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Norwegian University of
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Doctoral thesis

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Eksperimentelle og datavitenskapelige strategier for å identifisere synergistiske effekter av kreftmedisiner

Kombinasjoner av kreftmedisiner er forventet å forbedre effekten av behandling ved at disse kan svekke kreftens vekst gjennom flere ulike mekanismer. Synergistiske kombinasjoner, det vil si kombinasjoner som har større effekt enn hva man ville forvente ut fra effekten av enkeltmedisiner, er av spesiell interesse. Det store antallet av kjemoterapier og målrettede kreftlegemidler vil imidlertid innebære svært mange kombinasjoner og vanskeliggjør derfor identifisering av effektive kombinasjonsterapier. Arbeidet i denne avhandlingen omfatter (1) eksperimentelle høykapasitets-analyser som kan bidra til identifisering av effektive medikamentkombinasjoner i prekliniske laboratorieforsøk, og (2) strategier for å benytte datamodeller og datasimulering til å forutsi hvilke kombinasjoner som er attraktive for testing i en gitt celletype. Målet med doktorgraden er å danne et grunnlag for å teste eksperimentelt bare et utvalg av alle mulige kombinasjoner, men likevel finne så mange som mulig av de effektive kombinasjonene. Resultatene viser at våre datamodeller kan simulere kreftcellenes respons på kombinasjoner av kreftmedisiner på en måte som kan gi grunnlag for å redusere omfanget av eksperimentell screening og likevel identifisere flere synergistiske kombinasjoner. I tillegg presenterer jeg strategier som kan føre til forbedring av datamodell-genererte prediksjoner som igjen kan brukes som et grunnlag for videre forsking. Disse strategien omfatter optimalisering av datamodeller, av kalibreringsdata, og identifisering av datamodell-komponenter som er avgjørende i kalibreringsprosessen. Få kombinasjonsterapier som oppdages i laboratoriet blir godkjent som behandling for pasienter. En av grunnene til dette antas å være knyttet til at kreftsvulster i utilstrekkelig grad representeres i kreftmodellene som i dag brukes i preklinisk forskning: kreftcellelinjer dyrket som flate enkeltcellelag. En strategi for å forbedre prekliniske kreftmodeller, er å dyrke kreftcellelinjene i tredimensjonale sfæroider. Resultater i denne avhandlinga viser at medikament-kombinasjoner har ulik effekt i kreftcellekulturer dyrket som sfæroider sammenlignet med enkeltcellelag. Flere synergistiske kombinasjoner ble kun registrert i en av de to forskjellige dyrkningsmetoder for en og samme cellelinje.

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Abstract

Drug combinations are hoped to improve treatment response to anti-cancer drugs by targeting the cancers vulnerabilities at diverse trajectories. With a large number of approved agents of chemotherapeutic and targeted therapies and several anti-cancer drugs in development, the number of possible combinations extensively escalates. As drug combination effects are highly influenced by individual traits of the cancer or cancer model system, identification of effective compound combinations is challenging. High-throughput screening is used to uncover effective combination treatments by testing them in experimental systems relevant for a large range of cancer subtypes. However, efficient filtering of interesting combinations for testing, such as can be provided by predictive computational modelling, is needed to further economise screening efforts. In the scope of this thesis, we advanced the use of logical modelling to identify putative effective drug combinations for pre-clinical screening by demonstrating the successful calibration of cancer cell line specific models to predict drug combination effects for four different cell lines. Testing predictions against experimental observations for 153 drug combinations in our large drug combination screen, indicated that we could have reduced the screening load considerably and increased synergy detection rate among the proposed combinations by 2.6-fold. Improvements in modelling strategies contributed through the work in this thesis pertain to optimisation of model network topology, selection of baseline protein activity data for model calibration as well as to approaches that can be used to identify subsets of nodes in the model for which accurate acquisition of baseline activity data is most important.

Drug combination hits identified *in vitro* are met by low bench-to-bed translational efficiency, questioning the reliability of traditional planar (2D) cancer cell line cultures as model systems for drug screening. As part of this thesis, we have performed a high-throughput screen to systematically compare drug combination effects observed in planar (2D) and spherical (3D) cell line cultures. We identify combinations that act synergistically in only one but not the other culture mode. In spheroid cultures a lower number of synergistic drug combinations was identified with stronger dependency on MEK-signalling compared to 2D cultures. This indicates that future screening platforms should encompass more complex cancer models to capture a broader range of therapeutic synergy landscape as well as highlights the relevance of signalling dynamics and activities as markers for drug response.

This thesis offers strategies to support cell line specific logical modelling as a possible tool to economise pre-clinical screening efforts, and to enhance insights into design of high-quality high throughput combination screening for reliable detection of synergistic drug combination effects.

Acknowledgments

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List of Papers

Paper 1

Åsmund Flobak*, **Barbara Niederdorfer***, Vu To Nakstad, Liv Thommesen**, Geir Klinkenberg**, Astrid Lægreid**. A high-throughput drug combination screen of targeted small molecule inhibitors in cancer cell lines. Sci Data **6**, 237 (2019) doi:10.1038/s41597-019-0255-7

*These authors contributed equally to this work

**These authors jointly supervised this work

Paper 2

Barbara Niederdorfer, Vasundra Touré, Miguel Vazquez, Liv Thommesen, Martin Kuiper, Astrid Lægreid, Åsmund Flobak. Identification of strategies to enhance cell line specific drug synergy prediction using logic modeling.

Paper 3

Evelina Folkesson*, **Barbara Niederdorfer***, Vu To Nakstad, Liv Thommesen, Geir Klinkenberg, Astrid Lægreid, Åsmund Flobak. High-throughput screening identifies selective synergistic drug combinations in 2D and 3D colorectal cancer cell cultures

*These authors contributed equally to this work

Abbreviations

ctDNA	Circulating tumour DNA
CoLoMoTo	Consortium for Logical Models and Tools
CSS	Combination Sensitivity Score
DREAM project	Dialogue on Reverse Engineering Assessment and Methods project
EGFR	Epidermal growth factor receptor
HSA	Highest single agent
ICGC	International Cancer Genome Consortium
JNK	c-Jun N-terminal kinase
MRA	Modular response analysis
MS	Mass spectrometry
MuSyC	Multi-dimensional Synergy of Combinations
ODE	Ordinary Differential Equation
PD-1	Programmed death receptor-1
TCGA	The Cancer Genome Atlas
VEGF	Vascular endothelial growth factor
ZIP model	Zero Interaction Potency model

Introduction

With an aging population and increased prevalence of chronic diseases, there is an urgent need to develop effective and sustainable treatment options. Several decades of research have uncovered the broad landscape and complexity of many human diseases, including cancer, and it is apparent that treatment solutions must be developed beyond a "one size fits all" treatment option.

Different terms have been used to describe the current efforts in medicine that aim to improve diagnosis, prognosis, prevention and individualised therapy. Personalised medicine has become a buzzword and is often interchangeably used with precision medicine. P4 medicine (P4 = predictive, preventive, personalized and participatory) and systems medicine are aspiring similar goals, although they are not synonymous [1]. Personalised medicine seems to be a somewhat debatable term as medicine has always been focused towards providing "personalised" care for a specific patient. In 2013, Schleidgen et al. [2] performed a systematic review and came up with the following definition of this term offering a clearer understanding of the difference to traditional health care:

"[Personalised Medicine] *seeks to improve stratification and timing of health care by utilizing biological information and biomarkers on the level of molecular disease pathways, genetics, proteomics as well as metabolomics.*" [2]

While personalised medicine holds great promise to enable preventive and predictive care, concerns to its value and current strategies have been raised [3–5]. How will we identify which alterations associated with disease are clinically actionable? How can we select the right treatment? How do we ensure responsible data processing and sharing? While these are only a few of many challenges, they clearly point to the need for multi and cross- disciplinary efforts to provide good health care solutions [6].

Personalised medicine is pursued in all aspects of modern medicine. Hereafter this thesis will focus on the application of personalised medicine in oncology.

Personalised Oncology

Personalised oncology (reviewed in [7,8]) builds on the analysis of multiple data types to provide patient stratification. Efforts such as The Cancer Genome Atlas (TCGA) [9] and the International Cancer Genome Consortium (ICGC) [10] provide access to comprehensive data on patient tumour profiling, especially on the genomic and transcriptomic level. Systematic analysis of this data has greatly advanced our understanding of molecular processes in cancer, including amongst other the identification of driver genes [11] and shared molecular alterations across tumours types [12]. For a

comprehensive list of publications see [1]. Some of the identified alterations were found to be associated with survival and therapy response and could be implemented as biomarkers in the clinic. For example, an oestrogen receptor signature was established as an early indicator to identify breast cancer patients that require adjuvant therapy [13]. In recurrent ovarian cancer BRCA mutation status is used as an indicator for response to PARP inhibition as subsequent-line treatment [14]. The growing understanding of the clinical relevance of identified molecular alterations as possible actionable entities in the context of patient-specific molecular profiles has led to the emergence of mechanistically motivated therapies, known as targeted therapies.

Targeted Therapies

Targeted therapies are designed to target molecular alterations in cancer cells [7]. In contrast, chemotherapy and radiotherapy target all fast-growing cells. Immunotherapies are a recent addition, aiming at potentiating anti-tumour immunity [15].

Targeted therapies are mainly focused on monoclonal antibodies and small molecule kinase inhibitors. Monoclonal antibodies typically target extracellular proteins and interfere with cellular signalling by blocking the interaction between receptors and ligands. This can be mediated either through direct or indirect mechanisms. Small molecule inhibitors on the other hand are low molecular weight compounds (< 900 Da) able to diffuse into cells and block intracellular proteins [16]. Kinase inhibitors, a sub-class of small molecule inhibitors, can roughly be classified as (1) Type I inhibitors that competitively bind the ATP-binding pocket of proteins, and (2) Type II inhibitors which are non-ATP competitive inhibitors inducing a conformational change in the target [17]. For instance, the ATP-competitive kinase inhibitor vemurafenib targets V600E mutant BRAF. Other approved inhibitors include small molecule inhibitors pabloclicib and ribociclib targeting CDK4/6, and monoclonal antibodies pembrolizumab, targeting the immune checkpoints, and cemiplimab, targeting human programmed death receptor-1 (PD-1) [16,18].

While small molecule inhibitors are designed to target specific proteins, many of them target more than one protein due to their unspecific mode of action. This promiscuous behaviour is in part due to many kinase inhibitors targeting the highly conserved ATP-binding pocket. Whereas characterisation of inhibitor target profiles is crucial to uncover and understand their molecular and phenotypic actions, such characterisation is not yet routinely done in-depth for many of the clinically used or investigational kinase inhibitors [19]. This has also been recently highlighted by Lin et al. [20], showing

[1] https://gdc.cancer.gov/about-data/publications - last accessed 18/09/2019

that a panel of investigational and pre-clinical compounds exert phenotypic effects by off-target mode of action.

Existing kinase inhibitor profiling has mostly been performed using competition binding assays measuring physical interaction of a compound with a protein [19,21,22], or by biochemical assays measuring phosphotransferase activity of a tested kinase with the use of radioactive-labelled ATP. In the latter type of assays, the inhibition effects of Type I kinase inhibitors, i.e. ATP competitive inhibitors, can be more directly quantified. This method is applied by the MRC Protein Phosphorylation Unit Databases evaluating target profiles of Type I kinase inhibitors [23–25] and has also been used by others [26,27]. Database resources collecting target and bioassay information on compounds include www.chemicalprobes.org, PubChem [28], ChEMBL [29,30], and Library of Integrated Network-based Cellular Signatures (LINCS, http://lincs.hms.harvard.edu/kinomescan).

Targeted Therapies in Clinical Trials

Several clinical trials evaluate the applicability of targeted therapies and assess clinical feasibility of molecular profiling. In so-called basket-trials, treatment is given based on mutation status independent of cancer type. In contrast, in umbrella trials patients with the same cancer type but different mutations are treated with biomarker-informed drugs [7]. Trials evaluating clinical benefit of biomarker guided targeted therapies are among others:

- Initiative for Molecular Profiling in Advanced Cancer Therapy (**IMPACT**, NCT00851032, estimated completion: February 2021)
- **IMPACT/**Community Molecular Profiling in Advanced Cancers Trial (**COMPACT**, NCT01505400, estimated completion: February 2020) [31]
- Study of Personalized Cancer Therapy to Determine Response and Toxicity (UCSD_**PREDICT**, NCT02478931, estimated completion: September 2020) [32]
- Study of Molecular Profile-Related Evidence to Determine Individualized Therapy for Advanced or Poor Prognosis Cancers (**I-PREDICT**, NCT02534675, estimated completion: February 2025) [33]
- **Genomic Profiling in Phase I** (NCT02437617, estimated completion: July, 2020) [34]
- **Molecular Profiling Protocol** (SCRI-CA-001, NCT00530192, completed) [35]
- Molecular Screening for Cancer Treatment Optimization (**MOSCATO 01** [36], and **MOSCATO 02**, NCT01566019, estimated completion: October, 2019)
- **WINTHER** (NCT01856296, completed, no results available) [37,38]
- A Randomized Phase II Trial Comparing Therapy Based on Tumor Molecular Profiling Versus Conventional Therapy in Patients With Refractory Cancer (**SHIVA**, NCT01771458) [39]

- National Cancer Institute Molecular Profiling-Based Targeted Therapy in Treating Patients With Advanced Solid Tumors (**NCI-MPACT**, NCT01827384, estimated completion: May 2020) [40]
- Molecular Analysis for Therapy Choice (**MATCH**, NCT02465060, estimated completion: June 2022)
- N-of-1 Trial: Actionable Target Identification in Metastatic Cancer for Palliative Systemic Therapy (**MetAction**, NCT02142036, estimated completion: January 2022)
- LEE011 for Patients with CDK4/6 Pathway Activated Tumors (**SIGNATURE**, NCT02187783, completed)
- The Drug Rediscovery Protocol (DRUP Trial, **DRUP**, NCT02925234, estimated completion: December 2019)
- CPCT-02 Biopsy Protocol (CPCT-02, NCT01855477, estimated completion: August 2020)

While most of these trials are still ongoing, some few and sobering results have already been presented. The first comparative randomised trial with the aim of assessing the use of molecular matched therapy was the SHIVA trial. Approved targeted therapy was given to heavily pre-treated groups of patients outside of their indicated cancer type (off-label drug use) and based on genomic alterations in three signalling pathways (hormone receptor-, PI3K/AKT- and RAF/MEK- pathway). Progression-free survival did not differ between patients receiving matched therapy versus patients treated according to physicians choice [39]. Also in the IMPACT/COMPACT, PREDICT and Genomic Profiling in Phase I trial no statistical difference in overall survival was observed between patients in genotype-matched treatment group vs un-matched group [31,32,34].

SHIVA has been criticized for weak or incorrect drug-patient matches based on the present biomarker profile. Specifically the drug Everolimus, a mTORC1 inhibitor, was denounced as it only weakly affects the PI3K/AKT/mTOR pathway [41,42], as pointed out by the Le Tourneau et al. themselves. Another point of criticism was the use of only monotherapy rather than combination treatments. In cases in which several molecular alterations potentially relevant for treatment decision were identified, the authors tried to account for this by giving treatment based on the molecular target deemed to be the most clinically relevant by the Molecular Biology Board [39]. While this may account for mutations in the same pathway, this cannot account for mutations in parallel pathways, e.g. PI3K/AKT- and RAF/MEK- pathway, which potentially confer resistance to chosen therapy.

In the Genomic Profiling in Phase I study [34] each patient was assigned a matching score, quantifying the number of matched agents to number of detected gene alterations in order to account for confidence of assigned treatment. Treatments were further categorised into matched-direct

treatment (e.g.: BRAF inhibitor for patient with BRAF mutation) or matched-indirect treatment (e.g.: a PI3K inhibitor for patient with PTEN mutation), including both single and combination treatment. While this clinical trial could not document significant difference in overall survival and time-to-treatment failure between direct and indirect matches, patients with higher matching score showed significantly higher median time-to-treatment failure compared to patients with lower matching score, indicating that tailored targeted therapy may give clinical benefit when treatment is accounting for multiple alterations and high confidence drug-to-biomarker matches.

As tumours are not strictly comparable between patients, thus rendering direct comparison of treatment benefit impossible, several trials have started to compare the new treatment to the previous treatment in the same patient, i.e. using the patients as their own control. Using this approach, both in the Molecular Profiling Protocol study [35] and the MOSCATO 1 trial [36] the authors concluded that matched therapy improved progression-free survival in at least a subset of patients. Also, in the I-PREDICT study patient-free survival increased when comparing matched therapy to last prior treatment in patients with a high matching score of given treatment [33].

While one might be discouraged by these results, it is important to consider that (1) most of the included patients have advanced and heavily pre-treated diseases, (2) most of the above-mentioned studies perform tumour profiling solely by DNA sequencing, and (3) most of the above-mentioned studies solely relied on the use of monotherapies. Further, in order to generate comparable, interpretable and reproducible results from these clinical trials, some common guidelines should be followed when planning the studies [43,44], as well as when quantifying treatment benefit.

