DNA-PK in CLL Chemotherapy

L. Amrein, D. Davidson, R. Aloyz, and L. Panasci

1 Introduction

DNA is the principal target of many conventional anticancer agents, and inhibition of DNA repair is one of the most promising strategies in novel cancer therapy. Many studies demonstrated that nonhomologous end-joining (NHEJ) repair pathway proteins, especially DNA-dependent protein kinase (DNA-PK), is an attractive and effective target for the sensitization of cancer cells, including the most common type of leukemia in western countries, chronic lymphocytic leukemia (CLL), to DNA double-strand break (DSB)-inducing agents used in conventional cancer therapy. Nevertheless, promising results obtained *in vitro* cannot be translated to the clinic yet due to the nature of the DNA-PK inhibitors which are either nonspecific, for the first class of inhibitors, or degraded/eliminated from the human body before reaching the tumor site for the newer specific DNA-PK inhibitors.

2 CLL and Conventional Therapeutic Treatments

B-cell CLL is a complex disease characterized by actively dividing B-lymphocyte in the lymph nodes and bone marrow [1, 2] as well as the accumulation of quiescent lymphocytes in the peripheral blood of affected patients [3]. Although CLL has been described for a long time the cell of origin is unknown. This disease is the most common leukemia in western countries with approximately 15,500 new diagnoses and over than 4,000 deaths estimated per year in the United States only [4]. CLL cells express B-cell immunophenotypic markers, such as CD19, CD20, and CD23, along

L. Amrein • D. Davidson • R. Aloyz (✉) • L. Panasci
Lady Davis Institute, 3755 Cote ste Catherine, Montreal, QC, Canada
e-mail: raquel.aloyz@mcgill.ca

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with the T-cell marker CD5. CLL lymphocytes are also characterized by the expression of very low amounts of surface immunoglobulin. The clinical course is very heterogeneous with overall survival ranging from several months to more than 15 years [5]. Several biological factors have been linked with the clinical heterogeneity of CLL. These include the Rai/Binet stage, parameters of cell division, β 2-microglobulin, somatic mutations of immunoglobulin heavy-chain variable region (IGHV) genes, cytogenetic aberrations (deletion 11, deletion 17, deletion 13, and trisomy 12), and expression of CD38 and ZAP70 (reviewed in 3, 6). Although the majority of patients are asymptomatic at diagnosis, the incessant accumulation of B-CLL lymphocytes leads to symptomatic disease requiring therapy. Conventional chemotherapeutic drugs used in the treatment of CLL include nitrogen mustard analogue (chlorambucil (CLB), cyclophosphamide, and bendamustine) or the nucleotides analogue fludarabine. Chemo-immunotherapy combines chemotherapeutic drugs with monoclonal antibodies (immunotherapy) such as combination therapy with fludarabine, cyclophosphamide, and the CD20 monoclonal antibody rituximab (FCR) which is now a standard of care, offering good overall response rates (ORR), complete remission (CR) rates, and increased median progression-free survival (PFS) [7, 8]. However, FCR is not suitable for all patients, has significant side effects, and appears too toxic for some elderly patients. Given that CLL predominates in the elderly community, the potential toxicity of therapeutic regimens is an important issue. In addition, comparative clinical trial of fludarabine and cyclophosphamide (FC) against fludarabine alone suggested a higher incidence of chemotherapy-related myeloid neoplasia (a long-term toxicity) after FC than after fludarabine treatment [9]. Results from another clinical trial in CLL patients after initial therapy with CLB compared with fludarabine in patients over 65 years of age demonstrate that despite higher ORR and CR rates, this did not translate into improved PFS or overall survival [10]. However, due to the fact that therapeutic regimens come with toxic side effects, some progress has been achieved within the last decade. Nevertheless, another significant problem in treating CLL is that although patients often initially respond to conventional treatment, they eventually become resistant to the drugs and even if new strategies comprising chemotherapy combinations or chemo-immunotherapy have been used, CLL is still considered as an incurable disease [11].

