Phenotypic screening with 3D cell-based assays

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chapter 8

Appendices

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LIST OF ABBREVIATIONS

	2D	Two-dimensional
	2KW	Two-kidney weight
	3D	Three-dimensional
	8-Br-cAMP	Membrane-permeable cAMP
A	AC	Adenylyl cyclase
	AC-VI	Adenylyl cyclase 6
	ADP	Adenosine diphosphate
	ADPKD	Autosomal dominant polycystic kidney disease
	AMP	Adenosine monophosphate
	AMPK	5'AMP-activated protein kinase
	ANOVA	Analysis of variance
	ARPKD	Autosomal recessive polycystic kidney disease
	ArQ-197	Tivantinib
	ATM	Ataxia-telangiectasia mutated
	ATP	Adenosine triphosphate
	ATR	Ataxia telangiectasia and Rad3-related protein
	AVP	Antidiuretic hormone arginine vasopressin
B	BCA assay	Bicinchoninic acid assay
	BM	Basement membrane
	BSA	Bovine serum albumin
	BUN	Blood urea nitrogen
	BW	Bodyweight
C	c-Met	Tyrosine-protein kinase Met/HGF receptor
	cAMP	3'-, 5' Cyclic adenosine monophosphate
	Cdc2	Cell division cycle protein 2 (CDK1)
	CDDP	Cis- diamminedichloroplatinum(II), cisplatin
	CDK	Cyclin dependent kinase
	CFTR	Cystic fibrosis transmembrane conductance regulator
	CI	Combination index
	CREB	cAMP response element-binding protein
	Ctrl1	high affinity copper uptake protein 1
D	DMEM	Dulbecco's modified eagle medium
	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
	DNA-PK	DNA-dependent protein kinase
E	ECM	Extracellular matrix
	EDTA	Ethylenediaminetetraacetic acid
	EGF	Epidermal growth factor
	EGFR	Epidermal growth factor receptor
	EHS	Engelbreth-Holm-Swarm
	EMA	European medicines agency
	EMT	Epithelial- to mesenchymal transition

	ER	Endoplasmic reticulum
	ERK	Extracellular regulated kinase
	ESRD	End-stage renal disease
E	FBS	Fetal bovine serum
	FGFR	Fibroblast growth factor receptor
	Flt3	Fms-like tyrosine kinase 3, Fetal liver kinase 2, CD135
G	GB	Gigabyte
	GPCR	G-protein-coupled receptor
	GSK3	Glycogen synthase kinase 3
H	HCA	High-content analysis
	HCS	High-content screening
	HDM	Hanging-drop microtiter (plate)
	HER2	Human epidermal growth factor receptor 2
	HGF	Hepatocyte growth factor
	Hsp90	Heat shock protein 90
	HTS	High-throughput screening
I	IBMX	3-isobutyl-1-methylxanthine
	IGF-1(R)	Insulin-like growth factor 1 (receptor)
	IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
	IP3	Inositol triphosphate
	iPSC	Induced pluripotent stem cell
	IR	Insulin receptor
K	K-NN	K-nearest neighbor
	Klf4	Kruppel-like factor 4
	KNIME	Konstanz Information Miner
	KO	knockout
L	LDA	Linear discriminant analysis
M	MAD	Median absolute deviation
	MEK	Mitogen-activated protein-kinase/ERK kinase
	mIMCD3	Mouse inner medullary collecting duct 3
	mRNA	Messenger ribonucleic acid
	mTOR	Mammalian Target of Rapamycin
N	NF-κB	Nuclear factor kappa B
	NPI	Normalized-percent inhibition
	NVP-BEZ-235	Dactolisib
	NVP-BKM120	Buparlisib
O	OCT2	Organic cation transporter 2, SLC22A2
	Oct4	Octamer-binding transcription factor 4
P	PAS	Periodic acid-Schiff
	PBS	Phosphate-buffered saline
	PC	Principal component
	PC1	Polycystin-1

