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ATM, ATR, CHK1, CHK2 and WEE1 inhibitors in cancer and cancer stem cells†

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DNA inevitably undergoes a high number of damages throughout the cell cycle. To preserve the integrity of the genome, cells have developed a complex enzymatic machinery aimed at sensing and repairing DNA lesions, pausing the cell cycle to provide more time to repair, or induce apoptosis if damages are too severe. This so-called DNA-damage response (DDR) is yet considered as a major source of resistance to DNA-damaging treatments in oncology. Recently, it has been hypothesized that cancer stem cells (CSC), a sub-population of cancer cells particularly resistant and with tumour-initiating ability, allow tumour regrowth and cancer relapse. Therefore, DDR appears as a relevant target to sensitize cancer cells and cancer stem cells to classical radio- and chemotherapies as well as to overcome resistances. Moreover, the concept of synthetic lethality could be particularly efficiently exploited in DDR. Five kinases play pivotal roles in the DDR: ATM, ATR, CHK1, CHK2 and WEE1. Herein, we review the drugs targeting these proteins and the inhibitors used in the specific case of CSC. We also suggest molecules that may be of interest for preclinical and clinical researchers studying checkpoint inhibition to sensitize cancer and cancer stem cells to DNA-damaging treatments.

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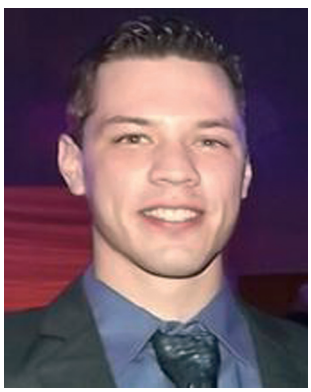
1. Introduction

DNA is under the constant assault of exogenous (UV-light exposure, irradiation or chemicals) and endogenous factors such as free radicals and alkylating agents naturally occurring during metabolic processes. This ensues damages, estimated at up to 10^5 lesions per cell per day, that may evolve



Cyril Ronco

Cyril Ronco received his PhD on cholinesterases ligands under the supervision of Prof. P.-Y. Renard at the University of Rouen. He was then awarded with a Humboldt postdoctoral fellowship to work on the total synthesis of complex natural products in Prof. H.-D. Arndt group at Jena University, Germany. After working on medicinal chemistry projects in Prof. Déprez's group (University of Lille), he joined in 2014 the group of Dr. Benhida as "Maître de Conférences" at the University of Nice Sophia Antipolis. His research focuses currently on the development of bioactive molecules to circumvent resistance mechanisms in the field of oncology.

Anthony R. Martin

Anthony R. Martin received his PhD from the University of Montpellier in 2011 under the supervision of Prof. Michael Smietana and Dr. Jean-Jacques Vasseur, working on the synthesis and self-assembly of borono (oligo)nucleotides. He then joined Prof. Steven Nolan's group (Saint-Andrews University) as a postdoctoral researcher to work on late transition metal coordination and their applications in homogeneous catalysis. In 2013, he moved to the Institute of Chemistry of Nice in Dr. Benhida's group where he was appointed Chargé de Recherche—CNRS in 2014. His researches focus on the design of new chemotherapeutics and encompass various aspects of nucleic acid chemistry.

into transcription and replication errors and ultimately lead to cell death or gene mutation if not repaired or mis-repaired.[1] Briefly, the two main DNA damage types encountered are: (i) double-strand breaks (DSB), which are considered as the most severe, and which are repaired through two different pathways, namely the non-homologous end joining (NHEJ) and the homologous recombination (HR);[2,3] (ii) single-strand breaks (SSB), a specific type of lesion occurring at stalled replication forks, but also a common intermediate formed during DSB repair. Therefore, to maintain genomic integrity, cells have developed throughout evolution a complex machinery called DNA-damage response (DDR) that senses and repairs DNA.[4] DDR consists in a set of responses with different groups of enzymes dedicated to specific types of lesions that can be classified into sensors, transducers and effectors (Fig. 1).[5] Together, they form a complex network of interconnected pathways, whose collaborative work allows the preservation of the genome integrity by initiating cell cycle arrest, repair processes and apoptosis induction (Fig. 1). Depending on the type of lesion, different pathways are involved. DSB are rapidly sensed by the Mre11–Rad50–NBS1 (MNR) complex. This ternary complex interacts with chromatin, and subsequently promotes the activation of Ataxia Telangiectasia Mutated (ATM) kinase by autophosphorylation. ATM relays the signal to a plethora of transducer enzymes, including Checkpoint kinase 2 (CHK2) and the transcription factor p53. SSB are sensed by the Rad9–Hus1–Rad1 complex. This complex, in cooperation with Rad17, Rfc2, Rfc3, Rfc4 and Rfc5 activates Ataxia Telangiectasia and Rad3-related kinase (ATR). The latter enzyme is directed by its subunit ATR interacting protein (ATRIP) to RPA (replication protein A) coated single-stranded DNA. Following this sensing step, Rad9 binds its partner protein TopBP1, which results in the stimulation of ATR-mediated CHK1 phosphorylation. CHK1 and CHK2 amplify the signals from the sensors, phosphorylating a variety of effectors. Depending on the severity of the damage, cells either transiently arrest cell cycle progression or enter the cell death pathway (apoptosis).

Despite the emergence of targeted therapy agents, DNA-damaging therapies are still among the most common cancer treatments. Their use relies on the fact that cancer cells are cycling more rapidly than healthy cells, and while they are associated with severe side-effects on normal tissues, they remain standard treatments for many cancers. DNA repair and checkpoint activation provide an important mean to survive DNA damages caused by irradiation or chemotherapeutics. It ensures the DNA damage repair and provides more time for this by pausing the cell cycle. DNA repair and particularly the checkpoint pathway activation are commonly admitted to play an important role in both radio- and chemoresistance.[1,6] Indeed, the repeated exposure to DNA-damaging agents after many cycles of chemotherapy causes cancer cells to enhance their DNA repair systems.[7] Therefore, targeting the checkpoint response by inhibiting some of its mains components may improve the global therapeutic efficacy of DNA damaging treatments and overcome resistance. Particularly interesting in this field is the concept of synthetic lethality which exploits the genetic defects which render cancer cells dependent on only one DNA damage response system.[8] For example, loss of the tumour suppressor p53 abolished the G1/S cell cycle checkpoint rendering cancer cells dependent on a functional G2–M arrest. Synthetic lethality exploits this weakness by inactivating the G2–M arrest in p53-deficient cancer cells.[9]

Herein, we review the inhibitors of five of the key regulators of the cell cycle checkpoints in cancer cells and in the

Luc Demange

Luc Demange is a medicinal chemist, graduated from "Ecole Nationale Supérieure de Chimie de Paris", who completed his PhD working on peptidyl-prolyl isomerases inhibitors (CEA/Saclay, 2001). He was post-doctoral fellow in Sherbrooke (synthesis of cardiac glycosides) then in Montpellier (design of growth hormone secretagogues). In 2006, he was recruited as professor assistant (Paris Descartes University), and he developed with Prof. Christiane Garbay CDKs-inhibitors and anti-angiogenic drugs with applications in cancer. In 2013, LD moved to the "Institut de Chimie de Nice" where his research interests are focused on the synthesis of new therapeutic agents to overcome drug resistances.

Rachid Benhida

Rachid Benhida is DR CNRS, Deputy-Director of the ICN Institute and leader of Bioactive Molecules team. He received his Ph. D. from the University-Paris XI (1992). After three years post-doc (Hoechst/Roussel-Uclaf, Germany and "Institut Curie-Paris", 1994), he joined the "Institut de Chimie des Substances Naturelles" as "ANRS young research award" and as CNRS researcher in the same year (1995). He moved to the University of Nice in 2002. His current researches focus on the development of synthetic methods and their applications in nucleic acid chemistry, chemical biology and the development of new strategies to overcome drug resistance using small molecules (specific targeting of nucleic acids and CSCs, identification and validation of new targets, etc.).

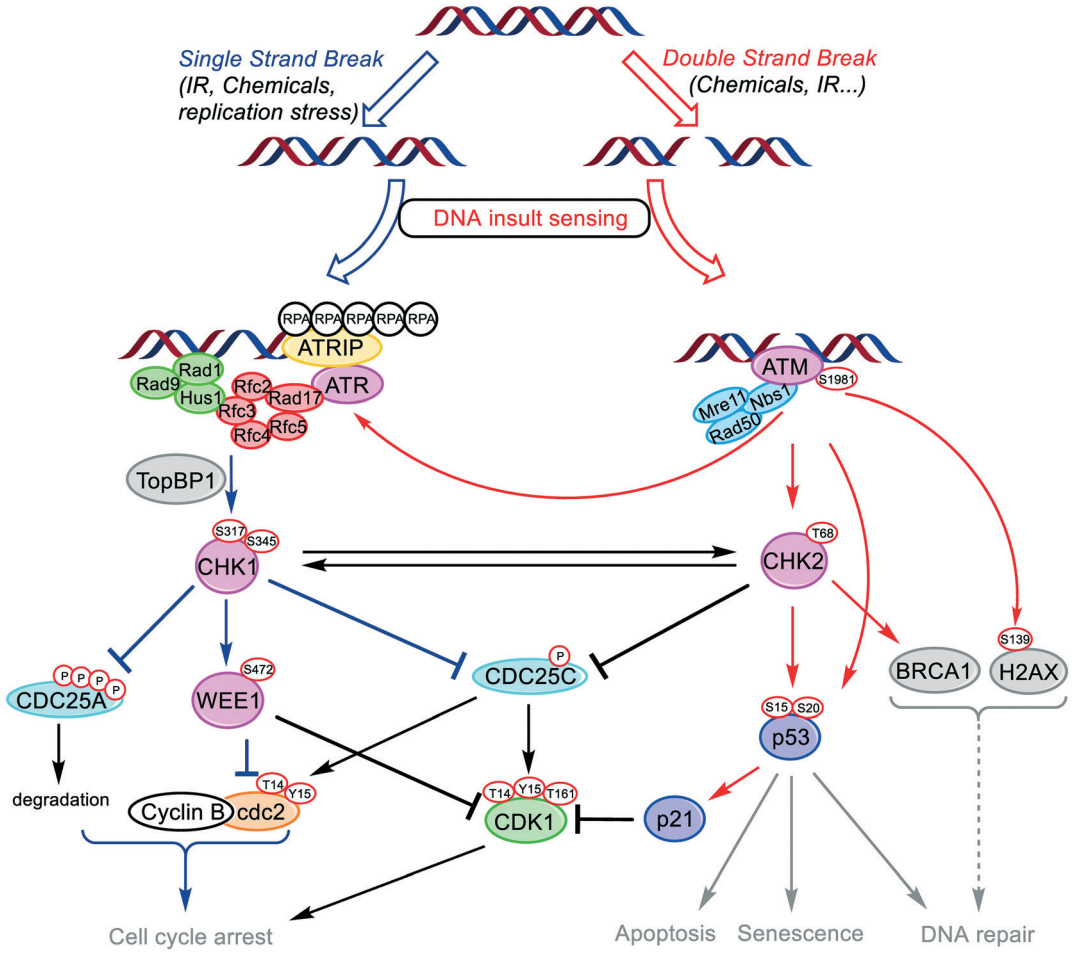


Fig. 1 Components of the DNA damage response pathways modulated by ATM, ATR, CHK1, CHK2 and WEE1 kinases.

particular settings of cancer stem cells: ATM and ATR, kinases that play apical roles in DDR; CHK1 and CHK2 kinases, respectively activated by ATR and ATM, that are central transducers towards cell cycle arrest, DNA repair and apoptotic pathways; and WEE1, which is a downstream effector of CHK1 and a key regulator of cell cycle progression.

1.1. Ataxia telangiectasia mutated (ATM)

ATM is a large 350 kDa serine/threonine kinase belonging to the phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKK), a family of 6 highly conserved enzymes playing pivotal roles in controlling cell homeostasis, including DDR (for ATM, ATR and DNAPKcs), cell growth (for mTOR), mRNA decay (for SMG1) and transcriptional regulation (for TRRAP).[10] ATM is present in all tissues, and plays a pivotal role in DDR. The kinase is recruited and activated by DSB, and initiates the DNA checkpoint response and promotes the repair of broken chromosomes by either NHEJ (non homologous end joining) or HR (homologous recombination).[2,3] ATM is not an essential protein under normal cell functioning[11] whereas it plays an essential role for cell survival after ionizing radiation (IR). Under normal conditions, the enzyme is kept inactive under the form of a homodimer.[3] In the presence of DSB, sensed by the MNR protein complex, ATM homodimers undergo a rapid autophosphorylation on Ser1981 and split into active monomers.[12] The signal transduction is provided through the activation of a plethora of enzymes,[13] and notably the important transducer CHK2 (Fig. 1). In addition to checkpoint kinases activation, ATM is the principal kinase for the phosphorylation of the breast cancer associated gene 1 (BRCA1) and p53, respectively leading to DNA repair and cell fate decision.[14] Of note, cytoplasmic ATM also plays a role in insulin signaling and glucose homeostasis, which should be taken into account when developing ATM inhibitors.[15] ATM inhibition, even transitory,[16] has been reported to efficiently enhance sensitivity of cancer cells to both IR and DNA damaging agents[17] whereas it proves less harmful for normal cells. In light of these results, ATM inhibition appears as an attractive approach for anticancer chemo- and radiosensitization, as well as to overcome resistance phenomena.[2,18] Therefore, ATM seems a relevant target for drug development.

