Cytotoxicity of anticancer drugs and PJ-34 (poly(ADP-ribose)polymerase-1 (PARP-1) inhibitor) on HL-60 and Jurkat cells

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Abstract

Background. The majority of the clinical trials with poly(ADP-ribose)polymerase-1 (PARP-1) inhibitors were conducted or are ongoing in patients with solid tumors, while trials with leukemia patients are less frequent. Surprisingly scarce data is available on the combinatory effects of PARP inhibitors with DNA damaging antitumor drugs in leukemic cells (primary cells or established lines).

Objectives. The aim of the present study was to assess the effect of PJ-34 (PARP-1 inhibitor) on the cytotoxicity of different antileukemic drugs with different DNA damaging mechanisms and potency (doxorubicin, etoposide, cytarabine and chlorambucil) in human leukemic Jurkat and HL-60 cells.

Material and methods. Different exposure scenarios were applied: 1) 72 h simultaneous incubation with PJ-34 (2.5 or 5 μM for Jurkat and HL-60 cells, respectively) and a drug used at a wide concentration range; 2) preincubation of the cells with PJ-34 for 24 h and then with a combination of PJ-34 + drug for an additional 48 h; 3) preincubation of the cells with the drug for 24 h with a subsequent incubation with a combination of PJ-34 + drug for an additional 48 h. Cytotoxicity was assessed using a WST-1 reduction test.

Results. It was determined that PJ-34, when used in all 3 scenarios, did not induce any significant enhancement of cytotoxicity of the drugs either in Jurkat or in HL-60 cells.

Conclusions. Although the results do not confirm the beneficial effects of PARP inhibition in combination treatment of the leukemic cells, we propose that future studies including an additional step with the inhibition of DNA repair by homologous recombination should provide promising results.

Key words: PJ-34, doxorubicin, cytarabine, chlorambucil, etoposide

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Poly(ADP-ribose) polymerases, also termed ADP-ribosyltransferases with diphtheria toxin homology (ARTDs) according to a new nomenclature, catalyze the polymerization of ADP-ribose units from NAD^+ on acceptor proteins, leading to the formation of linear or branched polymers of ADP-ribose. The PARP superfamily encompasses at least 18 enzymes involved in several biological processes, including transcriptional regulation, DNA repair, cell cycle regulation, hypoxic response, inflammation, spindle pole function, oncogene-related signaling and cell death.[1] Poly(ADP-ribose)polymerase-1 (PARP-1), an abundant and ubiquitous enzyme, is the best characterized member of the family. It accounts for 80–90% of detectable poly(ADP-ribose) synthesis following DNA damage.

Recently, PARP inhibitors were shown to selectively target DNA double-strand break (DSB) repair-deficient breast cancer type 1/2 susceptibility protein (BRCA1/2) null cells for killing.[2,3] The general understanding of this phenomenon, called synthetic lethality (i.e. when inactivation of either of 2 genes alone allows cell viability but simultaneous inactivation of both genes causes cell death), is that after inhibition of PARP-1 (a component of the DNA single-strand break (SSB) repair machinery), unrepaired SSB lesions are converted into DNA DSB during DNA replication and require activation of homologous recombination (HR) repair proteins (e.g. BRCA1/2) for their resolution. Hence, BRCA1/2 functionally null tumor cells treated with a PARP inhibitor accumulate DNA DSB and undergo cellular death. Besides its major role in detecting SSB, PARP-1 was shown to bind to and assist the repair of other damaged DNA structures, including stalled replication forks and DSB, having an effect on both HR and non-homologous end-joining repair processes (NHEJ). Assuming a high propensity of tumor cells towards genome instability and the complexity of PARP-related DNA repair routes, it is not surprising that PARP-1 inhibitors have raised many expectations as potential clinical anti-tumor drugs. These expectations have led to many trials with different generation PARP inhibitors used in monotherapy or in combination with other drugs (http://clinicaltrials.gov). Interestingly, so far the majority of the trials have been conducted (or are ongoing) in patients with solid tumors, while leukemia, myeloproliferative disorders or other hematological malignancies were less common. This situation is reflected by relatively scarce data in scientific literature on the combinatory effects of PARP inhibitors with other DNA damaging antitumor drugs in leukemic cells (primary cells or established lines).