3 DNA Damage and DNA Repair Mechanism

Cells are continuously subjected to numerous exogenous (radiation and environmental genotoxic compounds) and endogenous (intermediate products from normal metabolism and errors during replication process) sources of DNA damages. To overcome these threats, cells developed robust, complex, and highly conserved DNA-damage surveillance network, beginning with rapid and efficient detection of the lesions followed by the induction of complex protein signaling cascades leading to DNA repair mechanisms to ensure genomic integrity and stability [12]. Defects in signaling and repair of DNA damage are causally linked with the development of

genomic instability and human cancer. One of the most deleterious forms of DNA damage, the DNA double strand breaks (DSB) is repaired by two major DNA repair systems in eukaryotic cells, the homologous recombination (HR) and the NHEJ repair pathways [13, 14]. HR is error-free, depends on the presence of sister chromatids to provide a DNA template identical to the damaged one, and thus is active in late S and G2 phases of the cell cycle. NHEJ does not require a template, thereby; it is active throughout the cell cycle and is the predominant mechanism in higher eukaryotes [15, 16]. DNA-PK is a key component of the NHEJ pathway which plays an important role in V(D)J recombination and in the repair of DNA DSBs [17–20]. The carboxy-terminal region of DNA-PKcs contains a catalytic domain similar to the phosphatidylinositol 3-kinase (PI3K) superfamily involved in cell cycle control, DNA repair, and DNA damage responses [21]. DNA-PK acts as a sensor of DSB during NHEJ since it is activated to bind to the ends of DNA and targets other factors to the site of damage [22]. DNA-PK is a nuclear serine/threonine protein kinase comprising a DNA-binding subunit, the Ku autoantigen, and a large catalytic subunit (460 kDa), DNA-PKcs. The Ku autoantigen is a heterodimer of the Ku70 and Ku80 proteins that binds to DNA double-strand ends and recruits DNA-PKcs [23–25]. This active DNA-PK complex then acquires the capacity to phosphorylate many DNA-bound proteins containing Ser/Thr-Gln motif including c-jun, p53, Ku70, Ku80, X-ray cross-complementing group 4 (XRCC4), and DNA-PKcs itself [20, 26–30]. Mutations in either DNA-PKcs or in the Ku80 result in DSB repair defects that manifest themselves as X-ray sensitivity and impaired V(D)J recombination [31, 32]. In addition, previous reports showed that mutant cells deficient either in DNA-PKcs or in the Ku DNA-end binding activity also exhibit significant hypersensitivity to DSB-inducing agents [33, 34]. DNA-PKcs plays a central role in regulation of NHEJ since it remains quiescent until activation by DNA ends [24]. Many *in vitro* and *in vivo* phosphorylation sites of DNA-PKcs have been identified. The importance of DNA-PKcs autophosphorylation in the PQR cluster (Ser 2023–Ser 2056), the ABCDE cluster (Thr 2609–Thr 2647), Thr 3950, and Ser 3205 during the NHEJ process has been well defined [35–38].

4 DNA-PK Inhibitors

Wortmannin, vanillin, and quercetin are natural product classes inhibiting PI3K family members including DNA-PKcs [39, 40]. Wortmannin forms covalent adduct in a conserved lysine residue in the kinase domain of DNA-PKcs [41], while quercetine targets the ATP-binding site of the kinase resulting in irreversible inhibition of DNA-PK activity [42]. A more potent synthetic derivative of quercetine, LY294002 developed by Lilly Research Laboratories, also inhibits enzymatic phosphorylation of lipids and proteins [42]. These compounds were used *in vitro* to assess DNA-PK inhibition but due to their nonspecificity for this kinase a number of more specific DNA-PK inhibitors have been developed. As expected for specific DNA-PK inhibitors, compounds developed by ICOS Corporation (IC86621, IC486154, IC87102,

IC87261) directly inhibit the repair of DNA DSBs [43]. Research performed by KuDOS Pharmaceuticals Ltd led to the development of synthetic and specific DNA-PK inhibitors. They utilized LY294002 as a template and have identified several molecules including NU7026 and NU7441 with good selectivity for DNA-PK over other PI3K members. These inhibitors have demonstrated *in vitro* radio- and chemo-sensitization in several human tumor (including leukemia) cell lines [44–46]. Contrarily to wortmannin, the ICOS and KuDOS compounds target the DNA-PKcs ATP-binding pocket improving potency and selectivity for DNA-PK over other PI3K family enzymes. Also, wortmannin is an irreversible DNA-PKcs inhibitor while the inhibition by ICOS and KuDOS compounds is reversible [46].