Le Tourneau et al. [43] also accentuate that biopsy samples used for biomarker assessment only represent one section of the tumour and hence possibly only a part of the mutational landscape Alternative profiling methods like liquid biopsies encompassing the analysis of circulating tumour cells, circulating tumour DNA (ctDNA) [45] and extracellular vesicles, including exosomes, are of interest as complementary strategies to tumour biopsies and further allow for continuous monitoring. While blood-based cancer diagnostic on exosomes are available [46], there are still several unknowns when it comes to the composition and functional role of the different types of extracellular vesicles [47,48]. Feasibility of molecular profiling based on ctDNA was recently evaluated by the TARGET (Tumour chARacterisation to Guide Experimental Targeted therapy) study reporting a high overlap between mutations detected in tumour samples and ctDNA [49].

Cell Signalling in Personalised Oncology

Since nearly all targeted therapies are directed against signalling molecules, the importance of signalling biology in cancer is apparent. This is also pointed out by Yaffe [50] who states that “cancer

is primarily a signalling disease". Various signalling pathways, like PI3K/AKT- and RTK/RAS/MAPK, have been flagged for their relevance in cancer development and progression. Well known oncogenes in these pathways include the epidermal growth factor receptor (EGFR), KRAS and BRAF, associated with the RTK/RAS/MAPK signalling pathway, also being used as biomarkers, and PI3KCA, which is associated with the PI3K signalling pathway [51]. As illustrated in Figure 1, mutations can be detected in several members of the signalling pathway, although they vary in frequency between different patients and tumour types. Specific alterations, such as V600E in BRAF leading to constitutively active BRAF, can confer sustained growth-promoting signalling, which is one of the hallmarks of cancer [52]. Cancer cells show altered signalling dynamics arising from gene mutations but also other factors eliciting cancer-promoting rewiring of signalling transduction [50,52]. Pharmacological targeting of signalling pathways aims at blocking the cancer hallmark capabilities. However, due to high signalling crosstalk, where signals can be integrated at different crossing points, it can be conceived that blocking only single protein classes may result in no or only temporary response to treatment.

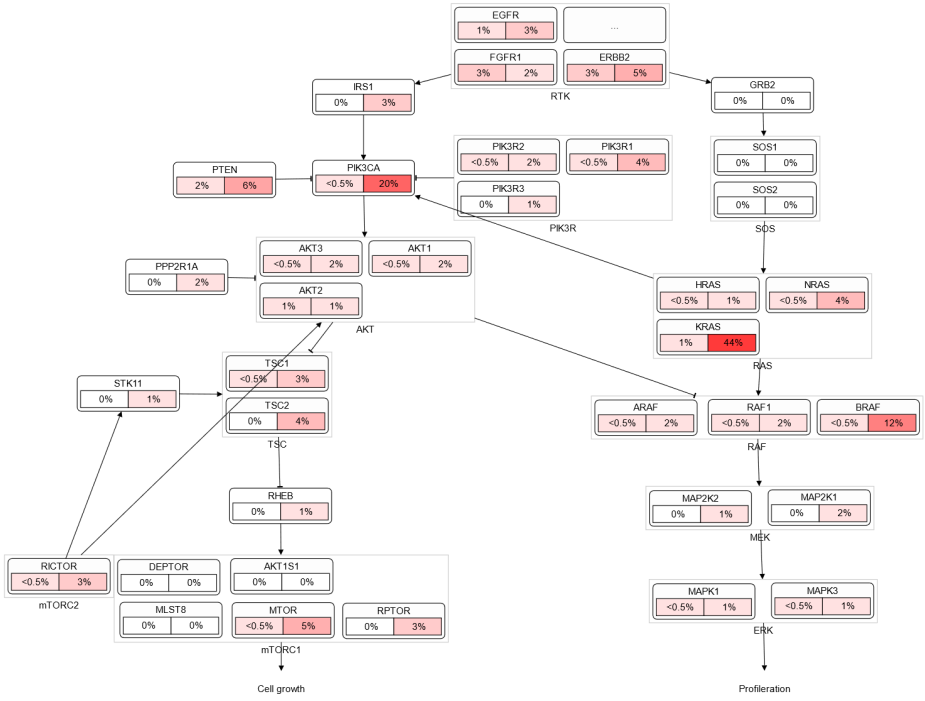

Figure 1 – Overview over the PI-3-Kinase and receptor-tyrosine kinase (RTK)/RAS/MAP-Kinase signalling pathway. Frequency of copy number alteration and mutation data from a metastatic colorectal cancer study [53] **are indicated below the respective genes. The figure was generated using PathwayMapper** [54]**.**

Drug Combinations – finding a needle in a haystack

Combination therapies were incorporated at an early stage into clinical practice, albeit comprising mainly chemotherapeutic agents. Rationales for combination of treatments are (1) to increase the effect of a treatment thus enabling reduced dosage and reduced toxicity, (2) to protect against off-target effects by another drug, (3) to attack multiple sites of a signalling pathway thus reducing the chance for resistance development and to (4) more efficiently targeting intra-tumour heterogeneity [55]. Improved observed effects of combination therapies might also be assigned to inter-tumour heterogeneity between patients purely based on a higher probability of patients responding to treatment [56]. In oncology, combination therapies have mainly focused on combined application of different chemotherapies, such as gemcitabine together with cisplatin a standard treatment for tumours such as bladder cancer and non-small cell lung cancer [55].

With the rise of small molecule inhibitors, combination treatments comprising of only targeted inhibitors or chemotherapies in combination with targeted drugs have become a research focus. A well-known example is the combination of different MEK inhibitors and BRAF inhibitors for the treatment of malignant melanoma [57–59] and lung adenocarcinoma [60]. Other clinically approved combinations include amongst others the monoclonal antibody bevacizumab against human vascular endothelial growth factor (VEGF) in combination with erlotinib, an inhibitor of EGFR for advanced lung cancer [61], and the combination of nab-paclitaxel (albumin-bound paclitaxel), a chemotherapeutic agent, in combination with atezolizumab, a monoclonal antibody against human programmed death ligand-1, for treatment of triple-negative breast cancer [62]. As discussed by Lopez and Banerji [63], successful implementation of combinations of targeted therapies in the clinic faces two major challenges; (1) Identification of effective combinations and (2) successful implementation of the treatment in the clinic.

Cancer Models for Drug Screening

A major bottleneck in the identification of effective drug combinations is the large number of targeted treatments that are possible with drugs available or currently in development [63]. For example, if one considers testing pairwise drug combinations of only the approximately 90 FDA-approved small molecule inhibitors for treatment of different cancer types[2], one would need to test 4 005 combinations. The number of possible drug combinations rises exponentially with increased number of compounds and with an even steeper curve if one considers higher order combinations. Hence, we need to rely on experimental systems that show characteristics similar to a patient's disease to test

[2] Targeted Cancer Therapies by the National Cancer Institute - https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet (last accessed 02/10/2019)

and prioritize drug combinations. Over the years, different experimental systems have been established to test sensitivity to drugs in single or combinatorial application and to infer markers for drug sensitivity and resistance (Box 1, Figure 2).

Box 1 – Cancer models

Established Cancer Cell Lines

Several well characterised cancer cell lines, like the NCI-60 panel, have been used over the years for single and combination drug screening and biomarker discovery [64–66]. Cancer cell lines can be easily cultivated and grown in large quantities over time. However, this has also led to selective homogeneity within the cell line population. Nevertheless, there is supporting evidence that a sufficiently large number of cell lines captures clinically relevant alterations and that pharmacogenomic screens can lead to the identification of putative drug sensitivity markers [51].

To better represent the tissue architecture of a tumour, methodologies to work with 3D cell cultures, also called spheroid cultures, or xenograft models have been established. Spheroid cultures are *in vitro* models that more closely mimic human tumours compared to planar cell cultures, e.g. by displaying different "zones" of cells dominated by either proliferative, quiescent or necrotic cells (reviewed in [67]). Studies comparing spheroid against monolayer (2D) cultures comprise report on comparable [68], reduced [69,70] or increased sensitivity [68,70,71] to drug treatments as well as well as changes in cellular signalling [69,71–75].

Patient-Derived Cancer Models

Patient-derived cancer models better represent the intratumor heterogeneity than established cancer cell lines. Patient-derived xenograft models, generated by inoculating or engrafting either patient-derived cell lines or tumour tissue, have been shown to faithfully recapitulate human tumour biology and been successfully applied in drug testing (example [76]). As maintenance of patient-derived xenograft models is high and material restrictions limit applicability for large-scale screens, also patient-derived xenograft cell lines have been established [77,78]. However, the initiation and propagation of patient-derived xenografts is demanding and time consuming with 2-4 months for establishment alone (reviewed in [79]).

A practical alternative to patient-derived xenografts are spheroid or organoid cultures of patient-derived cell lines or tumour tissue [80–84]. While there is no defined nomenclature for different 3D cultured models, spheroids are generally referred to as aggregates of cells grown in non-adherent conditions. The term organoid on the other hand describes mini-organlike structures containing multiple self-organised cell types [67,85].

Other Animal Models

Other categories of animal models besides cancer xenograft models generated by inoculating or engrafting cancer cell lines, comprise environmentally induced or genetically engineered animal models. Here cancer development is brought about by environmental factors like radiation or pathogens, or by genetic engineering using gene-targeting methodology to for example generate tumour suppressor knockout animals (reviewed in [86] for mouse).

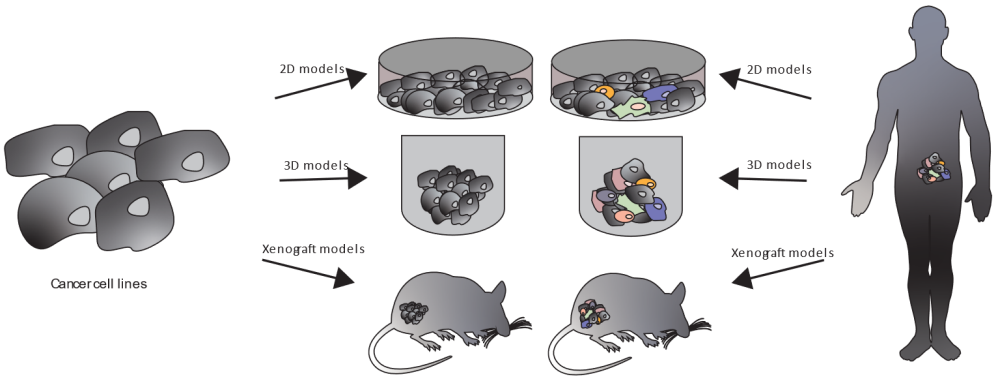

Figure 2 - Schematic representation of different cancer models.

Drug combination screens

For discovery of new effective drug combinations, high-throughput screens testing a large panel of compounds are performed, typically on cancer cell lines [63]. Drug combinations can be screened in an unbiased manner, i.e. all drug combinations are usually tested against each other (Table 1). Alternatively, high-throughput screens may be hypothesis-driven, i.e. a preselected inhibitor or a predefined molecular target is inhibited with one or several inhibitors and tested in combination with a drug library (examples [87–89]). Tested drug libraries often comprise a mixture of compound classes such as small molecule inhibitors and chemotherapeutic agents. Attractive targets for combination treatment may also be identified using RNAi (examples [90–93]), or CRIPR-based screens (examples [94,95]).

Table 1 – Overview of unbiased high-throughput drug combination screens in 2D cancer cell line models. Abbreviations: Sulphorhodamine B (SRB), Cyclin-dependent kinase 2 (CDK2), acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), fluorometric microculture cytotoxicity assay (FMCA). * In the study by Menden et al., a dataset of 137 screened cell lines was generated of which 85 cell lines had complete genomic data upon release of the study.

Study	Cell lines	Nr. of compounds and screening format	Read out	Ref.
Holbeck et al.	59 cell lines (NCI-60 panel)	105 drugs tested in 5,232 pairwise combinations at 5x3 or 3x3 matrix	Growth inhibition using either SRB or CellTiter-Glo® assay after 48h compared to 0h	[96]
Menden et al.	137* cancer cell lines	118 drugs tested in 910 combinations at 5x5 matrix	Cytotoxicity using SYTOX® Green after 120h	[97]
O'Neil et al.	39 cancer cell lines	38 drugs tested in 583 combinations at 4x4 matrix	Viability using CellTiter-Glo® after 96h	[98]

Study	Cell lines	Nr. of compounds and screening format	Read out	Ref.
Licciardello et al.	1 CML cell line	Primary screen: 308 drugs at 40,160 combinations at 1x1 Secondary screen: 20 combinations at 4x4 matrix	Viability using CellTiter-Glo® after 72h	[99]
Friedman et al.	36 melanoma cell lines	108 compounds in 5,778 combinations at 2 fixed-dose ratio rays	Cytotoxicity by high-content imaging after 72h	[100]
Langdon et al.	11 pancreatic carcinoma cell lines	40 compounds in 780 combinations at 3x3 matrix	Viability using CellTiter-Glo® after 72h	[101]

As alternatives to 2D growing cancer cell lines, drug combination screens have been performed in other cancer models. This includes screens performed in patient-derived xenografts [76], patient organoid cultures [81,102], cell line spheroid cultures [103] and transgenic animal models [104]. Limited patient material and a high number of potential drug combinations hamper the use of patient-derived screening models for comprehensive drug combination screening. However, the establishment of patient-derived xenograft and organoid libraries [77,105–109] as well as the development of techniques requiring low assay volume, such as presented by Eduati et al. [110], will allow large scale combination testing in patient-derived samples.

Screening format

Drug combination studies have been performed using several different designs, as is exemplified in Table 1. Cells may be exposed to compounds for different durations ranging usually from 48h – 120h and monitored with a variety of read-outs. Drugs may be applied at different dose combinations applied in e.g. a ray or matrix design (Figure 3). In the latter all possible combinations of drug concentrations are tested in an exhaustive screen. For example, if Drug A is tested at concentrations 0.5µM and 1µM and Drug B at 2µM and 5µM, the drug combination would result in four possible drug-concentration combinations. In a ray design either one drug is used as a fixed concentration, for example at IC50, while continuous doses of the other drug are applied. Alternatively, both doses are varied in each dose-combination. This would result in two dose concentrations in the above-mentioned example of Drug A and Drug B. While exploring a dose-response matrix provides a more comprehensive sampling of the combination, the number of possible data points increase exponentially with increasing number of drugs, samples and doses. For example, if one wanted to test eight cell lines with 19 compounds in all possible pairwise combinations, i.e. 171 combination, at five

doses a matrix design, this would result in 34 960 samples. In contrast, a ray design would result in 7 600 samples, i.e. approximately 22% of a complete matrix.

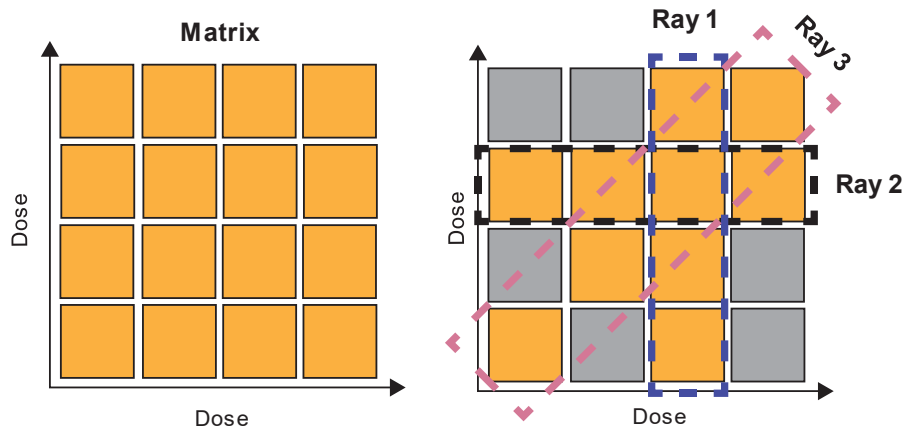

Figure 3 – Illustration of matrix and ray designed drug combination screen. For the ray design different vector of the dose-response matrix may be chosen as indicated by ray 1-3.

Metrics for Synergy Quantification

Different terminologies are used to describe drug combination effects as discussed in [111,112]. Generally, one distinguishes between synergy, additive and antagonistic effects. A combination is defined to act synergistically if the observed combination effect is greater than the expected additive effect. An antagonistic effect describes the effect being smaller than what is expected based on observed effects of each of the drugs alone. Definition of the expected combination effect differ depending on the "null" reference model used [113]. Numerous models having been proposed over time for the quantification of drug combination effects, with no general agreement on a standard reference model. Popular models comprise the Loewe additivity [114,115], Bliss independence [116], Highest single agent (HSA) model [117], discussed as followed and also reviewed in [111,112,118].