In our study, we decided to assess the influence of a well known PARP-1 inhibitor, PJ-34, on the cytotoxic effects of different antileukemic drugs showing different DNA damaging mechanisms and potency. To this end, we selected doxorubicin (DNA intercalation and inhibition of topoisomerase II, DNA and RNA polymerases, DNA alkylation, disruption of calcium homeostasis and generation of free radicals), etoposide (pure topoisomerase II inhibitor), cytarabine (antimetabolite incorporating into DNA and interfering with DNA and RNA synthesis) and chlorambucil (alkylating agent of the nitrogen mustard type). PJ-34 is a very potent PARP inhibitor with half maximal effective concentration (EC50) of 20 nM, which is 10,000 times lower than the EC50 of 3-AB[4]. For screening purposes, we selected Jurkat and HL-60 cells, which are well established human leukemic in vitro models. Although using a simultaneous coincubation of cells with different agents is currently the most common practice, in the present study, different exposure scenarios were applied. To this end, two general assumptions were made: 1) to preincubate the cells with PJ-34 for a longer time (24 h) to develop not only PARP inhibition, but also other recently-postulated potential PARP-independent effects (e.g. changes of cell cycle distribution[5,6] or activation of the cytoprotective phosphatidylinositol-3 kinase (PI3K)-Akt pathway), and 2) to superimpose PARP inhibition on a fully-developed DNA damage and DNA damage response/repair, i.e. commencing the co-exposure to PJ-34 after 24 h of preincubation of the cells with a drug.[5,6]

Material and methods

Chemicals and reagents

The doxorubicin was from Sequoia Research Products (#SRP04660d), chlorambucil was purchased from Enzo Life Science (#ALX-400-049-G001), Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, #11644807001) was obtained from Roche, RPMI 1640 + GlutaMAX culture medium (#61870) and Fetal Bovine Serum (#10270-106) were purchased from Life Technologies and the Mycoplasma Detection Kit - MycoProbe (#CUL001B) was from R&D Systems. All other chemicals including etoposide (#E1383), cytarabine (#C1768), PARP inhibitor VIII - PJ34 (#P4365), penicillin-streptomycin (#P0781) and trypsin-EDTA (#T4049) were from Sigma Aldrich.

Cell lines

The human T cell leukemia cell line (Jurkat - DSMZ #ACC 282) and the human acute myeloid leukemia cell line (HL-60 - DSMZ #ACC 3) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

The cells were cultured in suspension in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin 100 U/mL and 100 µg/mL streptomycin). The cells were incubated in a 5% CO_2 humidified atmosphere. They were screened for *Mycoplasma* spp. infection using a Mycoplasma Detection Kit.

Cytotoxicity assessment – WST-1 reduction test

The cytotoxicity of doxorubicin, etoposide, cytarabine, chlorambucil and PJ-34 on Jurkat and HL-60 cells was measured using a colorimetric WST-1 reduction test. The assay is based on the conversion by viable cells of light red tetrazolium salt WST-1 to the yellow formazan derivative, whose optical density is measured spectrophotometrically.

In brief, Jurkat cells (4×10^3 cells per well) or HL-60 cells (1.5×10^3 cells per well) were seeded in 50 µL into a 96-well plate (NUNC #167008) and exposed to test substances added as a ×2 concentrated solution in 50 µL of RPMI, for 24, 48 or 72 h. Then 10 µL of the WST-1 reagent was added to each well and the microplate was placed at 37°C for 1.5–2 h. After 1 min shaking, the optical density of the formazan product was determined using a Multiscan RC spectrophotometer (Labsystems Helsinki, Finland) with a 450 nm filter and 620 nm filter as a reference. The results were expressed as the percent of cell survival (OD of exposed vs OD of non-exposed cells (control)).

The effect of PAPR-1 inhibitor – PJ-34 on the cytotoxicity of doxorubicin, etoposide, cytarabine and chlorambucil on Jurkat and HL-60 cells was also studied. In these experiments the cells were exposed to a combination of a drug with PJ-34 (used at maximum non-cytotoxic concentration determined in preliminary experiments) for 72 h, or they were preincubated with PJ-34 or with the drugs for 24 h. After the preincubation, the cells were treated with a combination of drugs with the inhibitor for an additional 48 h. At the end of the exposure, the viability of the cells was assessed in a WST-1 reduction test.

