-filtration, and then, dilution with PBS, obtaining the stock solutions 2. Further dilutions were made from stock solutions 2 with PBS to obtain the required concentrations – ranging from 25 μM to 400 μM – in eppendorfs. Remaining stock solutions 2 were stored at 4°C. Additional dilutions were made in complete cell culture medium to obtain the concentration required on the well (2.5 μM – 40 μM).

Cell Culture

The U-87 MG human glioblastoma astrocytoma cell line was kindly offered by Professor Conceição Pedroso de Lima, from the Center for Neuroscience and Cell Biology of the University of Coimbra, Portugal. The cells were at the passage number 28, mycoplasma free and frozen on 28.10.2013.

Resuscitation of Frozen Cell Lines

In order to maintain the viability of the cell culture and enable it to recover more quickly, it is vital to thaw cells correctly. Since some cryoprotectants, such as DMSO, are toxic above 4 °C, the cells were thawed quickly and diluted in culture medium to minimise the toxic effects. Followed by centrifugation (to remove the cryoprotectant), the supernatant was removed and the cells were re-suspended in growth medium. Then, they were transferred to a 75 cm² flask and incubated at 37 °C, 5% carbon dioxide (CO₂). Cells were examined microscopically (phase contrast) after 24 hours and the medium replaced.

Cell Morphology & Cell Culture Conditions

The U-87 MG cells have an epithelial-like morphology (Figure 11), therefore growing in adherent mode.

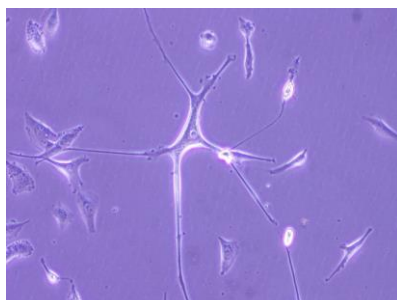


Figure 11 – U-87 MG cell line human glioblastoma astrocytoma. 100x total amplification, after 72h of seeding.

Cells were seeded at a 1.5×10^5 cells/mL (density) in 75 cm², 150 cm² or 175 cm² culture flasks and they were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mol penicillin and 100 µg/mL streptomycin. They were grown at 37 °C under humidified atmosphere containing 5% CO₂.

Subculture of Adherent Cell Lines

U-87 MG cells were splitted when sub-confluent (70-80 %) as recommended on the cell line data sheet from Sigma-Aldrich. Cells were brought into suspension by using tripsinisation. Upon centrifugation at 1100 for 5 minutes, cells were counted in a haemocytometer, using the Trypan blue colorimetric exclusion assay and then seeded at 1.5×10^5 cells/mL density.

Cell Quantification

For the majority of manipulations using cell cultures, for example cryopreservation and subculture routines, it is necessary to quantify the number of cells prior to use. Also, by doing that, cells maintain optimum growth, as well as, it helps to standardise procedures using cell cultures. This in turn gives results with better reproducibility (ECACC, 2013).

When required, cell quantification was performed after harvesting cells by tripsinisation as described above. Cells were re-suspended in a required volume of fresh culture medium and 20 µL were placed on an eppendorf with the same amount of Trypan blue dye (dilution factor 2). This cell suspension was then placed onto a haemocytometer chamber. Cell count was the following step (Figure 12) and the calculation of cell density was achieved by following the equation (ECACC, 2013):

$$\text{Viable Cell Count (live cells per millilitre)} = \frac{\text{Number Live Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution} \times 10,000$$

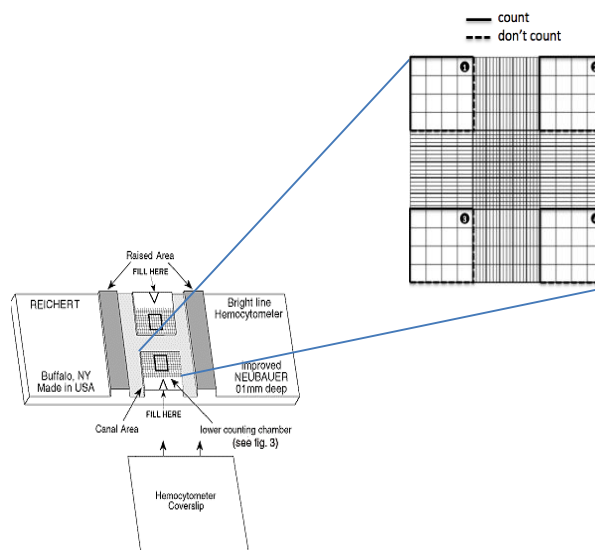


Figure 12 – Schematic representation of haemocytometer preparation and cell counting.

Cell Proliferation Studies & Doubling Time Analysis

In order to obtain the cellular growth profile of the U-87 MG cell line, cell proliferation studies (increase in the number of cells as a result of growth and division) and doubling time analysis (DTA) were performed. For the latter, the population doubling time (PDT) was also assessed and it can be defined as the time interval, calculated during the logarithmic phase of growth in which cells double in number. PDT can be determined by direct cell counting using a haemocytometer. It is important to have in mind that with each subculture the population doubling increases in relationship to the slip ratio at which the cells are plated (ATCC, 2012).

For these studies, cells were plated onto 6-well plates in triplicate at a density of 2.5×10^4 cells/mL in DMEM. After 6, 24, 48, 72, 96, 120 and 144 hours, cells were collected after trypsinization and counted using the following formula $= h \times \ln(2) / \ln(c_2/c_1)$ for DTA, here c is the number of cells at each time of collection and \ln is a neperian logarithm (Roth V. 2006 <http://www.doubling-time.com/compute.php>). Cell proliferation graph was performed on Excel 2013 © Microsoft Corporation.

Perturbation studies *in vitro*

Sulforhodamine B (SRB) Colorimetric Assay

The perturbation effect in the cell system level U-87 MG cell line human glioblastoma astrocytoma of two PDK1 inhibitor compounds (G51 and FC100) were evaluated by the Sulforhodamine B (SRB) colorimetric assay (Papazisis *et al.*, 1997). This relies on the uptake of the negatively charged pink aminoxanthine dye – SRB, comprising two sulfonic groups (Figure 13) – by basic amino acids residues under mild acidic conditions, and dissociate under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass (Vichai *et al.*, 2006).