Loewe additivity

The Loewe additivity model is based on the "sham mixture" experiment where a single drug is mixed with itself and no interaction effect should be observed. The model assumes the dose equivalence principle, meaning that one can achieve the same effect of a drug A with drug B at a certain dose of drug B and vice versa. Synergism, antagonism or additivity using the Loewe additivity model are calculated according to formula (1), where $a_{E(x)}$ and $b_{E(x)}$ are doses of drug A and B that produce an effect $E(x)$. The dose used of each of the single drugs that reaches the same effect $E(x)$ in the combination is described by $ap_{E(x)}$ and $bp_{E(x)}$, respectively. In other words, how much of drug A in the combination is needed to achieve the same effect as observed at a certain dose of drug A alone and vice versa for drug B.

$$\frac{a_{E(x)}}{ap_{E(x)}} + \frac{b_{E(x)}}{bp_{E(x)}} \begin{cases} = 1, \text{Additivity} \\ < 1, \text{Synergy} \\ > 1, \text{Anatagonism} \end{cases} \quad \textbf{(1)}$$

The Loewe model may be extended to quantify drug combination effects of more than pairwise combinations. The model relies on an accurate estimation of dose response curve, commonly modelled using the Hill model. Here the dose response relationship is modelled according to formula (2) where d is the dose of a drug and m is the slope of the curve. For the dose principle to hold when using the Hill model, a constant potency ratio, reflected in parallel log dose response curves, is assumed [119]. Thus, the potency ratio is the same of any point on the dose response curves of drug A and drug B. However, some compounds may not elicit a single-agent activity but show enhance effect in combination. This is for example intended for the use of a checkpoint kinase 1 inhibitors in combination with chemotherapy drugs [120].

$$E = \frac{E_{max}\left(\frac{d}{IC_{50}}\right)^{m}}{1 + \left(\frac{d}{IC_{50}}\right)^{m}} \quad \textbf{(2)}$$

Bliss independence

The Bliss independence is based on the theory of noninteraction between each of the drugs in a combination. The model assumes that a second drug B can only act on the unaffected fraction of a drug B. Thus, the expected affected fraction, e.g. % of killed cells, of a drug combination fa_{A+B} can be calculated according to formula (3), where fa_A is the affected fraction by drug A and fa_B is the affected fraction by drug B. As affected and unaffected fraction, e.g. % of viable cells, are complementary, i.e. $fa + fu = 1$, formula (3) can be written as formula (4) when measuring the unaffected population.

$$fa_{A+B} = fa_A + fa_B - fa_A * fa_B \quad \textbf{(3)}$$

$$fu_{A+B} = fu_A * fu_B \quad \textbf{(4)}$$

Whenever the observe effect of a drug combination is greater than the expected effect, or in other words when a higher fraction of the population is affected, then synergy is called. A drawback of the Bliss independence model is that is does not work with the “sham experiment” but results in a synergistic response if the drug shows a steep dose response curve.

Highest single agent

The HSA model, also known as Gaddum's non-interaction model, states that a combination is synergistic if the effect is greater than that achieved by any of the single drugs alone, resulting in formula (5). As for the Bliss independence model, whenever the observed effect of a combination is greater than the expected effect, the combination acts synergistically.

$$E_{a+b} = max(E_a, E_b) \quad \textbf{(5)}$$

Several extensions to the above-mentioned reference models have been proposed over the years. These include a combination of the mentioned models [121], Chou and Talalay's model [122,123], Multi-dimensional Synergy of Combinations (MuSyC) [124], Combination Sensitivity Score (CSS) [125], and the Zero Interaction Potency (ZIP) model [126].

From Bench to Bedside – Challenges in Personalised Oncology

Databases, such as the Cancer Genome Interpreter [127] and OncoKB [128] have curated information on biomarkers, mostly genomic alterations, associated with treatment responses for certain cancer types. As can be seen from these resources, little of the currently available massive tumour profiling data has been rendered actionable for clinical decision making. For example, of the listed biomarkers in the Cancer Genome Interpreter, only 91 alterations in 31 different genes/proteins are predictive of responsiveness to given drug(s), i.e. actionable event, per tumour type according to FDA guidelines[3]. These include BRAF (V600E), mutation in BRCA1 and 2 as well as different mutations in KRAS or EGFR. As few predictive biomarkers are available, similar or identical treatments are given to large patient groups, effectively resulting in a "one-size-fits-all" approach. This is especially problematic in highly heterogenous diseases such as pancreatic and colorectal cancer, where more biomarkers predictive of treatment response are urgently needed (reviewed in [129,130]).

Decoding Molecular Alterations

Molecular alterations associated with cancer, such as those identified from -omics analysis, may exert different effects, from having no function at all to inactivation of tumour suppressors or activation of oncogenes, with a large fraction of alterations not associated with any known functional significance. Understanding the molecular function of different tumour alterations can amongst others contribute to further identification of critical traits for biomarker-guided treatment. For example, in a follow up to the SHIVA study [39], the effect of several RTK/RAS/MAPK pathway protein variants of unknown significance were evaluated by *in vitro* reporter assay. Patients with positive survival predictions

[3] https://www.cancergenomeinterpreter.org/biomarkers - last accessed 02/10/2019

inferred from the *in vitro* responses to molecular agents showed significantly higher median progression-free survival than patients with negative predictions [131].

While the same actionable event may be shared among different cancers [11], due to distinct genetic background, context-specific signalling and other molecular features of individual tumours, these modifications can manifest in different treatment responses (reviewed in [132]). A well know example is the use of the BRAF inhibitors in BRAF mutant melanoma patients compared to BRAF mutant colorectal cancer patients. While melanoma patients with this mutation generally show initial response to this treatment, colorectal cancer patients harbouring the same mutations typically show no or limited treatment response. This treatment failure has been associated with activation of the EGFR receptor via feedback signalling. Due to the fact that this receptor is generally weakly expressed in melanoma tumours, inhibition of BRAF will not cause activation of EGFR as has been observed in colorectal cancer cells, and thus EGFR-triggered cancer growth is not observed [132,133].

Prospective of Phosphoproteomics profiling

Posttranslational modification of proteins, such as phosphorylation, is central in triggering molecular responses by relaying cellular signalling through the signalling network. Advances in mass spectrometry (MS) have opened up new avenues for assessment of signal transduction by shotgun phosphoproteomics. By enabling more direct and bulk quantification of protein kinase phosphorylation status without the need for antibodies, the phosphorylation status of thousands of phosphorylated peptides can be measured [134–136]. However, phosphoproteomics guided biomarker discovery is hampered by challenges related to reproducibility, sensitivity and functional characterization of phosphosites. The latter, necessary for inferring kinase activity status, is often based on kinase-substrate relationship information obtained from different databases and thus relies heavily on curated prior knowledge. Largely a distinct set of phosphorylated peptides is detected in each biological replicate due to stochastic sampling effects, related to the transient nature of phosphorylation's, making reliable quantification challenging [137,138].

Based on the idea of redundancy in cellular information fostered by proteins acting in signalling networks, targeted MS is pursued. While reducing the breadth of the analysis, by measuring a preselected subset of proteins and phosphosites using labelled peptides, robust and accurate quantification of selected phosphorylation sites can be achieved with small sample concentration. [139–141]. However, the selection of proteins to study by targeted MS still suffers under the bias for well-studied kinases as only in some cases, phosphorylation site-status has been deduced to protein activity. Needham et al. [137] indicated that there are many relevant phosphoproteins which have not yet been identified. Thus, there is a trade-off between quantity and quality; either one focuses on

studying few proteins for which activity can be accurately measured and inferred or one studies many to have a greater picture, albeit at reduced resolution.

Systems Medicine for Personalised Diagnosis and Treatment

In 2013, Yaffe stated that "the scientific community seem to be addicted to sequencing", calling for a combined genomic, proteomic and signalling analysis to advance interpretation capacity for improved cancer therapy [142]. A number of ongoing efforts use a systems medicine approach in order to infer drug response from tumour profiles [64,66,143]. While systems medicine pursues similar goals as personalised medicine, the former term reflects the use of a systems biology approach to understand and treat diseases. A system can be understood as a set of interacting objects that work together to make it function. Thus beyond studying multiple data types, e.g. genomics and proteomics, to provide patient stratification, the systems approach also focuses on strategies to tackle mechanisms and consequences of underlying molecular interactions [1].

Network Medicine

A focus area of systems medicine is network medicine, a term first introduced by Barabási in 2007 [144]. By studying causal and molecular interactions, network analysis allows for discovery of cancer driver genes and disease modules and pathways. Network medicine is hoped to advance personalised medicine by bridging the gap between the disease phenotype and disease-associated molecular alterations (molecular phenotypes) identified by -omics analysis [145–147]. For example, Hofree et al. [148] performed tumour stratification using mutation profiles guided by network information. This network-based stratification led to identification of tumour subtypes predictive of patient's survival for uterine, ovarian and lung cancers, while consensus clustering failed at identifying such subgroups. In another similar study by Wang et al. [149], network-based stratification was either highly correlated, weakly corresponding, or completely different compared to consensus tumour subtyping. This indicates that by studying mutations with respect to interaction networks, additional or new stratification clusters of prognostic value can be revealed.

The Connectivity Map project [150], extended by the NIH Library of Integrated Network-Based Cellular Signatures Program [151], aims at decoding the function of disease-associated genetic events by studying cellular signatures after systematic perturbations. To optimise this, the L1000 assay was developed measuring the mRNA abundance of 978 so-called landmark genes [152]. Identified drug perturbation signatures under the Connectivity Map initiative can be employed to determine associations and connections between diseases, drugs, genes and pathways (reviewed in [153]). Comparable to the L1000 Connectivity Map project, Abelin et al. [140] presented the P100 set for phosphopeptides to study the effects of molecular perturbations on protein phosphorylation involved

in cell signalling. Litichevskiy and colleagues [154] have recently used the P100 set together with the L1000-assay and global chromatin profiling to generate perturbation signatures to 90 small-molecule compounds. Disagreements between the different assays was often related to the mode of action of the tested compound. For example, for compounds of the class histone modifiers, positive connectivity was mostly inferred in the P100 dataset, while negative connectivity was frequently seen in the global chromatin profile. Thus, while each of these assays can give important biological insights, the best readout depends on the mode of action of tested compounds.

Prior Knowledge for Network Biology

The foundation for network-driven approaches in systems medicine is prior knowledge of causal and molecular interaction. Such knowledge is available in a comprehensive number of databases and scientific publications, fortifying network medicine approaches and generation of mechanistic models. Annotated pathways and causal interaction are based on manual curation, text mining and computed associations from high- or low-throughput data.

Databases with annotated causal interactions and pathways include (for a comprehensive list see [4]):

- *The SIGnaling Network Open Resource* (**SIGNOR**); reports on manually-annotated causal relationships between biological entities [155,156]
- **PhosphoSitePlus** [157]; entity focused database that has amongst others curated kinase-substrate relationships
- *Kyoto Encyclopedia of Genes and Genomes* (**KEGG**) Pathway; collection of manually created signalling pathways [158]
- The **Reactome** Knowledgebase; curated and peer-reviewed database of pathways [159]
- *Atlas of cancer signaling networks* (**ACSN**); collection of cancer-related signalling and metabolic pathways [160]
- **Pathway Commons;** combined knowledge from several resources including KEGG and Reactome [161]
- **IntAct** molecular interaction database [162]
- **SignaLink 2;** entity focused data base that incorporated pathway structure information thus reporting on multi-pathway proteins and connections between pathways [163,164]
- **OmniPath;** collecting data, information and knowledge from several resources [165]

[4] http://www.pathguide.org/ - last accessed 20/09/2019

Graph-based Properties

The field of network medicine has both contributed and sourced from biological network knowledge. Networks can be broadly classified into causal and association networks. Causal interaction between two entities, such as proteins and genes, reflect directed functional reactions having a cause and an effect. Associations on the other hand merely capture functional relatedness and correlative behaviour without the need for direct information transfer.

The study of network properties (Box 2), such as topological features, can give important insights into regulatory mechanisms and reveal amongst other potential drug targets (reviewed in [146,166,167]). For example, Jaeger et al. identified synergistic drug combinations for breast cancer by studying relative reduction of network efficiency upon removal of drug target nodes from a signalling network [168]. By studying frequently altered genes in a pathway-centric analysis, Sanchez-Vega et al. [169] identified that while tumours show multiple mutations, pairs of mutually exclusive and pairs of co-occurring alterations occur. These results indicate interdependencies between pathways and that one mutation per pathway functionally suffices to alter its activity with more might being disadvantageous. For example, alterations promoting the RTK/RAS/MAPK signalling pathway are not co-occurring with alterations in the PI3K signalling pathway.

The study of so-called disease modules, a subgraph containing all molecular determinants of a disease, can be useful to understand underlying molecular mechanisms as well as predict disease-disease relationships. Here a disease represents an altered version of an underlying disease-associated subgraph. This can amongst others reveal potential for drug repurposing [170–172].

Computational Modelling for Systems Medicine

Computational models often play a central role in systems medicine as they can be used to enhance understanding of complex systems. Applications may be based on purely data-driven approaches, i.e. stochastic/statistical models, or they may be based on network-driven approaches, i.e. mechanistic models [173,174]. Purely data-driven models have been used amongst other to identify drug response features or predict drug sensitivity from omics data [64,66]. Network-based models allow computational simulations of model behaviour and can be broadly classified into quantitative and qualitative models, with prominent examples being Boolean models and Ordinary Differential Equation (ODE) based models, respectively. Modelling of biological processes can be descriptive (recapitulating observed biological phenomena and data) or predictive (*in silico* perturbation analysis), allowing discovery of underlying molecular processes and generation of new hypotheses [174–176].

Box 2 – Network properties

Several different graph theory-based measurements can be used to describe node and network properties. These can be used to extract biological insight and identify important nodes or group of nodes in a network. Depending on the type of network and its edges, different type of analysis can be performed. Edges can be broadly classified into undirected (association) or directed (causal) edges which can also have a certain weight associated to them. Additionally, one can distinguish different types of networks such as protein-protein interaction networks, metabolic networks, signalling transduction networks and gene/transcription-regulatory networks. Below examples for informative network features are presented. [146,166,167]

Degree

The most general characteristic of a node is its degree, describing the number of edges (or links) a node has to other nodes in the network. When signed edges are used, one can distinguish between out-degree, number of outgoing edges, and in-degree, number of incoming edges. Highly connected nodes are also referred to as hubs. Some studies have shown that hubs are associated with disease genes, while others have indicated local hubs.

Betweenness centrality

Betweenness centrality of a node quantifies the fraction of shortest paths that pass through a node related to the total number of shortest paths linking the nodes in whose shortest path the node appears. Nodes with high betweenness centrality thus are important in conveying information through the network.

Closeness centrality

Closeness centrality quantifies how close a node is to other nodes by measuring how short the shortest path is to all other nodes in the network. Thus, one can express how fast the information's flows through a given node to other nodes.

Modules, motifs or cliques

Nodes may be associated with topological or functional communities. Topologically members of communities typically have more edges to each other compared to other nodes associated to other communities. While modules describe groups of functionally related nodes, motifs and cliques are defined by topological features, a clique being a maximally interconnected subnetwork. However, modules may exist of highly connected nodes and vice versa motifs and cliques may consist of functionally related nodes. For example, in signalling networks entities may be associated to one or several signalling pathways.

To foster collaborations and development of computational models to address biomedical questions, in 2005 the Dialogue on Reverse Engineering Assessment and Methods (DREAM) project was started (http://www.dreamchallenges.org/). Since then multiple teams have explored different modelling approaches to meet challenges of systems biology and personalised medicine. This has led to several insights for algorithms in the field of genetics, genomics and systems biology such as (1) inclusion of

prior knowledge such as biological pathways seems to improve performance, (2) integration of models across teams produces the most robust predictions – "wisdom of the crowd" phenomenon, (3) gene expression and phosphoproteomics data are the best individual data types, but (4) integration of multiple omics data types generally improves performance [97,177,178].

Biological Networks Models

Network-driven modelling approaches involve the construction of a network graph from prior knowledge, data-driven network inference or a combination of both. This is a cumbersome process due to the size, complexity and topological uncertainty of entity interactions. Especially network construction purely based on prior knowledge is challenging as database-annotated information regarding cell specific and context depended regulations and network connection logics is still scarce. Furthermore, networks that comprehensively capture complex biological come with increasing numbers of network entities, thus increasing the numbers of indeterminable parameters and hence, the degrees of freedom [174,179,180].

Data-driven network reconstruction methods can reduce network uncertainty and reveal novel regulatory relationships if experimental data is available [180,181]. An early approach is Modular response analysis (MRA) which infers both connections between signalling entities and connection strength from perturbation data [180,182,183]. As MRA requires perturbation of all nodes and such data is often hard to get by, several variants have been proposed addressing this limitation [184–187]. Also under the DREAM project umbrella, multiple groups have developed methods for network inference (examples [188–192]). Network inference has led to several insights such as (1) a large fraction of causal interactions is already known (for the studied cases), (2) core signalling networks are similar between cell lines, (3) subgraphs and edges can be context-, stimulus-, cell- and disease-specific.