Statistical analysis

The data was expressed as the mean ± SD from the indicated number of separate experiments. The Inhibitory Concentrations inducing 50% decrease in viability (IC50) with Confidence Intervals (CI) were calculated using GraphPad Prism v. 6.01 for Windows (GraphPad Prism Software, Inc., USA). After log transformation, the model of nonlinear regression (log(inhibitor) vs normalized response – variable slope) was applied.

Results

The cytotoxicity of PJ-34 (PARP-1 inhibitor) and selected drugs in Jurkat and HL-60 cells after 24-, 48- and 72-h exposure.

During 72-h incubation, the PJ-34 inhibitor decreased cell survival in a dose-dependent manner (Fig. 1). A higher sensitivity of Jurkat cells was observed, i.e. calculated IC50 values for all time-points were 2-fold less in comparison to HL-60 cells. Based on the results of further studies on the 72-h exposures, the PJ-34 concentration of 2.5 µM and 5 µM were selected for Jurkat and HL-60 cells, respectively.

The cell viability at these concentrations exceeded 70%. Prior to any studies on the inhibitory effects of PJ-34, a thorough dose-response cytotoxicity analysis of selected antitumor drugs on both cell lines was conducted. The data (Fig. 2) suggests a rather diverse potency of the drugs, with DOX and CYT showing the highest cytotoxic activity. In most cases (except CYT), after 72 h of incubation, the HL-60 cells were 2- (CHL) to 5- (ETO) fold more sensitive to the drugs in comparison to Jurkat cells.

Cytotoxicity of selected anti-cancer drugs in combination with PJ-34

Assuming different effects of PJ-34 on drug cytotoxicity depending on the extent of developing cellular damage and DNA damage response, 3 models of PJ-34-drug co-incubations were applied, i.e. 1) simultaneous co-incubation (scheme in Fig. 3), 2) 24-h preincubation with a drug or 3) 24-h preincubation with PJ-34 (scheme in Fig. 4). In each case, the cytotoxicity was determined after a total of 72 h of exposure.

Fig. 1. Viability of Jurkat and HL-60 cells after 24, 48 or 72-h exposure to PJ-34. WST-1 reduction test (n = 3–4). For each exposure, PJ-34 IC50 value has been calculated

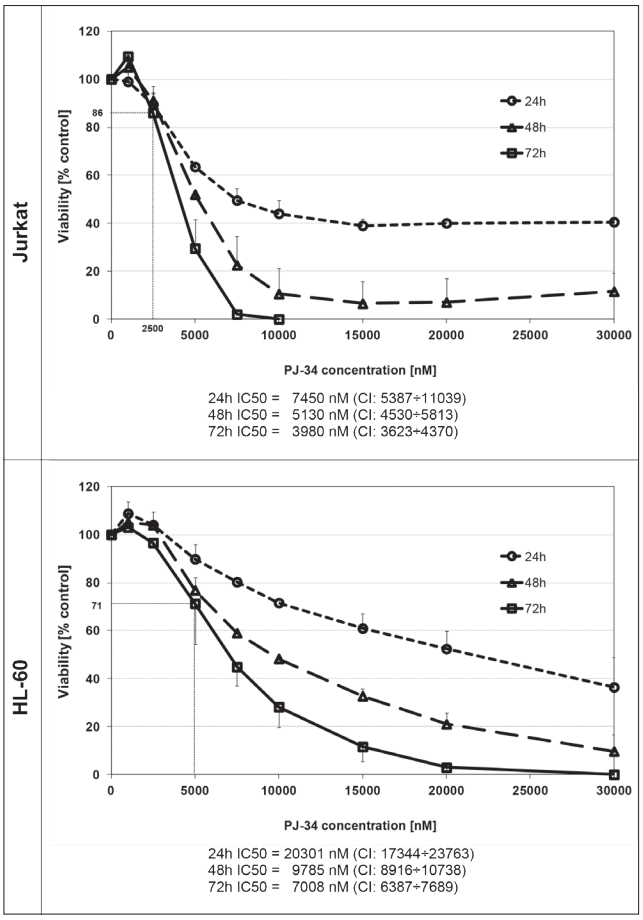


Fig. 2. Viability of Jurkat and HL-60 cells (% control; the Y axis) after the exposure to selected anti-tumor drugs at the concentrations indicated (nM; the X axis): doxorubicin, cytarabine, etoposide or chlorambucil for 24 h (circles), 48 h (triangles) or 72 h (squares). WST-1 reduction test (n = 3–4). For each exposure IC50 value has been calculated