1.2. Ataxia telangiectasia and Rad3-related (ATR)

ATR is another member of the PIKK family and one of the central kinases involved in the DDR. ATR plays an important

role in the enforcement of cell cycle checkpoints, particularly at intra-S-phase, both during normal progression and in response to DNA damage. ATR is activated in response to persistent SSB. Specifically, ATR in complex with ATRIP is attracted by long stretches of RPA-coated single-stranded DNA. Subsequently, ATR get activated by two different ways: through the canonical Rad17 pathway or alternatively in a Nbs1-dependent manner (non-canonical pathway).[19] Contrary to ATM, ATR is a necessary enzyme for cell survival, and its depletion leads to embryonic lethality in mouse and cell death in human cells.[20–23] ATR is generally considered to preserve genome integrity by phosphorylating a number of enzymes involved in DNA synthesis at replication forks, resulting in nucleotide levels regulation, fork progression, cell-cycle progression and DNA repair mechanisms activation. Many of ATR functions are mediated through its downstream target CHK1.[24] Like ATM, ATR inhibition affects both DNA checkpoint response and impairs global DSB repair, therefore enhancing the efficacy of IR and DNA-damaging drugs treatments. The principle of synthetic lethality can also be applied to ATR inhibition, as P53- or ATM-defective cells can only rely on ATR promoted cell cycle checkpoints to repair DNA. ATR inhibition leads selectively in such cells to an accumulation of DNA defects that result in mitotic catastrophe.[25,26] This makes ATR an attractive target for the development of molecular inhibitors to circumvent resistance to radio- and chemotherapies.

1.3. Checkpoint kinase 1 (CHK1)

CHK1 is a 54.4 kDa serine/threonine-specific protein kinase that plays, with CHK2, a pivotal role in maintaining DNA integrity. CHK1 is a substrate of ATR and its activation is therefore particularly important in the response to SSB sensing. The existence of a close crosstalk between CHK1 and CHK2 (substrate of ATM) has been evidenced and a significant overlap in both their activation and their substrates has been reported.[27] However, CHK1 seems to be an essential enzyme in contrast to CHK2, as $CHK1^{-/-}$ null mice embryos are not viable, whereas their $CHK2^{-/-}$ null pendant show normal development.[28] CHK1 activation results in the initiation of cell cycle checkpoints, later phase S and G2/M cell cycle arrest, DNA repair and possibly cell death. More precisely, it mediates the degradation of cell-division cycle 25A (CDC25A) phosphatase *via* polyphosphorylation on its serine residues, which in turn slows down the progression of DNA replication through the S-phase and provides time for resolution of the source of stress.[24] Moreover, CHK1 phosphorylates CDC25C, preventing cyclin-dependent kinase 1 (CDK1) activation, and stopping the cell cycle in the G2 phase. siRNA and knockdown experiments tend to show that CHK1 plays a more prominent role than CHK2. Because of the crosstalk between CHK1 and CHK2, the latter has been proposed to act in some cases rather as a backup kinase when CHK1 is non-functional.[29] Indeed, simultaneous knockdown of CHK1 and CHK2 did not improve DNA-damaging treatments efficacy compared with CHK1-knockdown alone.[30] However, the role of inhibiting simultaneously CHK1 and CHK2 over CHK1 alone is not yet clearly established. About 50% of human cancers display deficient p53 by mutation or inactivation.[31] These cancer cells cannot activate G1/S checkpoint and only rely on S and G2/M checkpoints controlled by CHK1. Selectivity towards these p53-deficient cancer cells can therefore be afforded by combination of CHK1 inhibition and DNA-damaging treatment, following the principle of synthetic lethality.[32]

1.4. Checkpoint kinase 2 (CHK2)

CHK2 is a 60.9 kDa serine/threonine kinase whose active site is structurally similar to those of CHK1. CHK2 is the second effector of the checkpoint response activation and it also plays a pivotal role in eliciting DNA repair, cell cycle arrest or apoptosis in the response to DNA damage. Mechanistically, it becomes activated by ATM through phosphorylation at residue Thr68 which induces CHK2 dimerization and autophosphorylation of the kinase domain.[33] Subsequently, CHK2 promotes the cell cycle arrest by phosphorylation of downstream targets including CDC25 phosphatases, responsible for dephosphorylation and activation of the CDK. As a downstream kinase of ATM, CHK2 is particularly important in the response to DSB. CHK2 also stimulates the repair of DSB through BRCA1 mediated processes. Moreover, CHK2 activates the transcription factor p53, which results in the cell cycle arrest through p21 or leads to apoptosis or senescence depending on damage severity.[27] Of note, the activation of p21 can also play a role in DNA repair by interacting with proliferating cell nuclear antigen (PCNA).[34] As a downstream kinase of ATM, CHK2 is a logical target for the development of radio- or chemosensitizers. Therefore, several small-sized inhibitors[35] have been proposed to precisely decipher its role in cell cycle arrest and tumourigenesis, and ultimately to validate its relevance as a target in oncology. Nevertheless, the advantage of using a dual CHK1/CHK2 inhibition over a CHK2 selective inhibition remains to be evaluated; thus, specific CHK2 inhibitors are under development.

1.5. WEE1

WEE1 is a 96 kDa dual-specific kinase which plays an important role in cell cycle progression, by phosphorylating CDK1 at tyrosine 15, a key modification that blocks G2–M transition.[36] WEE1 is activated by several enzymes including CHK1, chaperone protein Hsp90 and PP2A phosphatase in response to DNA damage accumulation.[37] WEE1 lengthens the G2 phase by controlling the activity of CDK1, thus allowing the DDR machinery additional time for DNA repair. During S and G2 phases, CDK1 is maintained inactivated by WEE1 phosphorylation at two different sites, Tyr15 and Thr14.[38] When DNA damage is repaired, WEE1 activity decreases while the inhibitory tyrosine 15 modification of CDK1 is removed by Cdc25 phosphatase. This leads to CDK1 reactivation and promotes entry into mitosis. Therefore, WEE1

can be seen as the gatekeeper of G2-arrest and acts as an inhibitor of mitosis.[39] This enzyme is essential for the development of mammals as WEE1 depletion leads to growth defects and cell death resulting from DNA damage. Indeed, WEE1$^{-/-}$ knockout (KO) mice are unable to survive after the 4th day of embryonic stage. WEE1 is overexpressed in many cancer lines, including breast cancer (35% of those),[40] glioma,[41,42] glioblastoma,[37] nasopharyngeal carcinoma (NPC),[41] as well as drug-resistant cancer cells.[43] Moreover, WEE1$^{-/-}$ KO reduces the viability of breast cancer cells, but not normal mammary epithelial cells.[44] This drew biochemists' attention on the inhibition of this enzyme to push cancer cells into mitotic catastrophe. Preclinical studies with cancer cells and animal models showed diminished cancer cell viability, reduced tumour growth and burden, and improved survival after WEE1 inhibition by siRNA or small-sized organic inhibitors. Moreover, their combination with conventional DNA damaging methods enhances these anti-cancer activities, thus sensitizing cancer cells to conventional therapy. As WEE1 is considered as one of the main gatekeepers of the G2 cell-cycle checkpoint, its inhibition is particularly effective in cells inherently presenting G1–S transition defects, typically those with deficient p53 signalling. Indeed, as p53 deficient cells present weakened G1/S DDR, Wee1 inhibition, that targets the G2/M DDR will act as combination therapy. It is noteworthy that the combined inhibition of WEE1 and CHK1/2 induces a synergistic decrease of cell viability and an increase of apoptosis in mouse xenograft models using melanoma cells from a patient.[45] Thus WEE1 inhibition has been considered as an effective sensitizer in combination with DNA-damaging therapy[46] and is a potential therapeutic target for radiosensitization of adult glioma[37] and various types of cancers.[47–49]

In this context, the five pivotal kinases introduced above appear as particularly relevant targets to develop therapeutic agents able to modulate the DNA checkpoint response in cancer cells.

2. Chemical inhibitors of ATM, ATR, CHK1, CHK2 and WEE1

2.1. ATM inhibitors

In parallel to ATM drug target validation, the development of ATM inhibitors has arisen these last 15 years, passing from early non-specific compounds to highly selective inhibitors that entered in pre-clinical studies (Fig. 2). Their main properties are listed in Table 1.

Wortmannin. Historically, several methylxanthine-derived drugs (theophylline, pentoxifyllin and caffeine) were reported to sensitize cells to radiations at low millimolar concentrations. However, the fungal metabolite **wortmannin** was the first compound proposed to target ATM.[50] Its radiosensitizing properties are attributed to an irreversible inhibition of several members of the PIKK family, including mTOR, DNA-PKcs, ATM (IC_{50} on isolated immune complexes ranging between 16 nM and 150 nM), as well as ATR to a lesser extent (IC_{50} = 1.8 μM). However, high dosages (≥10 μM) are necessary to obtain radiosensitizating effects in A549 lung cancer cells. This is two orders of magnitude higher than the dose required for PIKK inhibition. Therefore, the contribution of PIKK inhibition in the radiosensitizing activity remains unknown. Moreover, the intrinsic systemic toxicity relative to

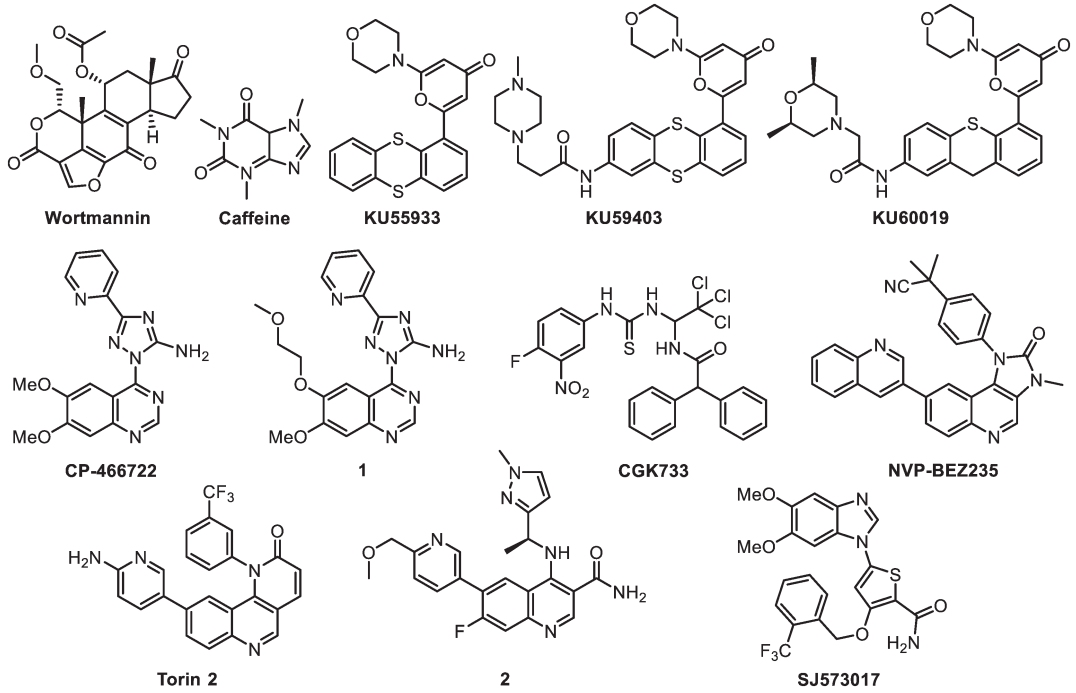


Fig. 2 ATM inhibitors.

Table 1 ATM inhibitors and their main properties

Compound	Enzymatic activity	*In vitro* selectivity	*In vitro* efficacy	Pharmacology	*In vivo* activity	Development stage
Wortmannin	IC_{50} = 150 nM (irreversible)	−	R+/−	− (toxicity)	N.D.	Discontinued at optimisation stage
Caffeine	IC_{50} = 0.2 mM	−	R+/−	− (toxicity)	N.D.	Discontinued at optimisation stage
KU55933	IC_{50} = 12.9 nM K_i = 2.2 nM	+	R+/− C++	− (solubility, distribution)	N.D.	Discontinued at research stage
KU59403	IC_{50} = 3 nM	++	C+	+	+	Preclinical stage
KU60019	IC_{50} = 6.3 nM	++	R+ C+	+	+	Preclinical stage
CP-466722	IC_{50} = 20 nM	+	M+ R+	− (stability)	N.D.	Research stage
1	N.D.	++	R+	+	N.D.	Research stage
CGK733	N.D.	−	M+/−	N.D.	N.D.	Optimisation stage
NVP-BEZ235	N.D.	−	M+ R++ C+	+/−	+	Clinical trials[a] phase II
Torin-2	IC_{50} = 28 nM	−	M− R+ C++	+	+	Preclinical stage
2	IC_{50} = 0.6 nM	++	C++	+	++	Preclinical stage
SJ573017	IC_{50} = 0.48 μM	−	M++ R+ C+	N.D.	N.D.	Optimisation stage

Selectivity: "−": non-selective (<2 fold); "+/−": moderately selective (2–50 fold); "+": selective (50–100 fold); "++": very selective (>1000 fold). ***In vitro* efficacy**: "M": monotherapy, as a single agent; "R": radiosensitizer; "C": chemosensitizer; "−": inefficient (IC_{50} > 30 μM as single agent or sensitization ratio < 1.2 fold); "+/−": moderately active (2 μM < IC_{50} < 30 μM as single agent or sensitization ratio: 1.2–2 fold); "+": active (100 nM < IC_{50} < 2 μM as a single agent or sensitization ratio: 2–10 fold); "++": very active (IC_{50} < 100 nM as single agent or sensitization ratio > 10 fold). **Pharmacology**: "−": poor, main issues in brackets; "+/−": moderate, main issues in brackets; "+": good. ***In vivo* activity**: "+/−": moderate tumour growth inhibition; "+": significant tumour growth inhibition; "++": tumour volume reduction. **Development stage**: "optimisation stage": *in vitro* properties to be optimised; "research stage": *in vitro* properties validated; "preclinical stage": *in vitro* and *in vivo* properties validated. N.D.: not determined. [a] All the clinical data presented in this review were retrieved on https://clinicaltrials.gov website.

covalent inhibitors (LD_{50} (mice) = 1 mg kg^{-1})[51] and the adverse side effects associated with the administration of **wortmannin** limited its utility. Of note, the use of **wortmannin** was revisited in 2012 with a nanoparticle drug delivery approach which helped to significantly reduce its toxicity.[52]

Caffeine. **Caffeine** was largely studied and used as radiosensitizing agent although its exact mechanism of action remains unclear. Its structure suggests that it might act at high dosage as a broad-spectrum kinase inhibitor; with probable inhibition of the phosphotransferase activity of a protein kinase involved in checkpoint signalling of DNA damaged cells. Inhibition of ATM and ATR activities were later reported at doses similar to radiosensitization (around 1 mM).[53] Nevertheless, the required dose to inhibit ATM activity (IC_{50} of 0.2 mM) is close to its LD_{50} *in vivo* and would be far too toxic to permit any use in animals.

