Ulm University Hospital
Surgery Center
Department of General and Visceral Surgery
Medical Director: Prof. Dr. med. Doris Henne-Bruns

Validation of new Casein Kinase 1 (CK1) small molecule inhibitor compounds and characterization of Inhibitors of Wnt Production (IWPs) as inhibitors of CK1δ

Dissertation submitted in partial fulfillment of the requirements for the
Doctoral Degree of Human Biology (Dr. biol. hum.)
at the Medical Faculty of Ulm University

submitted by

Balbina García Reyes
born in San José, Costa Rica

2017

Acting Dean:	Prof. Dr. Thomas Wirth
First reviewer:	Prof. Dr. Uwe Knippschild
Second reviewer:	PD Dr. Timo Burster
Day of Graduation:	05.05.2017

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List of abbreviations

A	adenine
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AML	acute myelogenous leukemia
APC	adenomatous polyposis coli
APS	ammonium persulfate
Arg	arginine
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	cytosine
Ci	curie
CK	casein kinase
CMGC	kinase family including cyclin-dependent kinases, mitogen-activated protein kinases, glycogen synthase kinases, and CDK-like kinases
DNA	deoxyribonucleic acid
DCIS	ductal carcinomas in situ
DDX3	DEAD-box RNA helicase 3
dH_2O	demineralized water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
DVL	dishevelled
E. coli	*Escherichia coli*
EC_{50}	half maximal effective concentration
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ePK	eukaryotic protein kinase
FACS	fluorescence assisted cell sorting

FAP	familial adenomatous polyposis coli
FCS	fetal calf serum
for	forward
FP	fusion protein
FZD	Frizzled
G	guanine
g	gram
GLI	glioma-associated oncogene
G/LOF	Gain/Loss of function
GSK3β	glycogen synthase kinase 3 beta
GST	glutathione-S-transferase
Hh	hedgehog
HPF	high power field
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horse-radish peroxidase
hrs	hour(s)
IC_{50}	half maximal inhibitory concentration
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
IWP	Inhibitor of Wnt Production
k_{cat}	turnover number
KD	kinase domain
kDa	kilodalton
l	liter
LATS	large tumor suppressor
LEF	lymphoid enhancing factor
Leu	leucine
Lys	lysine
M	molar
MDM2	mouse double minute 2 homolog
min	minute(s)
mRNA	messenger ribonucleic acid
MST	mammalian STE20-like protein kinase

mut	mutant
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
M_W	molecular weight
NLS	nuclear localization signal
OD	optical density
PCR	polymerase chain reaction
PD	Parkinson's disease
PER	period
pH	negative decimal logarithm of the hydrogen ion concentration
PKA	protein kinase A
PTCH	Patched
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time PCR
rev	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RQ	relative quantification
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDM	site-directed mutagenesis
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
SMAD	SMA/mothers against decapentaplegic
SMO	smoothened
STPK	Serine/Threonine Protein Kinases
T	thymine
TAZ	tafazzin
TBS	tris-buffered saline
TCF	T cell factor
TEAD	transcription factor TEA domain
TEMED	N,N,N',N'-tetramethylethylenediamine
Thr	threonine

TPK	Tyrosine Protein Kinase
TV	transcript variant
UICC	Union Internationale Contre le Cancer
V	volt
v/v	volume per volume
V_{max}	maximum velocity
w/v	weight per volume
Wnt	Wingless/Int-1
wt	wild type
YAP	Yes-associated protein
β-TrCP	beta-transducin repeat containing E3 ubiquitin protein ligase
µ	micro
°C	degree Celsius

1. Introduction

1.1 Protein phosphorylation and signal transduction

Posttranslational modifications such as phosphorylation, glycosylation, ubiquitination, nitrosylation, acylation, methylation, lipidation, and proteolysis affect the physiological and cellular processes that occur within organisms in many ways (Walsh *et al.* 2005). Among them, reversible phosphorylation is one of the most studied modifications. It is performed by protein kinases, which transfer the gamma phosphate of ATP onto different substrates, a process that in turn is reversed by phosphatases. Eukaryote protein kinases (ePKs) phosphorylate either tyrosine (TPKs, Tyrosine Protein Kinases), serine/threonine (STPKs, Ser-/Thr Protein Kinases) or both, tyrosine and threonine/serine residues (dual-specificity protein kinases) (Cohen 2001; Kennelly 2002; Kennelly 2003; Ubersax and Ferrell 2007; Fabbro *et al.* 2015). Phosphorylation plays an essential role in many cellular and extracellular processes. Unsurprisingly, anomalous phosphorylation patterns in eukaryotes are linked with a variety of disorders such as cancer, inflammatory diseases, neurodegenerative disorders, endocrine diseases, infectious diseases and cardiovascular disorders (Cohen 2002; Ubersax and Ferrell 2007; Fabbro *et al.* 2015).

The human genome encodes 518 ePKs, which can be classified into seven families of typical and seven families of atypical protein kinases, and most of them are STPKs (Hanks and Hunter 1995; Cohen 2001; Manning *et al.* 2002; Ubersax and Ferrell 2007). A small but well-studied fraction of ePKs is comprised by the TPKs, and their importance is highlighted by the various gain of function (GOF) and loss of function (LOF) mutations identified in this group of kinases (Lahiry *et al.* 2010; Fabbro *et al.* 2015). TPKs are further sub-classified into two classes: receptor TPKs and non-receptor TPKs. STPKs are divided into different families of typical ePKs: the TKL family (the Tyrosine Kinase-Like group), the CMGC family (including Casein Kinases 2), the AGC family (PKA, PKG, PKC), the CAMK family (Calcium/calmodulin-dependent Kinases), the STE20 family (including Mitogen-Activated Kinase Kinases) and finally the CK1 family (Casein Kinases 1). Furthermore, about 10% of the human protein kinases are pseudokinases: they are weakly active or presumed to be inactive, as the lack at least one of three motifs in the catalytic domain that are crucial for catalysis (Boudeau *et al.* 2006; Kannan and Taylor 2008). These pseudokinases are thought to have important regulatory functions, and they also can bind to ATP (Boudeau *et al.* 2006; Zeqiraj *et al.* 2009; Rajakulendran and Sicheri 2010).

In addition to transferring of the gamma phosphate of ATP onto hydroxyl groups of substrates, kinases have non-catalytic functions in signal cascades, such as scaffolding, allosteric effects, subcellular targeting, DNA binding and protein-protein interactions (Rauch *et al.* 2011). This variety of roles means that alterations in kinase activity influence many cellular processes (Cohen 2002; Chico *et al.* 2009; Lahiry *et al.* 2010; Knippschild *et al.* 2014). This makes kinases interesting targets for drug development for the treatment of various diseases.

1.2 The CK1 family

Members of the Casein Kinase (CK) 1 family and CK2 are some of the earliest described protein kinases (Hathaway and Traugh 1979; Ospina and Fernandez-Renart 1990; Rowles *et al.* 1991; Graves *et al.* 1993; Walczak *et al.* 1993; Tapia *et al.* 1994; Fish *et al.* 1995; Zhai *et al.* 1995). Both groups phosphorylate the milk protein casein *in vitro*, commonly serine or threonine residues located next to already phosphorylated N-terminal amino acid residues or acidic amino acids (Brunati *et al.* 2000). Members of the CK1 family as well as CK2 are STPKs: CK1 comprises its own phylogenetically-distinct kinase family while CK2 belongs to the CMGC family, which also includes cyclin-dependent kinases (CDK), mitogen-activated protein kinases (MAPK), CDK-like kinases (CLK), and glycogen synthase kinases (GSK3) (Manning *et al.* 2002).

The CK1 family is an independent and conserved group of monomeric kinases, their closest protein relatives are tau tubulin kinases 1 and 2 (TTBK1/2) and vaccinia-related kinases 1 to 3 (VRK1-3) (Lopez-Borges and Lazo 2000). CK1-homolog proteins have been isolated from organisms like yeast, basidiomycetes, plants, algae, and protozoa (DeMaggio *et al.* 1992; Robinson *et al.* 1992; Wang *et al.* 1992; Walczak *et al.* 1993; Dhillon and Hoekstra 1994; Kearney *et al.* 1994; Gross and Anderson 1998; Wang *et al.* 2011). Members of the CK1 kinase family are ubiquitously expressed and constitutively active, comprising seven distinct isoforms in mammals: α, β, γ1, γ2, γ3, δ, and ε, as well as various post-transcriptionally processed splice variants (transcription variants, TV) (Green and Bennett 1998; Fu *et al.* 2001; Burzio *et al.* 2002; Knippschild *et al.* 2005a). These isoforms are highly conserved within their kinase domains, with catalytic-domain-sequence-homology similarities reaching between 52 to 98%, whereby CK1δ and CK1ε show the highest homology level. In contrast, CK1 isoforms vary significantly in length and primary structure of their regulatory non-catalytic domains. The length of the different C-termini varies from 24 up to more than 200 amino acids, while their N-termini consists of 9-76 amino acids,

which results in a range of molecular weights between 32 kDa for CK1α to 52.2 kDa for CK1γ3 (Graves *et al.* 1993; Fish *et al.* 1995; Zhai *et al.* 1995; Gross and Anderson 1998; Knippschild *et al.* 2005b) (Figure 1A).

The kinase domains of CK1 isoforms are about 293 amino acid long and includes a kinesin homology domain (KHD). This domain is known to promote the interaction of kinases with components of the cytoskeleton-like kinesin motor proteins (Tuazon and Traugh 1991; Roof *et al.* 1992; Longenecker *et al.* 1998; Behrend *et al.* 2000b). A nuclear localization signal (NLS) sequence is located next to the ATP-binding site (Figure 1B), and in the case of certain CK1α splice variants, a second NLS can be found in its C-terminus (Rowles *et al.* 1991; Tuazon and Traugh 1991). This extra NLS is essential for the efficient translocation of the CK1α splice variant into the nucleus (Fu *et al.* 2001). CK1 family members show the typical bilobal structure of protein kinases, with a smaller N-terminal and a larger C-terminal lobe. A hinge region forms a catalytic cleft for ATP and substrate binding connects N-lobe, primarily consisting of β-sheets, and α-helical C-lobe (Xu *et al.* 1995) (Figure 1C and D).

1.3 CK1 substrate-specificity and consensus sequence

CK1 isoforms preferably recognize substrates with negatively-charged amino acids, due to the presence of several positively-charged residues within their substrate binding site in the CK1 kinase domain (Flotow and Roach 1991; Longenecker *et al.* 1996). Acidic or phosphorylated amino acids with the canonical consensus sequence motif pSer/Thr-X-X-(X)-Ser/Thr are the favored substrates of CK1 isoforms, where pSer/Thr is a phosphorylated serine of threonine, and X indicates any other amino acid except serine or threonine. These phosphor-primed residues can be replaced by negatively-charged amino acids, eliciting the kinase activity of CK1 isoforms (Agostinis *et al.* 1989; Flotow *et al.* 1990; Flotow and Roach 1991; Meggio *et al.* 1991; Graves *et al.* 1993). Other sequences can also be recognized and phosphorylated by CK1 family members, and these are termed non-canonical consensus sequences. A prominent example is the sequence Ser-Leu-Ser (SLS) present in β-catenin and nuclear factor of activated T-cells (NFAT) (Marin *et al.* 2003), and the Lys/Arg-X-Lys/Arg-X-X-Ser/Thr sequence of sulfatide and cholesterol-3-sulfate (SCS) binding proteins (Kawakami *et al.* 2008). Different consensus sequences have been reported for CK1 kinases in various cellular proteins. So far, over 140 targets of CK1 kinases have been described in the literature, *in vivo* and *in vitro* (Knippschild *et al.* 2014) (Table 1), indicating that CK1 family members hold relevant functions in a wide variety of cellular processes.

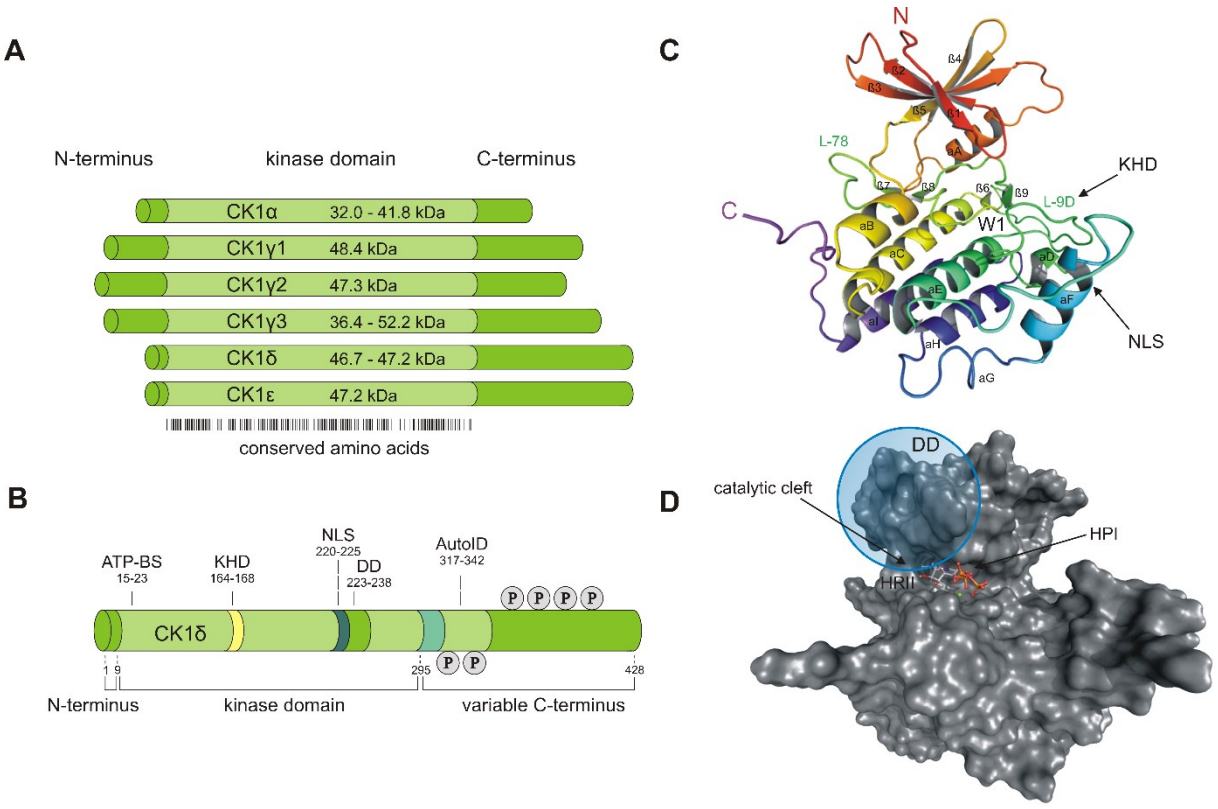


Figure 1: Structure of the Casein Kinase 1 (CK1) family. (A) Schematic alignment of human CK1 isoforms α, γ1-3, δ, and ε. Their molecular weight varies between 32 kDa (CK1α) and 52.2 kDa (CK1γ3). All CK1 isoforms are highly conserved within their kinase domains, but differ within their variable N- and C-terminal non-catalytic domains. Black bars indicate the positions of conserved amino acids (modified from Knippschild *et al.* 2005b). **(B)** Schematic representation of the rat CK1δ domain structure. Numbers indicate amino acid positions (ATP-BS: ATP binding site, KHD: kinesin homology domain, NLS: putative nuclear localization signal, AutoID: autoinhibitory domain, DD: putative dimerization domain, P: sites of phosphorylation). **(C)** Ribbon and **(D)** surface diagram of the molecular structure of CK1δ (PDB code 4HGT) modeled in complex with Mg2+-ATP at a resolution of 1.80 Å. The nomenclature is adapted from Xu *et al.* 1995 and Longenecker *et al.* 1998. For reasons of clarity and relevance only CK1δ is shown. The catalytic domain folds into two lobes primarily containing strands (N-terminal), respectively helices (C-terminal) forming a catalytic cleft between that represents the ATP binding pocket as well as a substrate binding site. The kinesin homology domain within L-9D. A putative dimerization domain (DD) containing various amino acids of β1, β2, β5, L-5B, β7, and αB, whereas the putative nuclear localization signal (NLS) sequence at the junction between L-EF and αF. A tungstate molecule binding site identifies a specific phosphate moiety binding motif (W1). The active site contains a deep hydrophobic pocket (HPI) and a spacious hydrophobic region (HRII). Figures 1B, 1C, and 1D are adopted from Knippschild *et al.* 2014.

Table 1: Reported substrates of CK1 family members *in vitro* and *in vivo*. Expanded from Knippschild *et al.* 2014.

Functional Groups	CK1 Substrates
cytoskeleton-associated proteins, adhesion factors, and scaffolding proteins	Myosin (Singh *et al.* 1982), troponin (Singh *et al.* 1982), ankyrin (Lu *et al.* 1985), spektrin 3 (Eder *et al.* 1986), filamin (Nakajo *et al.* 1987), vinculin (Cegielska *et al.* 1998), neurofilamentary proteins (Floyd *et al.* 1991; Link *et al.* 1993), dynein (Yang and Sale 2000), α-/β-tubulin (Behrend *et al.* 2000b), microtubule-associated proteins MAP1A (Wolff *et al.* 2005), MAP4 (Behrend *et al.* 2000a), stathmin (Behrend *et al.* 2000a), tau (Singh *et al.* 1995), keratin 17 (Gao *et al.* 2000), desmolein (Gao *et al.* 2000), annexin II (Gao *et al.* 2000), centaurin-α (p42IP4) (Dubois *et al.* 2002), neural cell adhesion molecule (NCAM) (Mackie *et al.* 1989), E-cadherin (Dupre-Crochet *et al.* 2007), RhoA, RhoB (Tillement *et al.* 2008), myelin basic protein (MBP) (Kawakami *et al.* 2008), kinesin-like protein 10A (KLP10A) (Mennella *et al.* 2009), galectin-3 (Szabo *et al.* 2009), End binding 1 (EB1) (Zyss *et al.* 2011), Sid4 (Johnson *et al.* 2013), RanBPM (Wolff *et al.* 2015)
receptors	β-subunit of the insulin receptor (Rapuano and Rosen 1991), TNFα-receptor (Darnay *et al.* 1997), muscarin M3-receptor (Tobin *et al.* 1997), Ste2p (α-factor-receptor) (Hicke *et al.* 1998), Ste3p (α-factor-rezeptor) (Feng and Davis 2000), platelet-derived growth factor (PDGF) receptor (Bioukar *et al.* 1999), retinoid X receptor (RXR) (Zhao *et al.* 2004), low density lipoprotein related receptor protein (LRP) 6 (Davidson *et al.* 2005; Zeng *et al.* 2005), type I interferon receptor (IFNAR1) (Liu *et al.* 2009), estrogen receptor α (ERα), amplified in breast cancer 1 (AIB1) (Giamas *et al.* 2009), calmodulin (CaM) (Papoff *et al.* 2010)
membrane transporters	erythrocytes anion transporter (Soong *et al.* 1987), uracil permease (*S. cerevisiae*) (Marchal *et al.* 2002), translocase of the outer mitochondrial membrane 22 (Tom22) (Gerbeth *et al.* 2013)
DNA-/RNA-associated proteins	RNA polymerase I and II (Dahmus 1981), non-histone chromatin proteins (Christmann and Dahmus 1981), topoisomerase Iiα (Grozav *et al.* 2009), Star-poly(A) polymerase (Star-PAP) (Gonzales *et al.* 2008), Rec8 (Ishiguro *et al.* 2010), DNA methyltransferase (Dnmt1) (Sugiyama *et al.* 2010), TAR DNA binding protein of 43 kDa (TDP-43) (Kametani *et al.* 2009), DEAD-box RNA helicase 3 (DDX3) (Cruciat *et al.* 2013), Ubiquitin-like, with PHD and RING finger domains 1 (UHRF1) (Chen *et al.* 2013)
ribosomal proteins	15 kDa, 20 kDa, 35 kDa ribosomal proteins Wodja *et al.* 1999), L4, L8, L13 ribosomal proteins (Gao *et al.* 2000), ribosomal protein S6 (rpS6) (Hutchinson *et al.* 2011), ENP1/BYSL and LTV1 (Zemp *et al.* 2014)
transcription and splice factors	p53 (Milne *et al.* 1992), cAMP responsive element modulator (CREM) (de Groot *et al.* 1993), Swi6 (Ho *et al.* 1997), nuclear factor of activated T-cells (NFAT) (Zhu *et al.* 1998), SR proteins (Gross *et al.* 1999), T-cell factor (Tcf) 3 (Lee *et al.* 2001), brain and muscle Arnt-like protein (BMAL) 1 (Eide *et al.* 2002), cryptochrome 1 (CRY) (Eide *et al.* 2002), β-catenin (Liu *et al.* 2002; Sakanaka 2002), Armadillo (Yanagawa *et al.* 2002), Smad 1-3 and 5 (Waddell *et al.* 2004), osmotic response element binding protein (OREBP) (Xu *et al.*

Functional Groups	CK1 Substrates
	2008), Cubitus interruptus (Ci) (Jia *et al.* 2005), forkhead box G1 (FoxG1) (Regad *et al.* 2007), SNAIL (Xu *et al.* 2010), Yes-associated protein (YAP) (Zhao *et al.* 2010), proliferator-activated receptor γ coactivator 1α (PGC-1α) (Li *et al.* 2011), NFkB (nuclear factor kappa-light-chain-enhancer of activated B-cells) subunit p65 (Wang *et al.* 2014)
translation factors	initiation factors (IF) 4B, 4E, (Haas and Hagedorn 1991, Meggio *et al.* 1992, Graves *et al.* 1993, Biswas *et al.* 2011)
viral proteins	Simian virus 40 large T-antigen (SV40 T-Ag) (Grasser *et al.* 1988), Hepatitis C virus non-structural 5A (NS5A) (Quintavalle *et al.* 2007), Human cytomegalovirus ppUL44 (Alvisi *et al.* 2011), Poa semi-latent hordeivirus triple gene block 1 (TGB1) (Makarov *et al.* 2012), Kaposi sarcoma-associated herpesvirus latency-associated nuclear antigen (LANA) (Woodard *et al.* 2012)
kinases and phosphatases	cyklin-dependent kinase 5 (CDK5) (Sharma *et al.* 1999), protein kinase C (PKC) (Okano *et al.* 2004), protein kinase D2 (PKD2) (von Blume *et al.* 2007), cell division cycle 25 (Cdc25) (Pal *et al.* 2008; Honaker and Piwnica-Worms 2010; Piao *et al.* 2011), PH domain and leucine rich repeat protein phosphatase 1 (PHLPP1) (Li *et al.* 2009)
inhibitors and modulators	inhibitor 2 of PPA 1 (Agostinis *et al.* 1989; Marin *et al.* 1994), dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32) (Desdouits *et al.* 1995), Dishevelled (Dvl) (Peters *et al.* 1999), mammalian period circadian protein (mPER) (Vielhaber *et al.* 2000), adenomatous polyposis coli (APC) (Rubinfeld *et al.* 2001), BH3 interacting domain death agonist (Bid) (Desagher *et al.* 2001), protein kinase C potentiated myosin phosphatase inhibitor of 17 kDa (CPI-17) (Zemlickova *et al.* 2004), nm23-H1 (Garzia *et al.* 2008), 14-3-3 proteins (Clokie *et al.* 2009), MDM2 (Winter *et al.* 2004), MDMX (Chen *et al.* 2005), FREQUENCY (FRQ) (He *et al.* 2006), WHITE COLLAR-1 (WC-1) (Huang *et al.* 2007), CARD containing MAGUK protein (CARMA1) / caspase recruitment domain (CARD11) (Bidere *et al.* 2009), SLR1 (Dai and Xue 2010), endogenous meiotic inhibitor 2 (Emi2) (Isoda *et al.* 2011), Chk1-activating domain (CKAD) of Claspin (Meng *et al.* 2011), PER2 (Shanware *et al.* 2011), Protein S (Stavenuiter *et al.* 2013)
metabolism-associated enzymes	acetyl-CoA carboxylase (Tipper *et al.* 1983), glycogen synthase (Kuret *et al.* 1985; Poulter *et al.* 1988), Ssy5 (Omnus and Ljungdahl 2013), NEDD4 (Liu *et al.* 2014)
vesicle and trafficking associated proteins	SV2 (Gross *et al.* 1995), β3A- and β3B-subunit of the AP-3 complex (Faundez and Kelly 2000), snapin (Wolff *et al.* 2006), ceramide transfer protein (CERT) (Tomishige *et al.* 2009)
receptor-associated proteins	Fas-associated death domain (FADD) (Alappat *et al.* 2005), receptor interacting protein 1 (RIP1) (Wang *et al.* 2008)
factors of neuro-degenerative diseases	presenilin-2 (Walter *et al.* 1998), tau (Singh *et al.* 1995), β-secretase (Walter *et al.* 2001), parkin (Yamamoto *et al.* 2005), α-synuclein (Okochi *et al.* 2000)
metastatic tumor antigens	Metastatic Tumor Antigen 1, short form (MTA1s) (Mishra *et al.* 2004)

1.4 Regulation of CK1 activity

CK1 kinases are ubiquitously expressed and constitutively active, found in many different organisms, tissues and cell lines, and show variation in their expression levels depending on cell type and tissue (Tuazon and Traugh 1991; Lohler *et al.* 2009; Utz *et al.* 2010). Unsurprisingly, various regulatory mechanisms have been discovered able to influence the expression and activity of this family of proteins. Certain factors seem to change the expression and activity of CK1, such as stimulation with insulin (Cobb and Rosen 1983) or gastrin (von Blume *et al.* 2007), viral transformation (Elias *et al.* 1981), treatment with topoisomerase inhibitors or other small molecules like camptothecin (Knippschild *et al.* 1997), γ-irradiation (Santos *et al.* 1996), or altered membrane concentrations of phosphatidylinositol-4,5-bisphosphate (PIP2) (Gross *et al.* 1995). At the posttranslational level, controlling mechanisms include structure-related regulation, subcellular localization, proteins-protein nteractions, and posttranslational modifications.

It has been proposed that one mode of negative regulation involves the formation of CK1 homodimers. X-ray crystallography revealed that CK1δ can form dimers, in which the specific intramolecular contacts of the dimerization domain occupy the adenine binding domain (Longenecker *et al.* 1998). Consequently, ATP is excluded from the active center of the kinase. This hypothesis is supported by the finding that a CK1δ dominant-negative mutant can downregulate the activity of wtCK1δ in simian virus 40 (SV40)-fully transformed cell lines resulting in a reversion of the maximally transformed phenotype to a minimally transformed phenotype (Deppert *et al.* 1991). Furthermore, this dominant-negative CK1δ also attenuates mammary tumorigenesis in WAP-mutCK1δ/WAP-T bi-transgenic mice resulting in prolonged survival of bi-transgenic animals compared to WAP-T transgenic mice (Hirner *et al.* 2012).

CK1 isoforms can also be regulated by subcellular localization and compartmentalization. For example, in *Saccharomyces cerevisiae* the CK1 homologs Yck1, Yck2, Yck3, Cki1, and Cki2 are anchored to the cytoplasmic membrane by an isoprenyl residue, while the Hrr25 isoform is located in the nucleus. Mutations in the NLS or the isoprenylation site of these homologs have been shown to alter their localization or even impair their functions (Vancura *et al.* 1994). Interestingly, it has been shown that the catalytic activity of CK1 is critical for its appropriate subcellular localization when the localization of CK1δ kinase-dead mutant had been studied (Milne *et al.* 2001).