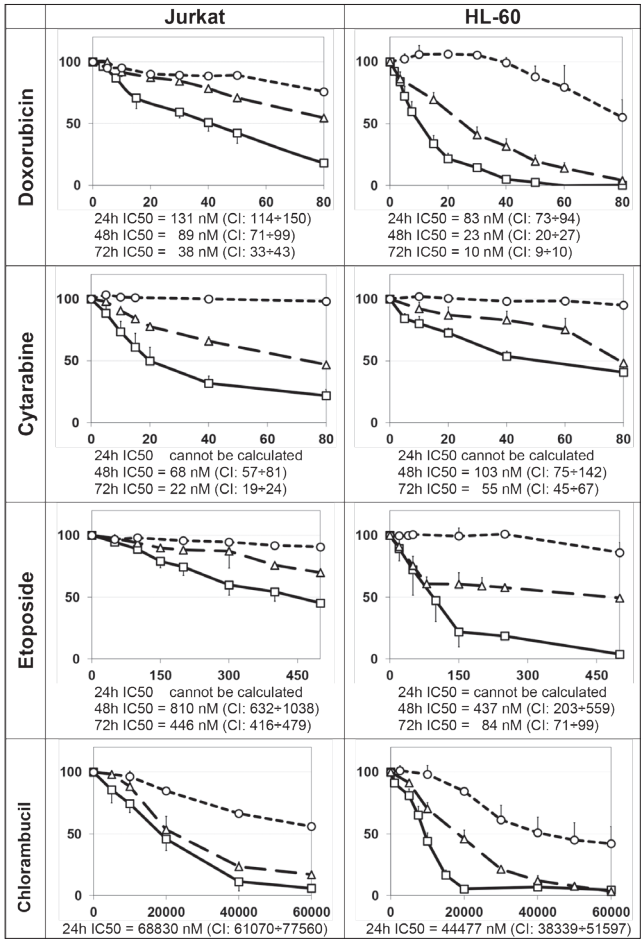
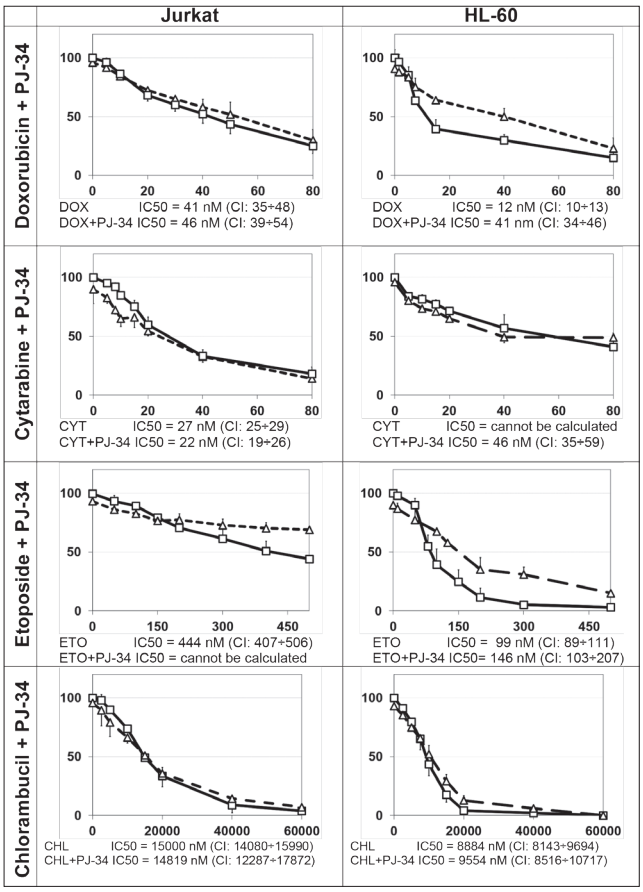


