Universidade do Algarve

Molecular biology techniques – A brief correlation between applied techniques in translational science and plant agriculture

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I want to thank you to everyone that somehow directly and indirectly made part of my life and had a major contribution for my personal and professional development.

So a big THANK YOU to them!

Abstract

This report aims to describe, how through my academic and professional life, I have developed a set of skills that allow me to apply for a "Hortofruticultura" Master's degree. After finishing my 5 years degree in 2005, I worked for Jardim Vista SA as an Agronomist, afterwards I went to the UK where I worked as a Research Technician/Research Assistant for 9 years. In September 2016 I started working as a lab technician in Universidade do Algarve for 6 months. More recently, in April 2017, I joined the Syngenta team in Moncarapacho as a Senior Grower and Quality Management Designate. In the UK, I took part in several scientific projects, mainly related to molecular biology. Considering that I have been involved in completely different scientific subjects in these 9 years, I decided to talk about some of the molecular biology techniques that I have learnt throughout these years and that are also used in Molecular Farming. With an increase in the world population, the impact of climate change and its effect on plant pathogens and plant diseases there is a need for healthy plants with a high production and better yields to fulfil those needs. Development of modified plants has progress through plant breeding and biotechnology that plays a major role in understanding how to modify plant genome or plant physiology to respond to those needs. By sequencing and studying the plants genome it is possible to understand and manipulate the characteristics of plants. Molecular farming, produces valuable proteins, peptides and small molecules through the use of both these techniques, plant molecular breeding and biotechnology.

Some routine molecular biology techniques used in plant biotechnology are common to the projects where I participated and are described in this report, namely DNA extraction, PCR, colony PCR, agarose gel electrophoresis, bacterial genetic transformation, protein expression, SDS-PAGE and Western Blot. These techniques are the base for the study of better ways to improve plants, preparing agriculture for the actual and future challenges.

Keywords: Molecular biology, biotechnology, agriculture, plant breeding, molecular farming.

Resumo

O objetivo deste trabalho é descrever a minha formação e aptidões adquiridas ao longo do meu percurso profissional, e como estas contribuíram para o desenvolvimento das minhas competências, as quais me permitem requerer o grau de Mestre. Após concluir o meu curso de 5 anos letivos em 2005 fui trabalhar como Engenheira Agrónoma para a empresa Jardim Vista SA. Posteriormente fui para o Reino Unido onde trabalhei como Técnica de Laboratório/Assistente de Laboratório durante 9 anos. Em Setembro de 2016 comecei a trabalhar como técnica de laboratório na Universidade do Algarve. Mais recentemente, em Abril de 2017 integrei a equipa da Syngenta em Moncarapacho como Senior Grower e Quality Management Designate. No Reino Unido, fui colaboradora de vários projetos científicos, os quais estavam maioritariamente relacionados com a área da biologia molecular. Considerando que durante estes 9 anos fiz parte de projetos que abordavam temas completamente diferentes, decidi abordar algumas das técnicas de biologia molecular que aprendi ao longo destes anos e que também são usadas na área da Agricultura Molecular e Melhoramento de Plantas. Com o nível de população a aumentar e o impacto das alterações climáticas, a necessidade de produzir plantas sem elementos patogénicos e destas serem mais produtivas e atingir elevadas produções, e eventualmente apresentarem resistência a herbicidas, assim como a produção de proteínas de interesse específico (Agricultura Molecular) também aumentou. O melhoramento de plantas em conjunto com a Biotecnologia têm um papel extremamente importante na compreensão e na descodificação da informação do DNA associado a estas necessidades, pois através da manipulação genética é possível alterar o genoma das plantas de modo a satisfazer as exigências atuais. Com o estudo do genoma das plantas é possível perceber e manipular as características que pretendemos alterar e melhorar. “Molecular farming” produz proteínas, péptidos e pequenas moléculas bastante valiosas, resultado dos estudos de melhoramento de plantas acoplado aos progressos na área da biotecnologia. Algumas das técnicas utilizadas em biotecnologia descritas nesta tese são a extração de DNA, o PCR, o colony PCR, a eletroforese, a transformação genética, a expressão proteíca, SDS-PAGE e Western Blot. Estas técnicas permitem-nos estudar métodos mais eficazes de melhorar as plantas para que a agricultura esteja melhor preparada para os desafios atuais e futuros.

Palavras-chave: Biologia molecular, biotecnologia, agricultura, melhoramento de plantas, agricultura molecular.

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Abbreviations

°C	Degrees centigrade
µ	Microlitres
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
CTAB	Cetyl trimethyl ammonium bromide
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
GFP	Green Fluorescent Protein
GM	Genetic modified
GMO	Genetic modified organism
L	Litres
LA	Luria-Agar
LDS	Lithium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBS-T	PBS plus 0.05% (v/v) Tween 20
PCR	Polymerase chain reaction
PVP	Polivinilpirrolidone
Ri plasmid	Root hair inducing plasmid
RNA	Ribonucleic acid
RNAases	Ribonucleases
SDS	Sodium Dodecyl Sulphate
TAE	Tris-acetate-EDTA
TALENs	Transcription Activator-like Effector Nucleases
T-DNA	Transfer DNA
TGS	Tris-Glycine-SDS
TMV	Tobacco mosaic virus
(w/v)	Weight by volume

I - Introduction

After approval from the "Comissão de Mestrado em Hortofruticultura" as a candidate for the Masters, it was decided that the candidate had to write and present her report, describing the professional competences acquired after a five-years degree completed at Universidade do Algarve. After finishing her degree the candidate worked for Jardim Vista SA as an Agronomist Engineer for almost 2 years. Afterwards the candidate went to work in the UK for 9 years as a Research Technician/Research Assistant in the University of Leicester, working on several projects (this will be explained in detail in section III). In September of 2016 the candidate started working as a lab technician in Universidade do Algarve. Due to the experience of the candidate in the laboratory, the theme chosen by the candidate was "Molecular biology techniques – A brief correlation between applied techniques in translational science and plant agriculture" that pretend to describe the acquired knowledge in molecular biology techniques, tools that are used in the lab to ameliorate the plants in a biotechnological context. A brief explanation on how biotechnology may be used to improve plants in the present and in the future, plant breeding and what is molecular farming and the issues around bio-safety will also be described.

In section II are described some of the technique the candidate has performed on the projects where she participated and that are relevant for plant biotechnology: DNA extraction, PCR, agarose gel electrophoresis, bacterial genetic transformation, protein expression, SDS-PAGE and Western Blot. In this section it will be discussed each of these techniques.

In section III it will be explained in detail the *curriculum vitae*, the projects in which the candidate participated, responsibilities and techniques learned by the candidate over the past twelve years. Articles where the candidate was co-author can also be found in section III.

II – Molecular biology techniques – A brief correlation between applied techniques in translational science and plant agriculture

1. Biotechnology role in crop development

Plants provide us with fibre, food, fuel and with one of the most important elements to sustain life, oxygen. But evolution to human population growth and the changing environmental conditions make us look at other alternatives for the use of plants. Climate changes is one of the most talked about and feared subject of the 21st century, and changes affect agriculture in many ways, and are notable in different systems in different regions of the world (Watkins, 2007). Agriculture is the most vulnerable sector to weather and climate change and in addition the majority of people involved in agriculture are poorer that urban population. Some of the major threats of global warming, are the unpredictable temperature and sea level rise. These threats affect plant agriculture in many ways, but biotechnology can help to fight those threats, through innovation, investment, sustainable agriculture development and trade policies. The role of biotechnology in the adjustment of agriculture to new climate changes is present to improve resistance to biotic and abiotic factors raised by climate changes. Biotechnology for example can help to improve resistance to abiotic factors by transferring a gene from a salt tolerant plant into susceptible crops to create salt crop resistance (Roy *et al.*, 2014), to improve drought tolerance in transgenic rice (Selvaraj *et al.*, 2017), to create plants tolerant to high temperatures (Bita and Gerats, 2013). Also, resistance to biotic factors (pests, diseases, weeds, etc), can be achieved by creating transgenic plants through biotechnology. Rice and soybean are relevant crops that have been transgenic modified to be resistant to virus, fungi, insects and herbicides, to overcome the global high losses produced by these biotic factors (Yapa, 2017). Genetic manipulation can also be used to modify fruit maturation through metabolic routes that lead to ethylene synthesis and degradation, modifying the plant organ maturation. But even so, we also need to create plants more space efficient. But plant agriculture also contributes to climate change, with the increasing number of greenhouses, and to a high polluted environment made by the uncontrolled and unknown use of chemicals to control pests and diseases. Economic and agronomic research proposes that the improvement of existing agricultural biotechnology can reduce greenhouse gas emissions by enhancing carbon sequestration on

cropland, decreasing the need for cropland expansion and reducing the use of carbon intensive inputs like fuel, insecticides and some herbicides.

By 2050, it is estimated that the global population will be around 9 billion. To sustain this population it will require food supply to double, demanding an increase of agriculture output. To address these future issues, genetic modifications can also be applied to produce plants for a high yield and seeds that are tolerant to new agriculture conditions (Searchinger *et al.*, 2014). It is important for countries to invest in technology to address food security rising problems, and see technology as a tool for economic growth. By producing the principal grains that feed most of the world, biofortified rice, wheat and corn, with increased mineral and vitamin content, the poorest countries can also improve their low variety diet (Hefferon, 2015). Biofortified foods can be obtained either through the generation of transgenic plants that have additional biosynthetic pathways, such as vitamin A enriched "Golden Rice" or by changing the general physiology of the plant in such a way that is able to extract more micronutrients from the soil. Some successful trials have be done to prove the efficiency of biofortified crops in micronutrient absorption on target populations, like enriched wheat with zinc, enriched beans and pearl millet with iron and enriched maize and cassava with Provitamin A (Bouis and Saltzman, 2017). Recently, new biotechnology techniques are revolutionizing the way food crops can be enhanced to respond to the actual humankind needs, by editing the genome. TALENs (Transcription Activator-like Effector Nucleases) and CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/Cas9 systems have been proven to be effective and reliable techniques for genome editing. These systems are relative simple and have a high efficiency in human, animal, and plant cells. Many genes have been targeted for mutations by TALENs in plants, including rice, barley and arabidopsis. For instances, TALENs was used to induce in rice disease resistance against *Xanthomonas oryzae* pv. *oryzae* (Li *et al.*, 2012). CRISPR-Cas 9 has been widely used due to its easy engineering. Some of the species with more targeted genes are Arabidopsis, rice, maize, *Nicotiana benthamiana* and tomato (Malzahn, 2017). Plant immunity against DNA viruses was achieved in *N. benthamiana* with CRISPR-Cas 9 (Baltes *et al.*, 2015).

To improve plants features, study plant gene expression and expression of foreign proteins, transformation with single genes is used. Nevertheless, in some situations transformation of plants with multiple genes is more suitable. Such as for, improving plants with multigenic traits, improving or changing metabolic pathways, expressing multimeric foreign proteins and expressing multiple enzymes involved in the synthesis of one or various compounds, such as

switchgrass (*Panicum virgatum* L.), produced for bioenergy due to high biomass production carrying multiple genes of interest (Ogawa *et al.*, 2014).

2. Plant genetic modification

Since the beginning of cultivation thousands of years ago plant breeding has been performed by humankind. Wild species were selected based on the advantageous characteristics (Vavilov, 1935), as flavor, size, resistance to seed or fruit drop, enhanced flavor, reduce dormancy, visual attractiveness, yield, resistance to abiotic and biotic factors. On the earlier 20th century Darwin's theory of evolution through selection and Mendel's laws of genetics led to modern plant breeding methods, where breeders/farmers use cross hybridization to combine genetic characteristics from different parental plants before selecting superior plants, meaning those with characteristics beneficial to humans, with new combinations of specific desirable traits among the progeny (Fu, 2015). Plant breeding can be performed using many different techniques, starting from the simple plant selection to the use of molecular biology techniques based on the genetics and chromosomes knowledge. The basic methods of classical plant breeding, backcrossing, inbreeding, hybrid breeding, mutation breeding and molecular-marker assisted selection where followed by new techniques, that allow to create further traits and to exchange features across species, that before were impossible. Plant breeding has been responsible for 50% or more of yield improvement in different crops, since 1930. In parallel other methods were or are being developed to interfere and specifically target plant DNA on a more efficient way. These methods include gene editing, ex: CRISPR-Cas9, cisgenesis, intragenesis, TALENs. Genetic engineering is intended to target a change in a plant gene sequence to effect a specific result through the use of recombinant DNA technology by using several approaches, ex: genetic transformation mediated by agrobacterium, agroinfiltration, particle bombardment and electroporation, RNA-dependent DNA methylation, oligonucleotide-directed mutagenesis, among others. Plant improvement programs frequently use a combination of molecular plant breeding and genetic engineering approaches, both making part of a long pathway on adapting crops to our needs.

With the development of plant biotechnology the possibility for the introduction of just one or several genes in crop plants, not only from the same plant, but also from other plant species and from completely different organisms has evolved, and a new branch of plant biotechnology –

Molecular Farming, was created for genetic modified plants that produce heterologous proteins and chemicals. Development of techniques based on natural methods as agrobacterium-mediated transformation or artificial methods as electroporation and particle bombardment, among others, gave a high contribution to plant biotechnology.

Stable genetic transformation through electroporation for plants is a technique that had a major importance in the 1980s and was widely adopted (Fromm *et al.*, 1985). Electroporation is a phenomenon where protoplasts are mixed with DNA in solution and subjected to a brief pulse of electrical current to create transient pores in the cell's membrane that allow DNA to enter efficiently. This allows yields of high frequency of both stable transformation and transient expression. Transformation by this method was developed for genetic transformation of monocotyledonous plants as corn (see review at Yadava *et al.*, 2016) and to plants recalcitrant to *Agrobacterium* mediated transformation (see below).

Gene delivery by particle bombardment or biolistic has become a widely used technique since the first studies by Christou (1988). This technique results when microprojectiles, usually tungsten or gold particles, coated with DNA, are propelled at the target cells, by acceleration. Microprojectile bombardment is optimized to deliver genes into the nuclear genomes of the plant cells. This technique may be applied to isolated cells or to apical meristems. Particle bombardment as well as electroporation may be limited by the regeneration capacity of the targeted cells or tissues (biolistic).

Genetic transformation also uses species of bacteria that are capable of transferring genes to higher plant species, namely Agrobacterium *tumefaciens* and *A. rhizogenes*, both widely used. *A. tumefaciens* is a naturally occurring soil microbe well known for causing crown gall disease on susceptible plant species. *A. rhizogenes* is responsible for hairy root disease in a broad range of dicotyledonous plants and some gymnosperms. These two *Agrobacterium* species elicits plant cell transformation by a complex series of chemical signals between the bacterium and the host that result in the movement and integration of a segment of T (transfer)-DNA to the nuclear genome of the host plant cells. This methodology allows the transfer of some genes (T-DNA) from an expression vector carried by *A. tumefaciens* into the genome of the host plant cells. Main advantages to use *Agrobacterium* to mediate transformation is the production of transgenic plants with single gene insertions. The soil bacterium *A. rhizogenes* induces the formation of adventitious roots by infecting the plant tissues. Hairy roots grow rapidly,

developing many branches without the need for *in vitro* exogenous plant growth regulators. Hairy roots culture are often used to produce secondary metabolites (Sharma *et al.*, 2013).

Functional analysis of transgenes and RNA silencing studies may be performed through agroinfiltration, a simple and effective method, involving the injection of *A. tumefaciens* into leaves or organs of a plant followed by the monitoring of transient transgene expression within the infiltrated tissue during the next days (Voinnet, 2001). Transgene delivery occurs through infiltration of *A. tumefaciens* carrying the transgene into the interstitial space of the mesophyll and then into the plant cells (Chen *et al*., 2013). This strategy involves the transfer of genes (T-DNA) into the nucleus of the host plant cells and transient expression through RNA synthesis and further production or not of protein. The leaves infiltration process can be done either with a syringe or by vacuum (Bashandy *et al*., 2015).

Agroinfiltration can be performed with a variety of expression vectors, including plant virus-based vectors. Transient expression with virus-based vectors can drive higher levels of protein accumulation in whole plants in a faster way. While many different plant viruses have been modified to function as expression vectors. As an example, *Tobacco mosaic virus* (TMV)-based vectors are used due to express the highest levels of foreign protein in plants (Lindbo, 2008). The vectors based on virus are used according to the plant species and where the protein expression should happen.

Continuous efforts must be made in plant breeding and cultivation techniques, to make plants production systems more efficient, sustainable and flexible. From a political and regulatory point of view, it's very important that innovation carries on getting sufficient opportunities and that considerations about new technologies are conducted truthfully with base on scientific data.

3. Molecular Farming

Biotechnology advances of the past few years have been tremendous, and nowadays plants are ideal candidates to produce heterologous proteins, that are used in pharmaceutical and industrial sectors (Ramalingam *et al.*, 2017). These proteins are obtained through recombinant DNA technology .*Hind* III was the first restriction endonuclease to be isolated in 1970 (Smith and Wilcox, 1970), and this finding led to recombinant DNA technology. In 1973 the first recombinant DNA experiment was published by Cohen and Boyer, twenty years after the

discovery of the DNA structure by Watson and Crick (Cohen *et al.*, 1973). In this experiment they cut a DNA fragment from an *E. coli* plasmid and transferred it into another *E. coli* DNA for gene expression. Nowadays, expression is not restricted to bacteria and recombinant techniques have been used with yeast, algae, plants, mammalian cell culture and to produce transgenic animals.

Molecular farming is described as the experimental application of biotechnology involving the production of proteins and chemicals by plants that are used for medical and commercial purposes. Molecular farming can be one of the options to produce biocompounds cheaper, safer, in bulk and that can be easily stored. Molecular farming makes use of distinct strategies for gene transfer that involve the stable genetic transformation or the transient expression of a nonintegrated transgene, to produce industrial enzymes, antibodies, vaccines, therapeutics, nutraceuticals and other pharmaceutical proteins (Moshelion and Altman, 2015).

Human insulin was the first recombinant protein approved by the United States Food and Drug Administration (FDA) to be used as a drug in 1982, after that the request for recombinant proteins, especially pharmaceutical proteins increased (Ma and Wang, 2012). A human growth hormone was the first plant-derived recombinant pharmaceutical protein to be produced in 1986 in transgenic tobacco (Barta *et al.*, 1986). The first plant-derived antibody was reported in 1989 (Hiatt *et al.*, 1989). In 1992, the first plant-derived vaccine, hepatitis B virus surface antigen, was expressed in transgenic tobacco (Mason *et al.*, 1992). The first plant-derived recombinant protein for commercial purposes was produced in 1997, avidin and egg protein was produced from maize (Hood *et al.*, 1997).

Some of the advantages of using plants are their low cost of production, considering that plant growth, transport and post-harvest are relatively economical, rapid scalability, absence of human pathogens reducing the risk of contamination, high biomass production and ability to produce high yield proteins accurately. One of the problems of using plants is the long timescale of production compared with microorganism or animal cell cultures.

For protein production in plants, theoretically any type of plant can be transformed, however it is wiser to use well studied and characterized plants for efficient protein production. Tobacco may be the ideal candidate to high-efficiency protein production in green tissue as it is a leafy plant that has a high capacity to produce biomass (Fischer *et al.*, 2004). In addition, maize, rice and wheat also have been used for recombinant protein production.

On section 5, it will be described biotechnology techniques that can be used during the process to obtain recombinant proteins from plants, and also to obtain modified plants needed to face the future.

4. Bio-safety

The development of genetically engineered plants brings a lot of benefits but with it also potential serious risks. Those risks are associated with the contact of genetic modified crops with wild species or other organisms, like bacteria and virus; the contact of crops resistant to pests and diseases with wild species, allows the opportunity for pests and diseases evolution leading to resistance. Thus the introduction of genetically modified crops raised public concern, in part due to lack of communication between authorities dealing with researchers, and also for the bio-safety and trade, leading to a delay in growth of molecular farming (Rastogi, 2013).
Work has been done to reduce environmental exposure to transgenics, by conducting controlled trials where environmental risk assessments are performed in order to follow the regulations and national laws for GMOs (Roberts *et al*., 2014), but we are still far away from a common solution to solve all the problems around regulations of molecular farming products.
It is very difficult to assess the long-term impact of molecular farming on the environment. One of the important concerns is the contamination of the food chain with pharmaceuticals plants. This can happen, just by using the same equipment for harvesting and processing of transgenic and food crops without proper decontamination and by growing food crops in the same field where transgenic crops where implemented previously without proper decontamination. To prevent this from happening, strict regulation needs to be implemented, including geographical restrictions on the location of transgenic crops, and harvesting and processing of these modified crops with designated equipment and proper decontamination if using the same equipment for food crops. The labeling of genetically modified products is essential, to inform and allow the customer to select according to their preferences.

The risks to human health by ingesting new proteins that didn't had a complete study on their impact at long-term consumption on the development of allergies or diseases brings a lot of public concern. The regulation of transgenic crops is a real challenge for regulatory agencies, each molecular product and each host system are a case that needs to be analyzed separately. The European Parliament and the Council of the European Union allowed the presence of 0.5% of transgenic material in non-transgenic food, in cases where the presence of transgenic material

is inevitable and the benefits are above the negative effects (EU, 2003). They are several ways of controlling gene flow among GMO's to prevent contamination of other species, mainly maternal inheritance, seed sterility and male sterility. By using sterile male traits and chloroplast transformation of plants, contamination of food crops can be prevented (Adem *et al.*, 2017). The chloroplast genome is maternally inherited, so the chances for transgene spread through pollen are controlled. Even so, they are no tactic that can be applied to all the crops, and a combination of strategies for controlling gene flow seems more effective.

5. Molecular Biology techniques

Some of the basic molecular biology techniques used to promote biotechnology related to molecular farming will be described in this report as DNA extraction, PCR, colony PCR, agarose gel electrophoresis, transformation, protein expression, SDS-PAGE and Western Blot. These techniques allow to study better ways to improve plants growth and production, helping agriculture meet future challenges. These techniques were applied by me in several experimental studies performed as a Research Technician/Research Assistant in the University of Leicester with bacteria and animal cells.

5.1. Standard stock and buffers

This section describes general protocols of the most common methodologies used in molecular biology. Table 1 describes, in detail, stocks and solutions used in the following molecular biology protocols.

Table 1 - Standard stocks and buffers.

Stocks and Buffers	Reagents	Reference
PBS❖(1x)	7.35 g NaCl 2.35 g Na_2HPO_4 1.3 g $NaH_2PO_4.2H_2O$ 1L dH_20 pH adjusted to 7.1	(Dulbecco and Vogt, 1954)
TAE buffer (50x)	242 g tris base 57.1 ml glacial acetic acid 37.2 g $Na_2EDTA.2H_2O$ 1L dH_20 pH adjusted to 8.5	(Ogden and Adams, 1987)

Coating solution	0.606 g Na_2CO_3 1.2 g $NaHCO_3$ 200 ml dH_2O pH adjusted to 9.6	(Engvall and Perlmann, 1971)
Blocking solution	0.5 g of milk 10 ml of 1x PBS	(Engvall and Perlmann, 1971)
Luria-Bertani broth❖	4 g tryptone 2 g yeast extract 2 g NaCl 400 ml dH_2O	(Luria and Burrous, 1957)
Luria-agar❖	Same as Luria-Bertani broth with addition of 1.5%(w/v) of agar	(Luria and Burrous, 1957)
SOC medium	SOB medium: 4 g tryptone 1 g yeast extract 0.1 g NaCl 200 ml dH_2O Plus: 50 µl 2M $MgCl_2$ 200 µl 1M glucose Filter-sterilized through 0.2 µM acrodisc syringe filter and added to 10 ml of SOB medium immediately before use.	(Sambrook *et al.*, 1989)

❖All media are autoclaved at 121°C, 15 psi for 20 minutes.

5.2. DNA extraction

The genomic DNA in plants is frequently larger than in animals (Bennett and Leitch, 2005) and compounds found in plant cells are not present in animal cells. These differences will dictate the different extraction methods, which influence the yield and purity of DNA. To extract genomic DNA from any prokaryotic or eukaryotic cell three central steps are needed: disruption of cell walls/membranes, separation of DNA from all other components (cell wall debris and metabolic substances) and maintain the integrity of DNA during the extraction. The extraction of genomic DNA is normally performed with a special extraction buffer and is purified by a phenol/chloroform extraction followed by isopropanol and/or ethanol precipitation (Fredricks and Relman, 1998), although other methodologies are available. Amounts of RNA and protein contaminants differ considerably between species, and these variations will dictate the selection and development of the cell lysis appropriate for the species that is being studied.