Protein-protein interactions play a central role in CK1 regulation, particularly the interaction with scaffold proteins, which influence partner proteins and their function. Some examples

of these protein scaffolds include CG-NAP/AKAP450 (Golgi N-kinase anchoring protein/ A-Kinase Anchoring Protein 450), which interacts with CK1δ and ε and mediates their centrosomal recruitment (Sillibourne *et al.* 2002). A prominent example of the influence of scaffolding proteins is the DEAD-box RNA helicase 3 (DDX3) (Gu *et al.* 2013). CK1 activity in Wnt signaling depends on DDX3 as a co-factor, as it directly interacts with CK1ε promoting the phosphorylation of Dishevelled (Cruciat *et al.* 2013). This protein is a regulatory subunit of various CK1 isoforms with the potential to increase the activity of CK1α, γ2, δ, and ε by up to five orders of magnitude (Cruciat *et al.* 2013).

Perhaps the most prominent regulatory mechanism of CK1 on a post-translational level is reversible phosphorylation, including intramolecular autophosphorylation, site-specific phosphorylation by cellular kinases and dephosphorylation by phosphatases. CK1α, γ3, δ, and ε have been shown to be capable of autophosphorylation, which mainly occurs in their C-terminal domains. In CK1δ and ε, sequences with the motif pSer/Thr-X-X-Y (Y: any amino acid except serine or threonine) can be generated by autophosphorylation events, blocking the catalytic center of the kinase (Graves and Roach 1995; Cegielska *et al.* 1998; Rivers *et al.* 1998; Gietzen and Virshup 1999; Budini *et al.* 2009). This kind of inhibition is supported by studies in which dephosphorylation of CK1 by phosphatases increases kinase activity *in vitro* (Cegielska *et al.* 1998; Bedri *et al.* 2007). Furthermore, proteolytic cleavage of the C-terminus has been shown to increase the activity of CK1 isoforms (Carmel *et al.* 1994; Cegielska *et al.* 1998). Modulation of CK1 activity by other cellular kinases has been reported, for example, CK1δ is phosphorylated *in vitro* by PKA (cAMP-dependent protein kinase), Akt (protein kinase B), CLK2 (CDC-like kinase 2), PKCα (protein kinase C, isoform α), CDKs, and Chk1 (checkpoint kinase 1) (Giamas *et al.* 2007; Bischof *et al.* 2013; Knippschild *et al.* 2014; Ianes *et al.* 2016; Meng *et al.* 2016).

1.5 Functions of CK1

CK1 family members are involved in a broad range of different cellular processes including circadian rhythm, membrane transport processes and intercellular interactions, cell cycle progression, microtubule-associated dynamics, and apoptosis (Price 2006; Cheong and Virshup 2011; Knippschild *et al.* 2014). Perhaps one of the best-known functions of CK1 refers to its role in the regulation of the circadian rhythms. CK1 isoforms can phosphorylate and regulate the clock proteins BMAL1 (brain and muscle ARNT-like protein 1) and CRY (cryptochrome) and change the expression of PHB2 (prohibitin 2) (Kategaya *et al.* 2012). Also, CK1δ and ε influence the stability and subcellular localization of PER (Period)

(Vielhaber *et al.* 2000; Eide *et al.* 2002; Etchegaray *et al.* 2009; Walton *et al.* 2009; Shanware *et al.* 2011). Furthermore, when protein phosphatase 5 (PP5) elevates the activity of CK1ε by dephosphorylation, the degradation of PER is increased, thereby altering the circadian rhythm (Partch *et al.* 2006).

CK1 isoforms are also involved in membrane transport and related processes. For example, there are CK1 homologs in *Saccharomyces cerevisiae* that are membrane-bound (Wang *et al.* 1992), and there is evidence of CK1-homologues participating in the regulation of yeast membrane transport processes (Panek *et al.* 1997; Murakami *et al.* 1999). Another example is the *Drosophila* CK1γ homolog Gilgamesh, which has a role in regulating vesicle trafficking and recycling (Gault *et al.* 2012). In higher eukaryotes CK1 has been shown to phosphorylate several proteins of the SNARE (soluble N-ethyl-maleimide-sensitive factor attachment protein receptor) complex in neuronal cells (Dubois *et al.* 2002; Wolff *et al.* 2006), which has an impact in synaptic vesicle exocytosis (Turner *et al.* 1999; Lin and Scheller 2000; Pyle *et al.* 2000). Regarding cell-to-cell contact, E-cadherin is a known substrate of CK1. Site-specific phosphorylation of E-cadherin acts as a negative regulator, leading to weakened cell-cell interactions. In cell culture, this interaction can be rescued by treatment with the CK1 inhibitor IC261 (Dupre-Crochet *et al.* 2007).

Different CK1 isoforms have been implicated in the regulation of cell cycle progression, spindle dynamics, chromosome segregation, and microtubule dynamics. CK1α, for example, has been shown to localize at the kinetochore, microtubule asters, and the centrosome, while CK1δ associates with the spindle apparatus during mitosis (Brockman *et al.* 1992; Wang *et al.* 2013a). As a response towards genotoxic stress, CK1δ directly regulates microtubule dynamics and the spindle apparatus through the phosphorylation of tubulin, stathmin, and the microtubule-associated proteins (MAPs) MAP4, MAP1A, tau, and Sid4, a delayer of cytokinesis (Behrend *et al.* 2000a; Li *et al.* 2004; Wolff *et al.* 2005; Hanger *et al.* 2007; Johnson *et al.* 2013; Leon-Espinosa *et al.* 2013). Another example of the role of CK1 at the centrosome is its interaction with the scaffolding protein AKAP450 (A-Kinase Anchoring Protein 450), also known as CG-NAP (Golgi N-kinase anchoring protein). AKAP450 interacts with CK1δ and ε and recruits them to the centrosome. Inhibition of these isoforms leads to functional impairment of several centrosome-associated proteins (Sillibourne *et al.* 2002). It has also been suggested that the interaction of CK1δ with AKAP450 is necessary for primary ciliogenesis (Greer *et al.* 2014). Dynein-dependent transport of membrane organelles along microtubules is regulated by CK1ε, which phosphorylates the dynein intermediate chain (DIC), as well as the DIC component IC138 (Yang and Sale 2000; Ikeda

et al. 2011). Finally, CK1δ associates to the spindle apparatus during mitosis and phosphorylates α-, β-, and γ-tubulin *in vitro*, thereby modulating microtubule's response to stress (Behrend *et al.* 2000b; Stoter *et al.* 2005).

A noteworthy function of CK1 family members is the modulation of the tumor suppressor protein p53 and its cellular counterpart Mouse double minute 2 homolog (MDM2). CK1α, δ, and ε phosphorylate certain N-terminal target sites of p53. As a response to stress, phosphorylation of p53 at Ser-15 and Thr-18 by CK1δ and ε results in decreased binding affinity between p53 and MDM2, leading to the accumulation of active p53 (Knippschild *et al.* 1997; Dumaz *et al.* 1999; Huart *et al.* 2009; Venerando *et al.* 2010). CK1δ and ε also phosphorylate MDM2 at Ser-118 and Ser-121, leading to its ubiquitination and proteasomal degradation, thus increasing even more the cellular levels of transcriptionally free p53 (Meek and Knippschild 2003; Winter *et al.* 2004; Kulikov *et al.* 2006; Huart *et al.* 2009; Inuzuka *et al.* 2010). It has been observed that CK1δ transcription is activated by genotoxic-stress-induced p53, and at the same time, p53 can be activated by CK1δ-mediated phosphorylation, creating an autoregulatory feedback loop between these two proteins (Knippschild *et al.* 1997). Furthermore, phosphorylation of MDM2 at several serine residues within the acidic domain enhances MDM2-p53 binding and subsequent degradation of p53 (Knippschild *et al.* 2014). Finally, CK1α physically interacts with MDM2 promoting p53 inhibition through degradation in unstressed cells (Huart *et al.* 2009; Huart *et al.* 2012).

Apoptosis is also a process in which CK1 plays a regulatory role. In Fas-mediated apoptosis, CK1 acts as a negative regulator by phosphorylating Bid (BH3-interacting domain death agonist). In its unphosphorylated state Bid is processed by caspase-8 and integrates into the mitochondrial membrane. Consistently, it has been observed that inhibition of CK1 induces Fas-triggered apoptosis, while overexpression of CK1 reduces the number of apoptotic cells (Desagher *et al.* 2001). Also, CK1α phosphorylates FADD (Fas-associated protein with dead domain), which leads to resistance to apoptosis induced by TRAIL (tumor necrosis factor-related apoptosis inducing ligand). It has been observed that inhibition of CK1 could increase the sensitivity towards chemotherapeutics, recruiting of FADD and procaspase 8 to the Death-Inducing Signaling Complex (DISC) (Izeradjene *et al.* 2004). Moreover, p75-mediated apoptosis is also negatively regulated by CK1 through the phosphorylation of p75 neurotrophin receptor (Beyaert *et al.* 1995).

1.6 CK1 role in the Wnt pathway

The Wnt pathway consists of a complex network of signal transduction cascades that integrate messages passed to the cell by the binding of a Wnt ligand to its receptor, thereby triggering a series of responses that alter fundamental processes such as embryonic development and tissue regeneration (Cruciat 2014) (Figure 2). The Wnt pathway is typically subdivided into the canonical and non-canonical, depending on if β-catenin is involved or not (Anastas and Moon 2013). In the canonical pathway, the Wnt ligand binds to a Frizzled receptor, which in turn forms a complex with the surface-bound lipoprotein receptor-related proteins 5/6 (LRP5/6). This complex activates Dishevelled (Dvl), and Dvl displaces GSK3β from the destruction complex integrated by Axin, Adenomatous Polyposis Coli (APC), CK1α, and GSK3β. In the absence of Wnt ligands, this destruction complex targets β-catenin for CK1α- and GSK3β-mediated phosphorylation and subsequent ubiquitination, leading to proteasomal degradation. When the destruction complex is inactivated, β-catenin is stabilized, accumulated, and subsequently translocated to the nucleus, where it fulfills a role as transcriptional regulator, by forming a complex with the transcription factor TCF/LEF (T-cell factor/lymphoid enhancing factor) (Moon *et al.* 1997; Logan and Nusse 2004; Clevers 2006; Niehrs 2012).

CK1 family members are involved in crucial steps of Wnt pathway, both as negative and positive regulators (Cruciat 2014; Knippschild *et al.* 2014). In the absence of Wnt stimulation, CK1α phosphorylates β-catenin, priming it for further phosphorylation by GSK3β, leading to its degradation (Elyada *et al.* 2011). The role of CK1 as a negative regulator of the Wnt pathway is further exemplified by the phosphorylation of APC by CK1δ and ε. Phosphorylated APC has a higher affinity to β-catenin, which causes β-catenin to be further downregulated by the destruction complex (Ha *et al.* 2004). It is also known that the phosphorylation of LRP5/6 by CK1ε has a negative regulatory function (Swiatek *et al.* 2006). CK1 isoforms also act as positive regulators of the Wnt pathway. Upon binding of a Wnt ligand to Frizzled, membrane-bound CK1γ phosphorylates LRP5/6 (Davidson *et al.* 2005) and CK1δ and ε phosphorylate Dvl (Peters *et al.* 1999; Sakanaka 2002; Greer and Rubin 2011). These two phosphorylation events are hallmarks of the activation of Wnt pathway. Phosphorylated LRP5/6 recruits the destruction complex through Axin binding, thereby inhibiting GSK3β (Mao *et al.* 2001). Axin acts as a scaffold that binds together the destruction complex, and its phosphorylation causes the dissociation of this complex. In the case of Dvl, CK1ε phosphorylates it with the help of the RNA-helicase DDX3, which acts as a regulatory subunit of CK1ε, promoting its kinase activity on Dvl (Cruciat *et al.* 2013).

This leads to the inhibition of GSK3β and the stabilization of β-catenin. CK1δ- and CK1ε-mediated phosphorylation of Dvl is crucial for both canonical and non-canonical Wnt signaling (Bryja *et al.* 2007; Greer and Rubin 2011). Furthermore, CK1ε phosphorylates TCF3, which promotes its binding to β-catenin (Lee *et al.* 2001).

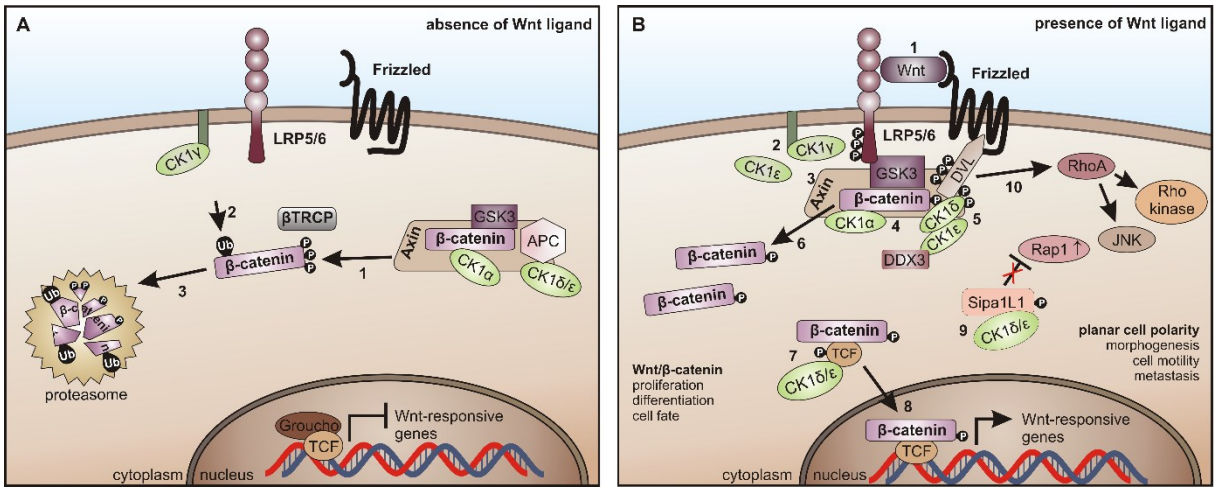


Figure 2: CK1 in Wnt signaling. (A) In absence of the Wnt, ligand β-catenin is progressively phosphorylated by CK1α and GSK3 (1), recruited to β-TrCP for ubiquitination (2) and thereby primed for proteasome-mediated degradation (3). **(B)** Upon binding of Wnt to FZD and LRP5/6 (1), LRP5/6 is phosphorylated by CK1γ (positive regulation) and CK1ε (negative regulation) (2). It subsequently recruits Axin and the β-catenin destruction complex to the membrane and inhibits GSK3 (3, 4). Wnt-activated CK1δ and ε phosphorylate DVL and Axin (5), induce a conformational change in the β-catenin destruction complex and initiate the dissociation of various components (6). CK1ε cooperates with DDX3 in phosphorylating DVL (7). In addition, TCF3 is phosphorylated by CK1δ and ε thereby increasing its binding affinity to β-catenin followed by the nuclear translocation of TCF3/β-catenin (8). The non-canonical Wnt pathway is positively regulated by CK1δ- and ε-dependent release of Rap1 from Sipa1L1 inhibition (9). The Rho/JNK signaling cascade is activated upon phosphorylation of DVL (10). (β-TrCP: beta-transducin repeat containing E3 ubiquitin protein ligase, CK1: Casein Kinase 1, DDX3: DEAD-box RNA helicase 3, DVL: Dishevelled, FZD: Frizzled, GSK3: glycogen synthase kinase 3, JNK: c-Jun N-terminal kinases, Wnt: Wingless/Int-1, LRP: low density lipoprotein-related receptor protein, Rap1: Ras-related protein 1, Sipa1Li: signal-induced proliferation-associated 1 like 1, TCF: T cell factor. Adopted from Knippschild *et al.* 2014. Copyright (2014) Knippschild, Krüger, Richter, Xu, García-Reyes, Peifer, Halekotte, Bakulev, and Bischof. This Figure is licensed under a Creative Commons Attribution 3.0 Generic License http://creativecommons.org/ licenses/by/3.0/

1.7 CK1 and diseases

The pleiotropic character of the CK1 family suggests that its aberrant expression and activity could lead to pathological processes. Indeed, CK1 isoforms have been linked to a wide range of diseases, such as Alzheimer's Disease, Parkinson's Disease, alterations in the circadian rhythm, and cancer. CK1 family members are perhaps better known for their role in sleeping disorders. The CK1ε *tau* mutation, the first discovered circadian mutation in mammals, is a gain of function (GOF) mutation that causes clock acceleration. Mice expressing CK1ε *tau* mutation present increased phosphorylation of PER1 and 2 which induces their degradation thus accelerating the circadian clock (Meng *et al.* 2008; Maywood *et al.* 2014). The PER3 polymorphism V647G, present in both CK1δ and ε, has been associated with Delayed Sleep Phase Syndrome (DSPS), while the mutation T44A in CK1δ is an established risk factor for Familial Advanced Sleep Phase Syndrome (FASPS) (Ebisawa *et al.* 2001; Toh *et al.* 2001). Furthermore, there is strong evidence that associates CK1 isoforms with neurodegenerative diseases, particularly in Alzheimer's Disease (AD). One of the hallmarks of end-stage AD in the brain is the presence of fibrillary lesions, in which there is an accumulation of hyperphosphorylated tau protein. Phosphorylated tau presents a conformation in which it dissociated from microtubules, provoking their destabilization and leading to neuron death. CK1δ is able to phosphorylate tau and other proteins linked to AD such as β-secretase and presenilin (Walter *et al.* 1998; Schwab *et al.* 2000). Elevated levels of both, CK1δ mRNA and protein levels, as well as increased kinase activity, have been observed (Ghoshal *et al.* 1999; Yasojima *et al.* 2000), suggesting that CK1 plays a major role in the abnormal levels of hyperphosphorylated tau. Finally, overexpression of CK1ε is correlated to increased production of the neurotoxic peptide amyloid beta (Aβ), which is also a hallmark of AD (Flajolet *et al.* 2007). CK1 phosphorylates the TAR (Transactivation Response Element) DNA binding protein of 43 kDa (TDP-43), a protein associated with motor neuron degenerative diseases such as amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and Parkinson's Disease (PD) (Kametani *et al.* 2009). CK1 also phosphorylates α-synuclein, which is found in the Lewy bodies found in PD patients (Okochi *et al.* 2000).

1.7.1 CK1 and tumorigenesis

As described above, altered CK1 expression and activity are linked to a broad range of pathologies. Most prominently, aberrant CK1 activity is associated with cancer development (Knippschild *et al.* 2005a; Cheong and Virshup 2011; Knippschild *et al.* 2014). Because of

their multiple roles in cellular processes, CK1 isoforms potentially have oncogenic features that promote proliferation, genome instability and inhibition of apoptotic processes. Many literature reports support this observation, as the expression of CK1 isoforms is altered in many tumor entities (Table 2). Although it is debatable whether to attribute the role of oncogenes or tumor suppressors to CK1 isoforms (Knippschild *et al.* 2014; Schittek and Sinnberg 2014), it is certain that they are associated with tumorigenesis.

Decreased CK1ε expression, loss of heterozygosity and a high frequency of CK1ε gene mutations have been found in breast cancer (Fuja *et al.* 2004; Foldynova-Trantirkova *et al.* 2010). CK1δ and CK1ε have been found in elevated levels in acute myeloid leukemia (AML), in ductal mammary carcinomas, and ductal pancreatic carcinomas (Fuja *et al.* 2004; Knippschild *et al.* 2005b; Brockschmidt *et al.* 2008). CK1δ and CK1γ3 expression is upregulated in choriocarcinoma (Stoter *et al.* 2005) and renal cell carcinoma (Masuda *et al.* 2003) respectively. Hyperproliferating colon epithelia exhibit changed CK1ε expression and mutations in the CK1δ C-terminal domain are known to promote the development of adenomas in the intestinal mucosa (Tsai *et al.* 2007). Expression of CK1ε in ovarian cancer has been correlated with survival in patients (Rodriguez *et al.* 2012). Survival has also been associated with reduced CK1δ and increased CK1ε mRNA levels in colorectal cancer patients (Richter *et al.* 2015; Richter *et al.* 2016). Loss of heterozygosity of CK1α in p53-deficient intestinal tissue leads to invasive carcinoma (Elyada *et al.* 2011).

It has been hypothesized that the key to the participation of CK1 isoforms in so many tumorigenic processes is connected to its ability to phosphorylate p53 and MDM2 (Knippschild *et al.* 1997; Meek and Knippschild 2003; Kulikov *et al.* 2006). CK1δ and ε phosphorylate p53 upon DNA damage, preventing its ubiquitylation by MDM2 (Li and Kurokawa; Sakaguchi *et al.* 2000; Venerando *et al.* 2010), and CK1δ phosphorylates MDM2, which in turn is targeted for proteasomal degradation (Winter *et al.* 2004). Phosphorylation of MDM2 increases its interaction with p53 (Haupt *et al.* 1997; Dumaz *et al.* 1999; Meek and Knippschild 2003), meaning that CK1 could also be involved in downregulation of p53.

Table 2: CK1 isoforms in different tumor entities Adopted from Knippschild *et al.* 2014.

Isoform	Characteristic Feature	Tumor Entity	Reference
CK1α	low/absent expression	primary/metastatic melanoma, lymphomas, ovarian, breast, and colon carcinomas	(Sinnberg *et al.* 2010)
CK1γ3	altered activity/expression	renal cell carcinoma	(Masuda *et al.* 2003)
CK1δ	increased expression levels	choriocarcinomas	(Stoter *et al.* 2005)
CK1δ	reduced immunostaining	poorly differentiated breast carcinomas and DCIS	(Knippschild *et al.* 2005b)
CK1δ/ε	elevated protein levels	high-grade ductal pancreatic carcinomas	(Brockschmidt *et al.* 2008)
CK1ε	reduced expression levels	pancreatic ductal adenocarcinoma	(Relles *et al.* 2013)
CK1ε	decreased immunoreactivity and increased immunoreactivity	invasive mammary carcinoma mammary DCIS	(Fuja *et al.* 2004; Utz *et al.* 2010)
CK1ε	overexpression	breast cancer	(Shin *et al.* 2014)
CK1ε	high gene expression	adenoid cystic carcinoma of the salivary gland	(Frierson *et al.* 2002)
CK1ε	overexpression	epithelial ovarian cancer	(Rodriguez *et al.* 2012)
CK1ε	overexpression	tumors of brain, head and neck, renal, bladder, lung, prostate, salivary gland, leukemia, melanoma, and seminoma	(Yang and Stockwell 2008)
CK1ε	overexpression	MYC-driven cancers (neuroblastoma, colon, lung, and breast cancer)	(Toyoshima *et al.* 2012)
CK1ε	loss of cytoplasmic expression	poor prognosis in oral cancer patients	(Lin *et al.* 2014)

1.8 CK1 isoforms as drug targets

Protein kinases are one of the most important groups subjected to drug development, as a multitude of kinases is involved in the pathogenesis if several diseases (Cohen 2002). It is a great challenge since kinases usually fulfill multiple roles within the cell, which means no kinase inhibitor is exempt from secondary effects. Factors such a drug resistance, unspecific inhibition, target validation and cellular availability are to be considered in drug development (Zhang *et al.* 2009). Regardless of how ambitious the goal is, kinase inhibition has a great therapeutic potential. Kinase activity inhibition can be accomplished at several levels, like gene expression, protein maturation, and folding, and phosphatase activation (Dancey and Sausville 2003). The most studied approach, however, is the direct inhibition of the kinase protein using small molecule inhibitors (SMIs).

Most kinase inhibitors compete with ATP by interacting with the ATP-binding site of the kinase through hydrogen bonds. In normal conditions, the adenine ring of ATP forms hydrogen bonds with different amino acid residues within the ATP-binding site, and SMIs are capable of mimicking this interaction (Traxler and Furet 1999; Liu and Gray 2006). SMIs can be classified into four categories. ATP-competitive inhibitors recognize either the active conformation of the kinase Asp-Phe-Gly (DFG) -in, Type 1 or the inactive conformation (DFG-out, Type 2). Type 3 inhibitors exert an allosteric effect, binding to the kinase without displacing the ATP in its active site but preventing kinase activity. Type 4 inhibitors bind covalently and irreversibly to the active site of the kinase (Zhang *et al.* 2009), preventing any further activity.

Small molecule kinase inhibitors have the advantage over classical chemotherapeutics of presenting less secondary effects, their mechanisms of action are usually known, and their administration can be done orally (Zhang *et al.* 2009). On the other hand, even if the compounds show a potent and selective inhibition of a kinase, it could be that its clinical administration is not possible due to toxicity or poor solubility, among other obstacles. During the last decade, a growing number of potential kinase inhibitors were evaluated in clinical trials, and some of them have received approval for therapeutic applications (Dancey and Sausville 2003; Zhang *et al.* 2009; Giamas *et al.* 2010).

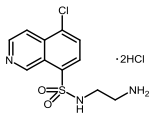
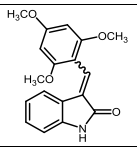
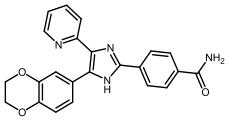
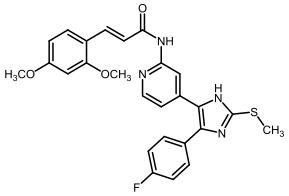
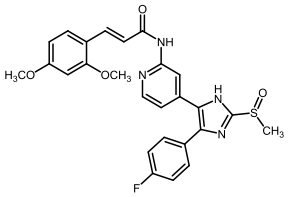
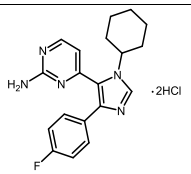
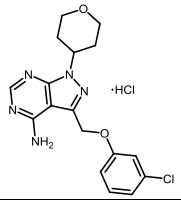
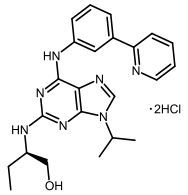
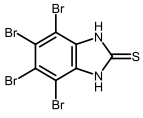
As CK1 is involved in different diseases, there is a special interest in developing potent and specific inhibitors of CK1 activity. Several SMIs have been described that present a therapeutic potential, all of them currently evaluated at a pre-clinical level (Table 3) (Knippschild *et al.* 2014). The first CK1-specific inhibitor described was CKI-7 (N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide), which is ATP competitive (Chijiwa *et al.*

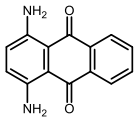
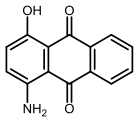
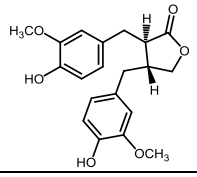
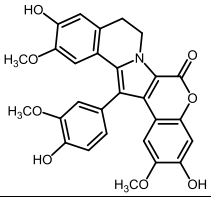
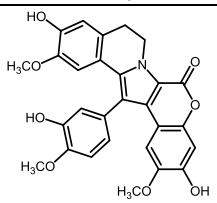
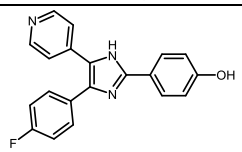
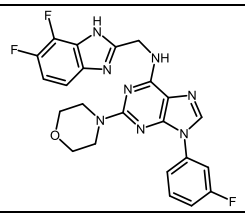
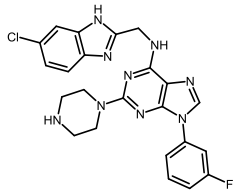
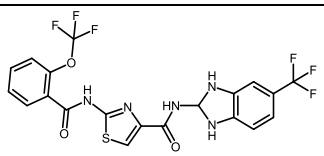
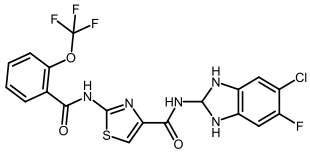
1989), followed by IC261 (3-[(2,4,6-trimethoxyphenyl)-methylidenyl]-indolin-2-one) benzamide), which targets CK1δ and ε and binds to their ATP binding pocket (Mashhoon *et al.* 2000). In a mouse xenotransplantation model for pancreatic cancer, IC261 showed to have efficacy (Brockschmidt *et al.* 2008). Unfortunately, IC261 has a known side effect: it binds to microtubules thereby inhibiting their polymerization, like the spindle poison colchicine (Cheong *et al.* 2011).