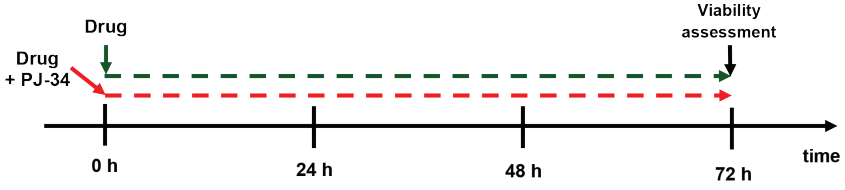
Fig. 5. Viability of Jurkat and HL-60 cells (% control; the Y axis) after the exposure to a combination of an anti-cancer drug (nM; the X axis): doxorubicin, cytarabine, etoposide or chlorambucil with PJ-34 (at 2.5 μM for Jurkat cells and 5 μM for HL-60 cells) for 72 h. WST-1 reduction test (n = 3–4). Squares – the drug, triangles – the combination of the drug with PJ-34. For each exposure IC50 value has been calculated



It was determined that PJ-34 when used simultaneously with the drugs did not induce any significant enhancement of cytotoxicity of the drugs either in Jurkat or in HL-60 cells (Fig. 5).

Similarly, PJ-34 did not significantly influence the cytotoxic potential of the drugs either when the cells were preincubated for 24 h with PJ-34 and then co-exposed with PJ-34 + drug for an additional 48 h (Jurkat cells: Fig. 6B; HL-60 cells: Fig. 7B) or when they were preincubated with a drug for 24 h and then co-exposed for an additional 48 h with drug-PJ-34 combination (Jurkat cells: Fig. 6A; HL-60 cells: Fig. 7A).

Fig. 3. Scheme of 72-h experiments – simultaneous incubation of Jurkat or HL-60 cells with a single drug (green line) or combination of an anti-cancer drug with PJ-34 (red line)



Discussion

In our study we hypothesized that increased DNA damage caused by the addition of PJ-34 to selected DNA-damaging drugs could produce a measurable increase in cytotoxicity in leukemic Jurkat and HL-60 cells. This concept is in line with current ideas of designing combined therapies where, by sensitizing tumor cells to cytotoxic agents, a lower dose could be given while maintaining the same relative efficacy and reducing the toxic side effects.

Although many reports indicate the usefulness of PARP-1 inhibitors in enhancing the cytotoxicity of DNA-damaging drugs in solid tumors, especially with BRCA1/2 deficiency, the experience with leukemic cells is relatively scarce. For many years, PARP-1 and PARP-2 have been recognized as central components of the Base Excision Repair/Single-strand break repair process (BER). However, recently PARP-1 has been found to be activated by other types

of lesions including DNA crosslinks, stalled replication forks and double-strand breaks.[7] PARP-1 can bind to and be activated by DSB both in vitro and in vivo.[8] It is predominantly involved in the HR-dependent repair of DSB at disrupted replication forks. While PARP-1 appears not to be involved in executing HR as such, some data indicates that it actively operates in the HR-dependent restart of stalled replication forks.[9] The current model for PARP1-mediated replication fork stability assumes that if SSB results in fork collapse, resulting in a one-ended DSB and SSB in the sister chromatid, then PARP-1 binds the SSB and/or DSB and recruits XRCC1, thereby promoting the SSB repair process to repair the sister chromatid.[10] PARP-1 may also repress Ku70/Ku80 binding at the one-ended DSB and thereby enabling HR-mediated template switching to promote fork

Fig. 4. Scheme of 24-h preincubation experiments. A) HL-60 or Jurkat cells were preincubated for 24 h with the drug, then for subsequent 48 h with a combination of the drug and PJ-34; B) HL-60 or Jurkat cells were preincubated for 24 h with PJ-34, then for subsequent 48 h with a combination of the drug and PJ-34