In the past few decades, scientists have developed numerous protocols and procedures for the extraction of DNA from different plant species, that are different based on the purpose of the isolation. Some of the most common procedures were described by Murray and Thompson, (1980), Doyle and Doyle (1987) and Rogers and Bendich (1988). However, there is not a universal protocol that can be used for all plant varieties. It can be difficult to obtain high quality DNA extractions from plants, being one of the key steps the preparation of tissues for extraction. Usually, liquid nitrogen is used to flash freeze followed by grinding the frozen tissue with a mortar and pestle. Some plant species can contain a lot of contaminants as polysaccharides, polyphenols and tannins that can inhibit some components of the solutions used during the extraction. During the initial centrifugation steps, the cell wall and other larger structures are removed, however polyphenols can still be present. DNA analysis by PCR can be affected by the variation in the lysis efficiency and DNA purity. To obtain the desired DNA quality, an existing protocol needs to be adapted, combined or developed. For example, to improve the DNA quality from strawberry leaves (*Fragaria ananassa* L.), Nunes *et al.* (2011) combined lyophilization and polivinilpirrolidone (PVP) that helped on the quality and amount of genomic DNA extracted from young leaves. The typical protocols for DNA extraction are not suitable for mango tree (*Mangifera indica* L.) due to the high content of secondary metabolites that can interfere with analytical applications, so Uddin *et al.* (2014) removed the undesirable polysaccharides, polyphenols and secondary metabolites from genomic DNA by modifying a traditional cetyl trimethyl ammonium bromide (CTAB) method by adding 0.4 M glucose.

Due to my long experience with plasmid DNA, below I will describe a protocol for DNA isolation based in extraction of plasmid DNA from bacteria. To extract plasmid DNA the right lysis method plays an important role to assure the separation from genomic DNA. As an example two commercial available kits NucleoSpin Plasmid (Macherey-Nagel, UK) and QIAprep Spin Miniprep (Qiagen, UK) are used. Both kits use the alkaline lysis method of Birnboim and Doly (1979).

To perform a DNA extraction of plasmid DNA, all the centrifugation steps should be performed at room temperature. From an overnight culture of *Escherichia coli* growing in LB plus required antibiotic, 10 ml are used to do DNA extraction. The culture is transferred into eppendorf tubes and centrifuged for 30 seconds. The cell pellets are ressuspended in the buffer provided by the kit containing RNase A (to a final concentration of 0.1 mg/ml and 0.4 mg/ml for the QIAprep Spin Miniprep kit and NucleoSpin® Plasmid kit, respectively). Sodium dodecyl

sulphate/alkaline lysis buffer provided by the kit is added to the tubes and carefully mixed. The tubes are incubated for 5 minutes at room temperature, until a clear and viscous lysate is visible. Guanidine hydrochloride buffer is added and incubate for 10 minutes to neutralize the lysates and allow the precipitation of denatured proteins, cell debris and other resulting products from lysis. The tubes are centrifuged for 10 minutes. The supernatant is transferred to the silica-membrane spin column and centrifuged for 1 minute, to allow for the DNA to bind to the column. The column is washed with an "ethanolic" solution, centrifuged for 1 minute, followed by another centrifugation for 1 minute to allow the membrane to dry. The DNA is eluted, by adding 50 µl of Tris-HCl (pH 8.5) buffer to the column and incubated at room temperature for 1 minute. The column is centrifuged for 1 minute and the plasmid DNA collected into a clean and sterile eppendorf tube.

5.3. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is used in agricultural biotechnology at different stages of product development. PCR allows the cloning of a definite gene and the specific detection of a DNA fragment from a complex range of DNA and its subsequent amplification. During product development PCR can be used for seed quality control, gene discovery, screening and characterization. One of the main applications for PCR currently in the agricultural sector is to verify the presence or absence of genetically modified (GM) material in a product and/or to quantify the amount of GM material present in that product. Rott *et al*. developed in 2004 a method to detect the presence of genetic modified soybean in sausages made from soybean Roundup Ready®. Also, Ma *et al*. developed the restriction site-anchored single-primer PCR method to obtain the unknown flanking sequences of transgene sequences in the maize transgenic plants (2014).

To perform a PCR reaction the following reagents are needed: template DNA, primers, nucleotides (adenine, thymine, cytosine and guanine), and DNA polymerase. The DNA polymerase links the nucleotides together to synthesize new DNA sequences that form the PCR product.

The primers detect the exact part of the sequence that we are looking for to amplify. DNA amplification occurs in three steps: denaturation, annealing and elongation. The thermal cycler machine lowers and raises the temperature of the block in precise, discrete and pre-programmed steps. The amplified copies can be detected by gel electrophoresis by their distinct size,

separated under electrical current. There are no significant differences between using a DNA template from animal or plants or bacteria. The PCR reagents may need to be adapted according to the segment size and DNA nucleotide composition that we want to amplify, as well as the cycling conditions. For the colony PCR an additional step is necessary to break the membrane from the bacteria so the DNA is released.

The PCR mixes are prepared on ice in a 50 µl reaction, using the following reagents:

- 5 µl 10x NH_4 reaction buffer: 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% (v/v) Tween-20 (Bioline).
- 1.5 µl of 50 mM $MgCl_2$ solution (Bioline).
- 1 µl deoxyribonucleotides triphosphate mix (dNTPs): 10 mM of each dATP, dCTP, dGTP, dTTP (Bioline). This mixture is prepared by adding 10 µl of each dNTP (100 mM) to 60 µl ultra nanopure H_2O. The solution is mixed, aliquoted and stored at -20°C.
- 25 pmol of each primer (forward and reverse).
- Up to 100 ng of DNA is used as a template. For the negative control, DNA is replaced by ultra nanopure H_2O.
- 1 µl (5 units) of thermostable BIOTAQ^{TM} DNA polymerase (Bioline).
- Ultra Nanopure H_2O, up to a final volume of 50 µl.

The PCR mixtures are centrifuged for 10 seconds and placed in a T-Gradient Thermal Cycler (Biometra, Germany). The thermal cycling program is performed using the following parameters:

Table 2 - Standard conditions for PCR.

DNA template			
Step	Temperature	Time	
Initial denaturation	95°C	1 minute	
Denaturation	94°C	30 seconds	30 cycles
Annealing	65°C	30 seconds	
Elongation	72°C	2 minutes	
Elongation hold	72°C	10 minutes	

The PCR products are analyzed by agarose gel electrophoresis (section 5.5).

5.4. Colony PCR

Colony PCR is a method to screen positive clones from ligation reactions, by determining the presence or absence of insert DNA in plasmid constructs. The PCR mix is prepared on ice in a 15 µl reaction, using the following reagents:

- Small amount of one (1) colony. In the negative control add water.
- 5 µl ultra Nanopure H_2O.

To each PCR tube containing nanopure water a small amount of one colony is added. To do this, a fine yellow pipette tip attached to the pipette lightly touches the side of one colony, the mixture is mixed by pipetting up and down. The mixture is placed in a T-Gradient Thermal Cycler (Biometra, Germany) for 10 minutes at 95°C. After that the following reagents are added:

- 1.5 µl 10x NH_4 reaction buffer: 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% (v/v) Tween-20 (Bioline).
- 0.45 µl of 50 mM $MgCl_2$ solution (Bioline).
- 0.3 µl deoxyribonucleotides triphosphate mix (dNTPs): 10 mM of each dATP, dCTP, dGTP, dTTP (Bioline). This mixture is prepared by adding 10 µl of each dNTP (100 mM) to 60 µl ultra nanopure H_2O. The solution is mixed, aliquoted and stored at -20°C.
- 7.5 pmol of each primer (forward and reverse).
- 0.3 µl (5 units) of thermostable Biotaq^{TM} DNA polymerase (Bioline).
- Ultra Nanopure H_2O, up to a final volume of 15 µl.

The PCR mixtures are centrifuged for 10 seconds and placed in a T-Gradient Thermal Cycler (Biometra, Germany). The thermal cycling program described in Table 2 is used and the products by agarose gel electrophoresis are analyzed (section 5.5).

5.5. Agarose gel electrophoresis

Electrophoresis is the most common method used to separate DNA fragments of different sizes. For the agarose gel electrophoresis, the size of the fragment that we are looking at is important to determine the percentage of agarose in the gel. Higher percentage of agarose increases

resolution of smaller bands, equally the lower the agarose percentage better resolution and separation of higher molecular-weight bands.

Considering for instance, that the size of the DNA fragments that we are looking for are approximately 1.5 Kb and 2.2 Kb, the PCR products should be run on a 0.9% (w/v) agarose gel to allow separation of fragments ranging from 0.5-7 Kb (as an example). For the gels preparation: 0.9 g of agarose (Bioline) is added to 100 ml of 1x TAE buffer pH 7.7 (from stock solution 50x TAE buffer). The mixture is melted in a microwave for a few minutes and left to cool down, ethidium bromide is added to a final concentration of 0.2 μg/ml. The gel is poured into a sealed tray with a well-forming comb and left to set. Next, the gel is transferred to the electrophoresis tank containing 1x TAE buffer, the comb is removed carefully and the samples are loaded. One of the wells is loaded with 1 Kb DNA ladder (MBI Fermentas, UK), to allow determining the size of the DNA fragments of interest. The DNA samples are diluted in 1x DNA loading dye (6x DNA loading dye - Fermentas, UK) and loaded into the gel wells. Electrophoresis is performed at 70 V, for approximately 20 to 30 minutes and the gel is visualized under a UV transilluminator. The image is captured, using for example an ImageQuant 100 (GE Healthcare) imaging system with the IQuant Capture 100 software (GE Healthcare).

5.6. Purification of PCR products

For the success of cloning it is important to purify the PCR products, to ensure the removal of short primers, dNTPs, enzymes, salts and short-failed PCR products that could interfere with the cloning steps. The silica membrane adsorbs the DNA when exposed to high concentration of salt, and allows the contaminants to pass through the column. The impurities are washed away, and the cleaned DNA is eluted with Tris buffer or water (www.qiagen.com).

Per each 1 volume of PCR product, 5 volumes of binding buffer is added and mixed. The mixture is transferred to a silica-membrane spin column and centrifuged for 30 seconds at 17,900 g, to allow the DNA to bind to the column. After that, an “ethanolic” buffer is added to the membrane to wash all the unwanted PCR products and impurities, centrifuged for 1 minute at the same speed, and spun again to dry out the membrane. Tris-HCl buffer (pH 8.5) is added to the column and left to incubate for 1 minute at room temperature, the column is spun again for 1 minute. The pellet containing the purified PCR product is collected into a clean and sterile eppendorf tube.

5.7. Cloning of targeted gene sequences into an expression vector

To clone a specific gene we need to take into account that the process differs from species to species and from gene to gene. The choice of an appropriate vector is an initial extremely important step. For easy manipulation, the vectors should be small molecules, capable of prolific replication in a living cell. They also should have restriction sites so the DNA that is to be cloned can be inserted.

For the cloning of targeted gene sequences the use of In-Fusion^{TM} Dry-Down PCR Cloning Kit is described as an example. This kit allows joining multiple pieces of DNA that have 15 base pairs of homology at their linear ends. This kit produces accurate constructs with inserts in desired orientation rapidly (www.clontech.com). The manufacturer's instructions were followed for the ligation reactions. The lyophilised reaction mixtures in a sealed 8-well strip are supplied with the kit. The purified PCR products are combined with the linearized vector at a molar ratio 2:1 in a final volume of 10 µl in ultra nanopure H_2O and the mixture is transferred to the reaction tubes supplied. For the cloning procedure, the tubes are incubated for 30 minutes at room temperature. After that, the tubes are transferred to ice and used in the cloning reactions in transformation of chemically competent cells (section 5.8).

5.8. Heat-pulse transformation of chemically competent bacterial cells- Transformation into BL21-Gold (DE3) pLysS competent cells

BL21-Gold (DE3) pLysS competent cell are an *E. coli* strain with a high-performance transformation efficiency promoting high levels of protein expression.

The bacterial transformation protocol recommended by the manufacturer Stratagene is described below. The competent cells are thawed on ice, mixed gently and aliquoted into 100 µl pre-chilled 14 ml falcon polypropylene round-bottom tubes (BD, UK). An additional tube is prepared with 100 µl to use as a transformation control. Approximately 50 ng (1-2 µl) of cloning reaction is added to the aliquots and 1 µl of pUC18 control plasmid to the transformation control tube. The tubes are swirled gently and placed on ice for 30 minutes. Each reaction is heat-pulse for 20 seconds in a 42°C water bath, followed by 2 minutes incubation on ice. Afterwards, 900 µl of SOC previously warmed at 42°C is added to the reactions. The tubes are incubated at 37°C for 1 hour on a shaker set at 220 rpm. The transformation reactions are plated onto LA plates

containing ampicillin and incubated overnight at 37°C. The following day randomly colonies are selected, and run through colony PCR (see section 5.4) and placed to growth on a new LA plate containing the plasmid encoded antibiotic resistance and incubated overnight at 37°C. Lyophilized and sent for sequencing the selected transformants.

5.9. Small scale protein expression

On average plant proteins are larger than bacterial proteins (Tiessen *et al.*, 2012).
Heterologous expression is when a protein is produced in a different organism than the natural one, often to enable large-scale production. The most common system used for heterologous protein production is *E. coli* due to its simplicity, low cost, compatibly and fast growth rate (Frommer and Ninnemann, 1995). This method allows the biochemical and biophysical analysis of plant protein expression and purification through microorganisms. Bacteria, yeast, insect cells and *Xenopus laevis* are the systems used for recombinant plant protein production (Yesilirmak and Zehra, 2009). Recombinant plants proteins are used in industrial purposes, like cellulases, for other biomedical purposes like frutalin known by the anti-tumor properties present in *Artocarpus incisa* L. (breadfruit) and produced in *E. coli* and *Pichia pastoris* (Oliveira *et al.*, 2014) and synthesis of a peptide against diabetes from ginseng (Yan *et al.*, 2003) in *Pichia pastoris*.
The method of heterologous protein production explained below is based on the production of recombinant proteins in prokaryotic cells.
E. coli carrying a recombinant expression vector with a specific gene sequence are inoculated into 10 ml of LB with antibiotic, with 220 rpm agitation at 37°C, overnight. The following day, 1 ml of overnight growing culture is added into new 10 ml LB with antibiotic and incubated at 37°C, with 220 rpm agitation, until the culture reached an OD600 of approximately 0.5-0.6. 1 mM filtered-sterilized IPTG (Melford laboratories, UK) is added and incubated until the culture reached an OD600 of approximately 1.5-1.6. The cells are collected in a PK131R Refrigerated Centrifuge (ALC International), at 2,324 x g for 15 minutes, at 4^{o}C. The pellet is ressuspended in 250 µl ice cold 1 x PBS and transferred to a clean and sterile eppendorf tube. To produce a crude cell extract containing the protein of interest, the cells are sonicated with a small sonicator probe (Soniprep). To prevent overheating from the sonication process, the tubes are kept all the time in a glass beaker filled with ice. During the sonication process intervals of 1 minute is made, and 5 seconds of pulse with amplitude of 6 microns is applied, followed by a “cool down”

period of 55 seconds. This step is performed 5-6 times or until the extract became clearer and less viscous. The tubes are centrifuged at 15,115 x *g* in a bench-top centrifuge at 4ºC for 10 minutes. The supernatant is collected into a clean and sterile eppendorf tube.

5.10. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate components of a protein mixture according to their molecular weight, based on their length. Being the most common method used to obtain high resolution analytical separation of proteins mixtures. An initial denaturation of the proteins mixture with an anionic detergent that binds to the proteins, charges the proteins negatively in proportion with their molecular weight. After this, the proteins are separated through a porous acrylamide gel by electrophoresis based on their molecular weight. This method has been used since the early 1970s, and works in applications that do not require retention of native features of protein structure or function (Laemmli, 1970). This method has been widely used to study proteins from organisms of all kingdoms since its discovery. As an example of its use for plant protein analysis, Chernyshenko *et al.*, in 2016 used this method to estimate the protein composition of roots and leaves of *Ophioglossum vulgatum*.The protocol described in this section needs to be adapted to the type and size of the proteins that we are looking for.
A 5% (w/v) stacking gel and 12% (w/v) resolving gel are used.

5.10.1. Gels preparation

For the casting frames, two glass plates are evenly aligned and clamped in the casting frames on the casting stands that are sealed by the casting gaskets on the bottom. In a falcon tube 15 ml of 12% (w/v) resolving gel is prepared (Table 3), by mix thoroughly and pipette into de gap between the glass plates, leaving up to 1 cm below the level of the comb teeth. The gap is filled with dH_2O to prevent dehydration and the gel left to jellify for 30-45 minutes.

Table 3 - Solutions for preparation of 12% (w/v) resolving gels and 5% (w/v) stacking gels for SDS-PAGE.

Solution	Resolving gel 12%	Stacking gel 5%
30% (w/v) acrylamide	6.0 ml	830 µl
1.5 M Tris-HCl , pH 8.8	3.8 ml	-
1.0 M Tris-HCl , pH 6.8	-	630 µl
10% (w/v) SDS	150 µl	50 µl
10% (w/v) ammonium persulfate	150 µl	50 µl
TEMED	6 µl	5 µl
Nanopure H_20	4.9 ml	3.4 ml
Final volume	≈15 ml	≈5 ml

In the meantime, 1x running buffer is prepared, by adding 100 ml of 10x Tris-glycine-SDS (TGS) electrophoresis grade buffer (National Diagnostics) into 1L of dH_2O. The water is discarded and the excess absorbed with filter paper. 5 ml of 5% (w/v) stacking gel (Table 3) is prepared, mixed thoroughly and pipetted into de gap between the glass plates. The well-forming comb without trapping air under the teeth is carefully insert, before polymerization begged. The gel is left to polymerize for 20-30 minutes. If the gels are not used on the same day, they are stored in 1x TGS at 4°C.

5.10.2. Sample preparation

To treat the samples, a reducing buffer is used in the presence of heat. The samples are mixed with 4x NuPAGE® LDS (lithium dodecyl sulphate) to a final concentration of 1x. A beaker is placed to boil with dH_2O on a hot plate. The lid of the tube samples is punctured with a needle to release the pressure inside the tube caused by temperature differential during boiling. After the beaker is boiling the samples are placed on a floating rack and transferred to the boiling water for 5 minutes.

5.10.3. Electrophoresis

The molding combs are gently remove after polymerization. The glass plates are removed from the casting frame and the gels placed into the electrophoresis tank, half-filled with 1x TGS. The protein marker (e.g.: Precision plus protein standard – all blue; Bio-Rad) is added to one well

alongside the samples and the electrophoresis chamber is filled with 1x TGS. The anodes of the Mini-PROTEAN 3 cell (Bio-Rad) tank are connected. The voltage is set at 40 mA and the gels run until the protein marker almost reaches the foot of the glass plates. After the run finished, the gel cassettes are gently removed and the gels carefully transferred into a large Petri dish. The gel is carefully washed with dH_2O and Coomassie blue staining solution (prepared by adding 1 part ethanol to 9 parts Protoblue safe – colloidal Coomassie blue G-250 stain from National Diagnostics) is added to the Petri dish and left to incubate overnight on a rocker plate. The following day, the gel is washed in dH_2O and analyzed. Alternatively, the gels are transblotted for later immunodetection (see section 5.11).

5.11. Western Blot

Western blot is an important technique widely used in cell and molecular biology to identify a specific protein in a sample mixture of proteins, that was introduced by Towbin *et al.*, 1979. This technique allows the identification of specific proteins from a complex mixture of proteins extracted from cells. Western blot can be divided into three parts: protein separation by size, transfer to a solid support and protein target marking with specific primary and secondary antibodies to identification. Western blot has been widely used as an analytical technique for studies of plant proteins, e.g. the detection of the presence of glutamine synthetase polypeptides on leaf extracts of tobacco (*Nicotiana tabacum* L.) (Carvalho *et al*., 1992), the identification and validation of reference rice proteins (Li e*t al.*, 2011) or to confirm the presence of recombinant CCL21 protein, a chemokine that can be used for anti-metastatic of cancer cell lines in *Solanum lycopersicum* (Beihaghi *et al.*, 2018). In the protocol described below some changes may be necessary considering the type of proteins that we are looking for. The antibodies should be specific for the protein that we are looking; incubation periods, reagents and gel running time may need to be adapted.

5.11.1. Transferring the protein from gel to the membrane

After protein samples electrophoresis through a 12% (w/v) SDS-PAGE (see section 5.10), the sandwich transfer is assembled. The gels are placed in 1x transfer buffer (5.9 g Tris, 2.9 g glycine, 200 ml methanol, 3 ml 10% (w/v) SDS, 800 ml distilled H_2O, pH 8.3) for 10 minutes. For the sandwich assemble, a foam pad is placed on one side of the cassette (negative side) and

one sheet of filter paper (Bio-Rad) on top of the pad, the gel is placed on top of the filter paper followed by nitrocellulose membrane (Amersham) placed on the positive side. The second sheet of filter paper is placed on top of the stack, followed by a second foam pad on top and the cassette holder is closed, making sure that no bubbles are trapped in the sandwich. The cassettes are transferred to the mini trans-blot tank (Bio-Rad) filled with ice-cold transfer buffer and an ice block is placed into the tank. The system is turned on for approximately 1 hour at 250 mA. After the transfer is complete, the nitrocellulose membrane is carefully removed with forceps. The membrane is blocked overnight with 5% (w/v) skimmed milk in PBS at 4 °C.

5.11.2. Immunodetection

The following day, the membrane is washed 3 times for 10 minutes with 0.05% (v/v) Tween 20 (PBS-T). The membrane is incubated with primary monoclonal antibody [1:1000 dilution in PBS-T plus 2% (w/v) skimmed milk], at room temperature and placed on a shaker for 90 minutes. After that, the membrane is washed four times for 10 minutes with PBS-T (10 minutes each wash), and incubated with the secondary antibody, goat anti-mouse IgG (Sigma) alkaline phosphatase conjugate [1:2000 dilution in PBS-T plus 2% (w/v) skimmed milk], at room temperature and placed on the shaker for 1 hour. Lastly, the membrane is washed 4 times for 10 minutes with PBS-T, and incubated with approximately 5 ml of BCIP/NBT liquid substrate system (Sigma) until the bands are visible and the detection of the antibody complex possible.

6. References

ADEM, M.; BEYENE, D.; FEYISSA, T. Recent achievements obtained by chloroplast transformation. **Plant Methods,** v. 13:30, 2017.

BAESHEN, N. A.; *et al*. Cell factories for insulin production. **Microbial Cell Factories** v. 13:141, 2014.

BALTES, N.; *et al*. Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. **Nature Plants**, 1, 2015.

BARTA, A.; *et al*. The expression of a nopaline synthase - human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. **Plant Molecular Biology,** v. 6, n. 5, p. 347-57, 1986.

BASHANDY, H.; JALKANEN, S.; TEERI, T. H. Within leaf variation is the largest source of variation in agroinfiltration of *Nicotiana benthamiana.* **Plant Methods,** v. 11, n. 47, p. 1-7, 2015.

BEIHAGHI, M.; *et al*. Transient Expression of CCL21 as Recombinant Protein in Tomato. **Biotechnology Reports**, v. 17, p. 10–15, 2018.

BENNETT, M. D.; LEITCH, I. J. Genome size evolution in plants. San Diego: Elsevier, 2005.

BIRNBOIM, H. C.; DOLY, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. **Nucleic Acids Research,** v. 24, n. 7, p. 1513-23, 1979.

BOUIS, H. E.; SALTZMAN, A. Improving nutrition through biofortification: A review of evidence from HarvestPlus, 2003 through 2016. **Global Food Security,** v. 12, p. 49–58, 2017.

CARVALHO, H., *et al*. Detection of a cytosolic glutamine synthetase in leaves of *Nicotiana tabacum* L. by Immunocytochemical Methods. **Plant Physiology,** v. 100, p. 1591-1594, 1992.

CHEN, Q.; LAI, H.; HURTADO, J.; STAHNKE, J.; LEUZINGER, K.; DENT, M. Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins. **Advanced Techniques in Biology & Medicine,** v. 1, n. 103, p. 1-21, 2013.

CHERNYSHENKO, M. S.; CHERNYSHENKO, V. O.; MUSATENKO , L. I. SDS-PAGE of *Ophioglossum vulgatum* proteins. **International Journal of Plant & Soil Science,** v. 9, n. 1, p. 1-7, 2016.

CHILTON, M. D.; TEPFER, D. A.; PETIT, A.; DAVID, C.; CASSE-DELBART, F.; TEMPÉ, J. A*grobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. **Nature,** v. 295, p. 432 – 434, 1982.

CHUPEAU, M. C.; *et al*. Transgenic plants of lettuce (*Lactuca sativa*) obtained through electroporation of protoplasts. **Nature Biotechnology**, v. 7, p. 503-508, 1989.

COHEN, S. N.; *et al*. Construction of biologically functional bacterial plasmids in vitro. **Proceedings of the National Academy of Sciences of the United States of America,** v. 70, n. 11, p. 3240-4, 1973.

BITA, C.; GERATS, T.; Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. **Frontiers in Plant Science** , v.4, p.273, 2013.

CHRISTOU, P. Strategies for variety-independent genetic-transformation of important cereals, legumes and woody species utilizing particle bombardment. **Euphytica**, v. 85, p. 13–27, 1995.

DOYLE, J. J.; DOYLE, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. **Phytochemical Bulletin,** v.19, p.11-15, 1987.

DULBECCO, R.; VOGT, M. Plaque formation and isolation of pure lines with poliomyelitis viruses. **The Journal of Experimental Medicine,** v. 31, n. 99(2), p. 167–182, 1954.