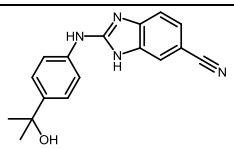
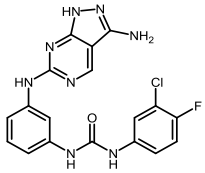
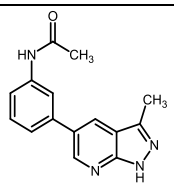
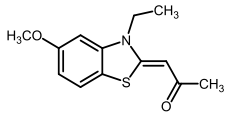
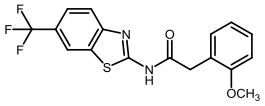
Pfizer Global Research and Development has developed two potential CK1 inhibitor compounds: PF670462 and PF4800567. PF670462 targets both CK1δ and ε, while PF4800567 shows a high selectivity against CK1ε (Badura *et al.* 2007; Walton *et al.* 2009). PF670462 has already shown positive results in the treatment of bipolar disorder (Arey and McClung 2012), addictive behavior (Perreau-Lenz *et al.* 2012) and perturbed circadian behavior (Meng *et al.* 2010). The compound D4476 (4-[4-(2,3-dihydro-benzo)[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl]-benzamide), first described as a receptor-like kinase 5 (ALK5)-inhibitor has been found to target CK1δ (Rena *et al.* 2004).

Other CK1 isoform inhibitors having been described include anthraquinone analogues (Cozza *et al.* 2008), roscovitine derivatives such as (R)-DRF053, which targets both CDKs and CK1 (Oumata *et al.* 2008) and several benzimidazole-based CK1-specific inhibitors like SR-3029, SR-2890 (Bibian *et al.* 2013), Bischof-5, Bischof-6 (Bischof *et al.* 2012) and further derivatives (Richter *et al.* 2014).

Table 3: CK1-specific small molecule inhibitors. First published in Knippschild *et al.* 2014. Copyright (2014) Knippschild, Krüger, Richter, Xu, García-Reyes, Peiffer, Halekotte, Bakulev, and Bischof. This table is licensed under a Creative Commons Attribution 3.0 Generic License http://creativecommons.org/ licenses/by/3.0/

Inhibitor	Structure	IC_{50}	[ATP]	Reference
CKI-7		CK1: 6 µM	100 µM	(Chijiwa *et al.* 1989; Rena *et al.* 2004)
IC261		CK1δ/ε: 2.5 µM	100 µM	(Mashhoon *et al.* 2000; Rena *et al.* 2004)
D4476		CK1δ: 0.3 µM	100 µM	(Rena *et al.* 2004)
Peifer-17		CK1δ: 0.005 µM CK1ε: 0.073 µM	100 µM	(Peifer *et al.* 2009)
Peifer-18		CK1δ: 0.011 µM CK1ε: 0.447 µM	100 µM	(Peifer *et al.* 2009)
PF-670462		CK1δ: 0.013 µM CK1ε: 0.080 µM	10 µM	(Badura *et al.* 2007; Walton *et al.* 2009)
PF-4800567		CK1δ: 0.711 µM CK1ε: 0.032 µM	10 µM	(Walton *et al.* 2009)
(R)-DRF053		CK1δ/ε: 0.014 µM	15 µM	(Oumata *et al.* 2008)
4,5,6,7-Tetrabromo-2-mercaptobenzimidazole		CK1: 2.2 µM	20 µM	(Andrzejewska *et al.* 2003)

Inhibitor	Structure	IC_{50}	[ATP]	Reference
1,4-Diaminoanthra-quinone		CK1δ: 0.3 µM	not reported	(Cozza *et al.* 2008)
1-Hydroxy-4-aminoanthra-quinone		CK1δ: 0.6 µM	not reported	(Cozza *et al.* 2008)
(-)-Matairenisol		CK1: 10 µM	10 µM	(Yokoyama *et al.* 2003)
Lamellarin 3		CK1δ/ε: 0.41 µM	15 µM	(Baunbaek *et al.* 2008)
Lamellarin 6		CK1δ/ε: 0.8 µM	15 µM	(Baunbaek *et al.* 2008)
SB-202190		CK1δ: 0.6 µM	50 µM	(Shanware *et al.* 2009)
SR-3029		CK1δ: 0.044 µM	10 µM	(Bibian *et al.* 2013)
SR-2890		CK1δ: 0.004 µM	10 µM	(Bibian *et al.* 2013)
Bischof-5		CK1δ: 0.04 µM CK1ε: 0.199 µM	10 µM	(Bischof *et al.* 2012)
Bischof-6		CK1δ: 0.042 µM CK1ε: 0.033 µM	10 µM	(Bischof *et al.* 2012)

Inhibitor	Structure	IC_{50}	[ATP]	Reference
Hua-1h		CK1γ: 0.018 µM	not reported	(Hua *et al.* 2012)
Yang-2		CK1: 0.078 µM	not reported	(Yang *et al.* 2012)
CK01	similar to PF-670462	not reported	not reported	(Arey and McClung 2012)
MRT00033659		CK1δ: 0.8935 µM	20 µM	(Huart *et al.* 2013)
TG0003		CK1δ: 0.4 µM CK1ε: 0.55 µM	not reported	(Muraki *et al.* 2004; Isojima *et al.* 2009)
Salado-34		CK1δ: 0.01 µM	10 µM	(Salado *et al.* 2014)

1.9 Aim of the study

The CK1 family is involved in various cellular processes, and its aberrant activity is known to contribute to pathogenic disorders. Targeting CK1 isoforms for inhibition is an interesting concept with possible therapeutic use, particularly with the use of small molecule inhibitors. Within the scope of this work, two facets of CK1δ inhibition are addressed:

(i) *Validation of newly synthesized CK1-specific inhibitors in vitro and in cell culture*. Development of effective and isoform-specific inhibitors targeting CK1 is a challenging and long process that requires various optimization and validation attempts. This work presents the results of the cooperation with an external research partner on efforts in the design and analysis of new small molecule inhibitors of the CK1δ isoform. Novel compounds are examined in this thesis through their capacity to inhibit substrate phosphorylation, and their mode of inhibition is further studied *in vitro* and *in silico*. Additionally, their effects on viability, growth, and proliferation of human tumor cell lines is analyzed, as the first step for their potential *in vivo* applications.

(ii) *Identification of IWP compounds as inhibitors of CK1δ kinase activity*. The widely used compound IWP-2 is part of a small molecule class of substances known to powerfully inhibit the Wnt pathway by targeting the MBOAT acyltransferase Porcupine. Because of its similarities with known CK1 inhibitors the question if IWPs also target CK1 was addressed. Here IWP-2 is validated as a specific CK1δ inhibitor, and its ATP-competitive properties are confirmed by *in vitro* kinase assays and *in silico* modeling. Its effects on cell culture are also explored, finding that IWP derivatives alone inhibit cancer cell proliferation. Finally, IWPs are shown to be interesting scaffolds for further CK1-inhibitor design, exemplified by newly developed IWP derivatives synthesized in collaboration with our research partners at the University of Kiel, Germany.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals and reagents used were purchased in analysis quality grades from the given companies

6x Loading	DyeThermo Fisher Scientific, Schwerte,Germany
Acetic acid	Sigma-Aldrich, St. Louis, USA
Acrylamide (Rotiporese® Gel 30)	Carl Roth, Karlsruhe, Germany
Agarose (SeaKem® LE Agarose)	Lonza, Rockland, USA
Ammonia (NH_3)	Sigma-Aldrich, St. Louis, USA
Ammonium bicarbonate (NH_4HCO_3)	Sigma-Aldrich, St. Louis, USA
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, St. Louis, USA
Ampicillin	Carl Roth, Karlsruhe, Germany
Aprotinin	Sigma-Aldrich, St. Louis, USA
APS	Sigma-Aldrich, St. Louis, USA
ATP	Sigma-Aldrich, St. Louis, USA
[γ-^{32}P]-ATP	Hartmann Analytic, Braunschweig, Germany
Bacto-Agar	BD Biosciences, San Jose, USA
Benzamidin	Sigma-Aldrich, St. Louis, USA
Brij-35	Sigma-Aldrich, St. Louis, USA
Bromphenol blue	Sigma-Aldrich, St. Louis, USA
BSA	Serva, Heidelberg, Germany
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, St. Louis, USA
α-Casein	Sigma-Aldrich, St. Louis, USA
Coomassie Brilliant Blue R-250	Waldeck/Chroma, Münster, Germany
DMEM (high glucose, glutamine)	Invitrogen (Gibco®), Carlsbad, USA
DMSO	Sigma-Aldrich, St. Louis, USA
DTT	Sigma-Aldrich (Fluka®), St. Louis, USA
EDTA	Sigma-Aldrich, St. Louis, USA
EGTA	Sigma-Aldrich, St. Louis, USA
Ethanol	Sigma-Aldrich, St. Louis, USA

Ethidium bromide	Sigma-Aldrich (Fluka®), St. Louis, USA
FCS	Biochrom, Berlin, Germany
Formaldehyde (37 %)	Sigma-Aldrich, St. Louis, USA
Glutathione Sepharose 4 Fast Flow	GE Healthcare, Chalfont St Giles, UK
Glutathione	Sigma-Aldrich, St. Louis, USA
Glycerol	Sigma-Aldrich, St. Louis, USA
Glycine	AppliChem, Darmstadt, Germany
Glutamine	Biochrom, Berlin, Germany
Hydrochloric acid (HCl, 37 %)	Merck, Darmstadt, Germany
Hydrochloric acid (HCl, 6 N)	Sigma-Aldrich (Fluka®), St. Louis, USA
IPTG	Fermentas, St. Leon-Rot, Germany
Isopropanol	VWR, Radnor, USA
IWP-2	Cayman Chemical, Ann Arbor, USA
IWP-2-V2	Cayman Chemical, Ann Arbor, USA
IWP-3	Cayman Chemical, Ann Arbor, USA
IWP-4	Cayman Chemical, Ann Arbor, USA
Leibovitz L-15	Invitrogen (Gibco®), Carlsbad, USA
McCoy's 5A	Invitrogen (Gibco®), Carlsbad, USA
Methanol	Sigma-Aldrich, St. Louis, USA
β-mercaptoethanol (MSH)	Sigma-Aldrich, St. Louis, USA
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, St. Louis, USA
Magnesium sulfate (MgSO4)	Merck, Darmstadt, Germany
Manganese chloride ($MnCl_2$)	Sigma-Aldrich, St. Louis, USA
MTT	Sigma Aldrich, Schnelldorf, Germany
Nonidet® P 40 (NP40)	Sigma-Aldrich (Fluka®), St. Louis, USA
PBS	Invitrogen (Gibco®), Carlsbad, USA
Peptone	Carl Roth, Karlsruhe, Germany
Penicillin/Streptomycin	Invitrogen (Gibco®), Carlsbad, USA
Poly(L-glutamic acid-L-tyrosine	Sigma-Aldrich, St. Louis, USA
Potassium phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Protein G sepharose	GE Healthcare, Chalfont St Giles, UK
RNase-free water	Qiagen, Hilden, Germany
RPMI-1640	Invitrogen (Gibco®), Carlsbad, USA
SDS	Carl Roth, Karlsruhe, Germany

Silver nitrate ($AgNO_3$)	Merck, Darmstadt, Germany
Sodium acetatae (NaOAc)	Sigma-Aldrich, St. Louis, USA
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich (Fluka®), St. Louis, USA
Sodium carbonate (Na_2CO_3)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Sigma-Aldrich, St. Louis, USA
Soidum fluoride (NaF)	Sigma-Aldrich, St. Louis, USA
Sodium hydroxide (NaOH, 1 M)	Sigma-Aldrich, St. Louis, USA
Sodium hydroxide (NaOH, pellets)	AppliChem, Darmstadt, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, St. Louis, USA
Sodium phosphate (Na_2HPO_4)	Merck, Darmstadt, Germany
Sodium thiosulfate ($Na_2S_2O_3$)	Sigma-Aldrich, St. Louis, USA
TEMED	Carl Roth, Karlsruhe, Germany
Tris	USB, Staufen, Germany
Triton X-100	Sigma-Aldrich (Fluka®), St. Louis, USA
Trypsin-EDTA	Biochrom, Berlin, Germany
Tween 20	Sigma-Aldrich, St. Louis, USA
Yeast extract	Sigma-Aldrich (Fluka®), St. Louis, USA

2.1.2 Consumables and kits

6-Well and 96- Well Micro Well Reaction Plate	Applied Biosystems, Carlsbad, USA
AffinityScript QPCR cDNA Synthesis Kit	Agilent, Munich, Germany
Bacteria tubes, 13 ml	Sarstedt, Nümbrecht, Germany
BCA protein assay kit	Thermo Fisher Scientific (Pierce®), Waltham, USA
BD Cycletest™ Plus DNA reagent kit	BD, Heidelberg, Germany
Cell culture dishes	Nunc, Wiesbaden, Germany
Cell culture scraper	Corning Costar, Bodenheim, Germany
Centrifuge tubes Falcon (15 and 50 mL)	Becton Dickinson, Heidelberg, Germany
DyNAmo Flash SYBR Green qPCR Kit	Thermo Fischer, Munich, Germany
Eppendorf Tubes® (1.5 mL, 2 mL)	Eppendorf, Hamburg, Germany
Glass flasks	Eppendorf®, Hamburg, Germany
Gloves (Nitril)	Kimberly Clark, Zaventem, Belgium

QuantiTect Primer Assay (200)	Qiagen, Stockach, Germany
RNeasy® Mini Kit	Qiagen, Stockach, Germany
Serological Pipets (Stripette 5 ml, 10 ml, 25 ml)	Corning Inc., Corning, USA
Sorvall Centrifuge tube 50 mL	Sorvall, Bad Homburg, Germany
Spatula	VWR International, Darmstadt, Germany
Sterile filters 0.22 and 0.45 µm	Sartorius, Göttingen, Germany
Tips for micropipettes	Standard Eppendorf, Hamburg, Germany
X-Ray Films Hyperfilm MP	GE Healthcare, Munich, Germany

2.1.3 Molecular weight markers

Gene Ruler 1kb DNA ladder	Thermo Fisher Scientific, Schwerte, Germany
Precision Plus Protein Dual Color Standard	BioRad, Hercules, USA

2.1.4 Enzymes

CK1ε	Invitrogen, Karlsruhe, Germany
CK1δKD	New England Biolabs, Frankfurt am Main, Germany
Lysozyme	Sigma-Aldrich, Munich, Germany
Taq polymerase	Hilden, Germany
TLK2	Proqinase, Freiburg, Germany
ZAP70	Proqinase, Freiburg, Germany

2.1.5 QRT-PCR primers

All primers for QRT-PCR were purchased from Qiagen, Stockach, Germany.

Hs_BCL2_1_SG QuantiTect Primer Assay (200)
Hs_CDK9_1_SG QuantiTect Primer Assay (200)
Hs_CDKN1A_1_SG QuantiTect Primer Assay (200)
Hs_CTNNB1_1_SG QuantiTect Primer Assay (200)
Hs_DYRK1B_1_SG QuantiTect Primer Assay (200)
Hs_GLI1_1_SG QuantiTect Primer Assay (200)

Hs_KRAS_1_SG QuantiTect Primer Assay (200)
Hs_MDM2_1_SG QuantiTect Primer Assay (200)
Hs_NANOG_1_SG QuantiTect Primer Assay (200)
Hs_NES_1_SG QuantiTect Primer Assay (200)
Hs_PITX2_1_SG QuantiTect Primer Assay (200
Hs_POU5F1_1_SG QuantiTect Primer Assay (200)
Hs_SOX2_1_SG QuantiTect Primer Assay (200)
Hs_TP53_1_SG QuantiTect Primer Assay (200)
Hs_CSNK1A_1_SG QuantiTect Primer Assay (200)
Hs_CSNK1D_1_SG QuantiTect Primer Assay (200)
Hs_CSNK1E_1_SG QuantiTect Primer Assay (200)
Hs_HPRT_1_SG QuantiTect Primer Assay (200)

2.1.6 Bacterial expression vectors

pGEX-2T-CK1α	FP296 (Milne *et al.* 1992)
pGEX-2T-CK1γ3	FP1054 (Sillibourne *et al.* 2002)
pGEX-2T-CK1δwt	FP449 (Knippschild *et al.* 1997)
pGEX-2T-CK1δmutM82F	FP1153 (Peifer *et al.* 2009)
pGEX6P-3-CK1δTV1wt	FP1417 (Richter *et al.* 2014)
pGEX6P-3-CK1δTV2wt	FP1410 (Richter *et al.* 2014)
pGEX6P-3-CK1δTV1mutT67S	FP1406 (Richter *et al.* 2014)

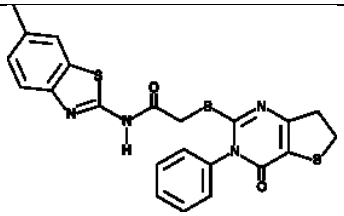
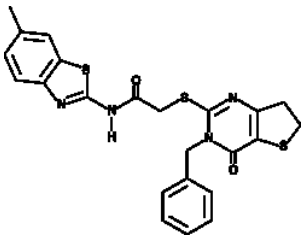
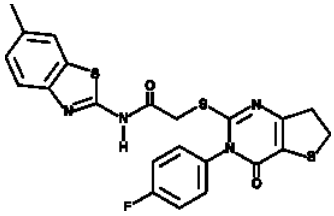
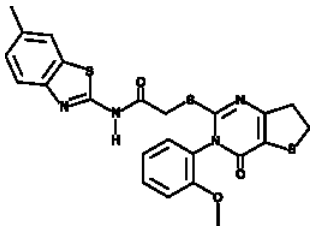
2.1.7 Inhibitor compounds

The CK1δ-specific inhibitor compounds 116-125 (Table 4) were designed, synthesized and provided by *4SC AG*, Planegg-Martinsried, Germany. The compounds originate from 2-Benzamido-*N*-(1*H*-benzo[*d*]imidazol-2-yl)thiazol-4-carboxamide derivatives further developed to inhibit CK1δ. Due to patent law restrictions, the compound structures cannot be published at the time of thesis publication. The Porcn inhibitors IWP-2, IWP-2-V2, IWP-3, and IWP-4 are commercially available (Table 5), and were purchased from Cayman Chemical (Ann Arbor, MI, USA). All inhibitor compounds were diluted in DMSO to a final concentration of 10 mM and stored at -20°C.

Table 4: Inhibitor compounds provided by 4SC and their molecular weight.

Compound Code	M_W
116	407.38
117	422.40
118	482.44
119	390.33
120	361.30
121	365.72
122	400.37
123	384.30
124	400.37
125	403.34

Table 5: Inhibitor compounds commercially available

Compound	Structure	M_W
IWP-2		466.60
IWP-2-V2		480.60
IWP-3		484.60
IWP-4		496.60

2.1.8 Bacteria tribe

SoluBL21™ Strain: F- ompT hsdSB (rB- mB-) gal dcm (DE3)† from Genlantic, San Diego, USA

2.1.9 Eukaryotic cell lines

All media were supplemented with 10 % fetal calf serum (FCS, Biochrom, Berlin, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco, Karlsruhe, Germany) and 2 mM glutamine. All cells were grown at 37°C in a humidified 5 % carbon dioxide atmosphere.

Table 6: Established cell lines with their respective origin and culture media.

Cell line	Type	Growth media	Reference
A818-6	Human pancreatic adenocarcinoma	RPMI	(Lehnert *et al.* 2001)
AsPc1	Human pancreatic adenocarcinoma	RPMI	(Chen *et al.* 1982)
BxPc3	Human pancreatic adenocarcinoma	DMEM:RPMI (1:1)	(Loor *et al.* 1982)
Capan-1	Human pancreatic cancer metastasis	RPMI	(Fogh and Trempe 1975)
HEK293	Human embryonic kidney	DMEM	(Graham *et al.* 1977)
HT29	Human colon adenocarcinoma	McCoy's 5A	(Fogh and Trempe 1975)
MCF7	Human breast adenocarcinoma	DMEM	(Soule *et al.* 1973)
MiaPaCa2	Human pancreatic carcinoma	DMEM	(Yunis *et al.* 1977)
Panc1	Human pancreatic carcinoma	DMEM	(Lieber *et al.* 1975)
Panc89	Human pancreatic adenocarcinoma	DMEM:RPMI (1:1)	(Okabe *et al.* 1983)
REF52	Rat embryo fibroblasts	DMEM	(Franza *et al.* 1986)
SW620	Human colorectal adenocarcinoma	Leibovitz L-15	(Leibovitz *et al.* 1976)

2.1.10 Equipment

Autoclave 32 2 3	KSG Sterilisatoren GmbH, Olching
Bacteria incubator (Certomat BS1)	Braun Biotech International, Melsungen, Germany
Centrifuge 5415C	Eppendorf®, Hamburg
Centrifuge 5417 R (Rotor FA-45 20-11)	Eppendorf®, Hamburg
Centrifuge 5430 (Rotor FA-45-24-11-HS)	Eppendorf®, Hamburg
Centrifuge 5810 R	Eppendorf®, Hamburg
Centrifuge Megafuge 1.0 R (Rotor 2705)	Heraeus, Hanau
Centrifuge Rotixa/ RP	Hettich, Tuttlingen
Centrifuge Sorvall RC-5B	Sorvall, Bad Homburg
Centrifuge Universal 32 R	Hettich, Tuttlingen
Flow cytometer (FACScan)	Becton-Dickinson, Franklin Lakes, USA
Hera freeze HFU 586 Basic	Heraeus, Hanau
Heraeus HeraCell incubator	Heraeus, Hanau
Kodak X- OMAT	Kodak, Rochester, USA
LightCycler® 480 Instrument II	Roche Diagnostics GmbH, Munich
LUNATM Automated Cell Counter	Logos Biosystems, Anyang-City, Korea
Micropipettes (0.1 µl – 5000 µl)	Eppendorf®, Hamburg
Microscope (Olympus IX81)	Olympus, Hamburg, Germany
Microscope (Olympus CK2)	Olympus, Hamburg, Germany
NanoDrop 1000 Spectrophotometer	Thermo Fischer, Munich, Germany
PCR apparatus (Mastercycler)	Eppendorf, Hamburg, Germany
PCR cycler (LabCycler)	SensoQuest, Göttingen, Germany
PCR electrophoresis apparatus	Gibco, Karlsruhe, Germany
Power Supply PAC 300	Biorad, Munich
Safety Cabinet (HERAsafe)	Heraeus, Hanau, Germany
Scintillation counter (LC6000IC)	Beckman Coulter, USA
Scale 440-35A	Kern und Sohn GmbH, Bailingen
Scale R180-D	Sartorius GmbH, Göttingen
SDS-PAGE electrophoresis apparatus	Biorad, Munich, Germany
Slab Gel Dryer 2000	ThermoSavant, USA
Sonication apparatus (Ultrasonic Sonifier 250)	Branson, Danbery, USA

TECAN Spectra II	TECAN, Männedorf, Swizerland
Thermomixer Comfort	Eppendorf®, Hamburg
Ultrasonic Sonifier 250	Branson, Danbury, USA

2.1.11 Software

Adobe Acrobat	Adobe Systems, Munich
Cell Quest	BD, Heidelberg
CorelDRAW 12	Corel, Ottawa, Canada
EndNote X1	Thomson Reuters, New York, USA
Excel 2013	Microsoft, Unterschleißheim
Graph Pad Prism 6	GraphPad Prism Software Inc., San Diego CA, USA
ImageJ	National Institutes of Health, Bethesda MD, USA
LightCycler® software	Roche Diagnostics GmbH, Munich
Magellan3	TECAN, Männedorf, Swizerland
Word 2013	Microsoft, Unterschleißheim

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 Total RNA purification from cells and first-strand cDNA synthesis

Total RNA was purified from treated cells per manufacturer's instructions for RNeasy® Mini Kit (Quiagen, Stockach). RNA was extracted in 30 µl of RNase free H2O and stored at -80°C. RNA concentration was measured using the NanoDrop 1000 Spectrophotometer (Thermo Fischer, Munich). 1 µg of total RNA was used to synthesize first-strand cDNA per manufacturer's instructions for the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Munich). cDNA was stored at -20°C until further use. 1 µg of total RNA was mixed with oligo(dT) primer (0.5 µg/µl) and RNase-free water and incubated at 65°C for 5 minutes. After cooling the reaction to room temperature, 1x AffinityScript RT buffer, 25 mM dNTP, 40 U/µl RNase Block Ribonuclease Inhibitor, and AffinityScript Multiple Temperature Reverse Transcriptase were added. Synthesis was performed at 55°C for 60 minutes in a temperature controlled thermal block. Termination of the reaction was done by incubation at 70°C for 15 minutes. The quality of the resulting cDNA was validated by PCR using primers specific for β-actin. The exon/exon standing β-actin primer pair β-actin F and β-actin R was used to check the quality of the cDNA while the β-actin intron/exon

standing primer pair β-actin I and β-actin E was used to detect contaminations with genomic DNA.

2.2.1.2 Polymerase chain reaction (PCR)

All PCR reactions were performed in a total volume of 25 µl. Template DNA (100 ng) was set in the reaction tube before addition of a master mix containing 1x PCR buffer, 200 µM dNTPs, 100 nM forward and reverse primers (β-actin primers 1 µM), and 2.5 U of Taq DNA polymerase. All reactions were set up on ice, centrifuged briefly and transferred to a thermal cycler (MasterCycler, Eppendorf, Hamburg, Germany or Labcycler, SensoQuest, Göttingen, Germany). The PCR conditions for β-actin amplification comprise a denaturation step of 3 minutes at 94°C, 35 annealing and elongation cycles of 40 seconds at 94°C, 40 seconds at 62°C and 1 minute at 72°C, followed by a final incubation at 72°C for 5 minutes. β-actin primers sequences (5' to 3') are as follow: β-actin F GGC ATC CTC ACC CTG AAG TA, β-actin R GTC AGG CAG CTC GTA GCT CT, β-actin I CGA GCA GGA GAT GGC CAC TGC and β-actin E GTG AGC TCT CTG GGT GCT GGG.

2.2.1.3 Agarose gel electrophoresis

PCR products and DNA fragments from endonuclease digestions were separated on 1% (w/v) agarose gels (1 g agarose in 100 ml of 1x TAE buffer) with 20 µg/ml ethidium bromide. Samples were loaded with the adequate volume of a 6x DNA loading buffer. A 1 kb DNA marker was also loaded to estimate the size of the DNA products. Electrophoresis was carried out at 120 V for 30 minutes (power supplied by PowerPac 200, BioRad, Hercules, USA). Detection of DNA was performed under UV light and photographs of the gels were taken using a fluorescent gel imager (Fluo-Link, Fröbel Labortechnik, Lindau, Germany).