KU55933. Since **caffeine** and **wortmannin** are neither specific nor useful *in vivo*, new ATM inhibitors have emerged. Since 1999, Smith's group is developing competitive specific inhibitors of DNA-PKcs and ATM kinases. From an initial screening, the 2-morpholino-8-phenyl-4*H*-chromen-4-one (LY294002) was identified as a hit, and this structure was optimized over the years to yield several lead compounds (Fig. 3). First, **KU55933** was obtained by substituting the chromen-4-one core by a pyran-4-one and replacing the phenyl by a thianthrene moiety. **KU55933** was the first ATM specific ATP-competitive inhibitor (IC_{50} = 13 nM) displaying selectivity over ATR, PI3K, DNA-PKcs, PI4K and m-TOR (all with IC_{50} > 2.5 μM).[17,54] At 1 μM, this compound showed slight IR sensitizing effects in HeLa cervical cancer, LoVo and SW620 colorectal cancer cell lines, while significant chemosensitizing effects were observed in combination with the marketed topoisomerase II inhibitor etoposide, at 10 μM. However, the pharmacokinetic parameters proved unfavourable, particularly the water solubility and the tissue distribution.

KU59403. **KU59403** is an upgraded version of **KU55933** in which the introduction of a 3-(4-methylpiperazin-1-yl)propanamide group led to an improvement of the pharmacokinetic parameters together with a gain of activity (IC_{50} = 3 nM) and selectivity (over 3 orders of magnitude for all other PIKK members tested). This allowed its use in animals (mice). **KU59403** was the first ATM specific inhibitor to show significant chemosensitization of campthotecin and irinotecan in mice xenografted with human colon cancer cells (HCT116 and SW620).[18]

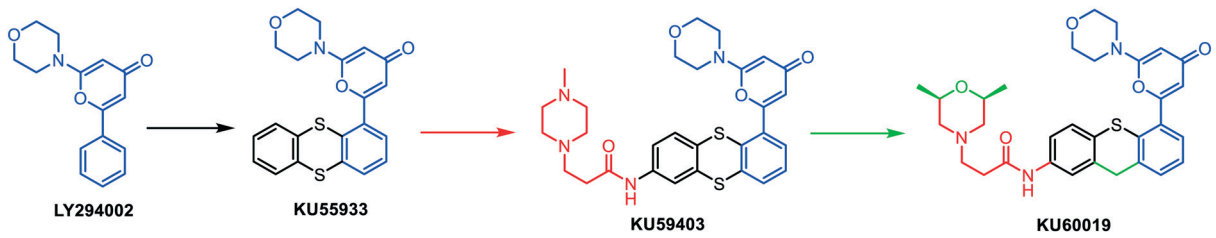


Fig. 3 Hit-to-lead optimization from **LY294002** to **KU60019**.

KU60019. **KU60019** is a variant of **KU59403** which encompasses a dimethylmorpholine and a thioxanthene group in replacement of the *N*-methylpiperazine and thianthrene moieties of the parent compound.[14,55] Compared with **KU59403**, it possesses very similar binding properties (IC_{50} = 6.3 nM), cellular activities (enhancement ratios of 2.1–2.9 at 0.6 μM) and pharmacokinetics with good drug exposition by intracranial injection. Moreover, **KU60019** proved highly selective with little to no off-target effects against a panel of 229 kinases at 1 μM, and limited toxicity towards healthy cells. Overall, the main particularity of this therapeutic agent is certainly its dual chemo- and IR-sensitization action, with submicromolar radiosensitization activity observed in glioma cells.[56] *In vivo*, **KU60019** exhibited selective anti-tumour properties against migration, invasion and cell growth, in xenografts p53-mutant glioma rodent models. These are probably due to the inhibition of prosurvival pathways. Interestingly, the combination of IR and **KU60019** promotes preferential killing of stem-like cells and specific sensitization to agents promoting DSB.[57] Efficacy and safety of **KU60019** treatments were validated in a detailed *in vivo* study that showed 2–3 fold survival increase in mice xenografted with orthotopic COMI glioblastoma cells, under IR.[58]

CP466722. **CP466722** was identified in 2008 as a new ATM inhibitor by screening a library of 1500 targeted kinase inhibitors. It proved selective over ATR, PI3K, DNA-PKcs and Abl kinase in hTERT-immortalized human fibroblasts.[16] **CP466722** leads to a rapid reversible inhibition of ATM function in cultured tissue, and presents antiproliferative effects (IC_{50} = 370 nM) as well as IR sensitizing properties similar to **KU55933**. However, *in vivo* studies could not be undertaken because of its rapid metabolic degradation ($t_{1/2}$ = 0.13 h in mice).

Methoxyquinazoline 1. In 2016, Min *et al.* up-graded the scaffold of **CP466722** by switching the C6-methoxy group by a methoxyethoxy one.[59] The resulting optimized molecule (**1**), exhibits a better metabolic stability than **CP466722** ($t_{1/2}$(microsomes) > 4 h *vs.* 0.47 h; $t_{1/2}$ (*in vivo*) = 19.8 h *vs.* 0.13 h), without significantly affecting its biological activity. Overall, this optimized compound possesses a good pharmacological profile to be evaluated *in vivo* as a radiosensitizer against non-small cell lung cancers (NSCLC).

CGK733. **CGK733**, a thiourea-containing compound was identified in 2008 as a dual ATM/ATR inhibitor[60] and tested as a cytotoxic drug in monotherapy (IC_{50} = 5–20 μM). However, this compound seems to have rather been used as a tool for studying the cellular functions of ATM/ATR than to prepare a clinical candidate. Indeed, neither binding assays nor activity assays on isolated kinase were reported. Therefore, the biological effects of **CGK733** might be due to cellular off-target activities, which remain elusive to date. Moreover, its pharmacological properties have not been reported yet.[61] Lastly, **CGK733** was reported not to inhibit ATM or ATR kinase in H460 human lung cancer cells.[62]

NVP-BEZ235. In 2011, a nanomolar non selective PI3K/mTOR/ATM/DNA-PKcs/Akt inhibitor was disclosed. Interestingly, this compound exhibits a good bioavailability, despite its apparent compact and lipophilic structure, and an ability to cross the blood brain barrier. Detailed experimentation revealed that the molecule blocks NHEJ and HR pathways, hence causing profound radiosensitization even at low doses (100 nM *vs.* 10 μM for **KU55933**). However, as described above, **NVP-BEZ235** is a multi-target compound, and the particular contribution of ATM inhibition in this activity has not been studied. Otherwise, this compound displays a strong synergy when administered in combination with IR or temozolomide (alkylating agent) in most of the treated gliomas, and afforded marked tumour volume reduction. As a consequence, **NVP-BEZ235** entered phase I/II clinical trials for the treatment of solid tumours and remains to date the only ATM inhibitor being clinically evaluated.[63–66]

Torin2. **Torin2** is a subnanomolar ATP-competitive inhibitor of mTOR (IC_{50} = 0.25 nM), also affecting ATM, ATR and DNA-PKcs with nanomolar activities (IC_{50} = 28, 35, 118 nM, respectively). Designed by lead optimization from the mTOR inhibitor Torin1 (which has no activity on ATM), it shares some structural analogy with **NVP-BEZ235**. **Torin2** exhibits nanomolar radiosensitization activities in cells (GI_{50} = 10–220 nM), encouraging pharmacokinetics and significant tumour growth inhibition in combination with the MEK inhibitor AZD6244, blocking the MAPK signalling cascade involved in cell proliferation and senescence. Of note, no tumour growth reductions were observed when these two compounds were administered alone.[67,68]

Fluoroquinoline 2. Very recently, a new 3-quinoline carboxamide derivative structurally related to **Torin2** has been described. This compound displays high cellular potency against ATM (IC_{50} = 33 nM) and excellent selectivity over ATR (IC_{50} > 19 μM) and other kinases. A good oral exposure was observed in rodents. Moreover, in combination with irinotecan, significant tumour volume reductions were observed in a SW620 colorectal cancer xenograft model.[69]

SJ573017. A new cell-based high-throughput screening (HTS) assay for ATM inhibitors was recently developed leading to the discovery of two hits. Among them, the most promising therapeutic agent is **SJ573017**. This compound possesses a submicromolar activity towards ATM (IC_{50} = 0.48 μM) but proved non-selective, particularly against PLK enzymes. Its growth inhibition activity (GI_{50} = 3–32 nM) higher than its IC_{50} on ATM is consistent with off-target effects.[70,71]

Overall, few selective ATM inhibitors have been described yet. The most interesting compounds are probably **KU59403** and **KU60019**, developed by Kudos Pharmaceuticals, and **2** developed by AstraZeneca, for which the preclinical studies have been validated. Thus, **KU60019** showed anticancer properties as a single agent whereas **KU59403** and **2** could be used as chemosensitizers. On the other hand, the improved version of **CP466722** (compound **1**) might also be interesting after *in vivo* validation. Lastly, the most advanced compound in the drug discovery process is currently the non-selective PI3K/mTOR/DNA-PKcs inhibitor **NVP-BEZ235**, currently in phase II clinical trials.

2.2. ATR inhibitors

Following the first use of non-selective PIKK inhibitors (like **caffeine** and **wortmannin**) as radiosensitizers, the availability of selective ATR competitive inhibitors was desired as tools to study its exact role in the DDR. This was accompanied with the hope to promote new therapeutically usable drugs targeting this kinase. The compounds that are reviewed herein as ATR inhibitors are represented below (Fig. 4) and their main properties are subsequently listed in Table 2.

The first molecule proposed for ATR inhibition is the natural product **schisandrin B**, isolated from *Fructus schisandrae*, and discovered by activity screening of herbal extracts. This tricyclic molecule shares structural analogy with **combretastatin A4**, a cytotoxic agent targeting the colchicine binding site of tubuline (Fig. 5). **Schisandrin B** displays moderate activity (IC_{50} = 7.25 μM) on purified ATR with some selectivity over ATM (IC_{50} of 1.74 mM), CHK1, PI3K, DNA-PKcs, and mTOR. However, it inhibits both ATM and ATR in cells, and therefore cannot be considered as a selective inhibitor. After UV exposure, **schisandrin B** exhibits ATR-dependent cytotoxic effects on cells at the dose of 30 μM. Moreover, owing to its similarity with **combretastatin A4**, it unsurprisingly acts as an anti-mitotic agent.[72] Of note, no pharmacological data have been provided for this compound, yet.

ETP46464. ETP46464 was identified in a cell-based screening assay from a PI3K/mTOR inhibitor library, and is structurally related to **NVP-BEZ235**. **ETP46464** efficiently inhibits ATR in cellular assays with an IC_{50} of 25 nM, and is selective over ATM and DNA-PKcs. However, the poor pharmacological properties reported in mice prevented its use for *in vivo* experimentation.[63]

NU6027. NU6027 was originally developed as a CDK2 inhibitor but showed low micromolar (IC_{50} = 6.7 μM) inhibition of cellular ATR activity. It impairs both G2/M arrest and HR thus increasing sensitivity to DNA-damaging agents and PARP inhibitors (Poly ADP ribose polymerase, a family of proteins mainly involved in DNA repair and apoptosis). CDK2 inhibition was also disclosed, but chemosensitization was confirmed to be due to ATR inhibition. However, the low water solubility of this compound impedes its *in vivo* evaluation.[73]

VE-821. The first ATR selective inhibitor, **VE-821**, was reported by Vertex Pharmaceuticals in 2011. **VE-821** stems from the structural optimization of a molecular hit identified by HTS. It shows low nanomolar inhibition values and good selectivity against a panel of kinases, particularly against PIKKs (ATM, DNA-PKcs, mTOR) and related PI3K; all of them having micromolar IC_{50}.[74,75] **VE-821** provided the proof of concept, supported by detailed studies on ovarian (SKOV3, OVCAR-8 cells),[76] pancreatic (PSN-1, MiaPaCa-2, PANC-1 cells)[77] and hypoxic tumour cancer cells (RKO cell line),[78] that ATR inhibition can improve the efficiency and the therapeutic index of IR but also of a set of marketed anticancer drugs with different modes of action (*e.g.* gemcitabine, temozolomide, cisplatin, topotecan or veliparib). It is worth noting that synthetic lethality was observed with this compound in ATM-, BRCA2-, XRCC3- and XRCC1-defective cells.[79] However, signs of cellular toxicity at 3 μM prompted the medicinal chemists to propose an optimized structure: **VE-822**.