	PC2	Polycystin-2
	PCA	Principal component analysis
	PCP	Planar cell polarity
	PDE (PDE1/4c)	cAMP-dependent phosphodiesterases
	PDGFR	Platelet-derived growth factor receptor
	PDX	Patient-derived xenograft
	PEG	Poly-ethylene glycol
	PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
	PKA	protein kinase A
	PKD	Polycystic kidney disease
	Pkd1	Gene encoding polycystin-1 (mouse)
	PKD1	Gene encoding polycystin-1 (human)
	Pkd2	Gene encoding polycystin-2 (mouse)
	PKD2	Gene encoding polycystin-2 (human)
	PKHD1	Gene encoding fibrocystin/polyductin (human)
	PLC	Phospholipase c
	px	Pixels
R	R&D	Research & development
	R788	Fostamatinib
	RNA	Ribonucleic acid
	ROCK	Rho kinase
	ROI	Regions of interest
S	SD	Standard deviation
	Sox2	SRY (Sex determining region Y)-box 2
	SSTR	Somatostatin receptor
	STAT3	Signal transducer and activator of transcription 3
	Syk	Spleen tyrosine kinase
T	TBS	This-buffered saline
	TBS-T	Tris-buffered saline/Tween 20
	TKI	Tyrosine kinase inhibitor
	TNFR	TNF receptor
	TNFα	Tumour necrosis factor alpha
	TRPP2	Transient receptor potential polycystic 2 (polycystin-2)
	TSC1	Hamartin
	TSC2	Tuberin
V	V1R	Vasopressin V1 receptor
	V2R	Vasopressin V2 receptor
	V2RA	V2R antagonist
	VEGFR	Vascular endothelial growth factor receptor
W	WNT	Refers to Wnt signalling pathway

ENGLISH SUMMARY

Traditional drug discovery approaches have been hampered by (*in vitro*) cell culture models that poorly represent the situation in the human body. Principally, cells grow in the body in a three-dimensional (3D) environment that cannot generally be captured using cell culture methods. For this reason, cell culture models have been developed where cells grow in a 3D environment, which allows them to form structures that are more comparable to tissue in the body. However, the full complexity of these advanced cell culture models can only be fully used for routine drug testing if the cell culture model can be used on a large scale (also termed high-throughput screening or HTS), and if the readout can capture all of the biological complexity reflected by the 3D-cultured cells (high-content screening or HCS), as discussed in chapter 2. Due to these technological limitations, 3D cellular models are not yet routinely applied in drug and drug-target discovery.

In order to underline the importance of measuring the biological complexity exerted by 3D-cultured cells, **chapter 3** describes the development and use of a 3D cell culture model that simulates the process of tumour cell invasion, a process that precedes metastasis. This model was used to test different types of molecules that may prevent this process through different mechanisms. By using phenotypic measurements on these 3D-cultured prostate cancer cells, the molecules could be classified by mechanism-of-action, and off-target activity. The classification of these molecules according to phenotype was confirmed to be correct by comparison with enzyme activity measurements.

In a similar approach, **chapter 4** describes the development of a 3D cell culture model that can mimic the development of renal cysts as observed in polycystic kidney disease (PKD). This disease is characterized by the development of fluid-filled renal cysts that cause a progressive decline of kidney function. As a result of the lack of relevant cell models, only one drug was recently admitted onto the market, and this is associated with side-effects that may limit its use. The model we developed uses 3D-cultured renal cells that form cysts. The enlargement of these cysts can be influenced or reduced by adding molecules that affect relevant disease targets. In order to find these relevant disease targets, this developed 3D cyst culture model was employed to test a library of molecules for which molecular targets were known. This allowed us to relate efficacy of the molecules at inhibiting growth of cysts to molecular targets, leading to the identification of mTOR, HER2, IGF-1R, CDK, Aurora A kinase and Syk, but surprisingly not PI3K and EGFR as relevant disease targets. Some of these molecular targets have been extensively described in literature (mTOR, CDK) for PKD, confirming the relevance of this approach.