TAE buffer: 40 mM Tris-acetate [pH 8.0], 1 mM EDTA

2.2.1.4 QRT-PCR

Transcription of genes was measured by QRT-PCR using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fischer, Munich). QuantiTect Primer Assay (200) for genes of interest (GOI) were purchased from Quiagen (Stockach). The LightCycler® 480 Instrument II and software (Roche Diagnostics GmbH, Munich) were used following the manufacturer's

instructions. Samples were denatured at 95°C for 7 minutes. 45 PCR cycles were performed as follows: 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds. HPRT was chosen as an endogenous control because it is constitutively expressed at the same level among all the samples analyzed. All experiments were done in duplicate. Results are shown as ΔCt values.

2.2.2 Bacteria methods

2.2.2.1 Storage of *E. coli*

The long-term storage of generated transformed *E. coli* tribes was done as glycerol preserved cultures. A certain volume of bacteria culture was mixed with an equal amount of a 1:1 (v/v) LB medium/glycerol mixture. After mixing the glycerol added culture was shock frozen in liquid nitrogen and stored at -80°C.

2.2.2.2 Transformation of competent bacteria

After thawing competent *E. coli* (SoluBL21) on ice, the bacteria were incubated with the appropriate pGEX vector encoding for the desired protein. Cells were permeabilized by heat shock at 42°C for 45 seconds and put back to ice for 2 minutes. Subsequently, the cells were suspended in 250 µL of LB-medium and incubated for 1 hour at 37°C, gently shaking at 300 rpm. Cells were plated at LB-Agar plates containing ampicillin and incubated overnight at 37°C.

LB-medium: 10 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone, 100 µg/mL ampicillin (added after autoclaving).
LB-Agar: LB-medium, 1.5 % BactoTM Agar

2.2.2.3 Bacterial expression and purification of recombinant GST-Fusion-Proteins

50 mL of LB-medium with 100 µg/mL ampicillin (LB/amp medium) were inoculated with recombinant SoluBL21 bacteria previously transformed with pGEX expression plasmids. The bacteria were incubated in a conical flask overnight at 125 rpm at 37°C and then stored at 4°C until further use. 400 mL of LB/amp medium were added and the culture was incubated for 1 hour at 125 rpm at 37°C. IPTG was added to a final concentration of 1.0 mM, to induce expression of GST-fusion-proteins. The samples were incubated overnight at 15°C. The cells were collected by centrifugation at 5000 rpm for 10 minutes and frozen

without supernatant at -80°C for 20 minutes. The pelleted bacteria were resuspended in 10 mL lysis buffer and transferred into a 30 mL Sorvall tube, which was placed then on ice. Approximately 2.5 g of lysozyme were added to the mixture and incubated for 30 minutes to lyse the cells. 10 mL of lysis buffer were added and the bacterial DNA was fragmented by sonication for approximately 2 seconds (level 3, Ultrasonic Sonifier 250, Branson, Danbury, USA). The samples were centrifuged for 30 minutes at 10000 rpm at 4°C. The pellet was discarded and the supernatant was mixed with 600 µL Glutathione-Sepharose beads (1:1 = 50% (v/v) Glutathione- Sepharose) and incubated for 3 hours. Proteins bound to the Glutathione-Sepharose were centrifuged at 3000 rpm for 3 minutes, washed three times with Washing Buffer I (Lysis Buffer with 300 mM NaCl) and washed twice with Washing Buffer II. GST-fusion-proteins were separated from the sepharose beads by addition of a reduced glutathione solution. For storage, glycerol was added to a final concentration of 10%, then the eluted proteins were shock-frozen in liquid nitrogen and stored at -80°C.

Lysis buffer: 20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 10% (v/v) glycerol, 0.5 % (v/v) NP-40, 2.5 mM DTT, 2.5 mM EDTA, 0.8 mM EGTA, 50 µM benzamidin, 1:200 Aprotinin

Washing buffer II: 20 mM Tris-HCl [pH 7.6], 50 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1:100 Aprotinin

Elution solution: 50 nM Tris-HCl [pH 7.0], 0.1% (w/v) reduced glutathione, 1 mM EDTA, 1:100 Aprotinin

2.2.3 Protein methods

2.2.3.1 BCA protein assay

To determine the protein concentration of cell extracts, BCA protein assays were performed (Thermo Fisher Scientific (Pierce®), Waltham, USA). A BSA protein stock was diluted to 0.1, 0.3, 0.5 and 0.7 µg/µl to generate a standard curve which was measured together with pre-diluted protein samples. In a 96-well plate, 10 µl of blank (water), standard, and the sample were mixed with 200 µl of the staining solution (1:50 dilution of solution B in solution A). After incubation at 37°C for 30 min, protein concentrations were automatically determined using a microplate reading photometer (Tecan Spectra II, Tecan, Männedorf, Switzerland).

Solution A:	contains bicinchoninic acid, sodium tartrate, Na2CO3, and NaHCO3 in 0.1 M NaOH
Solution B:	4 % CuSO4

2.2.3.2 SDS polyacrylamide gel electrophoresis

The separation of proteins was performed electrophoretically under denaturing conditions in discontinuous gel electrophoresis. The polyacrylamide gels used (measurements: 85x70x0.75 mm) consisted of an upper (collecting) gel, cast above a lower (separating) gel part. At a current of 25 mA/gel (PowerPac 200, BioRad, Hercules, USA) proteins were concentrated in the upper gel prior to separation in the lower gel for in total about 50 minutes. Before loading the samples were mixed with SDS sample buffer, boiled up at 99°C for 5 min and briefly centrifuged to collect the condensate.

Upper gel:	130 mM Tris-HCl [pH 6.8], 0.1 % (w/v) SDS, 4 % (v/v) acrylamide, just before casting the gel 0.09 % (w/v) APS and 0.3 % (v/v) TEMED were added.
Lower gel (12.5 %):	130 mM Tris-HCl [pH 8.8], 0.1 % (w/v) SDS, 12.5 % (v/v) acrylamide, just before casting the gel 0.05 % (w/v) APS and 0.12 % (v/v) TEMED were added.
Running buffer:	250 mM Tris, 1.9 M glycine, 1 % (w/v) SDS
5x SDS sample buffer:	250 mM Tris-HCl [pH 6.8], 25 % (v/v) β-mercapto-ethanol (MSH), 50 % (v/v) glycerol, 10 % (w/v) SDS, 0.5 % (w/v) bromphenol blue

2.2.3.3 Visualization of proteins in polyacrylamide gels

Proteins separated by SDS-PAGE were stained with Coomassie Brilliant Blue. The gels were incubated in a bath of Coomassie staining solution at room temperature on an orbital shaker for about 15 minutes. Gels were afterward incubated on destaining solution for at least 45 minutes. Lastly, gels were vacuum-dried at 80°C for at least 45 minutes (Slab Gel Dryer SGD 2000, Thermo Savant, New York, USA, Diaphragm vacuum pump, Vacuubrand GmbH and Co. KG, Wertheim, Germany).

Coomassie staining solution: 1.5 g Coomassie Brilliant Blue R-250 solved in 1 L methanol/dH2O/glacial acetic acid (5:5:1)
Coomassie destaining solution: 10 % (v/v) isopropanol, 10 % (v/v) acetic acid in dH2O

2.2.3.4 Autoradiographic detection of ^{32}P labeled proteins

X-ray films were exposed in the dark to dried gels containing ^{32}P-labeled proteins (Hyperfilm MP, GE Healthcare, Munich) for 30 minutes at -80°C, to visualize the phosphorylated proteins. A Kodak developing machine (Kodak X- OMAT, Kodak, Rochester, USA) was used to develop the films.

2.2.3.5 Quantification of proteins in polyacrylamide gels

For densitometric protein quantification, polyacrylamide gels were silver-stained in multiple steps. First, they were bathed in Fixation Solution 1 for 30-60 minutes, then in Fixation Solution 2 for 2 hours. Next, they were washed with demineralized water three times for 30 minutes. The gels were then soaked with Silver Salt Impregnation Solution for 30 minutes and afterward washed with 2.5 % sodium carbonate solution for 1 minute. The protein-bound silver ions were reduced to elementary silver, observed as a black band on the gel, by bathing the gels in Developing Solution for 10 minutes. This reaction was stopped by washing the gels in a 1 % glycine solution for 20 minutes. The gels were washed three times with demineralized water, 30 minutes each time, and finally preserved in a bath of 3% glycerol solution for 60 minutes. The gels were dried at 80°C for 45 minutes (Slab Gel Dryer SGD 2000, Thermo Savant, New York, USA, Diaphragm vacuum pump, Vacuubrand GmbH + Co. KG, Wertheim, Germany). Quantification of the silver-stained protein bands was performed using ImageJ software and protein amounts were adjusted accordingly by dilution with dH2O.

Fixation solution 1: 30 % ethanol, 10 % acetic acid in dH2O
Fixation solution 2: 30 % ethanol, 0.2 N sodium acetate, 100 mL glutaraldehyde, 400 µL thiosulfate solution (1g/ 50 mg) in dH2O
Silver salt impregnation solution: 0.2 % silver nitrate, 25 µL/100 mL formaldehyde (37 %) in dH2O
Developing solution: 2.5 % sodium carbonate, 40 µL/100 mL formaldehyde (37 %), 40 µL thiosulfate solution (1g/ 50 mL) in dH2O

2.2.3.6 *In vitro* Kinase Assay

In vitro kinase reactions were performed to test the activity of kinases in the presence of the different compounds. All steps were performed on ice unless otherwise indicated. In a 1.5 mL Eppendorf tube, a total reaction volume of 20 µL was prepared: 1µg of substrate protein α-casein was combined with 0.2 µL of [γ-^{32}P]-ATP (2 µCi). 2 µL of the respective 10x compound-stock (for concentrations see Table 7) was included in the reaction. DMSO was used as a control reaction.

Table 7: Concentrations of stock solutions for all compounds

#	Stock dilution (µM)	Dilution	Final concentration (µM)
1	100.00	1:100 (10 mM stock)	10.00
2	50.00	1:1 (#1)	5.00
3	25.00	1:1 (#2)	2.50
4	12.50	1:1 (#3)	1.25
5	6.25	1:1 (#4)	0.625
6	3.125	1:1 (#5)	0.313
7	1.563	1:1 (#6)	0.156
8	0.781	1:1 (#7)	0.078
9	0.391	1:1 (#8)	0.039
10	0.195	1:1 (#9)	0.020
11	0.098	1:1 (#10)	0.010
12	0.049	1:1 (#11)	0.005

Except for ATP-competition assays, all reactions had a final concentration of 10 µM ATP, which was included by the addition of 2 µL of 10x Kinase buffer (100 µM). To test ATP-competitive properties of the compounds, 2 µL of 10x Kinase buffer without ATP were used instead of the regular kinase buffer. Unlabeled ATP was added to reach a final concentration of 10, 50, 100, 250 or 500 µM ATP respectively. Finally, ddH20 was used to complete the reactions to a volume of 20 µL. The reactions were incubated for 30 minutes at 30°C and then stopped with 5 µL of 5x SDS Stop Buffer. The samples were incubated for 5 minutes at 99°C to allow the proteins to denature before separation by SDS-PAGE. For visualization of the proteins, the polyacrylamide gels were Commassie stained and dried at 80°C using a vacuum pump (Slab Gel Dryer SGD 2000, Thermo Savant, New York, USA, Diaphragm vacuum pump, Vacuubrand GmbH + Co. KG, Wertheim, Germany). The incorporated radioactivity levels on the proteins were visualized by autoradiography. Subsequently, the

dried proteins bands in the gel were cut out for Cherenkov counting (Cherenkov counter LS6500IC, Beckman Coulter, Brea, USA) to determine the amount of radioactivity incorporated into α-casein.

10x Kinase buffer: 1 mM ATP, 250 mM Tris-HCl [pH 7.5], 100 mM MgCl2, 1 mM EDTA
5x SDS stop buffer: 250 mM Tris-HCl [pH 6.8], 25% (v/v) ß-mercaptoethanol (MSH), 50% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromphenol blue
10x Kinase buffer - ATP: 250 mM Tris-HCl [pH 7.5], 100 mM MgCl2, 1 mM EDTA

2.2.3.7 High-Throughput Kinase Profiling

The residual activity of 321 eukaryotic kinases was measured by ProQinase GmbH (Freiburg, Germany) in the presence of compound IWP-2 (1 μM) or Compound 17 (1 μM). A dendrogram illustrating the phylogenetic relations of the kinases was generated using TREEspotTM Software Tool Image and reprinted with permission from KINOMEscan®, a division of DiscoveRx Corp., © DISCOVERX CORPORATION 2010.

2.2.4 Cell culture methods

2.2.4.1 Thawing and freezing of eukaryotic cell lines

Frozen cells preserved in liquid nitrogen were initially thawed at 37°C. Then cells were gently mixed with 10 mL of fresh growth medium and seeded into a 100 x 15 mL culture petri dish overnight at 37°C, then media was changed.
To freeze cells, cultures were detached with trypsin-EDTA solution and the reaction was stopped by the addition of culture medium. Cells were concentrated by centrifugation (5 min at 900 rpm), the cell pellet was resuspended in 1.5 ml freezing medium and transferred to a cryo tube on ice. The cells were first placed in the upper gaseous phase of a nitrogen tank for one hour and then overnight in the lower gaseous phase. Finally, the cells were stored in liquid nitrogen.

Freezing medium: complete growth medium, 10 % (v/v) DMSO

2.2.4.2 Culture of established cancer cell lines

Established cancer cell lines were cultured in appropriate growth media. Commercially purchased growth media were supplemented with 10 % (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.26 mg/mL L-glutamine. Cells were cultured at 37°C in 10 cm cell culture dishes at 5 % humidified carbon dioxide atmosphere (Heraeus HeraCell incubator, Thermo Scientific, Waltham, USA). Cells were split under sterile conditions twice a week: first, they were washed with 5 mL DPBS, then dissociated from each other and from the vessel with 1.5 mL of trypsin-EDTA solution for >5 minutes at 37°C. Finally, the cells were collected in 8.5 mL of medium. A cell dilution was seeded into a fresh cell culture dish and placed into the incubator.

2.2.4.3 Cell counting methods

When required, cells were counted using either a Neubauer Haemocytometer (counting chamber 1 mm2/ 0.1 mm depth) or a LUNA™ Automated Cell Counter. When the LUNA™ Automated Cell Counter was used, 10 µL of undiluted cell solution were loaded onto a disposable slide and then automatically counted.

2.2.4.4 MTT cell viability assay

Cells were seeded into sterile 96-well plates and incubated for 24 hours at 95 % humidity and 5 % carbon dioxide. Afterward, the media was replaced, either with treated or untreated fresh media. The treatments consisted of serial dilutions of the utilized compounds (10 µM, 5 µM, 2.5 µM, 1.25 µM, 625 nM and 313 nM) as well as DMSO control and untreated-medium control. After 48 hours of incubation, 10 µL of filtered MTT solution were added to each well and incubated for 4 hours at 37°C. The medium was then discarded, and the cells dissolved with 100 µL of acidic isopropanol. After 30 minutes of incubation, in the dark, at room temperature, the absorbance was measured with TECAN Spectra II Plate Reader using Magellan3 (TECAN) as software.

MTT solution:	5 mg/mL MTT in sterile PBS
Acidic isopropanol:	90 % isopropanol, 10 % 1N HCl

2.3.4.5 Compound treatment of cells for flow cytometry and qRT-PCR

Analyzed cells were seeded at a concentration of 50000 cells/mL into 6-well plates and incubated overnight at 37°C in an atmosphere with 95 % humidity and 5 % carbon dioxide. Then, media were changed, replacing it with treated media, DMSO controls or untreated control medium. Cells were then incubated for 48 hours and analyzed.

2.2.4.5 Flow cytometry assay for cell cycle analysis

After 48 hours of treatment, cells were gently washed with PBS and 1 mL of buffer solution (BD Cycletest™ Plus DNA reagent kit) was added to each well. Cells were harvested by gently scratching them off the petri dish and collected in a 1.5 mL Eppendorf tube. The BD Cycletest™ Plus DNA reagent kit was used to stain the nuclei with Propidium Iodide (PI) in three steps. First, cells were centrifuged at slow speed for 5 minutes. After removing the supernatant, the cells were gently suspended in 200 µL of Solution A, which contains trypsin, and incubated for 10 minutes at room temperature. Second, 180 µl of Solution B, which contains a trypsin inhibitor and RNAse, were added and incubated for 10 minutes at room temperature. Finally, 180 µl of PI Solution C were added and incubated up to 1 hour in the dark, on ice. PI is fluorescent and bounds nucleic acids, thus staining double stranded nuclear chromatin. The chromatin content of a cell is proportional to PI fluorescence and was indirectly measured by flow cytometry using a Becton Dickinson FACScan™ flow cytometer. Cell cycle distribution of the samples is displayed in DNA distribution histograms with the help of the CellQuest software.

2.2.5 Statistical analysis

For exploratory data analysis, MS Excel (Microsoft Corporation, USA) was used. Results for kinase activity determination are shown as normalized bar graphs. Results showing IC_{50} values are given as mean obtained from three independent measurements. The standard deviation is indicated by error bars. Data were normalized towards appropriate control measurements. IC_{50} values were determined using Prism 6 (GraphPad, San Diego, USA) including logarithmic transformation, normalization, and nonlinear regression (least squares fit) steps. Enzyme kinetic data were fitted to the Michaelis-Menten equation by nonlinear regression (least squares fit) using Prism 6.

3. Results

3.1. Validation of new CK1-specific small molecule inhibitor compounds

Protein phosphorylation is one of the most common forms of reversible post-translational modifications. It is calculated that about 50% of all proteins undergo this modification (Gross *et al.* 2015). Kinases regulate various processes involved in the so-called hallmarks of cancer, such as cell growth and viability (Hanahan and Weinberg 2011; Knippschild *et al.* 2014; Gross *et al.* 2015). Interest to develop CK1 specific inhibitors which can be used in new therapy concepts has enormously increased in the last five years due to CK1 isoforms' prominent role as regulators of cellular processes (Knippschild *et al.* 2014).

In this work, new specific inhibitors of CK1 are described. These substances have been part of a long process of compound development and optimization. Initially, a group of piperidinyl-thiazoles was found to be effective for inhibition of nuclear factor kappa B (NFκB) (Leban *et al.* 2007). This group of compounds was further modified by our cooperation partner 4SC Discovery GmbH (Planegg-Martinsried, Germany), to improve their inhibitory properties. However, the deletion of the piperidinyl residue resulted in a new group of substances that inhibited NFκB in a rather modest manner, while strongly inhibiting CK1 family members (Bischof *et al.* 2012). To improve the inhibitory potency of these compounds, further modifications were introduced, particularly regarding the benzimidazole group moiety (Richter *et al.* 2014). These alterations lead to an increase in the inhibitory ability of some of the compounds, as well as a higher specificity. New modifications were introduced to this group of substances to test them and evaluate their inhibitory potential. The results of *in vitro* experiments with this new set of compounds, their efficacy as inhibitors of CK1δ and ε, and their antiproliferative properties on a panel of established cancer cell lines are described in the following chapters.

3.1.1 Biological activity of the new compounds

The compounds numbered 116 up to 125, were first screened for their ability to inhibit bovine GST-CK1α, human GST-CK1γ3, rat GST-CK1δ, human CK1δKD, human GST-CK1δ TV1 and human CK1ε at a compound concentration of 10 µM, using α-casein as substrate (Figure 3). This initial screening showed that the compounds were mostly effective against the isoforms CK1δ and ε, while isoforms α and γ3 were only modestly inhibited. In the case of CK1α, only compound 125 decreased its activity by more than 39%, while the next best compound, 118, inhibits it only by 50% at the tested concentration of 10 µM. At best, CK1γ3 was inhibited to a 52% of residual activity by compound 122. In contrast, nine

out of ten compounds strongly inhibited CK1δ and CK1ε. CK1δ KD showed the lowest residual activity of all the assayed CK1δ variants. CK1δ KD was strongly inhibited to less than residual 10% activity by all compounds except 117 and 119, which reduced its kinase activity to 15% and 42% respectively. The other two CK1δ variants showed similar results, with compounds 117, 119 being the least effective inhibitors. CK1ε was inhibited strongly by compound 125, with an 8% of residual activity at the assayed concentration. Compounds 117 and 119 did not inhibit CK1ε. The rest of the compounds presented a good ability to inhibit CK1ε, albeit not as powerful as against CK1δ. These results indicate a high specificity of these substances against CK1δ and ε.

All compounds were subsequently characterized for their IC_{50} values against GST-CK1δ, CK1δKD, GST-CK1δTV1 and CK1ε via *in vitro* kinase assays at a wide range of compound concentrations (10 μM to 5 nM), using α-casein as substrate (Table 8). The compounds showed a range of IC_{50} values between 0.021 μM to 0.558 μM for GST-CK1δ, 0.010 μM to 0.371 μM for CK1δKD, 0.012 μM to 0.170 μM for GST-CK1δTV1 and 0.017 μM to 0.548 μM for CK1ε. Compounds 118, 122, 124 and 125 showed the most distinct inhibitory effects for both GST-CK1δ and CK1δKD, with IC_{50} values below 100 nM for both CK1δ variants. Compound 118 displayed the greatest inhibitory effect. It showed very low IC_{50} values against CK1δKD (0.01 μM) and GST-CK1δ (0.021 μM), similar to the values of the related compound Richter-1 (Richter *et al.* 2014). This indicates a very strong inhibitory ability against CK1δ. However, its IC_{50} value on CK1ε (0.017 μM) indicates that this compound is not specific to the delta isoform. In comparison, the compound Richter-1 showed a ten-fold increased IC_{50} value against CK1ε, indicating that it has a stronger selectivity towards CK1δ (Richter *et al.* 2014). Compounds 122 and 124 also show a similar trend, with strong inhibitory activity for CK1δ and ε. Compound 125 displays a more specific activity, with a 3-fold higher inhibitory ability against CK1δ over CK1ε.

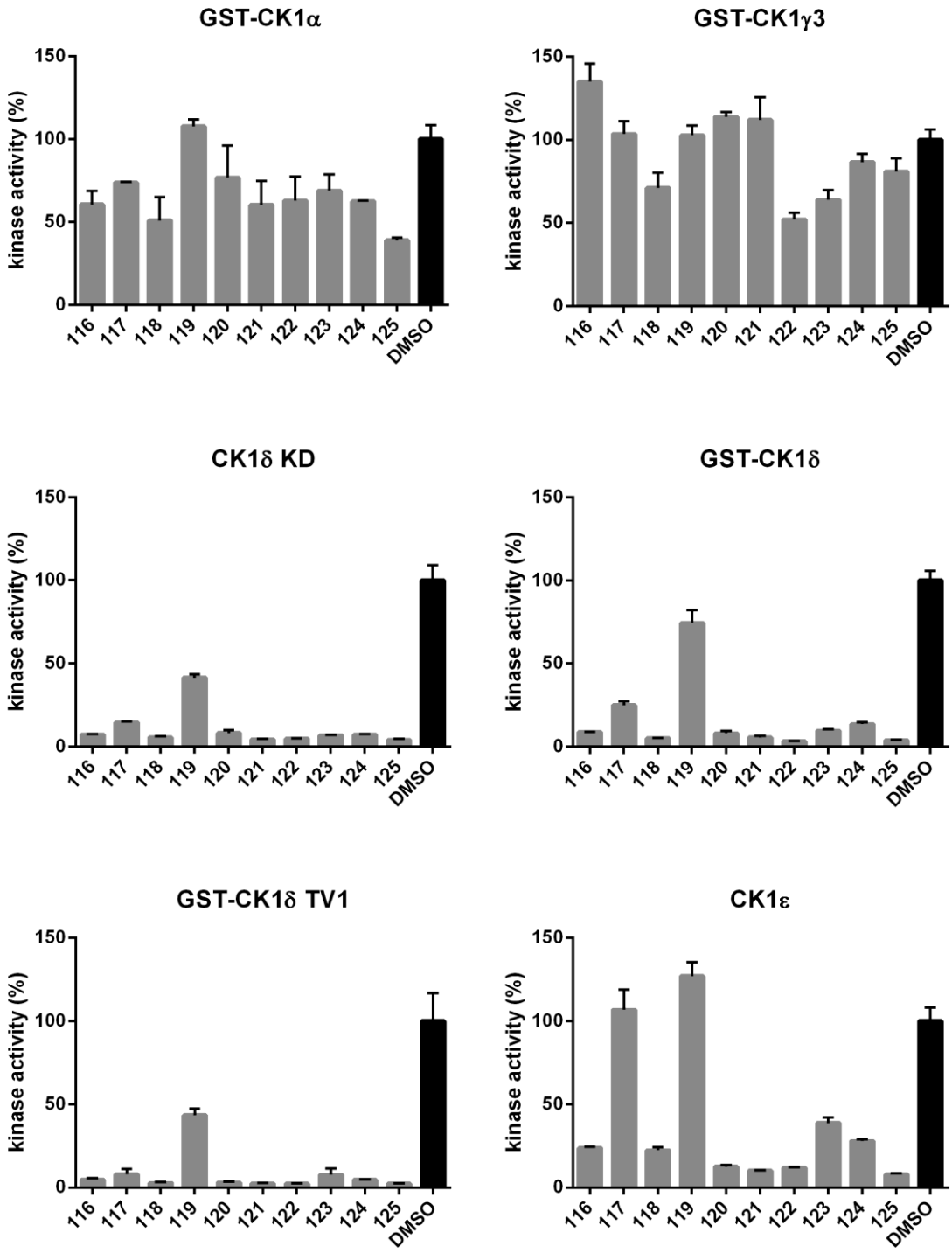


Figure 3: Inhibitory effects of compounds 118-125 on the kinase activity of different CK1 isoforms. *In vitro* kinase assays were performed in the presence or absence of compounds 116 to 125. Bovine GST-CK1α, human GST-CK1γ3, human CK1δ kinase domain (CK1δKD), rat GST-CK1δ, human GST-CK1δ TV1, and human CK1ε were used as sources of enzyme and α-casein as substrate. All compounds were used at a concentration of 10 µM. DMSO was used as a control. Kinase reactions were separated by SDS-PAGE and quantification of phosphate incorporation was performed by Cherenkov counting. Results are shown as normalized bar graphs using DMSO as a control for 100 % kinase activity (DMSO: Dimethyl sulfoxide).

Table 8: IC_{50} values determined for new inhibitor compounds. Determination of the 50% inhibitory concentration (IC_{50}) values of compounds 118 to 125 on the kinase activities of different CK1 isoforms using an inhibitor serial dilution ranging from 5 nM to 10 µM, the respective kinase and α-casein as substrate. DMSO was used as a control. IC_{50} values were calculated using GraphPad Prism 6. Values are presented in micromolar (µM) concentration as mean ± standard deviation (SD) from experiments performed in triplicate (KD: kinase domain, DMSO: Dimethyl sulfoxide) (*Experiments generated together with Ivana Sfarcic).

