A WEE1 family business: regulation of mitosis, cancer progression, and therapeutic target

Andrea Ghelli Luserna di Rorà, Claudio Cerchione, Giovanni Martinelli and Giorgia Simonetti*

Abstract

The inhibition of the DNA damage response (DDR) pathway in the treatment of cancer has recently gained interest, and different DDR inhibitors have been developed. Among them, the most promising ones target the WEE1 kinase family, which has a crucial role in cell cycle regulation and DNA damage identification and repair in both nonmalignant and cancer cells. This review recapitulates and discusses the most recent findings on the biological function of WEE1/PKMYT1 during the cell cycle and in the DNA damage repair, with a focus on their dual role as tumor suppressors in nonmalignant cells and pseudo-oncogenes in cancer cells. We here report the available data on the molecular and functional alterations of WEE1/PKMYT1 kinases in both hematological and solid tumors. Moreover, we summarize the preclinical information on 36 chemo/radiotherapy agents, and in particular their effect on cell cycle checkpoints and on the cellular WEE1/PKMYT1-dependent response. Finally, this review outlines the most important pre-clinical and clinical data available on the efficacy of WEE1/PKMYT1 inhibitors in monotherapy and in combination with chemo/radiotherapy agents or with other selective inhibitors currently used or under evaluation for the treatment of cancer patients.

Keywords: WEE1 family kinases, WEE1, PKMYT1, Cell cycle, DNA repair, Pseudo-oncogene, Tumor suppressor

Background

The WEE1 kinase family consists of three serine/threonine kinases sharing conserved molecular structures and encoded by the following genes: WEE1 (WEE1 G2 Checkpoint Kinase), PKMYT1 (membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase), and WEE1B (WEE2 oocyte meiosis inhibiting kinase). In eukaryotic somatic cells, WEE1 and PKMYT1 play a key role in cell cycle regulation and, in particular, they are involved in the entry into mitosis [1]. Their role as regulators is crucial during normal cell cycle progression and in response to DNA damages, as part of the DNA damage response (DDR) pathways. Similarly, WEE2 regulates cell cycle progression and, in particular, meiosis [2]. Briefly, WEE2 plays a dual regulatory role in oocyte meiosis by preventing premature restart prior to ovulation and permitting metaphase II exit at fertilization [3]. Despite the identification of WEE2 somatic mutations (1.9% of cases) and copy number (CN) alterations (22.5% of patients with CN loss and 22.5% with CN gain) across several cancer types (https://portal.gdc.cancer.gov), they have not been functionally linked to tumor development so far. Therefore, the following sections will be focused on WEE1 and PKMYT1 kinases that have a well-recognized role in oncology and hemato-oncology.

WEE1 and PKMYT1 in cell cycle regulation

WEE1 and PKMYT1 act as tumor suppressors in nonmalignant eukaryotic somatic cells. Similarly to other DDR-related kinases, their main biological function is to...
prevent replication of cells with altered DNA. The main downstream target of WEE1 family kinases is the cyclin-dependent kinase 1 (CDK1)-cyclin B1 complex, also known as mitotic-promoting factor (MPF). WEE1 phosphorylates CDK1 on Tyr15 while PKMYT1 has a dual activity on Tyr15 and Thr14 [4] (Fig. 1a). The phosphorylation of those residues keeps the MPF complex inhibited until the cell approaches mitosis. WEE1 is located in the nucleus, while PKMYT1 is associated with the endoplasmic reticulum and Golgi apparatus [5, 6], and regulates Golgi membrane reassembly following mitosis [7]. Together, WEE1 and PKMYT1 ensure that CDK1 remains inactive as it shuttles into and out of the nucleus [8]. Through its extra-nuclear localization, PKMYT1 can also promote CDK1 cytosolic segregation. At the G2/M border, if no DNA damage has been detected, CDK1 phosphorylation on Tyr15 and Thr14 is rapidly removed by CDC25C phosphatase. In the nucleus, the CDK-activating kinase (CAK) complex composed by cyclin-dependent kinase 7 (CDK7), cyclin H1, and MAT1 promotes MPF complex activation through the phosphorylation of CDK1(Thr161) [9, 10]. The active MPF complex is then imported into the nucleus through phosphorylation of cyclin B1 (Ser126, Ser128, Ser133, and Ser147) [11]. This event is required to enter mitosis. The relevance of WEE1 and PKMYT1 regulation of CDK1 has been recently confirmed by in vivo studies. Indeed, the replacement of the CDK1 inhibitory phosphorylation sites with non-phosphorylatable amino acids (CDK1 T14A/Y15F) was embryonic lethal in mice [12]. Once activated, the MPF complex can phosphorylate WEE1 and PKMYT1 to promote their inactivation via different cascades [5, 13, 14]. WEE1 is phosphorylated (Ser123) by CDK1 at the onset of mitosis, thereby generating a binding motif for polo like kinase 1 (PLK1) and casein kinase 2 (CK2), that in turn phosphorylate WEE1 (Ser53 and Ser121, respectively) [14, 15]. Together, the phosphorylation of the three Ser residues serves as a tag for the degradation of WEE1 by the ubiquitin ligase SCFβ-TrCP [13]. PKMYT1 is also phosphorylated by CDK1 and PLK1 and this event promotes its degradation [16]. In addition to the checkpoint function at the G2/M border, recent findings highlighted a role of WEE1 in the regulation of replication dynamics during S phase (intra S phase checkpoint). When cells reach the S phase, replication is initiated from a large number of replication origins triggered through the activation of the pre-replication complex [17] and following the activation of S phase specific CDK, primarily CDK2 [18, 19]. Similarly to CDK1, CDK2 regulation is controlled through Tyr15 phosphorylation status, that is balanced by WEE1 (Fig. 1a) and cell division cycle 25A (CDC25A) activity [20]. Both WEE1 and CDC25A/C have been shown to modulate unperturbed replication through regulating CDK1/CDK2 activity. Monoallelic expression of CDK1 T14A/Y15F induced replication stress and S phase arrest in mouse embryonic fibroblasts (MEFs), with substantial increase of γH2AX levels, chromosomal fragmentation, and DDR activation, as a consequence of intra-S phase DNA damage [12]. Moreover, unscheduled origin firing due to loss of WEE1 leads to exhaustion of the replication protein A1 (RPA1) pool and, as a consequence, to death during DNA replication.