In order to follow up on the identified molecular targets, in **chapter 5**, we tested another molecule library with known target specificity and affinity in the 3D cyst culture model. The phenotypic changes that the molecules induced on the 3D cultured cysts

were compared to the changes induced by a model compound for cytotoxicity. Using this approach, we were able to exclude potentially toxic molecules and retain molecules that only reduced cyst enlargement. We discovered that also these molecules are known to inhibit CDKs, but also again found Aurora A kinase, HER2 and IGF-1R inhibitors among the active molecules. Having confirmed our previous findings, we decided to compare the activity of these molecules on invasive tumour cells. This allowed us to select molecules that only influenced the growth of cysts, but not the phenotype of tumour cells. An IGF-1R and Akt1 inhibitor, VCC55, strongly reduced cyst swelling without killing cells, and could therefore be an interesting treatment candidate.

In **chapter 6**, we identified two effective molecules, pyrvinium pamoate and celastrol, after a large screen with 2320 molecules. These molecules showed desirable effects in our 3D cyst culture platform and were therefore tested in a mouse model of PKD. Whereas pyrvinium pamoate failed to show effect, celastrol potently reduced cyst burden and improved kidney function in these mice. It is currently unclear through which molecular mechanism celastrol influences cyst growth, but it likely involves multiple molecular targets. Celastrol is a molecule that is isolated from the thunder god vine, which is extensively described in (traditional Chinese-) medicine for its anti-inflammatory, anti-obesity and potential anti-cancer effects.

In summary, this thesis describes how 3D cell culture techniques, coupled with phenotypic profiling, can be used to advance drug discovery. The use of 3D cell-based assays can enhance physiological relevance of *in vitro* research and may thereby lead to improved drug selection and safety. In addition, by using more biologically relevant cell culture models, there may be a possibility to reduce the number of animal studies required, prior to clinical evaluation of drugs, due to increased success rates.

NEDERLANDSE SAMENVATTING

De ontwikkeling van nieuwe medicijnen wordt gelimiteerd door de beperkte fysiologische relevantie van traditionele celkweekmodellen in preklinisch *in vitro* onderzoek. In het menselijk lichaam groeien cellen in een driedimensionale (3D) omgeving die niet kan worden weergegeven door deze celkweekmodellen. Om over deze beperking heen te komen zijn 3D celkweeksystemen ontwikkeld, waarbij cellen een structuur kunnen vormen die fysiologisch relevanter is en meer lijkt op weefsels in het lichaam. Een probleem is dat om gebruik te maken van alles wat deze weefsels te bieden hebben, het celkweekmodel geschikt moet zijn voor het testen van medicijnen op grote schaal (high-throughput screening of HTS) en dat alle biologische complexiteit correct moet worden gemeten (high-content screening of HCS). Dit wordt verder besproken in **hoofdstuk 2**. Door deze problemen worden 3D celkweken nog niet routinematig gebruikt in het medicijnonderzoek.

Om het belang van het meten van de biologische complexiteit van 3D celkweken te benadrukken laat **hoofdstuk 3** de ontwikkeling van een 3D celkweek model zien dat het proces van kankercel-invasie simuleert (een proces dat voorafgaat aan het uitzaaien of metastaseren van kanker). We hebben dit celkweek model gebruikt om moleculen die dit proces op verschillende manieren beïnvloeden te testen. Door de structuur en vorm van de 3D prostaatkanker-celstructuren te bepalen konden deze moleculen geclassificeerd worden naar mechanisme en konden daarnaast moleculen geïdentificeerd worden die niet specifiek waren. De correctheid van deze classificatie aan de hand van het fenotype hebben we ten slotte bevestigd door het meten van enzymactiviteit.