Code	IC_{50} values µM (± SD)			
	GST-CK1δ	CK1δ KD	GST-CK1δ TV1*	CK1ε
116	0.324 ± 0.159	0.081 ± 0.006	n.d.	0.413 ± 0.141
117	0.437 ± 0.068	0.119 ± 0.027	n.d.	0.185 ± 0.035
118	0.021 ± 0.004	0.010 ± 0.002	0.075 ± 0.001	0.017 ± 0.004
119	0.207 ± 0.002	0.371 ± 0.035	n.d.	n.d.
120	0.218 ± 0.019	0.125 ± 0.006	0.147 ± 0.027	0.548 ± 0.110
121	0.558 ± 0.046	0.085 ± 0.019	n.d.	0.181 ± 0.005
122	0.088 ± 0.021	0.062 ± 0.012	0.170 ± 0.014	0.095 ± 0.026
123	0.147 ± 0.030	0.057 ± 0.009	0.096 ± 0.014	0.097 ± 0.015
124	0.060 ± 0.001	0.031 ± 0.002	0.012 ± 0.008	0.037 ± 0.004
125	0.096 ± 0.130	0.050 ± 0.011	0.091 ± 0.048	0.310 ± 0.158

Next, the ability of selected compounds to inhibit GST-CK1δ wt and the GST-CK1δ^{M82F} gatekeeper mutant was compared (Figure 4). Methionine 82 plays an essential role as gatekeeper residue in the docking mode of isoxazoles to the ATP binding pocket. In case the gatekeeper methionine 82 is mutated to phenylalanine the binding of that class of SMIs is blocked because a hydrophobic (selectivity) pocket beyond the gatekeeper position cannot be accessed by the compounds. However, the mutant kinase's ability to bind ATP is preserved (Peifer *et al.* 2009). The data presented in Figure 4 shows the normalized residual activity of both wild-type and mutated CK1δ against compounds 118, 119, 122, 123, 124, and 125 at their respective IC_{50} concentrations (see Table 8). *In vitro* kinase assays were performed in the absence or presence of the inhibitor compounds for GST-CK1δ wt or GST-CK1δ^{M82F}. α-casein was used as a substrate. The results indicate that wild-type GST-CK1δ activity was not distinct from the activity on the gatekeeper-mutated form of CK1δ. Only compound 119 showed a stronger inhibition on the mutant than on the wild type, with a

difference of only 18% of the normalized kinase activity. For all cases, it can be inferred that there is not a significant difference between both forms, meaning that this particular mutation does not interfere with the inhibitory ability of these substances. A similar result was observed for the compound Richter-1, with a reduction of about 20% of its inhibitory ability found when tested against the mutated form (Richter *et al.* 2014).

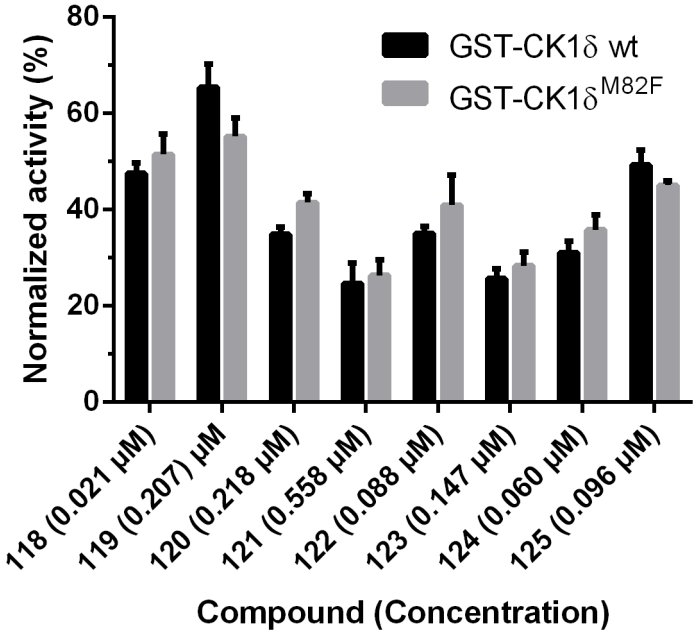


Figure 4: Comparison of the inhibitory ability of selected compounds against CK1δ wt or CK1δ^{M82F} gatekeeper mutant. Selected compounds (118, 119, 122, 123, 124, and 125) were used at the determined IC_{50} concentration to inhibit either GST-CK1δ wt or a GST-CK1δ^{M82F}. Kinase reactions were separated by SDS-PAGE and the phosphate incorporation into α-casein was measured by Cherenkov counting. Obtained data were normalized towards their respective DMSO control reactions. Bars represent the mean, and error bars represent the standard deviation (SD) (DMSO: Dimethyl sulfoxide).

The previously reported 2-benzamido-N-(1H-benzo[d]imidazol-2-yl)thiazole-4-carboxamide derived compounds were initially designed as ATP-competitive inhibitors (Bischof *et al.* 2012; Richter *et al.* 2014). We verified compounds 118, 122, 123, 124, and 125 at their respective IC_{50} concentration for CK1δKD at increasing concentrations of ATP to confirm their nature as ATP-competitive inhibitors (Figure 5). For each ATP concentration, the kinase activity was compared against a vehicle (DMSO) control. All these tested compounds showed decreasing inhibitory ability with higher ATP concentrations, suggesting that their mechanism of action is indeed ATP-competitive. The *in silico* model drawn by our industry research partner 4SC shows that the compounds bind to the ATP-binding pocket of CK1δ (data not shown).

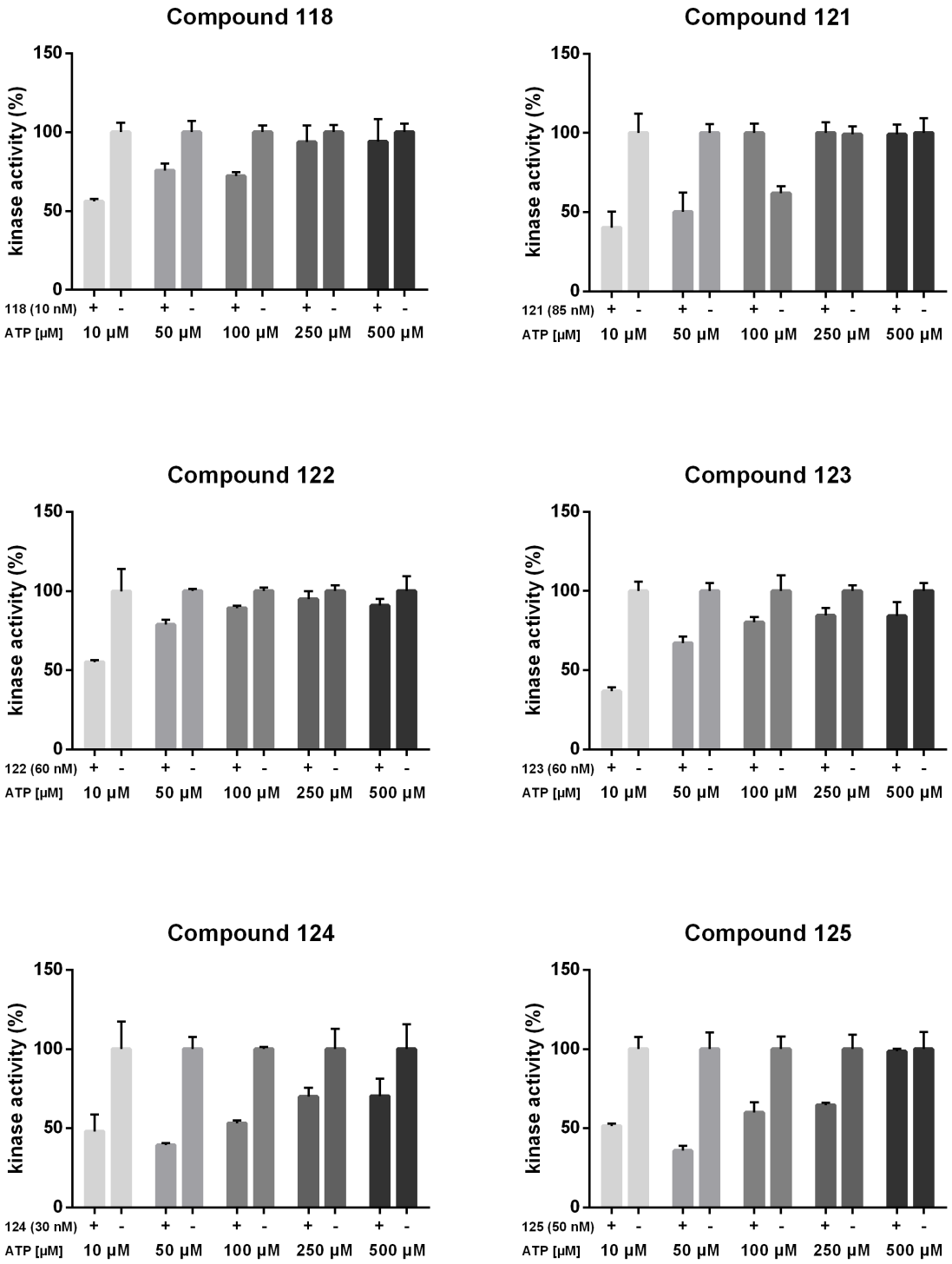


Figure 5: Selected compounds inhibit CK1δ in an ATP-competitive manner. Inhibitors 118 (10 nM), 121 (85 nM), 122 (60 nM), 123 (60 nM), 124 (30 nM) and 125 (50 nM) were assayed at their respective IC_{50} concentration in the presence of the indicated ATP concentrations (10, 50, 100, 250, and 500 µM) to demonstrate their ATP-competitive mode of action. Kinase assays were performed using CK1δ KD as enzyme and α-casein as substrate. Kinase reactions were separated by SDS-PAGE and the phosphate incorporation into α-casein was measured by Cherenkov counting. Obtained data were normalized towards their respective DMSO control reactions. Bars are shown as mean, and error bars represent the standard deviation (SD) (DMSO: Dimethyl sulfoxide).

3.1.2 Efficacy of compounds in cell culture

After assessing the *in vitro* ability of the compounds to inhibit CK1 isoforms, their ability to inhibit cell proliferation was analyzed. MTT assays were performed to determine the EC_{50} values of the different compounds for selected established tumor cell lines. Compounds 116, 117, and 119 were excluded, due to their lack of effect on viability displayed in preliminary experiments (data not shown). Though different cell lines might respond differently to the same treatment we found a rather constant response to each compound across the different cell lines (Table 9). For example, compounds 120, 121, 122 and 125 presented relatively high EC_{50} values in all treated cell lines, while compounds 123 and 124 consistently inhibited cell proliferation in a low range of concentrations for most analyzed cell lines. Compound 118 showed also an inhibitory effect, albeit less marked in comparison with compounds 123 and 124.

Table 9: EC_{50} values of most effective compounds for selected cell lines. Determination of half maximal effective concentration (EC_{50}) values of compounds 118, 120, 121, 122, 123, 124, and 125 on cell viability of different cell lines, using an inhibitor serial dilution. Compounds 116, 117 and 119 were not tested due to their poor inhibition ability determined in previous experiments. Cell viability was measured using MTT assay and EC_{50} values were calculated using GraphPad Prism 6. Data is presented in micromolar (μM) concentration as mean ± standard deviation (SD) (n.d.: not determined).

Cell Line	EC_{50} value (μM)						
	118	120	121	122	123	124	125
AsPc1	3.17 ± 0.58	5.43 ± 1.87	5.21 ± 0.25	3.90 ± 0.76	1.32 ± 0.06	0.56 ± 0.10	3.88 ± 0.76
BxPc3	1.11 ± 0.03	5.54 ± 0.95	5.75 ± 0.77	5.80 ± 0.18	0.71 ± 0.06	0.30 ± 0.01	4.36 ± 0.36
Capan-1	2.07 ± 0.20	8.44 ± 0.88	5.92 ± 0.85	4.37 ± 0.47	0.96 ± 0.15	1.01 ± 0.28	3.57 ± 0.83
MiaPaCa2	0.78 ± 0.17	5.28 ± 0.3	7.91 ± 1.02	5.19 ± 0.39	0.96 ± 0.09	0.43 ± 0.03	2.63 ± 0.96
Panc1	1.43 ± 0.11	7.89 ± 0.79	6.07 ± 1.22	5.82 ± 0.05	0.67 ± 0.07	0.29 ± 0.01	3.78 ± 0.62
Panc89	1.15 ± 0.07	5.97 ± 1.07	5.77 ± 0.73	2.67 ± 0.25	0.57 ± 0.07	0.63 ± 0.09	4.90 ± 1.00
HT29	1.65 ± 0.15	n.d.	n.d.	n.d.	0.38 ± 0.01	0.62 ± 0.08	n.d.
MCF7	1.10 ± 0.12	n.d.	n.d.	n.d.	0.63 ± 0.14	0.35 ± 0.01	n.d.
REF52	1.15 ± 0.21	n.d.	n.d.	n.d.	1.60 ± 0.08	0.40 ± 0.01	n.d.

The different cell lines showed different sensitivities towards different inhibitors. In the case of compound 118, the cell line displaying the most remarkable effects was MiaPaCa2, with an EC_{50} value of 0.78 μM. Panc89 and Panc1 were more responsive towards treatment with compound 123 (0.57 μM and 0.67 μM, respectively), though other cell lines showed similar sensitivity to this substance. Compound 124 presented, in general, the lowest EC_{50} values, affecting Panc1 and BxPC3 the most (0.29 μM and 0.30 μM respectively). The other tested compounds showed higher EC_{50} values, indicating that they do not possess the biological properties required to effectively inhibit cell growth effectively in cell culture. In the cases where we can compare with the previously reported compounds Richter-1 and Richter-2, similar EC_{50} values were observed, and in the case of Panc1 and MCF7, the EC_{50} result was slightly lower (Richter *et al.* 2014).

Additionally, FACS analysis was performed with different cell lines (HT29, MCF7, AsPC1, Panc89) in order to evaluate the effects on cell cycle progression of the most effective compounds: 118, 123, and 124. All cell lines were treated with the respective compound in two different concentrations (EC_{50} concentration and 3-fold of EC_{50} concentration) for 48 h, stained and then analyzed in a flow cytometer. In the case of compound 118, when cells were exposed to EC_{50} value concentrations, alterations in the cell cycle distribution were observed, in particular for cell line HT29. In the highest concentration, the most dramatic effects were observed, with most cells either arrested in G2 (HT29, MCF7, and AsPC1) or dead (Panc89) (Figure 6). Compounds 123 and 124 had nearly no effect on the cell cycle distribution (Figures 7 and 8).

The expression of 17 selected genes was measured by Real Time PCR analysis to preliminary evaluate the effects of the compounds on certain cellular processes. The transcribed RNA of control treated cells was compared to the transcribed RNA of a panel of pancreatic cancer cell lines treated with compounds 118, 123, 124, and 125 (Tables 10, 11, 12 and 13 respectively). Compound 118 induced the most marked and consistent effects across all tested cell lines in comparison with compounds 123, 124 and 125. Upregulation of stem cell marker genes such as SOX2, NANOG, and POU5F1 (oct4) was observed for compound 118. Other genes consistently upregulated upon treatment with compound 118 were BCL2, GLI1, PITX2, CKK9, and NES. In the case of TP53, it was found downregulated when cells were exposed to compounds 118 and 125. Genes like CTNNB1, KRAS and MDM2 were found downregulated when cells were treated with compounds 123 and 125. CK1 isoforms α, δ, and ε were slightly upregulated in cells treated with compounds

118 and 123. Compound 124 was found to induce a small upregulation of the expression of CK1ε.

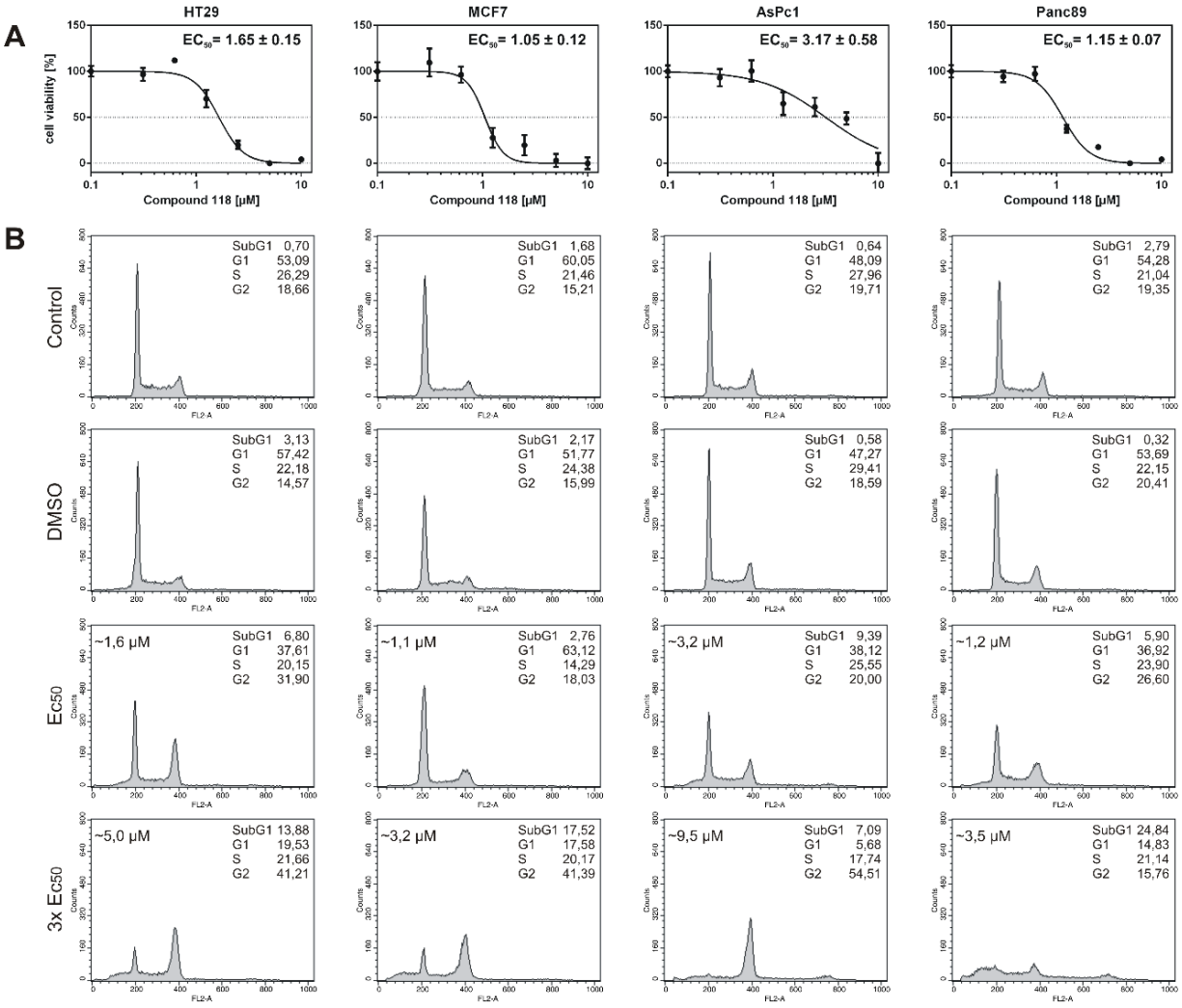


Figure 6: Activity of compound 118 in cell culture. Effects of compound 118 on cell viability and cell cycle distribution of MCF7, HT29, AsPC1, and Panc89 cells. **(A)** Cells were either untreated, treated with DMSO or with compound 118 in a range of concentrations. MTT assays were performed 48h after treatment. **(B)** Once EC_{50} values were obtained, FACS analyses of MCF7, HT29, AsPC1, and Panc89 cells were performed in the presence of different concentrations of compound 118 (EC_{50} concentration and 3-fold of EC_{50} concentration) for 48 h, then stained with propidium iodide, and analyzed in a flow cytometer using Cell Quest program. Control and DMSO-treated cells showed a normal cell cycle distribution of asynchronously proliferating cells. Treatment with compound 118 led to an increase in the number of dead cells as well as to an increased percentage of cells being arrested in G2 phase of the cell cycle in the case of MCF7, HT29, and AsPC1 cells (DMSO: Dimethyl sulfoxide) (all concentrations expressed as µM).

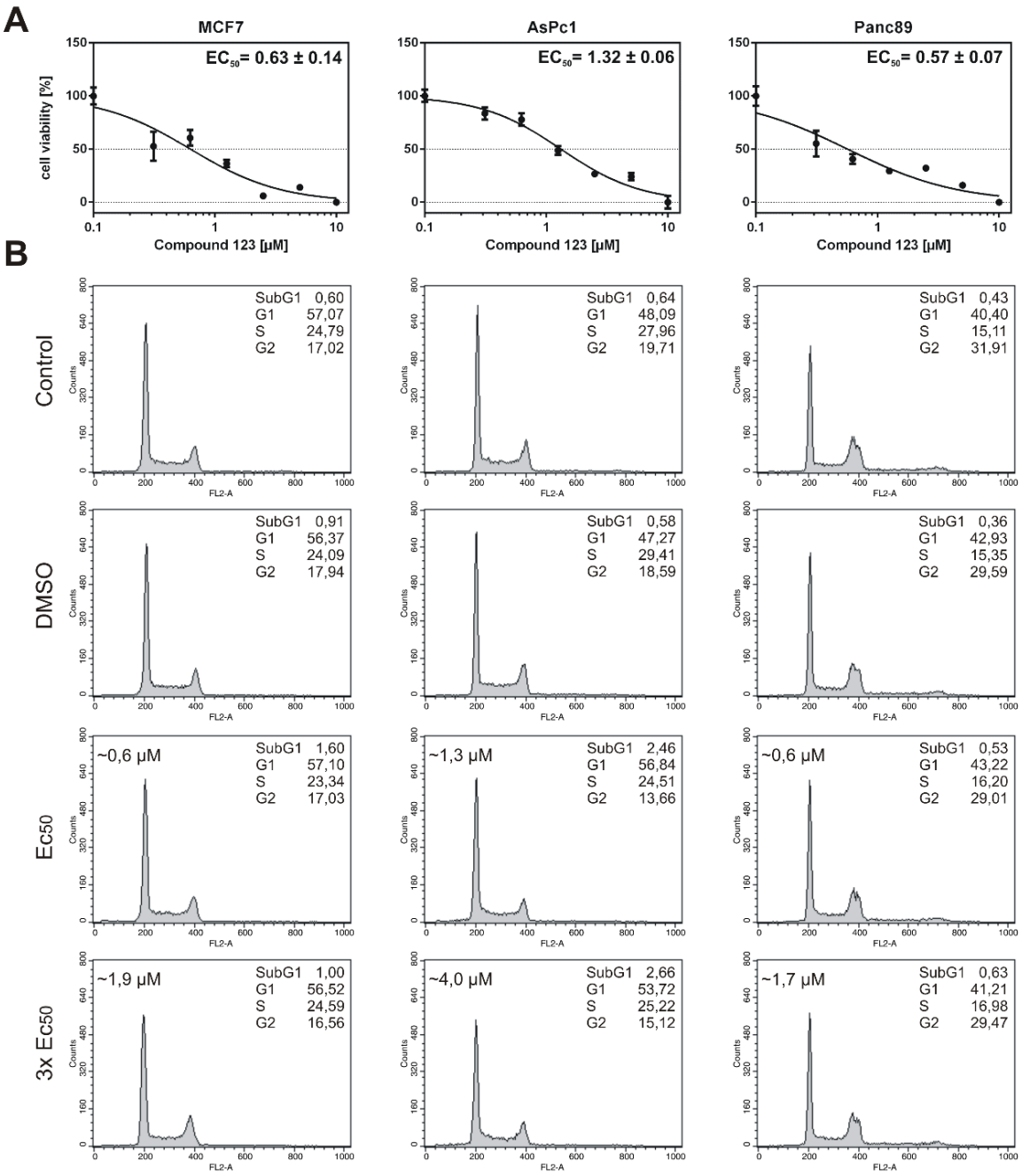


Figure 7: Activity of compound 123 in cell culture. Effects of compound 123 on cell viability and cell cycle distribution of MCF7, AsPC1, and Panc89 cells. **(A)** Cells were either untreated, treated with DMSO or with compound 123 in a range of concentrations. MTT assays were performed 48h after treatment. **(B)** Once EC_{50} values were obtained, FACS analyses of MCF7, HT29, AsPC1, and Panc89 cells were performed in the presence of different concentrations of compound 123 (EC_{50} concentration and 3-fold of EC_{50} concentration) for 48 h, then stained with propidium iodide, and analyzed in a flow cytometer using Cell Quest program. Control and DMSO-treated cells showed a normal cell cycle distribution of asynchronously proliferating cells (DMSO: Dimethyl sulfoxide) (all concentrations expressed as µM).

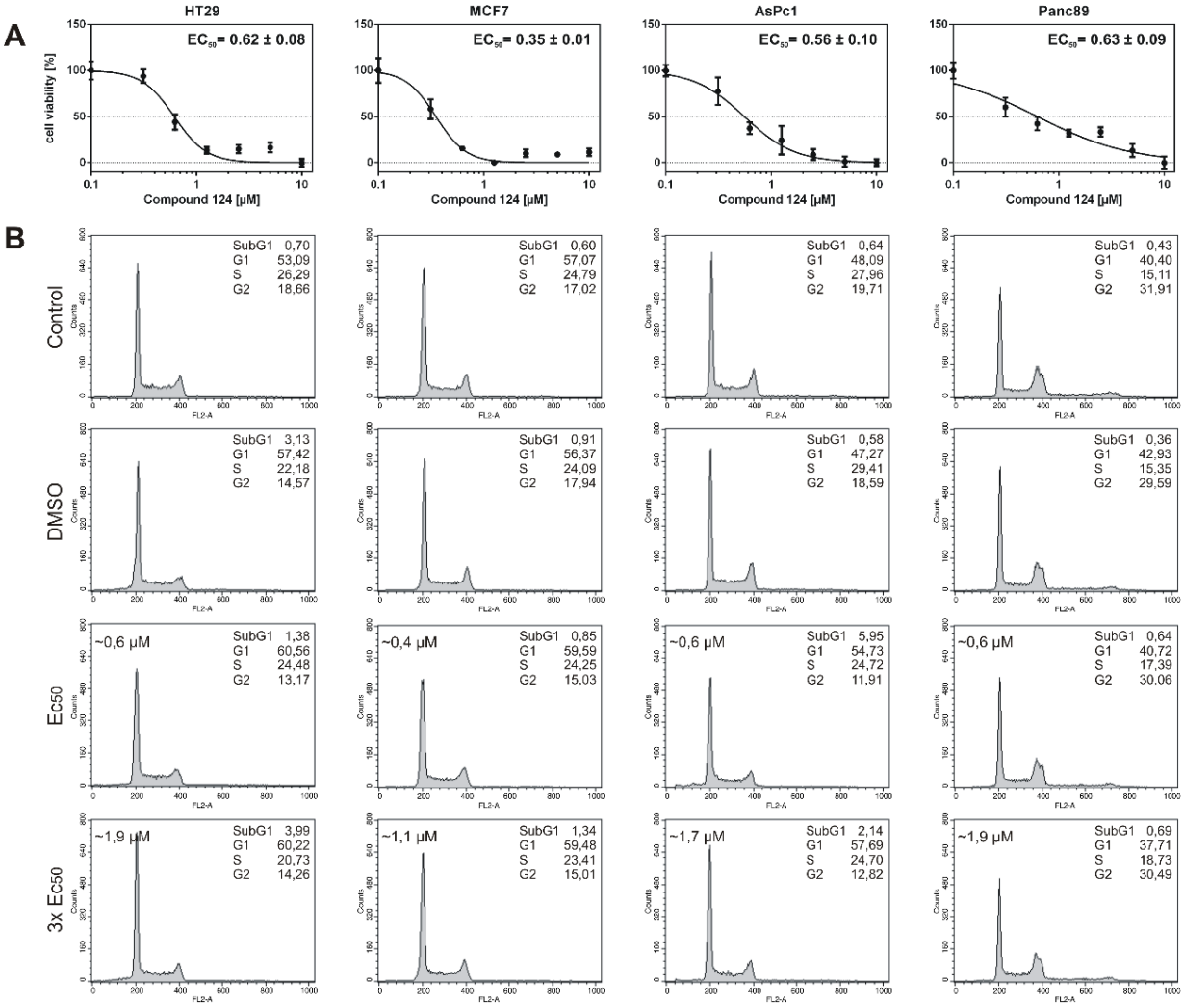


Figure 8: Activity of compound 124 in cell culture. Effects of compound 124 on cell viability and cell cycle distribution of HT29, MCF7, AsPC1, and Panc89 cells. **(A)** Cells were either untreated, treated with DMSO or with compound 124 in a range of concentrations. MTT assays were performed 48h after treatment. **(B)** Once EC_{50} values were obtained, FACS analyses of MCF7, HT29, AsPC1, and Panc89 cells were performed in the presence of different concentrations of compound 124 (EC_{50} concentration and 3-fold of EC_{50} concentration) for 48 h, then stained with propidium iodide, and analyzed in a flow cytometer using Cell Quest program. Control and DMSO-treated cells showed a normal cell cycle distribution of asynchronously proliferating cells (DMSO: Dimethyl sulfoxide) (all concentrations expressed as µM).