ENGVALL, E.; PERLMANN, P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. **Immunochemistry,** v. 8, n. 9, p. 871-874, 1971.

FISCHER, R.; *et al*. Plant-based production of biopharmaceuticals. **Current Opinion in Plant Biology,** v. 7, n. 152-158, 2004.

FREDRICKS D. N; RELMAN D.A. Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor Sodium Polyanetholesulfonate. **Journal Clinical Microbiology**, v. 36, p. 2810-2016, 1998.

FROMM, M.; TAYLOR, L. P.; WALBOT, V. Expression of genes transferred into monocot and dicot plant cells by electroporation. **Proceedings of the National Academy of Sciences**, v. 82, p. 5824–5828, 1985.

FROMMER, W. B.; NINNEMANN, O. Heterologous expression of genes in bacterial, fungal, animal, and plant cells. **Annual Review of Plant Physiology and Plant Molecular Biology,** v. 46, p. 419–444, 1995.

FU, YONG-BI. Understanding crop genetic diversity under modern plant breeding. **Theoretical and Applied Genetics**, v.128, n.11, p. 2131–2142, 2015.

GALLOIS, P.; LINDSEY, K.; MALONE, R. Electroporation of tobacco leaf protoplasts using plasmid DNA or total genomic DNA. Totowa, NJ: 1995.

HEFFERON, K. L.; Nutritionally enhanced food crops; Progress and perspectives. **International Journal of Molecular Sciences,** v.16, nº 2, p. 3895–3914, 2015.

HIATT, A.; CAFFFERKEY, R.; BOWDISH, K. Production of antibodies in transgenic plants. **Nature,** v. 342, p. 76–78, 1989.

HOOD, E. E.; *et al*. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. **Molecular Breeding,** v. 3, n. 4, p. 291–306, 1997.

HUIHUI, L.; *et al*. Fast-forwarding genetic gain. **Trends In Plant Science**, v. 23, n. 3, p. 184-186, 2018.

LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature,** v. 15, n. 227, p. 680–685, 1970.

LI, T.; *et al*. High-efficiency TALEN-based gene editing produces disease-resistant rice. **Nature Biotechnology**, v. 30, p. 390-392, 2012.

LI, X.; *et al*. Identification and validation of rice reference proteins for Western blotting. **Journal of experimental botany**, v. 62, p. 4763-4772, 2011.

LINDBO, J. TRBO: A high-efficiency tobacco mosaic virus RNA-Based overexpression vector. **Plant physiology**, v. 145, 2008.

LURIA, S. E.; BURROUS, J. W., Hybridization between *Escherichia coli* and *Shigella*. **Journal of Bacteriology**, v. 74, p. 461–476, 1957.

MA, J.; *et al*. Cloning of anking sequence in transgenic plants by restriction site-anchored single-primer polymerase chain reaction. **Genetics and Molecular Research,** v. 13, n. 4, p. 10556-10561, 2014.

MA, S.; WANG, A. **Molecular Farming in Plants: An Overview**. Springer, 2012.

MALZAHN, A.; LOWDER, L.; YIMPING, Q. Plant genome editing with TALEN and CRISPR. **Cell & Bioscience**, v. 7, n. 21, 2017.

MASON , H. S.; LAM , D. M.; ARNTZEN, C. J. Expression of hepatitis B surface antigen in transgenic plants. **Proceeding of the National Academic of Science of the United States of America,** v. 89, n. 24, p. 11745–11749, 1992.

MOSHELION, M.; ALTMAN, A. Current challenges and future perspectives of plant and agricultural biotechnology. **Trends in Biotechnology,** v. 33, n. 6, 2015.

MURRAY, M. G.; THOMPSON, W.F.; Rapid isolation of high molecular weight plant DNA. **Nucleic Acids Research**, v. 8, n. 19, p. 4321–4326, 1980.

NUNES, C. F.; *et al*. An improved method for genomic DNA extraction from strawberry leaves. **Ciência Rural,** v. 41, n. 8, p. 1383-1389, 2011.

OGAWA, Y.; *et al*. (2014). A simple and reliable multi-gene transformation method for switchgrass. **Plant cell reports**, v. 33,, n. 7, 2014.

OGDEN, R. C.; ADAMS, D. A. Electrophoresis in agarose and acrylamide gels. **Methods Enzymology,** v. 152, p. 61-87, 1987.

OLIVEIRA, C.; TEIXEIRA, J. A.; DOMINGUES, L. Recombinant production of plant lectins in microbial systems for biomedical application – the frutalin case study. **Frontiers in Plant Science,** v. 5, n. 390, 2014.

EU. E. Regulation (EC) No 1829/2003 of the European Parliament and of the council of 22 September 2003 on genetically modified food and feed., http://eurlex.europa.eu/LexUriServ/site/en/oj/2003/l_268/l_26820031018en00010023.pdf, 2003.

RAMALINGAM, K.; *et al*. (2017). Rapid production of therapeutic proteins using plant system. **Defence Life Science Journal**, v. 2. n. 2, p. 95-102, 2017.

RASTOGI, S. V. Genetically modified plants: Public and scientific perceptions. **ISRN Biotechnology**, p. 1-11, 2013.

ROBERTS, A.; *et al.* Environmental risk assessment of GE plants under low-exposure conditions. **Transgenic Research,** v. 23, n. 6 p. 971–983, 2014.

ROGERS, S. O.; BENDICH, A. J. Extraction of DNA from plant tissues. Plant molecular biology manual. In: GELVIN, S. V.; SCHILPEROORT, R. A.; VERMA, D. P. S. (Ed.).. cap.6, p.1-10. Dordrecht: Kluwer Academic, 1988.

ROTT, M. E.; *et al*. Detection and quantification of roundup ready soy in foods by conventional and real-time polymerase chain reaction. **Journal of Agricultural and Food Chemistry,** v. 52, n. 16, p. 5223-5232, 2004.

ROY, S.; NEGRÃO, S.; TESTER, M. Salt resistant crop plants. **Current Opinion in Biotechnology**, v. 26. p. 115–124, 2014.

SAMBROOK, J.; FRIRSCH, E. F.; MANIATIS, T. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, 1989.

SEARCHINGER, T.; *et al*. Creating a sustainable food future. A menu of solutions to sustainably feed more than 9 billion people by 2050. World resources report 2013-14. Interim findings, 2014.

SELVARAJ, M. G.; *et al*. Overexpression of an *Arabidopsis thaliana* galactinol synthase gene improves drought tolerance in transgenic rice and increased grain yield in the field. **Plant Biotechnology Jornal**, v. 15, p. 1465-1477, 2017.

SHARMA, P.; PADH, H.; SHRIVASTAVA, N. Hairy root cultures: A suitable biological system for studying secondary metabolic pathways in plants. **Engineering in Life Sciences**, v. 62, 2013.

SMITH, H. O.; WILCOX, K. W. A restriction enzyme from *Hemophilus influenzae*. Purification and general properties. **Journal Molecular Biology,** v. 51, n. 2, p. 379-91, 1970.

SPÖK, A. Molecular farming on the rise-GMO regulators still walking a tight rope. **Trends in Biotechnology,** v. 25, p. 74-82, 2007.

VOINNET, O. RNA silencing as a plant immune system against viruses. **Trends in Genetics ,** v.17, n. 8, p. 449–459, 2001.

TIESSEN, A.; PÉREZ-RODRÍGUEZ., P.; DELAYE-ARREDONDO, L. J. Mathematical modeling and comparison of protein size distribution in different plant, animal, fungal and microbial species reveals a negative correlation between protein size and protein number, thus providing insight into the evolution of proteomes. **BioMed Central Research Notes,** v. 5, n. 85, 2012.

TOWBIN, H.; STAEHELIN, T.; GORDON, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proceedings of the National Academy of Sciences of the United States of America,** v. 76, n. 9, p. 4350-4, 1979.

UDDIN, M. S.; *et al*. An improved method to extract DNA from mango M*angifera indica.* **Biologia,** v. 69, n. 2, p. 133–138, 2014.

VAVILOV, N. I. The origin, variation, immunity and breeding of cutivated plants. Translated by K. Staer Chester 1851. **Ronald Press**, New York, 1935.

WATKINS, K. Human development report 2007/2008. Fighting climate change: human solidarity in a divided world. United Nations Development Programme. New York: Palgrave Macmillan, 2007.

WHITELAM, G. C.; *et al*. Heterologous protein production in transgenic plants. **Biotechnology and Genetic Engineering Reviews,** v. 11, n. 1, p. 1-32, 1993.

YADAVA, P.; ABHISHEK, A.; SING, R.; SINGH, I.; KAUL, T.; PATTANAYAK, A.; AGRAWAL, P. K. Advances in maize transformation technologies and development of transgenic maize. **Frontiers in Plant Science,** v. 7, n. 1949, p. 1-12, 2016.

YAN, Y.; CHEN, J.; LI, J. Overexpression of a small medicinal peptide from ginseng in the yeast *Pichia pastoris*. **Protein Expression and Purification**, v. 29, p. 161-6, 2003.

YAPA, A. W. Transgenic plants: resistance to abiotic and biotic stresses. **Journal of Agriculture and Environment for International Development,** v. 111. p. 245-275, 2017.

YESILIRMAK, F.; ZEHRA, S. Heterelogous expression of plant genes. **International journal of plant genomics**, p. 1-16, 2009.

III – *Curriculum vitae*

On this chapter, I will present my academic qualifications, focusing on my professional career, present my publications and explain the relevance of my experience to obtain the "Hortofruticultura" Master's degree.

7. Academic pathway

I attended the 1st cycle of basic education from 1987 to 1991 in Escola E.B. 1 nº 1 de Tavira, and the 2nd cycle of basic education from 1991 to 1997, finishing the 3º cycle of basic education in 1999 with a final score of 15 out of 20. The internship was done on the final year of the degree in Viveiro Florestal do Algarve, Lda under the supervision of Prof. José Monteiro from Universidade do Algarve, with a final score of 16 out of 20, with the title "Actividade viveirística de espécies de jardim no Algarve. Germinação de sementes de *Ceratonia siliqua* L. e *Olea europaea* var. *sylvestris*."

I concluded the degree in Engenharia Agronómica – Ramo Hortofruticultura in 2005 at Universidade do Algarve (UALG) with a final score of 13 out of 20. In 2005 I conclude the Postgraduate course "Greenkeepers" with a final score of 13 out of 20.

8. Professional career

8.1. From April 2017 until present –Syngenta Flowers

I started working at Syngenta Flowers on the 3rd of April of 2017 as a Senior Grower and Quality Management Designate. Syngenta Flowers is dedicated to the breeding of pioneering flower varieties that offer outstanding performance in the professional greenhouse, at retail, in landscape and in the home garden. In addition to the vast experience in plant genetics, it supports industry professionals through world-class customer service, in-depth cultural information, flexible and exciting marketing programs, and a deep understanding of plants from many points of view.

8.1.1. Main activities and responsibilities

Lead and take part of the coordination and supervision of cultivation activities of all flower crops in the execution of crop production protocols within the assigned location such that final results meet the client specifications whilst ensuring knowledge transfer to local teams to build future capabilities. Assist the Head Grower in training and supervision of both permanent and temporary workers. Participate in the Quality Management system; write procedures/ work instructions / forms; participate in yearly review of documents. Utilize keen observation skills to monitor plant growth & nutritional status. Observe that all cultural activities happen in agreed deadlines.

8.1.2. Practical skills gained during this work

- Irrigation/Fertigation - preparation of fertilizer stock solution for all Flowers greenhouses.
- Climate control (through S-Monitor environmental control system, adjusting set points based on crop assessment and its phenological state; acting upon alarms provided by this system).
- Crop Protection (implementation of chemical or biological measures; taking part in chemical application; continuous monitoring of crop phyto conditions).
- Compliance with company Phytosanitary standards. Compliance with country legislation.
- Sampling (diseases and nutritional).
- Monitoring, calibration of equipment's (EC/pH meters, probes, injectors, scales).
- Keeping accurate and required records on crop production activities (including legal/regulatory requirements and global standards).
- Work across boundaries – capable of replacement of department's crucial functions during colleague's absences.

8.2. From September 2016 until March 2017 – Universidade do Algarve

The main goal of this work was to clone a fragment of each callose synthase and xylulose transglucosylase genes from *Citrus sinensis* (L.) Osbeck cv. Valencia late and *Citrus clementina* hort. ex Tanaka cv. Fina to further proceed to a qPCR gene expression analysis of these genes. I worked as a research technician on this project under the supervision of Prof. Natália Marques.

8.2.1. Main activities and responsibilities

- Citrus and *Thymus albicans* RNA extraction.
- Immunocapture/RT-PCR.
- Primer design.
- Amplification of gene sequences by PCR.
- Reverse transcription-polymerase chain reaction.
- cDNA synthesis.
- DNA quality assessment by spectrophotometry.
- Preparation of competent cells.
- Transformation of *E. coli*.
- Cloning of the sequences obtained into pGEM T-easy vector.
- To identify and analyse gene expression of cal S, cal S7 and xyl glu B.

8.2.2. Practical skills gained during this work

Knowledge about all the procedures of molecular biology related to the cloning procedure, as described in section 8.2.1.

8.3. From March 2014 until September 2016 – University of Leicester

I worked as a Research Assistant/Lab manager for a project funded by Kay Kendall Leukaemia Fund (KKLF), under the supervision of Professor Salvador Macip and Professor Martin Dyer. The project aim was to discover a drug or drugs combinations to treat patients with CLL (Chronic lymphocytic leukemia) and HCL (Hairy cell leukemia) leukemia types, based on animal models.

8.3.1. Main activities and responsibilities

- To prepare and organise the laboratory to meet the requirements of the research agenda. Ensuring that the work environment is maintained to the standard required to deliver the appropriate level of service to all those who use the facilities and adhering to the relevant COSHH regulations and safety standards affecting self and others at all times.
- Manage the cell bank and the DNA bank for the research group and perform key experiments (such as FACS, cell cultures, etc.) for the lab projects, as discussed with the line manager.
- Coordinate with other labs working on the same project.

- Responsible for the maintenance and organisation of the breeding colonies of mice for different projects ongoing in the lab (including xenografts, transgenic and ageing models).
- Operate a range of specialist equipment, providing instruction to others as required.
- To procure appropriate stock levels of lab consumables etc. to ensure co-workers can carry out their work without interruption, ensuring effective use of budgets within the financial constraints of the project
- Analyse and interpret research data and to present findings accurately.
- Attend group meetings in order to ensure up to date knowledge of the work of the research group and present own experimental data, as required.
- Liaise with other groups working on the same projects.
- Clarify work requirements with line manager as required.
- Order supplies and equipment to meet the requirements of the research to ensure stock levels are maintained.
- Plan own workload to ensure that requirements of the project are met.
- Perform activities to appropriate time and quality requirements.

8.3.2. Practical skills gained during this work

- Protein manipulation techniques including, protein expression, extraction and purification using the AKTA system, SDS gels, Western blotting and ELISA.
- Tissue culture of human cell lines and human primary cells.
- DNA manipulation, including PCR, transformation and DNA extraction.
- Flow cytometry (BD FACSCalibur and BD Canto II), full understanding of the technology with appropriate training for samples preparation; machine operation and data analysis.
- Full understanding of confidentiality and intellectual property protection considering that my role involved handling of commercially sensitive information.
- Training and supervision of students in key laboratory techniques.
- Maintaining laboratory equipment and organise repairs/servicing as required.
- Maintaining the equipment and facilities within the research laboratory.
- Maintaining breeding colonies of mice for the different research projects.
- Managing the cell bank and the DNA bank.
- Planning and delivering tasks to an agreed schedule.
- Training undergraduate, postgraduate students and visiting workers in key laboratory techniques and use of equipment.

8.4. From April 2010 until February 2014 – University of Leicester

The team from PNEUMOPEP (previous work – please see below) was awarded funding through the Seeding Drug Discovery initiative to identify new small molecules and through a programme of medicinal chemistry, combined with *in vitro* and *in vivo* testing, to identify lead compounds with appropriate efficacy, pharmacokinetics and toxicology. I worked on this project, called SDDI, as a Research Technician, under the supervision of Dr Rana Lonnen and Professor Peter Andrew. The aim was to give such molecules would reduce the number of patients that die or suffer handicap as a result.

8.4.1. Main activities and responsibilities

- Work in a team with one project manager, two Postdoctorals and another research technician.
- Regular production of writing and verbal reports for discussion with project manager.
- Continuous application of standard operating procedures (SOP) and assays, with deadlines, maintaining meticulously accurate records, according to Good Laboratory Practice (GLP).
- Maintenance of a clean and organised laboratory.
- Data statistical analysis and evaluation using Windows 7 and GraphPad Prism 5.
- Manage the level of stocks and assisting in purchasing chemicals, reagents and laboratory equipment.
- Labelling of chemicals, solutions and reagents to laboratory use, also batch data recording for traceability according with Good Laboratory Practice (GLP).
- Monitoring of animal model of infectious disease and post-mortem tissue collection and examination.
- Experience with bacterial pathogen *S. pneumoniae* and *E.coli*.
- Manage technical records.

8.4.2. Practical skills gained during this work

- Operating a range of lab equipment varying from microscopes, spectrophotometer, nanodrop, centrifuges, varioscan and flow cytometric machine (BD FACScalibur).
- Application of COSHH and BIOCOSHH.
- Experience in Good Laboratory Practice (GLP).

- Experience in a microbiology laboratory in the area of drug discovery, working with small molecules.
- Monitoring of animal model of infectious disease and post-mortem tissue collection and examination.
- Work under pressure and at flexible hours.
- Teaching skills, through instructing news laboratory members about the methods and techniques.

8.5. From November 2008 until March 2010 – University of Leicester

I worked as a Research Technician in the Commercial Amoebae Laboratory at University of Leicester, under the supervision of Dr James Lonnen. This lab main activities were the study of disinfectant and drug efficacy testing against microorganisms, in particular the testing of contact lens disinfecting solutions against bacteria, fungi, virus and *Acanthamoeba* according to ISO 14729. Also, the isolation and identification of pathogenic free-living amoeba and bacteria from water, environmental and clinical samples.

8.5.1. Main activities and responsibilities

- Conduct contact lens disinfectant evaluation (biocidal and regimen) assays in accordance with ISO/CD14729 against bacteria, fungi, virus and *Acanthamoeba*.
- Work in a team with the Lab Director, a Postdoctoral RA, two PhD students and another Research Technician.
- Regular production of written and verbal reports for discussion with the Lab Director to ensure that deadlines for studies funded by commercial clients were met.
- Perform assays for clients according to Good Laboratory Practice (GLP). Those assays followed strict Standard Operating Procedures (SOP´s).
- Maintenance of a clean and organised laboratory.
- Data statistical analysis and evaluation using SPSS, Excel and Graphpad.
- Manage level of stocks and assisted in purchasing chemicals, reagents and laboratory equipment. Prepare chemicals, solutions and reagents for laboratory use, according to standard protocols, also batch data record for traceability according to Good Laboratory Practice (GLP).

8.5.2. Practical skills gained during this work

- Operate and maintain a range of lab equipment including microscopes, centrifuges, PCR machines and an automated spiral plater.
- Application of COSHH and BIOCOSHH forms.
- Work to Good Laboratory Practice (GLP).
- Handling pathogenic microorganisms including bacteria, fungi, virus and protozoa.
- Experience of tissue culture using aseptic technique.
- Performing assays according to International Organization for Standardization (ISO) principles.

8.6. From November 2007 until November 2008 - University of Leicester

I worked as a Research Technician in the PNEUMOPEP project under the supervision of Dr Rana Lonnen and Professor Peter Andrew. This project was sponsored by European Comission – CORDIS (Community Research and Development Information Service). Undertaken in response to the need to find new methods of treatment of disease due to *Streptococcus pneumoniae*. This bacterium is a major cause of community-acquired pneumonia, meningitis, bacteraemia and otitis media and it exhibits high rates of multi-drug resistance in countries worldwide. More than ever before modern drug discovery is dependent on high-throughput screening. Therefore, the drug discovery process is shifting focus from identifying suitable candidate drugs - which remains an essential but time-consuming goal - to identifying suitable lead compounds in order to maximise the cost-effectiveness and speed of the subsequent lead optimisation process. The PNEUMOPEP project achieved its overall aim of finding lead compounds for treatment of pneumococcal diseases. Six molecules inhibited pneumolysin and were effective in vivo and one was found that acted against pneumococcal neuraminidase A.

8.6.1. Main activities and responsibilities

- Support and assist with the provision of an efficient histology service, working closely with senior academic and technical staff.
- Conduct *in vitro* efficacy assays with small molecules using hemolytic assays.
- Assist in performing *in vivo* efficacy assays for small molecules using murine models of bacteraemia and pneumonia.

- Maintain aerobic and anaerobic microbial cultures, as well as collecting, processing and analyzing data with various software programs.
- Conduct antimicrobial assays using small molecules by performing *in vitro* minimal inhibition concentration (MIC) assays.
- Discs susceptive assays impregnated in small molecules across a range of anaerobic and aerobic microorganisms.
- Laboratory maintenance responsibilities included: chemical inventory, preparing solutions and reagents for laboratory use according to defined protocols, safely disposing of waste material, maintaining a clean, safe work environment, and assisting in purchasing.

8.6.2. Practical skills gained during this work

-Experience in a microbiology laboratory in the area of drug discovery, working with small molecules.
- Monitoring of animal models of infectious disease and post-mortem tissue collection and examination.
- Work under pressure at unsociable hours.
- Experience with anaerobic (ex: *Streptococcus pneumoniae*) and aerobic (*ex: Pseudomonas aeruginosa*) bacterial pathogenic strains using aseptic technique.

8.7. From April 2006 until August 2007 – Jardim Vista S.A.

Jardim Vista S.A. is a Portugal leading company on the landscaping field. With a very high reputation, achieving top quality designs, construction and maintenance of gardens.

8.7.1. Main activities and responsibilities

- Control of pests and plant diseases.
- Quality control of garden plants.
- Negotiation and direct contact with European suppliers.
- Manage and coordinate teams for the collection and distribution of plants.
- Recruitment and supervision of both permanent and seasonal temporary staff.
- Coordination of irrigation and fertilization, supplies or other inputs according to the activities.
- Manage work on a number of projects simultaneously to meet deadlines.

8.7.2. Practical skills gained during this work

- Irrigation and fertilization knowledge.
- Teamwork and interpersonal skills.
- Time management and organization skills.
- Detection and identification of diseases and pests on ornamental plants.

9. Publications

9.1. Patent applications

Due to the commercial nature of the SDDI project, restrictions on the timing of publications were imposed by the Intellectual Property Management Group including the patent attorney, to avoid disclosure of commercially sensitive data. All my research contributions were written up into seven patent applications that were filed on 2011.

9.2. Published articles

A1. Chen Yixiang **Sandra Germano**, Chris Clements, Jesvin Samuel, Ghalia Shelmani, Sandrine Jayne, Martin J. S. Dyer and Salvador Macip. **2016**. Pro-survival signal inhibition by CDK inhibitor dinaciclib in Chronic Lymphocytic Leukaemia. British Journal Haematology. 175(4):641-651.

This work was based on inhibitor called Dinaciclib that is a cyclin-dependent kinase inhibitor with clinical potential in different cancers, including chronic lymphocytic leukaemia (CLL). In order to better understand its cytotoxic action, we characterized its effects on signalling pathways important for the survival of CLL cells. We found that dinaciclib induced apoptosis through the activation of caspases 8 and 9, which was independent of the presence of cytokines to mimic the environment of proliferation centres or IGVH mutation status. Moreover, treatment with dinaciclib led to the inhibition of oncogenic pathways normally activated in stimulated CLL cells, such as STAT3, NF-κB, p38, PI3K/AKT and RAF/MEK/ERK. Dinaciclib was also able to block the expression of anti-apoptotic proteins of the BCL2 family such as MCL1 and BCL-xL (also termed BCL2L1). Finally, we showed that low concentrations of dinaciclib enhanced cell sensitivity to ibrutinib and the BCL2 inhibitor ABT-199, two drugs with known effects on CLL. Taken together, our data show that dinaciclib targets multiple pro-

survival signalling pathways in CLL, which provides a mechanistic explanation for its potent induction of apoptosis. They also support a therapeutic application of cyclin-dependent kinase inhibitors in CLL in combination with other relevant targeted therapies.

A2. Yixiang Chen, **Sandra Germano**, Ghalia Shelmani, Diana Kluczna, Sandrine Jayne ,Martin J. S. Dyer and Salvador Macip. **2017**. Paradoxical activation of alternative pro-survival pathways determines resistance to MEK inhibitors in chronic lymphocytic leukaemia. British Journal Haematology. In press.

In this work we explored novel therapeutic opportunities for B-cell malignancies, we evaluated the effects of MEK inhibitors (MEKi) on chronic lymphocytic leukaemia (CLL). We found that none had a significant effect on CLL viability, despite inhibiting signaling. Paradoxically, MEKi promoted the accumulation of active MEK and CRAF, reduced the expression of negative regulators of the pathway and augmented AKT signaling. Combining MEKi and phosphoinositide 3-kinase (PI3K) inhibitors antagonized this effect and induced cell death. We propose that MEKi-mediated activation of pro-survival pathways explains their low toxicity in CLL and that inhibition of both RAF/MEK/ERK and PI3K/ AKT signaling could overcome single-agent resistance.

