Cancer Letters 383 (2016) 41–52

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Inhibition of ATR-dependent feedback activation of Chk1 sensitises cancer cells to Chk1 inhibitor monotherapy

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ARTICLE INFO

Article history:
Received 18 July 2016
Received in revised form
7 September 2016
Accepted 8 September 2016

Keywords:
Chk1
ATR
ATM
DNA-PK
Kinase inhibitor
Replication stress

ABSTRACT

The Chk1 and ATR kinases are critical mediators of the DNA damage response pathway and help protect cancer cells from endogenous and oncogene induced replication stress. Inhibitors of both kinases are currently being evaluated in clinical trials. Chk1 inhibition with V158411 increases DNA damage and activates the ATR, ATM and DNA-PKcs dependent DNA damage response pathways. Inhibiting ATR, ATM and/or DNA-PKcs has the potential to increase the therapeutic activity of Chk1 inhibitors. ATR inhibition but not ATM or DNA-PKcs inhibition potentiated the cytotoxicity of V158411 in p53 mutant and wild type human cancer cell lines. This increased cytotoxicity correlated with increased nuclear DNA damage and replication stress in a dose and time dependent manner. γH2AX induction following Chk1 inhibition protected cells from caspase-dependent apoptosis. Inhibition of ATR increased Chk1 inhibitor induced cell death independently of caspase activation. The effect of ATR, ATM and/or DNA-PK inhibition on Chk1 inhibitor induced replication stress was dependent on the concentration of Chk1 inhibitor. ATR inhibition potentiated Chk1 inhibitor induced replication stress and cytotoxicity via the abrogation of ATR-dependent feedback activation of Chk1 induced by Chk1 inhibitor generated replication stress. This study suggests that combining an ATR inhibitor to lower the threshold by which a Chk1 inhibitor induces replication stress, DNA damage and tumour cell death in a wide range of cancer types may be a useful clinical approach.

Introduction

Oncogene activation and/or the loss of tumour suppressor proteins drive tumour cell proliferation resulting in increased replication stress. Replication stress can arise through numerous mechanisms including a combination of deregulated origin firing, increased DNA damage through increased ROS production, collision of active replication forks with transcription factories, and the chromatin context of replicating DNA. Loss of the controls restricting the onset of S-phase results in an unscheduled and uncoordinated replication burst that is not matched by the supply of components necessary for replication fork progression resulting in replication fork stalling, fork collapse and the generation of DNA double strand breaks (DSBs) [11,14,46].

A series of sophisticated cell cycle checkpoint and DNA repair pathways (collectively termed the DNA damage response (DDR)) have evolved to allow cells to cope with the high levels of DNA damage sustained by the genome from endogenous and environmental sources on a daily basis [10,41,52]. The phosphatidylinositol 3-kinase-like kinase (PIKK) family members ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3 related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) along with the checkpoint kinases Chk1 and Chk2 are key signalling components of the DDR. ATR and Chk1 kinases are critical for the cellular response to replication stress. Replication fork stalling results in the generation of tracts of ssDNA as the replicative helicase continues to unwind DNA in front of the stalled DNA polymerase. Binding of ssDNA by RPA then recruits ATR and its regulatory subunit ATRIP along with additional regulatory factors including TOPBP1, Rad17, Claspin, 9-1-1 complex and Tim/Tipin. Activated ATR subsequently phosphorylates serine 317 and serine 345 in the Ser/Gln cluster domain of Chk1 [33,45]. These relieve the auto-inhibitory effect of the C-terminal CM2 domain on the Chk1 catalytic site allowing *cis* auto-phosphorylation on serine 296 [32] and subsequent downstream signalling by Chk1. In addition to control by ATR, Chk1 can be phosphorylated by AKT on serine 280 resulting in inhibition of Chk1 [21] and CDK1 on serine 286 and 301 thereby preventing its

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http://dx.doi.org/10.1016/j.canlet.2016.09.024
0304-3835/

activation by ATR [40]. Activation of ATR and Chk1 induces cell cycle arrest (through the degradation of Cdc25 phosphatases), fork stabilisation and inhibition of cleavage by the Mus81-Eme1-Mre11 nucleases, activation of homologous recombination repair and inhibition of new origin firing. Stabilisation and protection of replication forks allows fork restart once the source of fork arrest has been removed or bypassed by DNA damage mechanisms.