Table 10: Effect of compound 118 on the gene expression on different pancreatic cancer cell lines. Cells were treated with previously determined EC_{50} concentrations of compound 118, or DMSO as a control, for 48 h. The total RNA was isolated from cells and reversely transcribed to cDNA. Relative gene quantification was performed via QuantiTect Primer Assay and SYBR Green chemistry in a Roche Light Cycler 480II. Values represent the mean of relative fold changes in gene expression in respect to the gene control.

Gene	Relative change in gene expression per cell line					
	MiaPaCa2	Panc1	Capan1	Panc89	BxPC3	AsPC3
BCL2	5.91 ± 0.73	5.97 ± 1.29	3.33 ± 0.67	0.86 ± 0.14	16.91 ± 1.32	1.16 ± 0.12
CDKN1A	3.37 ± 0.68	1.53 ± 0.11	4.35 ± 0.26	1.63 ± 0.11	4.54 ± 0.27	2.10 ± 0.06
DYRK1B	0.68 ± 0.29	0.93 ± 0.07	0.66 ± 0.04	1.17 ± 0.09	1.4 ± 0.09	0.10 ± 0.10
GLI1	23.16 ± 13.6	6.62 ± 1.04	6.58 ± 0.61	1.09 ± 0.11	84.59 ± 4.99	1.08 ± 0.04
PITX2	5.54 ± 0.39	6.92 ± 0.51	4.38 ± 0.27	1.54 ± 0.25	25.87 ± 1.95	2.23 ± 0.24
SOX2	18.33 ± 2.66	7.8 ± 1.24	6.05 ± 0.38	2.58 ± 0.39	10.54 ± 0.81	2.37 ± 0.06
TP53	0.65 ± 0.02	0.77 ± 0.05	0.78 ± 0.18	0.32 ± 0.32	0.85 ± 0.05	0.45 ± 0.6
CDK9	1.63 ± 0.09	2.67 ± 0.18	1.28 ± 0.08	1.12 ± 0.07	2.36 ± 0.1	1.22 ± 0.04
CTNNB1	0.77 ± 0.05	0.95 ± 0.07	0.7 ± 0.04	0.79 ± 0.04	2.00 ± 0.09	0.81 ± 0.03
KRAS	1.31 ± 0.05	1.97 ± 0.12	0.94 ± 0.05	1.02 ± 0.05	2.57 ± 0.15	1.11 ± 0.1
MDM2	1.36 ± 0.18	1.24 ± 0.11	0.84 ± 0.06	0.83 ± 0.05	3.29 ± 0.48	1.18 ± 0.12
NANOG	10.96 ± 0.84	7.44 ± 0.98	5.57 ± 0.64	1.02 ± 0.15	39.37 ± 2.63	1.94 ± 0.28
NES	4.59 ± 0.76	2.43 ± 0.17	2.19 ± 0.09	1.11 ± 0.05	4.68 ± 0.28	1.08 ± 0.04
POU5F1	11.82 ± 1.30	8.88 ± 0.57		1.58 ± 0.11	24.44 ± 11.8	2.23 ± 0.13
CSNK1A1	1.90 ± 0.09	2.12 ± 0.09	1.24 ± 0.05	1.27 ± 0.09	3.80 ± 1.03	1.25 ± 0.05
CSNK1D	1.85 ± 0.15	1.52 ± 0.07	0.95 ± 0.39	1.32 ± 0.09	1.47 ± 0.08	
CSNK1E	2.09 ± 0.08	2.93 ± 0.13	2.14 ± 0.1	2.01 ± 0.15	1.8 ± 0.09	3.74 ± 0.09

Table 11: Effect of compound 123 on the gene expression on different pancreatic cancer cell lines. Cells were treated with previously determined EC_{50} concentrations of compound 123, or DMSO as a control, for 48 h. The total RNA was isolated from cells and reversely transcribed to cDNA. Relative gene quantification was performed via QuantiTect Primer Assay and SYBR Green chemistry in a Roche Light Cycler 480II. Values represent the mean of relative fold changes in gene expression in respect to the gene control.

Gene	Relative change in gene expression per cell line					
	MiaPaCa2	Panc1	Capan1	Panc89	BxPC3	AsPC3
BCL2	1.01 ± 0.11	0.28 ± 0.03	1.20 ± 0.27	0.88 ± 0.16	1.67 ± 0.24	0.78 ± 0.10
CDKN1A	1.80 ± 0.35	0.90 ± 0.04	1.41 ± 0.10	1.01 ± 0.08	2.08 ± 0.12	1.32 ± 0.05
DYRK1B	1.06 ± 0.45	1.32 ± 0.06	0.90 ± 0.07	1.12 ± 0.10	1.22 ± 0.09	1.10 ± 0.1
GLI1	1.98 ± 1.16	0.22 ± 0.02	1.54 ± 0.14	1.17 ± 0.16	3.21 ± 0.83	0.91 ± 0.07
PITX2	0.98 ± 0.11	0.40 ± 0.04	1.14 ± 0.09	0.95 ± 0.15	2.38 ± 0.16	1.06 ± 0.09
SOX2	1.58 ± 0.23	0.22 ± 0.04	1.53 ± 0.10	1.69 ± 0.32	1.33 ± 0.06	1.10 ± 0.06
TP53	0.78 ± 0.02	0.66 ± 0.03	1.20 ± 0.06	0.94 ± 0.07	1.03 ± 0.05	1.01 ± 0.07
CDK9	1.06 ± 0.01	1.13 ± 0.09	1.10 ± 0.05	0.94 ± 0.08	1.17 ± 0.04	1.39 ± 0.63
CTNNB1	0.82 ± 0.05	0.77 ± 0.06	0.94 ± 0.06	0.74 ± 0.06	1.50 ± 0.05	0.88 ± 0.04
KRAS	0.93 ± 0.03	1.05 ± 0.11	0.84 ± 0.04	0.92 ± 0.07	1.61 ± 0.06	0.82 ± 0.05
MDM2	0.67 ± 0.09	0.57 ± 0.06	0.62 ± 0.05	1.00 ± 0.09	2.38 ± 0.10	0.84 ± 0.03
NANOG	1.16 ± 0.13	0.40 ± 0.05	1.52 ± 0.19	0.91 ± 0.12	2.25 ± 0.16	0.86 ± 0.12
NES	1.15 ± 0.19	1.08 ± 0.08	1.48 ± 0.08	1.05 ± 0.09	1.44 ± 0.07	1.07 ± 0.04
POU5F1	1.26 ± 0.18	0.24 ± 0.03	1.37 ± 0.07	1.07 ± 0.14	1.82 ± 0.11	1.03 ± 0.08
CSNK1A1	1.11 ± 0.07	0.99 ± 0.05	1.20 ± 0.34	0.94 ± 0.06	1.92 ± 0.52	0.97 ± 0.04
CSNK1D	1.34 ± 0.08	1.38 ± 0.08	1.52 ± 0.20	1.06 ± 0.03	1.15 ± 0.06	
CSNK1E	1.57 ± 0.06	1.65 ± 0.10	1.93 ± 0.33	1.20 ± 0.04	1.39 ± 0.06	1.71 ± 0.05

Table 12: Effect of compound 124 on the gene expression on different pancreatic cancer cell lines. Cells were treated with previously determined EC_{50} concentrations of compound 124, or DMSO as a control, for 48 h. The total RNA was isolated from cells and reversely transcribed to cDNA. Relative gene quantification was performed via QuantiTect Primer Assay and Sybr Green chemistry in a Roche Light Cycler 480II. Values represent the mean of relative fold changes in gene expression in respect to the gene control.

Gene	Relative change in gene expression per cell line					
	MiaPaCa2	Panc1	Capan1	Panc89	BxPC3	AsPC3
BCL2	21.32 ± 3.00	0.19 ± 0.03	1.16 ± 0.18	0.80 ± 0.13	1.02 ± 0.05	0.87 ± 0.09
CDKN1A		0.90 ± 0.05	1.31 ± 0.08	0.94 ± 0.07	1.77 ± 0.10	1.18 ± 0.05
DYRK1B		1.28 ± 0.07	1.02 ± 0.06	0.93 ± 0.08	1.25 ± 0.06	
GLI1		0.13 ± 0.01	1.55 ± 0.15	1.00 ± 0.10	1.66 ± 0.09	0.01 ± 0.47
PITX2		0.28 ± 0.03	1.12 ± 0.12	0.81 ± 0.12	1.74 ± 0.15	0.80 ± 0.07
SOX2		0.09 ± 0.03	1.21 ± 0.08	0.87 ± 0.15	0.91 ± 0.04	0.80 ± 0.06
TP53		0.63 ± 0.04	0.83 ± 0.19	0.98 ± 0.07	0.83 ± 0.05	1.10 ± 0.08
CDK9	0.94 ± 0.02	1.05 ± 0.06	1.00 ± 0.05	0.89 ± 0.06	1.07 ± 0.03	0.96 ± 0.05
CTNNB1	0.51 ± 0.02	0.64 ± 0.03	0.71 ± 0.04	0.82 ± 0.06	0.83 ± 0.02	0.91 ± 0.04
KRAS	0.60 ± 0.01	0.77 ± 0.04	0.69 ± 0.04	0.95 ± 0.05	0.92 ± 0.01	0.91 ± 0.06
MDM2	0.28 ± 0.04	0.31 ± 0.02	0.47 ± 0.03	1.03 ± 0.09	0.99 ± 0.03	0.84 ± 0.13
NANOG	0.60 ± 0.04	0.32 ± 0.02	1.24 ± 0.14	0.81 ± 0.15	1.40 ± 0.10	0.85 ± 0.12
NES	0.76 ± 0.12	1.07 ± 0.06	1.28 ± 0.06	1.04 ± 0.06	1.35 ± 0.05	1.06 ± 0.04
POU5F1	0.58 ± 0.06	0.12 ± 0.01	1.42 ± 0.07	0.89 ± 0.05	1.29 ± 0.06	1.03 ± 0.06
CSNK1A1	0.82 ± 0.07	0.76 ± 0.05	0.83 ± 0.03		1.45 ± 0.38	1.01 ± 0.04
CSNK1D	1.24 ± 0.10	1.41 ± 0.07	1.40 ± 0.13		1.17 ± 0.05	
CSNK1E	1.30 ± 0.09	1.61 ± 0.09	1.79 ± 0.09	1.49 ± 0.10	1.43 ± 0.05	1.78 ± 0.06

Table 13: Effect of compound 125 on the gene expression on different pancreatic cancer cell lines. Cells were treated with previously determined EC_{50} concentrations of compound 125, or DMSO as a control, for 48 h. The total RNA was isolated from cells and reversely transcribed to cDNA. Relative gene quantification was performed via QuantiTect Primer Assay and SYBR Green chemistry in a Roche Light Cycler 480II. Values represent the mean of relative fold changes in gene expression in respect to the gene control.

Gene	Relative change in gene expression per cell line					
	MiaPaCa2	Panc1	Capan1	Panc89	BxPC3	AsPC3
BCL2	0.69 ± 0.10	0.20 ± 0.02	1.31 ± 0.23	0.82 ± 0.14	1.59 ± 0.16	1.19 ± 0.14
CDKN1A	1.43 ± 0.29	1.03 ± 0.04	1.14 ± 0.25	1.07 ± 0.08	2.40 ± 0.15	1.29 ± 0.12
DYRK1B	0.76 ± 0.32	1.32 ± 0.06	1.10 ± 0.10	1.25 ± 0.10	1.42 ± 0.09	0.02 ± 0.57
GLI1	1.11 ± 0.67	0.15 ± 0.01	1.04 ± 0.11	1.01 ± 0.12	2.88 ± 0.14	0.95 ± 0.04
PITX2	0.64 ± 0.12	0.38 ± 0.02	0.76 ± 0.09	1.45 ± 0.82	1.94 ± 0.09	1.46 ± 0.25
SOX2	0.55 ± 0.08	0.10 ± 0.02	0.88 ± 0.07	0.92 ± 0.14	1.13 ± 0.07	1.66 ± 0.21
TP53	0.49 ± 0.02	0.47 ± 0.03	0.61 ± 0.09	1.03 ± 0.09	0.83 ± 0.05	
CDK9	1.78 ± 0.79	1.04 ± 0.03	0.88 ± 0.04	1.04 ± 0.08	1.43 ± 0.03	1.15 ± 0.05
CTNNB1	0.48 ± 0.02	0.44 ± 0.01	0.34 ± 0.02	0.95 ± 0.06	0.95 ± 0.02	0.91 ± 0.04
KRAS	0.51 ± 0.01	0.54 ± 0.03	0.48 ± 0.02	0.98 ± 0.06	1.15 ± 0.02	0.96 ± 0.08
MDM2	0.22 ± 0.03	0.18 ± 0.03	0.23 ± 0.06	0.83 ± 0.06	1.14 ± 0.08	0.81 ± 0.03
NANOG	1.32 ± 0.42	0.34 ± 0.02	1.03 ± 0.18	0.74 ± 0.08	2.11 ± 0.24	1.30 ± 0.13
NES	2.35 ± 1.88	1.05 ± 0.03	1.34 ± 0.09	0.98 ± 0.06	1.57 ± 0.07	1.07 ± 0.04
POU5F1	0.89 ± 0.10	0.13 ± 0.02	1.21 ± 0.06	0.89 ± 0.05	1.90 ± 0.10	1.36 ± 0.09

3.2 IWP derivatives as inhibitors of CK1δ

In 2009, Chen and collaborators published the results of a synthetic chemical library screening, in which they searched for lead structures that would target Wnt-mediated cellular response (Chen *et al.* 2009). In this study IWPs (Inhibitors of Wnt Production), a class of compounds which targeted the N-palmitoyltransferase Porcupine (Porcn), was identified. Porcn is a member of the membrane-bound O-acyltransferases (MBOAT) protein family, which catalyzes the palmitoylation of Wnt ligands (Chen *et al.* 2009; Wang *et al.* 2013b). This modification is crucial for Wnt secretion and signaling. When Porcn is inactivated, Wnt3A is retained in the endoplasmic reticulum resulting in inhibition of the Wnt pathway (Takada *et al.* 2006).

Since IWPs show structural similarities to known CK1-specific inhibitors containing a benzimidazole scaffold (e.g. 2-benzamido-N-(1H-benzo[d]imidazol-2-yl)thiazole-4-carboxamide) (Bischof *et al.* 2012), IWP compounds could be additionally able to interact and inhibit CK1 isoforms (Figure 9). In this work, IWPs are validated as inhibitors of CK1 using *in vitro* kinase assays with different isoforms of CK1 in the presence of commercially available IWP derivatives. IWPs were also further modified by our research partners at the Christian-Albrechts-University in Kiel, to improve their inhibitory properties against CK1δ. Moreover, IWP-2 was tested at a concentration of 1 µM in a panel of 321 kinases to determine a selectivity profile. Docking studies of IWPs in the ATP binding pocket of CK1δ were also performed to reveal possible binding modes.

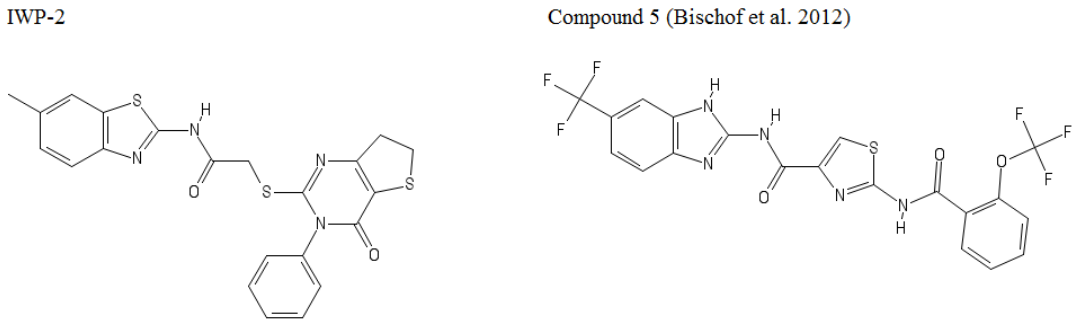


Figure 9: Chemical structures of IWP-2 and compound 5 (first published in Bischof *et al.* 2012 (Bischof *et al.* 2012)), a benzimidazole CK1 inhibitor. Structures were drawn using Pubchem Sketcher (Ihlenfeldt *et al.* 2009).

3.2.1 Characterization of IWPs as CK1 inhibitors *in vitro*

First, compounds IWP-2, IWP-2-V2, IWP-3, and IWP-4 were screened for their ability to inhibit bovine GST-CK1α, human GST-CK1γ3, rat GST-CK1δ, human CK1δKD, and human CK1ε at a compound concentration of 10 μM, using α-casein as substrate (Figure 10). This initial screening showed that the compounds inhibited the isoforms CK1δ and ε, while isoforms α and γ3 were only modestly inhibited. None of the IWP compounds inhibited CK1α to less than 90% of kinase activity when compared to the DMSO control. IWP-4 at 10 μM inhibited CK1γ3 to a 55% residual activity, IWP-2-V2 and IWP-3 to ~60% while IWP-2 did not show any inhibitory effect. CK1δ, both its full form and its kinase domain form, were consistently inhibited by different IWPs. IWP-2 inhibited CK1δ to 23% residual activity compared to the control, and CK1δ KD to 19% residual activity. IWP-4 also reduced CK1δ activity to less than 25%. CK1δ activity was also reduced by IWP-2-V2 and IWP-3, though not at the extent of the other tested compounds. Finally, CK1ε was only moderately inhibited by IWP-2 and IWP-4, to 35% and 43% residual activity respectively. These results suggest that IWP-2 and IWP-4 have a remarkable inhibitory effect on the activity of CK1δ.

Subsequently, the IC_{50} values of all compounds were determined for bovine GST-CK1α, human GST-CK1γ3, rat GST-CK1δ wild type, rat GST-CK1δ^{M82F}, human CK1δ KD, human GST-CK1δ Transcription Variant 1 (TV1), GST-CK1δ Transcription Variant 2 (TV2), GST-CK1δ TV2^{T67S}, and human CK1ε by *in vitro* kinase assays using α-casein as substrate, the different CK1 isoforms as enzymes, and a wide range of compound concentrations (10 μM to 5 nM) (Table 14). As expected, based on the initial screenings, CK1α and γ3 were not inhibited in the tested range of concentrations. The IWPs inhibited the wild type forms of CK1δ in a range of 0.93 μM to 1.89 μM, while the kinase domain of CK1δ (CK1δ KD) was inhibited in a lower range of 0.32 μM to 1.02 μM. Interestingly, the gatekeeper mutant CK1δ^{M82F} was significantly inhibited by all tested IWPs, with a range of IC_{50} values from 0.04 μM to 0.15 μM, several times lower than its respective value on the wild-type form (rat CK1δ wt). In the case of IWP-2-V2, the compound shows a difference of nearly 28-fold on its inhibitory ability against the gatekeeper mutant form of CK1δ (0.06 μM) in comparison to wild-type CK1δ. The human CK1δ transcription variants 1 and 2 (TV1 and TV2) were inhibited in low concentrations as well. The hyperactive CK1δ mutant CK1δ TV2^{T67S} showed a different sensitivity towards compounds IWP-3 and IWP-4 while responding to IWP-2 and IWP-2-V2 in a similar way as wild-type CK1δ TV2. CK1ε was only inhibited at high concentrations of IWPs, confirming the specificity towards CK1δ observed in the initial

screenings. Figure 11 shows the IC_{50} curves of each tested IWP compound on GST-CK1δ wt and GST-CK1δ^{M82F}.

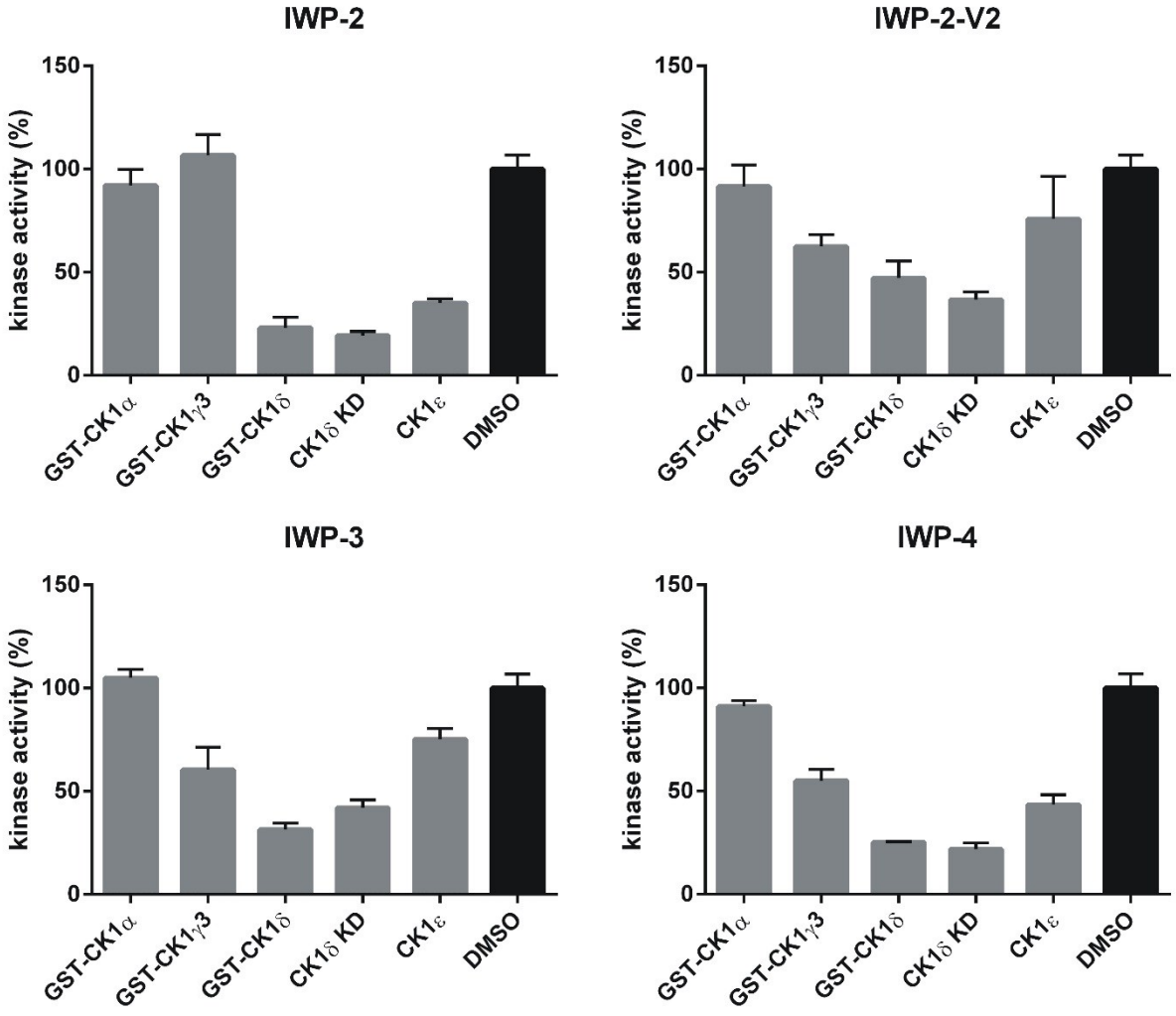


Figure 10: Inhibitory effects of IWP compounds on the kinase activity of different CK1 isoforms. *In vitro* kinase assays were performed in the presence or absence of IWP-2, IWP-2-V2, IWP-3 or IWP-4 (10 µM). Bovine GST-CK1α, human GST-CK1γ3, rat GST-CK1δ, human CK1δ kinase domain (CK1δKD), and human CK1ε were used as sources of enzyme and α-casein as substrate. All compounds were used at a concentration of 10 µM. DMSO was used as a control. Kinase reactions were separated by SDS-PAGE and quantification of phosphate incorporation was performed by Cherenkov counting. Results are shown as normalized bar graphs using DMSO as a control for 100 % kinase activity (DMSO: Dimethyl sulfoxide).

Table 14: IC_{50} values determined for IWP compounds. Determination of the 50% inhibitory concentration (IC_{50}) values of IWP compounds on the kinase activities of different CK1 isoforms using an inhibitor serial dilution ranging from 5 nM to 10 µM, the respective kinase, and α-casein as substrate. DMSO was used as a control. IC_{50} values were calculated using GraphPad Prism 6. Values are presented in micromolar (µM) concentration as mean ± standard deviation (SD) from experiments performed in triplicate (KD: kinase domain, DMSO: Dimethyl sulfoxide) (*Experiments generated together with Tanja Gehring).