VE-822. VE-822 (or **VX-970**) shares a high structural similarity with its parent compound, **VE-821**. **VE-822** displays a subnanomolar potency *in vitro* (K_i < 0.2 nM; 65-fold improvement) and a low nanomolar activity in cells (K_i = 19 nM). *In vitro*, it remains ATR-selective over ATM (>100-fold) and DNA-PKcs. Moreover, this molecule profoundly sensitizes tumours to chemotherapeutics, notably gemcitabine and cisplatin, at low concentration (80 nM *vs.* 1000 nM for **VE-821**), leading to 2–3 fold reduced cancer cell survival. It is noteworthy that **VE-822** has no effect when used as a single agent. The pharmacological data proved very promising; moreover, *in vivo* this compound significantly inhibits tumour growth in pancreatic MiaPaCa-2 mice xenografts without signs of toxicity.[80] Of note, in 2012, **VE-822** became the first ATR inhibitor to enter clinical trials.[81]

Chloropyrrolopyridine 3. Novartis proposed a series of tetrahydropyrazolo[1,5-*a*]pyrazines as selective ATR inhibitors

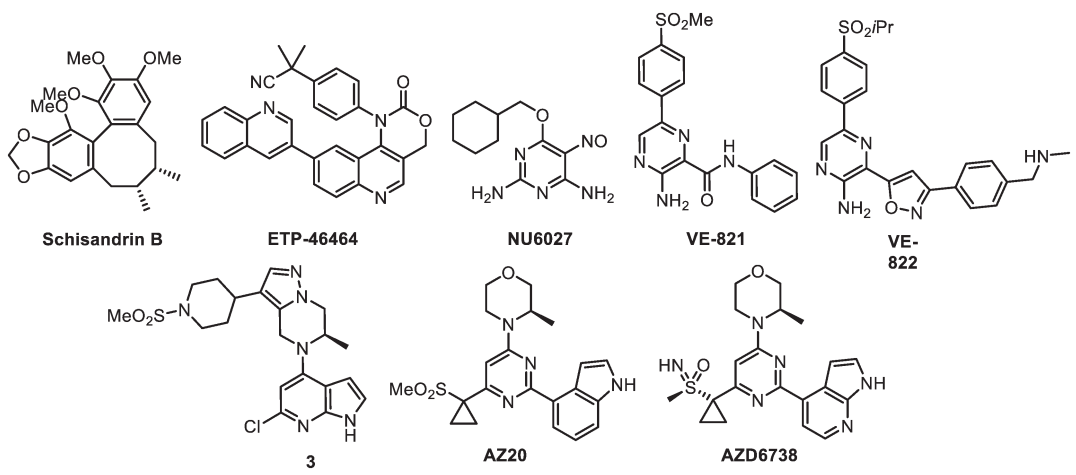


Fig. 4 ATR inhibitors.

Table 2 ATR inhibitors and their main properties

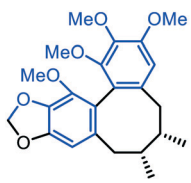
Compound	Enzymatic activity	*In vitro* selectivity	*In vitro* efficacy	Pharmacology	*In vivo* activity	Development stage
Schisandrin B	IC_{50} = 7.25 μM	–	R+/– C+/–	N.D.	N.D.	Optimisation stage
ETP-46464	N.D.	+	R+	—	N.D.	Optimisation stage
NU6027	N.D.	N.D.	R+/– C+	– (solubility)	N.D.	Optimisation stage
VE-821	K_i = 13 nM	+	R+ C+	– (toxicity)	N.D.	Discontinued at research stage
VE-822	K_i < 0.2 nM	+	M– R+ C++	+	+	Clinical trials phase I
3	IC_{50} = 0.4 nM	++	N.D.	+/– (toxicity)	N.D.	Research stage
AZ20	IC_{50} = 5 nM	+/–	M++	+/– (toxicity, solubility)	++	Clinical trials phase I
AZD6738	IC_{50} = 1 nM	++	M+ C++	+	+	Preclinical stage

Selectivity: "–": non-selective (<2 fold); "+/–": moderately selective (2–50 fold); "+": selective (50–100 fold); "++": very selective (>1000 fold). ***In vitro* efficacy**: "M": monotherapy, as single agent; "R": radiosensitizer; "C": chemosensitizer; "–": inefficient (IC_{50} > 30 μM as single agent or sensitization ratio < 1.2 fold); "+/–": moderately active (2 μM < IC_{50} < 30 μM as single agent or sensitization ratio: 1.2–2 fold); "+": active (100 nM < IC_{50} < 2 μM as single agent or sensitization ratio: 2–10 fold); "++": very active (IC_{50} < 100 nM as single agent or sensitization ratio > 10 fold). **Pharmacology**: "–": poor, main issues in brackets; "+/–": moderate, main issues in brackets; "+": good. ***In vivo* activity**: "+/–": moderate tumour growth inhibition; "+": significant tumour growth inhibition; "++": tumour volume reduction. **Development stage**: "optimisation stage": *in vitro* properties to be optimised; "research stage": *in vitro* properties validated; "preclinical stage": *in vitro* and *in vivo* properties validated. N.D.: not determined.

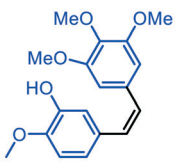
issued from HTS. A hit-to-lead strategy furnished the chloropyrrolopyridine **3**. It exhibits excellent *in vitro* activity (IC_{50} = 0.4 nM) and selectivity (>10^4 over PIKK, hERG and PI3K). A very high activity was retained in cells (IC_{50} = 37 nM) and a good pharmacological profile to probe ATR biology *in vivo* was obtained. However, potential safety concerns were reported because of *in vitro* inhibition of cytochrome CYP3A4, a major monooxygenase involved in drug metabolism. Compound **3** was proposed for its use as a chemosensitizer rather than as a monotherapy.[82]

AZ20. **AZ20** was discovered by AstraZeneca by a hit-to-lead strategy from a mTOR inhibitors library screening. It is a low nanomolar ATR inhibitor (IC_{50} = 5 nM), with remaining activity for mTOR (IC_{50} = 38 nM), but selective against all PI3K isoforms (IC_{50} > 10 μM). Interestingly, a better ATR selectivity was observed in cells. (IC_{50} = 50 nM for ATR and 2.4 μM for mTOR). Despite its low aqueous solubility, **AZ20** induced, as single agent, a significant tumour volume reduction (3 fold) in mice xenografted with LoVo colorectal cells. However, a time-dependent inhibition of cytochrome 3A was also reported, which led to elevated exposure in mice even at moderate doses.[83] Efforts to improve **AZ20** aqueous solubility resulted in decreased bioavailability.[84]

AZD6738. Finally, **AZ20** underwent further structural optimizations and eventually led to the discovery of **AZD6738**. This latter molecule is a very potent ATR inhibitor *in vitro* (IC_{50} = 1 nM) and *in cellulo* (IC_{50} = 74 nM, in H460 NSCLC).



Schisandrin B



Combretastatin A4

Fig. 5 Structure similarities between **Schisandrin B** and **Combretastatin A4**.

Moreover, **AZD6738** neither affects mTOR (IC_{50} > 23 μM), nor other PI3Ks (IC_{50} > 30 μM). Used at 1 μM concentration in combination with cisplatin, it leads to an important 20-fold decrease of cancer cells viability. This molecule exhibits an optimized pharmacokinetic profile, and is therefore orally bioavailable and active as a chemosensitizer. **AZD6738** induces cell death and senescence in H23 NSCLC xenografted mice and 85% tumour regression in combination with cisplatin. No apparent toxicity on mice was observed over the 2 week treatment.[11] Interestingly, **AZD6738** was also reported to be synthetically lethal with p53 and ATM defects.[25,26] Overall, this compound seems to be a valid drug candidate and is currently assessed in phase I clinical trials.

Among the molecules proposed for ATR inhibition, **AZD6738** and **VE-822** appear to be the only ones suitable for *in vivo* use, both being actually evaluated in phase I clinical trials. Compound **3** and **AZ20** could have been good drug candidates but safety concerns about cytochromes inhibition hinder their use *in vivo*. **ETP46464** could be used to probe ATR inhibition in cells, but its poor pharmacokinetic profile prevents its transfer to animals.

2.3. CHK1 inhibitors

The compounds that are reviewed herein as CHK1 inhibitors are represented below (Fig. 6) and their main properties are subsequently listed in Table 3.

UCN-01. **UCN-01** (7-hydroxystorausporine) is a natural product belonging to the staurosporine family. This is a non-selective multipathway inhibitor targeting CHK1 together with the PKC subfamily (a group of enzymes that play important roles in several signal transduction cascades), CDK1,[85,86] CDK2.[87] It also affects the p53/p21waf1 and CHK2/CDC25 pathways. **UCN-01** shows a nanomolar potency against CHK1 (K_i = 5.6 nM). The lack of selectivity of this inhibitor can by partly explained by the very high molecular rigidity of the staurosporine core. Indeed, it tightly binds the ATPase binding sites of a set of several kinases, as suggested by a co-

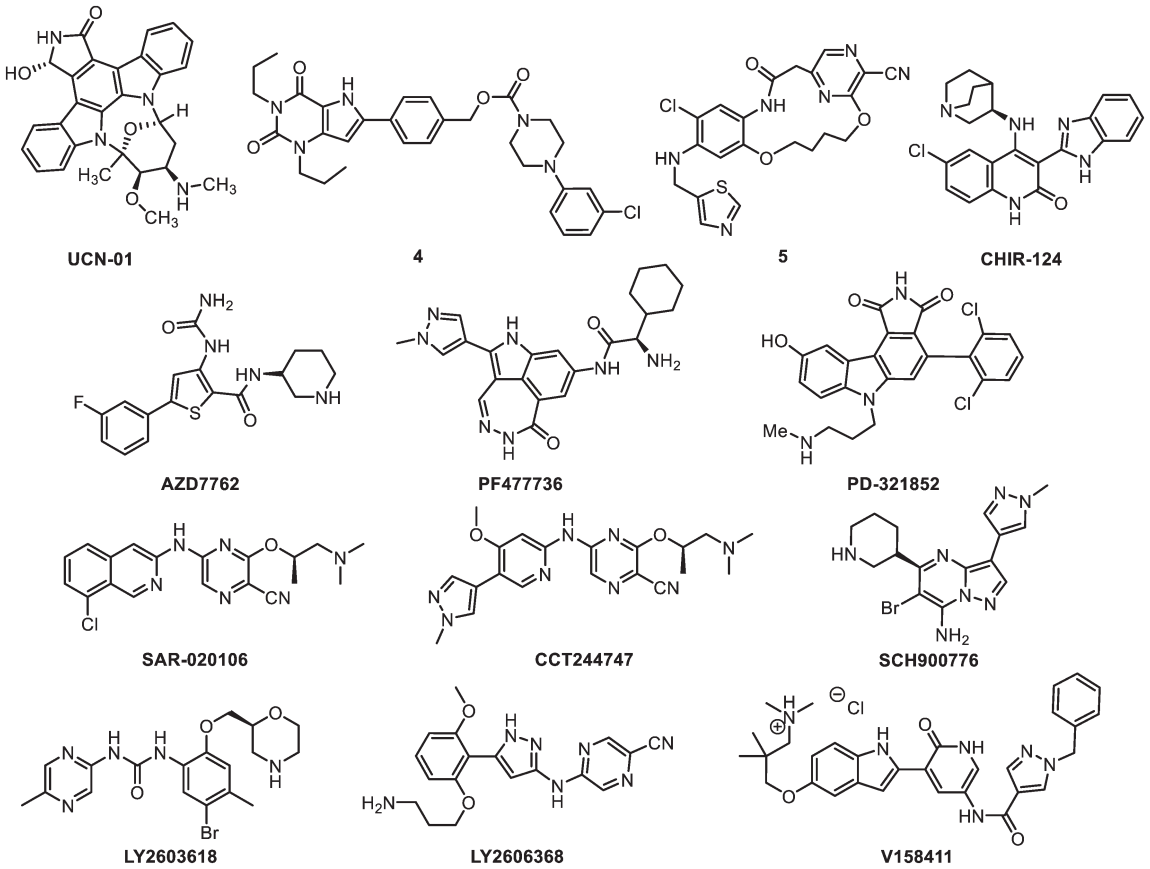


Fig. 6 CHK1 inhibitors.

crystallization study with CHK1.[88] **UCN-01** induces the cell growth inhibition of colon and hepatocellular carcinoma (Huh7, HepG2, Hep3B and HT29 cells), both *in vitro* and *in vivo*.[89] Remarkably, it also has this impact on glioblastoma stem cells (GSC) when a concomitant PDK1 inhibition is applied.[90] **UCN-01** has been evaluated in many different phase I and phase II clinical trials between 1995 and 2015, either as single a anti-neoplastic agent or in combination settings. Nevertheless, its use has always been hindered by poor pharmacokinetics, high binding to serum proteins and unfavourable toxicity profile.[91,92] **UCN-01** underwent structural optimizations to overcome its binding to serum proteins and nonspecific kinase inhibition. Eventually, the analogue **Gö6976** was proposed.[93] However, this simplified staurosporine proved 22-fold less active than **UCN-01** and poorly selective. In fact, **Gö6976** mainly inhibits the PKC kinases, together with numerous kinases, including CHK1 and possibly CHK2. It is noteworthy that other staurosporines, like **SB218078**,[94] were identified although **UCN-01** remains the spearhead of this family.