**Fig. 1** WEE1 and PKMYT1 biological functions. a Schematic representation of WEE1 and PKMYT1 involvement in cell cycle checkpoints. WEE1 regulates the activity of both CDK1 and CDK2 kinases (through phosphorylation of Tyr15) and is involved in the regulation of intra-S, G2/M, and M phase cell cycle checkpoints. PKMYT1 selectively regulates CDK1 (through phosphorylation of Tyr15 and Thr14) and is plays a role in the G2/M phase checkpoint. b Schematic representation of the regulation of MUS81-EME1/2 endonuclease complexes by WEE1 during S and G2/M cell cycle phases. By inhibiting CDK2 or CDK1, WEE1 prevents MUS81 activation and the generation of DNA damages during S phase, and chromosomes pulverization during G2/M phase.
The intra S phase activity of WEE1 is independent from PKMYT1 that is unable to phosphorylate CDK2 [5]. In addition, WEE1, but not PKMYT1, contributes to the control of mitosis exit. Indeed, WEE1-deficient MEFs showed mitotic defects (e.g., in the number and position of centrosomes) that induce arrest in mitosis or, in the majority of cells, mitotic slippage [21, 22]. At the end of mitosis, WEE1 inhibits CDK1 through phosphorylation of its Tyr15 residue (Fig. 1a). This event is dependent on the activation of the CTD phosphatase subunit 1 (FCP1) that dephosphorylates and activates WEE1 and other crucial component of the spindle assembly checkpoint (SAC) complex [23]. Although the precise mechanisms that regulate FCP1 activity is still unknown, it has been showed that FCP1 promotes the dephosphorylation of crucial SAC components, including cell division cycle 20 (CDC20) and ubiquitin specific peptidase 44 (USP44), thus promoting APC/C/Cdc20 activation and chromosome segregation [24–26]. Moreover, WEE1 directly interacts with APC/C components, including fizzy and cell division cycle 20 related 1 (CDH1), CDC20, cell division cycle 27 (CDC27), and its deletion enforced APC/C activity, resulting in alterations of the level of APC/C substrates and mitosis progression at the expense of genomic stability [21].

**WEE1 regulates replication forks and genome stability**

The activity of WEE1 through the cell cycle can explain its tumor suppressor function, at least in nonmalignant cells. This observation was confirmed and disentangled in preclinical studies. Indeed, conditional WEE1 heterozygous deletion in the murine mammary epithelium caused enhanced proliferation, with cells progressing into mitosis while still undergoing DNA replication, and consequent accumulation of DNA damage, resulting in genomic instability and, ultimately, in tumor development [21]. Biological processes such as DNA replication and homologous recombination involve the formation of branched DNA structures that physically link chromosomes. Such DNA structures needs to be disengaged prior to entry into mitosis, in order to ensure proper chromosome segregation. Eukaryotic cells evolved different mechanisms to identify and process branched DNA structures (Y-shape DNA) and the most important one involves the structure-selective endonuclease MUS81. MUS81 forms heterodimeric complexes with the non-catalytic subunits EME1 or EME2 and recognizes Y-shape DNA structures during DNA replication or during mitosis (homologous recombination). The activity of MUS81-EME1/2 complex is crucial to recover stalled replication forks, during prolonged S phase arrest, and to reset DNA junction between twin chromatids during homologous recombination [27]. In unperturbed cells, WEE1 protects replication forks and prevents the generation DNA damages and chromosome pulverization through an indirect inhibition of MUS81 functionality [28]. Indeed, WEE1 phosphorylates CDK1 and CDK2, thus preventing the CDK-mediated phosphorylation and activation of MUS81-EME1/2 complexes [29]. Lack of WEE1-dependent regulation of MUS81-EME1/2 endonucleases may lead to cleavage of unwanted DNA structure (excessive replication forks), which would slow down replication progression and increase genomic instability [27, 28] (Fig. 1b).

**WEE1 and PKMYT1 deregulation in cancer cells**

**WEE1 and PKMYT1 act like oncogenes**

The biological role of WEE1 and PKMYT1 in cancer cells is not fully understood. Reduced WEE1 expression has been detected in breast cancer compared with normal tissues, independently of the tumor grade [21]. However, most findings suggest that both kinases act like oncogenes rather than tumor suppressors. Indeed, they are frequently overexpressed in both solid and hematological tumors and a genome-wide CRISPR screen of 563 cancer cell lines, showed that they are essential for the cell viability of almost all cell lines [30]. The dependency of cancer cells on WEE1 family proteins may be linked to the following mechanisms (Fig. 2): (i) the high proliferation rate of cancer cells that follows the activation of driver oncogenes (e.g., RAS, MYC) needs to be sustained by a strong cell cycle regulation machinery; (ii) cancer cells frequently inactivate p53, which is a key gatekeeper of G0/G1 and S phases and, as a consequence, the regulation of cell cycle is sustained entirely by the G2/M checkpoint; (iii) the over-expression of DDR-related kinases is fundamental to maintain a tolerable level of genetic instability, an intrinsic feature of cancer cells [31, 32]. Therefore, we can speculate that, once the malignant transformation process has been induced, WEE1 upregulation exerts a pro-tumorigenic functions by securing a tolerable level of genetic instability in hematological and solid tumors.