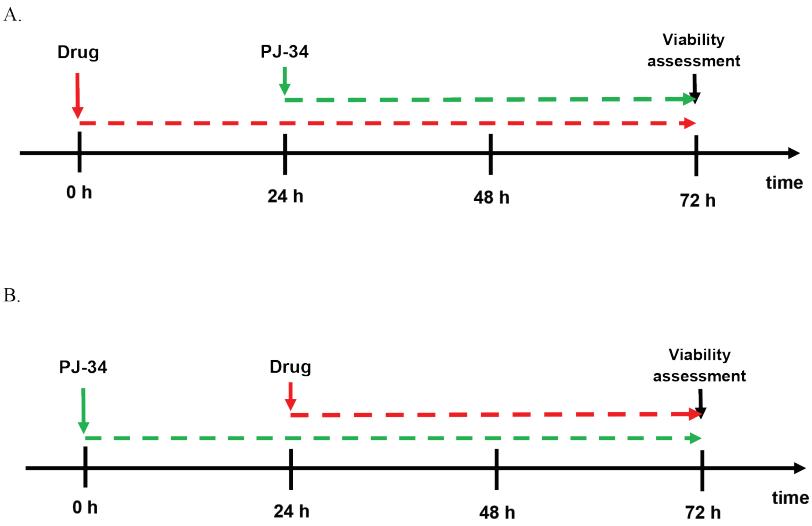


Table 1. Mechanisms of action and types of DNA repair induced by the selected drugs

Drug selected	Mechanisms of action	Type of repair of the DNA damage induced
Doxorubicin (DOX)	Topoisomerase II (Top2) inhibitor; "poisons" the enzyme by stabilizing the DNA cleavage complexes, resulting in DNA strand breaks[21] DNA intercalation, inhibition of DNA and RNA polymerases, DNA alkylation, disruption of calcium homeostasis, generation of free radicals; DOX-induced ROS generate mutagenic base modifications and drive the formation of additional bulky lesions in the form of DNA adducts and crosslinks, lesions which cause replication fork stall and collapse; the higher cytotoxicity of DOX compared to ETO may be due to a higher frequency of DNA double strand breaks and/or by the formation of more persistent cleavage complexes	Two major pathways are active in the repair of DNA DSB: NHEJ and HR repair; the phase of the cell cycle in which DNA damage is induced is critical in determining which of the response processes predominate: because HR requires a homologous DNA sequence it is prevalent in mitotic cells when a copy of the target DNA is available for exchange; DSB generated in the G1 phase of the cell cycle are repaired by NHEJ
Etoposide (ETO)	Potent and the most selective Top2 cleavage complexes-targeted drug currently in the clinic; does not intercalate DNA; Top2 cleavage complexes produced form in a monotonic manner without decrease at high drug concentration; the complexes are readily reversible upon drug wash out, in contrast to anthracyclines; despite the similar mechanisms of action of ETO and DOX, the kinetics of cleavage complex formation and recovery varies, the same as the ratio of single strand to double strand Top2 mediated DNA breaks[21]	
Cytarabine (CYT)	Primarily involves inhibiting DNA synthesis; after activation by phosphorylation, the triphosphates of CYT are incorporated into DNA opposite dG and inhibit DNA synthesis by stalling replication forks; when incorporated into DNA, CYT is also a potent inhibitor of topoisomerase I and II	– repair mechanism not clear; nuclear co-localization of Mre11, Rad50 and Nbs1 with phosphorylated ATM and H2AX, increases in response to CYT; function of ATM and MRN complex at sites of stalled replication forks is unknown, but they may prevent fork collapse, which otherwise could lead to DSB and chromosomal aberrations[24] – suggested DSB formed as a result of unresolved stalled replication forks may be repaired by HR or NHEJ
Chlorambucil (CHL)	Most probably alkylates the nitrogenous bases of DNA (e.g. formation of O6-chloroethylG, O4-chloroethylT adducts) and forms inter- and intrastrand crosslinks; formation of crosslinks results in uncoiling and twisting of the DNA helix[22,23] these structural changes in the DNA duplex result in the inhibition of DNA synthesis and DNA replication, DSB and finally cell death	– primary chloroethyl adducts at O6-G are repaired by direct base repair by O6-alkylG-DNA alkyltransferase – highly cytotoxic interstrand crosslinks require nucleotide excision repair (NER) factors (e.g. XPF-ERCC1) for incision and HR or NHEJ to complete repair – intrastrand crosslinks repaired by NER