Kinase	Compound (µM)			
	IWP-2	IWP-2-V2	IWP-3	IWP-4
GST-CK1α*	>10 µM	>10 µM	>10 µM	>10 µM
GST-CK1γ3*	>10 µM	>10 µM	>10 µM	>10 µM
CK1δ KD	0.32 ± 0.06	0.42 ± 0.06	0.55 ± 0.27	1.02 ± 0.13
GST-CK1δ wt	0.93 ± 0.15	1.66 ± 0.37	1.89 ± 0.07	1.06 ± 0.18
GST-CK1δ^{M82F}	0.04 ± 0.01	0.06 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
GST-CK1δ TV1*	0.43 ± 0.13	0.12 ± 0.03	1.37 ± 0.38	0.32 ± 0.06
GST-CK1δ TV2*	0.31 ± 0.01	0.24 ± 0.05	0.16 ± 0.04	0.20 ± 0.02
GST-CK1δ TV2^{T67S}*	0.25 ± 0.04	0.11 ± 0.01	1.81 ± 0.14	1.01 ± 0.27
CK1ε wt	4.03 ± 0.03	7.34 ± 2.58	>10 µM	7.07 ± 2.01

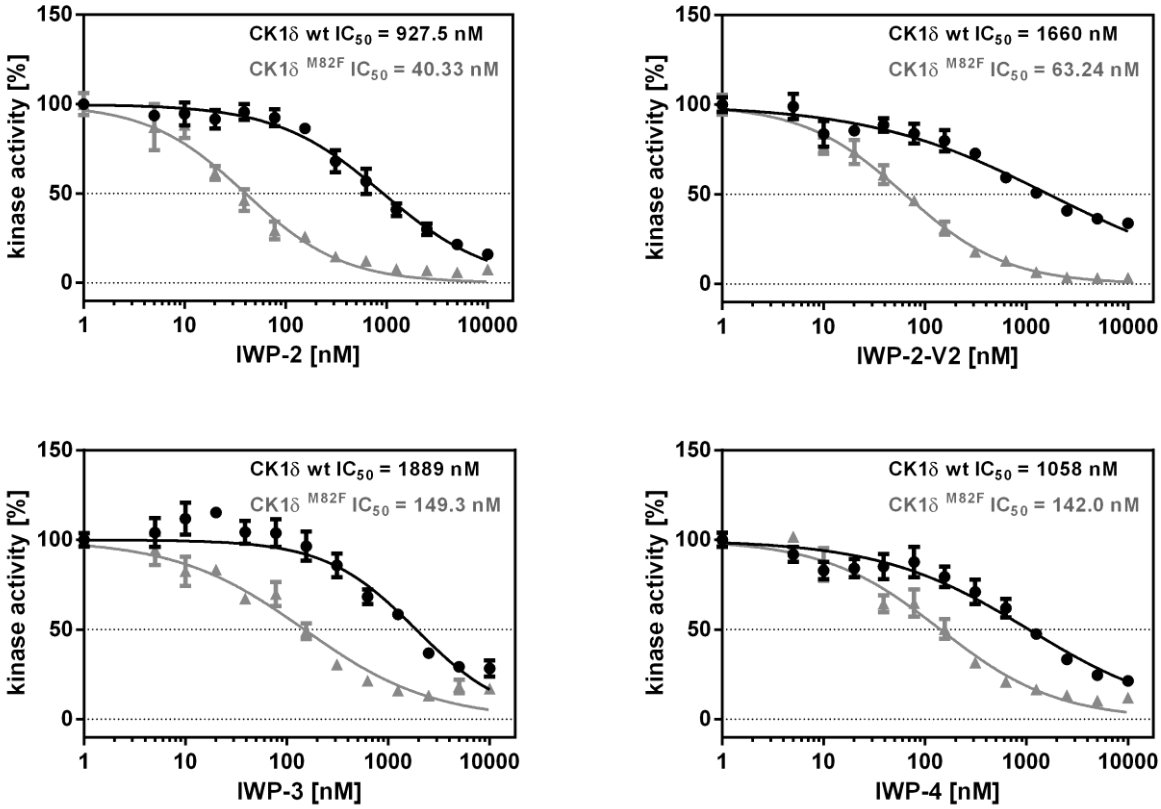


Figure 11: Comparison of the IC_{50} curves of IWP compounds against CK1δ wt or CK1δ^{M82F} gatekeeper mutant. IWP-2, -2-V2, -3 and -4 were used were used to inhibit either GST-CK1δ wt or a GST-CK1δ^{M82F}. Determination of the 50% inhibitory concentration (IC_{50}) curves of IWP compounds on the kinase activities using IWPs serial dilutions ranging from 5 nM to 10 µM, GST-CK1δ wt or GST-CK1δ^{M82F} as enzymes and α-casein as substrate. Kinase reactions were separated by SDS-PAGE and the phosphate incorporation into α-casein was measured by Cherenkov counting. Obtained data were normalized towards their respective DMSO control reactions. Dose-response analyses were performed using GraphPad Prism 6, curves are shown as mean, error bars represent the standard deviation (SD) (DMSO: Dimethyl sulfoxide).

Next, to analyze the role of CK1δ^{M82F} in detail IWP-2-V2 was docked into the ATP-binding sites of an apoprotein structure of wt CK1δ (pdb 4TW9) and its gatekeeper mutant structure (CK1δ^{M82F}) generated by homology modeling (Figure 12) (analysis was done in cooperation with Prof. Christian Peifer, Institute of Pharmaceutical Chemistry, Christian-Albrechts-University in Kiel). As result, the overall binding pose turned out to be quite similar. However, an analysis of detailed ligand-protein interactions regarding gatekeeper residue 82 revealed significant lipophilic π-stacking interactions towards F82 in comparison to M82 (Figure 12A). Thus, the measured high affinity of IWP compounds towards ^{M82F}CK1δ correlates with the modeled pose further indicating a valid calculated binding mode.

To characterize whether IWP-2 and IWP-2-V2 act as ATP-competitive inhibitors, *in vitro* kinase assays were performed in the presence and absence of IWP-2 or IWP-2-V2 using different concentrations of ATP, rat GST-CK1δ wild-type as enzyme, and α-casein as substrate (Figure 12B). Since the inhibitory capacity of both, IWP-2 and IWP-2-V2 was decreased at higher ATP concentrations, the mode of action could be confirmed as ATP-competitive.

In the next step, the specificity of IWP-2 to inhibit CK1δ was assessed. For this purpose, a panel of 321 eukaryotic protein kinases was assayed for their residual kinase activity in the presence of IWP-2 at a concentration of 1 μM (Figure 13). Three kinases were identified to be moderately inhibited: CK1δ (55% of residual activity), Tousled-like Kinase 2 (TLK2, 59% residual activity) and Zeta-chain-associated Protein Kinase 70 (ZAP70, 54% residual activity) (Figure 13A). No other CK1 family member nor their paralogs were inhibited to the same extent in this assay (Figure 13B). To validate the inhibitory effect of IWP-2 on TLK2 and ZAP70 *in vitro* kinase assays were performed with both kinases in the presence of different IWP-2 concentrations. However, no reduction of their kinase activity was observed in the tested range of IWP-2 concentrations (Figure 13C). These results indicate that the inhibitory effect of IWP-2 on CK1δ kinase activity is highly specific.

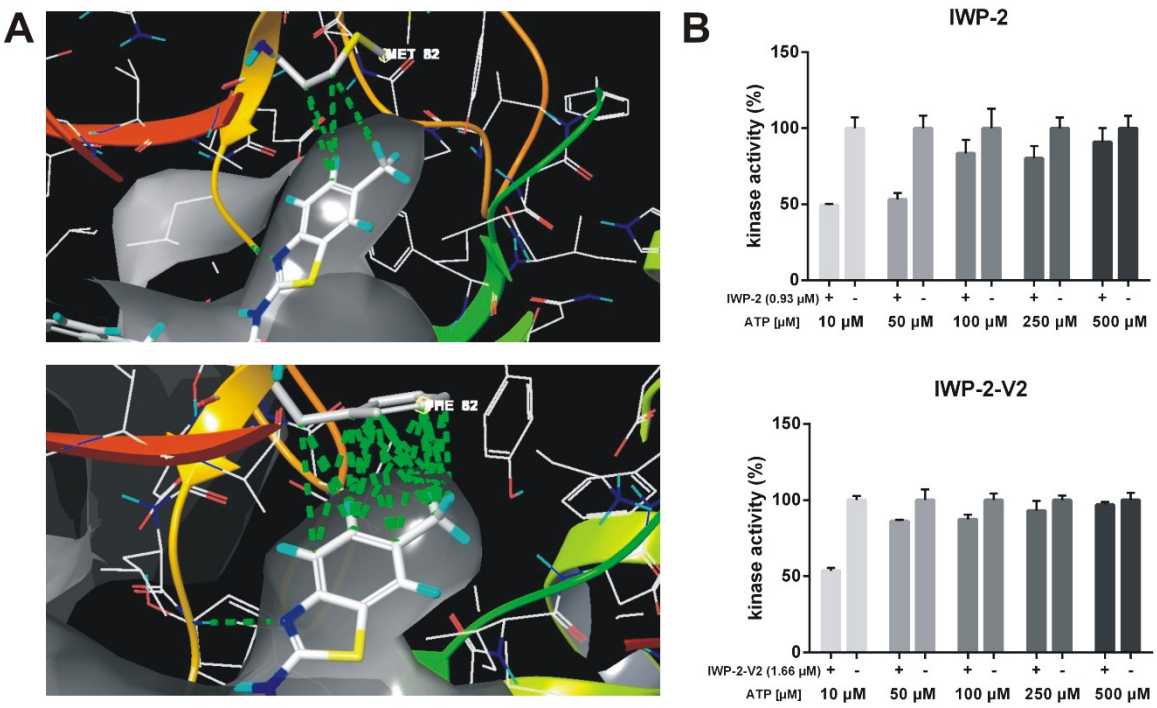


Figure 12: IWP compounds bind to the ATP-binding pocket of CK1δ and can be classified as ATP-competitive inhibitors. (A) Modeled binding modes of IWP-2-V2 in wt CK1δ (B, upper panel) and in a homology model of CK1δ^{M82F} (B, lower panel) presented as 2D LID. Protein structures are based on pdb 4TWC. Poses were determined by docking (Glide, Schrödinger) the ligands into the active site of apo structures. Key H-bond interactions of the benzthiazole moiety towards Hinge Leu 85 are shown. Focus on lipophilic interactions (green dotted lines) of the benzthiazole moiety of IWP-2-V2 towards gatekeeper residue CK1δ (Met 82) and CK1δ^{M82F} (Phe 82) in the hydrophobic pocket I. The phenylalanine residue in the mutant CK1δ^{M82F} forms significant π-stacking interactions when compared to wild type CK1δ. **(B)** Inhibitors IWP-2 and IWP-2-V2 were assayed at their respective IC_{50} concentration in the presence of the indicated ATP concentrations (10, 50, 100, 250, and 500 µM) to demonstrate their ATP-competitive mode of action. Kinase assays were performed using GST-CK1δ as enzyme and α-casein as substrate. Kinase reactions were separated by SDS-PAGE and the phosphate incorporation into α-casein was measured by Cherenkov counting. Obtained data were normalized towards their respective DMSO control reactions. Bars are shown as mean, and error bars represent the standard deviation (SD) (DMSO: Dimethyl sulfoxide).

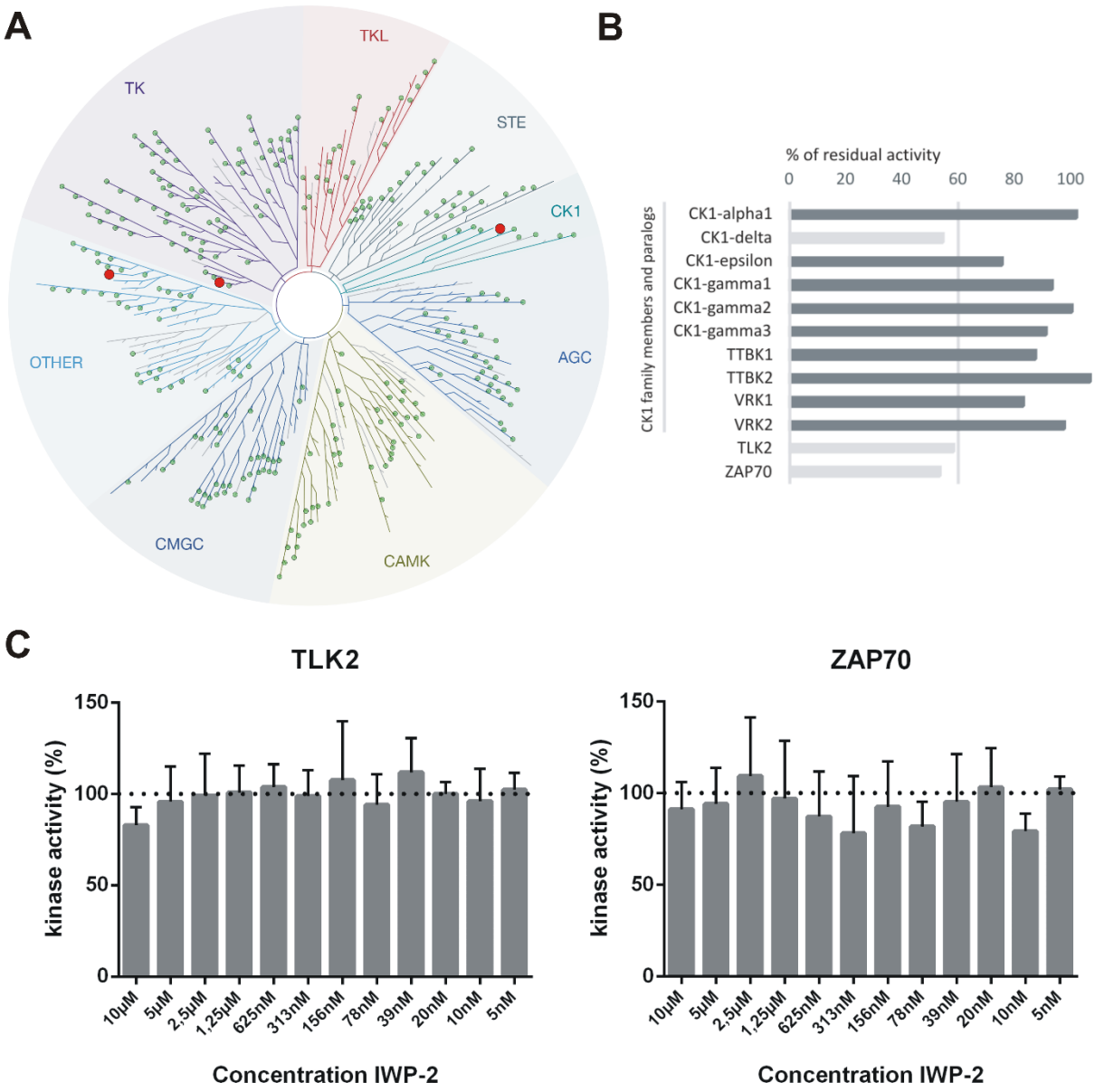


Figure 13: IWP-2 exhibits a high selectivity towards CK1δ. In order to determine target selectivity, a panel of 321 protein kinases was screened for inhibition by IWP-2 at a concentration of 1 µM. **(A)** Illustration of the phylogenetic relations of the tested kinases. Three kinases were inhibited by IWP-2, namely CK1δ, a member of the CK1 protein kinase family, ZAP70, a member of the Tyrosine-kinases (TK) family, and the Tousled-like kinase (TLK2) (shown as enlarged red spots). Image generated using TREEspot™ Software Tool and reprinted with permission from KINOMEscan™, a division of DiscoveRx Corporation, © DISCOVERX CORPORATION 2010. **(B)** Summary of the residual activity of CK1 family members, its paralogs, and the kinases ZAP70 and TLK2 against IWP-2 at 1µM. **(C)** *In vitro* kinase assays were performed using IWP-2 serial dilutions ranging from 5 nM to 10 µM against kinases TLK2 and ZAP70. α-casein was used as substrate for TLK2 and poly(L-glutamic acid-L-tyrosine) was used as substrate for ZAP70. Kinase reactions were separated by SDS-PAGE and the phosphate incorporation the substrates was measured by Cherenkov counting. Obtained data were normalized towards their respective DMSO control reactions (dotted line). Graph bars represent the mean, error bars represent the standard deviation (SD) (DMSO: Dimethyl sulfoxide). The results indicate that there is no substantial reduction of kinase activity at any tested concentration.

3.2.2 Efficacy of IWP compounds in cell culture

After assessing the *in vitro* ability of the compounds to inhibit CK1 isoforms, their ability to affect cell proliferation was analyzed. MTT assays were performed to determine the EC_{50} values of selected compounds for various established tumor cell lines. The results show a wide range of sensitivities towards different IWP derivatives (Table 15). In the case of IWP-2, EC_{50} values range from 1.90 µM to over 10 µM. The colon cancer cell line SW620 showed the lowest EC_{50} value, followed by the pancreatic cancer cell line Capan1 with an EC_{50} of 2.05 µM. The other pancreatic cancer cell lines Panc1 and MiaPaCa2 also showed increased sensitivity towards IWP-2 in comparison with the other cell lines. In contrast, the viability of the pancreatic cancer cell line A818-6 was only reduced at very high concentrations of IWP-2, with an EC_{50} value of 8.96 µM. IWP-2-V2 and IWP-3 showed less varying of results, and only inhibited cell growth at higher concentrations. In the case of IWP-4, cell growth was consistently and powerfully inhibited cell growth at low concentrations, ranging from EC_{50} values of 0.23 µM for MiaPaCa2 and Panc1 to 0.93 µM in A818-6.

Table 15: IWP-4 strongly inhibits cell proliferation in various cancer cell lines. Determination of half maximal effective concentration (EC_{50}) values of compounds IWP-2, -2-V2, -3, and -4 125 on cell viability of different cell lines, using an inhibitor serial dilution. Of all the analyzed IWP compounds, only IWP-4 consistently reduced the cell proliferation in different cell lines in the nanomolar range. Cell viability was measured using MTT assay and EC_{50} values were calculated using GraphPad Prism 6. Data is presented in micromolar (µM) concentration as mean ± standard deviation (SD) (n.d.: not determined).

Cell line	IWP-2	IWP-2-V2	IWP-3	IWP-4
A818-6	8.96 ± 2.49	>10	>10	0.93 ± 0.07
MiaPaCa2	2.47 ± 0.51	5.65 ± 0.16	5.34 ± 0.49	0.23 ± 0.01
Panc1	2.33 ± 0.22	>10	4.87 ± 1.03	0.23 ± 0.02
Panc89	3.86 ± 0.54	3.10 ± 0.70	5.07 ± 0.37	0.58 ± 0.12
HT29	4.67 ± 1.59	>10	>10	0.34 ± 0.10
HEK293	2.76 ± 0.65	n.d.	n.d.	n.d.
SW620	1.90 ± 0.28	n.d.	n.d.	n.d.
Capan	2.05 ± 0.44	n.d.	n.d.	n.d.

FACS analysis was performed using cell lines Panc1 and A818-6 to evaluate the effects of IWP-2 and IWP-4 on the cell cycle distribution. The cell lines were chosen to represent the both extremes of the range of EC_{50} values calculated for IWP-2, to observe the possible effects of the compound on cells with different sensitivities towards it (Figure 14): Panc1 growth was inhibited at relatively low concentrations, while A818-6 was only affected by IWP-2 at very high concentrations. The cell lines were treated with the respective compound for 48 h, stained, and then analyzed by flow cytometry analysis. No cell line showed significant changes in the cell cycle distribution upon any of the treatments.

An exploratory assessment of changes in gene expression pattern after IWP-2 treatment was performed using the cell line A818-6, exposed to EC_{50} concentrations of IWP-2. A panel of 17 selected genes was measured by Real Time PCR analysis. The transcribed RNA of control treated A818-6 cells was compared to the transcribed RNA of IWP-2-treated cells (Table 16). Stem cell markers SOX2, NANOG and POU5F1 (oct4) were upregulated in comparison with the control, as well as BCL2 and GLI1. The gene expression of CK1 family members α, δ, and ε was also found to be upregulated. TP53 and MDM2 were both downregulated upon IWP-2 treatment, and CTNNB1 (β-catenin) was somewhat downregulated as well.

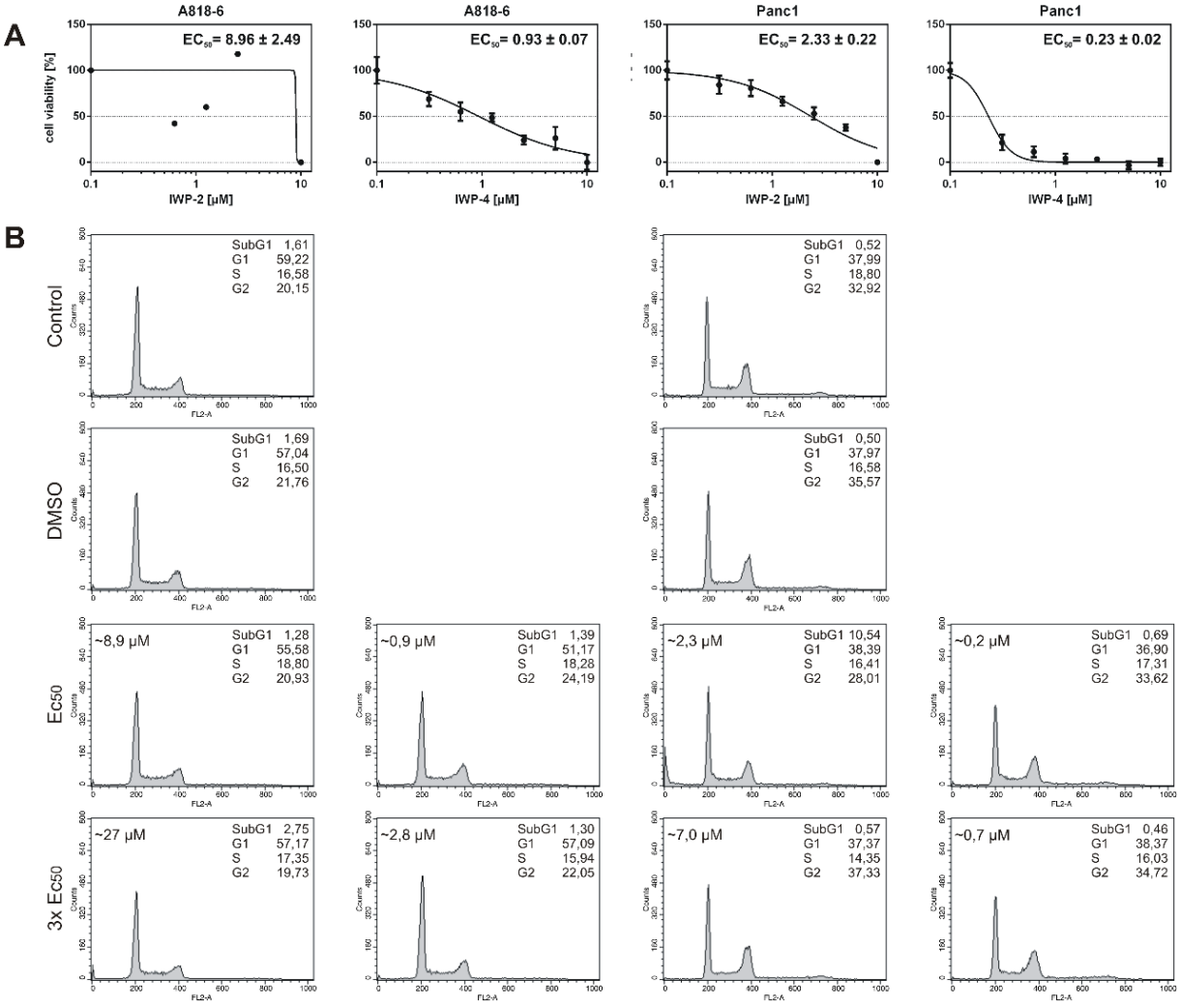


Figure 14: Activity of compounds IWP-2 and IWP-4 in cell culture. Effects of compounds IWP-2 and IWP-4 on cell viability and cell cycle distribution of A818-6 and Panc1 cells **(A)** Cells were either untreated, treated with DMSO or with the different IWPs in a range of different concentrations. MTT assays were performed 48h after treatment. **(B)** Once EC_{50} values were obtained, FACS analyses of A818-6 and Panc1 cells were performed in the presence of the calculated EC_{50} concentration and 3-fold of EC_{50} concentration of each IWP compound for 48h, then stained with propidium iodide, and analyzed in a flow cytometer using Cell Quest program. Control and DMSO-treated cells showed a normal cell cycle distribution of asynchronously proliferating cells (the same controls were used for both treatments). Treatment with IWP-2 did not cause notable changes in the cell cycle distribution (DMSO: Dimethyl sulfoxide) (all concentrations expressed as µM).

Table 16: Effect of IWP-2 on the gene expression of the cell line A818-6. A818-6 cells were treated with its previously determined EC_{50} concentrations for IWP-2 (8.96 µM) or DMSO as a control, for 48 h. Total RNA was isolated from cells and reversely transcribed to cDNA. Relative gene quantification was performed via QuantiTect Primer Assay and SYBR Green chemistry in a Roche Light Cycler 480II. Values represent the mean of relative fold changes in gene expression in respect to the gene control.

Gene	Relative change in gene expression	Gene	Relative change in gene expression
BCL2	1.37 ± 0.09	KRAS	0.98 ± 0.07
CDKN1A	0.89 ± 0.07	MDM2	0.45 ± 0.02
DYRK1B	0.63 ± 0.04	NANOG	2.53 ± 0.10
GLI1	2.18 ± 0.13	NES	0.91 ± 0.09
PITX2	1.24 ± 0.08	POU5F1	1.90 ± 0.11
SOX2	1.30 ± 0.06	CSNK1A1	1.45 ± 0.05
TP53	0.58 ± 0.02	CSNK1D	1.23 ± 0.15
CDK9	0.93 ± 0.03	CSNK1E	2.10 ± 0.27
CTNNB1	0.83 ± 0.01		

3.2.3 Development of novel IWP-2 and IWP-2-V2 derivatives

Based on the modeling results, different modifications were added to compounds IWP-2 and IWP-2-V2 in collaboration with Prof. Christian Peifer (Christian-Albrechts-University in Kiel), to obtain substances with an increased ability to inhibit CK1δ. These new compounds were termed Compound 17 (derived from IWP-2), Compound 18 (derived from IWP-2-V2), Compound 19, and Compound 20 (both derived from Compound 18). Their IC_{50} values against human CK1δ KD, rat GST-CK1δ wild type, rat GST-CK1δ^{M82F}, and human CK1ε were determined by *in vitro* kinase assays using α-casein as substrate (Table 17). A modest improvement in the inhibitory ability of the IWP derivatives on the wild-type forms of CK1δ was obtained. For example, compound 17 has an IC_{50} value of 0.66 µM (rat CK1δ wt), while IWP-2, its leading compound, shows an IC_{50} of 0.93 µM. Although the dissimilarity between the IC_{50}s of the gatekeeper mutant form remained lower with respect to the wild-type form, the differences were less marked. In comparison with the leading IWP compounds, the newly synthesized compounds showed less marked differences between the IC_{50} values of the wild type and the gatekeeper mutant form. In the case of Compound 17, it presented no improvement in its inhibition of CK1ε, preserving the specificity against CK1δ of IWP-2. Compound 18 and Compound 20 inhibited CK1ε moderately. Compound 19 inhibited CK1δ moderately better than it inhibited CK1ε. Additionally, the specificity of Compound 17 to inhibit CK1δ was assessed by calculating residual kinase activity in a panel of 321 eukaryotic protein kinases, using Compound 17 at a concentration of 1µM (Figure 15). The only kinase

inhibited in this panel was CK1δ (58% of residual activity) (Figure 15A). No other CK1 family members nor their paralogs were inhibited to the same extent in this assay (Figure 15B).

Table 17: IC_{50} values determined for novel IWP derivatives Determination of the 50% inhibitory concentration (IC_{50}) values of IWP compounds on the kinase activities of CK1δKD, CK1δ wt, CK1δ^{M82F} and CK1ε using an inhibitor serial dilution ranging from 5 nM to 10 µM, the respective kinase, and α-casein as substrate. DMSO was used as a control. IC_{50} values were calculated using GraphPad Prism 6. Values are presented in micromolar (µM) concentration as mean ± standard deviation (SD) from experiments performed in triplicate (KD: kinase domain, DMSO: Dimethyl sulfoxide) (*Experiments generated together with Ebru Karasu).