5 Importance of DNA Repair in CLL

As stated above, chemotherapeutic drugs used for clinical treatment of CLL patients are DNA-damaging agent. The primary response of cells with excessive DNA damage is to repair the lesions. Maintenance of the switching mechanisms that shift cells from DNA repair to apoptosis is of central importance for avoiding progression to malignancy. It has been proposed that enzyme-mediated repair of DSBs is a major mechanism of resistance to both ionizing radiation (IR) and drugs that cause DSBs as intermediates in repair processes [12]. *In vitro* experiments demonstrating cross resistance between nitrogen mustards and mitomycin C in B-CLL lymphocytes support the concept that cross resistance to different DNA-damaging agents involves accelerated DNA repair [47]. Also, B-CLL cells resistant to γ-radiation-induced apoptosis are completely resistant to apoptosis induced by neocarzinostatin and etoposide, compounds that specifically cause DNA DSBs [48]. Because DSBs are repaired by HR and NHEJ, inhibitors of key component of these two pathways have been investigated in combination with conventional drugs in B-CLL lymphocytes. For example, inhibition of c-abl (this non-receptor protein kinase phosphorylates and activates Rad51, a key component of HR) sensitizes B-CLL lymphocytes to CLB and fludarabine *in vitro* [49–51]. One of these investigations led to a phase I clinical trial in CLL patients where the combination of CLB and imatinib resulted in a 45 % response rate in a heavily pretreated population with minimal toxicity [52]. NHEJ, the other major DNA repair pathway, is also an attractive target to overcome resistance in B-CLL.

6 Role of DNA-PK for CLL Treatment

Despite many studies with various human cell lines, the first study of regulation of DNA-PK activity and DNA-PKcs protein expression in freshly isolated primary B-lymphocytes was done in 1997. It was demonstrated for the first time that DNA-PK activity could be measured in primary quiescent human B-CLL lymphocytes and that the level of DNA-PK activity varied considerably amongst CLL samples with higher

expression in previously clinically treated patient samples [53–55]. These results were concordant with our previous report demonstrating that lymphocytes from treated-resistant patients have an enhanced capacity to remove cross-links compared with those from untreated patients [56]. Similarly, changes in DNA-PK activity correlated with CLB resistance while sensitivity to topoisomerase II inhibitors (doxorubicin and etoposide) correlated with DNA-PKcs protein expression suggesting that DNA-PK plays an important role in regulating CLL response to DNA-damaging agents [54, 55, 57]. Also, inhibition of CLB-induced HR repair in CLL lymphocytes resulted in an increased DNA-PKcs autophosphorylation [51]. Major determinants of therapeutic resistance in B-CLL are deletion of p53 (chromosome 17), ATM (chromosome 11) gene, and/or mutation in p53 resulting in a dysfunctional p53-dependent DNA damage response pathway. B lymphocytes isolated from these CLL patients expressed higher DNA-PK activity than patient without these genetic abnormalities [58]. In accordance with the concept that regulation of DNA-PK activity occurs partially at the Ku level, the mechanism of regulation of DNA-PK activity in B-CLL lymphocytes proceeds initially through a variation in the Ku DNA end-binding activity and probably the expression of an altered form of the heterodimer. Furthermore, Ku expression and function in B-CLL cells play a pivotal role during the acquisition of resistance [53, 54]. These findings open the field for the investigation of NHEJ repair pathway inhibition to improve treatment and/or overcome the resistance to treatment in B-CLL patients.