XL-844. XL-844 (**EXEL-9844**) is a dual CHK1/CHK2 inhibitor whose structure remains undisclosed. It is a potent CHK1 inhibitor (K_i = 2.2 nM) but it exhibits a moderate selective over other kinases (K_i = 0.07 nM, CHK2). Few pharmacological details have been disclosed, nevertheless it proved orally available, and was shown to promote CHK1 and CHK2 specific radio- and chemosensitization at around 1 μM dosages.[95] *In vivo*, it led to 74% tumour growth inhibition in combination with gemcitabine on HT-29 xenografted mice. It entered phase I clinical trials for leukaemia in 2005, but the study has been closed due to slow enrolment. Its combination with gemcitabine has been assessed against lymphoma, although no results are available.[96]

Pyrimidinedione 4. Abbott laboratories developed a series of cyanopyridyl containing 1,4-dihydroindeno[1,2-*c*]pyrazoles as CHK1 inhibitors. The lead compound of the series (**4**), shows a subnanomolar activity (IC_{50} = 0.75 nM, CHK1), and is relatively selective against other Ser/Thr kinases. In HeLa cervical and H1299 lung cancer cells, this compound exhibits a weak anti-proliferative activity when used as a single agent (IC_{50} = 21 μM) and proved moderately active in combination with doxorubicin (EC_{50} = 0.54 μM). Efforts were devoted to improve its poor pharmacokinetics,[97] although **4** displayed a sufficient profile to be tested *in vivo*. No data in animals have been reported yet, but this compound might be used as a tool for *in vivo* evaluation of DNA-damaging agents in sensitized cells.[98]

Macrocycle 5. Macrocyclic ureas were proposed for the selective inhibition of CHK1 by structure-based design.[99] The lead compound (**5**) displays an IC_{50} of 4 nM against CHK1 and was highly selective among other kinases. It is noteworthy that it does not affect CHK2 (IC_{50} = 5.5 μM). As a single

Table 3 CHK1 inhibitors and their main properties

Compound	Enzymatic activity	*In vitro* selectivity	*In vitro* efficacy	Pharmacology	*In vivo* activity	Development stage
UCN-01	K_i = 5.6 nM	−	M+/− R+/− C +/−	− (distribution, toxicity)	+/−	Discontinued at clinical trials phase II
XL-844	K_i = 2.2 nM	−	M+/− R+/− C+	+	+	Discontinued at clinical trials phase I
4	IC_{50} = 0.75 nM	+/−	M+/− C+	+	N.D.	Optimisation stage
5	IC_{50} = 4 nM K_i = 4 nM	++	M− C++	+/− (exposition)	N.D.	Research stage
CHIR-124	IC_{50} = 0.3 nM K_i = 0.3 nM	+/−	M− R+/− C+	+	+/−	Preclinical stage
AZD7762	IC_{50} = 5 nM K_i = 3.6 nM	+/−	M+ C++	+	+	Discontinued at clinical trials phase I
PF477736	K_i = 0.49 nM	+	M− C+/−	+/− (distribution, metabolism, elimination)	+/−	Discontinued at clinical trials phase I
PD-321852	IC_{50} = 5 nM	+/−	C++	N.D.	N.D.	Research stage
SAR-020106	IC_{50} = 13.3 nM K_i = 10.9 nM	+	M− C++	+/− (distribution, metabolism)	+/−	Research stage
CCT244747	IC_{50} = 7.7 nM	++	M+ C+	+	+	Research stage
SCH900776	IC_{50} = 3 nM	+	R+ C++	+	+	Clinical trials phase II
LY2603618	IC_{50} = 7 nM	+	M+ C++	+/−	+	Clinical trials phase II
LY2606368	K_i = 0.9 nM	+/−	M++ C++	+	+	Clinical trials phase I
V158411	IC_{50} = 3.5 nM	−	M+ C+	N.D.	N.D.	Optimisation stage

Selectivity: "−": non-selective (<2 fold); "+/−": moderately selective (2–50 fold); "+": selective (50–100 fold); "++": very selective (>1000 fold). ***In vitro* efficacy**: "M": monotherapy, as single agent; "R": radiosensitizer; "C": chemosensitizer; "−": inefficient (IC_{50} > 30 μM as single agent or sensitization ratio < 1.2 fold); "+/−": moderately active (2 μM < IC_{50} < 30 μM as single agent or sensitization ratio: 1.2–2 fold); "+": active (100 nM < IC_{50} < 2 μM as single agent or sensitization ratio: 2–10 fold); "++": very active (IC_{50} < 100 nM as single agent or sensitization ratio > 10 fold). **Pharmacology**: "−": poor, main issues in brackets; "+/−": moderate, main issues in brackets; "+": good. ***In vivo* activity**: "+/−": moderate tumour growth inhibition; "+": significant tumour growth inhibition; "++": tumour volume reduction. **Development stage**: "optimisation stage": *in vitro* properties to be optimised; "research stage": *in vitro* properties validated; "preclinical stage": *in vitro* and *in vivo* properties validated. N.D.: not determined.

agent, a weak activity was observed in HeLa and H1299 cancer cell lines (EC_{50} = 59.3 μM, HeLa), whereas its combination with doxorubicine led to a moderate cytotoxicity (EC_{50} = 1 μM). Preliminary pharmacokinetic analyses showed a moderate plasma exposition. It seems that the development of this series has been discontinued as no further data were subsequently disclosed.

CHIR-124. **CHIR-124** is a potent and relatively selective quinolone-based molecule. It displays an IC_{50} of 0.3 nM against CHK1 and is selective over CHK2 (IC_{50} = 0.7 μM) and over a panel of 124 representative kinases (all IC_{50} > 100 nM). Nevertheless, it might target FLT3 (fms related tyrosine kinase 3, regulating hematopoiesis), PDGFR (platelet-derived growth factor receptor, regulating cell proliferation, cellular differentiation, cell growth and development) and GSK3 (glycogen synthase kinase 3, regulating cell proliferation, migration, glucose levels and apoptosis) as they display IC_{50} values of 5.8, 6.6 and 23.3 nM, respectively. No activity was observed as a monotherapy but radio- and chemosensitizing effects were reported at submicromolar concentrations in MDA-MD-435 melanoma cell lines. **CHIR-124** is orally bioavailable and potentiates topoisomerase I poisons (camptothecin, SN38, irinotecan) in mice xenografted with melanoma MDA-MB-435 cells (moderate tumour growth inhibition of 14–53%). Indeed, the immobilisation of topoisomerase I provokes collision with the replication fork and leads to DSB. Of note, no signs of toxicity or significant body weight loss were observed in animals.[100,101]

AZD7762. AstraZeneca dedicated a full medicinal chemistry program to develop the potent and selective CHK1 inhibitor **AZD7762** (IC_{50} = 5 nM, CHK1). Starting from a HTS screening and after multiple rounds of structure–activity relationship-driven structural optimizations and ADME profiling, **AZD7762** was discovered. This molecule presents chemo- and radiosensitizing effects in the 150–300 nM range and an EC_{50} value of 0.62 μM when used alone (SW620, MDA-MB-231 and HCT116 cancer cell lines). Good pharmacokinetics and 2–3-fold tumour growth inhibition were measured although a slight body weight loss (7%) was reported in mice (H460-DNp53 and SW620 xenografts). **AZD7762** entered phase I clinical trials, in combination with gemcitabine, a nucleoside analogue widely used as a chemotherapy agent. These studies were terminated after a full review of program data and assessment of the current risk–benefit profile. Nevertheless, this compound remains a valuable tool for the study of DNA checkpoint biochemistry.[29,102]

PF477736. In 2011, Pfizer developed the subnanomolar CHK1 inhibitor **PF477736** (K_i = 0.49 nM). It is selective over CHK2 (K_i = 47 nM) and retains a very potent activity in HeLa and HT29 cancer cell lines (EC_{50} = 38–42 nM). **PF477736**

displays a moderate chemosensitizing effects at 540 nM, in HT29 cell lines (31% with gemcitabine; 22% with camptothecin). It possesses a good toxicity profile but the pharmacokinetics proved ameliorable with a moderate distribution, a short half-life (2.9 h) and a low clearance. **PF477736** has no effect when administered alone, whereas it boosts up to +89% the anti-tumour activity of gemcitabine in mice xenografted with Colo205 human colon carcinoma cells. In the latter case, no enhancement of systemic toxicity of gemcitabine was observed.[103] **PF477736** entered phase I clinical trials, but these were discontinued for unknown reasons, which were not safety or efficacy related.

PD-321852. PD-321852 is a potent staurosporine analogue displaying an IC_{50} of 5 nM against CHK1 and a reasonable selectivity. It promotes a significant sensitization (ratios: 6.2–17) to gemcitabine in pancreatic cancer cells at 300 nM (BxPC3, M-Panc96 and MiaPaCa-2 cell lines). It was presented as a CHK1 antagonist but other kinases inhibition may contribute to the observed chemosensitization.[104] ADME and toxicity profiling have not been reported yet and may be established before *in vivo* use. An analogue of this compound, **PD-407824**, has been disclosed to inhibit CHK1 but prevalently affects WEE1; therefore it will be discussed in the dedicated section (*vide infra*).

SAR-020106. SAR-020106 is a nanomolar ATP-competitive and selective CHK1 inhibitor (IC_{50} = 13.3 nM against CHK1 and up to 10 μM against CHK2 and CDK1). It enhances irinotecan and gemcitabine anti-tumour activity in several colon cancer cell lines (HT29, SW620, and Colo205; enhancement ratios: 3–29). The pharmacokinetics proved moderate because of high binding to plasma proteins, short half-life and high clearance. Moreover, a moderate tumour growth delay was observed in mice xenografted with SW620 cells.[105] Therefore, **SAR-020106** underwent an additional round of structural optimization and eventually yielded **CCT244747**.

CCT244747. CCT244747 is as potent as **SAR-020106** against CHK1 (IC_{50} = 7.7 nM) although with a better kinase selectivity, particularly against CHK2 (IC_{50} > 10 μM). It displays a specific CHK1-dependent mechanism-of-action in cells at 300 nM, and good microsomal stability. Most importantly, it is the first CHK1-selective inhibitor that is orally bioavailable. However, the *in vivo* behaviour of **CCT244747** is not uniform. Varying levels of oral exposure and clearance are observed. Alike **SAR-020106**, it modulates the DDR pathways and shows anti-tumour activity in combination with gemcitabine and irinotecan in HT29 and SW620 colon cancers, and in Calu6 NSCLC. It is noteworthy that **CCT244747** also acts as a single agent.[106,107]

SCH900776. SCH900776 (or **MK8776**) is a potent and selective ATP-competitive CHK1 inhibitor (IC_{50} = 3 nM). It is effective at overcoming both the S- and G2-checkpoints caused by IR and various DNA-damaging agents. In contrast to **AZD7762** and **PF477736**, this compound displays a 500-fold selectivity over CHK2. However, **SCH900776** exhibits a lower selectivity over CDK2 (IC_{50} = 160 nM), which might be detrimental for its overall effectiveness since the inhibition of this cyclin-dependent kinase can induce cell cycle arrest and prevent checkpoint bypass. Nevertheless, **SCH900776** shows a significant tumour growth delay in AsPC-1 and MiaPaCa-2 pancreas cancer cell lines xenografts. In 2013, it entered phase II clinical trials alone or in combination with gemcitabine in subjects with relapsed acute myeloid leukaemia.[108–110]

LY2603618. LY2603618 potently inhibits CHK1 protein kinase activity *in vitro* (IC_{50} = 7 nM). It is the first highly selective CHK1 inhibitor, including over CHK2 (>10^3 fold), to enter clinical cancer trials. This compound is also selective over a panel of representative kinases (all with IC_{50} > 1 μM, most of them with IC_{50} > 20 μM). HeLa cells treated with **LY2603618** produce a cellular phenotype similar to the one reported for RNAi induced CHK1 depletions. In HCT116 and HT29 colon cell lines, the compound displays antiproliferative effects (EC_{50} = 430 nM) and significant chemosensitizing effects when used in combination with gemcitabine at 0.25 μM. It proved orally available with an acceptable pharmacological profile. Moreover, it does not increase the gemcitabine systemic toxicity, and xenograft experiments with Calu-6 lung cancer cells showed a significant reduction of CHK1 phosphorylation in the tumours. It was suggested that the anticancer property of **LY2603618** might be enhanced when used in combination with an autophagy inhibitor (chloroquine).[111] Phase I clinical trials in patients with advanced or metastatic cancers indicated an acceptable safety profile for **LY2603618**.[112,113]

LY2606368. LY2606368, another related compound, has a dual action of inducing DNA-damage by causing DSB,[114] and removing the protection of DNA-checkpoint by inhibiting CHK1 with a K_i of 0.9 nM. Ultimately, this compound leads to chromosome fragmentation at 33 nM concentration and replication catastrophe *in vitro* and *in vivo*. The compound is overall selective, but contrary to **LY2603618**, displays only a very slight selectivity (8 fold) over CHK2 and RSK family kinases. Strong nanomolar inhibition was retained in cells (9 nM, HeLa) and 4-fold tumour growth inhibition was reported in Calu-6 lung cancer xenograft models.[115] It is currently in phase I clinical trials for its use as single agent or in combination with various anti-neoplastic drugs.

V158411. Vernalis developed **V158411** as a novel and potent kinase-selective inhibitor of recombinant CHK1 (IC_{50} = 3.5 nM) and CHK2 (IC_{50} = 2.5 nM). *In vitro*, this compound is more active against leukaemia and lymphoma cell lines (GI_{50} = 0.17 μM) than against colon (IC_{50} = 2.8 μM) and lung (IC_{50} = 6.9 μM) cancer cell lines.[116,117] Pharmacological inhibition of CHK1 with **V158411** does not induce a definitive cell cycle arrest. The pharmacokinetics and the *in vivo* efficiency of the compound remain to be evaluated. If they are validated, it might be a promising candidate as both monotherapy and chemosensitizer of gemcitabine and irinotecan.

GDC0425 and GDC0575. GDC0425 and **GDC0575** are two novel CHK1 inhibitors recently developed by Genetech. **GDC0425** is a member of the 9*H*-pyrrolo[2,3-*b*:5,4-*c*′]dipyridine class whereas the structure of **GDC0575** has not been

disclosed. Very few information and biological data are available on these compounds, yet.[118]

Among the biological targets reviewed herein, CHK1 is the one whose inhibition has been the most studied. Following the identification of seminal non-selective CHK1 inhibitors, several ATP-competitive inhibitors with improved CHK1 selectivity were developed. The most promising compounds for clinical use are probably **CCT244747**, **LY2603618** and **SCH900776**. The two latter are currently evaluated in phase II clinical trials. **PF477736** displays a good selectivity and ADME profile but is lacking efficiency. Although not selective, **AZD7762** and **LY2606368** are valuable dual CHK1/CHK2 inhibitors.