Chk1 has been demonstrated to be important for replication origin firing [15,27,35], high rates of replication fork progression and replication fork stabilisation [42,44]. Chk1 inhibition results in increased CDK2 activity and unscheduled replication origin firing leading to replication slowing and stalling of forks. Such forks are normally repaired by homologous recombination (HRR) and subsequent cleavage by Mus81, Eme2 and Mre11 generates DNA double strand breaks (DSBs) [13,47]. Chk1 plays a critical role in regulating HRR by directing the localisation of RAD51 to the invading repair strand and inhibition of Chk1 therefore hampers the repair of the DNA DSBs [2,43].

The role of ATR, ATM and DNA-PKcs in an unperturbed S-phase is not fully understood. ATR functions upstream of, and activates, Chk1 and plays an important role in preventing genomic instability [13,23,34,44]. Proliferation promoting events such as Myc or Ras transformation render cells more sensitive to Chk1 or ATR inhibitors and appear critical in countering replication stress [19,31]. ATR prevents the global exhaustion of RPA by excess ssDNA by suppressing dormant origin firing [48] and co-ordinating RRM2 expression with origin firing [6]. Evidence for a role of ATM and DNA-PKcs in the replication stress response is more limited. ATM is activated by DSBs via the MRE11–RAD50–NBS1 (MRN) complex resulting in activation of Chk2 and p53. Whether DSBs generated by stalled replication fork cleavage activate ATM remains controversial though one study suggests that under these conditions ATM promotes HRR and is required for the recovery and restart of collapsed replication forks [49]. DNA-PKcs, in concert with Ku70 and Ku80, functions in DSB repair by the non-homologous end joining (NHEJ) pathway. DNA-PKcs can phosphorylate the RPA32 subunit of the heterotrimeric ssDNA binding complex RPA (a heterotrimer of RPA70, RPA32 and RPA14). Phosphorylation of RPA32 on serine 4 and 8 by DNA-PKcs as well as serine 33 by ATR regulates replication fork restart, new origin firing, HRR and replication catastrophe and cell survival in response to replication stress [1,24].

Chk1 and ATR inhibitors have demonstrated single agent activity in a range of cancer cell lines [3–5,7,9,38] and genetically engineered tumour models [12,31,50] characterised as harbouring defects in DNA repair pathways or with high levels of replicative stress. Inhibition of Chk1 induces a rapid (in under 1 h) decrease in pChk1 (S296) autophosphorylation [26] and is a robust biomarker suitable for monitoring target engagement in clinical studies [50]. In the absence of exogenous DNA damage, inhibition of Chk1 causes phosphorylation of ATR targets including Chk1 on serine 317 and 345 [4,44]. Inhibitors of Chk1 or ATR are in pre-clinical and clinical development with the focus predominantly on their ability to potentiate the cytotoxicity of genotoxic chemotherapy drugs (such as gemcitabine, irinotecan or cisplatin) or ionising radiation (reviewed in Refs. [8,28]). This approach is currently being evaluated in the clinic in a range of Phase I and II trials. Here we investigate the potential of small molecule inhibitors of ATR, ATM and DNA-PKcs to potentiate Chk1 inhibitor induced replication stress thereby increasing their therapeutic potential and clinical utility.

Materials and methods

Cell lines and cell culture

Cell lines were purchased from the American Type Culture Collection (ATCC), established as a low passage cell bank and then routinely passaged in our laboratory for less than 3 months after resuscitation. These were routinely cultured in media containing 10% FCS and 1% penicillin/streptomycin at 37 °C in a normal humidified atmosphere supplemented with 5% CO_2. Cells were authenticated by STR profiling (LGC Standards, Teddington UK).