**WEE1 and PKMYT1 genetic lesions in cancer**

WEE1 and PKMYT1 are rarely mutated in cancer patients, with an overall mutation frequency of 1.2% and 0.2%, respectively (https://portal.gdc.cancer.gov). The distribution of somatic mutations is highly heterogeneous across cancer types (WEE1: 0.2–7.6%; PKMYT1: 0.1–3.6%), with a higher frequency in uterine corpus endometrial carcinoma (UCEC) and tumors of the gastrointestinal tract (stomach and colon adenocarcinoma, Fig. 3a, b). In particular, WEE1 mutations have
been reported in 7.6% of UCEC cases. Moreover, PKMYT1 lesions have been also detected in 2.7% of diffuse large B cell lymphoma (DLBC). Conversely, both kinase genes are rarely mutated in brain lower grade glioma (LGG), ovarian serous cystadenocarcinoma (OV), prostate adenocarcinoma (PRAO), and sarcoma (SARC), with a frequency lower than 0.5%. Both genes are mainly targeted by missense mutations that preferentially cluster in the region encoding the WEE1 kinase domain and its surroundings (Fig. 3c), suggesting a potential gain of function effect of the kinase activity. Conversely, the mutations are scattered throughout the PKMYT1 sequence (Fig. 3d). Little is known about the functional consequences of WEE1 and PKMYT1 mutations. In the majority of cancer types, the transcript expression in the mutated cases is higher than the median value of the entire cohort (https://www.cbioportal.org), supporting once more an oncogenic function. In pancreatic adenocarcinoma (PA) patients and cell lines, an insertion was identified in the WEE1 poly-T track, which contains the binding site of the HuR RNA binding protein [33]. The insertion resulted in decreased WEE1 expression upon mitomycin-induced DNA damage, which would argue against a protective effect of the mutation. Copy number alterations (CNAs) represent a more frequent event compared with mutations, with the WEE1 gene being predominantly involved in CN loss (23.7% of cases versus 7.8% of patients with CN gains), while PKMYT1 showing a higher percentage of CN gain (15.9% versus 12.0% of CN loss, Fig. 3e, f). The predominance of WEE1 deletion events (6.3% versus 3.25% of cases with amplification) was also observed in breast cancer, in line with its reduced expression, as mentioned above [21]. Overall, cancer types showing the highest recurrence (> 10%) of CNAs were OV (27.7%), lung squamous cell carcinoma (LUSC, 14.8%), uterine carcinosarcoma (UCS, 12.5%), and SARC (11.2%) for WEE1 and OV (18.8%), bladder urothelial carcinoma (BLCA, 13.7%), and esophageal carcinoma (ESCA, 10.3%) for PKMYT1. Of note, OV and LUSC have been classified as tumors with multiple recurrent chromosomal gains and losses [34], which may suggest a bystander effect related to chromosomal instability in these tumor types, especially in the case of WEE1 deletion, that is unexpected, based on the general oncogenic function exerted by the kinase.

WEE1 and PKMYT1 functional role in hematological and solid tumors

Few studies have analyzed WEE1 and PKMYT1 expression in hematological malignancies. Our group showed that WEE1 kinase is highly expressed in acute lymphoblastic leukemia (ALL) cell lines and primary cells in comparison with normal hematopoietic cells, and that PKMYT1 is upregulated in relapsed ALL samples compared with nonmalignant hematopoietic cells [35]. Moreover, we demonstrated that ALL cells are dependent on WEE1 functionality for their survival and proliferation and that PKMYT1 levels may influence the sensitivity to the WEE1 inhibitor AZD-1775 [35]. Similar results on the role of WEE1 were obtained in multiple myeloma (MM), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and chronic lymphocyte leukemia (CLL) [36–39]. In AML cells, WEE1 and PKMYT1 are key gene discriminating between FLT3-ITD, FLT3-TKD, and NRAS-mutated samples. They were expressed at lower levels selectively in FLT3-ITD specimens in comparison with wild-type cells, suggesting either a tumor suppressor role in the leukemogenic process or a potential vulnerability in this AML subtype [40]. Pharmacological WEE1 inhibition alone or in combination with histone deacetylase inhibitors showed therapeutic potential in FLT3-ITD AML, confirming their dependency on WEE1 activity [41]. Since FLT3-ITD AML have intrinsic homologous recombination repair defects [42], WEE1 inhibition may exacerbate the cell genotoxic stress by disrupting multiple cell cycle checkpoints. WEE1 has been shown to be a valuable target also for lymphoma patients [43]. In parallel,
PKMYT1 proved to be essential for MM cell line viability, since its downregulation strongly decreased cell growth, while inducing apoptosis [44].

WEE1 and PKMYT1 are also over-expressed in solid tumors, including hepatocellular carcinoma, colon cancer, glioblastoma, non-small-cell lung cancer (NSCLS), neuroblastoma, and gastric cancers [31, 32, 45–47]. High WEE1 expression has been associated with negative prognostic factors including lymph node involvement, induction of metastasis, increased biomarkers of proliferation (CCND1, Ki67, or CCNA1) and resistance to treatments (radiotherapy or chemotherapy) [48–51]. Elevated PKMYT1 levels have been associated with tumor progression, a more aggressive disease, the induction of metastasis at least in NSCLS patients [45] and, generally, with poor prognosis. Depending on the cancer subtype, the expression of WEE1 and PKMYT1 has been linked with the activation of cellular pathways crucial for the specific disease. In melanoma cells, WEE1 silencing caused an increase of phospho p38 protein levels, indicating a role in the regulation of p38/MAPK pathway activation during p53-independent DNA damage response [49]. In hepatocellular carcinoma and colorectal cancers, PKMYT1 regulates epithelial-mesenchymal transition (EMT), a process relevant to tumor progression, invasion, metastasis, and drug resistance, through the activation of the beta-catenin/TCF

Fig. 3 WEE1 and PKMYT1 mutations and copy number alterations (CNAs) in cancer. a Frequency of patients with WEE1 or b PKMYT1 gene mutations across cancers from TCGA cohorts. c Distribution of mutations according to the WEE1 and d PKMYT1 amino acid (aa) sequence and protein domains (WEE1 transcript ENST00000450114, 646 aa; PKMYT1 transcript ENST00000262300, 499 aa). e Frequency of patients with copy number gain or loss in WEE1 or f PKMYT1 across cancers (https://portal.gdc.cancer.gov; ACC adrenocortical carcinoma, BLCA bladder urothelial carcinoma, BRCA breast invasive carcinoma, CESC cervical squamous cell carcinoma and endocervical adenocarcinoma, COAD colon adenocarcinoma, CHOL cholangiocarcinoma, DLBC diffuse large B cell lymphoma, ESCA esophageal carcinoma, GBM glioblastoma multiforme, HNSC head and neck squamous cell carcinoma, KICH kidney chromophobe, KIRK kidney renal clear cell carcinoma, KIRP kidney renal papillary cell carcinoma, LGG brain lower grade glioma, LIHC liver hepatocellular carcinoma, LUAD lung adenocarcinoma, LUSC lung squamous cell carcinoma, MESO mesothelioma, OV ovarian serous cystadenocarcinoma, PAAD pancreatic adenocarcinoma, PCPG pheochromocytoma and paraganglioma, PRAD prostate adenocarcinoma, READ rectum adenocarcinoma, SARC sarcoma, SKCM skin cutaneous melanoma, STAD stomach adenocarcinoma, TGCT testicular germ cell tumors, THYM thymoma, UCS uterine carcinosarcoma, UCEC uterine corpus endometrial carcinoma)
signaling [32, 46], while PKMYT1 has been reported to control Notch pathway in NSCLC [45]. In particular, crucial component of the pathway, including NOTCH1, p21, and HES1 are downregulated by chemical inhibition of PKMYT1 [45]. In neuroblastic tumors, PKMYT1 is required to stabilize MYCN protein, which is a crucial proto-oncogene for cancer types [52]. Moreover, in esophageal squamous cell carcinoma (ESCC) cell lines and primary cells, the expression of PKMYT1 is associated with and regulates the activation of the AKT/mTOR pathway [53] (Table 1). Taken together, this evidence suggests a broad role of WEE1/PKMYT1 besides the DNA damage response pathway that may increase the interest towards its therapeutic targeting.