Fig. 6. Viability of Jurkat cells (% control; the Y axis) preincubated with an anti-cancer drug at the concentrations indicated (nM; the X axis) for 24 h (A) or with PJ-34 at 2.5 μM (B) and then with the combination of both agents for subsequent 48 h. WST-1 reduction test (n = 3). Squares – the drug, triangles – the combination of the drug with PJ-34. For each exposure IC50 value has been calculated

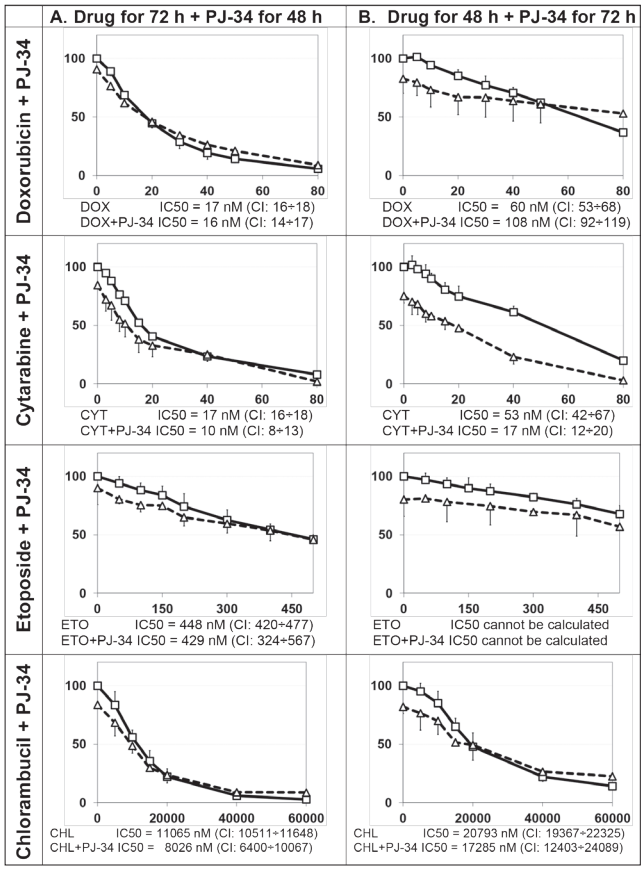
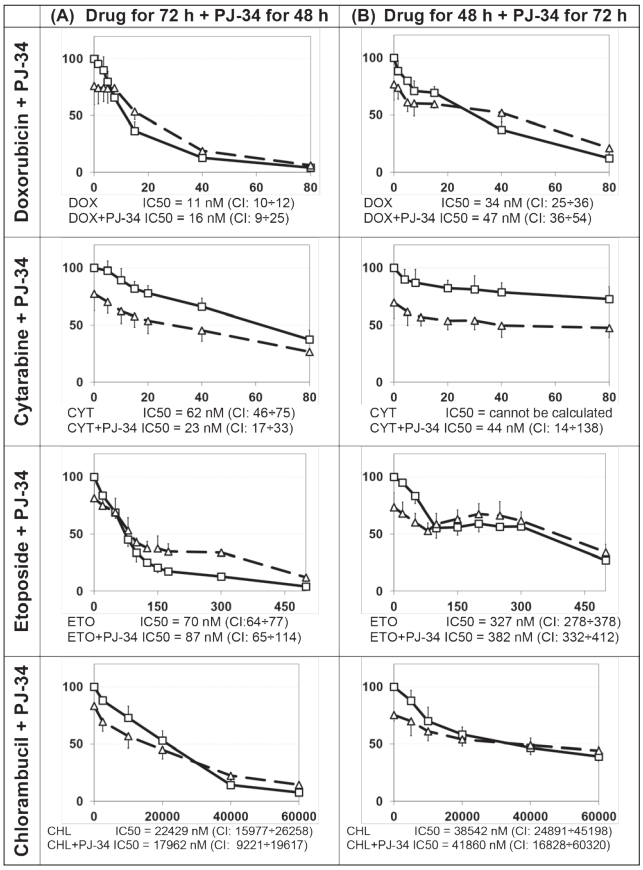