Compounds

Solid stocks of VX-970 (VE-822, 10 mM in DMSO), KU-60019 (10 mM in DMSO) and NU7441 (5 mM in DMSO) were purchased from Selleckchem and prepared as indicated. V158411 was from Vernalis Research and prepared as a 20 mM DMSO stock. Compounds were serially diluted in DMSO to 500× or 1000× then to 5× or 10× in complete media before addition to cells to yield a 1× final concentration.

Antibodies

Antibodies against Chk1, pChk1 (S317), pChk2 (T68), Chk2, pH2AX (S139), pCdc2 (Y15) and GAPDH, were purchased from Cell Signalling Technologies; pChk1 (S296) and RPA32 from Abcam; pRPA32 (S4/S8) from Bethyl Laboratories and pH2AX (S139) (clone JBW301) from Merck Millipore. Antibodies were used at the manufacturer's recommended dilutions.

Immunoblotting

Cells were washed once with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche). Protein concentration was determined using a BCA kit (Pierce). Equal amounts of lysate were separated by SDS-PAGE and western blot analysis conducted using the antibodies indicated above. Densitometric analysis was conducted with ImageJ software (NIH).

Single cell immunofluorescent imaging

Following compound treatment, cells were fixed in 3.7% paraformaldehyde in PBS at room temperature for 15 min, washed with PBS, blocked with 5% normal goat serum in 0.3% Triton X100 in PBS for 1 h at room temperature then incubated with primary antibody diluted in antibody dilution buffer (1% BSA, 0.3% Triton X100 in PBS) at 4 °C for 16 h. Cells were washed with PBS then incubated with an Alexa-labelled secondary antibody (1:500, Life Technologies) and Hoechst 33342 (1 μg/ml) in antibody dilution buffer at room temperature for 60 min. Following washing with PBS, cells were imaged with an Operetta high content imaging system (Perkin Elmer) at 10× or 20× magnification and analysed using Harmony software (Perkin Elmer).

Apoptosis

Cleaved caspase-3 (CC3) was detected in fixed cells using a monoclonal antibody to the amino-terminal residues adjacent to Asp175.

Cell proliferation assay

5000 cells per well were seeded in 96-well plates and incubated overnight. Cells were treated with a 10-point titration of compound for 72 h. The effect on cell proliferation was determined with sulphorhodamine B (SRB) after fixation with 10% trichloroacetic acid and read on a Victor plate reader (Perkin Elmer). GI_{50} values were calculated in Microsoft EXCEL using an XLFit software add-in (ID Business Solutions).

High content live cell imaging

Cells were seeded in 96 well CellCarrier plates (Perkin Elmer) and allowed to attach for 24 h before addition of compound. Images were acquired as indicated using the brightfield and digital phase imaging modalities on the Operetta high content imaging system at 10× magnification. Temperature was maintained at 37 °C and CO_2 at 5% with the live cell chamber module.

Cell confluency was determined from the brightfield images using the Find Texture Regions building block coupled with PhenoLOGIC texture based segmentation in the Harmony software. Cell number was determined by analysis of the digital phase images with the Find Cells building block in Harmony.

Results

Chk1 inhibition induces DNA damage and activates DDR signalling in human cancer cells

V158411 (Chk1i) is a potent, selective inhibitor of the checkpoint kinase Chk1 discovered using structure-based drug design and demonstrates activity both as a monotherapy and in combination with a range of cytotoxic chemotherapeutic agents [26,36]. Chk1 is activated through phosphorylation of S317 and S345 by ATR. Two other signalling components of the DDR are ATM which phosphorylates Chk2 on T68 and DNA-PKcs which phosphorylates RPA32 on S4/S8, the later a marker of increased replication stress. In HT29 and U2OS cells, Chk1i treatment induced a time dependent