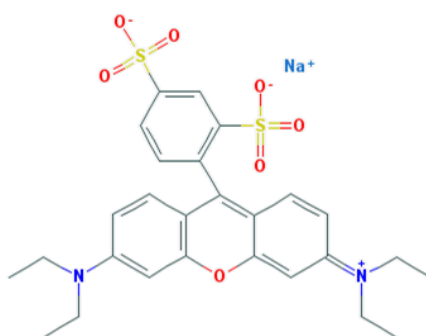


Figure 13 – Sulforhodamine B structure. The molecular formula and molecular weight of SRB are $C_{27}H_{29}N_2NaO_7S_2$ and 580.6 g/mol, respectively. From: PubChem Compound database, CID 9916275.

To perform the assay, cells were seeded at a 5.7×10^4 cells/mL density in 24-well plates, in triplicate, being allowed to adhere overnight. After that, cells were washed with PBS, and fresh culture medium was added. The test compounds were added at different required concentrations in order to achieve a desired final concentration in each well. A control was also prepared, for each time-point – 24, 48, 72 and 144 hours (C_{xh}), where x is the time point and h correspond to hours – that are treated with PBS, having the same volume of solution with perturbing agent. A control named C_0 , which just contain cells without any treatment, was performed as well. The plates were evaluated at each time-point mentioned above.

This type of cell proliferation assay can also be used to determine whether the effect is merely cytostatic (decreases cell growth), or cytotoxic (induces cell death) (Houghton *et al.*, 2007). Therefore, to do this evaluation, two sets of U-87 MG cells were exposed to the drug under the same conditions and for the same period of time. At the end of the exposure period – 72 hours – one set of cells was assayed whilst for the other, the medium containing the drug was discarded and replaced by fresh culture medium alone. The incubation continued for an additional 72 hours, and after this period the plate was assayed. The evaluation of the cytostatic or cytotoxic effect was done based as reported in the literature (Houghton *et al.*, 2007): If the drug has only a cytostatic effect, the cells will grow and undergo mitosis in the fresh medium but, if they have been killed during the initial exposure time, no such increase in number of cells will be observed upon drug removal (irreversible effect).

Regarding the cell fixation and staining step, the cells were fixed by adding 1 mL of cold 1% acetic acid/methanol (MeOH), followed by storage at 4 °C. Next day, fixed cell were thaw during 24h. 500 µL of SRB (0.5 % w/v) was added at each well and they were incubated at 37 °C during 1 hour. The plates were washed in 1% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solubilised with 1 mL of 10 mM Tris (pH 10) per well, shaken, and after that, the supernatant was transferred to 96-well plate and the absorbance was measured at 540 nm.

To parameterising the perturbation, the % SRB retention was computed by GraphPad Prism Software, Version 5.00 for Windows, using *One-Way ANOVA* method, followed by multiple comparison *Turkey's* test. Differences were considered statistically significant for $p < 0.05$; $p < 0.01$ and $p < 0.001$.

Multiparametric Dose-Response Analysis

In this project, the SRB colorimetric assay was used to yield the cellular density as the bases for parameterising multiparametric perturbation dose-response relationships. These relationships were performed in computational model approaches available on GraphPad Prism Software, Version 5.00 for Windows. Non-linear fit logistic curves to data on relative growth comprising a change in cell number after drug treatment normalized to the change in cell number in a untreated control well (C_0) were performed. Different dose-response parameters for each individual curve, including HS, E_0 , E_{inf} , GI_{50} and TGI were estimated. Additionally, it was calculated a parameter AUC

representing the area under the relative viability curve, defined as the sum of measured responses (relative viability) at all tested concentrations of the drug. Hence, AUC=9 corresponds to an inactive compound, whereas smaller AUC values correspond to higher drug activities in inhibiting cell proliferation and/or inducing cell death. The parameters IC_{50} was previously yield by Invitrogen Z'-LYTE® biochemical assay and the % Inhibition direct to PDK1 – testing at 10 μ M – were kindly provided by Dr. Simona Rapposelli (Department of Pharmacy, University of Pisa, Italy).

Cell Lysis and Protein Extraction

In order to future characterisations of the effects of PDK1 inhibitors in other system-level component, integrating the fate of cellular perturbations multiparametric dose-response relationship at specific relevant time-points for each compound, a cell lysis and protein extraction procedure was applied (Figure 14).