Development of WEE1 and PKMYT1 inhibitors

WEE1 and PKMYT1 inhibitors have single agent and chemo-sensitizer effects

Due to their potential oncogenic role, WEE1 and PKMYT1 have been investigated as therapeutic targets for hematological and solid tumors. Several pharmacological inhibitors have been designed and subsequently validated in different cancer models. The available literature highlights a common mechanism of action of WEE1/ PKMYT1 inhibitors in cancer cells either in single agent or in combination with DNA damaging agents (chemotherapy/radiotherapy). WEE1/PKMYT1 kinase inhibition causes G2/M cell cycle checkpoint override, premature mitotic entry, and cell death during mitosis, through a mechanism generally known as mitotic catastrophe (Fig. 4a). From a biological point of view, the inhibition of WEE1 kinase causes a significant reduction of phospho-CDK1 (Tyr15), thus promoting the accumulation of active CDK1-cyclin B1 complex and, consequently, mitotic entry. The beginning of mitosis is also associated with a progressive accumulation of DNA damages and the degeneration in mitotic catastrophe. The sensitivity to WEE1 kinase inhibitors in relation to TP53 mutational status remains controversial. Indeed, some studies reported increased sensitivity of TP53 mutant cell lines to WEE1 inhibitors in comparison to TP53 wild-type ones [62, 63], while others showed no association between p53 functionality and the effectiveness of WEE1 inhibition [35, 64]. These discrepancies may be linked to the intrinsic chromosomal instability of the analyzed tumors and to additional alterations deregulating the G1 checkpoint in TP53 wild-type cases that may enhance the sensitivity to WEE1 targeting.

Regarding the role of WEE1 inhibitors as chemosensitizer agents, a large number of studies demonstrated a synergistic activity between DNA damaging agents (chemotherapy including doxorubicin, cytarabine, methotrexate, cisplatin, clofarabine, etoposide, 5-fluorouracil, and radiotherapy) and different WEE1/PKMYT1 inhibitors in preclinical models [48, 56, 65–69]. The mechanism of action of the combination is based on the inhibition of the DDR pathway following induction of DNA damage induced by the chemotherapy or radiotherapy agents. In this scenario, cancer cells with damaged DNA fail to arrest cell cycle, continue to proliferate, and accumulate massive DNA damage until a point of no return (Fig. 4b). Indeed, several DNA damaging agent promote the indirect activation of WEE1 and PKMYT1 kinases, as shown mostly by the activation of cell cycle checkpoints (S and G2/M checkpoints) in cancer cells. We summarized in Table 2 the results of preclinical studies in which the effect of different chemotherapy agents or radiotherapy has been evaluated in terms of cell cycle perturbation and altered expression of WEE1 or PKMYT1 following in vitro or

| Gene   | Genetic alteration | Disease         | Effect/prognostic value                                      | Reference |
|--------|--------------------|-----------------|-------------------------------------------------------------|-----------|
| WEE1   | Over-expression    | ALL; AML; MM; CML; CLL; DLBCL | Crucial for cell viability of cancer cells (experimentally proven). | [35–40, 43, 54] |
| Copy number Gain | AML | Biological effect or prognostic value unknown | | [55] |
| PKMYT1 | Over-expression    | ALL; MM         | Crucial for cell viability of cancer cells (experimentally proven). | [35, 44] |
| Solid tumors |
| WEE1   | Over-expression    | GC; MaM; GL; OC; CC | Associated with lymph node involvement, induction of metastasis, increased biomarkers of proliferation (CCND1, KI67 or CCNA1), resistance to treatment and poor overall survival. | [48–51, 56–60] |
| Mutation | PA | Insertion causing decrease WEE1 expression upon DNA damage | | [33] |
| PKMYT1 | Over-expression    | HC; CC; GLB; NSCLC; N; GS | Associated with tumor progression, aggressive disease and poor overall survival. | [31, 32, 45–47] |
| Mutation | N | Biological effect or prognostic value unknown | | [61] |

ALL acute lymphoblastic leukemia, AML acute myeloid leukemia, MM multiple myeloma, CML chronic myeloid leukemia, CLL chronic lymphocyte leukemia, DLBCL diffuse large B cell lymphoma, GC gastric cancer, MaM malignant melanoma, GL gliomas, OC ovarian cancer, CC colorectal cancer, PA pancreatic adenocarcinoma, HC hepatocellular carcinoma, GLB glioblastoma, NSCLC non-small-cell lung cancer, N neuroblastoma
in vivo treatment. Taken together, the abovementioned data prove that WEE1 and PKMYT1 are ideal targets to override cell cycle checkpoint regulation and to improve the efficacy of DNA-damaging agents. In particular, tumors with a high level of chromosomal instability may respond to WEE1/PKMYT1 inhibition per se, while cases with a more stable genomic asset may benefit from the combination between DNA-damaging agents and WEE1 family kinase inhibitors. The following sections report the main preclinical and clinical findings obtained using small molecules inhibitors of WEE1 and PKMYT1 kinases.