2.4. CHK2 inhibitors

The compounds that are reviewed herein as CHK2 inhibitors are represented below (Fig. 7) and their main properties are subsequently listed in Table 4.

The pioneering CHK2 inhibitors are two marine natural products: **hymenialdisine** and **debromohymenialdisine** (**DHB**). These compounds are secondary metabolites isolated from the sponges *Axinella verrucosa* and *Acantella aurantiaca*.[119] **Hymenialdisine** was initially identified as a very potent mitogen-activated protein kinase kinase-1 (MEK-1) inhibitor (IC_{50} = 6 nM).[120] It was later shown that it also displays a potent CHK2 kinase inhibition (IC_{50} = 42 nM) with moderate selectivity over CHK1 (IC_{50} = 1.95 μM). Moreover, the chemical synthesis of this compound proved delicate at some steps, particularly for the regioselective bromination. **DHB** proved less potent (IC_{50} = 183 nM, CHK2) and less selective (IC_{50} = 725 nM, CHK1) than **hymenialdisine**, but was used in several studies to specifically target CSC populations in treatment-resistant tumours.

Indoloazepine 6. The synthesis of an indoloazepine derivative (**6**) of **hymenialdisine** allowed a slight 10-fold selectivity over MEK along with some gain in activity (IC_{50} = 8 nM). Nevertheless, a nanomolar inhibition of several other enzymes, including CDKs, NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex that controls DNA transcription), GSK-3β and CK1 (casein kinase 1, an enzyme regulating DNA repair and DNA transcription), has been observed. Compound **6** demonstrated a CHK2-mediated radioprotection effect in normal cells and p53 wild type cells, but not in p53 mutant cells at 30 μM (HCT116 colon cancer cells).[119] Several synthetic analogues were subsequently prepared, but they all showed significantly diminished activities.[121,122]

ABI. Johnson & Johnson proposed, in 2005, a 2-arylbenzimidazole (**ABI**) as potent CHK2 inhibitor (IC_{50} = 15 nM).[123] This compound is the first highly selective inhibitor over CHK1 ($IC_{50} > 10$ μM) and is now commercially available. Issued from HTS and hit-to-lead strategy, the key points of the structure are the presence of the amide and of the terminal chlorine. Indeed, the corresponding carboxylic acid without chlorine displayed an IC_{50} of 640 nM. Of note, **ABI** efficiency in cells was significantly lower (42% CHK2 inhibition at 5 μM) compared with enzymatic assay. However, used in the low micromolar range, it remains a valuable tool to probe CHK2 inhibition. Importantly, **ABI** could dose dependently protects CD4+ and CD8+ T-cells from apoptosis after IR.

NSC109555. **NSC109555** is a symmetric bis-guanylhydrazone diarylurea issued form a HTS preformed on a library of 100 000 compounds.[124] This compound has been co-crystalized in the CHK2 ATP binding site, and is a moderatly potent (IC_{50} = 240 nM) ATP-competitive inhibitor[121,125] displaying a promising kinase profiling. However, no activity could be measured at 100 μM in MCF7 breast cancer and HT29 colorectal cancer cells both as single agent or in combination with topotecan and campthotecin. Contrariwise, an additional study reported an activity in pancreatic cancer cells (MiaPaCa-2, CFPAC-1, Panc-1 and BxPC-3) at the dose of 5 μM, in combination with gemcitabine.[126,127]

PV1019. **PV1019** is a dissymmetric optimized analogue of **NSC109555** which shares the same guanylhydrazonearyl moiety.[128] This optimization yielded a 10-fold improvement in

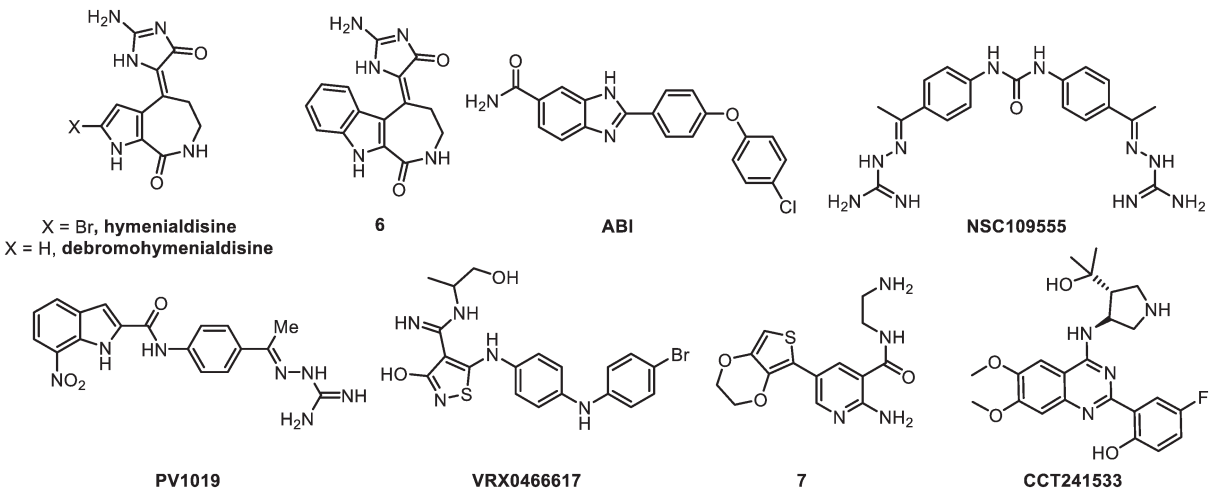


Fig. 7 CHK2 inhibitors.

Table 4 CHK2 inhibitors and their main properties

Compound	Enzymatic activity	*In vitro* selectivity	*In vitro* efficacy	Pharmacology	*In vivo* activity	Development stage
Hymenialdisine	IC_{50} = 42 nM	−	N.D.	N.D.	N.D.	Discontinued at optimisation stage
6	IC_{50} = 8 nM	+/−	R+/−	N.D.	N.D.	Optimisation stage
ABI	IC_{50} = 15 nM K_i = 37 nM	+	R+/−	N.D.	N.D.	Optimisation stage
NSC1095555	IC_{50} = 240 nM	+	M− C+/−	−	N.D.	Discontinued at optimisation stage
PV1019	IC_{50} = 24 nM	++	M+/− R+/− C+	N.D.	N.D.	Research stage
VRX0466617	IC_{50} = 140 nM K_i = 11 nM	+	M+/− R− C−	N.D.	N.D.	Research stage
7	IC_{50} = 28 nM	+/−	M+/−	N.D.	N.D.	Optimisation stage
CCT241533	IC_{50} = 3 nM K_i = 1.1 nM	+/−	M+ R++ C+	+	N.D.	Research stage

Selectivity: "−": non-selective (<2 fold); "+/−": moderately selective (2–50 fold); "+": selective (50–100 fold); "++": very selective (>1000 fold). ***In vitro* efficacy**: "M": monotherapy, as single agent; "R": radiosensitizer; "C": chemosensitizer; "−": inefficient (IC_{50} > 30 μM as single agent or sensitization ratio < 1.2 fold); "+/−": moderately active (2 μM < IC_{50} < 30 μM as single agent or sensitization ratio: 1.2–2 fold); "+": active (100 nM < IC_{50} < 2 μM as single agent or sensitization ratio: 2–10 fold); "++": very active (IC_{50} < 100 nM as single agent or sensitization ratio > 10 fold). **Pharmacology**: "−": poor; "+/−": moderate; "+": good. ***In vivo* activity**: "+/−": moderate tumour growth inhibition; "+": significant tumour growth inhibition; "++": tumour volume reduction. **Development stage**: "optimisation stage": *in vitro* properties to be optimised; "research stage": *in vitro* properties validated; "preclinical stage": *in vitro* and *in vivo* properties validated. N.D.: not determined.

CHK2 inhibition (IC_{50} = 24 nM) together with an increased selectivity over CHK1 (IC_{50} = 15.7 μM) and a over panel of 52 representative kinases (>10^2). Conversely to the parent compound, **PV1019** displayed indisputable effects as radio- and chemosensitizer with topotecan or camptothecin (OVAR-5 and MCF7 cell lines) and also a slight anti-tumour effect as a single agent (IC_{50} = 5–58 μM). However, the required dosages to achieve significant effects as mono- or combination therapies remain too high (10–25 μM). Therefore, further structural optimization of **PV1019** should be undertaken.

VRX046617. VRX046617 was identified by screening of a small kinase inhibitor library,[129] followed by a hit-to-lead optimization. This compound displays an IC_{50} of 140 nM against CHK2 and is selective over CHK1 (IC_{50} > 10 μM). In BJ-hTERT immortalized fibroblasts, no CHK1 inhibition was observed, and CHK2 inhibition with micromolar concentrations of the drug (10 μM) led to a weak anti-proliferative effect after 6 days (45% growth reduction). Moreover, neither IR, nor chemo-potentiation of doxorubicin or cisplatin cytotoxicity has been evidenced.[130] **VRX046617** is inactive in cells, and therefore may only be an interesting biological probe to study and decipher the CHK2-dependent pathways. This also raises the question of the correlation between CHK2 direct inhibition and the observed anti-proliferative effects.

Aminopyridine 7. Hilton *et al.* proposed 2-aminopyridine based compounds as CHK2 inhibitors.[131] Eleven hits were selected after a screening, against CHK2, performed on 7000 potential ATP-competitive kinase inhibitors. Subsequently, a hit-to-lead optimization afforded the aminopyrimidine **7**. This molecule is a potent CHK2 inhibitor (IC_{50} = 28 nM), and exhibits a moderate selectivity towards CHK1 (IC_{50} = 2.5 μM) and other kinases (only 18/24 displaying less than 40% at 1 μM). As a single agent, it inhibits CHK2 activity with IC_{50} values of 5–10 μM and showed a GI_{50} of 26 μM in HT29 colorectal cancer cells.

CCT241533. CCT241533 is a very potent (IC_{50} = 3 nM) CHK2 inhibitor which encompasses a 2-(quinazolin-2-yl)phenol scaffold.[132] This molecule is moderately selective over CHK1 (IC_{50} = 190 nM), and might be used as a single agent for monotherapy (GI_{50} = 1.7, 2.2 and 5.1 μM respectively on HT29, HeLa and MCF7 cells) or chemosensitizer with topotecan, etoposide and camptothecin (IC_{50} = 1–3 μM).[133] Interestingly, this compound displays good pharmacokinetics, oral bioavailability and is the only one that has been tested *in vivo* in a model of mouse isolated thymocytes. In this *in vivo* study, it showed a strongly enhanced apoptosis induction after IR exposure. **CCT241533** might be a good research tool for *in vitro* and *in vivo* pharmacological studies as well as a potential clinical candidate if *in vivo* studies are validated.

To conclude, only four reasonably selective CHK2 inhibitors have been described to date. Generally, CHK2 inhibitors proved significantly less active than ATM, ATR or CHK1 inhibitors as anti-proliferative compounds or at inducing mitotic catastrophe following IR or DNA-damaging treatment. **CCT241533** appears as the most promising compound, but *in vivo* studies are still needed. **ABI**, **VRX046617** and **PV1019** show a modest micromolar anti-proliferative activity whereas **NSC109555** is inactive in cells.

2.5. WEE1 inhibitors

The compounds that are reviewed herein as WEE1 inhibitors are represented below (Fig. 8) and their main properties are subsequently listed in Table 5.

PD0166285. The first compound that have been reported with an inhibitory activity against WEE1 is the non-selective 6-aryl-pyrido[2,3-*d*]pyrimidine derivative, **PD0166285.** This molecule has been identified through a screening performed on a compound library, and by means of tyrosine kinase assay. **PD0166285** shows a nanomolar activity (IC_{50} = 24 nM) on

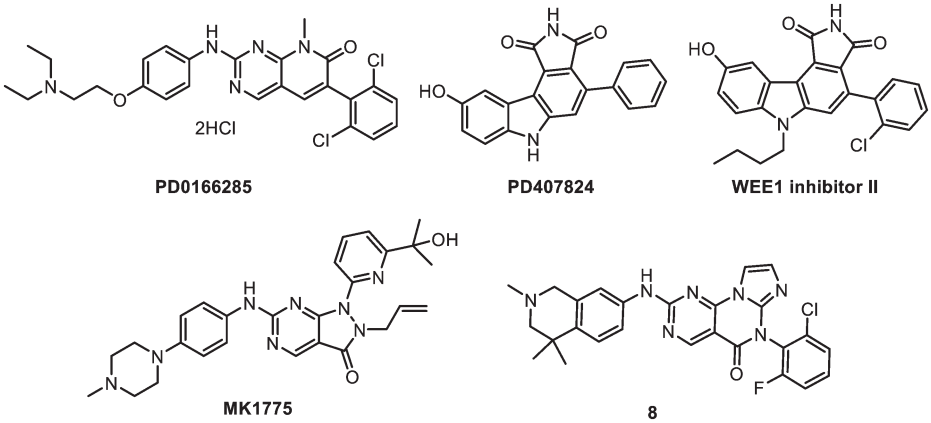


Fig. 8 WEE1 inhibitors.