Kinase	IC_{50} value (µM)			
	Compound 17	Compound 18	Compound 19	Compound 20
CK1δ KD wt	0.40 ± 0.09	0.52 ± 0.04	0.09 ± 0.02	0.23 ± 0.02
GST-CK1δ wt	0.66 ± 0.08	1.06 ± 0.13	0.41 ± 0.02	0.76 ± 0.21
GST-CK1δ^{M82F}	0.21 ± 0.03	0.27 ± 0.02	0.09 ± 0.01	0.21 ± 0.03
CK1ε wt	4.12 ± 0.80	1.41 ± 0.29	0.56 ± 0.09	1.23 ± 0.53

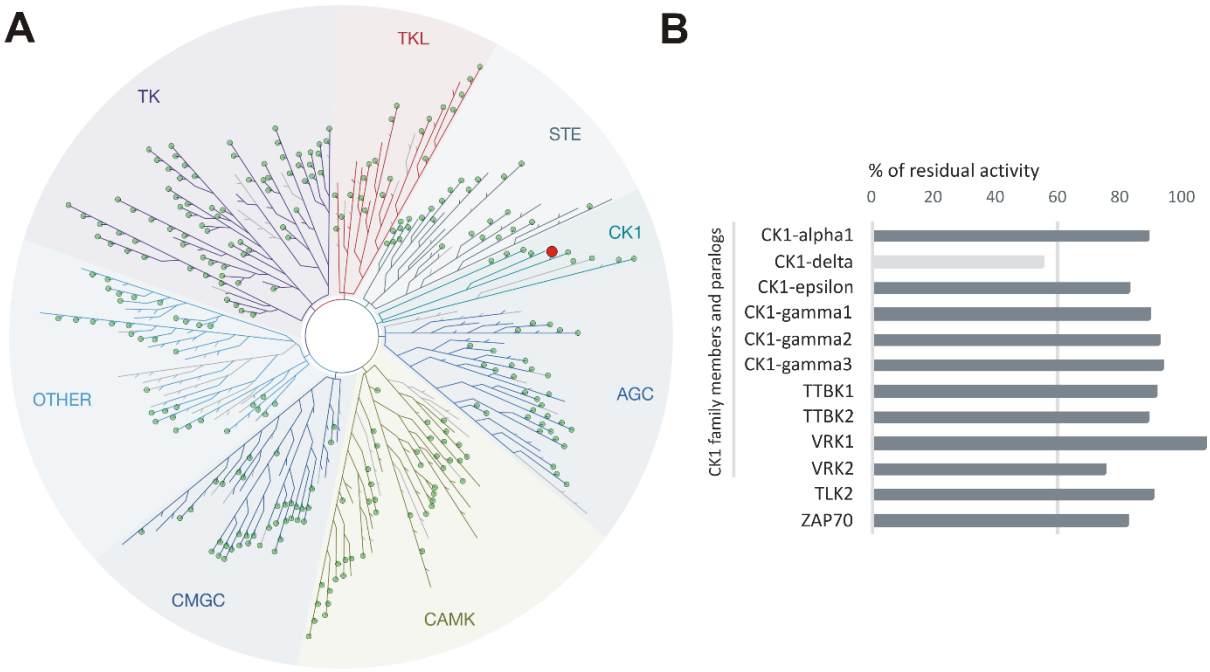


Figure 15: Compound 17 exhibits a high selectivity towards CK1δ. In order to determine target selectivity, a panel of 321 protein kinases was screened for inhibition by Compound 17 at a concentration of 1 µM. **(A)** Illustration of the phylogenetic relations of the tested kinases. Only CK1δ was inhibited by Compound 17 (shown as enlarged red spot). Image generated using TREEspot™ Software Tool and reprinted with permission from KINOMEscan™, a division of DiscoveRx Corporation, © DISCOVERX CORPORATION 2010). **(B)** Summary of the residual activity of CK1 family members, its paralogs, and the kinases ZAP70 and TLK2 against Compound 17 at 1µM.

4. Discussion

4.1 Validation of new CK1-specific small molecule inhibitor compounds

Members of the CK1 family play important roles in a wide variety of cellular pathways through their ability to phosphorylate key proteins. A prominent role of CK1 isoforms has been identified in tumorigenesis, where abnormal levels of its activity have been found (Cheong and Virshup 2011; Knippschild *et al.* 2014; Schittek and Sinnberg 2014). Deregulated activity of CK1 is frequently identified in different tumor entities, suggesting that the use of CK1-specific inhibitors could be of therapeutic value in the treatment of these diseases (Oumata *et al.* 2008; Perez *et al.* 2011; Bischof *et al.* 2012). This is exemplified by the positive results obtained by using the CK1δ inhibitor SR-3029 to reduce the growth of breast cancer in mouse models (Rosenberg *et al.* 2015). However, the establishment of clinically viable CK1 inhibitors remains challenging.

Here newly modified compounds were validated on their ability to inhibit CK1δ and to inhibit the growth of different tumor cell lines more efficiently than their previously described lead compounds described previously (Bischof *et al.* 2012; Richter *et al.* 2014). Several CK1δ/ε specific inhibitors exhibit IC_{50} values in the lower nanomolar range. As their lead compounds, these new substances act in an ATP-competitive manner. Some of these compounds were highly effective in inhibiting the growth of all analyzed tumor cell lines. These findings are further discussed in the following chapters.

4.1.1 Several compounds inhibit CK1δ and ε in the nanomolar range

Initial screenings of compounds 116-125 at 10 µM against bovine CK1α, human CK1γ3, human CK1δ kinase domain (CK1δ KD), rat CK1δ, CK1δ Transcription Variant 1 (CK1δTV1), and CK1ε showed that these compounds have retained their ability to inhibit CK1δ/ε, as it was found in the previously developed compounds (Bischof *et al.* 2012; Richter *et al.* 2014). Some of the compounds present a very strong inhibition potential, not unlike the previously described family of substances (Richter *et al.* 2014). At 10 µM concentration, none of the compounds inhibited CK1α or CK1γ3 more than 50%, except for compound 125. In contrast, most compounds reduced the activity of CK1δ and ε to very low levels. The great degree of homology between these two isoforms (Graves *et al.* 1993; Fish *et al.* 1995; Gross and Anderson 1998; Knippschild *et al.* 2005a), explains why most substances would have a similar effect on them.

The IC_{50} values of compounds 116-125 for rat GST-CK1δ wt and GST-CK1δTV1 were higher for the full-length form of CK1δ in comparison with its kinase-domain-only form.

This indicates that presence of the C-terminal region influences the kinetics of inhibition. Compounds 118, 122, 123, 124 and 125 showed IC_{50} values in a low nanomolar range with compound 118 showing the most remarkable inhibitory effects with IC_{50} values of 0.021 µM for GST-CK1δ, 0.010 µM for CK1δ KD and 0.017 µM for CK1ε. These values are comparable to those reported for the previous set of inhibitors (Richter *et al.* 2014). Established CK1δ inhibitors described in the literature have higher IC_{50} values than some of the compounds described in this work, such as CKI-7, IC261, D4476, PF-4800567, (R)-DRF053, and amino-anthraquinone analogs (see Table 3). This indicates that some of the compounds presented here are potent CK1δ inhibitors, although not all of them have the strong specificity shown by their predecessor compounds. The performed structural optimization yielded strong CK1 inhibitors, but the high specificity of the related compounds Bischof-5 and Richter-1 towards CK1δ (Bischof *et al.* 2012; Richter *et al.* 2014). was not observed. Compounds 118, 123 and 124 decreased the kinase activity of human CK1ε as potently as they decreased the activity of GST-CK1δ.

By performing kinase assays we also could demonstrate that compounds 118, 119, 122, 123, 124, and 125 inhibit the wild-type form of CK1δ and its correspondent gatekeeper mutant $CK1\delta^{M82F}$ to the same extent. This methionine 82 residue, when mutated to a bulkier phenylalanine, has been shown to affect the inhibitory ability of other compounds (Oumata *et al.* 2008; Peifer *et al.* 2009). As kinase inhibitor resistance is an important concern from a therapeutic point of view, compounds that equally inhibit mutated and wild-type kinases can be considered good candidates to develop safer and more effective drugs (Gross *et al.* 2015). Though it was not among the most potent inhibitors, compound 119 was also assayed, because the *in silico* analysis predicted that this compound would be slightly better against the mutated form (data not shown). We found that there are no remarkable differences in the inhibition level for the wild type and the mutated forms of CK1δ. Finally, The ATP-competitive properties of these newly developed compounds were confirmed, as expected by the properties already exhibited by their leading compounds

4.1.2 Compounds 118-125 inhibit the growth of various tumor cell lines

One of the challenges raised when characterizing SMIs is to prove their ability to cross the cell membrane to exert the effects they show *in vitro* (Mashhoon *et al.* 2000; Rena *et al.* 2004; Richter *et al.* 2014). Even if the compounds can permeate the cell membrane, intracellular ATP concentrations, time of binding in the ATP pocket, and drug export systems can reduce their inhibitory effect. Therefore, the validation process of any inhibitor

must include cell culture analysis. Compounds 116 and 117 were excluded due to their low capability of reducing CK1 activity *in vitro*. For the cell culture assessment, a panel of 9 cell lines was used, most of them of pancreatic cancer origin. An emphasis on pancreatic cancer cell lines is justified by the deficiency of pharmacological substances that effectively lower their proliferation, as well as the observed effects of another CK1 inhibitor, IC261, in reducing tumor growth of subcutaneous Panc-Tu1 cell xenografts in mice (Brockschmidt *et al.* 2008).

Cell line- and concentration- specific effects were observed in the cell culture characterization approach, as well as clear trends on results obtained by cell viability assays. Compounds 120, 121, 122 and 125 had little effect on cell proliferation and viability. In the case of compounds 123 and 124, low EC_{50} values were observed in most cell lines, comparable with the values for compound Richter-2 (Bischof *et al.* 2012; Richter *et al.* 2014). These compounds possibly have better permeability and therefore better intracellular availability compared with the other substances of the tested set. Compound 118 moderately inhibited cell proliferation, though not to the extent of compounds 123 and 124. These results were consistent when testing compounds 118, 123 and 124 against cell lines of non-pancreatic origin (HT29, MCF7, and REF52). The observed EC_{50} values for compounds 118, 123 and 124 are comparatively better to other established CK1 inhibitors, such as IC261, D4476, and DRF053 (Rena *et al.* 2004; Badura *et al.* 2007; Oumata *et al.* 2008). Direct comparisons on EC_{50} values obtained from different cell lines must be cautiously interpreted as the cellular background could differ, for example, the p53 status and Wnt signaling-related processes have been shown to have an important effect on the response to different treatments (Cheong and Virshup 2011; Knippschild *et al.* 2014). Also, more analysis is required to identify the possible off-target effects of these compounds. Though the precursors of the assayed inhibitors have been shown to be very selective (Bischof *et al.* 2012; Richter *et al.* 2014), the introduced changes might have changed this property, as suggested by the *in vitro* results. So far, compounds 118, 123, and 124 are potent inhibitors of cell proliferation in these cancer cell lines, but more research is required to fully understand their effect on cell growth on the molecular level.

Compounds 118, 123, and 124 were selected for further experimentation. When cell cycle distribution analysis was performed, compounds 123 and 124 showed no significant influence on the cell cycle. In contrast, compound 118 caused significant changes in the proportion of cells arrested in G2 for cell lines HT29, MCF7, AsPC1, while for cell line Panc89 the highest concentration treatment induced the death of a significant proportion of

cells. These results suggest that the compounds might act in different intracellular processes and that their inhibitory abilities have distinct consequences on cell proliferation. Members of the CK1 family of protein kinases are involved in several cellular signaling pathways, so a more sophisticated cell culture approach studying the cellular background is needed to reach more specific conclusions. Also, it would be of interest to elucidate why an SMI specifically targets certain cellular mechanisms, while a related chemical compound has a different effect on the cell.

Finally, selected gene expression patterns were assessed on different pancreatic cancer cell lines treated with compounds 118, 123, 124 and 125. Interesting trends were found, as well as different effects among the compounds. Again, compound 118 showed the most conspicuous results in comparison with compounds 123, 124 and 125. Upregulation of stem cell marker genes such as SOX2, NANOG and POU5F1 was observed for compound 118. This could be caused by the survival of cancer stem cells (CSC) to the treatment of compound 118: while other cells die in the presence of this compound, CSCs are enriched in the cell population, leading to an apparent increase in the expression of these markers. This effect has also been observed in pancreatic cancer cell lines treated with gemcitabine (Quint *et al.* 2012). A study by Hou and collaborators (2013) found that the CK1-specific inhibitor D4476 in combination with other small-molecule compounds induced pluripotent stem cells from mouse somatic cells showing an increase in the expression of stem cell markers (Hou *et al.* 2013). Further experiments would be necessary to confirm the mechanisms leading to the increment of stem cell marker expression upon treatment with compound 118. Other genes upregulated by this compound were GLI1, a known key regulatory protein of the Sonic Hedgehog pathway (Knippschild *et al.* 2014), and PITX2 which is usually induced by the activation of the Wnt pathway (Briata *et al.* 2003). The upregulation of CK1 isoforms α, δ, and ε by compounds 118 and 123 is less marked than the other observed trends. More analysis would be necessary to validate the gene expression differences found, and their meaning in the context of CK1 inhibition.

The results here presented are the conclusion of a long and successful process of compound design and optimization, leading to the identification of potent inhibitors of CK1δ and ε. Taken together with the studies previously performed (Bischof *et al.* 2012; Richter *et al.* 2014), a significant improvement both in kinase inhibition and cellular availability has been obtained. The comparative study of this set of substances could yield even more interesting data on the detailed mechanisms of action within the cell, and how different but related compounds can exert distinct effects. This is of interest in the context of therapeutical use of

SMIs targeting CK1 isoforms, an aspect of great consideration since CK1 family members have proven to be interesting targets for inhibition.

4.2 IWPs as inhibitors of CK1δ

IWP compounds are a class of small molecules that target the membrane-bound O-acyltransferase (MBOAT) family which catalyzes the palmitoylation of Wnt ligands (Chen *et al.* 2009; Wang *et al.* 2013b), a crucial post-translational modification required for Wnt secretion and signaling. For this reason, IWP compounds, are routinely used as pharmacological tools to inhibit the Wnt pathway, in particular IWP-2. Because IWPs exhibit similarities to CK1-specific small-molecule inhibitors (Bischof *et al.* 2012; Richter *et al.* 2014), their ability to inhibit CK1 isoforms was analyzed. The results are discussed in detail in the following chapters.

4.2.1 IWP compounds inhibit CK1δ

Initial screenings of compounds IWP-2, IWP-2-V2, IWP-3, and IWP-4 at a concentration of 10 μM against CK1α, CK1γ3, CK1δ (different variants), and CK1ε revealed that all IWP derivatives specifically inhibit CK1δ whereas CK1α, CK1γ3, and CK1ε were less inhibited. When more detailed analyses were performed, all analyzed CK1δ variants showed IC_{50} values in the nanomolar range, similar to those described for published CK1δ-specific inhibitors (see Table 3). For example, rat GST-CK1δ showed IC_{50} values ranging from 0.93 μM to 1.89 μM while the lowest IC_{50} value for CK1ε was 4.03 μM for IWP-2. The human splice variants of CK1δ were even stronger inhibited by IWPs than their rat counterpart. Interestingly, the gatekeeper mutant rat CK1δ^{M82F} was more potently inhibited by IWP compounds than its wild type form, indicated by its low IC_{50} value which was reduced approximately 28-fold in the case of IWP-2-V2.

IWPs exert their inhibition on CK1δ in an ATP-competitive manner. This postulated mode of inhibition was validated using different approaches. The *in vitro* approach confirmed the ATP-competitive properties of IWP-2 and IWP-2-V2 since inhibitory effects decrease at increasing ATP concentrations. In the second approach, *in silico* modeling was performed, by docking IWP-2-V2 into the ATP-binding sites of the protein structure of CK1δ wt and its gatekeeper CK1δ^{M82F} mutant. The overall binding pose is similar between both CK1δ forms, showing IWP-2-V2 is hydrogen-bonded with its amide group and with the benzthiazole moiety to the main chain of the hinge region and further stabilized by numerous van-der-Waals interactions between the benzthiazole moiety and atoms of the side chain residues of

Ile23 and Leu85. This benzthiazole moiety also interacts with the "gatekeeper" residue Met82, forming additional π hydrogen bonds of the compound to phenylalanine 82. This higher affinity of IWP-2-V2 compounds towards $CK1\delta^{M82F}$ explains the observed IC_{50} values *in vitro*.

As the previous experiments establish the ability of IWP compounds to inhibit kinase activity, a wider evaluation is necessary. The kinase inhibition profile of IWP-2 was evaluated in 321 eukaryotic protein kinases. This assay gives information about the potential of compounds to affect additional kinases leading to further signaling pathway alterations. The specificity of IWP-2 for CK1δ was confirmed in this kinome-wide screen. The isoform δ was the only CK1 family member, including CK1 paralogs, that was inhibited. Besides CK1δ only two additional kinases were inhibited in the same range: TLK2 and ZAP70, but further experiments failed to confirm any inhibition of IWP-2 on these kinases. These results indicate that the inhibitory effect of IWP-2 on kinase activity might be highly specific for CK1δ.

4.2.2 Effect of IWP derivatives on cell culture

Once it is recognized that a compound has inhibitory capabilities, its permeability through cell membranes must be evaluated. For IWP compounds it is known that they do permeate the cell membrane. IWP-2 is a compound routinely used in research to block the Wnt pathway. Modern stem cell differentiation protocols that rely on temporal modulation of this pathway make use of IWP-2, for example for the generation of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (Bauwens *et al.* 2016; Piccini *et al.* 2016; Zanella and Sheikh 2016) or in the conversion of mouse embryonic stem cells (ES) to epiblast-like stem cells (ten Berge *et al.* 2011). Its ease of use in cell culture makes this small molecule a powerful tool to investigate the role of the Wnt pathway in multiple biological processes, in combination with other treatments. In this work, the effect of IWPs alone on cell proliferation was evaluated.

IWPs inhibit CK1δ *in vitro*, and they could also inhibit cell proliferation, as many other established CK1-specific inhibitors (Bischof *et al.* 2012; Richter *et al.* 2014). The effect of IWPs alone was analyzed in cell culture, in a panel of cancer cell lines of different origin. In this biological screen, it was demonstrated that IWP compounds negatively affect the proliferation of several tumor cell lines. IWP-2, IWP-2-V2, and IWP-3 inhibit the proliferation of the investigated cell lines within the lower micromolar range. Interestingly, IWP-4 consistently inhibited cell proliferation in all tested cell lines with EC_{50} values in the

low nanomolar range. For example, the EC_{50} value obtained for IWP-2 on MiaPaCa was 1.90 µM, while IWP-4 was approximately 10-fold more effective on the same cell line (EC_{50} value 0.23 µM).

These results put into a new perspective how IWP-2 is currently used in research. Chen and collaborators initially found that IWP-2 inhibits the Wnt pathway on the cell line L-Wnt-STF at an IC_{50} concentration of 27 nM, while IWP-4 inhibited the pathway with an IC_{50} of 25 nM (Chen *et al.* 2009). Furthermore, when IWPs were used at 5 µM, Wnt-dependent phosphorylation of Lrp6 and Dvl2 were also blocked. Published protocols that use IWP-2 in the production of stem cells recommend the use of this compound at a concentration of 5 µM (Piccini *et al.* 2016; Zanella and Sheikh 2016). In the literature, IWP is found to be used at a range of concentrations from 0.5 µM to 30 µM, with most studies using it at a concentration of 5 µM. At these concentrations, IWP-2 inhibits CK1δ and exerts its own effect on cell proliferation. The use of IWPs at high concentrations might show effects associated with the repression of CK1 activity, simultaneous to the inactivation of the Wnt pathway.

It is likely that most research groups perform an evaluation of the effect of IWP compounds alone on their experiments, but very few studies explicitly describe their optimization process for the use of IWP-2. Few examples of IWP-4 were found in recent literature, but its ability to greatly reduce cell proliferation suggests that its use must also be carefully evaluated for cell culture applications. Further investigations are necessary to clarify to which extent gene expression is altered due to Porcn inhibition or CK1δ kinase activity inhibition.

4.2.3 Newly developed IWP derivatives as CK1δ-specific inhibitors

IWP derivatives showing the ability to inhibit CK1δ activity represent an interesting scaffold for the further development of new CK1-specific inhibitors. In partnership with the research group of Prof. Christian Peifer (Christian-Albrechts-University in Kiel), different modifications were added to compounds IWP-2 and IWP-2-V2, to improve the inhibitory properties and specificity of IWP compounds towards CK1δ. An improvement on inhibitory abilities was obtained for the IWP-2 derivative Compound 17, which showed an IC_{50} value of 0.66 µM, while its leading compound inhibited CK1δ at an IC_{50} of 0.93 µM. Differences were observed in the ability of the newly synthesized compounds to inhibit the wild-type and gatekeeper mutant form of CK1δ, although not to the same extent as observed with IWP-2 and IWP-2-V2. Furthermore, Compound 17 retained its specificity towards CK1δ,

inhibiting CKε at a concentration comparable to IWP-2. Compounds 18, 19, and 20 show a minor improvement on CK1δ inhibition, but they also inhibit CK1ε, thus losing the specificity displayed by their leading compound IWP-2-V2. This highlights the consequences of structural modifications on the interaction between small molecule inhibitors and kinases. Comparative studies of the binding properties of this newly developed compounds could be important to understand how protein structure impacts small inhibitor activity. Finally, Compound 17 was assayed at a concentration of 1 μM against a panel of 321 eukaryotic protein kinase, to confirm its specificity. CK1δ was inhibited in this assay to 58% of its activity, while no other kinase was affected to the same extent. This confirms that Compound 17 is highly specific to CK1δ.

In summary, IWP derivatives were identified as CK1δ inhibitors and using two different approaches it was confirmed that they act in an ATP-competitive manner. The interactions of IWP-2-V2 with the ATP-binding region are increased on the gatekeeper mutant form $CK1\delta^{M82F}$. IWP-2 was shown to specifically target CK1δ in a kinome-wide screening. IWPs were used as a leading compound for the development of new specific derivatives representing interesting substances aiming for CK1 inhibition. The ability of IWP compounds to reduce tumor cell proliferation is comparable to other established CK1 inhibitors. This suggests their potential in new therapeutical approaches towards tumorigenesis. Furthermore, IWP compounds reduce tumor cell proliferation like other established CK1 inhibitors, an effect that is not always addressed in the literature. Together with the ability to inhibit CK1δ, these results point out to unaccounted effects in cellular processes that have not been considered before, as the direct effects of IWP compounds on the Wnt pathway are not limited to the inhibition of Porcn, but could also affect pathways directly related to CK1δ.

5. Summary

Casein kinase 1 (CK1) proteins phosphorylate a broad range of substrate proteins and are involved in many physiological processes such as metabolism, transcription, cell cycle progression, apoptosis, and differentiation. The interest in targeting members of the CK1 family has increased in the past years since mutations and deregulation of their activity are associated with various diseases. The development and characterization of new potent CK1-specific inhibitors might be of interest for new therapeutical concepts in pathological conditions. So far, several CK1 inhibitor compounds have been identified and some of them have already been proven their efficacy in animal models. Still, designing powerful and isoform-specific inhibitors constitutes a challenge. CK1δ inhibition was accomplished in this work through two approaches: the informed design of specific CK1 inhibitors and the repurposing of a well-known small molecule commercially available.

As a result of a long-time collaboration with an industry research partner, a new set of small molecule inhibitors was developed from previously characterized scaffolds. These compounds were initially assayed to check for their ability to selectively inhibit CK1 isoforms. Most of the assayed compounds inhibited the activity of CK1δ and CK1ε in a nanomolar range. Compounds 118, 122, 124 and 125 showed the most distinct inhibitory effects. The compounds also showed to act in an ATP-competitive manner. When assayed against a gatekeeper mutant form of CK1δ, the compound did not show differences in inhibitory ability. Compounds 118, 123 and 124 were able to affect cell viability in various tumor cell lines. Cell cycle distribution analysis yielded compound- and cell line-specific differences. Treatment with compound 118 led to an increase in the number of dead cells as well as to an increased percentage of cells being arrested in G2, whereas compounds 123 and 124 had no noticeable effects on the cell distribution. Compound 118 also had the most conspicuous effects when the expression of selected genes was measured. Stem cell markers were found to be upregulated, an effect that has been previously observed for other small-molecule compounds. In conclusion, new CK1δ-specific inhibitor compounds with nanomolar inhibitory activity on tumor cell proliferation were successfully characterized in the first part of the study.

In the second part of this work, Inhibitors of Wnt Production (IWP) compounds were, for the first time, characterized as potent CK1δ inhibitors. These compounds were initially identified in a synthetic chemical library screening, in which Wnt signaling inhibitors were searched. IWPs target the N-palmitoyltransferase Porcupine (Porcn), a membrane-bound O-acyltransferases (MBOAT) catalyzing the palmitoylation of Wnt ligands. When Porcn is

inactivated, Wnt3A is retained in the endoplasmic reticulum resulting in inhibition of the Wnt pathway. Since these findings were published, IWP-2 became one of the most powerful and easiest ways to inhibit the Wnt pathway in cell culture. However, IWPs share structural similarities with CK1 inhibitors, so it was hypothesized that they could inhibit CK1 isoforms. Commercially-available IWP derivatives were initially assayed to check for their ability to selectively inhibit CK1 isoforms. They inhibited the activity of CK1δ, while only moderately affecting CK1ε. Interestingly, IWPs potently inhibit the gatekeeper mutant CK1δ^{M82F}. *In vitro* and *in silico* data revealed that IWPs act in an ATP-competitive manner, binding to the kinase's ATP pocket and establishing interactions with the gatekeeper residue. IWP-2 was assayed in a panel of 321 eukaryotic kinases, revealing a high specificity towards CK1δ. IWP-4 was found to be a powerful inhibitor of cell proliferation in various cancer cell lines. IWP-2 was also able to inhibit cell growth at concentrations lower than those used currently in research. However, IWP-2 did not cause noticeable changes in the cell cycle distribution. Selected gene expression patterns show that stem cell markers are upregulated upon IWP-2 treatment. Also, IWP-2 and IWP-2-V2 were used as scaffolds for further drug design, towards improving their ability to inhibit CK1δ. This was partially accomplished with Compound 17, a novel derivative of IWP-2 obtained with the collaboration of our research partners. Compound 17 showed moderately better IC_{50} values in comparison with its leading compound while retaining its specificity. This was confirmed by a panel of 321 eukaryotic kinases, where Compound 17 could only reduce the activity of CK1δ and no other assayed kinase. These results put into a new perspective the use of IWPs in research since they suggest that the direct effects of IWPs on the Wnt pathway are not limited to the inhibition of Porcupine, but also affect pathways directly related to CK1. Studying inhibitor compounds provides new insights into the mechanisms of kinase activity and highlights the potential of small molecule inhibitors for future drug development.

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Acknowledgements

The Acknowledgements Section was removed for data privacy protection reasons

List of Publications

García-Reyes B, Witt L, Karasu E, Gehring T, Trübenbach J, Leban J, Henne-Bruns D, Pichlo C, Brunstein E, Baumann U, Peifer C, Knippschild U. Discovery of IWP-2 and related compounds as selective ATP-competitive inhibitors of CK1δ (Submitted)

Wolff S*****, **García-Reyes B***, Henne-Bruns D, Bischof J, Knippschild U (2015). Protein kinase CK1 interacts with and phosphorylates RanBPM in vitro. J Mol Biochem 4:1 (*****authors with equal contribution).

Richter J, Bischof J, Zaja M, Kohlhof H, Othersen O, Vitt D, Alscher V, Pospiech I, **García-Reyes B**, Berg S, Leban J, Knippschild U (2015). Difluoro-dioxolo-benzoimidazol-benzamides as potent inhibitors of CK1delta and epsilon with nanomolar inhibitory activity on cancer cell proliferation. J Med Chem 57: 7933-7946.

Knippschild U, Krueger M, Richter J, Xu P, **García-Reyes B**, Peifer C, Halekotte J, Bakulev V, Bischof J (2014). The CK1 family: contribution to cellular stress response and its role in carcinogenesis. Front Oncol 4(96).