Preclinical studies of WEE1 and PKMYT1 inhibitors
Several targeted compounds showed an inhibitory activity on WEE1 and PKMYT1 kinases and their efficacy was proven in a number of tumor types. Table 3 shows the main preclinical studies that used WEE1/PKMYT1 inhibitors in single agent or in combination with chemo/radiotherapy agents in different tumor types.

PD0166285 is the first reported drug, with an inhibitory activity against WEE1, PKMYT1, and a range of other kinases including c-Src, EGFR, FGFR1, CHK1, and PDGFRb [151].

Adavosertib (AZD-1775) is the first highly potent and selective WEE1 inhibitor. A large number of preclinical studies evaluated its efficacy in single agent and in combinatorial approaches. Regarding the mechanism of action, adavosertib induces S and/or G2/M cell cycle checkpoints override, depending on cancer types, when used in monotherapy. Cell cycle perturbation is associated with a progressive accumulation of DNA damages and by the induction of apoptosis [35, 99, 119–122]. This last event is cell cycle phase-dependent and can occur (i) as a consequence of S phase checkpoint
override, when cancer cells start DNA replication even in the presence of DNA damages (replicative catastrophe); (ii) following G2/M phase checkpoint override, that results in forced entry into mitosis, even in the presence of DNA damages (mitotic catastrophe).

In combination strategies, adavosertib was able to enhance the cytotoxicity of chemo/radiotherapy agents, by inducing cell cycle checkpoint override, inhibition of DNA damage repair, and induction of apoptosis [35, 37, 38, 92, 121, 127–129]. The chemo-sensitizer efficacy of DDR inhibitors has been linked to drug scheduling [94, 152, 153]. Recently in pancreatic adenocarcinoma cells, it has been reported that the efficacy of a triple regimen combining gemcitabine, CHK1, and WEE1 inhibitors is strictly dependent on the timing of drug administration. Indeed, the maximum effect of the combination is

| Chemotherapy agents/radiotherapy | Intra S checkpoint | G2/M checkpoint | WEE1 and/or PKMYT1 experimentally proven involvement in cancer model |
|----------------------------------|-------------------|----------------|------------------------------------------------------------------|
| Actinomycin                      | No                | Yes [70, 71]   | WEE1 upregulation [71]                                           |
| Azacitidine                      | No                | Yes [72]      | NA                                                               |
| Bleomycin                        | No                | Yes [73]      | NA                                                               |
| Carboplatin                      | No                | Yes [74]      | NA                                                               |
| Cisplatin                        | No                | Yes [75, 76]  | WEE1 inhibition enhanced cytotoxicity [76, 77]                    |
| Cyclophosphamide                 | NA                | NA            | WEE1 upregulation [78]                                           |
| Cytarabine                       | Yes [79, 80]     | Yes [79, 80]  | WEE1 upregulation [38, 81]                                       |
| Clofarabine                      | Yes [35]         | No            | WEE1 inhibition enhanced cytotoxicity [35]                       |
| Daunorubicin                     | Yes [82]         | Yes [82]      | NA                                                               |
| Decitabine                       | No                | Yes [83]      | NA                                                               |
| Docetaxel                        | No                | Yes [84, 85]  | NA                                                               |
| Doxorubicin                      | No                | Yes [86]      | WEE1 upregulation [86]; WEE1 inhibition enhanced cytotoxicity [35] |
| Epirubicin                       | No                | Yes [87, 88]  | WEE1 inhibition enhanced cytotoxicity [89]                       |
| Epothilone                       | No                | Yes [90]      | NA                                                               |
| Etoposide                        | No                | Yes [91]      | WEE1 inhibition enhanced cytotoxicity [67]                       |
| Fluorouracil                     | Yes [92]         | No            | WEE1 inhibition enhanced cytotoxicity [92]                       |
| Fludarabine                      | Yes [80]         | No            | NA                                                               |
| Gemcitabine                      | Yes [80]         | No            | WEE1 upregulation [93]; WEE1 inhibition enhanced cytotoxicity [94] |
| Hydroxyurea                      | Yes [95]         | No            | WEE1 inhibition enhanced cytotoxicity [96]                       |
| Idarubicin                       | No                | Yes [97]      | NA                                                               |
| Irinotecan                       | No                | Yes [98]      | WEE1 inhibition enhanced cytotoxicity [99]                       |
|Mechloretamine                    | Yes [100]        | Yes [100]     | NA                                                               |
| Mercaptopurine                   | Yes [101]        | No            | NA                                                               |
| Methotrexate                     | Yes [102]        | No            | WEE1 inhibition enhanced cytotoxicity [103]                      |
| Mitoxantrone                     | No                | Yes [104]     | WEE1 inhibition enhanced cytotoxicity [78]                       |
| Oxiplatin                        | No                | Yes [105]     | WEE1 inhibition enhanced cytotoxicity [106]                      |
| Paclitaxel                       | No                | Yes [107]     | WEE1 inhibition enhanced cytotoxicity [108]                      |
| Pemetrexed                       | Yes [109]        | No            | WEE1 inhibition enhanced cytotoxicity [110]                      |
| Radiotherapy (ionizing radiation)| No                | Yes [111]     | WEE1 inhibition enhanced cytotoxicity [68]                       |
| Teniposide                       | Yes [112]        | Yes [112]     | NA                                                               |
| Thioguanine                      | Yes [113]        | Yes [113]     | WEE1 inhibition enhanced cytotoxicity [65]                       |
| Topotecan                        | No                | Yes [114]     | WEE1 inhibition enhanced cytotoxicity [115]                      |
| Vinblastine                      | No                | Yes [116]     | NA                                                               |
| Vincristine                      | No                | Yes [117]     | WEE1 inhibition enhanced cytotoxicity [118]                      |
obtained when gemcitabine and CHK1 inhibitors are 
 administered simultaneously (thus inducing replicative stress) and adavosertib is added at a later time [94]. Moreover, strong synergism has been observed by combining adavosertib with small molecules, including DDR-related inhibitors (CDK2 [89], CDK4-6 [149], etc.)
dates for a number of diverse therapeutic combinations. The activities exerted by the two kinases, and especially by CGK1 and PKMYT1, are suitable pharmacological targets for combination strategies in cancer. The broad spectrum of action of adavosertib is a suitable pharmacological target for combination strategies in cancer. The broad spectrum of activity exerted by the two kinases, and especially by WEE1, across the cell cycle, makes them good candidates for a number of diverse therapeutic combinations.