A benefit of network reconstruction approaches such as MRA is that model translation, verification, calibration and validation may be included in the network reconstruction process. However, with increasing network size, network reconstruction and training based on perturbation or stimulation of network nodes becomes impractical [180]. Large scale -omics data can help but is not always an efficient solution. For example, pathway reconstruction using data from standard shotgun proteomics techniques still suffers from this methodology's technical limitations such as low reproducibility [181].

Methodologies for Simulation of Biological Networks

Different mathematical and computational modelling approaches exist for translation of a network graph into a list of reactants and reactions to allow simulation of information flow. The choice of method depends on the extent of mechanistic detail that is required for the phenomenon being

investigated, the available data and the available computational resources. Available methods have been extensively covered in reviews and are shortly presented below [175,176,180,193].

Ordinary Differential Equation Modelling

The most commonly used quantitative modelling approach relies on ordinary differential equations (ODEs). Based on quantity changes as described by mass action kinetics, each species is represented by rates of production and consumption. Conventional ODE models are deterministic and do not consider compartments or time. In other words, it is assumed that all reactants have equal access to each other. In more complex mathematical model systems based on ODEs, spatial, temporal and/or stochastic aspects can be considered. A limitation of ODE models is the need for accurate kinetic parameter. However, simplifications such as the Michaelis-Menten approximation of enzyme-substrates kinetics have been proposed that require less data.

Logical Modelling

Logical modelling is a qualitative approach that was first introduced by Kauffman and Thomas [194,195]. In logical models the status of each entity in the network is given as a discrete value and represented by regulatory rules. In Boolean modelling, the simplest approach, entities can hold two different states: active (1) or inactive (0). The state of each entity is updated according to the activities of the regulating entities as defined in the regulatory rules. Regulatory rules follow the logical formalism *AND*, *OR* and *NOT*.

The global state of the system is defined by the activities of all entities in the network. Updating of the global state can occur either synchronously, where the state of all entities is updated simultaneously, or asynchronously, where only one node is updated at each timepoint. By updating the nodes' states started from an initial state, a logical model may reach a stable state, also referred to as fixed point where the activity of each node is stable, or a set of states that repeat themselves, i.e. cyclic attractors. In case of large model sizes, logical models can be computationally expensive to analyse, as the global state space is exponential to the number of entities in an asynchronous simulation.

Boolean models are especially useful when data is sparse and largely qualitative. Different tools have been developed that can optimise and calibrate a Boolean network, if experimental data is available [192,196]. Petri nets represent another type of discrete modelling, based on graph-based models containing two types of nodes: places and transitions, connected by edges. Places can hold "tokens", which can be transferred by transition nodes if the input place contains a minimum number of "tokens", representing dynamic signalling transduction of the system [197].

Discretization of nonbinary signalling data into qualitative states is challenging and reduces the accuracy. Approaches such as multistate discrete models, fuzzy logic models or logic-base ODEs overcome this, but also require more input data [180,198–200]. Probabilistic Boolean Networks can further account for stochasticity and uncertainty in the network [176,201]. Other tools take stochasticity of signal transduction into account and can be used to simulate the evolution of network states evolution over time as well as the probability of network state distributions [202]. In 2018, the Consortium for Logical Models and Tools (CoLoMoTo) provided an interactive notebook with access to several integrated logical modelling tools that can be accessed and used interchangeably [203].

While logical models are largely qualitative, they can provide valuable insights into biological process such as cell differentiation [204], DNA-damage response [205] and regulatory signalling mechanisms [206].

Application for Mechanistic Models for Personalised Oncology

In the field of personalised oncology, computational models can aid clinical decision making by identifying biomarkers as well as predicting effective therapies. Integration of clinical data with prior knowledge of signalling networks can reveal more complex matters that can amongst others lead to the identification of pathway biomarkers where no efficient genomic marker exists and pinpoint resistance mechanisms. As an example, Fey et al. [207] studied the c-Jun N-terminal kinase (JNK) pathway in neuroblastoma to identify response dynamics that be associated with survival. JNK signalling demonstrates a switch-like response that depending on stimulus and activation strength which can either promote cell survival and proliferation or apoptosis. The identified network structure that revealed both a positive feedback mechanism and AKT-mediated crosstalk, was translated into an ODE-based model. Patient-specific models calibrated with gene expression data could separate two groups according to JNK-signalling dynamics that corresponded with low and high patient survival, respectively. Jastrzebski et al. [208] constructed cell-line specific Bayesian-based models by integrating multiple baseline data sets as well as response to seven kinase inhibitors for a panel of breast cancer cell lines. By analysing the regulatory signalling in each cell lines in respect to each of the drug responses, the authors identify drug sensitivity and resistance mechanisms including both mutational events as well as protein expression levels.

In another study by Eduati et al. [209], logic ODE modelling was used to study the signalling dynamics of a panel of colorectal cancer cell lines and identify biomarkers for drug sensitivity. Cell specific models were parameterised using a drug perturbation dataset for seven single inhibitors and five stimulating agents. For roughly half of the investigated drugs, a significant association with a model parameter and drug efficacy could be identified. This included nine drugs for which no significant

association with genomic alterations could be obtained. Interestingly, identified pathway parameters underpinning network dynamics were informative for discovery of drug combinations targets suspending resistance mechanisms, and could therefore serve as *pathway dynamic biomarker*.

Fröhlich et al. [210] presented a computational approach that enabled parameterization of large-scale ODE-models with reduced computational cost compared to traditional approaches. Models calibrated to transcriptomic and genomic data were used to predict drug sensitivity for a panel of human cancer cell lines from 5 tissues. After application of the models to the initial data set, drug sensitivity for an additional panel of cell lines from different tissues as well as drug combination effect measured by O'Neil et al. [98] were predicted. A good correlation between predicted and observed drug responses was found and molecular mechanisms for sensitivity and resistance were identified.

Béal et al. [211] used stochastic logical models to stratify breast cancer patients. By integration of mutation, copy-number alteration and RNA expression data to a published logical model, probabilities of patient's outcomes for "proliferation" and "apoptosis" could be simulated. Patients predicted to have lower probability of "proliferation" had also better prognosis compared to patients with predicted higher probability of "proliferation". This was vice versa observed for "apoptosis" probability classifications. The presented approach thus can aid in identifying patients with higher chance of more aggressive progression of the disease and potentially suggest intervention points.

Computational methods further offer a reasonable alternative to preselect promising combinations from the large panel of all possible drug combinations both to economise preclinical screening efforts and eventually for clinical testing. Several groups have demonstrated the use of mechanistic models to predict drug combination effects using, amongst others, ODE-based [212,213], MRA-based [186] or logical model algorithms [209,214]. As an example, Shin et al. [215] used an ODE-based modelling approach to prioritize potent drug combinations from four potential targets for triple negative breast cancer. The model was calibrated using time-course and perturbation data for one triple negative breast cancer cell line. In a subsequent effort to adapt the models to predict patient response to one drug combination of interest, the authors integrated public gene expression data. The model successfully stratified patients into responders and non-responders.

In another study by Silverbush et al. [216], the authors used a logical model to predict single and combination responses for a panel of acute myeloid leukaemia cells. Cell-specific models were calibrated using phosphoprotein data under two perturbation conditions, and mutation data. By incorporating genetic alterations from other cell lines, the model could be further adopted to another cell context. The model successfully predicted cell-specific responses to single compound treatment

and was able to identify synergistic combinations from a panel of five single drugs across four cell lines.

In a previous effort by our group [217], logical modelling informed by baseline calibration data was used to predict drug combination effects of seven inhibitors in all possible pairwise combinations. The logical model fitted to the gastric AGS cell line, predicted five synergistic drug combinations, four of which were confirmed as synergistically inhibiting growth in subsequent experimental assay. Importantly, this approach did not rely on *a priori* perturbation experiments for model fitting and could in principle thus have significantly reduced the experimental load.

So far two DREAM-challenges have been launched toward prediction of drug combination effects [97,218]. In the first challenge, teams were asked to predict the effect of 91 combinations pairs by ranking them from most synergistic to least antagonistic. Teams were provided with single drug perturbation gene-expression data from the cell line under investigation. In the second challenge, teams were asked to predict synergistic drug combinations and biomarkers from a panel of 910 combinations across 85 cancer cell lines. Here the challenge was divided into multiple sub-challenges varying with respect to the provided data that could be used for synergy predictions. Teams were asked to predict 1.) continuous synergy scores and were provided with a training subset of quantified synergistic responses, 2.) continuous synergy scores using only mutation and copy number variation data, and 3.) binary synergy with no additionally provided training data. Submitted methods to both challenges encompassed mainly data-driven approaches. As noted also by Menden et al. [97], this might be related to challenges inherent to mechanistic models regarding network and model construction. An important aspect of the second DREAM challenge is that a specific sub-challenge was dedicated to prediction of drug combination effects using only baseline data. It is interesting to note that prediction performance from that sub-challenge was comparable to models from the first sub-challenge where drug combination training data was provided.

These examples collectively demonstrate the usefulness of models for prediction of drug combination effects although with varying success rate. They also show that different model platforms may offer different predictive capabilities, while this is not necessarily linked to the amount of training data used. Making use of prior knowledge while reducing the use of perturbation data for training, can allow a more economised approach to prioritization of drug combination and will be needed to offer optimized solutions countering the combinatorial explosion for pre-clinical screening and close the gap to what is possible in a clinical setting. This transition will not be trivial and will require amongst other comprehensive knowledge bases and optimised modelling strategies.

Objectives of the study

The work in this thesis was performed as part of the DrugLogics initiative[5], and as such, the overall objective of the thesis is aligned with the overall aim of the DrugLogics initiative: To investigate and demonstrate how systems medicine can be used to provide experimental-computational approaches that enable rational screening for synergistic drug combinations and in the long-term contributes to future clinical decision support with respect to anti-cancer combination therapies. Specifically, this thesis targets the following subobjectives:

- Analyse and report results from our comprehensive high-throughput drug combination screen comprising 19 targeted small-molecule inhibitors tested in 171 pairwise drug combinations in eight cancer cell lines in compliance with the FAIR principles to support reuse of the dataset
- Build a comprehensive pan-cancer prior knowledge network that can be used to predict drug combination effects across several cancer cell lines
- Investigate the drug synergy prediction performance of cell-line-specific models calibrated with use of baseline molecular data drug synergy predictions
- Identify methodological approaches and principles for network topology optimisation and logical model configuration
- Establish and evaluate a high-throughput drug combination screening platform for cancer cell spheroid cultures in cooperation with the High Throughput Screening facility at SINTEF, Trondheim
- Identify the relevance of spheroid cell cultures for combination screening by investigating differences in drug combination responses between planar and spheroid cultures cells

[5] https://www.druglogics.eu/ - last accessed 21/09/19

Summary of papers

Paper 1 - A high-throughput drug combination screen of targeted small molecule inhibitors in cancer cell lines (Å. Flobak and B. Niederdorfer et al.)

Combinatorial therapy is envisioned to enhance cancer therapy by targeting the altered signalling in multiple pathway trajectories and thus improving therapy response and halting resistance mechanisms. Despite the interest in drug combinations, only few large-scale screening studies have been performed with open-access data. In his study we specifically focused on small kinase inhibitors that target signalling proteins involved in well described signalling pathways, such as the PI3K/AKT-, TGF-beta-, RAS/MAPK- pathway. In an equimolar high-throughput screen eight cancer cell lines from seven tissue origins were profiled against a panel of 19 drugs and all possible 171 combinations. Drug effects were evaluated by viability inferred from measurements of ATP content. In a secondary screen, six drug combinations all involving the PI3K/mTOR inhibitor PI-103 were applied in a matrix design. Here cell confluency estimated by brightfield imaging was used as additional readout to ATP measurements. To quantify drug combinations effects, we used the Bliss independence model which was augmented with statistical significance estimation to correct for experimental variation and thereby to allow for high confidence synergy quantification. We rediscovered the synergistic drug combinations of co-targeting of PI3K/mTOR and TAK1 and MEK- and PI3K- signalling. Additionally, we identified that combined targeting of PI3K/mTOR and PDPK1 is potent across multiple cancer cell lines, recently reported as synergistic in bladder carcinoma cells.

Paper 2 - Analysis of logical model features that impact quality of drug synergy predictions across cancer cell lines (B. Niederdorfer et al.)

While combinatorial drug treatments offer an attractive strategy for cancer therapy, the sheer size of possible drug combinations poses a substantial obstacle to identification of effective treatment options and makes experimental testing of the complete combinatorial drug space infeasible. The aim of this paper was to investigate the use of logical modelling to filter out ineffective drug combinations for pre-clinical testing. Starting from a prior-knowledge network representing 144 nodes covering major cancer signalling pathways, cell-line specific models were configured based on baseline protein activity data from unperturbed cells in four different cancer cell lines. Predictions on drug combinatorial effects generated by model simulation were tested against observations from our high-throughput drug combination screen presented in **Paper 1**. Initial predictions showed that models calibrated towards baseline data leveraged from both omics-data inference and small-scale experiment observations reported in literature gave the best balance between true positive and false

negative predictions. Network and model refinement based on molecular mechanisms described in literature effectively decreased false negative predictions for both cell line-specific models. The cell-line specific models generated following the above strategies predicted ~22% of combination as synergistic in any of the cell line models, with three of the four models demonstrating an enrichment of observed synergies in the predicted subset compared to the unbiased screen. Specifically, we found that detection rate of synergies could have been increased by ~2.6-fold when combinations were pre-selected by *in silico* testing. Thus, our modelling approach effectively filtered out a substantial fraction of unpromising drug combinations. In ensuing *in silico*-based investigations mobilising large-scale simulation and perturbation analyses, we found that roughly one third to one half of the nodes in the models assert high-influence on prediction performance. Comparison of the performance of models calibrated to cell-specific fractions of high-influence nodes to the performance of models calibrated to the complete baseline data set indicated that calibration data can be focused on a pre-selected subset of nodes, thus offering a valuable strategy to economise experimental assessment of protein activity states in the cancer cell line to be modelled. In summary, our approach shows that (1) cell-specific models can be generated by calibration toward cell-specific baseline data and can prioritize drug combinations for screening by omitting unpromising combinations, (2) several relevant regulatory mechanisms are revealed in scientific literature calling for improved curation and annotation of both logic and context of signalling interactions, and (3) identification of high-influence nodes allows effective model calibration based on focused activity assessment on only a subset of proteins.

Paper 3 - High-throughput screening in 2D and 3D colorectal cancer cell cultures identifies synergistic drug combinations (E. Folkesson and B. Niederdorfer et al.)

More advanced clinical models are envisaged to improve translatability of drug testing for clinical use. In the here presented study, we have performed a high-throughput screen testing a total of seven drugs in all possible combinations in both planar (2D) and spherical (3D) cultured colorectal cancer cell lines (HCT-116, HT-29 and SW-620). Tested drugs include five targeted small-molecule inhibitors and two approved chemotherapeutic drugs. The drug combination screen was guided by an initial single dose screen were the potency of each drug was evaluated both in 2D and 3D cultured cells. Drugs were subsequently combined in 21 pairwise combinations in an IC20-guided 4x4 matrix. Effect of pairwise and single perturbations was assessed using viability measured by ATP content in both 2D and 3D cultured cells, complemented by assessment of confluency (2D) and spheroid size (3D). We find that some of the tested compounds and compound combinations show differences in sensitivity depending on cell culture format, such as reduced sensitivity of spheroid cultures to oxaliplatin and increased sensitivity of HCT-116 spheroids to PD0325901 (MEK inhibition). Drug combination effects,

as evaluated by Bliss independence showed that out of the 21 combinations tested, 13 were synergistic in at least one cell line cultured in 2D. Synergistic effects were less frequently observed in 3D cultured cells where only 8 combinations were found to have a higher than expected combinatorial effect. When taking both viability effect and synergistic strength into account, our results revealed that almost all effective and synergistic combinations involved the MEK inhibitor in 3D cultured cells. In contrast, only the top two of the effective and synergistic combinations in 2D cultured cells included MEK inhibition. The third most effective and synergistic combination in 2D cultured cells was co-targeting of PI3K/mTOR (PI-103) with TAK1 (5Z-7-Oxozeanol), which showed a markedly reduced synergistic effect in 3D. Longer exposure of cells to selected drug combinations revealed overall reduced synergistic response. This could be related to already strongly compromised viability by single inhibitor treatment. Prolonged exposure further indicated potential recovery from drug treatment in HCT-116 spheroids and SW-620 planar cultured cells when treated with the PI3K inhibitor (PI-103) in combination with the TAK1 inhibitor (5Z-7-Oxozeanol). In summary, by testing drug combinations in in 3D cultures in addition to 2D cultures, we were able to identify efficacious synergistic drug combinations that would have been left unidentified by solely relying on screening in 2D cultures. Furthermore, by studying multiple readouts, we uncovered additional synergistic drug combination effects not identified by conventional ATP-based viability assays. This highlights the importance of more advanced pre-clinical screening models for drug combination screening.