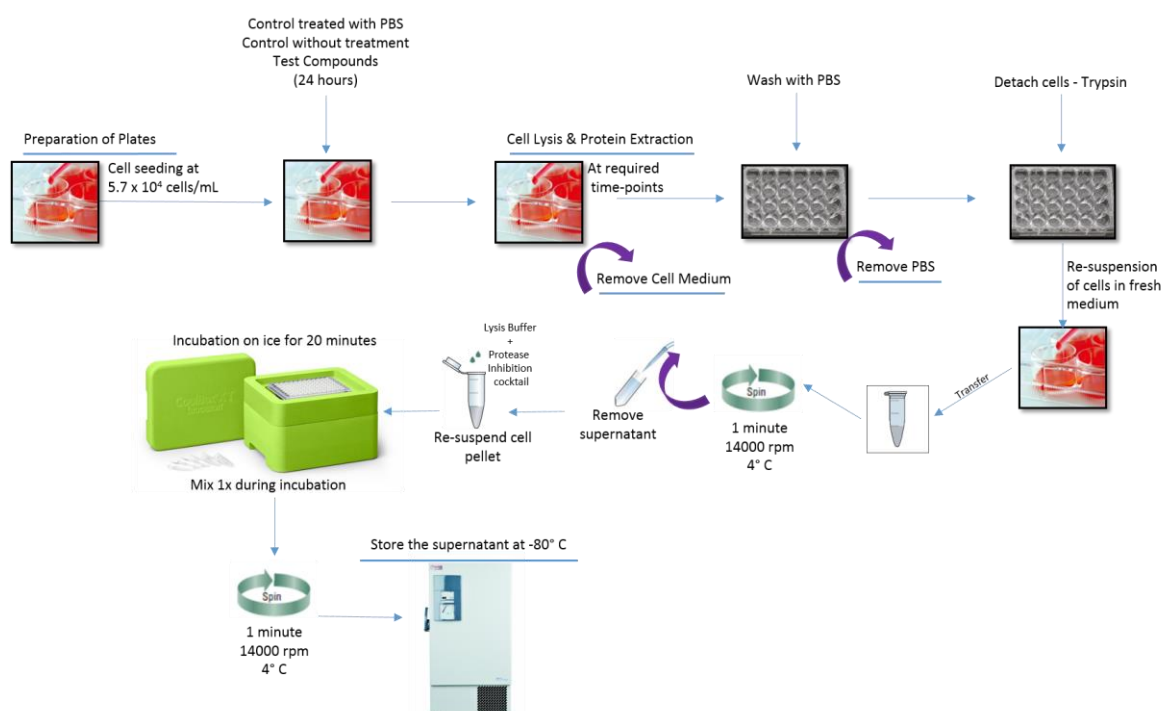


Figure 14 – Cell Lysis and protein extraction procedure. Cell lysis is the first step in protein extraction. The lysis buffer comprised NaCl, HEPES, $MgCl_2$ and glycerol. The protease inhibition cocktail included leupeptin hemisulfate, PMSF, pepstatin A and DTT.

Quality Control of the data

As important as get representative samples, choose and execute the technique adequately, it is important to calculate and express correctly the results. Hence, the researcher must know the mean of the evaluations which perform and be able to manage the uncertainties associated with these evaluations. Also, he should know how to detect and eliminate specific errors, as well as to discriminate between valid and spurious results (Sores, 2006). Therefore, in this study, a strong quality control of the data was performed. After raw data inspection – processed on Excel 2013 © Microsoft Corporation and GraphPad Prism Software, Version 5.00 for Windows – the analysis of average, amplitude of the sample, coefficient of variation (% CV), standard deviation (SD), standard error of the mean (SEM) and Dixon's Q test (90 % of confidence) were calculated. After computational model fitting, the goodness of fit was also evaluated by the coefficient of determination (R^2).

Results & Discussion

‘Business as usual’ is no longer an option in drug discovery & development (Csermely *et al.*, 2013). One of the major health challenges is the desperate need for innovative medicines to treat glioblastoma multiforme. Despite this fact, fast, affordable and precisely predictive drug response in drug development is still a vision that contrasts sharply with the current state of drug discovery (Jiguet *et al.*, 2014).

In a *new-age* pharmacology – Quantitative & Systems Pharmacology – computational modeling and simulation integrating complex multiparametric readouts of perturbation experiments with other associated experimental or clinical data are thought to be critical for better understanding both drug effects and disease. Therefore, the present study, and thereby, the results presently reported may contribute for the world global data regarding glioblastoma drug discovery and development.

Cellular Growth Profile of the U-87 MG cell line

To assess the cellular growth profile of the U-87 MG human cell line, cellular proliferation studies and doubling time analysis were carried out. This is a critical step before starting any experiments as alterations on the cellular growth can indicate a significant problem within cell line and if undetected can have detrimental effects on experimental results.

The U-87 MG growth curve is represented in Figure 15. The time-point 6 hours was performed to evaluate how the cells were adapting to the culture conditions, and as shown, at this time they are in Lag Phase, that lasts up to 24 hours. During this period the cells do not divide, even showing an insignificant decline on cell density, which states the period of adaptation to the new culture conditions. This cell line, shown exponential growth, and so, Logarithmic (Log) growth phase, from 24 hours to 72 hours after culturing. During this phase, the division rate systematically increase (Figure 15). Regarding the plateau (or stationary) phase, it was reached after 96 hours as

the cell population starts to becoming confluent. These results are in accordance with previously published growth curves for this same cell line (“Growth Profile for Cell Line: U-87 MG,” n.d.)(Jiguet *et al.*, 2014).