WEE1, but is also active on a range of kinases including proto-oncogene tyrosine-protein kinase Src (c-Src), myelin transcription factor 1 (MYT1), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 1 (FGFR1), CHK1 and beta-type platelet-derived growth factor receptor (PDGFRb) with IC_{50} values in the 8–85 nM range.[134] Several studies were undertaken in different cancer cell lines, including HCT116, HCT8, HT29, DLD-1, HeLa, H460, U2OS, MG-63, PA-1 cancer cell lines.[48,135–137] However, **PD0166285** monotherapy exhibited only a slight anti-proliferative effect (GI_{50} = 26 μM, HT29 cells) without promoting cell death. In combination, it acts as a modest p53 dependent radiosensitizer with a maximal enhancement ratio of 1.23. This is less than the impact of **caffeine** or **UCN-01** (respective enhancement ratios of 2.05 and 1.58 on HT29 cells). Moreover, cell toxicity was observed from 6 h exposure; thus the therapeutic window of cellular effectiveness *versus* cellular toxicity of the compound is lower than the one of **caffeine** or **UCN-01**. Attempts devoted to modify the structure to simultaneously improve both the absolute potency and selectivity towards WEE1 were unsuccessful. Indeed, the observed activities could not be separated from an even more potent inhibition of c-Src kinase.[138]

PD0407824. Following a HTS program, staurosporine analogues were later proposed for the inhibition of WEE1.[139] The representative hit of this screening is **PD0407824** which exhibits an IC_{50} of 97 nM on WEE1. However, this compound was more active on CHK1 (IC_{50} = 47 nM) and multiple derivatives were synthesised to achieve selectivity over this off-target protein.

WEE1 inhibitor II. Optimization of **PD0407824** resulted in the discovery of **WEE1 inhibitor II** that bears two additional substituents: a butyl chain on the indole nitrogen and a chlorine atom on the phenyl ring. This compound has an IC_{50} of 35 μM against CHK1, however its low solubility and cell permeability precluded it to present any activity in cells. Optimization of **WEE1 inhibitor II** was attempted but led to no significant improvement. Solubility problems persisted and no *in cellulo* activity was obtained.[140]

MK-1775. MK-1775 is the first highly potent and selective WEE1 inhibitor (IC_{50} = 5 nM); except over Yes1 kinase (IC_{50} = 14 nM).[141] It was discovered by Merck laboratories through the optimization of a HTS hit. **MK-1775** is also the first selective WEE1 inhibitor to be active in cells (including WiDr colon, H1299 lung and TOV21G ovary cancer cell lines), where it abrogates G2 DNA damage checkpoint and decreases cell

Table 5 WEE1 inhibitors and their main properties

Compound	Enzymatic activity	*In vitro* selectivity	*In vitro* efficacy	Pharmacology	*In vivo* activity	Development stage
PD0166285	IC_{50} = 24 nM	−	M− R+/− C+/−	− (toxicity)	N.D.	Optimisation stage
PD407824	IC_{50} = 97 nM	−	N.D.	N.D.	N.D.	Discontinued at optimisation stage
WEE1 inhibitor II	IC_{50} = 59 nM	+	N.D.	− (solubility, distribution)	N.D.	Optimisation stage
MK1775	IC_{50} = 5 nM	+	M+/− R+/− C+	+	+	Clinical trials phase II
8	K_i < 1.0 nM	+	M+ C+	+	+	Preclinical stage

Selectivity: "−": non-selective (<2 fold); "+/−": moderately selective (2–50 fold); "+": selective (50–100 fold); "++": very selective (>1000 fold). ***In vitro* efficacy**: "M": monotherapy, as single agent; "R": radiosensitizer; "C": chemosensitizer; "−": inefficient (IC_{50} > 30 μM as single agent or sensitization ratio < 1.2 fold); "+/−": moderately active (2 μM < IC_{50} < 30 μM as single agent or sensitization ratio: 1.2–2 fold); "+": active (100 nM < IC_{50} < 2 μM as single agent or sensitization ratio: 2–10 fold); "++": very active (IC_{50} < 100 nM as single agent or sensitization ratio > 10 fold); **Pharmacology**: "−": poor, main issues in brackets; "+/−": moderate, main issues in brackets; "+": good. ***In vivo* activity**: "+/−": moderate tumour growth inhibition; "+": significant tumour growth inhibition; "++": tumour volume reduction. **Development stage**: "optimisation stage": *in vitro* properties to be optimised; "research stage": *in vitro* properties validated; "preclinical stage": *in vitro* and *in vivo* properties validated. N.D.: not determined.

viability. **MK-1775** activity is selective towards p53 defective cells and without effect on p53+ cells.[141] It was tested at 100–300 nM concentrations in a number of different patterns: as IR sensitizer in glioma,[142] lung, breast and prostate cancers;[49] and as chemosensitizer in combination with gemcitabine, 5-fluorouracil, capecitabine, pemetrexed, camptothecin, doxorubicin, carboplatin, cisplatin, mitomycin C and paclitaxel.[143,144] Remarkably, no activity was observed as single agent, except in one study on sarcoma.[145] Additionally, **MK-1775** proved inefficient in combination with temozolomide for glioblastoma, putatively because of an insufficient distribution of temozolomide across the blood–brain barrier.[146] Of note, GSC were not strongly affected by this compound (*vide infra*).[142] *In vivo*, **MK-1775** potentiates the effects of DNA-damaging agents and induces the tumour growth inhibition of many xenograft models (HeLa, TOV21G-shp53, WiDr, MX-1, PANC198, PANC215, PANC185, *etc.*). Importantly, these co-treatments do not significantly increase the initial toxicity of the chemotherapeutics (gemcitabine or 5-fluorouracil) in mice.[141] Interestingly, the combined inhibition of CHK1 and WEE1 showed synergistic effects and is a significant example of synthetic lethality.[147] Treatment with non-toxic concentrations of a CHK1 inhibitor (**PF477736**) and **MK-1775** confirmed the marked synergistic effect in various human cancer cell lines (breast, ovarian, colon, prostate), regardless of p53 status. Similar results were obtained with the combination of **MK-1775** with another CHK1 inhibitor, **AZD-7762**, *in vitro* and *in vivo*, in melanoma,[45] neuroblastoma[148] and for mantle cell lymphoma, an aggressive and incurable disease characterized by a deregulated cell cycle.[149,150] In conclusion, **MK-1775** is the most potent and highly selective inhibitor of WEE1 and has recently reached phase I clinical trials, in combination with gemcitabine, cisplatin or carboplatin, and phase II in combination with paclitaxel and carboplatin in ovarian cancer.

Pyrimidopyrimidinone 8. Lastly, a pyrimidine-based tricyclic molecule was proposed in 2015 by AbbVie for the inhibition of WEE1.[151] This molecule has been designed by a rational hybridization between **MK-1775** and other WEE1 inhibitors. Among a series of potential WEE1 inhibitors, pyrimidopyrimidinone **8** emerged as the most promising molecule. This hit displays a very high potency ($K_i < 1.0$ nM, WEE1) and is selective on an 84 kinases profiling. Nanomolar efficiencies were retained in cells (LD_{50} = 310 nM, H1299 cell line), and the pharmacokinetics proved particularly good with 94% oral bioavailability, adequate half-life (2.8 h) and clearance values. Oral *in vivo* potentiation of irinotecan was evidenced in mice xenografted with H1299 cancer cell line, rendering this compound promising for further preclinical evaluation.

The first commercially available WEE1 Inhibitor **WEE1 inhibitor II** suffers from solubility and permeability issues that hinder its use in cells. Therefore, the standard drug for studying WEE1 inhibition *in vitro* and *in vivo* should be **MK-1775**, which is currently evaluated in phases I and II clinical trials. Pyrimidopyrimidinone **8** is an interesting molecule in the pipeline that could reach the preclinical stage in the near future.

3. ATM, ATR, CHK1, CHK2 and WEE1 inhibition in CSC

This last decade, growing interests have been dedicated to the so-called cancer stem cells (CSC) hypothesis. This model supports the idea that tumours, like adult tissues, arise from cells that exhibit the ability to self-renew as well as give rise to differentiated tissue cells. Most of the reported studies describe an increased DDR in CSC, in accordance with the "stemlike" feature of these cells. Indeed, stem cells have developed protection mechanisms and improved repair compared with differentiated cells.[55,152]

CSC employ several mechanisms to escape therapeutic assaults (drug efflux activation, anti-apoptotic signalling, hypoxia management…). Nevertheless, as the great majority of anti-cancer therapies (radio- and chemo-therapies) rely on DNA-damaging agents, tackling the enhanced DDR appears as a particularly attractive strategy to improve their efficiencies. Moreover, several studies suggest that CSC contribute to radio- and chemoresistance through enhanced activation of the DNA damage checkpoint response and increased DNA repair capacity. Furthermore, the CSC fraction within the tumour cell population typically increases after repeated cycles of DNA-damaging treatments that preferentially kill the non-stem cells. As a result, classical therapies become progressively ineffective towards these CSC-enriched tumours.[153] Therefore, targeting DNA damage checkpoint response and its underlying molecular pathways in CSC may sensitize these particular cells to DNA-damaging techniques and overcome these resistances. This re-sensitization might provide useful therapeutic models for malignant cancers.

In this context, we have reviewed the relevance of the five pivotal kinases ATM, ATR, CHK1, CHK2 and WEE1 as therapeutic targets in the particular case of CSC.

ATM. Despite rare contradictory reports,[154,155] most studies enlighten enhanced DDR *via* constitutively upregulated ATM signalling in CSC models compared with non-CSC.[24,56,156] This has been correlated with DNA-damaging treatment resistance. Therefore, ATM seems a relevant target for drug development to improve the efficiency of the current DNA-damaging treatments, and to counter resistance in CSC.

ATR. Higher levels of ATR have been reported in breast CSC (BCSC) models compared with differentiated tumour cells.[55] Moreover, ATR inhibition was shown to abrogate *in vitro* and *in vivo* tumourigenicity of human colon cancer cells by eliminating the cell population bearing the specific stem cell marker CD133.[157] This makes ATR an attractive target for the development of molecular inhibitors to circumvent resistance to classical radio- and chemotherapies.

CHK1 and *CHK2*. Several reports point out the interest of tackling CHK1 in the specific context of the CSC,[90,158–160] while, to the best of our knowledge, no examples of specific CHK2 inhibitors have been studied in CSC models so far.

WEE1. Apart from a study of WEE1-related radiosensitization in glioblastoma neurospheres, very few

reports on WEE1 inhibition in the particular case of cancer stem cells have been published.[37,161]

Overall, only a limited number of chemical modulators of ATM, ATR, CHK1, CHK2 and WEE1 have been tested in CSC or tumour-initiating cells. These inhibitors are presented in Table 6 and discussed afterwards. We also introduce a set of suggestions for the use and the development of new compounds to study cell cycle arrest, to induce a mitotic catastrophe and cell death in CSC.

3.1. ATM/ATR inhibitors in CSC

Five compounds were tested as sensitizers for the targeting of CSC: **caffeine**, **CP466722**, **KU55933**, **KU60019** and **NVP-BEZ235**.

In 2009, the first attempts to sensitize CSC were undertaken using **caffeine** in combination with etoposide. The **caffeine**/etoposide combo proved able to delay re-growth of A549 NSCL CSC in the tumour through ATM/ATR pathways inhibition. However, this potential is tarnished by the fact that **caffeine** also induced by itself a reversible growth arrest that is associated with increased fraction of CSC.[162] A selective targeting of CSC was obtained in human colon cancer cell lines (DLD1, Colo320, COGA-12 and RKO) models, where **caffeine**-promoted ATR inhibition preferentially depleted the chemo-resistant and exclusively tumorigenic CD133+ cell fraction. This depletion proceeds by apoptosis induction of CD133+ cycling cells. The remaining cells lose their tumorigenicity either *in vitro* or *in vivo*.[157] Moreover, the chemoresistance of those cells towards DNA inter-strand

Table 6 DNA-checkpoint response inhibitors used in CSC

Drug	Target	Cancer cell lines	Results	Ref.
Caffeine	ATR	NSCLC: A549	Re-growth delay alone or in combination with etoposide	157, 162
		Colon: DLD1, Colo320, COGA-12, RKO	Preferential apoptosis of the CD133+ cell fractions	
CP466722	ATM	Breast: BCSC MDA-MB-231, MDA-MB-453	CSC more sensitive to radiation than non-CSC because of lower ATM levels	155
KU55933	ATM	Glioblastoma: U87, U251 GSC, R10, S2, E2, G7 GSC	Comparable drug-induced radiosensitivity of GSC and GC Potent radiosensitization of GSC	156, 163
KU60019	ATM	Glioblastoma: BORRU, DR177, BORRU, VIPI, COMI, MPM176, DR177, DEMI, PERU, 2.11	Specific sensitization of GSC over GC 4/8 GSC are radiosensitized *in vivo*	57, 58
NVP-BEZ235	PTEN/PI3K/ Akt/mTOR	**Prostate:** DU 145, PC3 **Colon:** HCT-116, SW620	DDR-independent CSC growth inhibition DDR-independent sensitization of CSC to paclitaxel	164–167
		Glioblastoma: U87, U251, T98G, SHG44 **Glioma:** SU-2	Enhanced radiosensitivity of GSC	
DBH	CHK1/CHK2	**Glioma:** CD133+ D456MG, CD133+ D54MG, D456MG	Minimal impact alone. Disrupts radio-resistance of CD133+ GSC *in vitro* and *in vivo*	153, 168, 169
		Breast: 44+/CD24− MCF-7	Radiosensitization of both CD133− and CD133+ cells Significant radiosensitization of CSC	
UCN-01	ATR/CHK1/PDK1	**Colon:** CD133+ (DLD1, Colo320, RKO, COGA-12)	Significant decrease of CD133+ cells	90, 157, 170
		Glioblastoma: patient-derived GSC	Strong chemosensitization with temozolomide	
		Glioma: derived HNGC-2	No reduced viability CSC	
		HNSCC: SQ20B/CD44+/ALDH^{high}	Radiosensitization of CSC	
AZD7762	CHK1/	**Pancreas:** patient-derived CSC	Chemosensitization with gemcitabine and tumor-initiating capacity reduction	90, 158–160
	CHK2	**NSCLC:** patient-derived CSC	Dramatic reduction of survival *in vitro* and *in vivo*	
		Leukaemia: CD34+/CD38−/CD123+	Significant reduction of CSC without affecting normal SCs	
		Glioblastoma: patient-derived GSC	Ineffective chemosensitization with temozolomide	
PF477736	CHK1	**NSCLC:** NCI-H1299	Chemosensitization of CSC with gemcitabine	171,
		Pancreas: PANC-1, BxPC-3	Very slight tumour growth delay and enrichment of CSC	172
SCH900776	CHK1	**AML:** primary leukemic blasts, CD34+/CD38−/CD123+ CSC	Specific reduction of CSC fraction in combination with HDAC inhibitors	108
PD0166285	WEE1 c-Src/MYT1/EGFR/CHK1…	**Glioblastoma:** CD133+ (U251MG, U118MG, U87MG)	Specific significant radiosensitization of CSC	37
MK1775	WEE1	**Glioblastoma:** G179, G144 GNS	Effective sensitization of GBM non-SCs and normal SCs, but no radiosensitization of GNS	142

crosslinking agents like cisplatin could be overcome. This chemoresistance has been attributed to a preferential activation of the ATR/CHK1-dependent DNA-damage response in CD133+ cells.