Fig. 7. Viability of HL-60 cells (% control; the Y axis) preincubated with an anti-cancer drug at the concentrations indicated (nM; the X axis) for 24 h (A) or with PJ-34 at 5 μM (B) and then with the combination of both agents for subsequent 48 h. WST-1 reduction test (n = 4). Squares – the drug, triangles – the combination of the drug with PJ-34. For each exposure IC50 value has been calculated



restart, preventing aberrant NHEJ repair of the DSB. PARP-1 may also promote HR directly, e.g. by regulating Mre11 nuclease activity at the DSB.[11]

The data quoted strongly suggests that PARP-1 inhibitors may be potentially useful in neoplasms defective in a vast array of DNA damage repair pathways, not only in BER (SSB repair) or HR (DSB repair). For this reason, in the present study, drugs with different putative modes of DNA-damaging activity were used (Table 1). Moreover, assuming an as-yet unidentified effect of PARP-1 inhibition depending on the timing of DNA damage induction (hence the extent of the damage and development of signaling of the damage), we applied 3 different exposure scenarios. Such approach is fully supported by many clinical trials with PARP inhibitors in combination with chemotherapy, where it is often difficult to define an optimum combination dose and schedule that would improve the therapeutic ratio.

In spite of the extended investigations, we did not observe any satisfactory effect of PJ-34 on the drugs cytotoxicity in either treatment schedules. Available literature data indicates rather divergent effects of PARP inhibition in leukemic cells, ranging from increased sensitivity to the drugs, through no effects, to even increased resistance of the cells. For example, 5'-aza-2'-deoxycytidine (a DNA methyltransferase inhibitor) failed to increase the cytotoxicity of PARP inhibitors (KU-0058948 and PJ-34); in contrast, MS275 (a histone deacetylase inhibitor) potentiated the cytotoxic effect of KU-0058948 and PJ-34 in all PARP inhibitor-sensitive leukemic cells.[12] In human leukemia K562 cells, AG14361 (a PARP inhibitor) caused a 2-fold sensitization to camptothecin-induced cytotoxicity.[13] CEP-8983 (a novel PARP inhibitor) synergized with bendamustine (a nitrogen mustard derivative) in killing primary chronic lymphocytic leukemia cells in vitro.[14] Olaparib sensitized ATM null lymphoid tumor cells in vitro and in vivo to DNA-damaging agents.[15] On the other hand, the pre-treatment of HL-60 cells with 3-aminobenzamide (3-AB) or 6(5H)-phenanthridinone (PARP inhibitors), resulted in resistance to, rather than potentiation of, apoptotic death induced by DNA-damaging agents, idarubicin, etoposide and fludarabine.[16] As can be seen, in spite of the very attractive hypothesis of the synthetic lethality and theoretical usefulness of PARP inhibitors in enhancing the DNA-damaging effects of antitumor drugs, the above-mentioned divergent results in

leukemic cells are rather unexpected, with the underlying mechanisms probably being very complex.

The reasons for the lack of PJ-34 effects observed in the present study are unclear, however some potential explanations can be provided:

a) as we did not measure PARP activity, it cannot be excluded that the cells may have constitutively reduced protein expression, which may exert different effects compared to catalytic inactivation by PJ-34. Indeed, there are reports indicating that low PARP levels (and activity) attenuate responsiveness to PARP inhibitors.[17] In this case, decreased PARP protein might be selectively advantageous to withstand the "poisoning" activity of drug-induced DNA-PARP aggregates.[18] Quite similarly, the cytotoxic effect of topoisomerase inhibitors requires and is positively correlated with the levels and activity of topoisomerases;[19]

b) in our studies, we used the highest allowable concentration of PJ-34 which, after 72 h, did not induce a significant cytotoxicity in the cells. However, again, it cannot be excluded that a potential residual activity of PARP-1 in PJ-34-treated cells might suffice for rescuing the drug's cytotoxic effects. Even if the PARP activity in the cells was not fully inhibited, we believe that such a condition much better reflects the clinical situation, where full inhibition of PARP-1 with currently-used inhibitors is impossible to achieve because of over-toxicity;[20]

c) the cells may have an efficient DNA repair capacity via different paths, which was sufficient to repair DNA damage after exposure to the drugs. For example, it is well recognized that cells deficient in DNA DSB repair are highly sensitive to the chemical inhibitors of PARP, however, cells with intact DNA DSB-response pathways repair damage with high fidelity and accordingly show very little sensitivity to PARP inhibitors.

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