Op een vergelijkbare wijze laten we in **hoofdstuk 4** de ontwikkeling van een 3D celkweek model zien waarin we de ontwikkeling van cysten, zoals bij de humane ziekte cystenieren (ook wel PKD), simuleren. Bij deze erfelijke ziekte vormen tijdens het leven vocht-gevulde cysten in de nieren waardoor de nierfunctie sterk achteruit gaat. Doordat er voor het onderzoek naar geneesmiddelen voor deze ziekte geen relevante celkweken zijn die gebruikt kunnen worden om op grote schaal stoffen kunnen testen, is er nu slechts één geneesmiddel op de markt, dat ook lastige bijwerkingen heeft. Het model dat wij beschrijven in dit hoofdstuk gebruikt niercellen die in een 3D omgeving cysten kunnen vormen. Door moleculen toe te voegen aan deze cysten kunnen we onderzoeken of de moleculen die voor cystenieren relevante aangrijpingspunten (ook wel: moleculaire targets) hebben, cystegroei vertragen. Om te onderzoeken welke aangrijpingspunten binnen de cel relevant zijn voor cystenieren, hebben we dit 3D celkweek model gebruikt om een verzameling moleculen, waarvan de moleculaire targets bekend waren, te testen. Hierdoor was het mogelijk om het effect van de moleculen op cyste groei te relateren aan de moleculaire targets. Naar aanleiding hiervan identificeerden wij mTOR, HER2, IGF-1R, CDK, Aurora A kinase en Syk, maar tot onze verbazing niet PI3K of EGFR, als eiwitten die mogelijk van belang zijn bij het

remmen van cyste groei. Doordat een aantal van deze targets eerder beschreven waren in de literatuur (mTOR, CDK) voor cystenieren, bevestigt dit de relevantie van dit model.

Om volgens deze strategie verder te gaan, hebben we in **hoofdstuk 5** een andere verzameling moleculen getest, waarvan de precieze affiniteit voor de targets van bekend was. Hierbij hebben we de fenotypische veranderingen van de cysten na de behandeling met deze moleculen vergeleken met een toxische stof. Door moleculen, die een fenotype veroorzaakte dat erg lijkt op het fenotype geïnduceerd door de toxische stof, uit te sluiten hebben we moleculen kunnen selecteren die minder toxisch zijn. Ook hier vonden wij dat de moleculen die cyste groei remden CDK, Aurora A kinase, HER2 en IGF-1R als targets hadden. Doordat dit ook onze bevindingen in het vorige hoofdstuk bevestigde, hebben we de activiteit van deze moleculen vergeleken op een model voor kankercel invasie. Hierdoor konden we moleculen selecteren die alléén een effect hadden op cyste groei, en niet op deze andere cellen, waardoor er mogelijk een gerichter effect is op cystenieren. VCC55 is een IGF-1R en Akt1 remmer die cyste groei sterk verminderde zonder cellen te doden, waardoor dit mogelijk een kandidaat zou zijn voor vervolgonderzoek.

Hoofdstuk 6 beschrijft de ontdekking van twee zeer effectieve moleculen, pyrvinium pamoate en celastrol, na een grote screen met 2320 moleculen in het 3D celkweek model voor cystenieren. Door de sterke activiteit van deze moleculen in ons 3D celkweek model hebben we besloten deze stoffen te testen in een muismodel voor cystenieren, waarin we zagen dat pyrvinium pamoate geen gunstige effecten op het ziekteverloop had. Daarentegen bleek celastrol een zeer effectieve stof; na behandeling hadden de muizen een verbeterde nierfunctie en ook een kleiner aantal cysten. Op dit moment is onbekend waarom celastrol de groei van cysten vertraagt, maar dit komt waarschijnlijk door meerdere moleculaire targets. Celastrol kan worden geëxtraheerd uit *tripterygium wilfordii*, een plant die vaak in (traditionele Chinese-) geneeskunde wordt gebruikt ter genezing van ontstekingen en vanwege mogelijke werkingen tegen obesitas en tumoren.