**WEE1 inhibitors from bench to bedside**

Several clinical studies are currently evaluating the efficacy of adavosertib on different aggressive and advanced tumors (Table 4).

The results of phase I trials showed that adavosertib is well tolerated both in single agent and in combination. Depending on the study, the maximum tolerated dose (MTD) was established between 150 and 225 mg orally twice per day for 2.5 days per 2 weeks \([156–158]\). The most common adverse events reported in the abovementioned studies were fatigue, nausea, vomiting, diarrhea, and hematologic toxicity. Moreover, correaltive studies performed on tumor biopsies confirmed in vivo the mechanism of action of adavosertib. Indeed, immunohistochemistry analyses showed a reduction of phospho-CDK1 (Tyr15) and an increase of DNA damages (phospho-H2AX) in cancer cells \([156, 157]\).

The phase II studies confirmed that adavosertib sensitizes cancer patients to different chemotherapy agents. Interestingly, adavosertib showed efficacy when combined with carboplatin in TP53-mutated ovarian cancer patients, refractory or resistant to first-line platinum-based chemotherapy \([159]\). Similar results were reported in platinum-resistant primary ovarian cancer patients after treatment with the combination of adavosertib and a single chemotherapeutic agent (carboplatin, paclitaxel, gemcitabine, or pegylated liposomal doxorubicin) \([160]\).

### Primary resistance and predictive markers of response to WEE1/PKMYT1 based therapies

Several DDR inhibitors have proved their efficacy against different cancer types in the preclinical and clinical settings \([161–165]\). Among them, WEE1 inhibitor seems to be the most effective ones, also favored by a relative low off-target toxicity. However, despite the number of studies and the promising results, few predictive markers of response have been identified. Recently, cyclin E level has been linked to the efficacy of adavosertib in breast cancer models \([89]\), with cyclin E-high cells, that generally show elevated chromosome instability, being more sensitive compared with cyclin E-low ones. Despite the reported low levels of WEE1 expression in breast cancer, chromosome instability, that has also prognostic potential mainly in grade 2 tumors \([89]\), may explain the effectiveness of WEE1 inhibitors, as supported by the predictive role of cyclin E. Our group and others showed that high PKMYT1 expression associates with reduced sensitivity to adavosertib, indicating a potential compensatory effect \([35, 166]\). Moreover, high-throughput proteomic profiling demonstrated that small cell lung cancer and ovarian cancer models with primary resistance to adavosertib express high levels of AKT/mTOR pathway molecules and phosphorylated S6 ribosomal protein \([137, 138]\). In acute leukemia models, the sensitivity to adavosertib has been recently linked to HDAC and MYC regulation. Indeed, by generating adavosertib-resistant models, the researchers found that resistant acute leukemia cell lines are dependent on increased HDAC activity for their survival, partly due to increased KDM5A function. In addition, gene expression analyses demonstrated a HDAC-dependent expression of MYC in the adavosertib-resistant cell lines \([167]\). These observations support the success of preclinical studies combining WEE1 and HDAC \([41, 130, 131]\) or bromodomain inhibitors \([150]\).

### Conclusion

Thanks to a constantly growing amount of preclinical and clinical data, our knowledge on cancer biology is increasing and, consequently, the list of cancer hallmarks has been progressively expanding. Recent findings demonstrated that cancer cells are characterized by functional and molecular alterations in crucial genes involved in the DDR pathway, which is fundamental for cell cycle regulation, DNA damages recognition, and repair. Functional alterations of DDR-gene have a deep impact on tumor progression and on the clinical outcome of cancer patients. Indeed, the efficacy of
Table 4  Clinical trials evaluating WEE1/PKMYT1 inhibitor in monotherapy or in combination for cancer therapy