Discussion

Personalised oncology and systems medicine are anticipated to advance cancer therapy by providing biomarker-guided therapy. Rational approaches to effective treatments include application of drug combinations to enhance treatment response. Identification of effective drug combination treatments are a fundamental challenge due to the large number of possible drug combination High-throughput screen are routinely used to screen large panel of combinations and to identify effective putative drug combinations as well as markers for drug response. Computational models offer a potential complementary strategy by further economising screening efforts. In line with the overarching aim of the NTNU DrugLogics initiative, this PhD project explored the use of a systems medicine approach to identify effective drug combinations. In **Papers 1** and **3** we carried out high-throughput screening for identification of drug combination effects. In **Paper 3** we specifically investigated drug combination effects in planar (2D) versus spheroid (3D) cultured cells. Additionally, we explored different phenotypic readouts for studying drug combination effects. In **Paper 2** we investigated the use of logical modelling as a tool to economise drug combination screening efforts in a cancer cell line model specific manner, using the observations from the screen in **Paper 1** to test the predictive performance of the models.

Drug synergies are a rare find with typically only a relatively minor fraction of all tested compound combinations showing synergistic responses, as also exemplified in our studies. In **Paper 1** we identified 55 'drug combination-cell line' conditions where synergistic reduction of cell viability was observed. Relating this to all 1368 tested 'drug combination-cell line' pairs this translates to a synergy prevalence of 4%, and with approximately 13% of all 171 tested drug pairs displaying synergistic effects in at least one cell line. In **Paper 3**, approximately 25% of all tested 'drug combination - cell line' pairs were found to act synergistically. Interestingly, in the 2D cultures almost twice as many synergistic combinatorial effects were observed compared to 3D cultures, with 14% and 38% of all 21 tested combinations identified to be synergistic in at least one cell line in 2D and 3D, respectively. O'Neil et al. [98] reported a miniscule fraction of only ~0.05% of all tested 538 'drug combination – cell line' conditions across all drug pairs and 39 cell lines to display synergistic effect. Approximately 50% of all 583 tested combinations were synergistic in at least one of the 39 screened cell lines. In the NCI-ALMANAC study by Holbeck et al. [96], 1,898 of 5,232 tested drug pairs showed greater than additive response in at least 1 cell line (~36%). Thus, as shown in our and previous studies, a large number of drug pairs exhibit interesting combinatorial effects, but many of them are found to act synergistically in only few cell lines. Observations that only few combinations generally act synergistically and that drug combination effects are highly influenced by individual traits of the cancer

or cancer model system, further corroborates the challenge of identifying effective drug combinations. This also hints to challenges of combination therapy selection in personalised medicine.

The variation in prevalence of synergies seen in our and other studies [96,98], is influenced among others by different classes of drugs tested (e.g. targeted therapies vs chemotherapeutic agents) as well as by different metrics and thresholds used for synergy calling. Vlot et al. [219] recently demonstrated that the choice of synergy model can influence which combinations are identified as synergistic with different metrics demonstrating agreements but also disagreements of synergy quantifications when applied to the same dataset. This study [219] thus raises the importance of selecting an appropriate synergy metric for quantification of drug combinations.

While there is no consensus in the scientific community on which theoretical synergy metric gives the best estimation of greater than expected additive effects and thus highest validity for synergistic combinations, there are scenarios where not all models are suitable and thus cannot be used interchangeably. Use of the Highest single agent (HSA) model has been criticized for low specificity thus giving a higher rate of false positive synergy callings compared to other theoretical synergy models [219]. However, in the early stages of pre-clinical screening it may be more important to detect potentially synergistic drug combinations with as high sensitivity as possible, at the cost of lower specificity, even if a high amount of non-yielding combinations may need to be tested in the ensuing pre-clinical screens. The extra cost can be justified by a higher total of discovered synergies. In contrast, in a clinical setting seen from the patient-doctor's point of view, it is most important to be able to identify *verifiably* effective drug combination with high specificity and thus to avoid subjecting patients to inefficient treatment. Since we in **Paper 2** investigate the use of logical models to prioritise drug combinations for pre-clinical screening efforts, we relied on the HSA model for quantifying drug combination effects in the cell line screen used to test model performance to detect synergistic combinations with high sensitivity.

As reproducibility between drug screens is heavily debated [220–223], this also questions the reliability of identified synergistic drug combinations and thus their validity. Especially since such a low percentage of drug combinations is found to act synergistically, it is important that both positive as well as negative hits can be identified with confidence. So far, no comprehensive comparisons between independent drug combination screens have been performed, possibly due to the fact there is yet very little data available for drug combinations being tested across the same cell lines and in the same dose range compared to data available for single drug screens. Menden et al. [97] have however specifically investigated reproducibility of synergy effects within screen replicates as well as compared compliance of identified synergies among the limited number of combinations tested in another

screen. They found an inter-replicate Spearman correlation of 0.56 for their own data set and 0.63 for the dataset performed by O'Neil et al. [98]. In comparison, in our screen from **Paper 1**, we observe an average inter-replicate Spearman correlation of 0.41 and 0.60 in the primary and secondary screen, respectively. The lower correlation in our primary screen, can be related to the fact that most combinations received a Bliss synergy score close to 0, thus the ranking of the numbers may vary even though the absolute scores may be very similar. A closer inspection of correlation between mean Bliss excess scores (see Figure 4) confirms this notion and shows that while ranking between replicates vary, a combination that is found to have a greater than expected effect in one biological replicate is reidentified in the other. This can be seen from the accumulation of negative Bliss excess scores (Synergy strength) in the left lower corner of the scatter plots in Figure 4. These findings further suggest a high confidence between synergy calling at least between biological screening replicates.

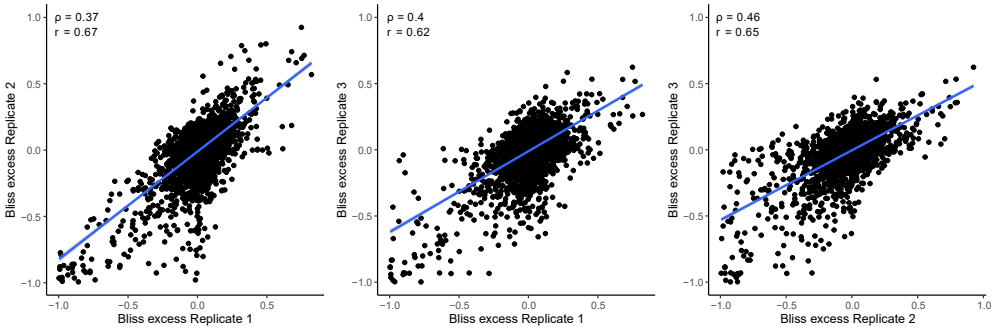

Figure 4 – Spearman (ρ) and Pearson (r) correlation between mean Bliss excess scores in the primary screen in Paper 1 across the three biological replicates.

In order to further assess the synergy calling between two independent screening efforts, Menden et al. [97] reported that for the nine 'drug combination-cell line' conditions tested in both their own and in the NCI-ALMANAC study [96], eight drug combinations found to be efficient in one study were also found to have a greater than expected effect in the same cell line in the respective other study. While the high concordance in synergy calling between the two screens is promising, with only nine combinations tested in the same dose range and cell line conditions, the comparison between the two screens is far from comprehensive and should thus be interpreted with caution. Currently, we are unable to broadly investigate the reproducibility of results from our screen to other independent studies, since to our knowledge no other high-throughput studies testing the same drug combinations and in this set of cell lines has been published. To nevertheless evaluate reliability of identified synergies in our high-throughput screening effort from **Paper 1**, we compared 'drug combination-cell line' conditions tested in both the primary and secondary screen. These screens have been performed roughly 2 years apart, with new batches of compounds for the secondary screen, and by different

group members. Comparison of drug combinatorial effects assessed in both the primary and secondary screen, identifies a Spearman correlation of 0.53 for mean Bliss excess across biological replicates, indicating a similar reproducibility as observed between biological replicates.

These results collectively indicate that synergy calling is reproducible both between biological replicates as well as different screening efforts, at least if the same synergy definition, namely metric and threshold, is used. To further substantiate reproducibility of synergy calling between independent drug combination screens, more comprehensive and rigorous studies with many data points allowing for in depth comparisons are needed. Such data may also aid in formulation of standards to generate findable, accessible, interoperable, and reusable (FAIR) drug combination data, and to improve comparability between different screening efforts, as well as to economise resources invested in experimental screens. A first contribution in this directions has recently been made by Niepel et al. [220], albeit focused on single drug screening. Here, to enable evaluation of factors influencing reproducibility between screening facilities, five different laboratories tested sensitivity towards a selection of eight compounds in one cancer cell line. Although the number of tested conditions was relatively small, the authors detected several sources affecting reproducibility of drug response and formulated best practices for dose-response experiments to improve assay precision and reproducibility also of relevance to support reproducible and comparable in drug combination screening efforts, such as standardisation of reagents which include cell line identify confirmations, as well as standardisation of data processing.

In pursuing standardisation of drug combination screening and comparison of drug effects across cancer models and testing sites, a central investigation is the significance of different readouts used to evaluate drug response effects as a basis for selection of relevant treatments. Substantial work remains to comprehensively identify molecular processes that should be targeted to cure cancer [224]. It is important to note that efforts to kill cancer cells can create selective bottlenecks, exemplified by subclonal resistant cells that proliferate because they are the “fitter” subpopulation when the cancer cells which respond to treatment are killed [225]. Additionally, several other phenotypes such as invasive and metastatic potentials of cancer cells are possible therapeutic targets of interest, thus calling for readouts additional to cell viability estimations that are routinely performed.

Findings by us and Gautam et al. [226], clearly demonstrate that the drug combination effects may be distinctly observed with some readouts but not by others, highlighting the importance of inclusion of additional readouts beyond cell viability. Comparable to our study, Gautam et al. [226] also reported that several of the tested single compounds reduced cell viability while not affecting cell death.

Together with the observation of a strong correlation between confluency and cell viability, this indicates a mostly cytostatic rather than a cytotoxic response towards the tested compounds (**Paper 3**). Of note, Horn et al. [224] have indicated that higher-order combinations of compounds (n > 3) targeting signalling pathways associated with cell growth are needed to effectively induce cell death. This is in accordance with our results from the follow-up screen, where we tested 3 pairwise drug combinations for an extended treatment interval and observed that both single and pairwise drug treatment more strongly affected cell confluency compared to their effect on cell death.

Although it is an intriguing question whether high-order drug combinations may be the answer to more effective treatment response, it is important to note that drug effects are also highly cell, time and dose dependent. This is also apparent from our results and findings by Horn et al. [224]. Four of our five targeted inhibitors induce apoptosis at higher concentrations in certain cell lines. Some of these compounds such as PD0325901 (MEK inhibitor) and palbociclib (CDK4/6 inhibitor) treatment showed maximum increases of apoptosis at the end of our treatment window (48h), other compounds such as PI-103 (PI3K/mTOR inhibitor) showed maximum signal at 24h. Both HT-29 planar cultures as well as HCT-116 spheroids showed strong induction of apoptosis when treated with the MEK inhibitor for an increased duration (96h).

Together the above-mentioned findings indicate the need for more advanced screening methods in regard to assessed phenotypic responses as well as time points. While inclusion of additional readouts in high-throughput screening will inevitably affect assay cost and lead to, amongst other, more labour-intensive data analysis, these efforts will be worth it if they result in improved understanding of drug effects both *in vitro* as well as in vivo.

Challenges in translation of *in vitro* drug testing into clinical actions, such as the failing of drugs in clinical trials, have seeded concern regarding the applicability of different models for pharmacology screening and personalised medicine. Several models have been developed to better recapitulate the physiology of a tumour, including spheroids, organoids, organ-on-a-chip and 3D-printed tissues [227,228]. Multiple studies have further shown that 2D and 3D cultured cancer cells show differences in sensitivity profiles to a range of compounds [229,230].

As multicellular architecture affects single drug responses as well as cellular signalling, it can be expected that drug combination effects also differ between 2D and 3D formats. In other words, choice of culturing format will affect which drug combinations are identified as synergistic in pre-clinical screening efforts and are thus deemed potentially attractive for further explorations. In **Paper 3**, we compared responses to seven single compounds and all their pairwise drug combinations in both 2D and 3D cultured colorectal cancer cell lines. Indeed, we observed several combinations that showed a

synergistic response in either 2D or 3D cultured cells. In 2D, the most effective combinations comprised a considerable variety of different compound pairs, while in spheroids, the MEK inhibitor participated in all the top five effective synergies. Several studies have previously reported that there is increased dependency of spheroid models on RAS/MAPK-signalling and reduced basal activity of PI3K/AKT-signalling to their respective 2D cultures [69,72,74,229], which could explain their higher sensitivity to MEK inhibitor combinations.

What does this mean for drug combination screening? Are the drug combinations identified as synergistic hits only in 2D cultures but not recapitulated in spheroids in **Paper 3** dismissible for follow-up? Spheroid-based models allow for a closer resemblance to tissue structures and may thus be useful to pursue for solid neoplastic diseases. However, even though monoculture spheroid cultures are considered to represent higher order architectures of solid non-vascularized tumours [231], they are still highly simplified tumour model systems and do not account for multicellular aspects such as those found in the tumour microenvironment. The risk of dealing with inadequate cancer models associated with the simplifications in monocultures can be mitigated by co-culture models of cancer cell lines with stromal cells and endothelial cells [232]. Short-term spheroid cultures of primary tumour cells [80] or organoid cultures [81,82] have also been used to test responses to anti-cancer agents. The establishment of patient-derived cell- and organoid- culture biobanks is further expected to eventually remedy the problem of limited material and augment the possibilities for access to more clinically relevant models for drug testing. It is a timely question, whether drug sensitivity inferences from spheroid-models will fulfil the anticipation of being more easily translatable to *in vivo* responses.

While spheroid-based models more closely resemble a solid tumours physiological condition, I would argue that planar cell cultures are still important for drug combination screening. In a systematic study, Iorio et al. [51] reported that planar cultured cancer cell lines are clinically relevant models for pharmacogenomic screens. Further drug discovery and large-scale drug combination screening will continue to rely on the use of high-throughput platforms where thousands of compounds can be tested in standardised assay conditions and at manageable cost. While not all cancer cell lines can form spheroids, we and others have established 3D models that are compatible with robotic screening platforms for several cancer cell lines [228–231,233,234]. To fully make use of spheroid systems, high-content imaging platforms for real-time monitoring of drug response are of interest even though our own work has demonstrated challenges that remain to be solved related to the fact that with increasing size the uptake of molecular dyes is hampered, and the excitation and emission light are lower in the core than in the periphery. Thus, while we can expect to see an increasing number of single drug and drug combination screens performed in spheroid-cultures, work remains to fully implement them in automated screening approaches.

To come back to my initial question regarding relevance of drug combinations identified as synergistic hits only in 2D but not in 3D cultures in **Paper 3** for further follow-up; at this stage, synergistic drug combinations even if observed in only one of the culture formats are of interest. Differences in drug sensitivity between planar and spheroid cultures of the same cancer cell line rather highlight the importance of capturing a large variety of cellular signalling dynamics and activities in assaying drug sensitivity. This pushes for the use of pathway biomarkers as a complementary strategy to the currently genomics dominated field of biomarker discovery for clinical decision making.

In our efforts to develop *in silico* approaches to contribute to identification of effective drug combinations and to economise screening efforts, we built on a previous study by our group where logical modelling was used to discover synergistic drug combinations in the AGS gastric adenocarcinoma cell line [217]. In **Paper 2**, we pursued the use of logical modelling to predict cell line specific synergistic drug combinations across a panel of four cell lines from three different origins. Predictive models that can reduce the combinatorial multiplicity of potentially interesting drug combinations are needed to prioritize drug combinations for testing. When applying mechanistic models, these models need to be tailored to a biological system of interest, such as cell lines, and need to represent the signalling pathways targeted by the drugs to be tested.

Several studies have highlighted differences in signalling dynamics between cell lines [186,190,209,235]. Thus, in order to achieve cell line specific modelling, these models should reflect decisive aspects of the status of intracellular signalling in each of the cells. Studies by our group [217] and by Silverbush et al. [216] have shown that cell-line specific modelling can be achieved by accounting for cell-specific molecular switches (such as e.g. represented by specific signalling proteins) in carefully curated prior knowledge networks. To efficiently reduce experimental cost associated measurement of model parameters, we continued to rely on molecular baseline data from unperturbed systems to generate cell line specific models, for which we have previously demonstrated good model performance [217]. The results presented in this thesis show that by informing models with baseline activity data, we were able to generate predictions that can successfully eliminate true negative drug combinations. The baseline activity profiles used for model calibration comprised of omics-data, inferred from copy number variations and gene expression data using the activity inference method PARADIGM [236], and signalling protein activity status obtained by literature curation. The model-generated predictions, if they had been used to prioritise drug screening, would have enabled an average enrichment by 2.6-fold in observed synergistic drug combinations across all four cell lines included in the study. Interestingly, we also observed high model plasticity between cell lines with only roughly half of all predicted synergistic combinations to be identical for three or more cell line models. We thus corroborate previous findings of baseline data as a useful source for model

calibration [217] and further show that cell line specific models for cell lines originating from different organs can be generated by taking one mechanistic prior knowledge network as a starting point for the logical models.