9.3. Submitted articles

SA1. Diana Kluczna, Ghalia Shelmani, Yixiang Chen, Mireia Casulleras, Laura Castilla, Jason Rodencal, **Sandra Germano**, Sandrine Jayne, Ben Kennedy, Simon Wagner, Costas Balotis, Martin J. S. Dyer and Salvador Macip. **2017**. **Submitted to**: British Journal Haematology.

This article focus is about NOTCH1 mutations poor prognostic factor for Chronic Lymphocytic Leukaemia (CLL), associated with resistance to rituximab and increased risk of Richter's transformation. To characterize the role of *NOTCH1* mutation in drug sensitivity, we first genotyped exon 34 (including the 3'-UTR) in 147 CLL patients. We found mutations in 13/106 (12.3%) of a random cohort and 13/41 (26.8%) of patients with trisomy 12. The most common mutation, seen in 92.3%, was the CT deletion in the PEST domain. We also found two previously unreported frame-shift mutations (a 38bp deletion and a nucleotide insertion) in the transactivation domain, resulting in loss of the PEST domain. We next treated *NOTCH1* mutated and wild type patient cells with different targeted inhibitors. No differences were observed with most of the drugs tested. However, a subgroup of *NOTCH1* mutated patients

showed increased resistance to BCL2 inhibitor venetoclax. To extend our study, we compared Rec-1, a malignant B cell line that harbours a *NOTCH1* mutation, with wild-type cell lines JVM3 and Mec-1. We found that Rec-1 were more sensitive to inhibition of NF-κB and γ-secretase. Moreover, combination of ibrutinib with either γ-secretase or BCL2 inhibitors showed synergy in the mutated cells. Our results show that NOTCH1 mutation in CLL defines a subgroup of disease with specific sensitivities to combinations of targeted agents that may allow new therapeutic approaches.

10. Conferences and seminars

- 5th of September 2012: Society for General Microbiology (SGM). Autumn Conference at University of Warwick.
- 6th of November 2008: Quarterly meeting of the EU consortium, Oral presentation of my work on Novel antimicrobials.
- 25th of May de 2005 – "Problemática dos Incêndios: o que nos reserva o futuro?" seminar at Universidade do Algarve.
- 19th of May 2004 –"Jornadas de Construções de Jardins e Ornamentais", at Universidade do Algarve.
- 12th of March 2004 – "1.º Encontro com a Terra "A Mobilização do Solo e a Erosão", in Tavira.
- 29th of March 2001 – "Seminário de Saúde Ambiental", at Universidade do Algarve by Associação Internacional de Estudantes de Agricultura.
- 19th and 20th of October 2000: "Agricultura Sustentável e Actividades Complementares" at Universidade do Algarve.

11. Other competences

11.1. Courses and Training

- Level 3 HACCP Training Course (2016).
- Holder of a personal Home Office License, Module 1-5, Animals (Scientific procedures), Act 1986 (2015).
- Good clinical practices Level 1 (2014).
- Certificado de competências pedagógicas (CCP) (2007).

- Commercial trade, in CEGOC (2006).

11.2. IT skills

Excellent Proficiency in Microsoft Office, GraphPad Prism and SPSS.

11.3. Languages

	Understanding				Speaking				Writing	
	Listening		Reading		Spoken interaction		Spoken production			
English	C1	Proficient user	C1	Proficient user	C1	Proficient user	C1	Proficient user	C1	Proficient user
Spanish	C2	Proficient user	C2	Proficient user	C2	Proficient user	C2	Proficient user	C2	Proficient user

12. Critical point of view about professional evolution

The degree in Engenharia Agronómica – Ramo Hortofruticultura at the Universidade do Algarve, where technical aptitudes where acquired were vital for professional performance of the functions mentioned in this report. Subsequently, the professional experience allowed the acquisition of technical knowledge essential to the proper performance of the functions executed during all these years. These knowledge acquisition also enabled a development of critical analysis of various subjects. There were also relevant capacities developed as team leader, the ability to solve problems in short periods of time and the ability to work as a team or independently with defined deadlines.

Working in Jardim Vista S.A. from 2006 until 2007 it helped developing skills in fertilization, chemical control and organization. In November 2007 I moved to the UK where I worked in University of Leicester for 9 years as Research Technician/Research Assistant in areas such as molecular biology and microbiology. I participated in several UK and European research projects, from those projects I contributed to several patent applications and articles. In September 2016, I moved back to Portugal where I worked as a Research Technician in Universidade do Algarve on the project “Cloning of fragments of the genes callose synthase and xyloglucan endotransglucosylase from *Citrus* x *clementina* hort. ex Tanaka “Fina” and *Citrus sinensis* (L.) Osbeck “Valencia late”. In April 2017 I joined Syngenta in Moncarapacho, as a Senior Grower.

I have a very diversified profile in science, considering that I worked on projects related with respiratory infection diseases, water quality and cancer, where I learn many different lab techniques, protocols and worked with a lot of different lab equipment.

From 2007 until 2016 my experience was based on science related to infectious diseases and oncology, even so all the subjects that I had lecturers at Universidade do Algarve help me to know and to better understand all the molecular biology techniques required for these jobs.

Thus, and in addition to the technical skills acquired, others were also relevant, among which can be broadly highlighted, the capacity of planning and organisation, in accordance with the resources available, and with the priorities and deadlines defined and reassessed in the face of unforeseen changes. Given the need to define priorities in order to meet the objectives stipulated and adequately manage the working time related to each task were also developed competences relevant to the level of the guidance for results. From the professional point of view various skills where developed, relevant knowledge, practical experience and sense of responsibility. It is also worth mentioning the experience gained during these years in statistical programs, namely GraphPad and SPSS, and in the preparation of technical reports and as coauthor of scientific articles. In the University of Leicester I had the opportunity of supervise students.

At the level of professional experience, some constraints on the evolution of professional experience can also be pointed out, which can be considered weak points or threats.

It can be pointed out as a weak point that I performed functions centred mainly in a very specific area, not covering other areas in the field of Agronomic Engineering.

It also adds as a weak point the lack of willingness to exercise tasks considered less priority but still with some importance in good professional performance. Even if the projects that I collaborate, at University of Leicester weren't related to agriculture, all the techniques learnt are used in plant biotechnology.

Since I finished my degree until today, all the experiences gained during that time gave me support and knowledge to hold now a position as a Senior Grower at Syngenta Flowers.

The master's degree in "Hortofruticultura" will allow me to continue my work, as well as to promote and deepen my knowledge in a specific way and at the same time help me to better execute my most recent functions.

Appendix

Pro-survival signal inhibition by CDK inhibitor dinaciclib in Chronic Lymphocytic Leukaemia

Yixiang Chen,[1,2] Sandra Germano,[1,2] Chris Clements,[1,2] Jesvin Samuel,[1,2] Ghalia Shelmani,[1,2] Sandrine Jayne,[2,3] Martin J. S. Dyer[2,3] and Salvador Macip[1,2]

[1]Mechanisms of Cancer and Ageing Laboratory, Department of Molecular and Cell Biology, University of Leicester, [2]Ernest and Helen Scott Haematological Research Institute, University of Leicester, and [3]Department of Cancer Studies, University of Leicester, Leicester, UK

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Summary

Dinaciclib is a cyclin-dependent kinase inhibitor with clinical potential in different cancers, including chronic lymphocytic leukaemia (CLL). In order to better understand its cytotoxic action, we characterized its effects on signalling pathways important for the survival of CLL cells. We found that dinaciclib induced apoptosis through the activation of caspases 8 and 9, which was independent of the presence of cytokines to mimic the environment of proliferation centres or IGVH mutation status. Moreover, treat-ment with dinaciclib led to the inhibition of oncogenic pathways normally activated in stimulated CLL cells, such as STAT3, NF-jB, p38, PI3K/AKT and RAF/MEK/ERK. Dinaciclib was also able to block the expression of anti-apoptotic proteins of the BCL2 family such as MCL1 and BCL-xL (also termed BCL2L1). Finally, we showed that low concentrations of dinaciclib enhanced cell sensitivity to ibrutinib and the BCL2 inhibitor ABT-199, two drugs with known effects on CLL. Taken together, our data show that dinaciclib targets multiple pro-survival signalling pathways in CLL, which provides a mechanistic explanation for its potent induction of apoptosis. They also support a therapeutic application of cyclin-dependent kinase inhibitors in CLL in combination with other relevant targeted therapies.

Keywords: dinaciclib, CDK inhibitors, chronic lymphocytic leukaemia,

Chronic lymphocytic leukaemia (CLL) is a B-cell malignancy characterized by a progressive accumulation of functionally incompetent B lymphocytes in the peripheral blood, lymph nodes and bone marrow (Chiorazzi et al, 2005; Mittal et al, 2014). Recent therapeutic strategies in CLL have significantly improved the survival of patients, although it remains incurable. Thus, efforts to develop effective treatments are still necessary (Dyer et al, 2013). Although CLL cells in peripheral blood are mostly non-dividing and easily targeted by many inhibitors, a small number of cells residing in the bone marrow and lymphoid nodes have higher chemoresistance and proliferative capacity (Meads et al, 2008). A variety of cytokines, including T lymphocyte-expressed CD40 ligand (CD40LG or CD154), BAFF (also termed TNFSF13B) and interleukin 4 (IL4), are produced within this microenvironment niche and, together with stromal cells, nurse-like cells and T cells, are thought to be the major cause of resistance CLL therapy (ten Hacken & Burger, 2014). To mimic this microenvironment in vitro, a number of models have been established, including stimulation with soluble CD154/IL4, and co-cultures with CD154-expressing fibroblasts together with IL4 (Willimott et al, 2007a). Under these conditions, the expression of anti-apoptotic members of the BCL2 fam-ily, particularly BCL-xL (also termed BCL2L1) and MCL1, are greatly increased, which contributes to the resistance to spontaneous- and drug-induced apoptosis in CLL (Willimott et al, 2007a,b; Vogler et al, 2009).

B-cell receptor (BCR) signalling can be induced by ligand-independent stimulation and/or ligation of auto-antigens present in the microenvironment. Once engaged, it activates pro-survival signals such as the RAF/mitogen-activated protein kinase-extracellular signal-regulated kinase/extracellular signal-regulated kinase) (RAF/MEK/ERK), phosphatidylinositol 3-kinase/a serine/threonine kinase (PI3K/AKT), p38 (also termed MAPK14), nuclear factor (NF)-jB and signal transducer and activator of transcription 3 (STAT3 pathways,

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which play a pathogenic role in CLL (Kawauchi et al, 2002; Ogasawara et al, 2003; Pickering et al, 2007; Ringshausen et al, 2002; Hazan-Halevy et al, 2010; Cunı et al, 2004; Sainz-Perez et al, 2006; Rozovski et al, 2014). Nearly half of CLL cases have high levels of constitutively phosphorylated ERK1/2 (Muzio et al, 2008) and in freshly isolated CLL cells, phosphorylated p38 is predominately activated (Sainz-Perez et al, 2006). PI3K is also frequently activated in CLL cells and its inhibition induces apoptosis in some models (Cunı et al, 2004). PI3K/AKT activates NF-jB and the loss of AKT and NF-jB is also associated with apoptosis in these cells (Cunı et al, 2004). NF-jB RelA/p65 activation correlates chemoresistance and poorer clinical outcome in CLL (Fur-man et al, 2000; Cunı et al, 2004; Hewamana et al, 2008). Moreover, NF-jB activation can be regulated by STAT3 (Liu et al, 2011), which is constitutively phosphorylated at serine 727 in CLL cells (Hazan-Halevy et al, 2010; Rozovski et al, 2014).

Disruption of activated pro-survival signals such as these has become a field of major interest in the development of antineoplastic drugs, and many small molecular kinase inhibitors that selectively target them have already been developed (Dyer et al, 2013). Finding treatments that target multiple signalling pathways simultaneously in diseases such as CLL is important to avoid recurrences and eventually provide a curative strategy (Dyer et al, 2013).

Cyclin-dependent kinases (CDKs) are essential in the regulation of cell division, cell-cycle progression and gene transcription, and therefore deregulated in many cancers (Malumbres & Barbacid, 2009). Their inhibition represents a promising therapeutic approach in blood malignancies (Blachly et al, 2016). Although initial clinical studies with the CDK inhibitor, Alvocid, showed high toxicity due to hyperacute tumour lysis, there have been successful trials with subsequent inhibitors (Blachly et al, 2016). Dinaciclib (MK-7965, formerly SCH727965), a novel, selective CDK1-4/7/9 inhibitor with improved therapeutic index, was found to induce apoptosis in CLL cells (Johnson et al, 2012). It has already been tested in initial trials for solid tumours (Nemu-naitis et al, 2013), myeloma (Kumar et al, 2015) and refrac-tory CLL (Flynn et al, 2015). Encouraging Phase I and II results provide a rationale for the use of dinaciclib alone or in combination with immunotherapies in CLL and lym-phoma (Blachly et al, 2016). However, the molecular events that contribute to dinaciclib-induced apoptosis of malignant B cells are not fully understood.

Here, we studied the effects of dinaciclib on the pro-survival signals present in CLL to better understand the mechanisms involved defining its therapeutic potential. We show that dinaciclib simultaneously targets several pro-survival signalling pathways and suppresses expression of anti-apoptotic proteins to induce a caspase-dependent cell death. We also present evidence that supports the hypothesis that dinaciclib could be of therapeutic use in CLL, alone or in combination with other targeted drugs.

Methods

Cell culture and reagents

MEC-1, ESKOL and JVM3 cell lines were cultured in complete medium (RPMI-1640 medium with 10% fetal bovine serum, 2 mmol/l L-glutamine, 50 u/ml penicillin and 50 mg/ml streptomycin) at 37°C in incubator containing 5% CO_2. Peripheral blood samples were obtained from CLL patients attending clinics at the Leicester Royal Infirmary (Leicester, UK) following informed consent and approval from the local Research Ethics Committee and in accordance with the Declaration of Helsinki. All patients, diagnosed according to the International Workshop on Chronic Lymphocytic Leuksemia 2008 guidelines (Hallek et al, 2008), had been treatment-free for at least 6 months and had a white blood cell count >50 9 10^9/l. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density centrifugation. Heparinized whole blood was diluted 1:1 with phosphate-buffered saline (PBS) and gently layered onto 15 ml Ficoll (Histopaque 1077, Sigma-Aldrich, Poole, UK) prior to centrifugation at 400 g for 30 min. The mononuclear cell layer was removed from the interphase, washed and resuspended in RPMI-1640 supplemented with 10 ng/ml IL4 and CD154 (CD40LG) to maintain cell survival in vitro. Alternatively, mouse fibroblast L cells (NTL) and NTL cells stably expressing CD154 (NTL/CD154) were used in co-culture experiments to support CLL cell survival/proliferation. NTL/CD154 cells were irradiated with 30 Gy and then co-cultured with CLL patient cells at a ratio of 1:25. The inhibitors dinaciclib (SCH727965), ibrutinib (PCI-32765), Entospletinib (GS-9973), Ver155008, BX-912, RAF265, Sorafenib, Vemurafenib (PXL4032), Trametinib (GSK1120212), MG-132, Fedratinib and ABT-199 used in this study were obtained from Selleckchem (Houston, TX, USA). Z-Vad-Fmk was bought from Sigma-Aldrich.

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay

The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to estimate cell viability and proliferation, according to the manufacturer's instructions. Briefly, 100 ll 0 25 9 10^6/ml cells from cell lines or 2 9 10^6/ml CLL cells were seeded in 96-well plates. Inhibitors were added at different concentrations for 48 h before 20 ll MTS was added to each well. After incuba-tion for 1–3 h at 37°C, the absorbance at 490 nm was recorded on a TECAN infinite F50 reader (Labtech Interna-tional, Uckfield, UK). Cell viability was calculated based on the reducing potential of the viable cells using a colourimet-ric reaction. Assays were performed in triplicate and repeated on at least two independent occasions and results were plot-ted and analysed using GraphPad Prism 6 (GraphPad Soft-ware Inc., San Diego, CA, USA).

Cell-cycle analysis

Cells were harvested, fixed with precooled 70% ethanol at 20°C overnight, and then stained with propidium iodide (PI; Sigma-Aldrich) diluted in PBS containing RNase A at 37°C for 30 min in the dark. Cell-cycle distribution was then determined using a FACS Canto II cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell death analysis

Cells were harvested and stained with Annexin V (Sigma-Aldrich) for 15 min at 4°C. Percentages of apoptotic cells (posi-tive for Annexin V) were determined by flow cytometry using a FACS Canto II cytometer (BD Biosciences) following the manufacturer's instructions. Alternatively, cell death was measured by staining the cells with PI for 30 min at 4°C and the percent-age of PI-positive cells (dead) determined by flow cytometry.

Western blotting

Total protein was prepared in radioimmunoprecipitation assay lysis buffer [10 mmol/l Tris pH 7 4, 150 mmol/l NaCl, 1% TritonX-100, 0 1% sodium deoxycholate, 0 1% sodium dodecylsulfate (SDS), 5 mmol/l EDTA] supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Proteins were separated with SDS-polyacrylamide gel electrophoresis and incubated with specific antibodies. Protein bands were visualized and quantified with an Odyssey system (Pierce, Waltham, MA, USA). The antibodies used were: ERK, phospho-ERK (Thr202/Tyr204), AKT, phosphor-AKT (Ser473), IjBa, phosphor-IjBa (Ser32/36), STAT3, phosphor-STAT3 (Tyr705), p38, phosphor-p38 (Thr180/Tyr182), PARP, caspase-8 and caspase-9 (Cell Signaling Technology, Danvers, MA, USA); BCL2 (Dako, Santa Clara, CA, USA); b-actin (Millipore); BCL-xL/S, MCL1 and NF-jB(p65) (Santa Cruz Biotechnology, Dallas, TX, USA). Fluorescent-conjugated secondary anti-rabbit or anti-mouse antibodies were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Results

CLL cells are highly sensitive to dinaciclib

In order to better understand the sensitivity of CLL cells to the CDK inhibitor dinaciclib, we compared its effects on the viability of MEC-1, an Epstein–Barr virus-positive B-cell line derived from a patient with CLL that is often used as a model for this disease (Stacchini et al, 1999), to that of different targeted inhibitors of pro-survival pathways often activated in leukaemia. We selected other drugs that have been studied in the context of leukaemia, such as MG-132 [a proteasome inhibitor (Guo & Peng, 2013)], ibrutinib [a BTK inhibitor (Byrd et al, 2013; Danilov, 2013)], vemurafenib [a $BRAF^{V600E}$ inhibitor (Samuel et al, 2014)], ver155008 [a HSP70 inhibitor (Reikvam et al, 2013)], BX-912 [a PDK1 inhibitor targeting the PI3K/AKT pathway (Feldman et al, 2005)] and fedratinib [a Jak/STAT pathway inhibitor (Pardanani et al, 2007)]. Dinaciclib demonstrated more than 100-fold greater inhibitory effects on the metabolic activity of MEC-1 than any of the other drugs tested (Fig 1A), confirming its potential as a therapeutic approach for CLL and consistent with recent results that showed the strong cytotoxic effects of CDK inhibitors in CLL (Johnson et al, 2012; Paiva et al, 2015; Sylvan et al, 2016). We determined that the effects of dinaciclib on cell viability were the consequence of a dose-dependent increase in cell death but not cell cycle arrest, as tested by PI staining (Fig 1B). Specifically, we found that this was due to the induction of apoptosis (Fig 1C). Cell death induction was progressive and reached a maximum after 48 h (Fig 1D). Of note, these figures show that a fraction of cells were resistant to even the highest concentration of dinaciclib tested, for rea-sons that will need to be elucidated. A similar response to these drugs was also observed in other malignant B-cell lines, such as ESKOL and JVM3 (Fig S1A).

We next confirmed these results in primary CLL cells (Table S1) cultured in the presence of CD154 and IL4 to mimic the protective microenvironment of proliferation centres. Dinaciclib was also able to reduce cell viability at low concentrations under these conditions (Fig 1E), showing a higher effect than the other inhibitors. This was also dose-dependent (Fig 1F), with a 50% inhibitory concentration below 5 nmol/l, and not determined by patient IGVH mutation status (Fig 1G). We could not find any clinical parameters that strongly determined a change in sensitivity to dinaciclib, except perhaps a higher ratio of deletions in more resistant cells (Table S2). Similar results were also observed in the primary CLL patient cells co-cultured with mouse fibroblast L (NTL) cells stably expressing CD154 and supplemented with IL4, an alternative stimulation system (Fig S1B). Taken together, these data support the hypothesis that dinaciclib is highly toxic for malignant B cells at sub-micromolar concentrations, with effects stronger than any of the inhibitors of pro-survival signals tested, and that this is due to the induction of apoptosis. Also, we showed that dinaciclib can strongly affect cell viability even in different culture conditions that mimic protective microenviron-ments, which underscores its potential to target resistant CLL cells.

Dinaciclib-induced toxicity in CLL is dependent on caspase activation

We further explored the apoptotic effects of dinaciclib on CLL cells by analysing the activation of the caspase pathway. Our data showed that dinaciclib can activate caspase 8 and caspase 9, and induce PARP cleavage in CLL patient cells (Fig 2A), which indicates induction of apoptosis with involvement of both the intrinsic (mediated by caspase 9)

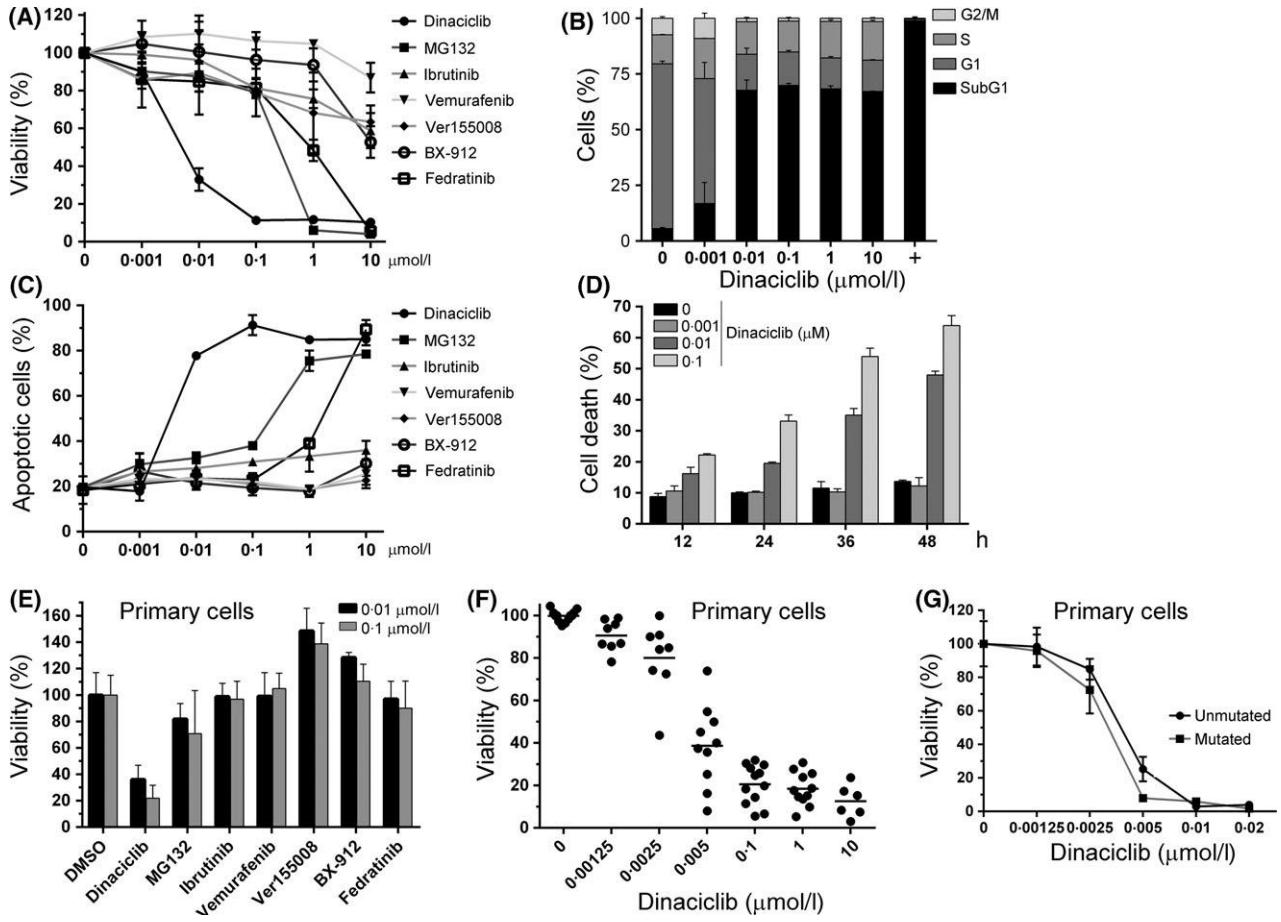

Fig 1. MEC-1 cells are highly sensitive to dinaciclib. (A) MEC-1 cell viability measured by MTS assay. Cells were treated with various small molecule inhibitors (0 001 to 10 lmol/l) or dimethylsulfoxide (0 lmol/l) for 48 h. Experiments were performed in triplicate and repeated at least two times. Graphics show mean values and error bars represent standard deviation, as in all the other panels of this figure. (B) Percentage of cells in each phase of the cell cycle, as measured by propidium iodide (PI) staining of MEC-1 cells treated with dinaciclib for 72 h. +: positive control (0 1% Triton X). Experiments were performed in triplicate and repeated on at least two independent occasions. (C) Induction of apoptosis in MEC-1, as measured by FACS analysis of Annexin V-stained cells. Cells were treated with inhibitors for 72 h. Graphics show percentage of apop-totic cells (Annexin positive). (D) Time-course analysis the induction of apoptosis in MEC-1 cells treated with different concentrations of dinaci-clib, as measured by PI staining. Experiments were performed in triplicate and data were expressed as mean standard deviation in all the panels of this figure. (E) CLL patient cell viability measured by an MTS assay. Cells were cultured for 24 h in the presence of 10 ng/ml IL4 and CD154 and then treated with 0 01 and 0 1 lmol/l of different inhibitors for 48 h. Between 3 and 10 samples were tested for each inhibitor, and experiments were performed in triplicate. Graphics show mean values and error bars represent standard deviation. (F) Primary cells (from Patients 2, 5, 8, 9, 10, 14, 16, 20, 26, 27, 28, 31 and 32 in Table S1) were cultured as above and treated with dinaciclib at various concentrations for 48 h. (G) Viability of primary patient cells treated with different concentrations of dinaciclib for 48 h, as measured by MTS assay. Patient 9 (IGVH mutated) and Patient 32 (unmutated) are shown as representative cases. Experiments were performed in triplicate and repeated on at least two independent occasions. Data are expressed as mean standard deviation.

and extrinsic (mediated by caspase 8) pathways (Parrish et al, 2013). We next treated CLL cells with the pan-caspase inhibitor Z-Vad-Fmk before exposing them to dinaciclib. The presence of the caspase inhibitor partially reduced the PARP cleavage and apoptosis induced by dinaciclib (Fig 2B), suggesting that the cytotoxicity of dinaciclib in CLL is, at least, partly dependent on the activation of the caspase cascade.