| Study ID       | Study title                                                                 | Tumor | Interventions                                                | Status | Phase |
|---------------|------------------------------------------------------------------------------|-------|-------------------------------------------------------------|--------|-------|
| NCT02610075   | Phase Ib Study to Determine MTD of AZD1775 Monotherapy in Patients With Locally Advanced or Metastatic Solid Tumours. | S     | AZD1775                                                     | C      | 1     |
| NCT03668340   | AZD1775 in Women With Recurrent or Persistent Uterine Serous Carcinoma       | S     | AZD1775                                                     | R      | 2     |
| NCT02482311   | Safety, Tolerance, PK, and Anti-tumour Activity of AZD1775 Monotherapy in Patients With Advanced Solid Tumours | S     | AZD1775                                                     | C      | 1     |
| NCT02207010   | A Phase 0 Study of AZD1775 in Recurrent GBM Patients                         | S     | AZD1775                                                     | NA     | 1     |
| NCT03315091   | Phase I Study to Assess the Effect of Food on AZD1775 Pharmacokinetics in Patients With Advanced Solid Tumours | S     | AZD1775                                                     | C      | 1     |
| NCT01748825   | AZD1775 for Advanced Solid Tumors                                            | S/H   | AZD1775                                                     | ANR    | 1     |
| NCT02511795   | AZD1775 Combined With Olaparib in Patients With Refractory Solid Tumors      | S     | AZD1775 + Olaparib                                          | C      | 1     |
| NCT03313557   | AZD1775 Continued Access Study to Assess Safety and Tolerability for Patients Enrolled in AZD1775 Clinical Pharmacology Studies | S     | AZD1775                                                     | C      | 1     |
| NCT02593019   | Phase II, Single-arm Study of AZD1775 Monotherapy in Relapsed Small Cell Lung Cancer Patients | S     | AZD1775                                                     | NA     | 2     |
| NCT02688907   | Phase II, Single-arm Study of AZD1775 Monotherapy in Relapsed Small Cell Lung Cancer Patients With MYC Family Amplification or CDKN2A Mutation Combined With TP53 Mutation | S     | AZD1775                                                     | T      | 2     |
| NCT02087176   | A Placebo Controlled Study Comparing AZD1775 + Docetaxel Versus Placebo + Docetaxel to Treat Lung Cancer | S     | AZD1775 + Docetaxel                                         | T      | 2     |
| NCT03012477   | CISPLATIN + AZD-1775 In Breast Cancer                                        | S     | AZD1775 + Cisplatin                                         | ANR    | 2     |
| NCT02341456   | Phase Ib Study AZD1775 in Combination With Carboplatin and Paclitaxel in Adult Asian Patients With Solid Tumours | S     | AZD1775 + Carboplatin or Paclitaxel                        | C      | 1     |
| NCT02791919   | Wee1 Kinase Inhibitor AZD1775 and Combination Chemotherapy in Treating Children, Adolescents and Young Adults With Relapsed or Refractory Acute Myeloid Leukemia | H     | AZD1775 + Cytarabine or Filgrastim or Fludarabine Phosphate | W      | 1     |
| NCT02513563   | AZD1775 Plus Carboplatin-Paclitaxel in Squamous Cell Lung Cancer            | S     | AZD1775 + Carboplatin or Paclitaxel                        | R      | 2     |
| NCT03718143   | AZD1775 in Advanced Acute Myeloid Leukemia, Myelodysplastic Syndrome and Myelofibrosis | H     | AZD1775 + Cytarabine                                        | T      | 2     |
| NCT02585973   | Dose-escalating AZD1775 + Concurrent Radiation + Cisplatin for Intermediate/High Risk HNSCC | S     | AZD1775 + Cisplatin + Radiation                            | R      | 1     |
| NCT02087241   | Ph II Trial of Carboplatin and Pemetrexed With or Without AZD1775 for Untreated Lung Cancer | S     | AZD1775 + pemetrexed or carboplatin                      | T      | 2     |
| NCT02381548   | Phase I Trial of AZD1775 and Belinostat in Treating Patients With Relapsed or Refractory Myeloid Malignancies or Untreated Acute Myeloid Leukemia | H     | AZD1775 + Belinostat                                        | T      | 1     |
| NCT03333824   | Effects of AZD1775 on the PK Substrates for CYP3A, CYP2C19, CYP1A2 and on QT Interval in Patients With Advanced Cancer | S     | AZD1775                                                     | C      | 1     |
| NCT02906059   | Study of Irinotecan and AZD1775, a Selective Wee1 Inhibitor, in RAS or BRAF Mutated, Second-line Metastatic Colorectal Cancer | S     | AZD1775 + Irinotecan                                        | R      | 1     |
| NCT02037230   | Dose Escalation Trial of AZD1775 and Gemcitabine (+Radiation) for Unresectable Adenocarcinoma of the Pancreas | S     | AZD1775 + Gemcitabine+Radiation Therapy                    | C      | 1,2   |
| NCT02617277   | Safety, Tolerability and Pharmacokinetics of AZD1775 (Adavosertib) Plus MEDI4736 (Duvalumab) in Patients With Advanced Solid Tumours | S     | AZD1775 + Durvalumab                                        | ANR    | 1     |
| NCT02666950   | WEE1 Inhibitor AZD1775 With or Without Cytarabine in Treating Patients With Advanced Acute Myeloid Leukemia or Myelodysplastic Syndrome | H     | AZD1775 + Cytarabine                                        | C      | 2     |
| NCT01047007   | A Dose Escalation Study of MK1775 in Combination With 5-FU or 5-FU/CDDP in Patients With Advanced Solid Tumor (1775-005) | S     | AZD1775 + 5-FU or S-FU/CDDP                                | T      | 1     |
| NCT01164995   | Study With Wee-1 Inhibitor MK-1775 and Carboplatin to Treat                  | S     | AZD1775 + carboplatin                                       | NA     | 2     |
| Study ID   | Study title                                                                 | Tumor                        | Interventions                                | Status | Phase |
|-----------|------------------------------------------------------------------------------|------------------------------|----------------------------------------------|--------|-------|
| NCT02448329 | Study of AZD1775 in Combination With Paclitaxel, in Advanced Gastric Adenocarcinoma Patients Harboring TP53 Mutation as a Second-line Chemotherapy | S                             | AZD1775 + paclitaxel                         | R      | 2     |
| NCT02508246 | WEE1 Inhibitor MK-1775, Docetaxel, and Cisplatin Before Surgery in Treating Patients With Borderline Resectable Stage III-IVB Squamous Cell Carcinoma of the Head and Neck | S                             | AZD1775 + Cisplatin + Docetaxel              | C      | 1     |
| NCT03253679 | AZD1775 in Treating Patients With Advanced Refractory Solid Tumors With CCNE1 Amplification | S                             | AZD1775                                    | R      | 2     |
| NCT01076400 | A Study of MK-1775 in Combination With Topotecan/Cisplatin in Participants With Cervical Cancer (Mk-1775-008) | S                             | AZD1775 + Topotecan or Cisplatin             | T      | 1,2   |
| NCT02196168 | Cisplatin With or Without WEE1 Inhibitor MK-1775 in Treating Patients With Recurrent or Metastatic Head and Neck Cancer | S                             | AZD1775 + Cisplatin                         | T      | 2     |