The construction of mechanistic models encompassing signalling pathways of interest is hampered by the incompleteness of molecular signalling knowledge and lack of biological context annotations in the knowledge bases. Thus, annotations are still largely lacking information regarding for example cell- and tissue-specific, or stimulus-depended regulations as well as the logics in which the regulations occur, needed to select regulatory interactions relevant for the studied system. In the case of Boolean models, the latter is further important for formulation of logical rules that determine model dynamics. If for example a node, representing a signalling protein, has multiple positive regulators, these may activate the node independently of each other, translating to the Boolean *OR* operator. Alternatively, the protein represented by the node may only be active if all or a certain number of positive regulators are active. Similar considerations apply for negative regulators. Additionally, some positive regulators may alleviate the negative regulation of a node. Another challenge related to current representation of signalling knowledge in databases is highlighted by Invergo and Beltrao [181] who point out that databases suffer from study bias of well-characterized proteins being favoured for studies by the research community. This leads to increased coverage of regulatory mechanisms for favoured proteins. When the information in existing pathway resources is used to generate networks, the biological entities which are disproportionally highly covered may appear as signalling hubs which will influence formulation of new hypotheses for further research and potentially clinical decision making.

The remedy of using drug perturbation data for model optimization has been proven successful in overcoming some of the challenges related to uncertainties in curated regulatory interactions and has also been demonstrated to introduce cell line and stimulus specificity, in order words biological context in signalling interactions and dynamics [186,209,213]. However, due to high experimental cost, optimisation of causal regulatory networks based on perturbation data is not scalable to all contexts and perturbation conditions of interest. In **Paper 2** we thus turned to literature to source more knowledge on signalling interactions and demonstrated the feasibility to identify several causal interactions deemed relevant for our model to improve our network graph and reduce false negative predictions. Our results show that network refinement by focused literature curation can improve predictive model performance. In a parallel study by Tsirvouli et al. [237], we effectively extended and successfully improved our prior knowledge network using multi-omics data from the TCGA Colorectal Adenocarcinoma (COAD) cohort which pinpointed causal interactions relevant for this cancer type. We observe that this aspect of our work also exemplifies the fact that relevant regulatory interactions have been discovered and reported in scientific literature, while the curation of this information into

signalling databases is still sparse. Thus, it is important to advance efforts to increase translation coverage of scientific literature into standardised computable knowledge [238] to support construction of mechanistic models

We note that several model predictions in **Paper 2** classified as false negatives for one of the cell line model, were predicted as synergistic by one or several of the other cell specific models. This may be related to uncertainties in model calibration data and/or elicited by causal interactions specific to a certain cell line. Both hypotheses are equally relevant and are supported by the observation that cell line-specific models could not be fully fitted to agree with entire calibration data. To mitigate uncertainty in both baseline calibration data and uncertainty of signalling interactions, ensemble of models could be generated to optimise fitting to calibration data while allowing for variation in the underlying prior knowledge graph, as presented by Thobe et al. [239].

Targeted phosphoproteomics mass spectrometry is a methodology that can be used to obtain accurate and reproducible measurements for phosphorylation status of cellular proteins and thus to subsequently estimate their activity [139–141]. As such, this method offers to contribute to reduce uncertainty in model calibration by providing accurate activity estimates for a priori specified number of protein kinases. Since experimental costs of targeted phosphoproteomics increases with the number of phosphosites measured, the essential question arises: which nodes in a model would benefit most from high quality activity data as benchmarked by predictive performance? Previous research has shown that a subset of nodes harbour higher information gain than others and can significantly reduce the network entropy by determining the global state of the network [240–244]. To contribute to optimisation of model training and calibration, we pursued *in silico* explorations where we evaluated changes in synergy predictions upon sequential fixations or inversion of activity for each node in the network one node at a time. This enabled us to determine which nodes in our model were of high influence for synergy predictions and would thus benefit most from high quality protein activity data. Analogous to work by others [240–244], we succeeded in identifying a subset of nodes that display the highest information gain in our models. It is interesting to note that our investigations demonstrate that predictions of models informed with baseline data for only the most highly ranked cell line specific high influential nodes, representing roughly a quarter of all nodes in the model, achieved predictive performance comparable to or even superior to models informed with the complete baseline data. These findings support the hypothesis that logical models can be successfully calibrated to baseline activity data focused on a subset of high influence nodes and further emphasize the relevance to continue pursuing approaches to identify such nodes for optimisation of predictive modelling in pre-clinical and clinical settings.

Interestingly, we identified that roughly a quarter of all nodes in our network were of high influence across all cell specific models with a similar fraction of low importance. This could be related to the fact that some synergies were observed across all cell lines or network nodes showing the same activity and logical rules between two models. Alternatively, this could point to certain prior knowledge network characteristics that emerge as a feature influencing the importance of a node. Indeed, when investigating high influential nodes with respect to features relating to network traits or biological function, we observed that nodes with high betweenness centrality, pathway-crosstalk inhibition index and closeness centrality are overrepresented among high influential nodes. While our high influential nodes were not found to be associated with specific biological processes or pathways, Puniya et al. [240] reported that influential nodes identified in their study were enriched in essential genes.

Interestingly, in a later publication by the Helikar group [241] previously identified influential nodes were found to be overrepresented among nodes with high determinative power, defined by a quantification of mutual information. Mutual information is defined both by the network structure as well as the Boolean functions determining the network dynamics. Pentzien et al. [241] further found that nodes with high determinative power often also hold a high out-degree. In accordance with our findings, the authors also observed a non-absolute association between the highlighted network features and influential nodes. This indicates that nodes with high information flow offer attractive targets for accurate activity assessment when the model dynamics are not known or cannot be studied, although some influential nodes may be missed.

Target control can be also applied to propose putative drug targets and resistance mechanisms as presented by Puniya et al. [240] and Yang et al. [243], further highlighting that mechanistic insights can be drawn from network topology. Thus, high-influential nodes such as those identified in **Paper 2,** may be further studied to identify synergy mechanisms, define biomarkers for drug combination effects as well as propose putative high-order drug combinations. In addition to controlling nodes, one could also consider controlling edges in the network. This could mitigate the effects of network damage induced by fixing a node to either ON or OFF, as this inevitably affects all nodes regulated by the node in question, with the benefit of being still applicable to large networks [245]. Applied to prediction of drug effects, this could be used to identify relevance of specific protein-protein interactions and signalling cascades as biomarkers for drug response.

As calling of experimentally observed synergistic drug combinations is not trivial, it can be argued that there are also uncertainties associated with their use as validation sets for testing model predictions. As discussed above and by others [219,226], both the choice of readout and the choice of synergy

metric affect which combinations are considered to be synergistic. Thus, model-based predictions that are classified as false positive or false negative related to one specific combination screen, may be deemed true positives or negatives when tested against an alternative screen where different set ups for the cell assays were used. Hence, for a broadest possible basis for evaluation of model predictive performance and thus further development of modelling approaches, it is important to have access to experimental drug screening data based on several experimental read-outs and carefully chosen synergy calling approaches.

The effect of drugs in single or combinatorial applications depends on the whole spectrum of their actions, i.e. both on their effect on recognised "main targets" and on their off-target effects. As pointed out by Lin et al. [20] such off-target effects may be involved in the underlying mechanisms of the drugs potency. Hence, any significant off-target effects can influence observed combination effects. This circumstance also has consequences for model-based prediction of drug combination effects. Especially mechanistically focused models, such as logic Boolean models, which rely heavily on accurate knowledge regarding compound specificity, should ideally be able to account for their off-target effects for successful simulation of cellular effects caused by the compounds' inhibition of signalling proteins. While some drugs are highly specific, others may target several other kinases and proteins [19]. This is also clearly seen in the compiled information on target profiles for the inhibitors studied in the **Paper 1** screen. While the information for most inhibitors generally indicates high specificity, for four inhibitors, Toxoflavin (STAT3i), BI-D1870 (RSKi), PRT 062607 (SYKi), Doramapimod (MAPK14i), considerable off-target effects were documented. Besides affecting abilities to predict effect of single and higher-order perturbations, also network inference algorithms are dependent on accurate knowledge of target profiles when reconstructing signalling networks from perturbation data. This might thus further confound our knowledge on signal transductions when some of the observed effects are caused by off-target activities. While a wide target spectrum of a drug does not necessarily preclude its progression into the clinic [19], both biomarker guided drug selection based on molecular tumour profile as well as model-based predictions of drug- and drug combination effects are highly dependent on accurate knowledge regarding the drugs' target spectra. Target deconvolution by improved curation and profiling of a compound's targets will further support not only predictions of target combinations but also predictions of drug combinations.

It is commonly known that models are simplified representations of an investigated system. While we in **Paper 2** focused on categorising combinations into synergistic and non-synergistic combinations using a mainly Boolean logical model approach, the curated prior knowledge network could be translated to a more advanced model to better account for biological complexity. An interesting and feasible advancement would be the use of a multi-valued logical model approach, which may also

alleviate challenges of binarization of biological data. For this, similar as applied by Silverbush et al. [216], baseline data could be discretized into a more fine-grained activity scale than just active (1) and inactive (0) to possibly achieve improved predictions regarding sensitivity to single drugs and combination treatments. A limitation of our approach is that we, also due to the large model topology, relied on stable state computation for evaluating perturbation effects. Thus, our model approach did not account for oscillating behaviour and represent time-evolution of signal propagation. To allow for more advanced simulations as well as for large number of targets of interest, drug combination simulations may be performed on combinations of modular subgraphs or pathway-centred, similar to Jaeger et al. [168].

Conclusion and Future Perspectives

High-throughput drug combination screens in combination with computational models are central to handle the large amount of possible treatments and drug combinations that are approved or currently in development. In this work, both experimental as well as computational strategies have been explored to support identification of synergistic drug combinations of anti-cancer compounds. Our findings show that calibration of cell line specific logical models can be used to enrich for synergistic drug combinations among the drug pairs suggested for clinical screening. Investigations on optimisation of drug predictions by network refinement and by focussing application of baseline calibration data to a subset of high influential model nodes indicated the need for comprehensive knowledge databases and corroborated that a subset of nodes in the network have high information gain over the state of the system. This offers possibilities for focused baseline activity assessment to obtain high quality training data sets for configuration of cancer cell line specific model dynamics. In our screening efforts we identified new synergistic drug combinations and validated drug combinations found to be synergistic also by others. Additionally, we described differences in drug combination effects between planar and spheroid cultures and demonstrated that some drug combination effects are observed to be synergistic in only a subset of phenotypic readouts. This suggests the need for screening platforms encompassing multiple readouts and a variety of culturing modes to effectively capture a broad range of drug combination effects.

Drug combination screens as performed in **Paper 1** and **3** are valuable resources both for identification of putative clinically relevant drug combinations and as testing data for validating computational modelling approaches. **Paper 3** contributes to further development of the spheroid (3D) format for high-throughput drug response screening. Investigations from **Paper 2** offer strategies for optimisation of logical modelling aspects to predict drug synergies for given cancers.

In future efforts we are planning to further implement screening on patient-derived spheroid models for testing of cancer drug combinations. When it comes to implementing mechanistically based *in silico* models to prioritise drug combinations to be tested in experimental screening platforms, integrated automated computational pipelines are required to serve the large spectrum of drugs and pre-clinical cancer models. Our group has developed an automated logical modelling pipeline, headed by Åsmund Flobak, that can be used to generate computational predictive models for any drug combination and any cancer model of interest, given sufficient available information on drug target profiles and baseline activity status of cancer model. This automated pipeline can potentially contribute both to economise drug combination screening efforts as well as to support clinical decisions on cancer treatment. Important aspects of the pipeline comprise automated construction

of network topology for drug targets of interest as well as training of the models to different biological systems such as different cancer cell lines and eventually patients.

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Paper I

SCIENTIFIC DATA

OPEN

DATA DESCRIPTOR

A high-throughput drug combination screen of targeted small molecule inhibitors in cancer cell lines

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While there is a high interest in drug combinations in cancer therapy, openly accessible datasets for drug combination responses are sparse. Here we present a dataset comprising 171 pairwise combinations of 19 individual drugs targeting signal transduction mechanisms across eight cancer cell lines, where the effect of each drug and drug combination is reported as cell viability assessed by metabolic activity. Drugs are chosen by their capacity to specifically interfere with well-known signal transduction mechanisms. Signalling processes targeted by the drugs include PI3K/AKT, NFkB, JAK/STAT, CTNNB1/TCF, and MAPK pathways. Drug combinations are classified as synergistic based on the Bliss independence synergy metrics. The data identifies combinations that synergistically reduce cancer cell viability and that can be of interest for further pre-clinical investigations.

Background & Summary

Treatment of non-resectable cancer has previously mainly relied on cytotoxic chemotherapy that indiscriminately kills all rapidly dividing cells. With the discovery of molecular mechanisms driving cancer, a new generation of molecularly targeted drugs has entered the market over the last two decades. A remaining challenge in the envisaged transition from 'one-size-fits all' therapeutic approach to personalised treatment is the development of resistance to single-targeted drug treatment. Combinatorial therapy may be able to overcome this by co-targeting multiple mechanisms involved in cancer cell growth and survival[1,2].

Already compelling results have been achieved with some combinations of cancer signalling inhibitors, e.g. the synergistic combination of MAP2K1/2 (MEK) and BRAF inhibitors for malignant melanoma[3–5] and lung adenocarcinoma[6]. This has sparked a wide interest in identifying novel efficient pairs of small molecule targeted drugs. However, our knowledge about beneficial targeted drug combinations is still limited, partly due to the combinatorial complexity.

Few high-throughput screens testing multiple targeted drugs in combinations have been published with open access data. Examples include combinatorial screens on ovarian cancer cell lines[7], melanoma cell lines[8], sarcoma cell lines[9] and lung-cancer-patient-derived cell culture models[10]. In a study performed by O'Neil *et al.*, combinations of around 12 targeted drugs were screened across a panel of 39 cancer cell lines. In total 22 experimental drugs were tested in all possible combinations and in combination with 16 approved drugs[11]. The National Cancer Institute (NCI) screened pairs of 104 FDA-approved cancer drugs against the NCI-60 cell line panel. Around 30 of the tested compounds can be classified as small molecule targeted therapies[12]. In the AstraZeneca's drug combination data set of the latest DREAM challenge, 118 drugs, including 59 targeted therapies, were tested in 910 pairwise combinations against 85 cancer cell lines[13].

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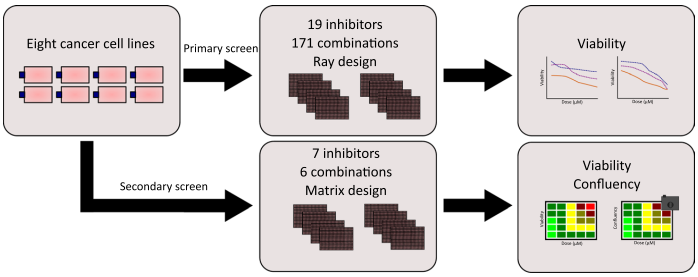

Fig. 1 Schematic representation of study design. Eight human cancer cell lines from different tissue origins were used in this study. Cells were incubated overnight prior to drug addition. In the primary screen, cells were screened against 19 small-molecule inhibitors in single and double application, with a total of 171 combinations. After 48 hours of drug exposure the assay was terminated, and cell viability was measured using CellTiter-Glo 2.0 (Promega). In the secondary screen, cells were screened against 7 single small-molecule inhibitors and 6 combinations for a duration of 48 hours. Drug effect was measured using automated brightfield imaging of confluency and CellTiter-Glo 2.0 (Promega).

In this paper, we report results from a high-throughput screen testing the effects of combining 19 small-molecule inhibitors on cancer cell viability. The drugs target signalling proteins involved in several well described signalling pathways, including PI3K/AKT, NFkB, JAK/STAT, CTNNB1/TCF, and MAPK pathways. Inhibitors were selected for high specificity and a minimum of known off-target activities, as judged from characterizations available from the MRC PPU lab (https://www.ppu.mrc.ac.uk/)[14] for 15 of 19 drugs. Eight cancer cell lines were treated with single inhibitors and 171 pairwise combinations at equimolar concentrations from 10–0.16 µM. Cell viability was determined after 48 hours from ATP content using CellTiter-Glo 2.0 (Promega). Several of the synergistic combinations involved the PI-103 inhibitor. Six of these combinations were investigated in a secondary screen. We here applied a matrix design allowing for synergy assessment outside the equimolar range and determined inhibitor effects from both viability (ATP content) and cell confluency (brightfield imaging). Figure 1 gives a schematic overview of the study design.