Dinaciclib inhibits pro-survival signals in CLL cells

To further investigate the molecular events that determine the effect of dinaciclib on CLL cell survival, we measured the activation status of multiple pro-survival oncogenic signalling pathways that have been reported to be upregulated in B cell malignancies, such as STAT3, NF-jB, p38, PI3K/AKT and RAF/MEK/ERK (Kawauchi et al, 2002; Ringshausen et al, 2002; Ogasawara et al, 2003; Cunı et al, 2004; Sainz-Perez et al, 2006; Pickering et al, 2007; Hazan-Halevy et al, 2010; Rozovski et al, 2014). The phosphorylation level of STAT3 (Tyr705), IjBa (Ser32/36), p38 (Thr180/Tyr182), AKT (Ser473) and ERK (Thr202/Tyr204) were assessed as markers of activation of these pathways in MEC-1 cells. As shown in Fig 3A (0 1 lmol/l dinaciclib) and Fig S2A (0 01 lmol/l dinaciclib), in treated cells there was a marked decrease in

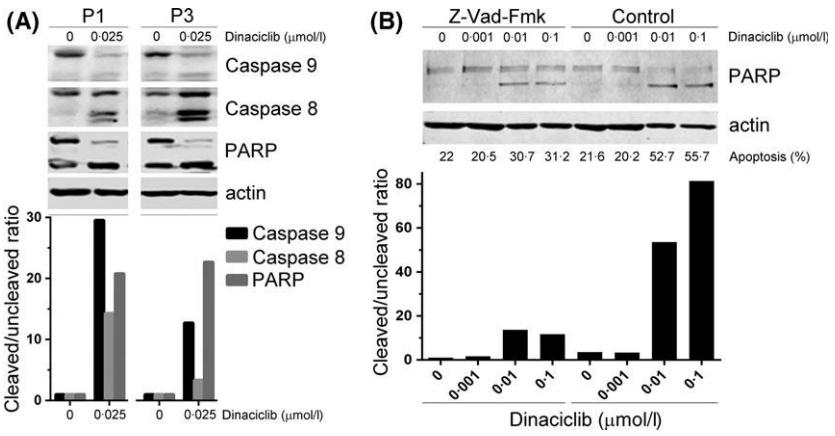

Fig 2. Cell death induced by Dinaciclib in primary cells is dependent on caspase cleavage. (A) Western blot showing caspase 9, caspase 8 and PARP cleavage in CLL primary cells (P1 and P3). Cells were cultured as above and treated with dinaciclib for 16 h. b-actin served as a loading control. The graph shows quantitation of these two blots, expressed as the ratio of cleaved to uncleaved proteins, normalized to b-actin. (B) Patient cells (P22) were cultured as above and treated with 25 lmol/l caspase inhibitor Z-Vad-Fmk for 1 h before being treated with different concentrations of dinaciclib for 16 h. Numbers show percentage of apoptotic cells in the same experiment, as measured by Annexin staining.

the phosphorylation of STAT3 and IjBa, together with a more gradual decrease in the phosphorylation of p38. On the other hand, AKT and ERK phosphorylation started to decline after 8 h, following an initial increase. A substantial reduc-tion in all these signals was evident after 12 h of treatment (Fig 3B; Fig S2B). Of note, the levels of phosphorylated pro-teins did not change in untreated cells cultured for the same time, showing that the inhibition of the signalling pathways was indeed due to the effects of dinaciclib and not an arte-fact determined by spontaneous protein degradation (see Fig-ure S2B). Moreover, these changes in the signalling pathways occurred before PARP cleavage and the induction of apopto-sis, which started after 12 h of exposure to dinaciclib (see Fig 3A and Fig S2A), suggesting they are an early event in the cellular response to the drug.

Next, we confirmed these results in primary CLL cells. We found that the phosphorylation of STAT3, IjBa and p38 decreased after 12 h exposure to dinaciclib in cells cultured in the presence of CD154/IL4 (Fig 3C). Moreover, we observed that P-ERK, P-AKT (Fig 3D) and NF-jB (Fig 3E) were also inhibited by dinaciclib in primary cells. Of note, although a similar response was observed in ESKOL cells, JVM3 cells showed reduction in the phosphorylation of AKT

but not in that of ERK, perhaps due to an over-activation of this pathway driven by the $BRAF^{K601N}$ mutation present in these cells (Fig S2C).

We also observed that the levels of MCL1, an anti-apopto-tic protein of the BCL2 family, quickly decreased in MEC-1 after treatment with dinaciclib in a time and concentration dependent manner, which correlated to PARP cleavage and induction of apoptosis (see Fig 3A and Fig S2A). This is con-sistent with recent reports showing that MCL1 expression was decreased by dinaciclib in various cancer cells (Fu et al, 2011; Chen et al, 2015; Gregory et al, 2015; Varadarajan et al, 2015). We confirmed that dinaciclib inhibited both MCL1 and BCL-xL in stimulated primary cells and MEC-1 (Fig 3F). However, its effects on BCL2 were patient-depen-dent (Fig 3G). As the levels of BCL2-related proteins, such as MCL1, BCL-xL and BCL2, have been linked to the resistance of malignant B cells to apoptosis (Kitada et al, 1998; Pepper et al, 2008), this inhibition could contribute to the cytotoxic-ity of dinaciclib. Taken together, our data shows that dinaci-clib has an inhibitory effect on several oncogenic signalling pathways usually activated in malignant B-cells, which pro-vides a mechanism to explain its potent effects in these cells.

Positive effects of dinaciclib in combination therapies

Despite their strong apoptotic effects on malignant B cells, the efficacy of CDK inhibitors as monotherapy has not been as successful as expected (Bose et al, 2013). Dinaciclib has only a 54% overall response rate in relapsed/refractory CLL patients and a tumour lysis syndrome is sometimes induced (Flynn et al, 2015). This suggests that combination with other targeted therapies could improve its clinical impact. To explore this possibility, we assessed the combination of dinaciclib with low concentrations of other specific inhibitors relevant to B cell malignancies, such as ABT-199 (BCL2 inhi-bitor), iburitinib (BTK inhibitor) and entosplentinib (SYK inhibitor). The effects of ibrutinib and ABT-199 on MEC-1 survival were increased by combination with nanomolar con-centrations of dinaciclib, while there was no variation with entospletinib (Fig 4A). The greatest effect was seen with ABT-199, which could trigger only low levels of cell death (~20%) when used alone, induced over 80% apoptosis when combined with 0 01 lmol/l dinaciclib. This is consistent with previous reports showing that CDK inhibitors sensitize CLL cells to BH3 mimetics, such as ABT-737 (Paiva et al, 2015). For ibrutinb, the increase in cell death was from ~50 to ~90%. A similar response was observed in stimulated

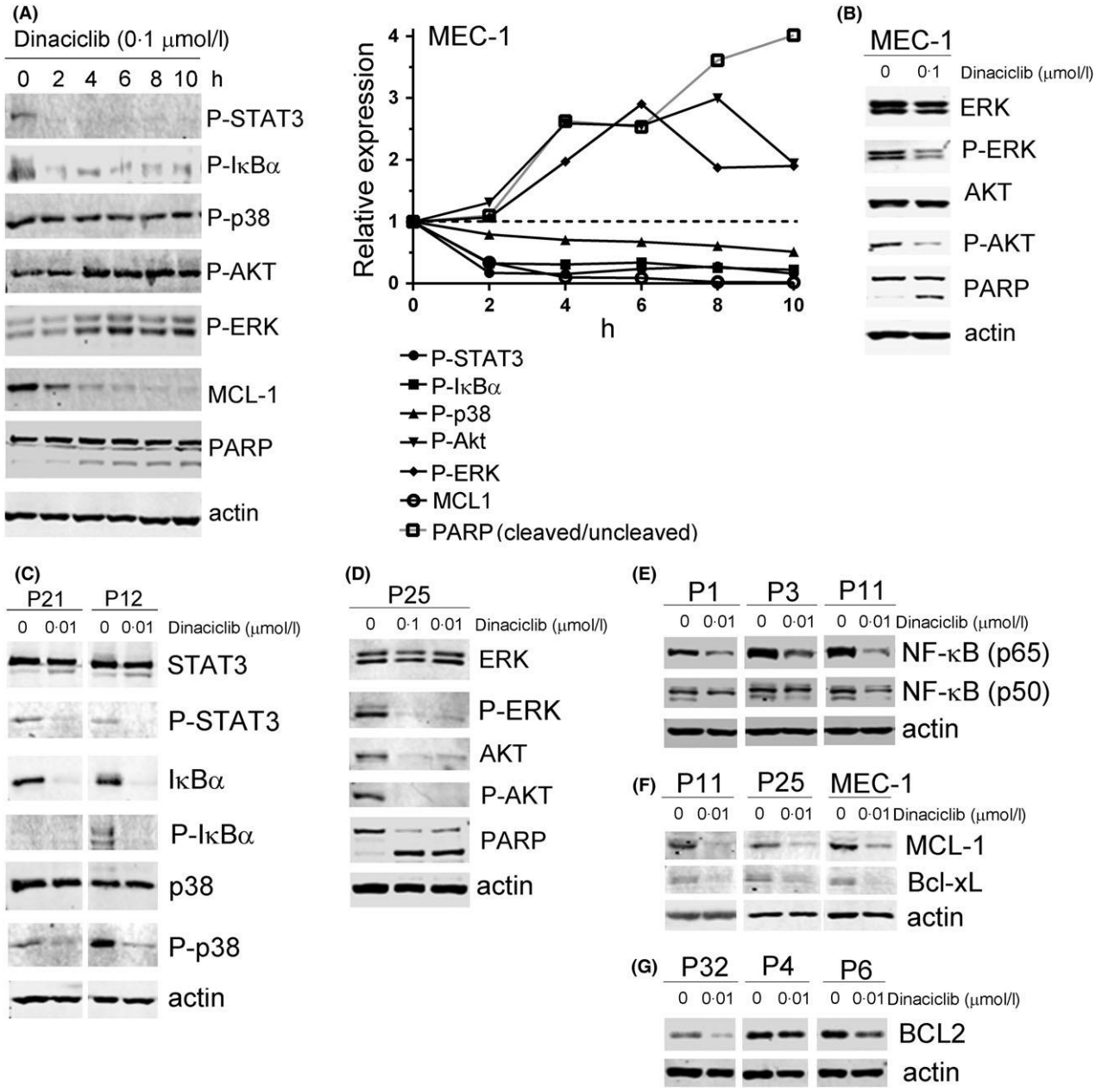

Fig 3. Dinaciclib inhibits pro-survival signals in CLL cells. (A) Western blot performed on lysates of MEC-1 cells treated with dinaciclib at 0 1 lmol/l concentration and collected at 2, 4, 6, 8 and 10 h after treatment. Levels of activation of signalling pathways was measured by the phosphorylation of Erk (Thr202/Tyr204), Akt (Ser473), IjBa (Ser32/36), STAT3 (Tyr705) and p38 (Thr180/Tyr182). MCL1 (MCL-1) and PARP levels were also measured. Right-panel graph shows quantitation the Western blot bands normalized to b-actin and expressed as relative to con-trol (0 h) treatment. (B) Representative Western blot of MEC-1 cells treated with dinaciclib for 12 h. Levels of total and phosphorylate Erk and Akt were measured, as well as PARP cleavage. (C) Representative Western blot of patient CLL cells (P21 and P12) cultured as described and trea-ted with dinaciclib for 12 h. Levels of total and phosphorylated STAT3, IjBa and p38 were measured. (D) Same as above, measuring the levels of P-ERK, ERK, P-AKT and AKT, as well as PARP cleavage. (E) Same as above, showing levels of NF-jB (p65 and p50). (F) Same as above, showing levels of MCL1 and Bcl-xL in CLL patient cells and MEC-1 treated with dinaciclib. (G) Same as above, showing levels of BCL2.

primary cells (Fig 4B). The increase in apoptosis induced in this case by ibrutinib was from ~60 to ~90%, and by ABT-199 from ~80 to ~90%. These results suggest that nanomolar concentrations of dinaciclib could sensitize CLL cells to ibrutinib or BCL2 inhibitors, potentially enhancing their effects and reducing chemoresistance.

Discussion

Despite having been developed many years ago, CDK inhibitors have only recently been considered for antineoplastic therapies. First-generation CDK inhibitors, such as flavopiridol (PHA-793887) and roscovitine (CYC202), had a narrow

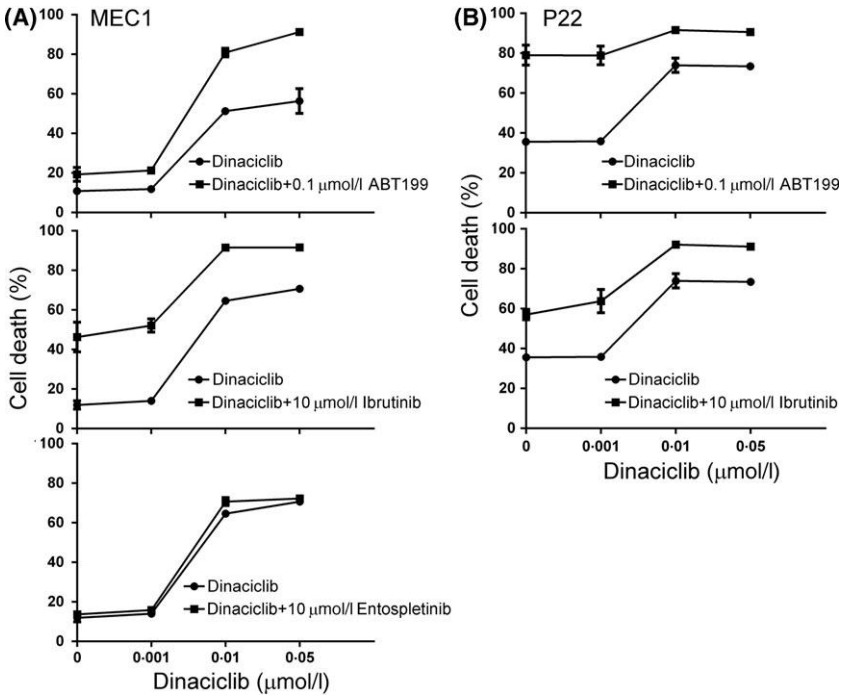

Fig 4. Dinaciclib enhances sensitivity of CLL cells to ibrutinib and ABT-199. (A) Percentage of apoptotic MEC-1 cell in cells treated with different concentrations of dinaciclib for 48 h, either alone or in combination with 0 1 lmol/l ABT-199, 10 lmol/l ibrutinib or 10 lmol/l entospletinib, as measured by propidium iodide staining. Graphs show average of three independent experiments. (B) Same for combinations of dinaciclib with 0 1 lmol/l ABT-199 and 10 lmol/l ibrutinib on CLL patient cells (P22) cultured for 24 h in the presence of 10 ng/ml IL4 and CD154. Graphs show average of two independent experiments.

therapeutic window and low selectivity (Blachly & Byrd, 2013; Blachly et al, 2016) Dinaciclib was designed to maximize the therapeutic index and minimize the toxicity associated with this drug type. It specifically shows a strong and selective inhibition of CDK1, CDK2, CDK5 and CDK9 and was shown to induce death in various cancer cells (Parry et al, 2010; Paruch et al, 2010). Dinaciclib has already been evaluated in clinical trials and results show that it has encouraging activity in relapsed/refractory CLL (Fabre et al, 2014; Flynn et al, 2015).

In this study, we explored the effects of dinaciclib on pro-survival signalling pathways in CLL cells. Our data confirmed that malignant B cells have a high sensitivity to dinaciclib, even in the presence of stimulatory cytokines that recapitu-late the protective features of the microenvironment in proliferation centres. Reaching these cells is necessary for a curative effect in CLL, because they are less sensitive to therapies than circulating cells and may thus be the cause of resistant disease (Dyer et al, 2013). Importantly, the concentration of dinaciclib needed to exert an effect on B cell survival is several orders of magnitude below that of other small molecule inhibitors we tested. All this greatly supports the clinical relevance of dinaciclib as a treatment for CLL. However, our results also show a percentage of refractory cells (10–25%), even at concentrations above therapeutic limits (see Figs 1A–C), which suggest that dinaciclib may not be a monotherapy of choice.

It has been previously reported that CDK inhibitors promote apoptosis in different cancer cells via suppression of Rb phosphorylation (Parry et al, 2010; Fu et al, 2011) or in a JNK/p38-dependent manner (Paiva et al, 2015). Our data showed that this requires the activation of caspase 8 and 9, suggesting an involvement of both the extrinsic and intrinsic pathways. The use of patients in our experiments with TP53 mutations or deletions (see Table S1) indicates that dinaciclib-induced apoptosis can also be TP53-independent.

We show that treating CLL cells with dinaciclib has wide ranging effects and interferes with at least five major oncogenic pathways that are often hyperactivated in these cells: RAF/MEK/ERK, p38, PI3K/AKT, NF-jB and STAT3. Given the fact that these are well known pro-survival signals, it is reasonable to assume that their inhibition would greatly contribute to the induction of cell death. Moreover, these are early effects, observed before the activation of the apoptotic machinery and thus would play a role in the downstream events. It is likely that the strong effect of dinaciclib on CLL survival is partly mediated by the combined inhibition of two or more of these oncogenic pathways.

According to our results, this molecular response of CLL cells to dinaciclib follows two different phases. The early effects include an immediate inhibition of STAT3 and NF-jB signalling. In parallel, the ERK, p38 and AKT pathways show a more gradual inhibition. Eventually, all these signals are suppressed, and then apoptosis begins. This suggests that the first three pathways may be directly inhibited by dinaciclib, while the latter two may be an indirect or secondary response. Of note, dinaciclib could effectively induce cell death in JVM3 cells despite the fact ERK phosphorylation was not inhibited, suggesting that either this effect may be dispensable in triggering apoptosis or inhibiting other path-ways simultaneously is sufficient. The importance of sig-nalling pathways in cell responses to dinaciclib is likely to

vary in different CLL patients. For instance, not all patients have a high level ERK activation (Muzio et al, 2008) and AKT Serine 473 phosphorylation, a marker of its activation, was very low in unstimulated CLL cells (Ringshausen et al, 2002). These data support the view that the signalling networks in CLL cells are very diverse and complex and that drugs that can target more than one of these signals are likely to be effective at treating a greater number of patients.

It remains to be determined which of these pathways needs to be inhibited for dinaciclib to induce cells apoptosis and which inhibition may be dispensable. Our data suggests that induction of apoptosis is linked to an early inhibition of STAT3, NF-jB and p38 signalling, which is then followed by blocking of the PI3K/AKT and RAF/MEK/ERK pathways. NF-jB has been recently shown to be essential for CDK inhibitors to induce apoptosis in CLL cells (Cosimo et al, 2013). This is consistent with the fact that cytokines expressed in the microenvironment of proliferation centres induce NF-jB transcriptional activity to up-regulate chemokines, cell cycle regulators and anti-apoptotic proteins that further increase the resistance of CLL cells to apoptosis. Similarly, STAT3 regulates the expression of anti-apoptotic, pro-proliferative and immune response genes, therefore playing a role in the interaction between cancer cells and their microenvironment (Grivennikov & Karin, 2010). Although this supports their importance in the cellular response to dinaciclib, further experiments will be needed to confirm this.

Dinaciclib also has an inhibitory effect on anti-apoptotic proteins, which is likely to reinforce its induction of CLL cell death. Indeed, we observed that MCL1 and BCL-xL levels decreased upon dinaciclib treatment. Similar results were observed with other CDK inhibitors, such as Seliciclib/R-roscovitiine/CYC202 (Hallaert et al, 2007). However, the levels of BCL2 showed a variable response in primary cells. Specific inhibition of BCL2 greatly enhanced the effects of dinaciclib (see Fig 4), which suggests that the anti-apoptotic effects of BCL2 could provide cellular resistance to dinaciclib in certain patients. The downregulation of these and other proteins by dinaciclib, including AKT, IjBa and NF-jB, could be due to its ability to regulate transcription (Alvi et al, 2005; Natoni et al, 2011), particularly, the inhibition on CDK9, which tar-gets RNA polymerase II activity (Oelgeschl€ager, 2002). Thus, repression of transcription has been considered as a mechanism that explains how CDK inhibitors kill cancer cells (Alvi et al, 2005; Natoni et al, 2011).

Dinaciclib is being tested in clinical trials, alone or in combination with drugs like rituximab, especially in the context of relapsed or refractory CLL (Fabre et al, 2014; Flynn et al, 2015; Blachly et al, 2016). Our results suggest that dinaciclib is unlikely to be a useful monotherapy, due to its toxicity and the existence of a percentage of refractory cells, but it could be combined with other specific small molecular inhibitors, like ibrutinib or ABT-199, and be highly effective at nanomolar concentrations. Ibrutinib is an irreversible inhibitor of Bruton tyrosine kinase (BTK), which has shown robust clinical activity in CLL (Herman et al, 2011; Byrd et al, 2013) and has been approved by the US Food and Drug Administration (FDA) for the treatment of CLL. Resis-tance to ibrutinib monotherapy in CLL patients appears to be due to the mutation of cysteine to serine at position 481 of BTK (Woyach et al, 2014). According to our data, combi-nation with low concentrations of dinaciclib could potentiate the effects of ibrutinib and reduce chemoresistance. Similarly, ABT-199, a third-generation BCL2 inhibitor, has shown clinical potential in a wide variety of haematological malignancies, including CLL (Scarfo & Ghia, 2013; Souers et al, 2013; Pan et al, 2014). However, it selectively targets BCL2 but not MCL1. This may confer resistance to this drug, since MCL1 can compensate for the loss of BCL2 function (Moulding et al, 2000). Consistent with this, down-regulation of MCL1 increases the lethality of the BCL2 inhibitor ABT-737 in human leukaemia cells (Chen et al, 2007). We showed that the capacity of ABT-199 to induce apoptosis in CLL cells was improved by combining it with dinaciclib, perhaps mediated by its effects on MCL1. This supports the hypothe-sis that dinaciclib could sensitize CLL cells to BCL2 inhibi-tors and enhance its therapeutic effects.

In summary, our data proposes a mechanistic explanation for the effects of dinaciclib on CLL cell survival and support the idea that using CDK inhibitors in combination with other specific inhibitors could improve current treatments of CLL.

Acknowledgments

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Author contribution

YC and SM designed the experiments, analysed the data and prepared the manuscript, with contributions from MJSD, SJ and SG. YC performed the experiments, with help from SG, CC, JS, GS and SJ. All authors reviewed the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. (A) Same as Fig 1A, using Eskol and JVM3 cells. (B) Viability of unmutated and mutated CLL cells co-cultured with NTL/CD154/IL-4 for 48 h, before being treated with 0.1 or 0.01 μmol/l of different drugs for 48 h, as

measured by MTS assay. Patient cells used: P11, P4, P5, P7, P8, P15, P20, P23, P28, P29, P31, P32.