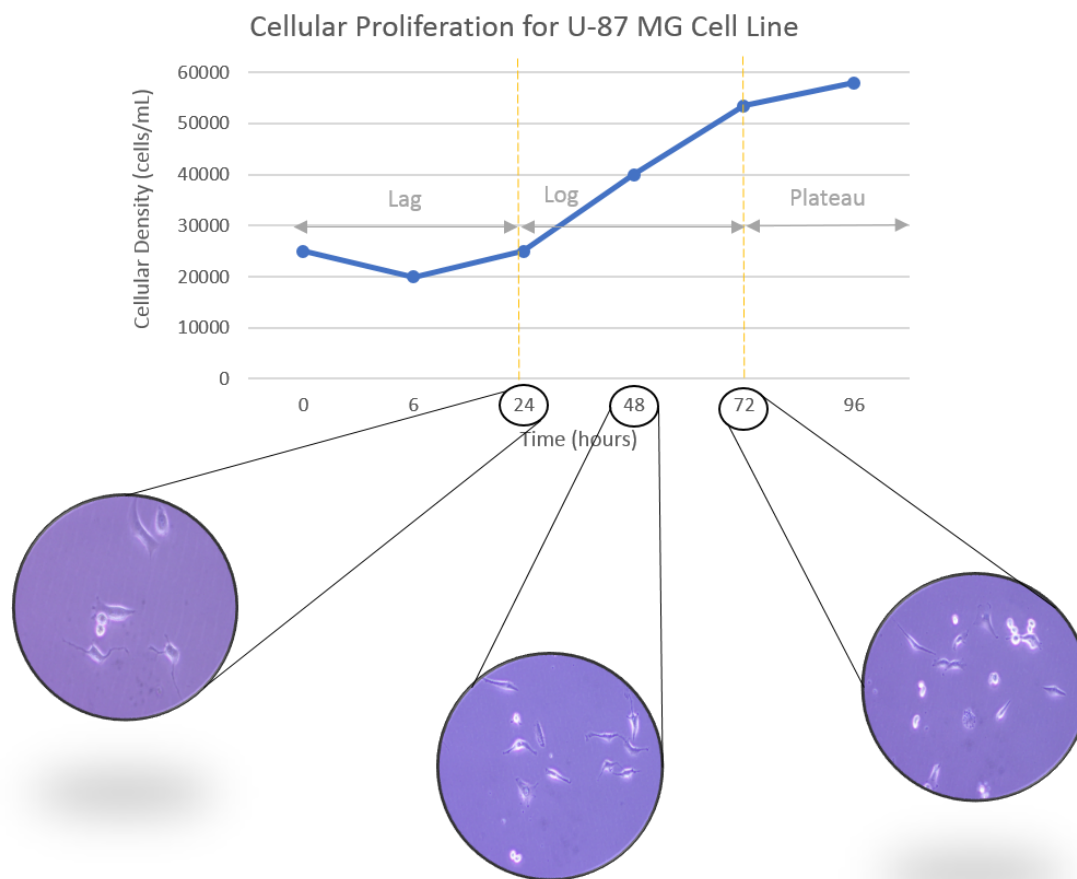


Figure 15 – Graphical representation of the cell proliferation profile for the U-87 MG cell line and morphological representation of the cells during the logarithmic phase. The cellular proliferation of U-87 MG cell line human glioblastoma astrocytoma was assayed, the number of cells counted and cellular density calculated at 0, 6 hours time points and then every 24h. Data from an experiment out of three repetitions is shown, representing the cellular density of viable cells. Furthermore, phase-contrast images of cells at 100x total amplification are also shown.

As reported elsewhere (ECACC, 2013), the Log phase is the optimal period to determine the population doubling time (PDT), therefore, the double time analysis were performed during this phase. And so, the PDT for U-87 MG cell line was 45.6 hours, with a growth rate – number of doublings that occur per unit of time – of 1.53×10^{-2} . In this study, the value of PDT is slightly higher than other values (29 - 34 hours) published in the literature (Ramão *et al.*, 2012; ATCC, 2012; Khoei *et al.*, 2011). This may be due to the high number of the cellular subculture (passage #31).

***In vitro* Perturbation studies**

There is a growing recognition that supporting drug discovery and development integration from *in vitro* to *in vivo* scales and from preclinical to clinical studies is particularly critical. The promise of multiscale modeling is to assess the functional impact of molecular perturbations – drug dose and exposure, but also genotypic and environmental variations – across changes in scales. This will allow cross-scale effects assessment or physiological consequences (most often drug action, broadly defined, and measured by biomarkers and disease metrics) predictions. Thereby, this type of approach should facilitate the focus on treatments and reduce this process cost. The overall context of perturbation studies is the new generation quantitative and systems pharmacology.

In this project, the induced *in vitro* perturbation on the U-87 MG cell line was evaluated by the SRB colorimetric assay, followed parameterisation *via* multiparametric dose-response analysis.

Sulforhodamine B (SRB) Assay

The SRB method was chosen to assess the cellular perturbation by PDK1 inhibitors regarding its advantages over other standard techniques, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability test. The SRB assay is sensitive, simple, reproducible, more rapid than the formazan-based assays and gives better linearity, a good signal-to-noise ratio, apart from having a stable end-point that does not require a time-sensitive measurement, as does the MTT assay (Houghton *et al.*, 2007). Nonetheless, some compounds can directly interfere with MTT reduction, while SRB staining is rarely affected by this type of interference (Vichai *et al.*, 2006).

The present study measured the perturbation at the cellular system-level of U-87 MG cells by a range of dosages of two PDK1 inhibitors, G51 and FC100. The perturbation effect by G51 drug can be found on Figure 16.

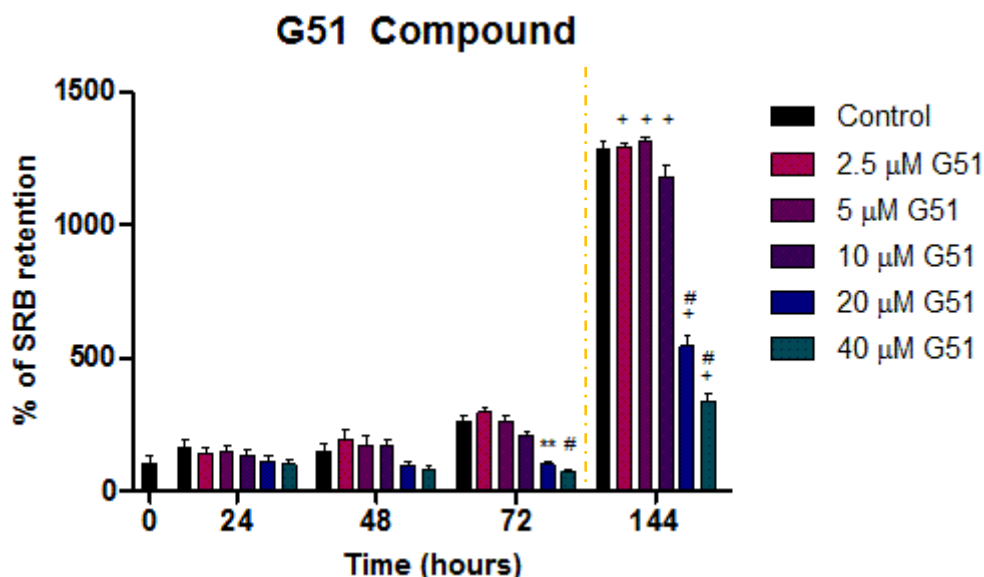


Figure 16 – Perturbation effect of G51 drug at the cellular system-level of U-87 MG cell line. The perturbation effect was generated from the percentage of SRB retention, and so, in terms of cellular density. The effect of the drug treatment was measured during 72 hours. The vertical dotted line at 72 hours represents the drug removal and substitution by new culture medium, allowing the evaluation of the reversibility of the drug effect. The results are presented as mean values of triplicates and for n=3 (3 independent samples from one independent experiment). The bars represent \pm SEM. **p < 0.01 vs control, #p < 0.001 vs control, *p < 0.001 vs 72 hours treatment.