In the here presented dataset we rediscovered previously reported synergistic combinations and discovered several new synergies. The synergistic reduction of cell viability resulting from jointly targeting PI3K/mTOR and TAK1, previously reported by us for the AGS cell line[15] and later by others[16], is observed in two of the tested cell lines. Furthermore, we rediscovered the synergistic effect of jointly targeting MEK and PI3K[17–19]. Recently, Sathe *et al*. have reported on increased effect of combined inhibition of PI3K/mTOR and PDPK1 in bladder carcinoma cells[20]. We identify that combined targeting of PI3K/mTOR and PDPK1 is potent across multiple cancer cell lines. The PI3 kinase is one of the canonical activators of PDPK1, and our dataset thus identifies this 'vertical synergy' intervention by targeting multiple points in one cascade as a potential chemical intervention for inhibiting PI3K-dependent cancer growth across cell line models representing different tissue types. Co-targeting of multiple vertical points in one cascade is also the rationale for the clinically verified 'vertical synergy' observed by jointly targeting MEK and BRAF[3–6]. Moreover, PI3K and PI3K/mTOR inhibitors tested in a clinical setting (e.g. pictilisib[21] and apitolisib[22]) have suffered from toxic effects[23,24]. Thus, identification of compounds that increase the therapeutic index for PI3K inhibitors could enable new uses for PI3K inhibition in oncology.

The outcomes of our work contribute to expanding the available data sets on novel combinations of targeted drugs across a variety of cancer cell lines and can benefit the fields of cancer therapeutics, molecular signalling interventions and molecular cancer biology.

Methods

Cell lines and culturing. The following eight cancer cell lines were used in this study: A498 (kidney cancer), AGS (gastric adenocarcinoma), COLO 205 (colorectal cancer), DU-145 (prostate cancer), MDA-MB-468 (breast cancer), SF-295 (glioblastoma), SW-620 (colorectal cancer), and UACC-62 (melanoma). All cell lines used in the study are of human origin. Seven of the cell lines were obtained from the NCI-DCTD Repository, Frederick MD, and are part of the DTP 60 cell line panel (NCI-60 collection). The AGS cell line was obtained from ATCC. AGS cells were cultured in HAMS's F12 (GIBCO, 21765-029) supplemented with 10% FBS (GIBCO, 10270-106), 10 µg/ml penicillin-streptomycin (GIBCO, 15140-122) and 1 µg/ml fungizone (GIBCO, A11138-03). MDA-MB-468 were cultured in RPMI 1640 (GIBCO, 31870-025) supplemented with 5% FBS (SIGMA, F7524), 2 mM L-Glutamine (SIGMA, G7513) and 100 U/ml penicillin-streptomycin (GIBCO, 15140-122). All other cells were cultured in RPMI 1640 (GIBCO, 31870-025) supplemented with 10% FBS (SIGMA, F7524), 2 mM L-Glutamine (SIGMA, G7513) and 100 U/ml penicillin-streptomycin (GIBCO, 15140-122). Cells were cultured for approximately two weeks before assay and discarded after a maximum of two months in culture. The day before seeding out cells for the high throughput assay, cells were sub-cultured 1:2. Due to technical reasons, this was not performed for biological replicate 2 and 3 of the follow-up secondary screen.

Primary screen - ray designed drug treatment. Cell lines were treated for 48 hours with 19 small-molecule inhibitors in single and pairwise applications, comprising a total of 171 drug combinations. All

Cell line	Tissue of origin	Cells/well (384-well plate)	Population doubling time (h)	Growth Media during drug screening	Term Accession Number	Term Source REF
A498	kidney cancer	972	66.8	RPMI 1640 10%FBS	BTO:0003769	BTO
AGS	gastric adenocarcinoma	800	20	HAMS's F12 5%FBS	BTO:0001007	BTO
COLO 205	colorectal cancer	3000	23.8	RPMI 1640 10%FBS	BTO:0000179	BTO
DU-145	prostate cancer	2100	32.3	RPMI 1640 10%FBS	BTO:0001332	BTO
MDA-MB-468	breast cancer	8100	62	RPMI 1640 5%FBS	BTO:0001570	BTO
SF-295	glioblastoma	1050	29.5	RPMI 1640 10%FBS	BTO:0004213	BTO
SW-620	colorectal cancer	3750	20.4	RPMI 1640 10%FBS	BTO:0000675	BTO
UACC-62	melanoma	945	31.3	RPMI 1640 10%FBS	BTO:0004152	BTO

Table 1. Cell lines used in screen. Cell line density and growth media used are indicated. All media were supplemented with L-glutamine and penicillin-streptomycin. Population doubling time as annotated by provider. Cell lines are identified by BRENDA tissue/enzyme source (BTO, Version: 2016-05-05).

drugs were pre-diluted in DMSO to a stock concentration of 20 mM. Drugs were applied in an equimolar ray design from 10 µM to 0.016 µM using the following procedure: First, 2.0 mM solutions of each inhibitor and inhibitor combination were prepared. These were then serially diluted in DMSO (four 5-fold serial dilutions). The solutions were further diluted in medium (8 µl inhibitor in DMSO in a total volume of 228 µl), of which 5 µl were added to each cell culture well. Total cell culture volume per well was 35 µl. Highest final concentrations were thus 10 µM in each well. All dilutions and liquid handling were performed with robotic equipment using sterile and pyrogen free pipette tips. The inhibitors are listed in Online-only Table 1. Final DMSO concentration in assay wells was 0.5%.

Secondary screen - matrix designed drug treatment. In the secondary screen six combinations were screened in a matrix designed across all cell lines. As the PI inhibitor was identified as one of the top drugs involved in synergistic combinations, it was chosen as the anchor drug. The inhibitor was combined with BI, D1, JN, G2, PD, and 5Z. Cell lines were treated for 48 hours with the seven small-molecule inhibitors in single application and six drug combinations in all possible combinations of doses 10, 2, 0.4, 0.08 and 0.02 µM. Drugs, prepared as stocks of 20 mM in DMSO, were first diluted to 400x final concentration in DMSO. The solutions were further manually mixed 1:1 with either DMSO, for single drugs, or combination drug and applied to a 96 well plate. Further liquid handling was performed with robotic equipment. The solutions were diluted in medium (28.5x), of which 5 µl was added to each cell culture well leading to a total volume of 35 µl. We note that a new set of inhibitors was ordered for this screen.

High-throughput screen. Cells were seeded in 384-well plates (CORNING, 3712) at densities shown in Table 1 and allowed to attach overnight, before they were treated with small-molecule inhibitors and incubated for another 48 hours. The cell seeding densities were selected such that the cells were in a sub confluent stage at the time of drug addition. The variances in doubling time between the cell lines were taken into account and seeding densities were adjusted so that cell cultures in wells were either in late log phase or early plateau at the end of the incubation period. Cell viability was measured using the commercially available CellTiter-Glo 2.0 assay (Promega). In the secondary screen confluency was included as an additional readout and monitored by brightfield imaging using (SpectraMax i3x MiniMax 300 Imaging Cytometer, 2 views per well). A set of control wells with cells treated with 0.5% DMSO was included on all plates. The assay was performed with three biological replicates, if not stated otherwise.

For the first two biological replicates of the primary screen, two technical replicates are reported. Two of the four technical replicates assayed were excluded due to insufficient mixing of drug dilutions for these replicates. For the third biological replicate all four technical replicates assayed are included. For MDA-MB-468 and AGS cells, one or two biological replicates were performed, respectively. Some data points for the AGS cell line had to be excluded due to erroneous drug addition within the robotic script.

For the first biological replicate in the secondary screen, some technical replicates had to be excluded for the SW-620 cell line as too little drug volume was added. For the MDA-MB-468 cell line the first biological replicate was excluded due to low cell viability in the control wells. For the first, second and third biological replicate some technical replicates had to be excluded due to non-efficient cell attachment caused by an air bubble below the cell suspension. Additionally, some wells were excluded in the cell confluency data due to condensation of water on the bottom of the plate.

Data processing and representation. *Viability data - Primary screen.* For each 384-well plate, viability data of treated cells was normalised to the DMSO control cells measured in the same plate. Normalised viability data for each biological replicate where processed using "R-script-average-of-biological-replicates.R". The average viability score for each technical replicate as well as the average viability score across the biological replicates were calculated and exported as tab-separated values (tsv) files. Standard deviation across biological replicates was calculated using "R-script-calculate-mean-STDV.R". Dose response curves were generated using "R-script-sensitivity-of-drugs-across-cell-lines-plot.R" (Fig. 2, and in figshare deposit[25]: Drug_cell_response.pdf). Drug combination plots were generated from average viability scores with standard deviations using

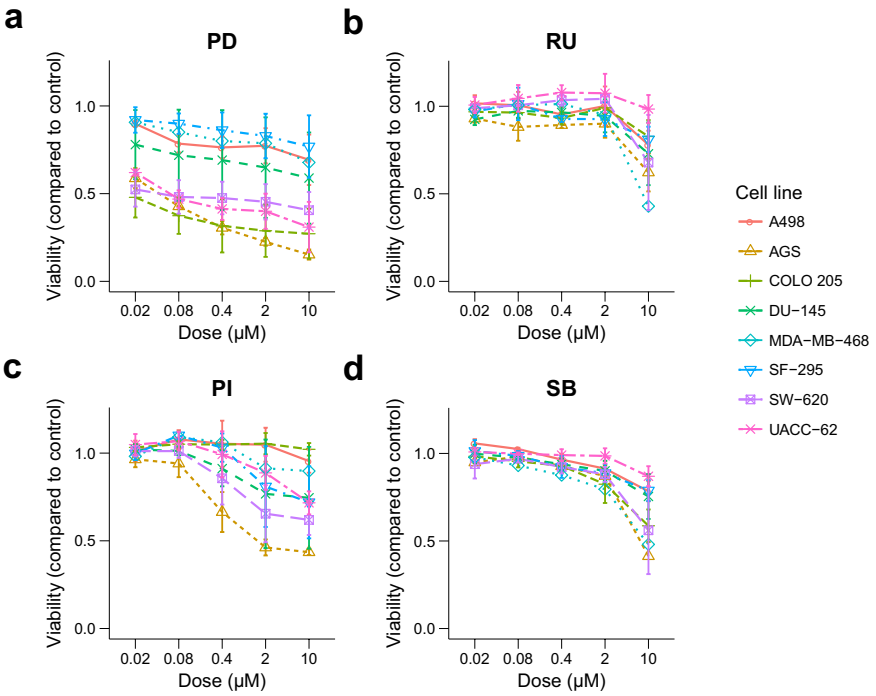

Fig. 2 Single dose response data of 4 out of 19 tested drugs across eight cell lines. The plot shows the viability of the eight tested cancer cell lines exposed to four different inhibitors in single application in a 5-fold dilution series from 10–0.016 μM, with standard deviation indicated by error bars. Viability was normalised to DMSO control. Abbreviations: PD-0325901 (PD); Ruxolitinib (RU); PI-103 (PI); SB-505125 (SB).

"R-script-plotting-drug-combination.R" (figshare deposit[25]: Combination_plots_primary_screen.pdf). Data points with missing values (14%) are represented as *NA* values.

Viability and confluency data - Secondary screen. For confluency data brightfield images were analysed using the SoftMax® Pro Software field analysis setting. For this, the region of the image covering the well was selected and confluency was reported. We note that the absolute values for confluency for very flat and long cells (e.g. A498, SF-295) may be underestimated while absolute confluency for round cells (e.g. MDA-MB-498) may be overestimated. We further want to indicate that COLO 205 are growing sub-confluent. For completeness of the data set, confluency is reported for this cell line. For each 384-well plate, viability and confluency data of treated cells was normalised to the plate-internal DMSO control. Technical replicates and averages of biological replicates are reported as comma-separated files (csv). These were further processed using "Biological_Average_secondary_screen.R" to calculate the biological average, standard deviation and coefficient of variation. Drug combination plots were generation from average viability scores with standard deviations using "Combination_plots_secondary_screen.R" (figshare deposit[25]: Combination_plots_secondary_screen_viability.pdf). The same script was used to generate drug combination plots for confluency data (figshare deposit[25]: Combination_plots_secondary_screen_confluency.pdf).

Calculation of synergy and statistical significance of quantified synergy. Bliss excess was evaluated for all doses and biological replicates per drug combination and cell line. We first computed all possible Bliss expectations of the reported technical replicates per dose per drug combination. Each technical replicate of combination dose responses was then compared to the average of computed Bliss expectations per technical replicate per dose in same biological replicate. For calculation of Bliss viability values were capped at one.

$$\textit{Bliss excess}: \ \textit{viability}(\textit{drug A} + \textit{drug B}) - \textit{viability}\ (\textit{drug B}) * \textit{viability}\ (\textit{drug A})$$

In the primary screen statistical significance of the synergistic response was evaluated. For this the computed Bliss excesses for each drug combination-cell line was compared to a zero-centred normal distribution. T-tests were performed to test for statistically significant deviations from a zero-centred distribution. When the null hypothesis was rejected the alternative hypothesis was accepted, which states that the distribution is not zero-centred, i.e. either antagonistic or synergistic. A conservative Bonferroni correction was done to adjust p

values for multiple hypotheses. Combinations with average Bliss excess values ≤ -0.08 and p-value ≤ 0.05 were classified as synergies. Bliss excess and significance classifications were performed using "p_values_Bliss.R". A synergy score heatmap and volcano plot visualising mean Bliss excess was generated using "R-script-heatmap.R" (Fig. 3a,b). For the secondary screen average Bliss excesses per dose, combination and cell line were computed using "Bliss_synergy_secondary_screen.R". A cumulative Bliss score was calculated using the same script by taking the mean over the computed Bliss excesses per dose. Combinations with average Bliss excess value < 0 were considered as synergies. The lower cut-off was motivated by the fact that five times more combination concentrations were tested in the secondary compared to the primary screen. Example plots of mean Bliss excess $\pm$ standard deviation for two combination tested in the secondary screen are illustrated in Fig. 3c.

Drug target annotation. Online-only Table 1 lists inhibitor abbreviation, name and unique identifiers (InChiKey and PubChem CID) along with the provider and primary targets as given by provider. CHEBI IDs are provided when available. We provide extensive target profile information in the form of bioassay information extracted from PubChem (last accessed on 24-06-2019) and kinase profiling data from the MRC PPU International Centre for Kinase Profiling - Kinase Profiling Inhibitor Database (http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors, last time accessed 24-06-2019) available in our figshare deposit[25].

For the inhibitors SF1670, BI605906 and 10058-F4 no bioassay information supporting targets given by the provider were found in PubChem or MRC PPU Inhibitor Database (proteins inhibited below 10% activity). For these inhibitors, we performed an exhaustive literature search to provide annotated information[25].

Data Records

All data is available at figshare[25], https://doi.org/10.6084/m9.figshare.9810719. Below, the file names for the different data sets are listed.

Viability and confluency data. The raw luminescence data from the three biological replicates from the primary screen can be found in NTNU_HTS_Repl[1-3]_raw.txt within figshare[25]. Relative viability data per biological replicate is available in [Cell line].NTNU_HTS_Repl[1-3]_processed.tsv[25]. Mean viability data of technical and biological replicates are depicted in [Cell line].tech-mean_Effect.tsv[25] and [Cell line].mean_Effect.tsv[25]. Example plots for drug sensitivity across the eight cell lines are shown in Fig. 2, while all plots can be found in Drug_cell_response.pdf[25]. In Fig. 4, the combination effect of selected drug combinations is visualised. Visualised drug combinations are based on significance and strength of synergy (see section for *Calculation of synergy and statistical significance of quantified synergy*, Fig. 3). Figure 4b shows average viability of SF-295 cells and A498 cells treated with Doramapimod (BI) in combination with PI-103 (PI), and SF-295 cells treated with BI-D1870 (D1) in combination with PI from the primary and secondary screen. The complete file with all drug combination plots can be found in Combination_plots_primary_screen.pdf[25] for the primary screen and in Combination_plots_secondary_screen_viability.pdf and Combination_plots_secondary_screen_confluency.pdf for the secondary screen for viability and confluency response, respectively[25].

The raw luminescence data and relative viability data from the three biological replicates from the secondary screen can be found in NTNU_HTS_secondary_viability_REPL[1-3].csv[25]. Raw as well as relative confluency data can be found in NTNU_HTS_secondary_confluency_REPL[1-3].csv[25]. Mean viability and confluency across the three biological replicates are depicted in NTNU_HTS_secondary_viability_Biological_Average.tsv[25] and NTNU_HTS_secondary_confluency_Biological_Average.tsv[25], respectively.

Synergy data. The Bliss excess values for each combination tested in the primary screen across the eight cell lines are represented in bliss_significance.tsv[25]. Synergistic drug combinations are further presented in bliss_synergies.tsv[25]. A visual representation of Bliss excess is depicted in Fig. 3a. Cell lines were clustered according to Euclidean distances of quantified synergy strengths. 4% synergies were recorded according Bliss Independence model.

Bliss excess values for each combination per dose tested in the secondary across eight cell lines are represented in bliss_synergies_secondary_screen_viability.tsv[25] and bliss_synergies_secondary_screen_confluency.tsv[25] from viability and confluency data, respectively. A visual representation of Bliss excess $\pm$ standard deviation from the viability data is shown in bliss_synergies_secondary_screen_plots_viability.pdf[25] and in bliss_synergies_secondary_screen_plots_confluency.pdf[25] from the confluency data. Cumulative bliss scores for the viability data can be found in Mean.Bliss.excess.matrix.tsv[25].