Fig S2. (A) Same as Fig 3A, using 0.01 lmol/l dinaciclib. (B) Western blot of MEC-1 cells treated with DMSO ( ) or 0.1 lmol/l dinaciclib (+) for the indicated hours, showing levels of different phosphorylated proteins. (C) Same as Fige 3B, using ESKOL and JVM3.

Table S1. Relevant clinical information for the primary CLL samples used. #: patient number used in the text; nd: not determined; U: unmutated; M: mutated.

Table S2. List of patient cells most sensitive and less sensitive to dinaciclib, according to the results shown in Fig 1F. Patient number, sex (M/F) and cytogenetics are included.

References

Alvi, A.J., Austen, B., Weston, V.J., Fegan, C., Maccallum, D., Gianella-Borradori, A., Lane, D.P., Hubank, M., Powell, J.E., Wei, W., Taylor, A.M., Moss, P.A. & Stankovic, T. (2005) A novel CDK inhibitor, CYC202 (R-roscovitine), overcomes the defect in p53-dependent apoptosis in B-CLL by down-regulation of genes involved in transcription regulation and survival. Blood, 105, 4484–4491.

Blachly, J.S. & Byrd, J.C. (2013) Emerging drug profile: cyclin-dependent kinase inhibitors. Leukaemia & Lymphoma, 54, 2133–2143.

Blachly, J.S., Byrd, J.C. & Grever, M. (2016) Cyclin-dependent kinase inhibitors for the treatment of chronic lymphocytic leukemia. Seminars in Oncology, 43, 265–273.

Bose, P., Simmons, G.L. & Grant, S. (2013) Cyclin-dependent kinase inhibitor therapy for hematologic malignancies. Expert Opinion on Investigational Drugs, 22, 723–738.

Byrd, J.C., Furman, R.R., Coutre, S.E., Flinn, I.W., Burger, J.A., Blum, K.A., Grant, B., Sharman, J.P., Coleman, M., Wierda, W.G., Jones, J.A., Zhao, W., Heerema, N.A., Johnson, A.J., Suk-buntherng, J., Chang, B.Y., Clow, F., Hedrick, E., Buggy, J.J., James, D.F. & O'Brien, S. (2013) Targeting BTK with Ibrutinib in relapsed chronic lymphocytic leukemia. New England Journal of Medicine, 369, 32–42.

Chen, S., Dai, Y., Harada, H., Dent, P. & Grant, S. (2007) Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. Cancer Research, 67, 782–791.

Chen, X.X., Xie, F.F., Zhu, X.J., Lin, F., Pan, S.S., Gong, L.H., Qiu, J.G., Zhang, W.J., Jiang, Q.W., Mei, X.L., Xue, Y.Q., Qin, W.M., Shi, Z. & Yan, X.J. (2015) Cyclin-dependent kinase inhibitor dinaciclib potently synergizes with cisplatin in preclinical models of ovarian cancer. Oncotarget, 6, 14926–14939.

Chiorazzi, N., Rai, K.R. & Ferrarini, M. (2005) Chronic lymphocytic leukemia. New England Journal of Medicine, 352, 804–815.

Cosimo, E., McCaig, A.M., Carter-Brzezinski, L.J., Wheadon, H., Leach, M.T., le Ster, K., Berthou, C., Durieu, E., Oumata, N., Galons, H., Meijer, L. & Michie, A.M. (2013) Inhibition of NF-jB-mediated signaling by the cyclin-dependent kinase inhibitor CR8 overcomes prosurvival stimuli to induce apoptosis in chronic lymphocytic leukemia cells. Clinical Cancer Research, 19, 2393–2405.

Cunı, S., Perez-Aciego, P., Perez-Chacon, G., Vargas, J.A., Sanchez, A., Martın-Saavedra, F.M., Ballester, S., Garcıa-Marco, J., Jorda, J. & Durantez, A. (2004) A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia, 18, 1391–1400.

Danilov, A.V. (2013) Targeted therapy in chronic lymphocytic leukemia: past, present, and future. Clinical Therapeutics, 35, 1258–1270.

Dyer, M.J., Vogler, M., Samuel, J., Jayne, S., Wagner, S., Pritchard, C. & Macip, S. (2013) Precision medicines for B-cell leukaemias and lymphomas; progress and potential pitfalls. British Journal of Haematology, 160, 725–733.

Fabre, C., Gobbi, M., Ezzili, C., Zoubir, M., Sablin, M.P., Small, K., Im, E., Shinwari, N., Zhang, D., Zhou, H. & le Tourneau, C. (2014) Clinical study of the novel cyclin-dependent kinase inhi-bitor dinaciclib in combination with rituximab in relapsed/refractory chronic lymphocytic leukemia patients. Cancer Chemotherapy and Phar-macology, 74, 1057–1064.

Feldman, R.I., Wu, J.M., Polokoff, M.A., Kochanny, M.J., Dinter, H., Zhu, D., Biroc, S.L., Alicke, B., Bryant, J., Yuan, S., Buckman, B.O., Lentz, D., Ferrer, M., Whitlow, M., Adler, M., Finster, S., Chang, Z. & Arnaiz, D.O. (2005) Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. Journal of Biological Chemistry, 280, 19867–19874.

Flynn, J., Jones, J., Johnson, A.J., Andritsos, L., Maddocks, K., Jaglowski, S., Hessler, J., Grever, M.R., Im, E., Zhou, H., Zhu, Y., Zhang, D., Small, K., Bannerji, R. & Byrd, J.C. (2015) Dinaciclib is a novel cyclin-dependent kinase inhibitor with significant clinical activity in relapsed and refractory chronic lymphocytic leukemia. Leukemia, 29, 1524–1529.

Fu, W., Ma, L., Chu, B., Wang, X., Bui, M.M., Gemmer, J., Altiok, S. & Pledger, W.J. (2011) The cyclin-dependent kinase inhibitor SCH 727965 (dinacliclib) induces the apoptosis of osteosarcoma cells. Molecular Cancer Therapeu-tics, 10, 1018–1027.

Furman, R.R., Asgary, Z., Mascarenhas, J.O., Liou, H.C. & Schattner, E.J. (2000) Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. The Journal of Immunology, 164, 2200–2206.

Gregory, G.P., Hogg, S.J., Kats, L.M., Vidacs, E., Baker, A.J., Gilan, O., Lefebure, M., Martin, B.P., Dawson, M.A., Johnstone, R.W. & Shortt, J. (2015) CDK9 inhibition by dinaciclib potently suppresses Mcl-1 to induce durable apoptotic responses in aggressive MYC-driven B-cell lymphoma in vivo. Leukemia, 29, 1437–1441.

Grivennikov, S.I. & Karin, M. (2010) Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine & Growth Fac-tor Reviews, 21, 11–19.

Guo, N. & Peng, Z. (2013) MG132, a proteasome inhibitor, induces apoptosis in tumor cells. Asia-Pacific Journal of Clinical Oncology, 9, 6–11. ten Hacken, E. & Burger, J.A. (2014) Molecular pathways: targeting the microenvironment in chronic lymphocytic leukemia–focus on the B-cell receptor. Clinical Cancer Research, 20, 548–556.

Hallaert, D.Y., Spijker, R., Jak, M., Derks, I.A., Alves, N.L., Wensveen, F.M., de Boer, J.P., de Jong, D., Green, S.R., van Oers, M.H. & Eldering, E. (2007) Crosstalk among Bcl-2 family members in B-CLL: seliciclib acts via the Mcl-1/Noxa axis and gradual exhaustion of Bcl-2 protection. Cell Death and Differentiation, 14, 1958–1967.

Hallek, M., Cheson, B. D., Catovsky, D., Caligaris-Cappio, F., Dighiero, G., Dohner, H., Hillmen, P., Keating, M. J., Montserrat, E., Rai, K. R. & Kipps, T. J. & International Workshop On Chronic Lymphocytic Leukemia. 2008. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood, 111, 5446–5456.

Hazan-Halevy, I., Harris, D., Liu, Z., Liu, J., Li, P., Chen, X., Shanker, S., Ferrajoli, A., Keating, M.J. & Estrov, Z. (2010) STAT3 is constitutively phosphorylated on serine 727 residues, binds DNA, and activates transcription in CLL cells. Blood, 115, 2852–2863.

Herman, S.E., Gordon, A.L., Hertlein, E., Ramanunni, A., Zhang, X., Jaglowski, S., Flynn, J., Jones, J., Blum, K.A., Buggy, J.J., Hamdy, A., Johnson, A.J. & Byrd, J.C. (2011) Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. Blood, 117, 6287–6296.

Hewamana, S., Alghazal, S., Lin, T.T., Clement, M., Jenkins, C., Guzman, M.L., Jordan, C.T., Neelakantan, S., Crooks, P.A., Burnett, A.K., Pratt, G., Fegan, C., Rowntree, C., Brennan, P. & Pepper, C. (2008) The NF-kappaB subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lympho-cytic leukemia and represents a promising thera-peutic target. Blood, 111, 4681–4689.

Johnson, A.J., Yeh, Y.Y., Smith, L.L., Wagner, A.J., Hessler, J., Gupta, S., Flynn, J., Jones, J., Zhang, X., Bannerji, R., Grever, M.R. & Byrd, J.C. (2012) The novel cyclin-dependent kinase inhi-bitor dinaciclib (SCH727965) promotes apopto-sis and abrogates microenvironmental cytokine protection in chronic lymphocytic leukemia cells. Leukemia, 26, 2554–2557.

Kawauchi, K., Ogasawara, T. & Yasuyama, M. (2002) Activation of extracellular signal-regulated kinase through B-cell antigen receptor in B-cell chronic lymphocytic leukemia. International Journal of Hematology, 75, 508–513.

Kitada, S., Andersen, J., Akar, S., Zapata, J.M., Takayama, S., Krajewski, S., Wang, H.G., Zhang, X., Bullrich, F., Croce, C.M., Rai, K., Hines, J. & Reed, J.C. (1998) Expression of apoptosis-regu-lating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemore-sponses. Blood, 91, 3379–3389.

Kumar, S. K., Laplant, B., Chng, W. J., Zonder, J., Callander, N., Fonseca, R., Fruth, B., Roy, V., Erlichman, C., Stewart, A. K. & Consortium, M. P. (2015) Dinaciclib, a novel CDK inhibitor, demonstrates encouraging single-agent activity in patients with relapsed multiple myeloma. Blood, 125, 443–448.

Liu, Z., Hazan-Halevy, I., Harris, D.M., Li, P., Ferrajoli, A., Faderl, S., Keating, M.J. & Estrov, Z. (2011) STAT-3 activates NF-kappaB in chronic lymphocytic leukemia cells. Molecular Cancer Research, 9, 507–515.

Malumbres, M. & Barbacid, M. (2009) Cell cycle, CDKs and cancer: a changing paradigm. Nature Reviews Cancer, 9, 153–166.

Meads, M.B., Hazlehurst, L.A. & Dalton, W.S. (2008) The bone marrow microenvironment as a tumor sanctuary and contributor to drug resis-tance. Clinical Cancer Research, 14, 2519–2526.

Mittal, A.K., Chaturvedi, N.K., Rai, K.J., Gilling-Cutucache, C.E., Nordgren, T.M., Moragues, M., Lu, R., Opavsky, R., Bociek, G.R., Weisenburger, D.D., Iqbal, J. & Joshi, S.S. (2014) Chronic lym-phocytic leukemia cells in a lymph node microenvironment depict molecular signature associated with an aggressive disease. Molecular Medicine, 20, 290–301.

Moulding, D.A., Giles, R.V., Spiller, D.G., White, M.R., Tidd, D.M. & Edwards, S.W. (2000) Apoptosis is rapidly triggered by antisense depletion of MCL-1 in differentiating U937 cells. Blood, 96, 1756–1763.

Muzio, M., Apollonio, B., Scielzo, C., Frenquelli, M., Vandoni, I., Boussiotis, V., Caligaris-Cappio, F. & Ghia, P. (2008) Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. Blood, 112, 188–195.

Natoni, A., Murillo, L.S., Kliszczak, A.E., Catherwood, M.A., Montagnoli, A., Samali, A., O'Dwyer, M. & Santocanale, C. (2011) Mechanisms of action of a dual Cdc7/Cdk9 kinase inhibitor against quiescent and proliferating CLL cells. Molecular Cancer Therapeutics, 10, 1624–1634.

Nemunaitis, J.J., Small, K.A., Kirschmeier, P., Zhang, D., Zhu, Y., Jou, Y.M., Statkevich, P., Yao, S.L. & Bannerji, R. (2013) A first-in-human, phase 1, dose-escalation study of dinaciclib, a novel cyclin-dependent kinase inhibitor, administered weekly in subjects with advanced malignancies. Journal of Translational Medicine, 11, 259.

Oelgeschl€ager, T. (2002) Regulation of RNA poly-merase II activity by CTD phosphorylation and cell cycle control. Journal of Cellular Physiology, 190, 160–169.

Ogasawara, T., Yasuyama, M. & Kawauchi, K. (2003) Constitutive activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase in B-cell lymphoproliferative disorders. International Journal of Hematology, 77, 364–370.

Paiva, C., Godbersen, J.C., Soderquist, R.S., Rowland, T., Kilmarx, S., Spurgeon, S.E., Brown, J.R., Srinivasa, S.P. & Danilov, A.V. (2015) Cyclin-dependent kinase inhibitor P1446A induces apoptosis in a JNK/p38 MAPK-dependent manner in chronic lymphocytic leukemia B-cells. PLoS ONE, 10, e0143685.

Pan, R., Hogdal, L.J., Benito, J.M., Bucci, D., Han, L., Borthakur, G., Cortes, J., Deangelo, D.J., Debose, L., Mu, H., Dohner,€ H., Gaidzik, V.I., Galinsky, I., Golfman, L.S., Haferlach, T., Harutyunyan, K.G., Hu, J., Leverson, J.D., Marcucci, G., Muschen,€ M., Newman, R., Park, E., Ruvolo, P.P., Ruvolo, V., Ryan, J., Schindela, S., Zwei-dler-Mckay, P., Stone, R.M., Kantarjian, H., Andreeff, M., Konopleva, M. & Letai, A.G. (2014) Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. Cancer Discovery, 4, 362–375.

Pardanani, A., Hood, J., Lasho, T., Levine, R.L., Martin, M.B., Noronha, G., Finke, C., Mak, C.C., Mesa, R., Zhu, H., Soll, R., Gilliland, D.G. & Tefferi, A. (2007) TG101209, a small molecule JAK2-selective kinase inhibitor potently inhibits myeloproliferative disorder-associated JAK2V617F and MPLW515L/K mutations. Leukemia, 21, 1658–1668.

Parrish, A. B., Freel, C. D. & Kornbluth, S. 2013. Cellular mechanisms controlling caspase activa-tion and function. Cold Spring Harbor Perspectives in Biology, 5, 1–24.

Parry, D., Guzi, T., Shanahan, F., Davis, N., Prabhavalkar, D., Wiswell, D., Seghezzi, W., Paruch, K., Dwyer, M.P., Doll, R., Nomeir, A., Windsor, W., Fischmann, T., Wang, Y., Oft, M., Chen, T., Kirschmeier, P. & Lees, E.M. (2010) Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. Molecular Cancer Therapeutics, 9, 2344–2353.

Paruch, K., Dwyer, M.P., Alvarez, C., Brown, C., Chan, T.Y., Doll, R.J., Keertikar, K., Knutson, C., McKittrick, B., Rivera, J., Rossman, R., Tucker, G., Fischmann, T., Hruza, A., Madison, V., Nomeir, A.A., Wang, Y., Kirschmeier, P., Lees, E., Parry, D., Sgambellone, N., Seghezzi, W., Schultz, L., Shanahan, F., Wiswell, D., Xu, X., Zhou, Q., James, R.A., Paradkar, V.M., Park, H., Rokosz, L.R., Stauffer, T.M. & Guzi, T.J. (2010) Discovery of Dinaciclib (SCH 727965): a potent and selective inhibitor of cyclin-dependent kinases. ACS Medicinal Chemistry Letters, 1, 204–208.

Pepper, C., Lin, T.T., Pratt, G., Hewamana, S., Brennan, P., Hiller, L., Hills, R., Ward, R., Starczynski, J., Austen, B., Hooper, L., Stankovic, T. & Fegan, C. (2008) Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers. Blood, 112, 3807–3817.

Pickering, B.M., de Mel, S., Lee, M., Howell, M., Habens, F., Dallman, C.L., Neville, L.A., Potter, K.N., Mann, J., Mann, D.A., Johnson, P.W., Stevenson, F.K. & Packham, G. (2007) Pharmacological inhibitors of NF-kappaB accelerate apoptosis in chronic lymphocytic leukaemia cells. Oncogene, 26, 1166–1177.

Reikvam, H., Nepstad, I., Sulen, A., Gjertsen, B.T., Hatfield, K.J. & Bruserud, Ø. (2013) Increased antileukemic effects in human acute myeloid leukemia by combining HSP70 and HSP90 inhibitors. Expert Opinion on Investigational Drugs, 22, 551–563.

Ringshausen, I., Schneller, F., Bogner, C., Hipp, S., Duyster, J., Peschel, C. & Decker, T. (2002) Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. Blood, 100, 3741–3748.

Rozovski, U., Wu, J.Y., Harris, D.M., Liu, Z., Li, P., Hazan-Halevy, I., Ferrajoli, A., Burger, J.A., O'Brien, S., Jain, N., Verstovsek, S., Wierda, W.G., Keating, M.J. & Estrov, Z. (2014) Stimulation of the B-cell receptor activates the JAK2/STAT3 signaling pathway in chronic lymphocytic leukemia cells. Blood, 123, 3797–3802.

Sainz-Perez, A., Gary-Gouy, H., Portier, A., Davi, F., Merle-Beral, H., Galanaud, P. & Dalloul, A. (2006) High Mda-7 expression promotes malignant cell survival and p38 MAP kinase activation in chronic lymphocytic leukemia. Leukemia, 20, 498–504.

Samuel, J., Macip, S. & Dyer, M.J. (2014) Efficacy of vemurafenib in hairy-cell leukemia. New Eng-land Journal of Medicine, 370, 286–288.

Scarfo, L. & Ghia, P. (2013) Reprogramming cell death: BCL2 family inhibition in hematological malignancies. Immunology Letters, 155, 36–39.

Souers, A.J., Leverson, J.D., Boghaert, E.R., Ackler, S.L., Catron, N.D., Chen, J., Dayton, B.D., Ding, H., Enschede, S.H., Fairbrother, W.J., Huang, D.C., Hymowitz, S.G., Jin, S., Khaw, S.L., Kovar, P.J., Lam, L.T., Lee, J., Maecker, H.L., Marsh, K.C., Mason, K.D., Mitten, M.J., Nimmer, P.M., Oleksijew, A., Park, C.H., Park, C.M., Phillips, D.C., Roberts, A.W., Sampath, D., Seymour, J.F., Smith, M.L., Sullivan, G.M., Tahir, S.K., Tse, C., Wendt, M.D., Xiao, Y., Xue, J.C., Zhang, H., Humerickhouse, R.A., Rosenberg, S.H. & Elmore, S.W. (2013) ABT-199, a potent and selective BCL-2 inhibitor, achieves antitu-mor activity while sparing platelets. Nature Med-icine, 19, 202–208.

Stacchini, A., Aragno, M., Vallario, A., Alfarano, A., Circosta, P., Gottardi, D., Faldella, A., Rege-Cambrin, G., Thunberg, U., Nilsson, K. & Caligaris-Cappio, F. (1999) MEC1 and MEC2: two new cell lines derived from B-chronic lymphocytic leukaemia in prolymphocytoid transformation. Leukemia Research, 23, 127–136.

Sylvan, S.E., Skribek, H., Norin, S., Muhari, O., Osterborg, A. & Szekely, L. (2016) Sensitivity of chronic lymphocytic leukemia cells to small targeted therapeutic molecules: an in vitro comparative study. Experimental Hematology, 44, e1.

Varadarajan, S., Poornima, P., Milani, M., Gowda, K., Amin, S., Wang, H.G. & Cohen, G.M. (2015) Maritoclax and dinaciclib inhibit MCL-1 activity and induce apoptosis in both a MCL-1-dependent and -independent manner. Oncotarget, 6, 12668–12681.

Vogler, M., Butterworth, M., Majid, A., Walewska, R.J., Sun, X.M., Dyer, M.J. & Cohen, G.M. (2009) Concurrent up-regulation of BCL-XL and BCL2A1 induces approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia. Blood, 113, 4403–4413.

Willimott, S., Baou, M., Huf, S. & Wagner, S.D. (2007a) Separate cell culture conditions to promote proliferation or quiescent cell survival in chronic lymphocytic leukemia. Leukaemia & Lymphoma, 48, 1647–1650.

Willimott, S., Baou, M., Naresh, K. & Wagner, S.D. (2007b) CD154 induces a switch in pro-survival Bcl-2 family members in chronic lym-phocytic leukaemia. British Journal of Haematol-ogy, 138, 721–732.

Woyach, J.A., Furman, R.R., Liu, T.M., Ozer, H.G., Zapatka, M., Ruppert, A.S., Xue, L., Li, D.H., Steggerda, S.M., Versele, M., Dave, S.S., Zhang, J., Yilmaz, A.S., Jaglowski, S.M., Blum, K.A., Lozanski, A., Lozanski, G., James, D.F., Barrientos, J.C., Lichter, P., Stilgenbauer, S., Buggy, J.J., Chang, B.Y., Johnson, A.J. & Byrd, J.C. (2014) Resistance mechanisms for the Bru-ton's tyrosine kinase inhibitor ibrutinib. New England Journal of Medicine, 370, 2286–2294.

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Paradoxical activation of alternative pro-survival pathways determines resistance to MEK inhibitors in chronic lymphocytic leukaemia

In order to explore novel therapeutic opportunities for B-cell malignancies, we evaluated the effects of MEK inhibitors (MEKi) on chronic lymphocytic leukaemia (CLL). We found that none had a significant effect on CLL viability, despite inhibiting signalling. Paradoxically, MEKi promoted the accumulation of active MEK and CRAF, reduced the expression of negative regulators of the pathway and augmented AKT signalling. Combining MEKi and phosphoinositide 3-kinase (PI3K) inhibitors antagonized this effect and induced cell death. We propose that MEKi-mediated activa-tion of pro-survival pathways explains their low toxicity in CLL and that inhibition of both RAF/MEK/ERK and PI3K/ AKT signalling could overcome single-agent resistance.

The RAF/MEK/ERK signalling cascade is activated in nearly half of CLL patients, suggesting a pathogenic role (Muzio et al, 2008). Selective inhibitors have been developed, including MEK1/2 inhibitors (MEKi) that are being clinically assessed as a monotherapy or in combination. However, MEKi have a lower efficiency in cancers not driven by mutant BRAF or KRAS, due to resistance mechanisms. This has limited the clinical use of these compounds.

To test the effects of blocking the RAF/MEK/ERK pathway in CLL, we used different MEKi (U0126, PD0325901, selumetinib and trametinib) on the MEC-1 cell line, often used as a model for CLL, in which we observed increased RAF/MEK/ERK signalling (Chen et al, 2016). We used sorafenib (pan-RAF inhibitor) and dabrafenib (BRAF inhibitor) for comparison. Materials and methods are detailed in the Supplementary material. MEKi did not affect the viability (Fig 1A), survival (Figure S1A), cell cycle profile (Figure S1B), Mcl-1 levels or PARP cleavage (Figure S1C) in MEC-1, and only very high concentrations of sorafenib decreased Mcl-1 expression, cleaved PARP and induced death in MEC-1 (Fig 1A, S1A–C). Similar results were observed in JVM3, a

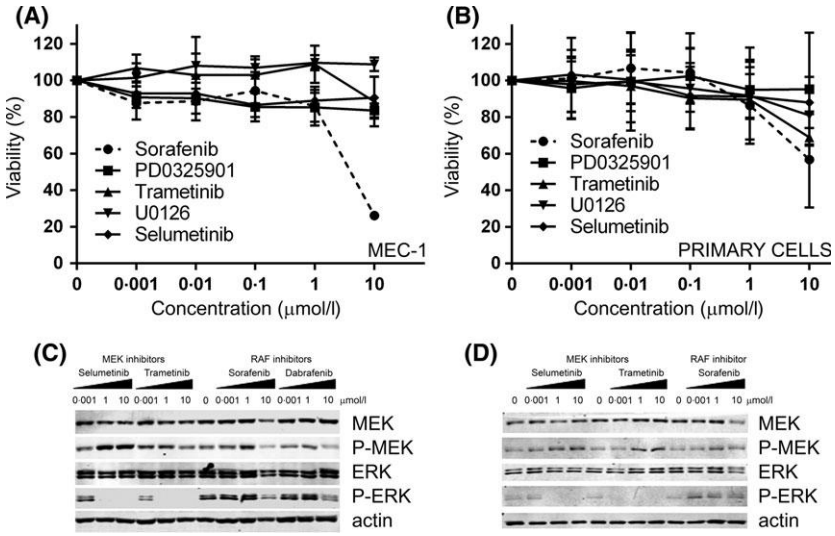

Fig 1. Inhibition of MEK does not affect CLL cell survival. (A) Percentage cell viability of MEC-1 cells at 48 h after treatment with 0 to 10 lM of various inhibitors, as measured by an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Experiments were performed in triplicate and repeated at least twice. Graphics show mean values and error bars represent standard deviation, as in all the other panels of this figure. (B) Percentage cell viability of CLL patient cells cultured for 24 h in the presence of 10 ng/ml IL4/CD154, 48 h after treatment with the same inhibitors. Eight to 19 different samples were tested for each inhibitor, and experiments were performed in triplicate. (C) Representative Western blots using lysates of MEC-1 treated with different concentrations of MEK or RAF inhibitors for 16 h. Pro-tein levels of ERK, phospho-ERK (P-ERK), MEK and phospho-MEK (P-MEK) were detected with specific antibodies. (D) Representative Western blots using lysates of primary cells obtained patients. Cells were cultured as described and treated with different concentrations of selumetinib, trametinib or sorafenib for 16 h. The total and phosphorylated levels of ERK and MEK were measured with specific antibodies.