The most significant perturbation in the cellular system-level was verified after 72 hours of incubation with the drug for the higher dose – 40 μM. Also, the cellular system shows a very significant perturbation at 20 μM. At 24 and 48 hours there is no significant meaning of system perturbation, since no difference of response is observed between the C_{24h} and the other treatments at this time-point. However, as the main intent of the systematic perturbation is to explore diverse aspects of the signaling response and to maximize the information regarding the cell response profiles for model inference, it may be relevant to carry out a detailed analysis of all variations of the cellular system occurring as a function of both concentration and time of incubation. These results, comprised in Table 10 show that the dosage seems to be more important

than the duration of treatment for the induction of a significant perturbation: when comparing the lower concentrations at 72 hours to doses in the range 10 μM – 40 μM at the lower time points of treatment, there is a higher effect for the latter. Thus, it may suggest that small variations in the PDK1 pathway on GBM does not induce an important cell response between 24 and 48 hours, but random related events might be perturbed, including biochemical reactions, binding interactions, macromolecule complex assembly, transport reactions, conformational changes, and post-translational modifications. Additionally, for 72 hours of incubation there are no significant differences between the C_{72h} and the 2.5 μM , 5 μM and 10 μM doses tested. Further functional interactions analysis would help to understand what is happening within this system, but this was not the scope of the present study.

Table 10 – Multiple comparison analysis of the G51 induced perturbation in the cellular system-level U-87 MG cell line.

Turkey's Multiple Comparison Test	Mean difference of perturbation	Significance
10 μM 24h vs 2.5 μM 72h	-0.3646	$p < 0.01$
10 μM 24h vs 5 μM 72h	-0.2924	$p < 0.05$
20 μM 24h vs 2.5 μM 72h	-0.4041	$p < 0.001$
20 μM 24h vs 5 μM 72h	-0.3320	$p < 0.01$
40 μM 24h vs 2.5 μM 72h	-0.4334	$p < 0.001$
40 μM 24h vs 5 μM 72h	-0.3613	$p < 0.01$
20 μM 48h vs 2.5 μM 72h	-0.4407	$p < 0.001$
20 μM 48h vs 5 μM 72h	-0.3686	$p < 0.01$
40 μM 48h vs 2.5 μM 72h	-0.4722	$p < 0.001$
40 μM 48h vs 5 μM 72h	-0.4001	$p < 0.001$
2.5 μM 72h vs 20 μM 72h	0.4317	$p < 0.001$
2.5 μM 72h vs 40 μM 72h	0.5006	$p < 0.001$
5 μM 72h vs 20 μM 72h	0.3596	$p < 0.01$
5 μM 72h vs 40 μM 72h	0.4284	$p < 0.001$
10 μM 72h vs 40 μM 72h	0.3124	$p < 0.05$

Exploring if the induced perturbation leads to a cytotoxic or cytostatic effect, the results shown in **Figure 16** evidence that as the cells grow and undergo mitosis 3 days after removal of the

G51 compound – time point 144 hours – the effect is cytostatic with an extremely significant mean for the 20 μM and 40 μM doses. Still analysing that effect, treating the cells with 40 μM dosage, the cellular system seems to be more perturbed when compared with 20 μM dosage (C_{144h} vs 20 μM 144h – 1.647 mean difference; C_{144h} vs 40 μM 144h – 2.114 mean difference; 20 μM 144h vs 40 μM 144h – 0.4670 mean difference). This highlights again the importance of the dose on the cellular system-level perturbation.

As it is reported, PDK1 pathway has a critical role on both cytostatic and cytotoxic effects through different interactions within the cellular system (Wu *et al.*, 2010; Nesi, 2011b; Nesi, 2011a; Velpula *et al.*, 2013). Therefore, it is suggested that the cytostatic effect of this PDK1 inhibitor may be associated with the *Gap 1* and *Synthesis* progression (PDK1/Akt/forkhead box transcription factors (FOXO) pathway) and cell cycle progression (PDK1/Akt/Raf Murine Sarcoma Viral Oncogene Homolog (Raf) kinase pathway) (Wu *et al.*, 2010).

As to the FC100 drug, its perturbation at the cellular system-level U-87 MG cell line is represented in the **Figure 17**.

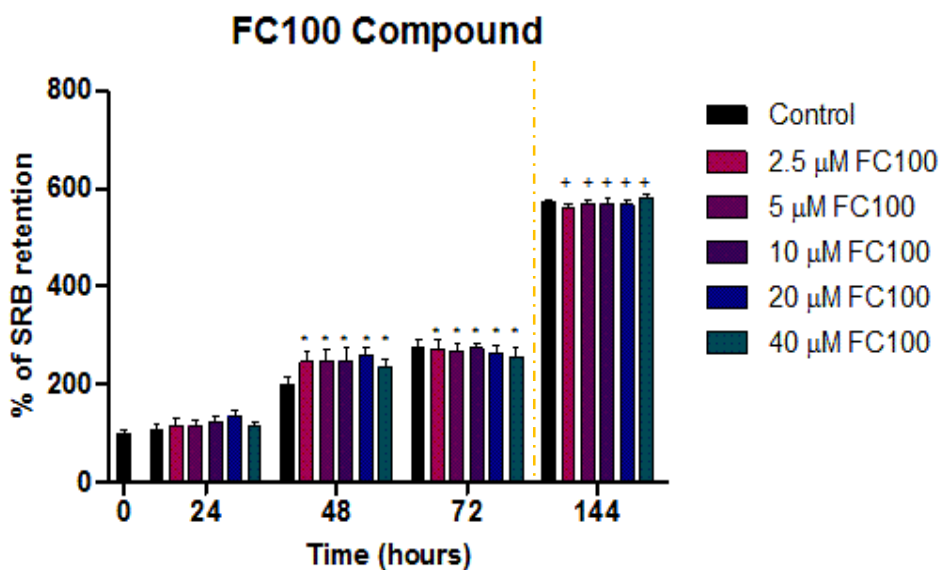


Figure 17 - Perturbation effect of FC100 drug at the cellular system-level of U-87 MG cell line.