Technical Validation

Cell line identity. The seven NCI-cell lines used were received from the NCI-DCTD Repository and cultivated no more than 25 passages after reception in the lab. All cell lines were validated by STR profiling performed by Cell Line Authentication Service at ATCC. Eight core STR loci (TH01, D5S818, D13S317, D7S820, D16S539, CSF1PO, vWA and TPOX) plus amelogenin were matched to database profiles obtained from ATCC and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). For two of the cell lines, SF-295 and UACC-62, no database profile was available for comparison from ATCC and DSMZ. However, both cell lines were found to match their STR profile listed on the ExPASy website (https://web.expasy.org/cellosaurus). All 7 cell lines were found to be identical or similar to their database profiles with percent matches at 95–100%. The AGS cell line was validated by STR profiling performed by the in-house Genomics Core Facility. Eight core STR loci (TH01, D5S818, D13S317, D7S820, D16S539, CSF1PO, vWA and TPOX) plus amelogenin had a match of 81% to the ATCC profile (CRL-1739).

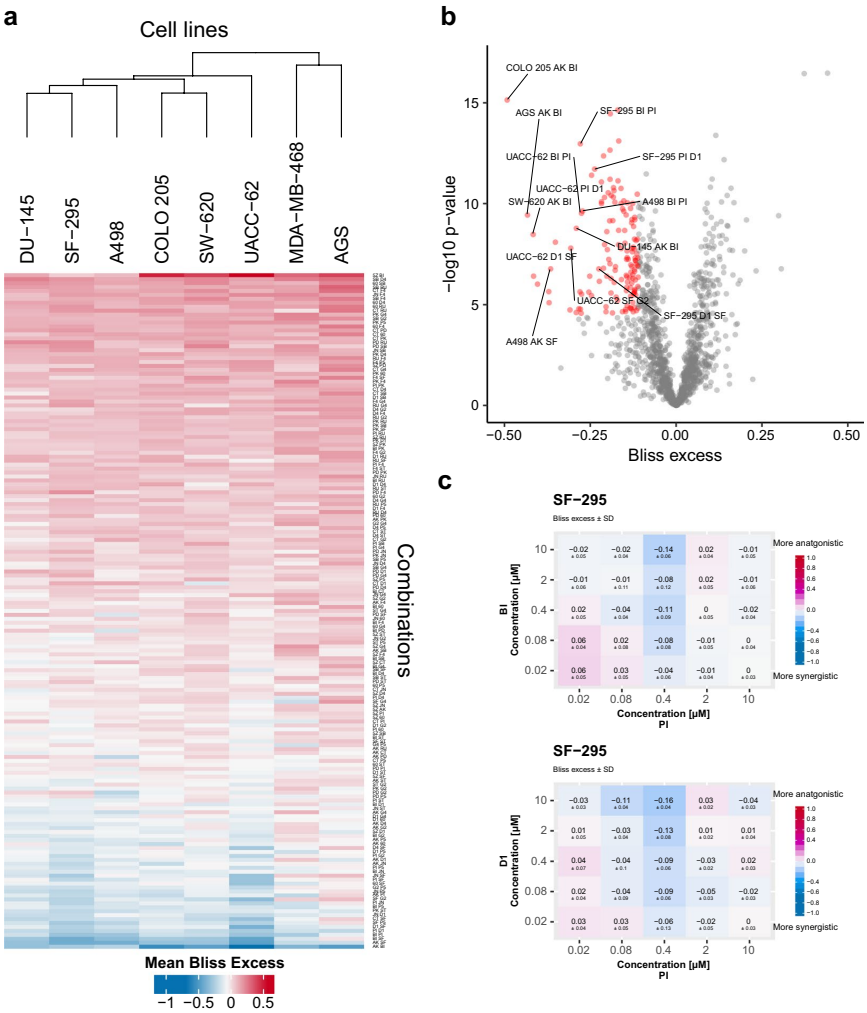

Fig. 3 Overview of synergy scores of all drug combinations across all tested cell lines. (**a**) The heatmap shows Bliss excess across the eight cell lines tested in this study, where a negative value indicates a stronger synergy. Bliss excess ≤ -0.11 are coloured in blue (synergy), while values ≥ 0.11 are coloured in red (antagonism). Cell lines are clustered using Euclidian distances of synergy strengths, while rows are sorted according to mean Bliss excess across all cell lines. (**b**) Volcano plot showing synergy strength (Bliss excess) vs significance scores of synergistic responses. (**c**) Average Bliss excess ± standard deviation for SF-295 cells treated with Doramapimod (BI) in combination with PI-103 (PI) and BI-D1870 (D1) in combination with PI from secondary screen.

Viability assay performance and variation. The performance of the viability assays was monitored using a sample set of small molecule positive controls. In the ray design nine concentrations of digitonin ranging from 100 µg/ml to 0.02 µg/ml was included in each biological replicate. In the matrix design a sample set with three concentrations of the positive controls digitonin, ranging from 26 µg/ml to 3 µg/ml, and staurosporine, ranging from 1.6 µM to 0.18 µM, were included in each plate. The standard deviation of the plate internal reference groups was also monitored and was less than 10% at maximum (on average below 5%) in the ray designed screen. Overall, we observed a mean coefficient of variation of 8% across all biological replicates in the primary screen and 7% in the secondary screen for both the viability and confluency data.

To assess reproducibility between biological replicates, we calculated Pearson correlation coefficients for all measured data points (normalised). We observe a high correlation between all comparisons with a correlation of R = 0.89 between Replicate 1 vs Replicate 2, R = 0.88 between Replicate 1 vs Replicate 3 and R = 0.87 between Replicate 2 vs Replicate 3 of the primary screen (See Fig. 5a–c). In the viability data of the secondary screen we

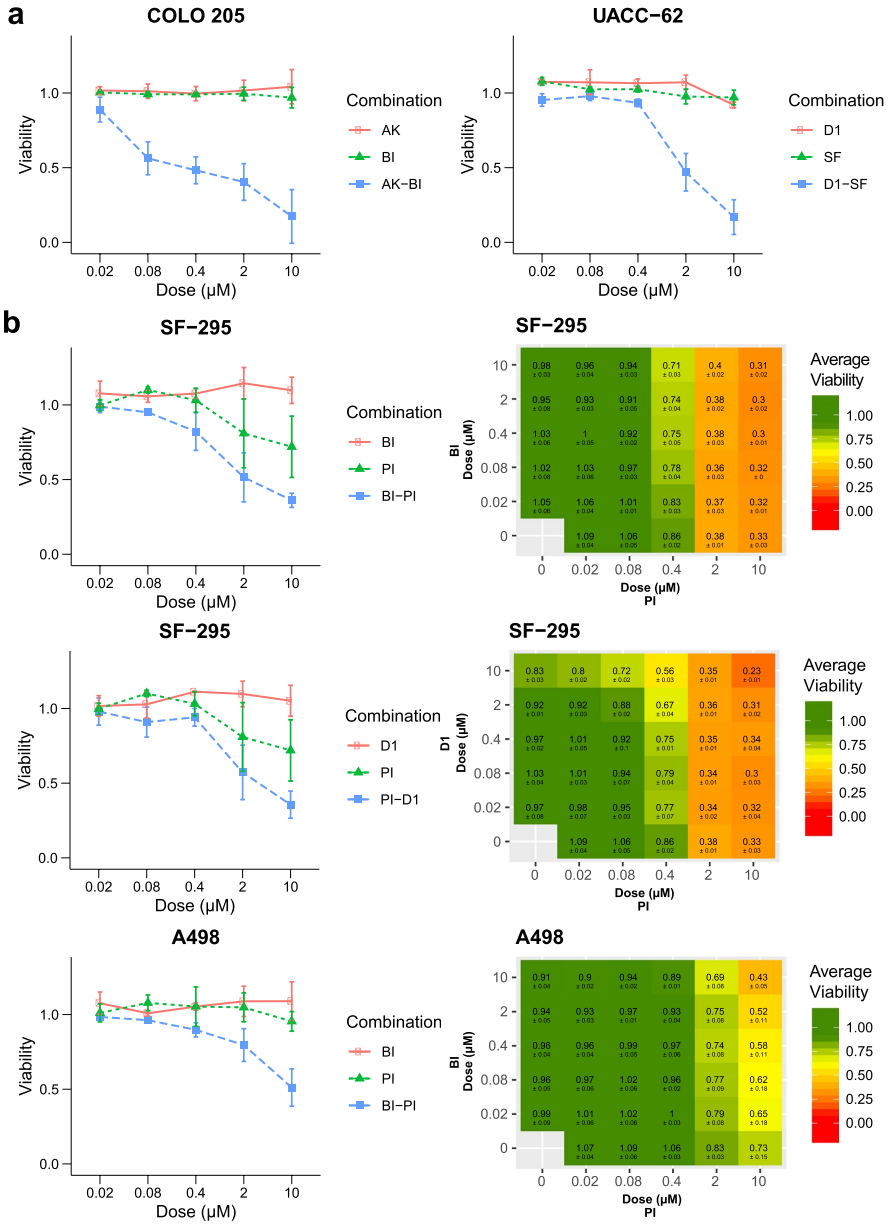

Fig. 4 Example drug combination plots for drug combinations found to be highly synergistic according to Bliss excess and p-value. (**a**) The graphs show viability data with standard deviation for COLO 205 cells tested Doramapimod (BI) and Akt Inhibitor VIII (AK) and UACC-62 cells tested with BI-D1870 (D1) and SF1670 (SF) in the primary screen. (**b**) The graphs show viability data with standard deviation for SF-295 cells treated with drug combinations Doramapimod (BI) - PI-103 (PI) and BI-D1870 (D1) - PI, and A498 cells treated with BI - PI in the primary and secondary screen. The data is normalised to DMSO control.

observed a correlation of R = 0.98 between Replicate 1 vs Replicate 2, R = 0.97 between Replicate 1 vs Replicate 3, and R = 0.98 between Replicate 2 vs Replicate 3. In the confluency data of the secondary screen we observed a correlation of R = 0.90 between Replicate 1 vs Replicate 2, R = 0.88 between Replicate 1 vs Replicate 3, and

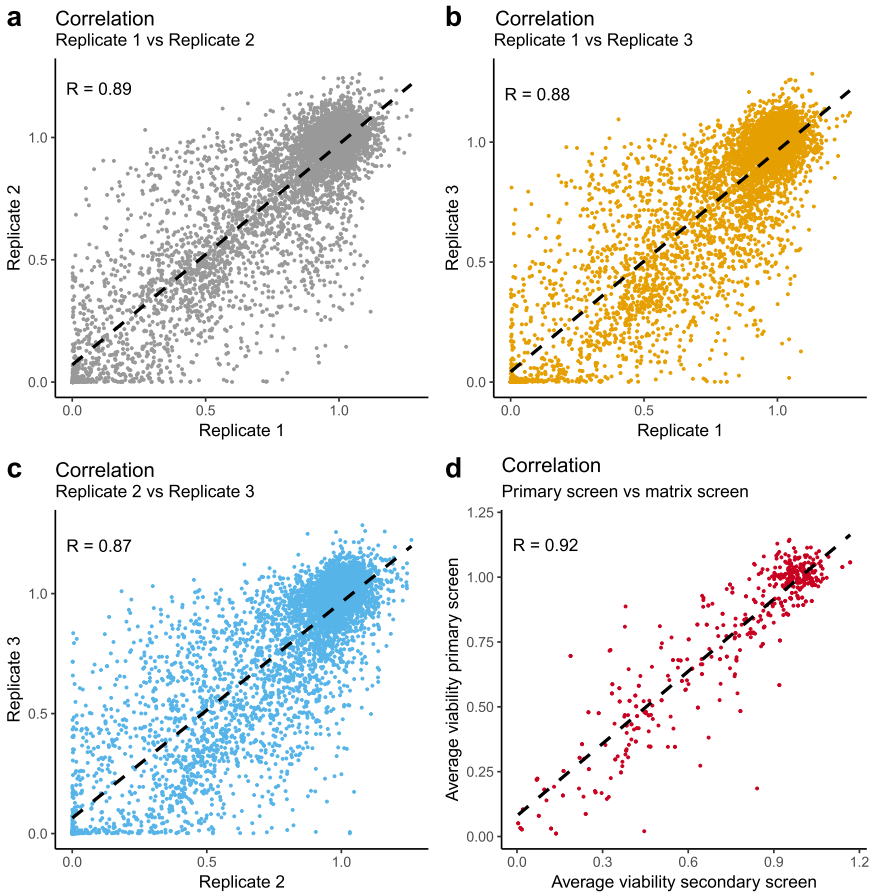

Fig. 5 Scatter plots and computed Pearson correlation values for biological replicates. (**a**) 1 vs 2, (**b**) 1 vs 3 and (**c**) 2 vs 3. The correlation between shared conditions of primary and secondary screen are shown in (**d**).

R = 0.95 between Replicate 2 vs Replicate 3. The average viability of shared data points between the primary and secondary screen showed a correlation of R = 0.92 (Fig. 5d). This variation is within the expected variation in high throughput cellular viability assays[11,26].

Synergistic combinations in primary screen. The primary screen led to the identification of 55 synergies according to the chosen cut-off from 1368 total combinations for all cell lines. An analysis of the list showed that 23 unique combinations were classified as synergistic across seven unique cell lines, with no synergistic combination in the MDA-MB-468 cell line. The inhibitors PI (targets PIK3CA/B/D, mTOR and DNA-PK) and SF (target PTEN) were identified to be involved in the majority of synergistic combination. The PI inhibitor was identified as synergistic with BI (target MAPK14) and with D1 (target RPS6KA1/2/3/6) in more than half of the tested cell lines. Further synergistic drug pairs sorted by occurrence across cell lines are G2 (target PDPK1), JN (targets MAPK8/9/10), P5 (target SYK) and AK (targets AKT1, AKT2, AKT3).

Synergistic combinations in secondary screen. As the PI inhibitor was identified as one of the top drugs involved in synergistic combinations, it was chosen as the anchor drug for the secondary screen. We combined the PI inhibitor with the top four synergistic drug pairs judged by occurrence across cell lines. We also included PI in combination with PD (targets MAP2K1/2), to investigate synergistic behaviour of drug concentrations outside the concentration ratios tested in the primary screen. This was motivated by the fact that drugs targeting PI3K and MAP2K1/2 have been previously reported to be synergistic in several cell lines[18,19]. Additionally, we tested PI in combination with 5Z a combination previously identified as synergistic in the AGS cell line in a previous study by our group[15] and Morris *et al.*[16].

Results from the secondary screen confirmed combinations as being synergistic or antagonistic in most cell lines. We confirmed the synergistic effect of PI-G2 in SF-295, SW-620 and UACC-62 cells. Additionally, we observed a synergistic response in A498 and AGS cells in the secondary screen. The combination PI-BI was also redetected as synergistic in five of the cell lines, while for two cell lines the combination did not show a greater effect compared to single drug treatment in the secondary screen. For two combinations, that were not classified as synergistic in the primary screen, PI-5Z and PI-PD, we could observe synergistic responses in the secondary matrix design screen. The PI-5Z combination was synergistic in two cell lines including AGS cells, confirming previous findings[15,16]. The PI-PD combination (targets PIK3CA/B/D, mTOR and DNA-PK - MAP2K1/2) was found to be synergistic in five cell lines and non-synergistic in three cell lines including the two colorectal cancer cell lines SW-620 and COLO 205. While colorectal cancer cell lines have been frequently observed to display synergistic response to combined inhibition of MAP2K1/2 (MEK1/2) and PI3K[27–30], weaker synergy has previously been observed in SW-620 and COLO 205 cells[28].

Usage Notes

Other users may investigate alternative methods for synergy quantification or another threshold using the provided data. To assist other researchers in visualising and analysing the dataset from the primary screen described herein we have prepared files that can be uploaded to the web-service CImbinator (http://rbbt.bsc.es/CImbinator/CombinationIndex) from which several synergy metrics can be computed and visualised[31].

Code availability

The data was processed using R 3.5.2 and RStudio Version 1.1.463. The following packages were used for data processing "tidyr" (0.8.3), "dplyr" (0.8.1), "ggrepel" (0.8.1) and "stats" (3.5.2); and data visualization "RColorBrewer" (1.1-2), "gplots" (3.0.1.1), "ggplot2" (3.2.0), "ComplexHeatmap"[32] (1.20.0), "ggpubr" (0.2) and "gridExtra" (2.3).

All code applied for processing and visualising the data is provided at the figshare deposit[25].

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Author contributions

Å.F. designed and performed experiments, analysed data and prepared figures. B.N. designed and performed experiments, analysed data, prepared figures and drafted the first version of the manuscript. V.T.N. performed experiments and provided experimental expertise. L.T. and A.L. supervised experiments and provided experimental expertise. G.K. supervised and performed experiments and provided experimental expertise. All authors contributed to writing the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Paper II

Paper III

The remaining supplementary files are included only in the online version of the thesis.

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