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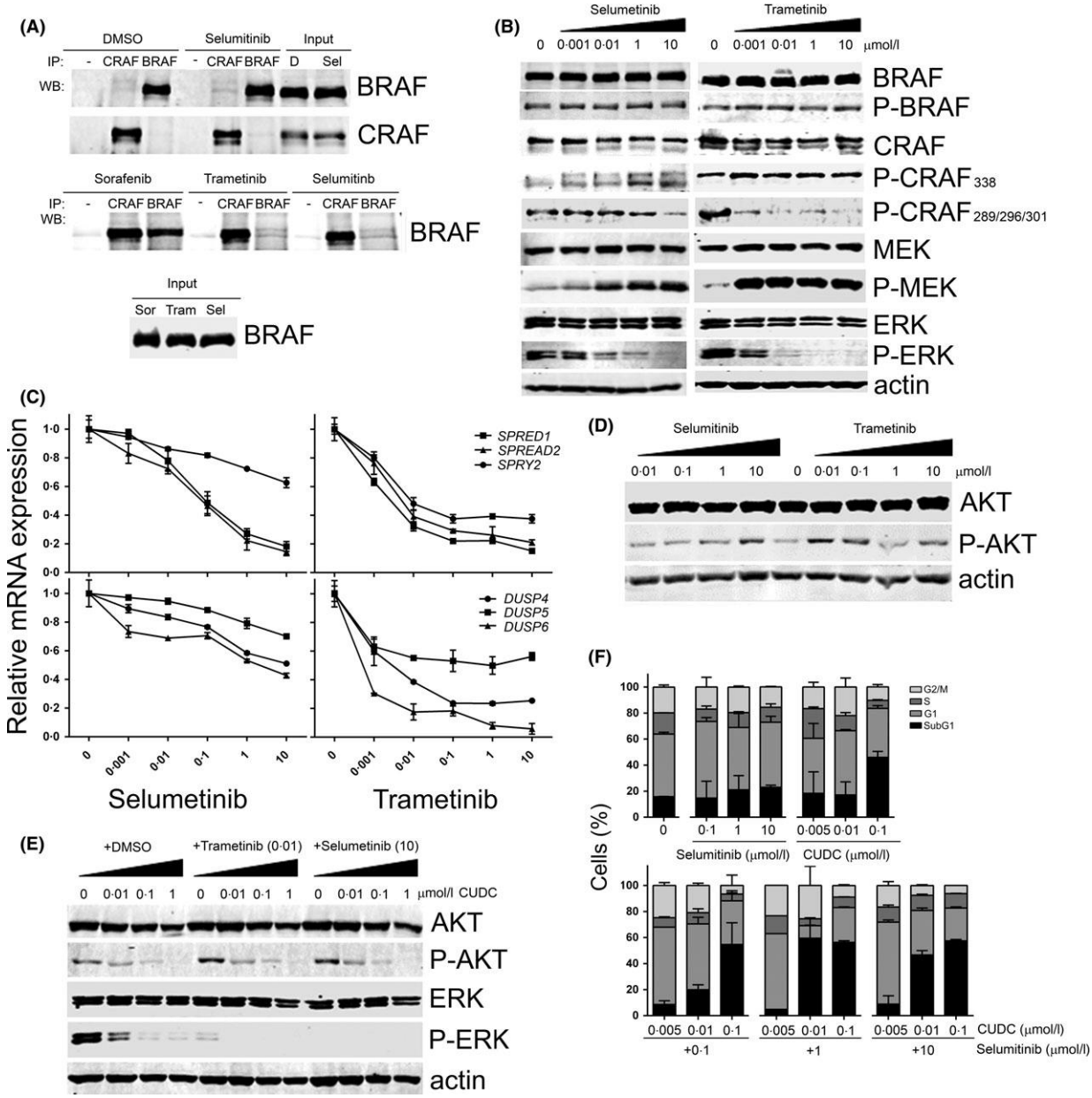

Fig 2. (A) Co-immunoprecipitation measuring the levels of BRAF-CRAF dimerization in MEC-1 cells treated with 5 lmol/l selumetinib, 0 1 lmol/l trametinib or 0 5 lmol/l sorafenib for 16 h. Cell lysates were incubated with BRAF or CRAF antibodies, following protein A-Sephar-ose beads precipitation. Precipitated BRAF and CRAF proteins were measured by Western blotting. (B) Representative Western blots using MEC-1 cells treated with different concentrations of selumetinib or trametinib for 16 h. Detection of subject P-BRAF (Ser445), P-CRAF (Ser338) and P-CRAF (Ser289/296/301) was done using specific antibodies. (C) Top Left: mRNA expression of SPRY2, SPRED1 and SPRED2, which encode members of the SPRY family of proteins that have been reported to be negative regulators of RAF/MEK/ERK signalling, in MEC-1 cells treated with different concentrations of selumitinib for 16 h. The expression of each of these genes was normalized to GAPDH and values are shown as relative to untreated cells. Experiments were performed in triplicate and repeated at least 3 times. Top right: mRNA expression of SPRY2, SPRED1 and SPRED2 in MEC-1 cells treated with different concentrations of trametinib for 16 h. Bottom left: mRNA expression of DUSP4/5/6 in MEC-1 cells treated with different concentrations of selumitinib for 16 h. Bottom right: mRNA expression of DUSP4/5/6 in MEC-1 cells trea-ted with different concentrations of trametinib for 16 h. (D) Representative Western blots using lysates of MEC-1 cells treated with different con-centrations of selumetinib or trametinib for 16 h. Total and Ser473phosphorylated (active) levels of AKT were measured. (E) Representative Western blots using lysates of MEC-1 treated with different concentrations of CUDC907 (CUDC) alone (+dimethyl sulphoxide, DMSO) or in combination with 0 01 lmol/l trametinib or 10 lmol/l selumetinib for 16 h. Total and phosphorylated levels of AKT and ERK were measured using specific antibodies. (F) Top: Cell cycle profiles of MEC-1 cells treated with different concentrations of selumetinib or CUDC907 for 48 h, as measured by propidium iodide staining and fluorescence-activated cell sorting analysis. Bottom: Cell cycle profiles of MEC-1 cells treated with a combined treatment of selumetinib (0 1, 1 or 10 lmol/l) and CUDC907 (0 005, 0 01 or 0 1 lmol/l) for 48 h. SubG1 events are indicative of the percentage of cell death. Results show average of 3 independent experiments and error bars represent standard deviation.

B-prolymphocytic leukaemia cell line (Figure S1D). In contrast, the viability of SIG-M5, an acute myeloid leukaemia cell line with a BRAF mutation, was reduced (Figure S1D), consistent with the known sensitivity of BRAF-driven malignancies to RAF/MEK/ERK inhibition. In primary cells stimulated with CD40 ligand (CD40L)/interleukin 4 (IL4) to mimic the microenvironment of proliferation centres, MEKi did not reduce viability either (Fig 1B). This suggests that MEKi do not affect survival of CLL cells at pharmacologically relevant doses.

We found that the downstream phosphorylation of ERK (on Thr202 and Tyr204) in MEC-1 was abolished by MEKi, as expected (Fig 1C). However, MEKi induced Ser218/222-phosphorylated MEK, suggesting an upstream reactivation of the pathway. This was also the case in patient cells stimulated with CD40L/IL4 (Fig 1D) or co-cultured with mouse fibroblast L cells (NTL)/CD154/IL4 (Figure S2A). Of note, sorafenib and dabrafenib reduced the phosphorylation of both MEK and ERK (Fig 1C and D).

BRAF-CRAF dimerization reactivates ERK signalling in BRAF-wild type cells treated with BRAF-specific inhibitors. Moreover, BRAF-CRAF dimerization is required for RAF activation. Thus, we reasoned that MEKi-triggered MEK phosphorylation could be due to RAF reactivation. However, MEKi did not promote dimer formation, although sorafenib was able to enhance it (Fig 2A). MEKi treatment did not change the activating phosphorylation of BRAF (Ser445), but it either increased phosphorylated Ser338 in CRAF, which is related to activation, and/or decreased its inhibitory Ser289/296/301 phosphorylation (Fig 2B). This is consistent with ERK being inhibited in response to MEKi. MEKi did not change the expression of protein phosphatase 2A (PP2A), which regulates RAF/MEK/ERK by dephosphorylating CRAF (Figure S2B). This shows that MEKi reactivate CRAF, which could mediate the accumulation of MEK. We next examined the effects of MEKi on negative regulators of the pathway. We found that MEKi decreased SPRED1/2, SPRY2 and DUSP4/5/6 in a dose-dependent manner (Fig 2C). Downregulation of all these modulators could also contribute to the reactivation of upstream RAF/MEK/ERK signalling.

We also found that selumetinib activated AKT (Fig 2D), as did trametinib, although the effect was reversed at higher concentrations. This suggested that AKT could contribute to MEKi resistance. To test this, we used CUDC907, which inhibits histone deacetylases and PI3K. The combination of MEKi and CUDC907 suppressed ERK and AKT signalling, as expected (Fig 2E). Low concentrations of CUDC907 did not induce cell death in MEC-1 (Fig 2F), but when combined with non-toxic concentrations of selumetinib, cell death increased to 40–50%. This shows that a non-lethal suppres-sion of PI3K turns MEK inhibition into apoptosis in CLL. This was confirmed with trametinib and idelalisib, a specific PI3K d inhibitor (Figure S2C). The effect was not observed when two RAF/MEK/ERK inhibitors were combined (Figure S2D).

We propose that MEKi induce upstream RAF/MEK/ERK signalling in CLL by reactivating RAF through downregulation of SPRY and DUSP proteins, leading to phosphorylation of CRAF. This results in activation of pro-survival pathways, including AKT, and drug resistance. Precision medicines have changed the prognosis of blood malignancies but their use is hampered by resistance, hence the need to find parameters that allow the stratification of patients (Dyer et al, 2013). We propose that understanding ERK-dependent signalling feedback is important for designing new CLL therapies. The use of MEKi as single agents should be discouraged because the activation of pro-survival signals may compensate the effect. Combination therapies of MEKi with PI3K inhibitors could, instead,provide a novel therapeutic strategy.

Acknowledgments

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Author contribution

YC and SM designed the experiments, analysed the data and prepared the manuscript, with MJSD and SJ. YC performed the experiments, with SG, DK, GS and SJ. All authors reviewed the manuscript.

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Keywords: CLL, MEK inhibitors, MAPK, CRAF, AKT

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Clinical data available for the patients studied.
Table SII. List of primers used in the qRT-PCR experiments.

Fig S1. (A) Induction of cell death in the same cells as Fig 1A, as measured by PI staining and FACS analysis.

Graphics show percentage of non-permeabilised cells positive for PI fluorescence (dead). (B) Cell cycle profiles of MEC-1 cells treated with different concentrations of MEKi or sorafenib for 48 h, as measured by PI staining and FACS analysis. (C) Representative Western blots of lysates of MEC-1 treated with different concentrations of selumetinib, trametinib or sorafenib for 16 h. The membranes were probed with antibodies against Mcl-1 (an anti-apoptotic protein) and PARP (the cleavage of which is associated with induction of apoptosis). b-actin was used as a loading control, as in all the other Western blots in this study. (D) Percentage cell viability of JVM3 and SISG-M5 cells 48 h after being treated with 0–10 lmol/l of various inhibitors, as measured by an MTS assay.

Fig S2. (A) Representative Western blots using lysates of CLL patient cells co-cultured with NTL/CD154/IL-4 for 48 h before being treated with 0.001 or 1 lmol/l selumetinib or trametinib. Total and phosphorylated levels of MEK and ERK were measured. (B) mRNA expression of PP2A in MEC-1 cells treated with different concentrations of selumitinib or trametinib for 16 h. The expression of each of these genes was normalized to GAPDH and values are shown as relative to untreated cells. Experiments were performed in triplicate and repeated at least 3 times. (C) Percentage cell viability of MEC-1 cells 48 h after being treated with trametinib and/or idelalisib, as measured by an MTS assay. Although idelalisib (first group of bars) or trametinib (black bars) alone do not decrease significantly viability, the combination of both reduces it up to 60%. Graphics show mean values of three experiments and error bars represent standard deviation. (D) Top: Cell cycle profiles of MEC-1 cells treated with different concentrations of selumetinib or CUDC907 for 48 h, as measured by PI staining and FACS analysis. Bottom: same, using a combined treatment of selumetinib (0.1, 1 or 10 lmol/l) and CUDC907 (0.005, 0.01 or 0.1 lmol/l) for the same amount of time. SubG1 events are indicative of the percent-age of cell death. Results show average of 3 independent experiments and error bars represent standard deviation.

References

Chen, Y., Germano, S., Clements, C., Samuel, J., Shelmani, G., Jayne, S., Dyer, M.J. & Macip, S. (2016) Pro-survival signal inhibition by CDK inhibitor dinaciclib in Chronic Lymphocytic Leukaemia. British Journal of Haematology, 175, 641–651.

Dyer, M.J., Vogler, M., Samuel, J., Jayne, S., Wagner, S., Pritchard, C. & Macip, S. (2013) Precision medicines for B-cell leukaemias and lymphomas; progress and potential pit-falls. British Journal of Haematology, 160, 725–733.

Muzio, M., Apollonio, B., Scielzo, C., Frenquelli, M., Vandoni, I., Boussiotis, V., Caligaris-Cappio, F. & Ghia, P. (2008) Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. Blood, 112, 188–195.

A3

NOTCH1 mutations influence Chronic Lymphocytic Leukaemia sensitivity to targeted inhibitors

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Keywords: CLL, Notch, Bcl2 inhibitors, stratified medicine.

Short title: Drug sensitivity of NOTCH1 mutant malignant B cells

ABSTRACT

NOTCH1 mutations are a poor prognostic factor for Chronic Lymphocytic Leukaemia (CLL), associated with resistance to rituximab and increased risk of Richter's transformation. To characterize the role of *NOTCH1* mutation in drug sensitivity, we first genotyped exon 34 (including the 3'-UTR) in 147 CLL patients. We found mutations in 13/106 (12.3%) of a random cohort and 13/41 (26.8%) of patients with trisomy 12. The most common mutation, seen in 92.3%, was the CT deletion in the PEST domain. We also found two previously unreported frame-shift mutations (a 38bp deletion and a nucleotide insertion) in the transactivation domain, resulting in loss of the PEST domain. We next treated *NOTCH1* mutated and wild type patient cells with different targeted inhibitors. No differences were observed with most of the drugs tested. However, a subgroup of *NOTCH1* mutated patients showed increased resistance to BCL2 inhibitor venetoclax. To extend our study, we compared Rec-1, a malignant B cell line that harbours a *NOTCH1* mutation, with wild-type cell lines JVM3 and Mec-1. We found that Rec-1 were more sensitive to inhibition of NF-κB and γ-secretase. Moreover, combination of ibrutinib with either γ-secretase or BCL2 inhibitors showed synergy in the mutated cells. Our results show that NOTCH1 mutation in CLL defines a subgroup of disease with specific sensitivities to combinations of targeted agents that may allow new therapeutic approaches.

INTRODUCTION

CLL is a highly heterogeneous disease, in which some patients have an indolent course while others suffer aggressive forms with short life expectancy. Heterogeneity is also evident at the genetic level [1-4]. As the condition progresses, the number of genetic variations increases. For example, TP53 mutations are present in 5-10% patients at diagnosis and can increase to 60% in advanced cases [1,5,6]. IgHV status, stereotyped B-cell receptor subgroups, cytogenetic abnormalities as well as a number of recurrent mutations have also been linked to prognosis and response to treatments [2,7-9].

Around 12% of CLL patients harbour NOTCH1 mutations, one of the most recurrent in this disease and a poor prognostic factor [4,10]. The frequency of mutations increases in refractory patients and Richter syndrome (20% and 30% respectively) [11]. CLL with NOTCH1 mutation progresses faster and is classed to have an intermediate-risk [9,12,13]. The most common NOTCH1 mutations occur in the PEST domain of the intracellular domain (NICD), which targets the protein for degradation [14]. Mutations make the domain more resistant, leading to accumulation of the NICD.

Notch signalling is a treatment target in a number of cancers, such as breast, brain, pancreatic as well as haematological malignancies, in particular T-cell leukaemia [15], but has not been considered in the context of CLL. In order to explore this possibility, we sequenced a local cohort for NOTCH1 mutations in exon 34, a mutation hotspot, and performed a pre-clinical evaluation of effectiveness of commercially available small molecule inhibitors. We show that NOTCH1 mutations influence the response to drugs, which provides a rationale to use them as stratification factors in CLL.

MATERIALS AND METHODS

Patient samples. A total of 147 patients were used in this project and were categorised into two sets. Set #1 included 106 randomly selected patients from the database (Random set) and set #2 included 41 patients who were known to have clones harbouring Trisomy 12 (Trisomy 12 set). Only patients with Trisomy 12 in 50% or more of their tumour population were eligible for the second set. The summary of patients' characteristics is outlined in Table S1. Peripheral blood samples were obtained following an informed consent and approval from the Local Research Ethics Committee and the Research and Development Office of the University Hospitals of Leicester NHS Trust. To prepare B-cell samples for treatment efficacy testing, white blood cells were separated from whole blood samples using density separation, as previously described [16]. White blood cells were then carefully collected and washed in media. The pellet was then resuspended and cultured in RPMI-1640 supplemented with 10ng/ml CD40L/IL-4 to mimic the microenvironment of proliferation centres and maintain cell survival in vitro [16,17]. Cells were cultured at 37 °C in an incubator containing 5% CO_2.

Cell lines. A NOTCH1 mutated cell line (Rec-1) and two NOTCH1 wild-type cell lines (Mec-1 and JVM3) were used. All of them were originally derived from B cell malignancies. Cells were maintained in complete medium (RPMI-1640 for Rec-1 and JVM3 and IMDM for Mec-1) with 10% foetal bovine serum 2 mmol/l L-glutamine, 50 u/ml penicillin and 50 mg/ml streptomycin. All cell lines were cultured at 37 °C in an incubator containing 5% CO_2.

DNA Sequencing. DNA was extracted following treatment with Proteinase K and RNAse. DNA samples were PCR amplified and then purified using SureClean. To sequence each sample, two sets of primers were used (Table S2). As a result, two overlapping DNA fragments

were sequenced, covering around 1,500 base pairs of the exon 34 of NOTCH1 gene, including a known mutation hotspot region. Purified PCR products of DNA concentration of at least 15 ng/µl and their corresponding primers were send to Beckman Coulter Genomics (Genewiz) for Sanger DNA Sequencing. Chromatogram files were then analysed manually and the sequence was also analysed using nucleotide BLAST. All samples were compared to *Homo sapiens* notch 1 (NOTCH1) reference sequence (Sequence ID: ref|NG_007458.1). All variations were compared to NOTCH1 gene ENSG00000148400, Transcript NOTCH1-001 on Ensembl database. Expasy translation tool was used to compare amino acid sequences based on FASTA sequence.

Cell viability assay. Cell lines and primary cells were treated with inhibitors targeting the gamma-secretase complex of the Notch1 protein (RO4929097 and PF-03084014), a BCL2 inhibitor (ABT199), a NFκB pathway inhibitor (IMD 0354) and a BTK inhibitor (ibrutinib). The suppliers of each inhibitor are listed in the Table S3. All the inhibitors were diluted in DMSO. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to estimate cell viability and proliferation after 48 hours of treatment, according to the manufacturer's instructions. Cell lines and primary CLL cells were seeded at a concentration of 1 x 10^6 cells/ml and 3-4 x 10^6 cells/ml, respectively. Following the treatment period, the MTS reagent was added to each well, incubated for 1-2 hours and the absorbance at 490nm was recorded on a TECAN infinite F50 reader (Labtech International, Uckfield, UK). Cell viability was calculated based on the reducing potential of the viable cells using a colourimetric reaction. Assays were performed in triplicate and repeated on at least two independent occasions and results were plotted and analysed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

In order to characterise potential new treatments for CLL, 106 randomly selected and 41 trisomy 12 CLL patients were sequenced for mutations in exon 34 of NOTCH1 (Figure 1A). We found that 12.3% patients in the random set and 29.3% in the trisomy 12 set had NOTCH1 mutations (SNPs not included). The most common mutation was a known CT deletion in the PEST domain of the gene, c.7541_7542delCT (Figure S1A), which accounted for 92% of all coding mutations found (84.6% in the random set and all in the trisomy 12 set). In 91.3% of all cases it was a heterozygous mutation, while one patient had a sub-clonal population and another a homozygous mutation (Figure S1B). Of note, two previously unreported non-synonymous coding mutations were found, both contained within the TAD domain of NOTCH1. One patient had a 38 base pair deletion (c.6906-6943del) and one a new G insertion (c.6877insG) (Figure S1C). Additionally, a known synonymous silent mutation (c.6870C>T) was found in the TAD of a patient with trisomy 12 set (Figure S1C). A number of SNPs were found, however they haven't been shown to be associated with the disease or function of Notch 1 protein (data not shown). In summary, patients with trisomy 12 were 3.2 times more likely to have NOTCH1 mutations than patients without the abnormality, consistent with previous reports [18].

We next studied cell sensitivity to different clinically relevant inhibitors in relation to their NOTCH1 status. To this end, we used Rec-1 (a Mantle cell lymphoma cell line with NOTCH1 mutation), Mec-1 (CLL cell line with wild type NOTCH1) and JVM3 (a B-cell prolymphocytic leukaemia with wild type NOTCH1). Since Notch signalling leads to activation of the NF-κB and BCL2 pathways [19], the drugs chosen were inhibitors of γ-secretase (RO4929097 and PF03084014), BCL-2 (ABT199) and NF-κB signalling (IMD0354), as well as BTK inhibitor (ibrutinib), currently used to treat CLL. We found that the NOTCH1 mutated

cell line was sensitive to RO4929097 while the wild type cells were not (Figure 1B and Table S5). However, this was not the case with PF03084014, which showed an unexpected heterogeneous response to γ-secretase inhibitors. Rec-1 was also more sensitive to IMD0354, but there were no differences of responses linked to NOTCH1 status in ibrutinib. Further, Rec-1 showed some resistance to ABT199 compared to the wild type cells, which was also evident in primary cells. Patient cells treated with the same drugs showed similar trends in mutated and wild type cells, although NOTCH1 mutants were more resistant to low concentrations of ABT199 or high concentrations of ibrutinib (Figure 2A). Of note, we did not see any differences sensitivity of NOTCH1 mutated primary cells to any of these drugs when stimulated to mimic the microenvironment in proliferation centres [16] (data not shown), suggesting that microenvironmental signals may help cells bypass the protective effects of mutant NOTCH1.

Finally, we look at sensitivity of Rec-1 cells to combination therapies. As shown in Table S5, many of the combinations increased cell sensitivity to drugs when compared to single agent treatments. Of them, the sum of ibrutinib and RO4929097 or ABT199 were found to be synergistic (Figure 2B and Table S6). Contrary to this, we found antagonism between RO4929097 and IMD0354. These results together suggest that NOTCH1 mutated cells may be sensitive to certain γ-secretase inhibitors and could respond to inhibitors of NFκB as single agents, but they actually may be more sensitive to combination therapies, especially with ibrutinib.

Cancer treatments are entering the era of precision medicine, in which therapeutic interventions can be tailored to individual patient's characteristics [20]. Although it has been accepted that the genetics of the CLL cells has a big impact on the response to treatment, little is known on the specific effects of NOTCH1 mutations on drug sensitivity, despite being a known unfavourable prognostic factor [13,21,22]. Consistent with previous reports [2,4,10,11], we

confirmed that NOTCH1 mutations are common in our cohort and their frequency increases significantly with trisomy 12. Moreover, we found three new mutations in the TAD domain: a deletion, an insertion and a silent mutation. Based on their nature and location, we predict that the effects of the two frameshift mutations would be similar to the commonest NOTCH1 mutation, a 2-base pair deletion. This could also lead to an increased resistance to intracellular NOTCH1 degradation, its consequent accumulation in the cell and a constitutive pro-survival signalling activation. It is important to highlight the identification of one patient harbouring both NOTCH1 and SF3B1 mutations, which were believed to me mutually-exclusive in the past. Although rare, in the recent years there have been reports of co-existence of the two [23].