7 DNA-PK Inhibitors to Improve CLL Treatment

Inhibition of DNA-PK and the consequent inhibition of DSB repair were speculated to be the mechanisms whereby wortmannin potentiates the cytotoxicity of ionizing radiation in a Chinese Hamster Ovary cell line [59]. In primary B-CLL lymphocytes, wortmannin enhanced CLB cytotoxicity and γ-radiation-induced apoptosis in cells sensitive and most importantly in lymphocytes resistant to DSB-inducing agent. Sensitivity to these DNA-damaging agents was associated with inhibition of DNA repair and in resistant lymphocytes, the increase in CLB sensitivity correlated with the ability of wortmannin to inhibit DNA-PK activity [48, 55]. Vanillin, another natural but nonspecific DNA-PK inhibitor, sensitizes B-CLL cells from drug-sensitive and -resistant lymphocytes to fludarabine but the authors did not find any correlation between either DNA-PKcs expression and fludarabine sensitivity or DNA-PKcs expression and inhibitor sensitization [60]. Nevertheless, wortmannin and vanillin inhibit all the PI3K family members rendering it difficult to determine the exact role of DNA-PK and the drug sensitization induced by these agents in B-CLL lymphocytes. Synthesis of specific DNA-PK inhibitors made possible studies of the real impact of DNA-PK inhibition on drug resistance and its potential advantage in CLL therapy. Although NU7026, a specific DNA-PK inhibitor, was not toxic by itself in primary B-CLL lymphocytes and a B-CLL cell line, when combined with γ-irradiations or CLB treatment, NU7026 inhibited NHEJ-mediated DNA repair and DNA-PKcs phosphorylation leading both sensitive and resistant cells to undergo

apoptosis after DNA damage [48, 61]. These data confirmed results obtained with wortmannin suggesting that DSB end-ligation activity was dependent on DNA-PK activity in these cells. Importantly in primary B-CLL cells, NU7026 inhibits CLB-induced DNA-PKcs autophosphorylation but did not affect CLB-induced ATM (another PI3K family member implicated in DSB repair pathway) phosphorylation, suggesting that at the doses used, NU7026 is a specific DNA-PK inhibitor in these cells [61]. NU7441, another DNA-PK inhibitor developed from LY294002, increased CLB and fludarabine-induced DNA damage and apoptosis resulting in B-CLL cell sensitization to these conventional drugs [58, 60]. Furthermore, simultaneous inhibition of both the HR and the NHEJ (by specific inhibition of DNA-PK) pathways potentiated the synergistic effect of either inhibitor alone on CLB cytotoxicity in CLL lymphocytes and was associated with an increase in CLB-induced DNA damage and decreased DNA repair [51].

8 Limitation for DNA-PK Therapy

All the studies stated above demonstrated that DNA-PK inhibition enhances the effects of DNA-damaging compounds by preventing repair through the NHEJ pathway in primary B-CLL lymphocytes *in vitro*. All these results have clinical interest and can potentially increase therapeutic treatment for CLL patients. Unfortunately, natural compounds such as wortmannin and vanillin are not specific enough and current specific DNA-PK inhibitors such as NU7026 have poor *in vivo* bioavailability, largely due to rapid oxidative metabolism in the liver [62].

9 Conclusion

The primary response of cells to DNA damage is to repair the lesions. The balance between DNA repair and apoptosis is of central importance for avoiding the occurrence of cancer. The various mechanisms of DNA repair, which are important to maintain healthy cells, ironically can become the front line of resistance for malignant cells. Indeed, there is a dynamic interaction between the two major DNA repair pathways, HR and NHEJ, in CLL lymphocytes in response to drug-induced DNA damage and overactive NHEJ DSB repair allows human B-CLL cells to escape apoptosis in the presence of chemotherapy-induced DNA damage. The development of specific inhibitors of key proteins of DNA repair pathway, especially DNA-PK inhibitors, has helped circumvent the problem of resistance to drugs treatment at least *in vitro* and has important clinical implications. However, the problem which faces us is now to translate these discoveries from the bench to the bed side. The current step is to be able to optimize the structure of existing DNA-PK inhibitors to improve their *in vivo* properties for clinical administration.

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