The perturbation effect was generated from the percentage of SRB retention, and so, in terms of cellular density. The effect of the drug treatment was measured during 72 hours. The vertical dotted line at 72 hours represents the drug removal and substitution by new culture medium, allowing the evaluation of the reversibility of the drug effect. The results are presented as mean values of triplicates and for n=3 (3 independent samples from one independent experiment). The bars represent \pm SEM. * $p < 0.01$ vs control, # $p < 0.001$ vs control, + $p < 0.001$ vs 72 hours treatment.

The perturbation of the cellular system U-87 MG by FC100 fails to produce a specific cytostatic or cytotoxic effect, irrespective of the concentration tested. In fact, even showing a significant difference between cells treated at 72 hours and cells counted 3 days after FC100 removal (time-point 144 hours), which could indicate a cytostatic effect, there is no significant reduction in the percentage of SRB retention of these cells, when compared to the control, for any of the time-points considered in the study (24, 48 and 72 hours). However, the higher significant mean difference, comparing the time-points 72 and 144 hours was for the dose 40 μM (-1.654), whereas, it was not too much different from the effect of the other doses (Table 11).

Furthermore, there is no evidence of a cytotoxic effect induced by perturbation as no significant cell density reduction was verified when compared to C_0 . However, curiously enough, FC100 was found to induce a higher cellular perturbations in the first 24 hours of treatment when compared to 48 and 72 hours. Also interesting is the fact that there is no significant perturbation differences between the time-points 48 and 72 hours upon treatment with the drug, which might indicate that the almost negligible perturbation due to FC100 is induced immediately – shown at 24 hours – on this cellular system, and then, somehow the U-87 MG cell line turns to be resistant to this compound. Inclusively, during the first 24 hours the cell response to FC100 seems to be similar for all the dosages tested (Table 11).

Table 11 - Multiple comparison analysis of the FC100 induced perturbation in the cellular system-level U-87 MG cell line.

Turkey's Multiple Comparison Test	Mean difference of perturbation	Significance
2.5 μM 24h vs 2.5 μM 48h	-0.6551	$p < 0.001$
2.5 μM 24h vs 5 μM 48h	-0.6629	$p < 0.001$
2.5 μM 24h vs 10 μM 48h	-0.6608	$p < 0.001$
2.5 μM 24h vs 20 μM 48h	-0.7239	$p < 0.001$
2.5 μM 24h vs 40 μM 48h	-0.5951	$p < 0.001$
2.5 μM 24h vs 2.5 μM 72h	-0.7807	$p < 0.001$
2.5 μM 24h vs 5 μM 72h	-0.7722	$p < 0.001$
2.5 μM 24h vs 10 μM 72h	-0.7988	$p < 0.001$
2.5 μM 24h vs 20 μM 72h	-0.7377	$p < 0.001$
2.5 μM 24h vs 40 μM 72h	-0.7068	$p < 0.001$
5 μM 24h vs 2.5 μM 48h	-0.6672	$p < 0.001$

5 μ M 24h vs 5 μ M 48h	-0.6750	$p < 0.001$
5 μ M 24h vs 10 μ M 48h	-0.6729	$p < 0.001$
5 μ M 24h vs 20 μ M 48h	-0.7360	$p < 0.001$
5 μ M 24h vs 40 μ M 48h	-0.6072	$p < 0.001$
5 μ M 24h vs 2.5 μ M 72h	-0.7928	$p < 0.001$
5 μ M 24h vs 5 μ M 72h	-0.7843	$p < 0.001$
5 μ M 24h vs 10 μ M 72h	-0.8109	$p < 0.001$
5 μ M 24h vs 20 μ M 72h	-0.7498	$p < 0.001$
5 μ M 24h vs 40 μ M 72h	-0.7189	$p < 0.001$
10 μ M 24h vs 2.5 μ M 48h	-0.6200	$p < 0.001$
10 μ M 24h vs 5 μ M 48h	-0.6278	$p < 0.001$
10 μ M 24h vs 10 μ M 48h	-0.6257	$p < 0.001$
10 μ M 24h vs 20 μ M 48h	-0.6888	$p < 0.001$
10 μ M 24h vs 40 μ M 48h	-0.5600	$p < 0.001$
10 μ M 24h vs 2.5 μ M 72h	-0.7456	$p < 0.001$
10 μ M 24h vs 5 μ M 72h	-0.7371	$p < 0.001$
10 μ M 24h vs 10 μ M 72h	-0.7637	$p < 0.001$
10 μ M 24h vs 20 μ M 72h	-0.7026	$p < 0.001$
10 μ M 24h vs 40 μ M 72h	-0.6717	$p < 0.001$
20 μ M 24h vs 2.5 μ M 48h	-0.5659	$p < 0.001$
20 μ M 24h vs 5 μ M 48h	-0.5737	$p < 0.001$
20 μ M 24h vs 10 μ M 48h	-0.5716	$p < 0.001$
20 μ M 24h vs 20 μ M 48h	-0.6347	$p < 0.001$
20 μ M 24h vs 40 μ M 48h	-0.5059	$p < 0.001$
20 μ M 24h vs 2.5 μ M 72h	-0.6914	$p < 0.001$
20 μ M 24h vs 5 μ M 72h	-0.6830	$p < 0.001$
20 μ M 24h vs 10 μ M 72h	-0.7096	$p < 0.001$
20 μ M 24h vs 20 μ M 72h	-0.6484	$p < 0.001$
20 μ M 24h vs 40 μ M 72h	-0.6176	$p < 0.001$
40 μ M 24h vs 2.5 μ M 48h	-0.6598	$p < 0.001$
40 μ M 24h vs 5 μ M 48h	-0.6676	$p < 0.001$
40 μ M 24h vs 10 μ M 48h	-0.6654	$p < 0.001$
40 μ M 24h vs 20 μ M 48h	-0.7286	$p < 0.001$