| NCT02101775 | Gemcitabine Hydrochloride With or Without WEE1 Inhibitor MK-1775 in Treating Patients With Recurrent Ovarian, Primary Peritoneal, or Fallopian Tube Cancer | S                             | AZD1775 + Gemcitabine                       | ANR    | 2     |
| NCT03028766 | WEE1 Inhibitor With Cisplatin and Radiotherapy: A Trial in Head and Neck Cancer | S                             | AZD1775 + Cisplatin + Radiotherapy          | ANR    | 1     |
| NCT01357161 | A Study of MK-1775 in Combination With Paclitaxel and Carboplatin Versus Paclitaxel and Carboplatin Alone for Participants With Platinum-Sensitive Ovarian Tumors With the PS3 Gene Mutation (MK-1775-004) | S                             | AZD1775 + paclitaxel + carboplatin          | C      | 2     |
| NCT03284385 | Testing AZD1775 in Advanced Solid Tumors That Have a Mutation Called SETD2 | S                             | AZD1775                                    | R      | 2     |
| NCT00648648 | A Dose Escalation Study of MK-1775 in Combination With Either Gemcitabine, Cisplatin, or Carboplatin in Adults With Advanced Solid Tumors (MK-1775-001) | S                             | AZD1775 + Gemcitabine or Cisplatin or Carboplatin | C      | 1     |
| NCT02194829 | Paclitaxel Albumin-Stabilized Nanoparticle Formulation and Gemcitabine Hydrochloride With or Without WEE1 Inhibitor MK-1775 in Treating Patients With Previously Untreated Pancreatic Cancer That Is Metastatic or Cannot Be Removed by Surgery | S                             | AZD1775 + Gemcitabine + paclitaxel         | ANR    | 1,2   |
| NCT02576444 | Olaparib Combinations                                                      | S                             | AZD1775 + olaparib                          | ANR    | 2     |
| NCT04197713 | Testing the Sequential Combination of the Anti-cancer Drugs Olaparib Followed by Adavosertib (AZD1775) in Patients With Advanced Solid Tumors With Selected Mutations and PARP Resistance, STAR Study | S                             | AZD1775 + olaparib                          | ANR    | 1     |
| NCT01922076 | Adavosertib and Local Radiation Therapy in Treating Children With Newly Diagnosed Diffuse Intrinsic Pontine Gliomas | S                             | AZD1775 + Radiation Therapy                 | ANR    | 1     |
| NCT03579316 | Adavosertib With or Without Olaparib in Treating Patients With Recurrent Ovarian, Primary Peritoneal, or Fallopian Tube Cancer | S                             | AZD1775 + olaparib                          | R      | 2     |
| NCT02095132 | Adavosertib and Irinotecan Hydrochloride in Treating Younger Patients With Relapsed or Metastatic Solid Tumors | S                             | AZD1775 + Irinotecan or Irinotecan Hydrochloride | R      | 1,2   |
| NCT03345784 | Adavosertib, External Beam Radiation Therapy, and Cisplatin in Treating Patients With Cervical, Vaginal, or Uterine Cancer | S                             | AZD1775 + Cisplatin + Radiation (External Beam Radiation Therapy) | R      | 1     |
| NCT01849146 | Adavosertib, Radiation Therapy, and Temozolomide in Treating Patients With Newly Diagnosed or Recurrent Glioblastoma | S                             | AZD1775 + Radiation Therapy + Temozolomide | R      | 1     |
| NCT02937818 | A Phase II, Study to Determine the Preliminary Efficacy of Novel Combinations of Treatment in Patients With Platinum Refractory Extensive-Stage Small-Cell Lung Cancer | S                             | AZD1775 + carboplatin                        | ANR    | 2     |
| NCT02546661 | Open-Label, Randomised, Multi-Drug, Biomarker-Directed, Phase 1b Study in Pts w/ Muscle Invasive Bladder Cancer | S                             | AZD1775 + Duvalumab                         | ANR    | 1     |
| NCT02659241 | Adavosertib Before Surgery in Treating Patients With Advanced High Grade Ovarian, Fallopian Tube, or Primary Peritoneal Cancer | S                             | AZD1775                                    | R      | 1     |
| NCT02272790 | Adavosertib Plus Chemotherapy in Platinum-Resistant Epithelial Ovarian, Fallopian Tube, or Primary Peritoneal Cancer | S                             | AZD1775 + Paclitaxel or Carboplatin          | ANR    | 2     |
standard of care chemo/radiotherapy regimens depends on the generation of DNA damages in proliferating malignant cells. In this scenario, the overexpression or uncontrolled activation of DDR pathways has been showed to protect cancer cells from the therapeutic effect of DNA damaging agents. Moreover, a large number of preclinical studies highlighted that cancer cells depend on the functionality of DDR-gene functionality. Preclinical data has proven the efficacy of DDR inhibition in different kinds of hematological and solid tumors, both as monotherapy and in combination with a wide number of DNA damaging agents. Among DDR inhibitors, the most effective once are those targeting PARP1 and WEE1 family kinases. The effectiveness of PARP1 inhibitors is however dependent on homologous recombination (HR) repair deficiency while WEE1 family kinases inhibitors seems to have a widespread efficacy independently from a specific the genetic background. Indeed, cancer cells seem to be strictly dependent on the functionality of WEE1/PKMYT1 kinases to survive, especially those with alterations targeting the G1 checkpoint. WEE1/PKMYT1 kinases are involved in different biological processes and they seem to play diverse roles in nonmalignant and in cancer cells. Indeed, they control cell cycle regulation and genetic stability in nonmalignant cells and for these reasons act as tumor suppressor genes. Conversely, their ability of promote DNA damages repair and cell cycle control makes them act as pseudo-oncogenes in cancer cells. Several molecular studies showed that malignant cells have high expression level of WEE1 and PKMYT1, which has become a good prognostic biomarker for chemo/radiotherapy regimens. However, we currently lack information regarding predictive markers of response to WEE1/PKMYT1 inhibitors. Large preclinical and clinical studies should be conducted in order to identify specific molecular backgrounds in which the use of WEE1/PKMYT1 inhibitors may be recommended. The identification of molecular vulnerabilities in cancer patients will be fundamental to design novel therapeutic regimens using WEE1/PKMYT1 inhibitors in a chemo/radiotherapy-free, synthetic lethality-based approach.

Abbreviations
ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; BC: Breast cancer; C: Chemotherapy; CC: Colorectal cancer; CN: Copy number; CNA: Copy number alteration; CLL: Chronic lymphocyte leukemia; CML: Chronic myeloid leukemia; DDR: DNA damage response; DLBCL: Diffuse large B cell lymphoma; ES: Esophageal cancer; GC: Gastric cancer; GL: Gliomas; GLB: Glioblastoma; HC: Hepatocellular carcinoma; HR: Homologous recombination; HNSCC: Head and neck squamous cell carcinoma; LC: Lung cancer; M: Monotherapy; MaM: Malignant melanoma; MCL: Mantle cell lymphoma; MM: Multiple myeloma; MPF: Mitotic promoting factor; N: Neuroblastoma; NSCLC: Non-small-cell lung cancer; OC: Ovarian cancer; PC: Pancreatic cancer; R: Radiotherapy; SAC: Spindle assembly checkpoint; TNBC: Triple-negative breast cancer

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AGLDR, CC, and GS drafted the first version of the manuscript and created the figures. GM critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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GM has competing interests with Novartis, BMS, Roche, Pfizer, ARIAD, and MSD. The other authors declare that they have no competing interests.
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