Samenvattend; in dit proefschrift laten we zien hoe 3D celkweek modellen, gecombineerd met fenotypische analyse, gebruikt kan worden om het *in vitro* medicijnonderzoek te verbeteren, zodat de bevindingen relevanter zijn voor diermodellen en voor patiënten, met als resultaat mogelijk veiligere geneesmiddelen met minder bijwerkingen. Doordat deze nieuwere celkweek methoden kunnen bijdragen aan een grotere kans op succes voor geneesmiddelen, is het mogelijk dat uiteindelijk het aantal dierproeven, dat nodig is voordat geneesmiddelen in mensen getest kunnen worden, kan worden teruggedrongen.

LIST OF PUBLICATIONS

High-throughput phenotypic screening of kinase inhibitors to identify drug targets for polycystic kidney disease
Booij, T.H., Bange, H., Leonhard, W.N., Yan, K., Fokkelman, M., Kunnen, S.J., Dauwerse, J.G., Qin, Y., van de Water, B., van Westen, G.J.P., Peters, D.J.M., Price, L.S.
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Journal of Biomolecular Screening, Oct. 2016; 21(9): 912-22

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Manuscript submitted and under revision

Getting the most out of 3D cell based assays with high content image analysis and phenotypic profiling
Booij, T.H., Herpers, B., Yan, K., Price, L.S.
Manuscript in preparation

Phenotypic profiling of 3D-cultured micro-tissues to identify selective inhibitors of cyst growth
Booij, T.H., Kaczmarczyk, A., Bange, H., van Asten, S.D., Yan, K., van de Water, B., Peters, D.J.M., Price, L.S.
Manuscript in preparation

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CURRICULUM VITAE

Tijmen Harmen Booij was born on November 26th, 1988 in Den Helder, the Netherlands. Between 2001 and 2007 he went to the Murmellius Gymnasium in Alkmaar, also in the Netherlands.

Greatly fascinated by (cell-) biology and medicine, he decided in 2007 to pursue his Bachelor´s degree in Bio-Pharmaceutical Sciences at the University of Leiden, the Netherlands. During his Bachelor's studies, he was first introduced to cell culture and 3D-cell culture technologies, the latter of which were, back then, used only in small-scale experiments.

Fascinated by the improved physiological relevance of complex multicellular structures that developed when cells were cultured in extracellular matrix, and by the potential of such cell culture assays to transform and enhance preclinical drug research, Tijmen pursued his Master's degree in Bio-Pharmaceutical Sciences at the University of Leiden from 2010. He joined the Division of Toxicology of the Leiden Academic Centre for Drug Research (LACDR) for his master's internship, where he miniaturized 3D cell culture assays to enable their use for high-throughput drug evaluation under supervision of Dr. Leo S. Price and Prof. Dr. Bob van de Water. For this work, Tijmen later received the Suzanne Hovinga Award for best master's internship project at the LACDR in 2013. During his master's studies, he also worked as a student assistant in this laboratory to screen molecule libraries in close collaboration with pharmaceutical industry. He later joined the Eidgenössische Technische Hochschule (ETH) in Zürich, Switzerland, to study the role of matrix metalloproteases on wound healing as part of his master's education.

Tijmen's great interest in developing physiologically relevant *in vitro* culture systems that can improve the quality of *in vitro* drug research and may eventually reduce or replace required animal experiments, motivated him in 2013 to pursue his PhD research at the division of Toxicology of the LACDR under supervision of Dr. Leo S. Price, Prof. Dr. Dorien J.M. Peters and Prof. Dr. Bob van de Water. During his PhD studies, he developed 3D cell culture-based screening assays with many different cell types. His PhD studies have resulted in the establishment of 3D cell-culture based phenotypic screening technology that can be used to identify new drug targets or potential new drug candidates as described in this thesis. During his PhD studies, Tijmen received two awards to attend scientific conferences (SLAS Tony B. Travel Awards 2013 and 2016).

After his PhD studies in 2017, Tijmen will combine his interests in developing (physiologically relevant) cell-based assays, laboratory automation technology and compound screening, as lab automation and screening specialist at NEXUS Personalized Health Technologies at the ETH in Zürich.