40 μ M 24h vs 40 μ M 48h	-0.5998	$p < 0.001$
40 μ M 24h vs 2.5 μ M 72h	-0.7853	$p < 0.001$
40 μ M 24h vs 5 μ M 72h	-0.7769	$p < 0.001$
40 μ M 24h vs 10 μ M 72h	-0.8034	$p < 0.001$
40 μ M 24h vs 20 μ M 72h	-0.7423	$p < 0.001$
40 μ M 24h vs 40 μ M 72h	-0.7114	$p < 0.001$
2.5 μ M 72h vs 2.5 μ M 144h	-1.474	$p < 0.001$
5 μ M 72h vs 5 μ M 144h	-1.524	$p < 0.001$
10 μ M 72h vs 10 μ M 144h	-1.489	$p < 0.001$
20 μ M 72h vs 20 μ M 144h	-1.545	$p < 0.001$
40 μ M 72h vs 40 μ M 144h	-1.651	$p < 0.001$

Taking together the results gathered for FC100 compound with the literature on the protein interaction network for GBM, the effect measured for this compound might be related with a quick response of the cellular system to the perturbation, possible associated to deoxyribonucleic acid (DNA) repair mechanisms, through PDK1/Akt/tumour protein p53 (TP53) pathway perturbation. Further tests at higher dosage levels may provide more definite conclusions on the mechanisms involved (Wu *et al.*, 2010). Also, the fact of the perturbation by FC100 drug does not translate in a measurable effect on the cellular viability, this does not mean that the other random process were not perturbed. Moreover, the hypothetical resistance at the cellular system-level may be due to a high metabolism of the compound within the cells. Also, FC100 may not perturb enough the cellular system to inhibit random events that promotes cell growth and resistance. For example, it was reported recently that EGFR phosphorylation might interact and activate PDK1 in glioblastoma multiforme, promoting cancer cell metabolism and tumour progression (Velpula *et al.*, 2013).

Multiparametric Dose-Response Analysis

Patient-to-patient variability in drug response is a primary challenge facing development and clinical use of new medicines. Therefore, understanding and parameterising this variability early during the drug research process, by analysing a variety of drug parameters responses across different types of cell lines representative of the disease model, might reveal and predict the

systematic variation in response to cancer drugs in a real therapeutic context (Fallahi-Sichani *et al.*, 2013). In this project, variation in features other than potency were analysed, namely GI_{50} , TGI, HS, E_{inf} , E_{max} and AUC (Table 12). Additionally, the results provided by the collaborating R&D group from the University of Pisa were taken together with those presently gathered for U-87 MG cell line.

Table 12 – Multiparametric dose-response parameters for G51 and FC100 compounds.

Parameters	Results						System-Level
	G51			FC100			
% Inhibition							
PDK1 Direct (Z'LYTE® Assay)	92			97			PDK1 kinase*
IC₅₀ (Z'LYTE® Assay)	9.80 x 10 ⁻⁷ M			3.10 x 10 ⁻⁷ M			PDK1 kinase*
Time Points	24h	48h	72h	24h	48h	72h	
GI₅₀ (M)	2.49 x 10 ⁻⁵	1.64 x 10 ⁻⁵	1.38 x 10 ⁻⁵	3.50 x 10 ⁻⁶	1.28 x 10 ⁻¹⁰	1.98 x 10 ⁻⁵	U-87 MG Cell Line
TGI (M)	3.70 x 10 ⁻⁵	1.95 x 10 ⁻⁵	1.95 x 10 ⁻⁵	Not Reached			U-87 MG Cell Line
HS	-0.9575	-9.056	-2.924	1.79	~1.340	~ -29.32	U-87 MG Cell Line
E_{inf}	-0.6315	-0.1703	-0.3594	1.082	~0.6732	0.89	U-87 MG Cell Line
E_{max}	0.9817	1.41	1.09	3.282	1.456	0.9875	U-87 MG Cell Line
AUC	2.27	4.41	2.58	13.9	7.28	4.76	U-87 MG Cell Line

*Results from the collaborating R&D group from the University of Pisa (Italy).

An overall analysis of the results, the importance of each calculated parameter to characterise the cellular response to the perturbing agent show that the most important difference between drugs or between sensitive and resistant cells does not rely on the IC_{50} only, in accordance with previously reported data (Fallahi-Sichani *et al.*, 2013). In fact, sole analysis of the IC_{50} values,

would lead to the conclusion that FC100 compound would be the most promising compound to treat glioblastoma multiforme since its IC_{50} is much lower than that determined for G51 (3.10×10^{-7} M vs 9.80×10^{-7} M, respectively). However, other analysed parameters do not corroborate this conclusion: the smaller values of AUC for G51 as compared to FC100 reflect a higher drug activity for the former, in contrast to the corresponding IC_{50} values. The higher values of AUC for FC100 compound, also means that this cell line might be resistant to this drug. In addition, during the different time points, the AUC observed for these compounds match well with the results from the cellular perturbation shown before. Besides, the results obtained for TGI agree with AUC data, even showing that FC100 fails to completely inhibit cell growth up to $40 \mu\text{M}$, thus confirming the poor activity of this compound towards this cellular system.

Talking about the steepness – HS – of the dose-response, it can be graphically visualised in Figure 18.