Similar results have been observed by comparing the efficiency of the ATM-selective inhibitor **CP466722** on MDA-MB231 and MDA-MB453 BCSC with their corresponding non-stem cells. In this model, BCSC were more sensitive to radiation than non-BCSC. The authors imply that this higher sensitivity might be due to lower ATM intracellular levels and DNA repair capacity in BCSC, which constitutes a controversy relative to most other reports.[155]

On the other hand, Carruthers *et al.* reported a potent radiosensitization of GSC after treatment with the ATM inhibitor **KU55933**.[163] An additional study, described by Zhou *et al.*, confirmed this potent radiosensitization in GSC.[156] Surprisingly, the same level of radiosensitization was observed in GC and GSC although ATM activity is significantly higher in the latter cell type. Indeed, in response to radiation, both GC and GSC treated with **KU55933** had an enhanced proportion of cells in G2 phase and a decreased proportion of cells in G1 phase. Additionally, an enhanced apoptotic rate, relative to the untreated cells, was observed.[156]

Contradictory, Raso *et al.* reported a specific sensitization of BORRU GSC compared to BORRU GC, when using ATM-selective inhibitors **KU55933** and **KU60019**. Furthermore, no sensitization was observed in DR117 GSC, which display a less pronounced stem phenotype than BORRU cells. This suggests that ATM inhibition may specifically sensitize GSC while sparing non-stem cells.[57] Consistently, GSC radioresistance could be overcome *in vivo* using **KU60019** in four GSC lines (BORRU, VIPI, COMI and MPM176), whereas four others were protected (DR177, DEMI, PERU and 2.11) under the same conditions. However, among the several markers that were followed, the positive response to **KU60019** rather correlated with high PI3K expression, than with the "stemness" of the cell lines. This underlines the high heterogenic behaviour of CSC towards ATM-specific inhibition.[58]

The multi-target PI3K/mTOR/ATM/DNA-PKcs/Akt inhibitor **NVP-BEZ235** was reported to inhibit the growth of prostate CD133+/CD44+ CSC,[164] to decrease CD133+ colon CSC proliferation and survival,[165] and to sensitize SW620 colorectal CSC to paclitaxel.[166] However, these specific effects seem in all cases to be unrelated to DNA-checkpoint response, but rather depending on the PTEN/PI3K/Akt/mTOR pathway. Contrarily, in the case of SU-2 GSC, an enhanced radiosensitivity induced by **NVP-BEZ235** was observed with a concomitant decrease of DNA repair capacity. The correlation between these two events has not been fully deciphered yet.[167]

3.2. CHK1/CHK2 inhibitors in CSC

Checkpoint kinases inhibition has been studied in the case of CSC. Indeed, CHK1-mediated DNA checkpoint response was shown to be more robust in CSC than non-stem cells.[158] Strikingly, normal embryonic stem cells seem to fail in activating CHK1 in response to DNA replication stress. This non-activation of checkpoints is thought to be a mechanism of preservation of genome integrity, by eliminating damaged cells through apoptosis, rather than trying to repair them through DDR.[173] Therefore, CHK1 inhibition was studied as potential therapeutic approach for sensitizing resistant tumour cells. Five inhibitors were assayed in several CSC tumours: **UCN-01**, **AZD7762**, **PF477736**, **SCH900776** and **DBH**.

The dual CHK1/CHK2 inhibitor **DBH** was the first compound employed to try to overcome IR resistance in CD133+ cells. Although it had a minimal impact on CD133− and CD133+ cells, it displayed a potent synergy with radiation. Notably it has the ability to reverse specifically resistance in CD133+ cells *in vitro* and *in vivo*.[153] On the contrary, it radiosensitizes both CD133− and CD133+ cells in a D456MG glioma model.[168] Otherwise, in a recent *in vitro* study, **DBH** treatment in addition to radiation significantly reduced the stem cell proportion of MCF-7 breast cancer cells.[169]

Treatment of colon cancer cells with **UCN-01** results in significant decreased CD133+ expressing cells fraction and consecutive loss of *in vitro* and *in vivo* tumorigenicity of the remaining cells. Mechanistic studies suggested that either inhibition of CHK1 or upstream ATR are responsible of the observed sensitizing effects.[157] Otherwise, **UCN-01** induced radiosensitization of SQ20B/CD44+/ALDH^{high} head and neck squamous cell carcinoma stem cells; this could be improved by co-treatment with all-trans retinoic acid as ALDH inhibitor.[170] In contrast, **UCN-01** was found inactive on glioma derived HNGC-2 stem cells.[174]

Interestingly, Signore & Ricci-Vitiani demonstrated that most GSC are resistant to specific inhibition of the major signalling pathways involved in cell survival and proliferation. For example, the specific CHK inhibition with **AZD7762** proved scarcely effective in temozolomide chemosensitizing. On the contrary, the multipathway inhibitor **UCN-01** displayed remarkable inhibition of GSC growth in the same *in vitro* and *in vivo* experience patterns.[90] The authors then deciphered that the combined inhibition of PDK1 (3-phosphoinositide dependent protein kinase-1, a major kinase downstream of PI3K required for the activation of Akt) and CHK1 was responsible of the strong anti-tumour effects observed, thus paving the way to a new potential therapeutic approach to reduce the growth of human glioblastoma.

In NSCLC-SC, the dual CHK1/CHK2 inhibitor **AZD7762**, combined with the chemotherapy agents gemcitabine, paclitaxel and cisplatin significantly reduced *in vitro* and *in vivo* tumour growth, whereas chemotherapy alone was almost ineffective.[159] Similar results were observed in leukaemia, where co-administration of **AZD7762** with genotoxic agents (*e.g.* gemcitabine) was reported to significantly reduce tumour cell progenitors bearing CD34+/CD38−/CD123+ stem cell markers without affecting normal hematopoietic progenitors;[160] the same phenomenon was observed in patient-derived pancreas cancer cells (using CD24, CD44 and ESA markers). In this latter case, the combination **AZD7762**/gemcitabine significantly delayed tumour initiation.[158]

A contradictory study reported that the combination of gemcitabine with CHK1-selective inhibitor **PF477736** leads to very slight tumour growth delay and enriched CSC in PANC-1 and BxPC-3 pancreatic cell lines ductal adenocarcinoma models.[172] In this study, the authors proposed the selectivity of **PF477736** towards CHK1 (100 fold over CHK2) as an explanation. Otherwise, in a NCI-H1299 non-small cell lung cancer model, **PF477736** enhances the anti-proliferative effect of gemcitabine against both the parental and the CSC-like cell populations; although CSC-like cells exhibited resistance to apoptosis whereas non-CSC remained sensitive.[171]

Interestingly, the combination of the CHK1-selective **SCH900776** and a HDAC inhibitor also proved beneficial to kill AML primary leukemic blasts and CD34+/CD38−/CD123+ leukaemia initiating cells (regardless of p53 status) whereas it did not promote substantial toxicity towards normal CD34+ hematopoietic cells.[108]

3.3. WEE1 inhibitors in CSC

The selective WEE1 inhibitor **MK-1775** was tested against U251, U87, and T98G glioblastoma cells where it showed a potent G2-checkpoint abrogation and enhanced radiosensitization. Human normal astrocytes were also sensitive when submitted to the same conditions. In contrast, G179 and G144 glioblastoma neural stem (GNS) cells were slightly affected, displaying only an attenuation of the accumulation of cells into G2–M phase, and no radiosensitization effect.[142] Therefore, these initial findings suggest that although WEE1 inhibition is highly effective in radiosensitization, targeting only this pathway might be insufficient to modulate radiation response in this focused GNS population.

Otherwise, opposite results were reported the same year on a primary glioblastoma (GBM) neurospheres model with the non-selective inhibitor **PD0166285**. In this study, radiation resistant CD133+ GBM cells were efficiently killed after treatment with this compound, suggesting that radiation resistance could be overcome by WEE1 inhibition.[37] However, **PD0166285** is a multi-target compound inhibiting a panel of other enzymes in the nanomolar range. Whether this radioresistance reversion is or is not due to WEE1 inhibition has not been established yet; thus it would be very interesting to collect more data to assess this purpose.

Overall, disparate results have been obtained for the sensitization potential of DNA-checkpoint modulators in CSC. However, several points can be highlighted:

i) Several DNA-damage checkpoint inhibitors are active against CSC *in vitro* and *in vivo*.

ii) There is no clear selectivity of these drugs on CSC over non-CSC; the activity being either higher, lesser or comparable depending on the cases.

iii) For a defined drug and a defined cancer, the sensitivity of CSC depends on the cell lines.

iv) Importantly, among cancers, glioma stem cells which have been largely studied prove particularly resistant compared to other CSC. To a lesser extent, pancreas stem cells seem difficult to target.

v) Generally, at first sight, ATM/ATR inhibitors appear to have more impact on CSC than CHK1, CHK2 and WEE1 specific inhibitors, even if more data using other selective drugs are needed to confirm this trend.

vi) Non-selective multi-pathways inhibitors (*e.g.* **caffeine**, **UCN-01**, **NVP-BEZ235**, **PD0166285**,…) usually display a better activity on CSC than target-selective compounds. In the particular case of **NVP-BEZ235**, its growth inhibition activity on various CSC even seems DDR-independent. Additional results are also required to clarify the most relevant pathways to target for CSC sensitization.

4. Future prospects of ATM, ATR, CHK1, CHK2 and WEE1 inhibitors as CSC sensitizers

Target enzymes. From several pioneering reports released these last 5 years, the specific CSC targeting is an area of growing interest with many questions that remain to be addressed. On the five enzymes involved in the cell cycle arrest we have focused on ATM and ATR, which are validated targets for the sensitization of drug-resistant cancers cells as well as their respective CSC populations. Importantly, the most resistant GSC seem to be sensitizable, at least for several cell lines. CHK1 is a valid oncotarget for the sensitization of cancer cells with already five compounds having reached clinical phases. However, only one study using a selective CHK1 inhibitor (**PF477736** on NSCLC) has been reported for the sensitization of CSC.[171] Other studies with other specific drugs and other cancer cells are urgently needed to validate this enzyme in the particular case of CSC. The role of CHK2 in oncology is less clear. As for CHK1, it was at first sight legitimate to target this protein to afford sensitization to DNA-damaging agents as it represents the second main actor of the checkpoint response activation. However, accumulating evidence was latter disclosed that CHK2, in spite of having a role in checkpoint signalling especially in response to radiation, is not a necessary kinase and its depletion has no effect on the sensitization of gemcitabine-treated cells.[175–177] In the case of WEE1, very few data are available, with only one negative report using a selective inhibitor, and a positive one using a multi-target drug.

Utilizable drugs. In all cases, more data need to be accumulated to decipher the potential, scope and limitations of DNA-damage checkpoint response inhibitors to target CSC. To this end, many potent and selective recent drugs are available and have not been tested yet. They may serve as interesting chemical tools to probe the biology of these enzymes in oncology, and in particular case of CSC. This encompasses ATM inhibitors: **KU60019** on which preliminary results on CSC have been disclosed, its not yet tested analogue **KU59403**, and the promising new drugs **1** and **2**. For ATR inhibitors, **VE-822** and **AZD6738**, which are very potent *in vitro*

and *in vivo* on differentiated cancer cells, might be highlighted. Within all compounds described to target CHK1, five of them might be considered for their potential use in CSC: **macrocycle 5**, **SAR020106**, **CCT244747**, **SCH900776** and **LY2603618**. Concerning CHK2, its inhibition alone does not seem efficient in the sensitization of DNA-damaging processes. For example, **VRX046617**, a potent *in vitro* and *in vivo* CHK2 inhibitor with no CHK1 activity, did not potentiate doxorubicin or cisplatin activity.[130] On the contrary, the only potent molecule described as CHK2 inhibitor, **CCT241533**, displays a moderate selectivity, raising the hypothesis of off-target effects. Therefore, if the advantage to afford a dual CHK1/CHK2 inhibition over a CHK1 inhibition alone remains to be evaluated, there is apparently no reason that a specific CHK2 inhibition will result in chemo- or radiosensitization in the particular case of CSC.[178] Very few data are available on WEE1-specific inhibition in CSC, and one new compound (**8**) might be very interesting to assay.

Finally, targeting DNA-damage checkpoint response in CSC seems an overall good strategy as it is generally admitted that CSC have superior drug-resistance mechanisms than non-CSC. However, the best therapeutic target to induce mitotic catastrophe by G2-abrogation remains elusive, whether ATM, ATR, MYT1, CHK1, Hsp90, PP2A, WEE1, or other unknown G2–M transition targets or combinations thereof.[46]

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