We also found that the NOTCH1 mutation status has an effect on the sensitivity of CLL cells to inhibitors of pro-survival pathways. Although γ-secretase inhibitors have been shown to target NOTCH1-mutant cells [24,25], we observed a different response to the two inhibitors tested, suggesting that there may be unknown off-target effects that may contribute to the induction of cell death. Moreover, mutant cells were more sensitive to inhibitors of NFκB, a pathway found to be upregulated in virtually all CLL patients, which highlights a specific dependence that these cells may have on these signals. The COSMIC database on drug sensitivity suggested that NOTCH1 mutated cells would be more sensitive to BCL2 inhibitors, but we found the opposite in both cell lines and primary cells. However, we identified two synergies a NOTCH1 mutant cell line: RO4929097 (γ-secretase inhibitor) and ABT199 (BCL2 inhibitor) both with ibrutinib. As these drugs are currently used in clinical practice, these results could potentially provide improved therapeutic strategies in NOTCH1-mutated patients.

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AUTHOR CONTRIBUTION

DK, MJSD and SM designed the experiments, analysed the data and prepared the manuscript. DK performed the experiments, with help from GS, YC, MC, LC, JR, SG and SJ. BK, SW and CB obtained the patient samples and provided clinical advice. All authors reviewed the manuscript.

REFERENCES

1. Schuh A, Becq J, Humphray S, et al. Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. *Blood*. 2012;120(20):4191-4196.
2. Strefford JC. The genomic landscape of chronic lymphocytic leukaemia: biological and clinical implications. British Journal of Haematology. John Wiley & Sons Ltd. Cancer Genomics, Academic Unit of Cancer Sciences, Faculty of Medicine, University of Southampton, Southampton, UK. 2014(169):14-31.
3. Messmer BT, Albesiano E, Efremov DG, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. *The Journal of Experimental Medicine*. 2004;4(200):519-525.
4. Quesada V, et al. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet*. 2011;44:47-52.
5. Foà R, Giudice ID, Guarini A, Rossi D, Gaidano G. Clinical implications of the molecular genetics of chronic lymphocytic leukemia. *Haematologica*. 2013;98(5):675–685.
6. Gaidano G, Foa R, Dalla-Favera R. Molecular pathogenesis of chronic lymphocytic leukaemia. *The Journal of Clinical Investigation*. 2012;122(10):3432.
7. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig VH Genes Are Associated With a More Aggressive Form of Chronic Lymphocytic Leukemia. *Blood*. 1999;94(6):1848.

(C) Longo PG, Laurenti L, Gobessi S, et al. The Akt signaling pathway determines the different proliferative capacity of chronic lymphocytic leukemia B-cells from patients with progressive and stable disease. *Leukaemia*. 2007(21):110-120.
(D) Rossi D, Rasi S, Spina V, et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *International Journal of Hematologic Oncology*. 2013;121(8):1403-1412.
(E) Stilgenbauer S, Schnaiter A, Paschka P, Zenz T. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood*. 2014;123(21):3247-3254.
(F) Fabbri G, Rasi S, Rossi D, et al. Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. *J Exp Med*. 2011;208(7):1389-1401.
(G) Rossi D, et al. Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia. *Blood*. 2012;119(2):521-529.
(H) Villamor N, Conde L, Martínez-Trillos A, et al. NOTCH1 mutations identify a genetic subgroup of chronic lymphocytic leukemia patients with high risk of transformation and poor outcome. *Leukemia*. 2013;27:1100-1106.
(I) Kovall RA, Blacklow SC. Mechanistic insights into Notch receptor signaling from structural and biochemical studies. . *Current Topics in Developmental Biology*. 2010(93):31-
(C)
M. Espinoza I, Miele L. Notch inhibitors for cancer treatment. *Elsevier*. 2013;139:95-100.
N. Samuel J, Jayne S, Chen Y, et al. Posttranscriptional Upregulation of p53 by Reactive Oxygen Species in Chronic Lymphocytic Leukemia. *Cancer Res*. 2016;76(21):6311-6319.
O. Ahearne MJ, Willimott S, Pinon L, et al. Enhancement of CD154/IL4 proliferation by the T follicular helper (Tfh) cytokine, IL21 and increased numbers of circulating cells resembling Tfh cells in chronic lymphocytic leukaemia. *British Journal of haematology*. 2013;162:360-370.
P. Balatti V, Bottoni A, Palamarchuk A, et al. NOTCH1 mutations in CLL associated with trisomy 12. *Blood*. 2012;119(2):329-331.
Q. Xu Z, Zhang J. Constitutive activation of NF-κB signaling by NOTCH1 mutations in chronic lymphocytic leukemia. *Oncology Reports*. 2015;33:1609-1614.
R. Dyer MJ, Vogler M, Samuel J, et al. Precision medicines for B-cell leukaemias and lymphomas; progress and potential pitfalls. *Br J Haematol*. 2013;160(6):725-733.
S. Rossi D, et al. The genetics of Richter syndrome reveals disease heterogeneity and predicts survival after transformation. *Blood*. 2011;117(12):3391.
T. Tsimberidou AM, Keating MJ. Richter Syndrome: Biology, Incidence, and Therapeutic Strategies. *Cancer*. 2005;103(2):216-228.
U. Larrayoz M, Rose-Zerilli MJJ, Kadalayil L, Parker H, Blakemore S, Forster J. Non-coding NOTCH1 mutations in chronic lymphocytic leukemia; their clinical impact in the UK CLL4 trial. *Leukaemia*. 2017;31:510-514.
V. van Es JH, van Gijn ME, Riccio O, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature*. 2005;435(7044):959-963.
W. O'Neil J, Calvo J, McKenna K, et al. Activating Notch1 mutations in mouse models of T-ALL. *Blood*. 2006;107(2):781-785.

FIGURE LEGENDS

Figure 1. (A) Charts showing cytogenetic characteristics of patients from the Random (left) and Trisomy 12 (right) sets, including NOTCH1, SF3B1, IgHV, Trisomy 12, deletions of the long arm of chromosome 13 (13q del) and 11 (11q del) and short arm of chromosome 17, and recognised SNPs within NOTCH1 (126G>A and 6853G>A), with percentages over the total number of patients. Trisomy 12 patients were found to less likely to have a deletion in the long arm of chromosome 13 (odds ratio= 0.0452). No other associations were detected. **(B)** MTS viability assay of JVM3, Mec-1 and Rec-1 following 48 hours treatment with RO492907, PF 03084014, IMD 0354, ABT199 and ibrutinib at different concentrations. The statistical significance between JVM3 and Rec-1 cell line is shown by bottom line of stars and between Mec-1 and Rec-1 by the top line. Graph represent means of 3 experiments. Error bars represent standard deviation. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

Figure 2. (A) MTS viability assays of primary CLL cells after 48 hours of treatment with RO4929097, PF03084014, IMD 0354, ABT 199 or ibrutinib. The graphs show mean values of 2 measurements per patient and error bars represent standard deviations. Each circle and triangle represents a different NOTCH1-mutated or wild-type patient, respectively. **(B)** MTS viability assay of Rec1 treated with combinations of ibrutinib and ABT199 or RO492907. Error bars represent standard deviation. The statistical significance between single agent treatment and combinations are shown by the stars at the bottom. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

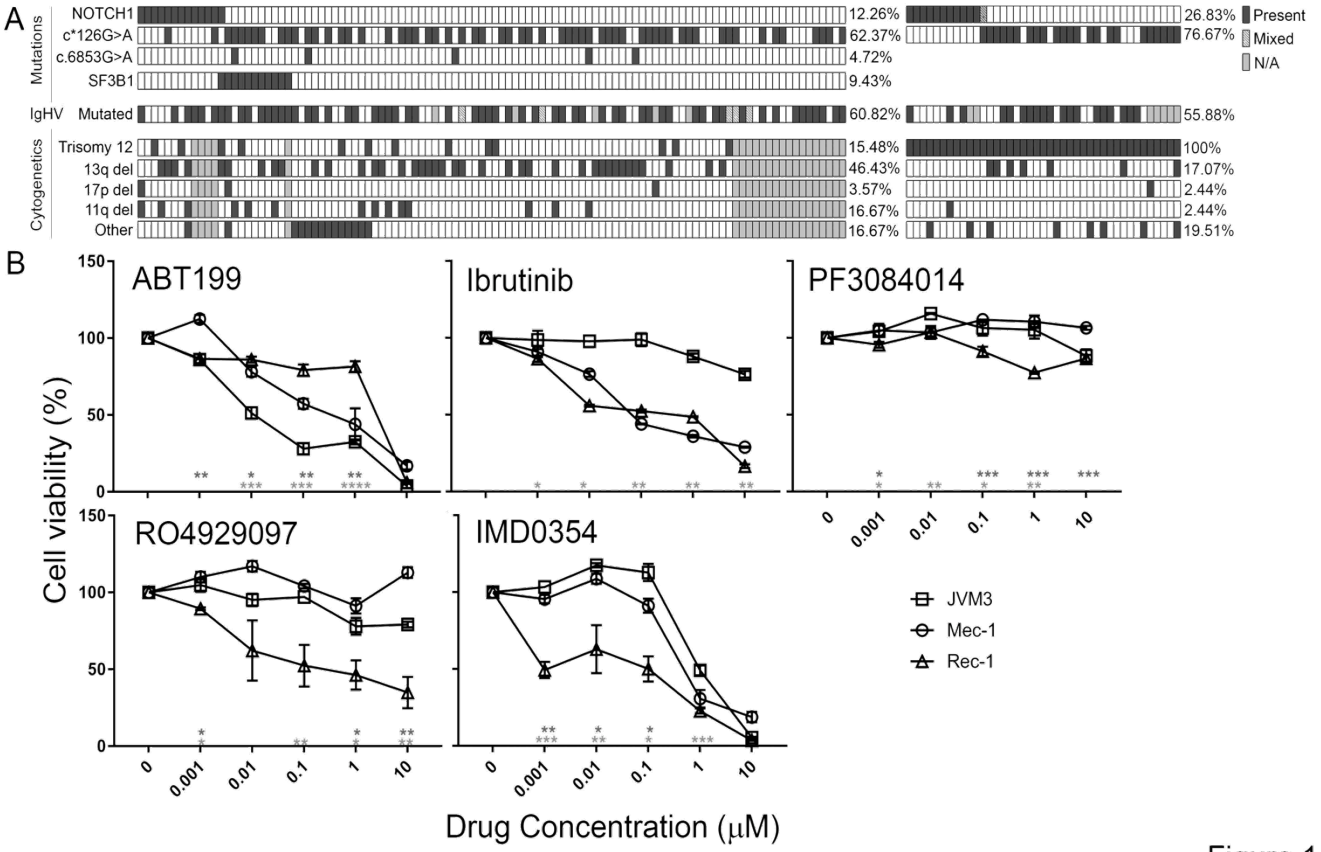
A
Mutations
NOTCH1
c*126G>A
c.6853G>A
SF3B1
12.26%
62.37%
4.72%
9.43%
26.83%
76.67%
Present
Mixed
N/A
IgHV
Mutated
60.82%
55.88%
Cytogenetics
Trisomy 12
13q del
17p del
11q del
Other
15.48%
46.43%
3.57%
16.67%
16.67%
100%
17.07%
2.44%
2.44%
19.51%
B
ABT199
Ibrutinib
PF3084014
RO4929097
IMD0354
Cell viability (%)
Drug Concentration (μM)
JVM3
Mec-1
Rec-1

Figure 1

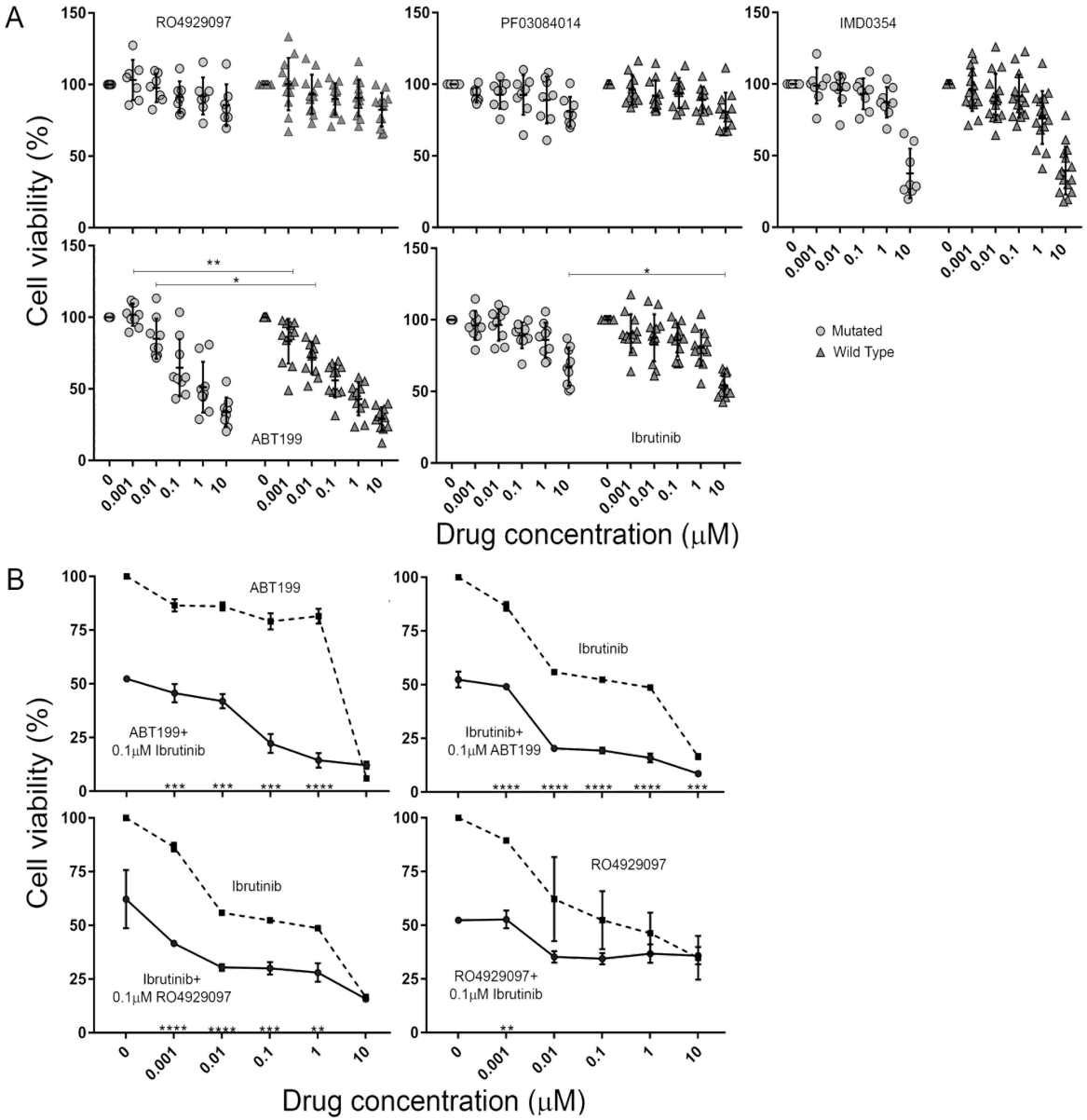
A
RO4929097
PF03084014
IMD0354
Cell viability (%)
ABT199
Ibrutinib
Mutated
Wild Type
Drug concentration (μM)
B
ABT199
ABT199+
0.1μM Ibrutinib
Ibrutinib
Ibrutinib+
0.1μM ABT199
Ibrutinib
Ibrutinib+
0.1μM RO4929097
RO4929097
RO4929097+
0.1μM Ibrutinib
Cell viability (%)
Drug concentration (μM)

Figure 2

SUPPLEMENTARY INFORMATION

	Random set	Trisomy 12 set
Number of patients	106	41
Female to male ratio	1:1.625	1:1.6
Median age at diagnosis	62 years	68 years
Age range	33 to 86 years	42 to 92 years
Mutated IgHV	60.82%	55.9%
Chromosomal abnormalities	85%	100%
13q del	46.43%	17.1%
Trisomy 12*	15.48%	100%
11q del	16.6%	2.4%
17p del	3.57%	2.4%
Other chromosomal abnormalities	16.67%	19.51%

Table S1. Characteristics of the sets of patient samples used in the study. Cytogenetic data was only available for 84 out of the 106 patients of the Random set.

NOTCH1 Primers		
Set 1	Forward	5'- ATGGCTACCTGTCAGACGTG
	Reverse	5'- TCTCCTGGGGCAGAATAGTG
Set 2	Forward	5'- GAGCTTCCTGAGTGGAGAGC
	Reverse	5'- CCTGGCTCTCAGAACTTGCT

Table S2. Primers used for sequencing NOTCH1 mutations.

Inhibitor	Target	Supplier
RO4929097	Gamma-Secretase Complex	Generon (A4005)
PF-03084014		Selleckchem (S8018)
ABT 199	BCL2	Selleckchem (S8048)
IMD 0354	NFκB pathway (IκBβ phosphorylation inhibitor)	Stratech (S2864)
Ibrutinib	BTK	Stratech (S2680)

Table S3. Targeted inhibitors used in this study.

	JVM3	Mec-1	Rec-1
RO4929097	>10μM	> 10μM	**5.51 μM**
PF 03084014	>10 μM	>10 μM	>10 μM
IMD 0354	5.05 μM	5.13 μM	**1.54 μM**
ABT 199	1.90 μM	4.57 μM	**4.67 μM**
Ibrutinib	>10 μM	4.65 μM	3.65 μM

Table S4. Table summarising the IC50 values for inhibitors tested in each cell line, with the values suggesting an increased sensitivity in NOTCH1 mutant cells highlighted in green and decreased sensitivity in red.

Constant inhibitor / Variable inhibitor	RO 4929097 (0.1uM)	PF 03084014 (0.1uM)	IMD 0354 (0.1uM)	ABT 199 (0.1uM)	Ibrutinib (0.1uM)
RO 4929097	**5.51**	>10	>10	5.53	1.41
PF 03084014	>10	**>10**	>10	5.56	<0.001
IMD 0354	4.23	3.51	**1.54**	3.9	0.54
ABT 199	2.76	2.99	4.93	**4.67**	<0.001
Ibrutinib	<0.001	1.57	3.28	<0.001	**3.6**

Table S5. Chart showing IC50 values (μM) of drugs in combination therapies in Rec-1, using a constant inhibitor at 0.1 μM (top row) and a variable inhibitor (left-hand column). IC50 values for each drug as a single agent are highlighted in bold (white background). Increased sensitivity to the variable drug in comparison to IC50 of the drug alone is highlighted in green, a decreased sensitivity in red, no relevant change in grey.

Variable inhibitor Constant inhibitor	RO	PF	IMD	ABT199	Ibrutinib
RO	n/s	n/s	n/s	0.815	0.068
PF	n/s	n/s	n/s	n/s	n/s
IMD	n/s	n/s	n/s	n/s	n/s
ABT199	4.4	n/s	n/s	n/s	0.032
Ibrutinib	0.436	n/s	n/s	0.082	n/s

Table S6. Combination Index (CI) values calculated using the CalcuSyn software. CI is used as an indication of synergy, as follows: < 0.1, Very strong synergism; 0.1–0.3, Strong synergism; 0.3–0.7, Synergism; 0.7–0.85, Moderate synergism; 0.85–0.9, Slight synergism; 0.90–1.1, Nearly additive; 1.1–1.2, Slight antagonism; 1.2–1.45, Moderate antagonism; 1.45–3.3, Antagonism; 3.3–10, Strong antagonism;>10, Very strong antagonism. In green, synergistic combinations. In red, antagonistic combinations. n/s: not significant.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. (A) Chromatograms showing sequencing of the NOTCH1 PEST domain. The 2 base pair CT deletion in the PEST domain (c.7541_7542delCT) shown by the red arrow was the most frequent (#1). The frameshift mutation causes amino acid change and a premature stop codon to be inserted. The mutation was heterozygous in 91.3% of the patients, as seen in this example. The CT deletion was also seen at a sub-clonal population level (#2) and as a homozygous mutation (#3). A chromatogram of a patient with wild-type NOTCH1 is shown as reference (#4). **(B)** Chromatogram showing NOTCH1 sequencing results of patient with a silent single cytosine (C) to thymine (T) nucleotide substitution (red arrow). **(C)** Chromatogram showing NOTCH1 sequencing of a patient with a 38 base pair deletion (c.6906-6943del CAGTTTGAATGG TCAATGCGAGTGGCTGTCCCGGCTGC) (top) and patient with c.6877insG (bottom). The original nucleotide sequence and the shifted sequence are shown below the chromoatograms.

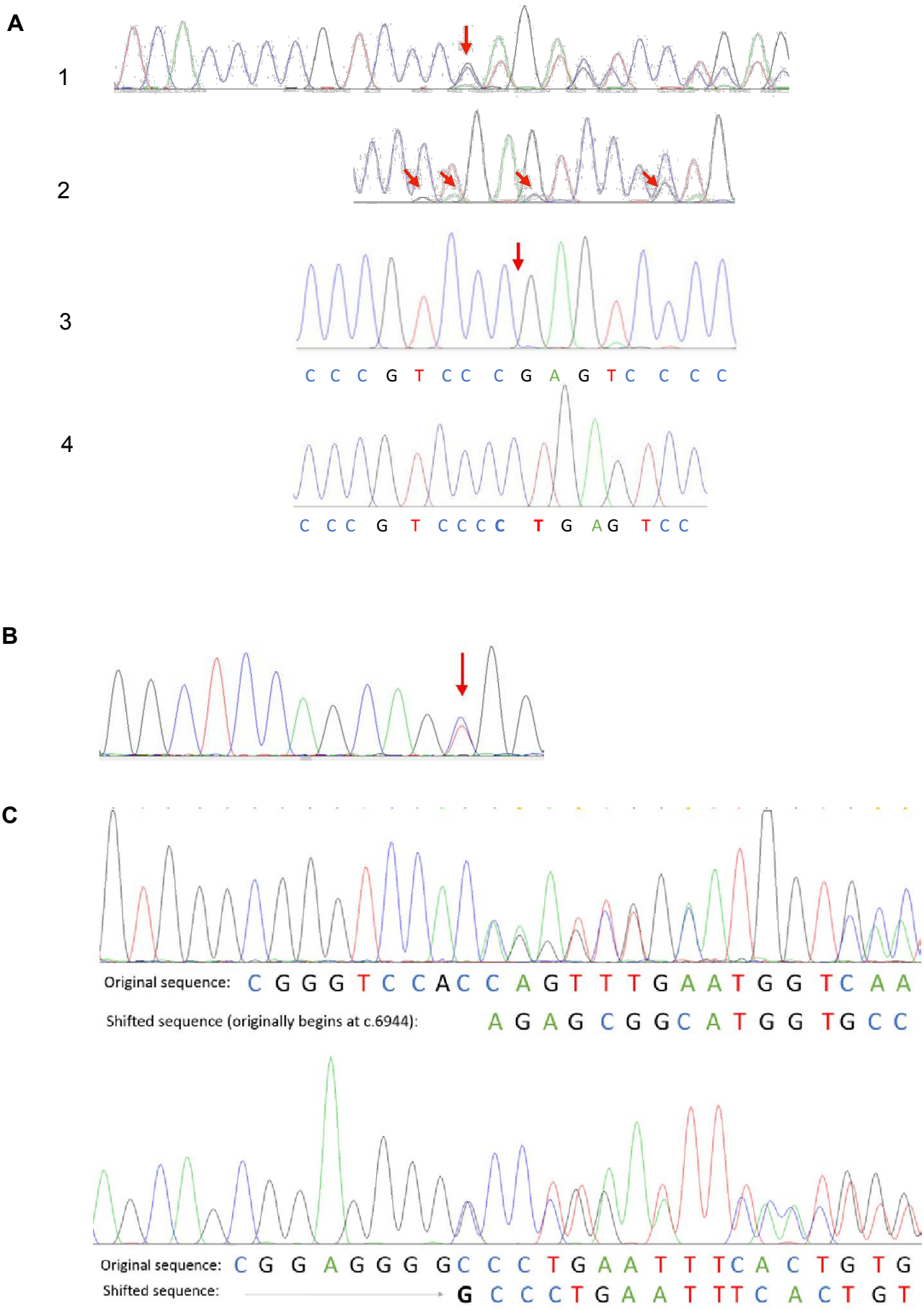
A
1
2
3
C C C G T C C C G A G T C C C C
4
C C C G T C C C C T G A G T C C
B
C
Original sequence: C G G G T C C A C C A G T T T G A A T G G T C A A
Shifted sequence (originally begins at c.6944): A G A G C G G C A T G G T G C C
Original sequence: C G G A G G G G C C C T G A A T T T C A C T G T G
Shifted sequence: G C C C T G A A T T T C A C T G T

Figure S1