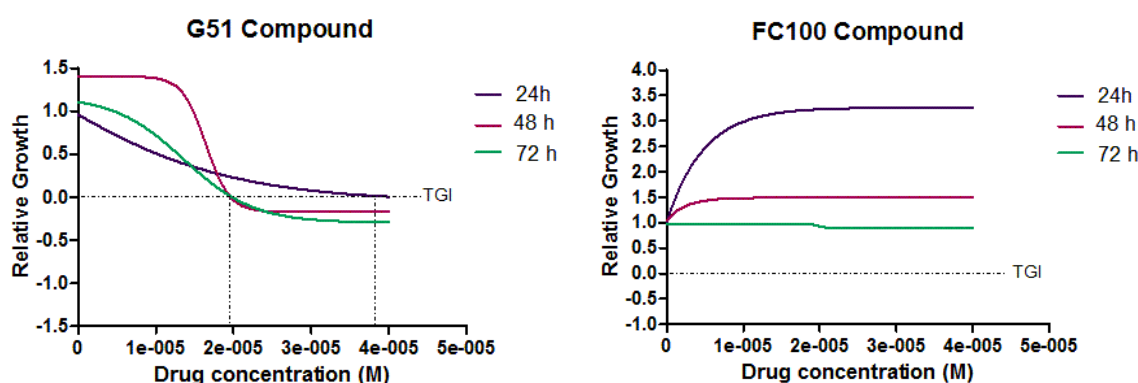


Figure 18 – Multiparametric dose-response curves for G51 and FC100 compounds. Curves were generated by fitting logistic curves to data on relative growth comprising a change in cell number after drug treatment, normalized to the change in cell number in an untreated control well. The total growth inhibition concentration (TGI) is represented.

HS is expected to vary in a consistent way with drug class (Fallahi-Sichani et al., 2013). However, in this study two drugs of the same class (acting on PDK1) were tested, yielding apparently different HS profiles. Although there is no reported a pharmacological explanation for this fact. But, as these compounds have difference degrees of affinities for the receptor, other targets than PDK1 might be influenced in a different way by these drugs.

The probable suggested acquired resistance of the cellular system to the perturbation by FC100 (detected experimentally), may be confirmed by the very low HS values after 72 hours of treatment (-29.32) since $HS \ll 1$ (shallow dose-response curve) is correlated with high cell-to-cell

variability in target inhibition compared to drugs for which $HS \sim 1$ (Fallahi-Sichani *et al.*, 2013). Therefore, this type of response to FC100 means a higher fraction cell killing when compared to the G51. In spite of this might be happening for both compounds, the effect is marked for FC100. As a result, it indicates that this cellular system contains a subpopulation of cells that were simple unaffected at high drug dosages, corroborating the theory of cellular and molecular biology heterogeneity of glioblastoma multiforme (Olar *et al.*, 2014; Hofer *et al.*, 2014; Dunn *et al.*, 2012).

GI_{50} values are lower for FC100 compound when compared to G51 compound, whereas, the first one seems not to induce a high enough perturbation to produce a total growth inhibition of U-87 MG cell line.

Regarding the measurement of drug efficacy, for anticancer drugs the high maximal effect is obtained when $E_{max} \sim E_{inf} \sim 0$, which correspond to death of all of the cells (Fallahi-Sichani *et al.*, 2013). Analysing our data, G51 compound shows closer values to 0 when compared to FC100. This support the HS conclusion about the theory of fractional cell killing, also agreeing with the poor drug activity verified for FC100 and its failure to reach TGI values. Additionally, the differences of E_{max} between these pair of drugs confirms that this parameter could be used to distinguish virtually drugs as reported (Fallahi-Sichani *et al.*, 2013). As well as, E_{inf} shows different values between this pair of drugs, and so, it might could be further evaluated to see if it happen when applying different compounds and cell lines.

Intriguingly and curiously enough, when comparing the drug-likeness score for both drugs, FC100 appears to display the ideal properties for a drug, while G51 has undesirable properties. Nevertheless, as shown in this study, these computed analyses of physicochemical properties are sometimes erroneous, and may exclude compounds with a significant perturbation at cellular level and/or having therapeutic effects. It is sometimes reported that these type of predictions based on the drug-likeness score might be a hard cut-off of promising agents, and may lead to inaccurate conclusions.

Conclusions

Twenty first century drug discovery and development welcomes a shift in its scientific background since many promising drugs were suddenly followed by disappointment, especially in cancer drug discovery and particularly for glioblastoma multiforme, which remains a largely fatal disease. Some approaches, such as multidimensional optimization and translational research, are helping to increase the success rate at both preclinical and clinical stages, but the high price of clinical development requires that even greater efforts are made to ensure that compounds entering clinical trials cross the finishing line (Abou-Gharbia *et al.*, 2014).

In this dissertation many strategies were described to improve the drug discovery and development process, and some of them were applied to this masters' project. For instance, this study brought together several academic institutions (from Portugal and Italy) showing that such interdisciplinary collaborations are desirable and beneficial to this type of research activity.

With a view to analyse the effect of the PDK1 inhibitors under investigation, a novel concept – parameterisation of dose-response perturbations – was successfully applied. This approach is based on Quantitative and Systems Pharmacology, which intends to improve the drug discovery and development process by promoting a more detailed characterization of both the disease and the drug response, integrating these effects at different levels.

Within this type of strategy, it was possible to conclude that the perturbation induced by the G51 – a PDK1 inhibitor – has a cytostatic effect against the human cellular system U-87 MG, whereas the FC100 compound shows no evident effect on these cells and cellular resistance might occur. In addition, the importance of the multiparametric analysis other than potency – IC_{50} – was proven: It is obviously an important goal to reduce IC_{50} values, but this may not be enough for an agent to be considered a promising drug and it cannot provide sufficient information to properly understand the variability of cellular responses to a drugs. Hence, other parameters, such as steepening the dose-response relationships, increase the maximum effect and the activity of the drug might important parameters to focus when developing new drugs.

A limitation of this study is the reduced reported similar studies, as so far only one analogous work has been performed, to the author's knowledge in this specific area of QSP. On

other hand, it is a proof of the high level of innovation of the present investigation as it is the first of its kind to be carried out in Portugal, applying this innovative concept to parameterise the perturbation induced by PDK1 inhibitors at the cellular system-level U-87 MG.

In further studies, it would be interesting understand the processes associated to the perturbation profile induced by these PDK1 inhibitors on the U-87 MG cellular system, particularly the functional mechanisms related to the cellular resistance proposed for the FC100 compound. Furthermore, it would be useful to translating this results for other system-levels in order to understand the effects of the perturbation verified.

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